

UNIVERSIDAD COMPLUTENSE DE MADRID

Facultad de Ciencias Biológicas



TESIS DOCTORAL

**Métodos moleculares aplicados a la conservación de la
ictiofauna de la cuenca del Duero**

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Andrea Corral Lou

DIRECTOR

Ignacio Doadrio Villarejo

Madrid, 2022

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PROGRAMA DE DOCTORADO EN BIOLOGÍA

La presente Tesis Doctoral ha sido financiada por:

- Ayudas para contratos para la formación de investigadores en empresas 2017 (Doctorados Industriales) de la Comunidad de Madrid (IND2017/AMB-7699), con la colaboración de la Universidad Complutense de Madrid, Consultores en la Biología de la Conservación S.L. y el Museo Nacional de Ciencias Naturales (CSIC).
- Los estudios también contaron parcialmente con el apoyo de una comisión de gestión financiada por la Confederación Hidrográfica del Duero en España (Análisis y estudios filogeográficos y de necesidad genética de endemismos ibéricos de ciprínidos: Life13 nat/es/000772 “Actuaciones para la protección y conservación de ciprínidos ibéricos de interés comunitario”).

*A toda mi familia,
la de sangre y la elegida*

AGRADECIMIENTOS

Me gustaría empezar agradeciendo a Nacho, mi director de tesis. Primero de todo gracias por confiar en mí y darme la oportunidad de hacer una tesis contigo, eres una persona admirable y una fuente inagotable de conocimiento, no hay río, pez o montaña que se te escape, aunque, si tengo que destacar una cualidad tuya es la de identificar alevines desde la orilla, simplemente, guao. Admiro tu pasión por la ciencia, tu generosidad y tu humor (creo que ahí nos entendemos muy bien), siempre dispuesto a ayudarme y guiarme. En definitiva, mil gracias.

Lourdes, Silvia, Miriam, Paloma y Joserra, gracias por existir. Lu, admiro tu paciencia, tu conocimiento, tu buen carácter y tu ser pragmático. Gracias por enseñarme y transmitirme tu adoración por el laboratorio donde tantas horas hemos pasado (y reído) juntas. Se que nunca me perdonarás el que no te dejase desayunar en el Huebra, pero que buenos ratos hemos pasado!. Silvi, que bien haber podido compartir contigo una gran parte de mi tesis, me siento muy afortunada. Eres la definición de bondad (nunca había visto a nadie tratar así a una pequeña cobitis) y todo un ejemplo a seguir y del que aprender, muchas gracias por todo. Miri, me dijiste que me inspiras en anécdotas que había vivido con las personas, y contigo son kilómetros y kilómetros de anécdotas (y horas y horas de filtrado), pero si tengo que elegir una, desde luego me quedo con la de Canelita. Gracias por todo tu apoyo emocional y laboral, tu implicación con todo, tus consejos, repletos de sabiduría y de mucho cariño. Paloma, eres energía y alegría, que más se puede pedir? muchas gracias por reírte conmigo y estar cuando te he necesitado (muchas gracias por las clases del maldito BEAST te estaré eternamente agradecida jaja). José Ramón, mi mañico, durante estos años me has hecho que Madrid pareciese un poco más mi casa, y ya con eso estoy muy agradecida, pero además, me has ayudado y motivado, ya fuese con café, con capazos o con risas. Has sido cordura y paciencia, y aunque alguna vez me has mandado a escaparrar, siempre has estado dispuesto a ayudarme sin pedir nada a cambio. Gracias maño.

Gracias a Obe y Alba, siempre me habéis facilitado todo con una amabilidad extraordinaria. Gracias a Diego y Ainhoa, gracias a ellos tuve la oportunidad de hacer la tesis en el museo. Gracias a Sara y Eva, vosotras me adentrasteis en el maravilloso mundo del ADN ambiental. Gracias a Melinda por sus múltiples correcciones y revisiones impecables. Gracias a todos mis compañeros

y compañeras del museo, los que en algún momento me habéis dedicado vuestro tiempo y vuestro cariño y de los que, sin duda, me llevo un buen recuerdo, gracias a Violeta, María, Lucia, Eva, Mariana, Alberto, Martí, Paula, Ernesto, Goyo, Karen, Jorge, Dan, Fernando, Jonathan, Guille, Iker, Juanes, Samu, Mabel e Iván, hemos pasado muy buenos ratos. Gracias a Olga, ha sido un verdadero placer ser contagiada diariamente de tu energía y estar contigo todos estos años. Gracias a Belén, Ana, Tessa y Alfonso, vuestra aparición en la última etapa de mi tesis ha venido cargada de muy buenos ratos y de mucha energía.

Gracias a mis amigas, a Elia y Lorena, gracias por cada jueves de charretas de desahogo, de terapia, de carcajadas y de quesos, muuuuchos quesos. Gracias por saber escucharme y comprenderme, desde luego sois el mejor cargador que he podido tener. Gracias a mis Lauras, los audios interminables y las visitas express, que suerte tengo de poder contar con vosotras cuando sea y dónde sea. Muchas gracias a mis quintas (Patry, Eva, Sara, Isa y Noemi), hacéis que mi vida sea más increíble con vuestra presencia, que suerte tengo de formar parte de un todo perfecto. Leyre y Maña, me habéis estado acompañando desde que me caí en Madrid, compartiendo nuestras dos pasiones, la biología y las tartas de queso, muchas gracias por seguir ahí. Gracias a Andrea, solo hizo falta un año para convertirnos en inseparables, eres la amiga más lejana y a la vez la más cercana, adoro hablar contigo de ciencia y de lo que sea, vuelve pronto.

Gracias a toda mi familia. A mis padres (la Pili y el Pepe), gracias por haberme apoyado de esa manera tan incondicional y con tanto cariño en cualquier decisión que tomaste, hacéis que la vida no dé tanto vértigo, y no es porque sea vuestra hija, pero sois lo mejores padres del mundo. También gracias por los tupperts de comida, creo que gracias a eso aun sigo viva. Quien tiene una hermana tiene un tesoro (y más ropa en el armario), pues yo tengo dos tesoros!!!. Victoria y Sara, sois geniales, unas grandes luchadoras y un verdadero ejemplo a seguir. Gracias por vuestra manera de ver las cosas, por ayudarme a relativizar y hacer que no pierda continuamente los nervios. Gracias por escucharme y comprenderme, sé que cualquier persona no podría aguantar oírme hablar de lo mismo por tantas horas seguidas. Sois un gran apoyo y me siento muy feliz de teneros. Por cierto, gracias por los tupperts de pure Sara. Gracias a Lisa, me has dado mucho amor/odio durante muchos años. Gracias a mi abuelo José, tus mensajes de texto eternos me hacen sentir muy afortunada.

Por último, Ale, gracias por ser, estar y querer como has sido, estado y querido a lo largo de todo este tiempo (además gracias por tu soporte técnico, dirás que no lo eres, pero como informático lo bordas). Gracias por tu apoyo, por quererme como soy, por aguantarme en mis días grises y acompañarme en los de mil colores. Gracias por tus ánimos, tus consejos y tu sonrisa. Te quiero.

*“Sale, entre las ramas que crecen en la vida y las hojas que se renuevan cada primavera,
bolas de laurel y olivo para que tengas presente que el triunfo y la amargura crecen en el
mismo árbol”*

José Corral Valiente

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Métodos moleculares aplicados a la conservación de la ictiofauna de la cuenca del Duero

Debido a la escasa capacidad de dispersión de los peces primarios de agua dulce, su distribución suele estar limitada a la cuenca hidrográfica donde están presentes. Como consecuencia de ello, tanto la endemidad como la diversificación de la ictiofauna continental de la Península Ibérica y los patrones evolutivos asociados han sido relacionados con la compleja historia hidrogeomorfológica y las oscilaciones climáticas que ocurrieron en el pasado. Todo ello ha dado lugar a que la ictiofauna dulceacuícola ibérica se caracterice por pocos, pero muy diversificados géneros de peces. Una excepción es el género *Phoxinus*, donde sólo la especie *P. phoxinus* había sido descrita en la Península Ibérica, encontrándose ampliamente distribuida en pequeñas cuencas cantábricas y catalanas, en la cuenca del Ebro y en la cuenca del Duero, donde la especie fue trasladada.

Un número de especies representativas de la ictiofauna primaria ibérica puede ser encontrada en la cuenca del Duero, la cuenca más grande de la Península Ibérica, la cual alberga el 70% de los géneros y el 30% de las especies de peces primarios de agua dulce de toda España. De todas las especies de peces primarios de agua dulce presentes en la cuenca del Duero el 64% se encuentran catalogadas bajo alguna de las tres categorías de amenaza de la lista roja de la IUCN, estando catalogadas como En Peligro de Extinción las especies *A. salmantinum* y *C. vettonica* las cuales poseen un área de distribución muy reducida y, en el caso de *A. salmantinum* limitada a la cuenca del Duero. Por lo tanto, la cuenca del Duero actúa como una unidad imprescindible para la conservación de las especies de peces de España.

Los principales objetivos de la tesis fueron, conocer la diversidad genética de *P. phoxinus*, *A. salmantinum* y *C. vettonica*, aumentar el conocimiento sobre los principales patrones que han afectado a su diversificación, proponer recomendaciones, ajustadas a las necesidades de cada especie y por último desarrollar una técnica no invasiva de detección para la ictiofauna presente en la cuenca del Duero. Por un lado, el estudio genético de las

diferentes poblaciones de las especies fue llevado a cabo a través de muestras de tejido, cubriendo toda su área de distribución conocida. El análisis genético fue llevado a cabo a través de un marcador mitocondrial (el gen del citocromo b; *MT-CYB*), un marcador nuclear (el gen nuclear reactivador de recombinación 1; *RAG1*) y además para *A. salmantinum* y *C. vettonica* también se utilizó una batería de polimorfismos de un solo nucleótido (SNPs). Por otro lado, el estudio de la biodiversidad de la ictiofauna del Duero se llevó a cabo a través de la tecnología de metabarcoding con muestras de ADN ambiental, usando como marcador el gen mitocondrial ribosomal 12S (*12S*).

A través de diferentes aproximaciones filogeográficas y filogenéticas aportamos conocimiento sobre la diversificación de las diferentes especies estudiadas. Así mismo, el estudio genético del género *Phoxinus* reveló la existencia de tres especies coexistiendo en la Península Ibérica (*P. bigerri*, *P. dragarum* y *P. septimaniae*). Mientras que para *P. dragarum* discutimos su origen en la Península Ibérica y se plantea la posibilidad de un origen natural, para la especie *P. septimaniae* aportamos evidencias para pensar que su origen en la Península Ibérica fue consecuencia de una introducción artificial en las cuencas catalanas y en la parte baja de la cuenca del Ebro, donde, además, detectamos una zona de hibridación entre *P. dragarum* y *P. septimaniae*. Para las especies *C. vettonica* y *A. salmantinum* descubrimos una fuerte estructura genética poblacional, la cual no se correspondía con la configuración actual de las cuencas hidrográficas y establecimos las diferentes Unidades Operativas de Conservación (OCUs) para cada especie, discutiendo sobre la conservación de cada una de ellas. Además, para todas las especies explicamos los diferentes procesos hidrogeomorfológicos y climáticos del pasado que podrían haber condicionado la estructura genética actual detectada. Por último, el monitoreo de peces ibéricos de agua dulce a través de ADN ambiental usando *metabarcoding* fue más eficiente para la detección de especies que los métodos tradicionales, resultando en una técnica potente y no invasiva de seguimiento para la biodiversidad de la ictiofauna continental ibérica.

ABSTRACT

Molecular methods applied to the conservation of the ichthyofauna of the Duero Basin

The low dispersal abilities of primary freshwater fish species often limits their distribution to the hydrographic basins where they are present. As a consequence, both the endemism and the diversification of the Iberian continental ichthyofauna, and the patterns related to them, are associated with the region's complex hydrogeomorphological history and past climatic oscillations. The complex patterns of diversification shaped by these events have given rise to a group of fishes comprised of few but highly diversified genera. An exception to this is the genus *Phoxinus*: only the species *Phoxinus phoxinus* is thought to occur in the Iberian Peninsula, specifically in small Cantabrian and Catalan basins, the Ebro Basin and, due to translocation, the Duero Basin.

Around 70% of the genera and 30% of the species of Spain's primary freshwater fishes are found in the Duero Basin, the greatest basin of the Iberian Peninsula. Approximately 64% of these species are classified under one of the three threatened categories of the IUCN Red List. Among them are the endangered species *Achondrostoma salmantinum* and *Cobitis vettonica*, both of which have a highly restricted distribution area. In the case of *A. salmantinum*, its distribution is limited to only the Duero Basin. As a result, the Duero Basin acts as an essential unit for the conservation of fish species in Spain.

The main aims of this thesis were to (1) determine the genetic diversity of *P. phoxinus*, *A. salmantinum* and *C. vettonica*, (2) increase our understanding about the main patterns that have affected the diversification of these species, (3) propose recommendations specific to the conservation needs of each species and, lastly, (4) develop a non-invasive detection technique to monitor the composition of the ichthyofauna present in the Duero Basin. For the first three aims, populations across the entire known distribution area of each species were surveyed, and tissue samples were collected for molecular analysis. For each species, a variety of population genetics analyses were performed using the mitochondrial marker cytochrome b (*MT-CYB*) and the nuclear marker recombination activator gene 1 (*RAG1*); for *A. salmantinum* and *C. vettonica*, a battery of single nucleotide

polymorphisms (SNPs) was also used. For the last aim, a metabarcoding approach based on environmental DNA (eDNA) samples, and the mitochondrial ribosomal gene 12S (*12S*) as a marker, was undertaken to assess the current biodiversity of the Duero ichthyofauna.

In addition, various phylogeographic and phylogenetic approaches were used to make inferences about the diversification of the studied fish species. The genetic study of *Phoxinus* revealed the existence of three species coexisting in the Iberian Peninsula: *P. bigerri*, *P. dragarum* and *P. septimaniae*. In the case of *P. dragarum*, we argue that it is native to the Iberian Peninsula. For *P. septimaniae*, we provide evidence indicating that its presence in the peninsula is due to its artificial introduction into Catalan and lower Ebro basins, where we also detected a zone of hybridization between *P. dragarum* and *P. septimaniae*. For the species *C. vettonica* and *A. salmantinum*, we discovered a strong genetic population structure that does not correspond to the current configuration of the hydrographic basins. For these species, we established ‘operational conservation units’ (OCUs) and discuss factors related to their conservation. In addition, for all the species, we discuss past hydrogeomorphological and climatic processes that likely shaped the current genetic structure of their populations. Finally, we showed that, compared with traditional methods (e.g., electrofishing), the eDNA metabarcoding approach is more efficient at species detection, making it a powerful non-invasive tool for monitoring the biodiversity of the Iberian freshwater fish fauna.

LISTA DE PUBLICACIONES

La presente tesis doctoral ha dado lugar a las siguientes publicaciones científicas

Estudios publicados:

Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926-941.

Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585-600.

Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12(3), e8635.

Estudios pendientes:

Corral-Lou, A. & Doadrio, I. (2022). Environmental DNA metabarcoding of water samples as a tool for monitoring Iberian freshwater fish composition. *Under Review*.

INTRODUCCIÓN GENERAL

La biodiversidad es la variabilidad de los organismos vivos de cualquier fuente, incluidos, entre otros, los ecosistemas terrestres, marinos y otros sistemas acuáticos, y las funciones ecológicas de las que forman parte; comprende la diversidad dentro de cada especie, entre las especies y de los ecosistemas. La biodiversidad global se encuentra actualmente altamente amenazada, ya que una gran parte de la misma está desapareciendo de forma acelerada en el Antropoceno. De hecho, el último informe IPBES 2019 (Plataforma Intergubernamental sobre Biodiversidad y Servicios de los Ecosistemas) determinó que alrededor de un millón de especies de animales y plantas están ahora en peligro de extinción, más que nunca en la historia de la humanidad.

Aunque la extinción forma parte de los principales motores que mueven la evolución, se ha estimado que la tasa de extinción se ha incrementado entre 100 y 1000 veces como consecuencia de las actividades humanas (Raup, 1995; The Royal Society, 2003) perdiendo así una gran cantidad de especies, de poblaciones y de ecosistemas (WCMC 1992). A este fenómeno se le conoce como la extinción masiva del Holoceno, la sexta extinción masiva o la extinción del Antropoceno (Leakey y Lewin 1995; Eldredge 1999; Pimm et al., 2014). La biodiversidad posee valores estéticos, éticos, morales, utilitarios y ecológicos irremplazables garantizando el equilibrio y el buen funcionamiento de los ecosistemas. Así mismo, desde su origen en la Conferencia de las Naciones Unidas sobre Medio Ambiente y Desarrollo de 1992, el Convenio de las Naciones Unidas sobre la Diversidad Biológica (CDB) lucha por la protección de la biodiversidad siendo su conservación uno de sus tres objetivos principales.

La conservación de la biodiversidad puede ser abordada tanto desde diferentes disciplinas como a distintos niveles. En esta tesis doctoral se destaca la conservación genética, la filogenia, la filogeografía y el monitoreo de especies como herramientas fundamentales para la conservación de la biodiversidad. En primer lugar, la genética de la conservación es la disciplina que se encarga de estudiar los factores genéticos que afectan al riesgo de extinción y los métodos de manejo requeridos para minimizarlo. Dentro de las principales cuestiones que trata la genética de la conservación están: reducir la pérdida de diversidad genética, identificar las poblaciones de interés, resolver las incertidumbres taxonómicas

y la estructura poblacional, definir las unidades de manejo dentro de las especies, detectar la hibridación, la elaboración de muestreos no intrusivos para análisis genéticos, definir sitios para las reintroducciones y elegir la mejor población para estas últimas (Frankham et al., 2002). En segundo lugar, la filogenia y la filogeografía han desempeñado un papel fundamental para la conservación de la biodiversidad. Por un lado, para la planificación de la conservación de las especies es importante identificar los diferentes linajes evolutivos a fin de mantener la máxima diversidad genética e incorporar información sobre los procesos históricos de cada población (Avice, 1989; Brooks et al., 1992; Moritz, 1994; Moritz, 1995). Por otro lado, la filogeografía permite comprender los procesos y patrones que dieron lugar a esos linajes evolutivos actuales y además nos permite elaborar diferentes modelos biogeográficos que nos pueden ayudar a la interpretación de la historia evolutiva de otros taxones (Simberloff, 1988; Buhay et al., 2009). De esta manera, conocer los procesos evolutivos a nivel de población es primordial para la conservación de la biodiversidad (Avice, 1995; Carvalho, 1998; Hewitt, 2001; Moritz, 2002). Por último, el monitoreo es una herramienta fundamental para combatir la pérdida de biodiversidad, recopilando información útil, la cual puede ser utilizada para desarrollar una política de conservación, evaluar los resultados de la gestión y guiar las decisiones de manejo (Dallmeier, 1996; Kull et al., 2008; Marsh y Trenham, 2008). En varias ocasiones la falta de datos sobre la biodiversidad ha limitado la toma de decisiones en la gestión y conservación de los recursos. Por lo tanto, el conocimiento sobre la composición de los organismos de un determinado ecosistema es fundamental para la elaboración de estrategias de conservación más fiables y ajustadas a cada ecosistema.

La pérdida de biodiversidad se ha visto más acelerada en las poblaciones animales pertenecientes a los ecosistemas de agua dulce que a esas que viven en los ecosistemas marinos o terrestres (WWF 2014). Los ecosistemas de agua dulce abastecen múltiples necesidades humanas lo que resulta en una gran explotación de estos y se prevé un mayor aprovechamiento de este recurso como consecuencia del crecimiento poblacional (Alcamo et al., 2008; Dudgeon, 2010; Strayer y Dudgeon, 2010). Los principales factores de amenaza para los ecosistemas de agua dulce están asociados a la regulación del caudal, presas y captación de agua, la contaminación, el cambio de uso del suelo, la sobreexplotación de los recursos biológicos y la presencia de especies invasoras, todo ello inmerso y acentuado bajo un escenario de cambio climático (Dudgeon, 2019). Además, estos ecosistemas representan únicamente el 0.03% del volumen total de agua y por lo

tanto su vulnerabilidad se ve más acentuada por la escasez de este recurso (Shiklomanov y Linz, 1991; Gleick, 1993; Gleik, 1998). A su vez, los ecosistemas de agua dulce albergan una gran biodiversidad, con una representación del 9.5% de las especies de animales conocidas, siendo un tercio de ellas vertebrados (Dudgeon et al., 2006; Balian et al., 2007). Dentro de los vertebrados de agua dulce, los peces representan el grupo más abundante, contribuyendo a una gran parte de la biodiversidad encontrada en estos ecosistemas y a su vez, debido a la fuerte amenaza de estos ecosistemas, los peces de las aguas continentales se consideran uno de los grupos más amenazados de la Tierra (Cambray y Bianco, 1998; Geist et al., 2011; Tierno de Figueroa et al., 2013; Freyhof et al., 2014).

La cuenca mediterránea presenta una gran diversidad de peces de agua dulce, siendo considerada uno de los 25 puntos calientes (hotspot) de biodiversidad de importancia mundial (Myers et al., 2000). De esta manera, los peces de agua dulce forman el grupo de vertebrados más amenazado en la cuenca mediterránea con el 36% de las especies dentro de una de las tres categorías de amenaza de la Lista Roja de la UICN (En Peligro Crítico; CR, En Peligro; EN y Vulnerable; VU; Tierno de Figueroa et al., 2013). Este hecho es aún más remarcable para la ictiofauna de la Península Ibérica, ya que aproximadamente el 80% de los peces de agua dulce, se encuentran dentro de alguna de las tres categorías de amenaza de la Lista Roja de la UICN (Doadrio et al., 2011).

La Península Ibérica posee una de las ictiofaunas con un mayor grado de endemismo del mundo (Smith y Darwall, 2006; Kottelat y Freyhof, 2007). Concretamente de las 51 especies presentes, 41 son endémicas y muchas de las que no son consideradas endémicas, tienen su área de distribución restringida a una pequeña parte del sur de Francia (Doadrio et al., 2011). Los peces de agua dulce poseen una capacidad de dispersión reducida, limitada a la cuenca hidrográfica donde están presentes (Rosenzweig, 1995; Burridge et al., 2008; Hugueny et al., 2010) y es por ello por lo que la distribución y la riqueza de sus linajes tiende a reflejar la historia de las cuencas fluviales. De esta manera las peculiaridades asociadas a la endemismo y a la diversificación de la ictiofauna continental ibérica han sido asociadas tanto a la compleja historia geomorfológica como a las oscilaciones climáticas durante el Cenozoico (Carmona et al., 2000; Filipe et al., 2009).

La composición actual de la ictiofauna de agua dulce de la Península Ibérica, dominada por los cipriniformes, tiene su origen en el Oligoceno y sustituye a la ictiofauna Mesozoica de la Península Ibérica (Cabrera y Gaudant, 1985; De la Peña, 1995; Böhme y Ilg, 2003). Desde entonces y a lo largo del Cenozoico la Península Ibérica queda aislada, lo que origina un empobrecimiento en cuanto al número de linajes evolutivos, pero a una gran endemidad y diversificación específica. Dos de los principales eventos que afectaron al aislamiento de las cuencas ibéricas fueron, por un lado, la ausencia de contacto de ésta con respecto a las cuencas europeas debido al levantamiento de los Pirineos, lo cual formó una importante barrera para la dispersión de los peces de agua dulce desde el Oligoceno y por otro lado, la separación con el continente africano como consecuencia de la reapertura del Estrecho de Gibraltar a finales del Mioceno hace 5,3 Ma (Doadrio, 1990; Zardoya y Doadrio, 1998; Krijgsman et al., 1999; Krijgsman et al., 2000; Machordom y Doadrio, 2001; Perdices y Doadrio, 2001; Doadrio y Perdices 2005; Calvet et al., 2021). Además, durante la Orogenia Alpina hace 13.8-7.2 Ma se generaron una variedad de barreras geográficas que afectaron a la geomorfología fluvial de los ríos y por lo tanto a la biota que vive en ellos (Muñoz et al., 1986; Martínez et al., 1989; Puigdefàbregas et al., 1992; Alonso et al., 2007; Ortuño et al., 2013; Perea et al., 2016; Stange et al., 2016).

La fuerte diversificación de los peces de agua dulce ibéricos también se vio impulsada por las diferentes fases de formación de las actuales cuencas hidrográficas, tanto desde su fase de sistemas endorreicos en el interior de la Península Ibérica, principalmente durante el Mioceno, hasta su transición a cuencas exorreicas y finalmente a la culminación de la formación de las cuencas actuales. Esta evolución paleohidrológica dio lugar a reordenamientos hidrogeomorfológicos más locales además de diferentes episodios de capturas fluviales entre las distintas cuencas durante el Plioceno-Pleistoceno (Feio, 1952; Doadrio, 1988; Calvo Sorando et al., 1993; Rodríguez Vidal et al., 1993; Vargas et al., 1998; Perdices y Doadrio, 2001; Sanjur et al., 2003; Perdices et al., 2005; Doadrio y Carmona, 2006; Robalo et al., 2006; Perea et al., 2016; Sousa-Santos et al., 2019). Además, el periodo del cuaternario se caracteriza por oscilaciones climáticas con un patrón de ciclos glaciales e interglaciares que ha dominado el clima global desde hace 2.5 Ma hasta nuestros días (Pillans y Naish, 2004; Gibbard et al., 2010). Aunque la Península Ibérica ha sido considerada como un refugio glacial (Hewitt, 1996) con los glaciares existente limitados al noroeste peninsular y sin evidencia de glaciares fuera de las cadenas

montañosas (Alberti et al., 2004; Oliva et al., 2019), las condiciones locales fluctuaron dramáticamente durante este periodo (Fernandez et al., 2007). Es de esta manera que muchos eventos tanto de expansión, fragmentación y contactos secundarios entre especies o poblaciones aisladas fuesen promovidos durante este periodo como consecuencia de los cambios glacio-estáticos, de los procesos glaciares y periglaciares en cabeceras de alta montaña y de los cambios climáticos locales y regionales (Sanjur et al., 2003; Mesquita et al., 2005; Santisteban y Schulte, 2007; Sousa-Santos et al., 2007; Gante et al., 2009; Perea y Doadrio, 2015; Perea et al., 2016).

Por todo ello la evolución de la ictiofauna continental española presenta una gran complejidad, siendo muchas veces incongruentes los patrones encontrados tanto a nivel poblacional como de distribución con la configuración actual de las ceuncas hidrográficas (Doadrio, 1988; Carmona et al., 2000; Doadrio y Carmona, 2004; Filipe et al., 2009; Levy et al., 2009; Perea et al., 2021). Es de esta manera que la estructura poblacional de muchas especies no sea intuitiva, lo cual resalta la necesidad de estudios genéticos específicos. Por lo tanto, el estudio genético de las especies de peces de agua dulce ayuda a mejorar el conocimiento sobre los diferentes patrones hidrogeográficos que tuvieron lugar en el pasado resultando en la configuración actual.

En esta tesis se seleccionaron tres especies de peces primarios de agua dulce pertenecientes a las dos familias más abundantes de la Península Ibérica, las cuales fueron: *Phoxinus phoxinus* Kottelat, 2007 y *Achondrostoma salmantinum* Doadrio y Elvira, 2007, pertenecientes a la familia Leuciscidae compuesta por aproximadamente 31 especies (también la familia más abundante a nivel global; Nelson, 2006), y *Cobitis vettonica* Doadrio y Perdices, 1997, perteneciente a la familia Cobitidae compuesta por 3 especies (Doadrio et al., 2011). A través del estudio genético de estas tres especies se quiere aumentar el conocimiento sobre la diversificación, la variabilidad genética y los diferentes procesos que dieron lugar a la configuración actual de las poblaciones. Esto además nos proporcionará información clave tanto para la obtención de diferentes modelos biogeográficos, como para la elaboración de diferentes estrategias de conservación.

***Phoxinus phoxinus* Kottelat, 2007 (piscardo)**

La especie *P. phoxinus* es un pequeño leuciscidae de entre 6-12 cm de longitud estándar. Esta especie es endémica de España y del suroeste de Francia y suele vivir en los tramos

medios y altos de los ríos en aguas oxigenadas, limpias, frescas y con fondos pedregosos (Kottelat y Freyhoff, 2007; Doadrio et al., 2011). Dentro de la Península Ibérica se distribuye de manera autóctona en las cuencas del Ebro y en pequeñas cuencas cantábricas y quizás en alguna de las cuencas catalanas (Kottelat, 2007; Kottelat & Freyhof, 2007; Doadrio et al., 2011) y de manera introducida en la cuenca del Duero, en pequeñas cuencas gallegas y en lagos de montaña del norte (Lozano-Rey, 1935; Doadrio y Garzón, 1986; Doadrio, 2001; Sánchez-Hernández et al., 2012; Miró y Ventura, 2015). Sin embargo, su uso como cebo vivo o para favorecer la alimentación de la trucha y las múltiples translocaciones, dificultan saber en qué cuencas son autóctonas y en cuales no (Doadrio y Garzón, 1986). La existencia de varias especies del género *Phoxinus* Rafinesque, 1820 dentro de la Península Ibérica ha sido sugerida anteriormente (Kottelat y Freyhof, 2007; Palandačić et al., 2017), sin embargo no hay estudios previos enfocados a resolver esta cuestión. Por otro lado, aunque esta especie esté considerada bajo el criterio de Preocupación Menor en la Lista Roja de la IUCN, tampoco hay estudios genéticos sobre la estructura poblacional y la variabilidad genética. El estudio genético de las poblaciones ibéricas del género *Phoxinus* además ayudaría a la elaboración de modelos biogeográficos abordando una gran parte del norte peninsular, el cual no había sido abordado con anterioridad con peces de agua dulce de la familia leuciscidae.

***Achondrostoma salmantium* Doadrio y Elvira, 2007 (sarda)**

La especie *A. salmantinum* es un pequeño leuciscidae que llega a alcanzar como máximo los 10-11 cm de longitud estándar. Esta especie es endémica de España y vive en ríos estacionales con abundante vegetación acuática de macrófitos, fondos limosos y arenosos y con escasa corriente acuática (Elvira et al., 1990; Velasco et al., 1997). Su área de distribución se encuentra restringida a unos pocos ríos pertenecientes a tres pequeñas subcuencas de la parte suroccidental de la cuenca de Duero dentro de la provincia de Salamanca (Doadrio y Elvira, 2007). La especiación de *A. salmantinum* se asocia al Mioceno cuando la paleocuenca de Ciudad Rodrigo quedó aislada del resto de la cuenca del Duero, previo a la configuración actual de la cuenca (Berggren et al., 1995; Carmona et al., 2000; Doadrio y Elvira, 2007). La especie *A. salmantinum* se encuentra dentro de la categoría de En Peligro en la Lista Roja de la IUCN (<https://www.iucnredlist.org/species/184445/8277728>) y un declive poblacional ha sido detectado para sus poblaciones, habiendo llegado a desaparecer de algunos puntos históricos, como es el caso del río Turones (Doadrio et al., 2011). Sin embargo, no hay

estudios moleculares previos sobre la estructura y variabilidad genética ni para esta especie ni para otros organismos dulceacuícolas en esta área que puedan proporcionar un modelo biogeográfico que ayude a la elaboración de planes de manejo y conservación.

***Cobitis vettonica* Doadrio y Perdices, 1997 (colmilleja del Alagón)**

La especie *C. vettonica* es un pez bentónico, perteneciente a la familia Cobitidae con un tamaño de entre 4-8 cm de longitud estándar en los ejemplares adultos (Doadrio y Perdices 1997). Esta especie es endémica de la Península Ibérica y vive en ríos de aguas claras y generalmente someras, con fondos de cantos rodados y gravas y comúnmente se encuentra en las cabeceras de los ríos donde vive (Carmona et al., 1999; Doadrio et al., 2011; Perdices y Coelho, 2020; Collares-Pereira et al., 2021). Su área de distribución está restringida a unas pequeñas subcuencas del centro-oeste peninsular pertenecientes tanto a la cuenca del Duero como del Tajo, localizadas en ambos países Portugal y España (Doadrio y Perdices, 1997; Perdices y Coelho, 2020). Al igual que ocurre con la especie *A. salmantinum* (con la cual comparte una parte de su área de distribución), la especiación de *C. vettonica* ha sido asociada al aislamiento de la paleocuenca de Ciudad Rodrigo con respecto a la cuenca del Duero, previo a su configuración actual (Doadrio et al., 2011). La especie *C. vettonica* se encuentra dentro de la categoría de En Peligro en la Lista Roja de la IUCN (<https://www.iucnredlist.org/species/60347/12355688>) y un declive poblacional ha sido detectado para sus poblaciones, aunque en algunas áreas es abundante, habiendo llegado a desaparecer de algunos puntos históricos, como es el caso del río Turones (Doadrio et al., 2011). Aunque estudios moleculares previos para otros cipriniformes han sido llevados a cabo en esta área (i.e *Squalius carolitertti*, *S. alburnoides*, *S. pyrenaicus* y *L. bocagei*; Doadrio et al., 2002; Cunha et al., 2004; Sousa-Santos et al., 2007; Perea et al., 2021), las especies del género *Cobitis* Linnaeus, 1758 presentan una capacidad de dispersión más limitada, siendo un buen modelo para estudiar la biogeografía de esta área. Por otro lado, aunque hay algunos estudios moleculares para esta especie, estos no han tenido en cuenta toda su área de distribución. Por lo tanto, el estudio de *C. vettonica* a lo largo de toda su área de distribución nos podría ayudar a elaborar un modelo biogeográfico más detallado además de un conocimiento necesario para su conservación a través de la estructura y diversidad de sus poblaciones.

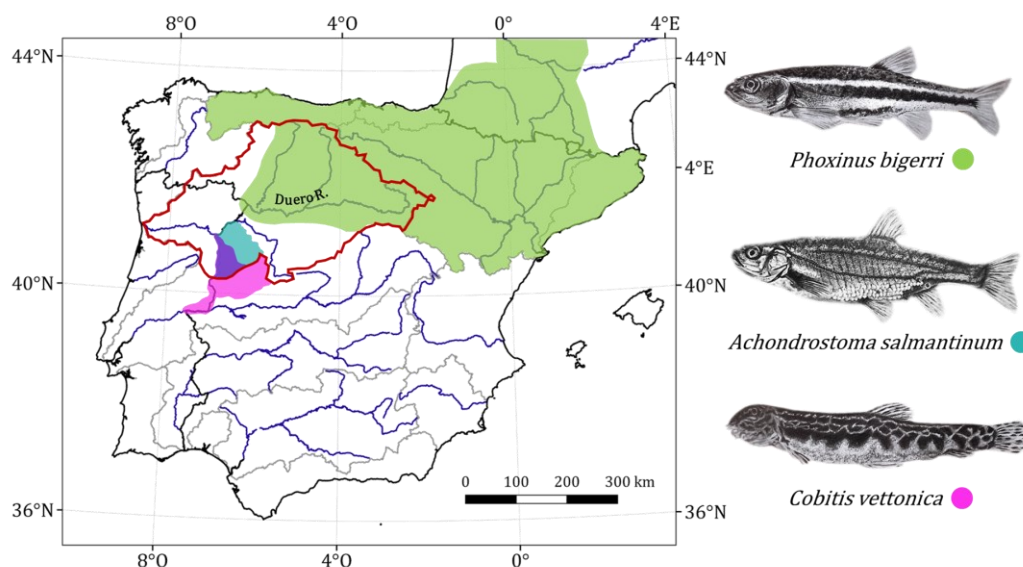


Figura1. Mapa de distribución conocida de las especies *P. bigerri* (antes de esta tesis doctoral), *A. salmantinum* y *C. vettonica*. Delineadas en gris están las principales cuencas hidrográficas de la península ibérica. Delineada en color rojo está marcada la cuenca del Duero.

De las tres especies seleccionadas, la especies *P. bigerri* y *C. vettonica* presentan una parte de su área de distribución en la cuenca del Duero, mientras que la totalidad del área de distribución de *A. salmantinum* se encuentra dentro de esta cuenca. La cuenca del Duero es la cuenca más grande de España con 97.290 km² de superficie y alberga aproximadamente el 70% de los géneros y el 30% de las especies de toda España. De todas las especies presentes en la cuenca del Duero un 64% se encuentran dentro de alguna de las tres categorías de amenaza de la UICN viéndose la biodiversidad de la ictiofauna primaria de esta área comprometida por diversos factores (Doadrio et al., 2011). En primer lugar, ha habido una reducción sustancial de los recursos hídricos en la cuenca del Duero durante los últimos 50 años en relación tanto con las variables atmosféricas como con los cambios en la cobertura del suelo, y además se prevé que esta reducción se acentúe aún más en el futuro como consecuencia del cambio climático (Ceballos-Barbancho et al., 2008, Morán-Tejeda et al., 2010; Morán-Tejeda et al., 2012). Por otro lado, la cuenca del Duero muestra una gran variedad de especies exóticas, siendo la presencia de estas, una de las principales amenazas que afectan a la viabilidad de las especies autóctonas (Dudgeon, 2019). Además, la cuenca del Duero mostró la proporción más baja, en comparación con todas las cuencas hidrográficas españolas, del número de

especies nativas por el total de especies, incluyendo tanto las especies de peces nativas como las exóticas (Elvira, 1995). Aunque muchas veces se desconoce el impacto real de las especies invasoras frente a las especies autóctonas, éstas pueden alterar a la fauna autóctona por varios factores, ya sea por la competencia de nicho o de los recursos, depredando la fauna autóctona, transfiriendo patógenos, alterando significativamente el hábitat o dando lugar a la introgresión genética, lo que lleva a la pérdida de diversidad genética (Rincón et al., 1990; Elvira y Almodóvar, 2001; Almodóvar et al., 2006).

Por lo tanto, el monitoreo de especies tanto autóctonas como alóctonas presentes en la cuenca del Duero parece imprescindible para priorizar y llevar a cabo las diferentes estrategias para la conservación de la ictiofauna ibérica. Normalmente el monitoreo de las especies de peces ha sido llevado a cabo por diferentes métodos intrusivos, los cuales conllevan la captura directa de los individuos, tales como la pesca eléctrica y las redes (Bonar et al., 2009). La captura directa, puede comprometer la viabilidad de las especies, especialmente si se encuentran en peligro de extinción, y además necesitan la identificación morfológica de un experto (Snyder, 2003; Roy et al., 2018; Bylemans et al., 2019; Goutte et al., 2020). En los últimos años, los enfoques de ADN ambiental (eDNA) de muestras de agua, es decir, el material genético que liberan los organismos a través de esas células y tejidos en los ecosistemas (Taberlet et al., 2012), se han utilizado ampliamente para monitorear la biodiversidad de comunidades de peces (Goldberg et al., 2015; Valentini et al., 2016; Cilleros et al., 2019; Sales et al., 2021). Esta técnica ha demostrado, entre otros factores, una mayor sensibilidad para detectar especies de baja abundancia que los métodos tradicionales además de un menor esfuerzo económico y de tiempo (Hinlo et al., 2017; Sigsgaard et al., 2020). Sin embargo, esta técnica no ha sido previamente desarrollada para la caracterización de la ictiofauna ibérica.

Por todo lo mencionado anteriormente, resulta imprescindible conocer tanto la composición de la biodiversidad ictiológica como la variabilidad genética de las especies, así como los procesos que han dado lugar a los diferentes patrones biogeográficos de las distintas especies presentes en la cuenca del Duero. Con ello se pretende contribuir a la conservación de la ictiofauna ibérica y a entender los procesos históricos que la originaron. Así como poner a punto una técnica de seguimiento de la ictiofauna continental que contribuya a su conservación.

OBJETIVOS GENERALES

Los tres objetivos principales de la Tesis Doctoral fueron:

Objetivo 1: Conseguir mejorar los planes de conservación y manejo para la especie *A. salmantinum* y *C. vettonica*, dos especies amenazadas con áreas de distribución muy reducidas.

Objetivo 1.1: Definir las Unidades Operativas de Conservación, mediante el análisis genético de su estructura espacial a lo largo de toda su área de distribución utilizando diferentes marcadores moleculares.

Objetivo 1.2: Analizar la variabilidad genética de cada Unidad Operativa de Conservación.

Objetivo 1.3: Evaluar los resultados obtenidos y proponer recomendaciones ajustadas a las necesidades de cada especie.

Objetivo 2: Aumentar el conocimiento sobre los principales patrones que han afectado a la diversificación de la ictiofauna continental ibérica en diferentes regiones de la Península Ibérica utilizando como modelo las especies *P. bigerri*, *A. salmantinum* y *C. vettonica*.

Objetivo 2.1: Elaboración de análisis biogeográficos, de modelización de nicho con proyecciones al pasado y estimación de tiempos de divergencia entre los diferentes linajes obtenidos.

Objetivo 3: Desarrollar una técnica no invasiva de detección de especies para la ictiofauna presente en la cuenca del Duero.

Objetivo 3.1: Análisis de ADN ambiental con muestras de agua mediante marcadores moleculares.

Objetivo 3.2: Elaborar una base de datos para el marcador molecular seleccionado.

Objetivo 3.3: Evaluar los resultados obtenidos comparándolos con los registros históricos y con métodos tradicionales de pesca.

MATERIALES Y MÉTODOS GENERALES

Recolección de muestras

El muestreo de los peces se realizó mediante la técnica de pesca eléctrica con las correspondientes autorizaciones de las administraciones pertinentes. En cada punto de muestreo se realizó pesca eléctrica en un tramo de aproximadamente 100 metros. El mismo transecto se realizó dos veces con un tiempo total invertido entre 40-50 minutos cubriendo todos los mesohábitats disponibles para tener en cuenta toda la heterogeneidad del hábitat. A algunos ejemplares se les cortaron las aletas y luego fueron devueltos al río. Las aletas se conservaron en etanol absoluto. Algunas muestras de tejido fueron obtenidas directamente de la Colección de Tejidos y ADN del Museo Nacional de Ciencias Naturales (MNCN-CSIC).

La obtención de muestras de ADN ambiental (eDNA) se realizó mediante la filtración de agua del río a través de los portafiltros reutilizables con receptor Nalgene™ utilizando un filtro de membrana Supor®-200 (Pall Corporation, Life Sciences, Ann Arbor, MI, EE. UU.) de 0,45 y/o 0,2 µm de tamaño de poro.

Extracción de ADN

El ADN de las muestras de tejido se extrajo con el kit Qiagen DNeasy® Blood and Tissue (Qiagen, Inc.) siguiendo el protocolo del fabricante. Se utilizó la reacción en cadena de la polimerasa (PCR) para amplificar un fragmento del gen mitocondrial del citocromo b (*MT-CYB*), del gen activador de recombinación nuclear 1 (*RAG1*) o del gen mitocondrial ribosomal del 12S (*12S*). Las condiciones de ciclo térmico variaron en función del gen, del marcador y del grupo taxonómico. Los productos de PCR se revisaron en un gel de agarosa al 1,5 o 2% y las amplificaciones positivas se purificaron con ExoSAP-IT (USB) y se secuenciaron usando un secuenciador 3730xl de Macrogen Inc.

Todas las secuencias obtenidas fueron revisadas manualmente a través de Geneious 10.1.3 (Kearse et al., 2012). Las matrices para el *MT-CYB*, *RAG1* y *12S* fueron alineadas usando el algoritmo MAFFT (Katoh y Standley, 2013) o Clustal W (Thompson et al., 1994) implementado en Geneious 10.1.3. Los alelos del fragmento del *RAG1* fueron

separados usando el algoritmo PHASE (Stephens et al., 2001; Stephens y Donnelly, 2003) implementado en DnaSP v.6.10.01 (Rozas et al., 2017). Algunas secuencias fueron descargadas directamente de GenBank.

Preparación de librerías y filtrado bioinformático para SNPs y metabarcoding

La preparación de las librerías para el estudio de SNPs fueron llevada a cabo a través de *double digest restriction-site associated DNA* (ddRAD-seq), siguiendo el protocolo de Kess et al. (2015). Las muestras fueron secuenciadas a través de un secuenciador Illumina NovaSeq 6000 PE150 o HiSeq 2500 PE100. Las lecturas se limpiaron y filtraron a través de Trimmomatic 0.36 (Bolger et al., 2014) y de diferentes módulos del programa STACKS 2.4 (Catchen et al., 2013; Catchen et al., 2017).

La preparación de librerías para el estudio de metabarcoding se realizó a través de las muestras de eDNA extraído para un fragmento del *12S* utilizando unos cebadores universales para peces (Miya et al., 2015). El conjunto se secuenció en una fracción de una ejecución de MiSeq PE300 (Illumina). El filtrado y la limpieza de las secuencias obtenidas se realizó a través de diferentes programas tales como FLASH2 v.2.2.00 (Magoč y Salzberg, 2011), CUTADAPT v.1.2.1 (Martin, 2011), VSEARCH (Rognes et al., 2016) y diferentes módulos de QIIME (Caporaso et al., 2010). La asignación taxonómica se realizó a través del script *assign_taxonomy.py* de QIIME.

Análisis filogenéticos

La mejor combinación de esquemas de partición y modelos de sustitución de nucleótidos para el *MT-CYB* se determinó con PartitionFinder 1.1.1 (Lanfear et al., 2012) o jModelTest 2.1.10 (Darriba et al., 2012). La reconstrucción filogenética se llevó a cabo para *MT-CYB* mediante el análisis de Máxima Verosimilitud (ML) implementado en MrBayes v.3.2 (Ronquist et al., 2012) y/o Inferencia Bayesiana (BI) implementado en RAxML v8.2.8 (Stamatakis, 2014) ejecutados en CIPRES Science Gateway v3.3 (Miller et al., 2010). Las relaciones filogenéticas a través de los SNPs fueron llevadas a cabo a través del método de ML en el programa RAxML v8.2.8 implementando en CIPRES Science Gateway v3.3.

Se utilizó el programa MEGA v.6.0 (Tamura et al., 2013) para calcular las distancias genéticas absolutas sin corregir (*p-uncorrected*) del *MT-CYB*.

La diferenciación genética para el *12S* fue calculado a través del porcentaje de similitud calculado en Geneious 10.1.3.

Estructura genética

La estructura genética poblacional fue observada utilizando redes de haplotipos usando el algoritmo de *median-joining* (Bandelt et al., 1999) implementado en PopART (Leigh y Bryant, 2015) tanto para *MT-CYB* como para *RAG1*.

La fuente de variación genética fue observada a través del análisis de la varianza molecular (AMOVA) implementado en Arlequin v.3.11 (Excoffier y Lischer, 2010). La diferenciación genética fue observada a través del parámetro Φ_{st} (Hudson et al., 1992) implementado en Arlequin v.3.11.

Para los SNPs, los loci bajo selección fueron evaluados a través de BayeScan v.2.0 (Foll y Gaggiotti, 2008) y eliminados para los análisis de estructura genética. El programa STRUCTURE (Pritchard et al., 2000) fue usado para analizar la estructura genética de los SNPs. El número de subpoblaciones más probable fue seleccionado posteriormente en base a los métodos ΔK (Evanno et al., 2005) y/o Puechmaille (Puechmaille, 2016).

Los patrones de covarianza genética para los SNPs fueron examinados a través de un análisis de componentes principales (PCA) usando el paquete SNPRelate v.1.12.0 implementado en R (Zheng et al., 2012). La diferenciación genética para los SNPs fue observada a través del índice de fijación (F_{ST}) implementado en GenoDive (Meirmans y Van Tienderen, 2004).

Diversidad genética y demografía

La diversidad genética para el *MT-CYB* y el *RAG1* fue examinada a través de los siguientes parámetros: número de haplotipos, número de alelos, diversidad haplotípica, diversidad alélica, diversidad nucleotídica y número de sitios polimórficos a través del programa DnaSP v.6.10.01.

Para los SNPs, los parámetros de diversidad genética estudiados fueron: número de alelos privados, número de sitios variantes, número de sitios polimórficos, porcentaje de sitios polimórficos, heterocigosidad observada, heterocigosidad esperada, diversidad nucleotídica y coeficiente de consanguinidad a través del módulo *populations* implementado en STACKS 2.4.

Para evaluar los cambios históricos acontecidos en la demografía, se estimaron las desviaciones respecto del modelo de mutación neutral para *MT-CYB* y *RAG1* calculando la F_s de Fu (Fu, 1997) y la D de Tajima (Tajima, 1989) con Arlequin v.3.11.

Tiempos de divergencia y delimitación de especies

La estimación de los tiempos de divergencia se realizó con un reloj molecular relajado usando un modelo de coalescencia o un modelo de especiación de muestreo incompleto nacimiento-muerte implementado en el programa BEAST v.1.8.4 (Drummond et al., 2012). Cuando fue posible el reloj molecular se calibró usando una tasa de mutación y/o fósiles. Los valores posteriores de los prior fueron revisados en Tracer v1.6 (Rambaut et al., 2015).

La delimitación de especies fue llevada a cabo para *MT-CYB* usando un modelo Poisson Tree Processes (PTP) implementado en el servidor web bPTP (Zhang et al., 2013)

Modelización de nicho y reconstrucción de área ancestral

La modelización de nicho ecológico para el presente y la proyección al pasado se realizó mediante el software MaxEnt v.3.4.1 (Phillips et al., 2017). Las variables bioclimáticas fueron descargadas de WorldClim (Fick y Hijmans, 2017). La proyección del nicho potencial al pasado se llevó a cabo para distintos periodos de tiempo: Presente (1970-2000), Último Máximo Glacial (LGM, hace 22.000 años) y Último Interglaciario (LIG, hace 120.000-140.000 años). La calidad de los modelos fue evaluada a partir del parámetro AUC (área debajo de la curva).

El área ancestral fue reconstruida usando los métodos S-DIVA (Yu et al., 2010) y S-DEC (Beaulieu et al., 2013; Ree y Smith, 2008) implementados en RASP v.4.2 (Yu et al., 2015; Yu et al., 2020).

RESULTADOS

En esta sección se incluye el conjunto de trabajos que forman parte de esta tesis junto con el material suplementario de los mismos:

CAPÍTULO I: Filogeografía y delimitación de especies del género *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) en la Península Ibérica

Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926-941.

CAPÍTULO II: Alta diferenciación genética en *Achondrostoma salmantinum* Doadrio y Elvira, 2007, un pez de agua dulce endémico y en peligro de extinción de España, revelado por un marcador mitocondrial y SNPs

Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585-600.

CAPÍTULO III: Cambios geomorfológicos y climáticos cuaternarios asociados a la diversificación de los peces ibéricos de agua dulce: el caso del género *Cobitis* (Cypriniformes, Cobitidae)

Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12(3), e8635.

CAPÍTULO IV: El uso de la herramienta de *metabarcoding* de ADN ambiental de muestras de agua para el seguimiento de la composición de peces ibéricos de agua dulce

Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Environmental DNA metabarcoding of water samples as a tool for monitoring Iberian freshwater fish composition. *Under Review*.

I

CAPÍTULO

**Filogeografía y delimitación de especies del
género *Phoxinus* Rafinesque, 1820
(Actinopterygii: Leuciscidae) en la Península
Ibérica**



Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926-941.

Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula

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Funding information

Industrial Doctorate of Community of Madrid, Grant/Award Number: IND2017/AMB -7699; Duero Hydrographic Confederation, Grant/Award Number: Life13 nat/es/000772

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Abstract

The genus *Phoxinus* is comprised of at least 15 currently recognized species inhabiting Eurasia. Morphological traits have been traditionally used to delineate species in *Phoxinus*; however, the high level of phenotypic plasticity observed in the genus has confounded this process. Molecular genetic analyses have revealed a higher than expected genetic structure within *Phoxinus*. Here, we analyzed both nuclear and mitochondrial molecular genetic markers to infer the phylogeography and divergence times of *Phoxinus* in the Iberian Peninsula. Our results show that the Iberian lineages of *Phoxinus* were polyphyletic. They also support the co-existence of three species in the Iberian Peninsula, two corresponding to two previously recognized species (*Phoxinus bigerri* and *Phoxinus septimaniae*) and a third undescribed species (*Phoxinus* sp.). *Phoxinus bigerri* is structured into western Cantabrian, eastern Cantabrian, and Artibai basins. We hypothesize that this structure is a consequence of glaciation–deglaciation cycles during the Pleistocene. While the presence of *P. septimaniae* in the Iberian Peninsula is possibly the result of human translocation, that of *Phoxinus* sp. in lower Ebro rivers may be attributed to past fluvial captures. Our study represents the first report to show a relationship among *Phoxinus* populations from central Pyrenean rivers of Spain and France. Furthermore, we found genetic hybridization between *Phoxinus* sp. and *P. septimaniae* in the shared localities, a likely consequence of anthropogenic activities. Overall, our findings provide insight into the genetic structure of Iberian *Phoxinus* populations, including the presence of an undescribed species and the putative introduction of some species that may have implications for conservation.

KEY WORDS

Iberian Peninsula, MT-CYB, *Phoxinus*, phylogeography, RAG1

1 | INTRODUCTION

The freshwater fish fauna in the Mediterranean region is characterized by endemic species with geographical distributions restricted to a limited number of basins. Highly restricted endemic species are especially noted in the Iberian Peninsula as aquatic systems in

this region were isolated from others in Europe mainly during the Miocene with the end of formation of the Pyrenees (Martínez, 1989; Muñoz, Martínez, & Vergés, 1986; Puigdefàbregas, Muñoz, & Vergés, 1992). In addition, the Alpine orogeny formed successive mountains, most running parallel to the Pyrenees, that have generated a variety of geographical barriers and that have affected the

Material Suplementario

Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926-941.

Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula

Authors:

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Supporting Information

Table S1. This table corresponds to all individuals studied referencing in Table 1. The following information is include in the table: voucher number, species, river, locality, basin, biogeographical region, coordinates, N_{MT-CYB} (marked with an “X” if the individual has been sequenced for *MT-CYB*), N_{RAG1} (marked with an “X” if the individual has been sequenced for *RAG1*), the *MT-CYB* haplotype, the alleles for *RAG1*, accession number for *MT-CYB* and accession number for *RAG*.

Table S2. *Phoxinus* samples available from GenBank used in this study. Different references for each sample is also included in the table. The following information is reported in the table: the number of the sample, the clade to which it belongs *sensu* Palandačić *et al.* (2017) and the study from which it has been taken.

Table S3. Polymorphisms in the mitochondrial gene (*MT-CYB*) in *Phoxinus* populations from the Iberian Peninsula and southern France.

Table S4. Polymorphisms in the nuclear gene (*RAG1*) in *Phoxinus* populations from the Iberian Peninsula and southern France.

Table S5. Uncorrected p -distances between the phylogenetic clades are represented below the diagonal. Standard errors of the uncorrected p -distances are indicated above the diagonal.

Table S6. Values of Φ_{st} between localities. Only significant values after Bonferroni correction are shown. $\Phi_{st} < 0.3$ in red, $\Phi_{st} > 0.3$ but < 0.6 in orange, $\Phi_{st} > 0.6$ but < 0.8 in yellow and $\Phi_{st} > 0.80$ in green.

Table S1. This table corresponds to all individuals studied referencing in Table 1. The following information is include in the table: voucher number, species, river, locality, basin, biogeographical region, coordinates, N_{MT-CYB} (marked with an “X” if the individual has been sequenced for $MT-CYB$), N_{RAG1} (marked with an “X” if the individual has been sequenced for $RAG1$), the $MT-CYB$ haplotype, the alleles for $RAG1$, accession number for $MT-CYB$ and accession number for RAG .

MNCN /ADN	Species	River	Locality	Basin	Biogeographical region	Coordinates	N_{MT-CYB}	N_{RAG1}	CYTB haplotype	RAG1 Alleles	accession number cytb	accession number RAG1
103085	<i>P. septimaniae</i>	Bévère	Sospel	Bévère	East-Rhône	43.877708, 7.451893	X	X	Hap_78	Allele_16 and allele_16	MK787813	MK788099
103086	<i>Phoxinus sp.</i>	Garonne	Clarac	Garonne	Garonne	43.098089, 0.628983	X	X	Hap_45	Allele_34 and allele_34	MK787811	MK788097
103087	<i>Phoxinus sp.</i>	Garonne	Clarac	Garonne	Garonne	43.098089, 0.628983	X	X	Hap_76	Allele_35 and allele_35	MK787810	MK788096
103088	<i>Phoxinus sp.</i>	Garonne	Montespan	Garonne	Garonne	43.094564, 0.841614	X	X	Hap_75	Allele_14 and allele_14	MK787809	MK788095
103089	<i>Phoxinus sp.</i>	Garonne	Montespan	Garonne	Garonne	43.094564, 0.841614	X	X	Hap_75	Allele_36 and allele_36	MK787808	MK788094
103090	<i>P. septimaniae</i>	Hérault	Valleraugue	Hérault	Hérault	44.059093, 3.685357	X	X	Hap_77	Allele_37 and allele_37	MK787812	MK788098
58710	<i>P. septimaniae</i>	Perles	Canelles	Ebro	Lower Ebro	42.176203, 1.350161	X		Hap_52		MK787683	
102724	<i>P. bigerri</i>	Arlanza	Vilviestre del Pinar	Duero	Duero	41.969887, -3.080887	X	X	Hap_39	Allele_21 and allele_21	MK787618	MK787948
102725	<i>P. bigerri</i>	Arlanza	Vilviestre del Pinar	Duero	Duero	41.969887, -3.080887	X		Hap_39		MK787617	
102726	<i>P. bigerri</i>	Arlanza	Vilviestre del Pinar	Duero	Duero	41.969887, -3.080887	X	X	Hap_39	Allele_21 and allele_21	MK787616	MK787947
102727	<i>P. bigerri</i>	Arlanza	Vilviestre del Pinar	Duero	Duero	41.969887, -3.080887	X	X	Hap_39	Allele_21 and allele_21	MK787615	MK787946

102728	<i>P. bigerri</i>	Arlanza	Vilviestre del Pinar	Duero	Duero	41.969887, - 3.080887	X			Hap_40		MK787614	
102729	<i>P. bigerri</i>	Cares	Mier	Deva	Deva	43.319046, - 4.675282	X	X		Hap_1	Allele_19 and allele_19	MK787485	MK787851
102730	<i>P. bigerri</i>	Cares	Mier	Deva	Deva	43.319046, - 4.675282	X	X		Hap_1	Allele_20 and allele_20	MK787484	MK787850
102731	<i>P. bigerri</i>	Cares	Mier	Deva	Deva	43.319046, - 4.675282	X			Hap_1		MK787483	
102732	<i>P. bigerri</i>	Cares	Mier	Deva	Deva	43.319046, - 4.675282	X	X		Hap_1	Allele_19 and allele_19	MK787482	MK787849
102733	<i>P. bigerri</i>	Zardón	Cangas de Onís	Sella	Sella	43.394887, - 5.095277	X	X		Hap_1	Allele_19 and allele_19	MK787481	MK787848
102734	<i>P. bigerri</i>	Zardón	Cangas de Onís	Sella	Sella	43.394887, - 5.095277	X	X		Hap_4	Allele_19 and allele_75	MK787480	MK787847
102735	<i>P. bigerri</i>	Zardón	Cangas de Onís	Sella	Sella	43.394887, - 5.095277	X	X		Hap_3	Allele_19 and allele_75	MK787479	MK787846
102736	<i>P. bigerri</i>	Zardón	Cangas de Onís	Sella	Sella	43.394887, - 5.095277	X	X		Hap_1	Allele_19 and allele_19	MK787478	MK787845
102737	<i>P. bigerri</i>	Zardón	Cangas de Onís	Sella	Sella	43.394887, - 5.095277	X	X		Hap_1	Allele_19 and allele_19	MK787477	MK787844
102738	<i>P. bigerri</i>	Pigueña	Belmonte	Narcea	Narcea	43.3078, - 6.234761	X	X		Hap_1	Allele_19 and allele_65	MK787476	MK787843
102739	<i>P. bigerri</i>	Pigueña	Belmonte	Narcea	Narcea	43.3078, - 6.234761	X			Hap_1		MK787475	
102740	<i>P. bigerri</i>	Pigueña	Belmonte	Narcea	Narcea	43.3078, - 6.234761	X			Hap_1		MK787474	
102741	<i>P. bigerri</i>	Pigueña	Belmonte	Narcea	Narcea	43.3078, - 6.234761	X			Hap_2		MK787473	
102742	<i>P. bigerri</i>	Pigueña	Belmonte	Narcea	Narcea	43.3078, - 6.234761	X	X		Hap_1	Allele_19 and allele_19	MK787472	MK787842
102743	<i>P. bigerri</i>	Esva	Brieves	Esva	Esva	43.482687, - 6.442144	X			Hap_2		MK787471	

102744	<i>P. bigerri</i>	Esva	Brieves	Esva	Western Cantabria	43.482687, - 6.442144	X	X	Hap_2	Allele_19 and allele_19	MK787470	MK787841
102745	<i>P. bigerri</i>	Esva	Brieves	Esva	Western Cantabria	43.482687, - 6.442144	X	X	Hap_1	Allele_19 and allele_19	MK787469	MK787840
102746	<i>P. bigerri</i>	Esva	Brieves	Esva	Western Cantabria	43.482687, - 6.442144	X	X	Hap_1	Allele_19 and allele_19	MK787468	MK787839
102747	<i>P. bigerri</i>	Esva	Brieves	Esva	Western Cantabria	43.482687, - 6.442144	X	X	Hap_1	Allele_33 and allele_33	MK787467	MK787838
102748	<i>P. bigerri</i>	Agüera	Villaverde de Trucios	Agüera	Eastern Cantabria	43.260127, - 3.264195	X	X	Hap_19	Allele_1 and allele_1	MK787533	MK787891
102749	<i>P. bigerri</i>	Agüera	Villaverde de Trucios	Agüera	Eastern Cantabria	43.260127, - 3.264195	X	X	Hap_19	Allele_1 and allele_1	MK787532	MK787890
102750	<i>P. bigerri</i>	Agüera	Villaverde de Trucios	Agüera	Eastern Cantabria	43.260127, - 3.264195	X	X	Hap_19	Allele_1 and allele_1	MK787531	MK787889
102751	<i>P. bigerri</i>	Agüera	Villaverde de Trucios	Agüera	Eastern Cantabria	43.260127, - 3.264195	X		Hap_19		MK787530	
102752	<i>P. bigerri</i>	Agüera	Villaverde de Trucios	Agüera	Eastern Cantabria	43.260127, - 3.264195	X		Hap_19		MK787529	
102753	<i>P. bigerri</i>	Asón	Udalla	Asón	Eastern Cantabria	43.315162, - 3.447179	X	X	Hap_18	Allele_8 and allele_8	MK787528	MK787888
102754	<i>P. bigerri</i>	Asón	Udalla	Asón	Eastern Cantabria	43.315162, - 3.447179	X		Hap_18		MK787527	
102755	<i>P. bigerri</i>	Asón	Udalla	Asón	Eastern Cantabria	43.315162, - 3.447179	X	X	Hap_17	Allele_8 and allele_8	MK787526	MK787887
102756	<i>P. bigerri</i>	Asón	Udalla	Asón	Eastern Cantabria	43.315162, - 3.447179	X		Hap_16		MK787525	
102757	<i>P. bigerri</i>	Asón	Udalla	Asón	Eastern Cantabria	43.315162, - 3.447179	X		Hap_15		MK787524	
102758	<i>P. bigerri</i>	Pas	Entrasbasmest as	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_14	Allele_75 and allele_75	MK787523	MK787886

102759	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_11	Allele_75 and allele_75	MK787522	MK787885
102760	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_13	Allele_75 and allele_75	MK787521	MK787884
102761	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X		Hap_7		MK787520	
102762	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_12	Allele_75 and allele_8	MK787519	MK787883
102763	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_10	Allele_75 and allele_75	MK787518	MK787882
102764	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_10	Allele_75 and allele_8	MK787517	MK787881
102765	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_7	Allele_75 and allele_75	MK787516	MK787880
102766	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_7	Allele_75 and allele_76	MK787515	MK787879
102767	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X		Hap_10		MK787514	
102768	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_12	Allele_75 and allele_75	MK787513	MK787878
102769	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_10	Allele_75 and allele_75	MK787512	MK787877
102770	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_11	Allele_75 and allele_75	MK787511	MK787876
102771	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_10	Allele_75 and allele_77	MK787510	MK787875
102772	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_9	Allele_75 and allele_75	MK787509	MK787874
102773	<i>P. bigerri</i>	Pas	Entrasbasmes tas	Pas	Western Cantabria	43.171297, - 3.882203	X	X	Hap_8	Allele_75 and allele_75	MK787508	MK787873

102774	<i>P. bigerri</i>	Pas	Entrasbasmest as	Pas	Western Cantabria	43.171297, - 3.882203	X	X	X	Hap_7	Allele_75 and allele_75	MK787507	MK787872
102775	<i>P. bigerri</i>	Pas	Entrasbasmest as	Pas	Western Cantabria	43.171297, - 3.882203	X	X	X	Hap_7	Allele_75 and allele_75	MK787506	MK787871
102776	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787505	MK787870
102777	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_1	Allele_19 and allele_19	MK787504	MK787869
102778	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_64	MK787503	MK787868
102779	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787502	MK787867
102780	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_6	Allele_19 and allele_19	MK787501	MK787866
102781	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_20 and allele_20	MK787500	MK787865
102782	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787499	MK787864
102783	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787498	MK787863
102784	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787497	MK787862
102785	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X		Hap_1		MK787496	
102786	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_1	Allele_19 and allele_19	MK787495	MK787861
102787	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787494	MK787860
102788	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, - 4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787493	MK787859

102789	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_1	Allele_19 and allele_19	MK787492	MK787858
102790	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787491	MK787857
102791	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_5	Allele_19 and allele_64	MK787490	MK787856
102792	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787489	MK787855
102793	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787488	MK787854
102794	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_5	Allele_19 and allele_19	MK787487	MK787853
102795	<i>P. bigerri</i>	Nansa	Muñorodero	Nansa	Western Cantabria	43.35378, -4.493688	X	X	X	Hap_1	Allele_19 and allele_19	MK787486	MK787852
102796	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_10 and allele_96	MK787785	MK788072
102797	<i>Phoxinus sp.</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_56	Allele_97 and allele_98	MK787784	MK788071
102798	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_100 and allele_99	MK787783	MK788070
102799	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_101 and allele_11	MK787782	MK788069
102800	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_102 and allele_102	MK787781	MK788068
102801	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X		Hap_52		MK787780	
102802	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_38	Allele_103 and allele_74	MK787779	MK788067
102803	<i>Phoxinus sp.</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_74	Allele_34 and allele_48	MK787778	MK788066
102804	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_38	Allele_104 and allele_2	MK787777	MK788065
102805	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_71	Allele_74 and allele_74	MK787776	MK788064

102806	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_38	Allele_48 and allele_48	MK787775	MK788063
102807	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_38	Allele_103 and allele_105	MK787774	MK788062
102808	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_73	Allele_106 and allele_107	MK787773	MK788061
102809	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_48 and allele_95	MK787772	MK788060
102810	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_71	Allele_10 and allele_97	MK787771	MK788059
102811	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_108 and allele_109	MK787770	MK788058
102812	<i>Phoxinus sp.</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_56	Allele_79 and allele_82	MK787769	MK788057
102813	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.190373, 2.197644	X	X	X	Hap_52	Allele_110 and allele_111	MK787768	MK788056
102814	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_56	Allele_12 and allele_12	MK787743	MK788034
102815	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_52	Allele_12 and allele_38	MK787742	MK788033
102816	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_59	Allele_39 and allele_39	MK787741	MK788032
102817	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_52	Allele_12 and allele_40	MK787740	MK788031
102818	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_52	Allele_41 and allele_41	MK787739	MK788030
102819	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_56	Allele_42 and allele_43	MK787738	MK788029
102820	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_59	Allele_44 and allele_45	MK787737	MK788028
102821	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_52	Allele_46 and allele_47	MK787736	MK788027
102822	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X		Hap_52		MK787735	
102823	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	X	Hap_52	Allele_14 and allele_48	MK787734	MK788026

102824	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_52		MK787733	
102825	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	Hap_59	Allele_12 and allele_49	MK787732	MK788025
102826	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_52		MK787731	
102827	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_52		MK787730	
102828	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	Hap_52	Allele_50 and allele_50	MK787729	MK788024
102829	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	Hap_52	Allele_51 and allele_52	MK787728	MK788023
102830	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_59		MK787727	
102831	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X	X	Hap_52	Allele_14 and allele_14	MK787726	MK788022
102832	<i>P. septimaniae</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_52		MK787725	
102833	<i>Phoxinus sp.</i>	Llobrega t	La Rabeia	Llobregat	Catalonia	41.880224, 1.88945	X		Hap_59		MK787724	
102834	<i>P. bigerri</i>	Garona	Yeste	Ebro	Upper Ebro	42.385072, - 0.672432	X		Hap_39		MK787666	
102835	<i>P. bigerri</i>	Garona	Yeste	Ebro	Upper Ebro	42.385072, - 0.672432	X		Hap_33		MK787665	
102836	<i>P. bigerri</i>	Garona	Yeste	Ebro	Upper Ebro	42.385072, - 0.672432	X	X	Hap_33	Allele_2 and allele_9	MK787664	MK787977
102837	<i>P. bigerri</i>	Garona	Yeste	Ebro	Upper Ebro	42.385072, - 0.672432	X	X	Hap_33	Allele_24 and allele_6	MK787663	MK787976
102838	<i>P. bigerri</i>	Garona	Yeste	Ebro	Upper Ebro	42.385072, - 0.672432	X	X	Hap_39	Allele_21 and allele_21	MK787662	MK787975
102839	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	Hap_33	Allele_9 and allele_9	MK787661	MK787974
102840	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	Hap_33	Allele_9 and allele_9	MK787660	MK787973
102841	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X		Hap_33		MK787659	

102842	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_2 and allele_21	MK787658	MK787972
102843	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_9 and allele_9	MK787657	MK787971
102844	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_39	Allele_21 and allele_25	MK787656	MK787970
102845	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_6 and allele_6	MK787655	MK787969
102846	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_39	Allele_21 and allele_21	MK787654	MK787968
102847	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X			Hap_33		MK787653	
102848	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_39	Allele_6 and allele_6	MK787652	MK787967
102849	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_9 and allele_9	MK787651	MK787966
102850	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_26 and allele_27	MK787650	MK787965
102851	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_9 and allele_9	MK787649	MK787964
102852	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_9 and allele_9	MK787648	MK787963
102853	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_6 and allele_6	MK787647	MK787962
102854	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_28 and allele_28	MK787646	MK787961
102855	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_6 and allele_6	MK787645	MK787960
102856	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X	X	X	Hap_33	Allele_21 and allele_21	MK787644	MK787959
102857	<i>P. bigerri</i>	Aragón	Jaca	Ebro	Upper Ebro	42.557021, - 0.616949	X			Hap_33		MK787643	
102858	<i>P. bigerri</i>	Arga	Puente la Reina	Ebro	Upper Ebro	42.6708, - 2.154796	X			Hap_40		MK787642	
102859	<i>P. bigerri</i>	Arga	Puente la Reina	Ebro	Upper Ebro	42.6708, - 2.154796	X			Hap_40		MK787641	

102860	<i>P. bigerri</i>	Arga	Puente la Reina	Ebro	Upper Ebro	42.6708, - 2.154796	X	X	X	Hap_33	Allele_21 and allele_21	MK787640	MK787958
102861	<i>P. bigerri</i>	Arga	Puente la Reina	Ebro	Upper Ebro	42.6708, - 2.154796	X	X	X	Hap_43	Allele_6 and allele_6	MK787639	MK787957
102862	<i>P. bigerri</i>	Arga	Puente la Reina	Ebro	Upper Ebro	42.6708, - 2.154796	X			Hap_42		MK787638	
102863	<i>P. bigerri</i>	Aragón	Marcilla	Ebro	Upper Ebro	42.320789, - 1.729991	X	X	X	Hap_33	Allele_21 and allele_21	MK787637	MK787956
102864	<i>P. bigerri</i>	Aragón	Marcilla	Ebro	Upper Ebro	42.320789, - 1.729991	X	X	X	Hap_33	Allele_29 and allele_30	MK787636	MK787955
102865	<i>P. bigerri</i>	Aragón	Marcilla	Ebro	Upper Ebro	42.320789, - 1.729991	X	X	X	Hap_40	Allele_2 and allele_21	MK787635	MK787954
102866	<i>P. bigerri</i>	Aragón	Marcilla	Ebro	Upper Ebro	42.320789, - 1.729991	X	X	X	Hap_40	Allele_21 and allele_31	MK787634	MK787953
102867	<i>P. bigerri</i>	Aragón	Marcilla	Ebro	Upper Ebro	42.320789, - 1.729991	X	X	X	Hap_39	Allele_9 and allele_9	MK787633	MK787952
53678	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X			Hap_35		MK787590	
53679	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_2 and allele_8	MK787589	MK787929
53680	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X			Hap_34		MK787588	
102868	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_6 and allele_6	MK787587	MK787928
102869	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_23 and allele_23	MK787586	MK787927
102870	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X			Hap_36		MK787585	
102871	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_6 and allele_6	MK787584	MK787926
102872	<i>P. bigerri</i>	Ugarana	Dantxarinea	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X			Hap_33		MK787583	

102873	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_23 and allele_23	MK787582	MK787925
102874	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_23 and allele_23	MK787581	MK787924
102875	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_114 and allele_115	MK787580	MK787923
102876	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_34	Allele_2 and allele_9	MK787579	MK787922
102877	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_6 and allele_6	MK787578	MK787921
102878	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_35	Allele_2 and allele_23	MK787577	MK787920
102879	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X	X	Hap_34	Allele_23 and allele_6	MK787576	MK787919
102880	<i>P. bigerri</i>	Ugarana	Dantxarina	Nivelle	Eastern Cantabria	43.290884, - 1.505634	X	X		Hap_33		MK787575	
102881	<i>P. bigerri</i>	Baztan	Arbayoz	Bidasoa	Eastern Cantabria	43.14438, - 1.563267	X	X		Hap_35		MK787594	
102882	<i>P. bigerri</i>	Baztan	Arbayoz	Bidasoa	Eastern Cantabria	43.14438, - 1.563267	X	X		Hap_37		MK787593	
102883	<i>P. bigerri</i>	Baztan	Arbayoz	Bidasoa	Eastern Cantabria	43.14438, - 1.563267	X	X		Hap_35		MK787592	
102884	<i>P. bigerri</i>	Baztan	Arbayoz	Bidasoa	Eastern Cantabria	43.14438, - 1.563267	X	X		Hap_33		MK787591	
102885	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X		Hap_31		MK787569	
102886	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	X	Hap_28	Allele_2 and allele_2	MK787568	MK787917
102887	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X		Hap_28		MK787567	

102888	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X		Hap_27		MK787566	
102889	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X		Hap_28		MK787565	
102890	<i>P. bigerri</i>	Oka	Mugica	Oka	Eastern Cantabria	43.261569, - 2.684819	X	X	Hap_20	Allele_1 and allele_17	MK787574	MK787918
102891	<i>P. bigerri</i>	Oka	Mugica	Oka	Eastern Cantabria	43.261569, - 2.684819	X		Hap_32		MK787573	
102892	<i>P. bigerri</i>	Oka	Mugica	Oka	Eastern Cantabria	43.261569, - 2.684819	X		Hap_32		MK787572	
102893	<i>P. bigerri</i>	Oka	Mugica	Oka	Eastern Cantabria	43.261569, - 2.684819	X		Hap_20		MK787571	
102894	<i>P. bigerri</i>	Oka	Mugica	Oka	Eastern Cantabria	43.261569, - 2.684819	X		Hap_24		MK787570	
102895	<i>P. bigerri</i>	Butrón	Fruiz	Butrón	Eastern Cantabria	43.327042, - 2.783595	X	X	Hap_24	Allele_17 and allele_18	MK787547	MK787900
102896	<i>P. bigerri</i>	Butrón	Fruiz	Butrón	Eastern Cantabria	43.327042, - 2.783595	X	X	Hap_26	Allele_17 and allele_18	MK787546	MK787899
102897	<i>P. bigerri</i>	Butrón	Fruiz	Butrón	Eastern Cantabria	43.327042, - 2.783595	X	X	Hap_25	Allele_1 and allele_1	MK787545	MK787898
102898	<i>P. bigerri</i>	Butrón	Fruiz	Butrón	Eastern Cantabria	43.327042, - 2.783595	X		Hap_24		MK787544	
102899	<i>P. bigerri</i>	Butrón	Fruiz	Butrón	Eastern Cantabria	43.327042, - 2.783595	X		Hap_23		MK787543	
102900	<i>P. bigerri</i>	Barbadún n	Muskiz	Barbadún	Eastern Cantabria	43.31509, - 3.120076	X		Hap_20		MK787538	
102901	<i>P. bigerri</i>	Barbadún n	Muskiz	Barbadún	Eastern Cantabria	43.31509, - 3.120076	X	X	Hap_20	Allele_1 and allele_1	MK787537	MK787895
102902	<i>P. bigerri</i>	Barbadún n	Muskiz	Barbadún	Eastern Cantabria	43.31509, - 3.120076	X	X	Hap_20	Allele_9 and allele_9	MK787536	MK787894

102903	<i>P. bigerri</i>	Barbadú n	Muskiz	Barbadún	Eastern Cantabria	43.31509, - 3.120076	X	X	X	Hap_20	Allele_1 and allele_1	MK787535	MK787893
102904	<i>P. bigerri</i>	Barbadú n	Muskiz	Barbadún	Eastern Cantabria	43.31509, - 3.120076	X	X	X	Hap_20	Allele_1 and allele_1	MK787534	MK787892
102905	<i>P. bigerri</i>	Altube	Ziorraga	Nervión	Eastern Cantabria	43.02603, - 2.913997	X	X		Hap_22		MK787542	
102906	<i>P. bigerri</i>	Altube	Ziorraga	Nervión	Eastern Cantabria	43.02603, - 2.913997	X	X	X	Hap_20	Allele_1 and allele_1	MK787541	MK787897
102907	<i>P. bigerri</i>	Altube	Ziorraga	Nervión	Eastern Cantabria	43.02603, - 2.913997	X	X		Hap_21		MK787540	
102908	<i>P. bigerri</i>	Altube	Ziorraga	Nervión	Eastern Cantabria	43.02603, - 2.913997	X	X	X	Hap_20	Allele_66 and allele_67	MK787539	MK787896
102909	<i>P. bigerri</i>	Ebro	La Puebla de Labarca	Ebro	Upper Ebro	42.495421, - 2.565839	X	X		Hap_39		MK787632	
102910	<i>P. bigerri</i>	Ebro	La Puebla de Labarca	Ebro	Upper Ebro	42.495421, - 2.565839	X	X	X	Hap_39	Allele_9 and allele_9	MK787631	MK787951
102911	<i>P. bigerri</i>	Ebro	La Puebla de Labarca	Ebro	Upper Ebro	42.495421, - 2.565839	X	X		Hap_39		MK787630	
102912	<i>P. bigerri</i>	Ebro	La Puebla de Labarca	Ebro	Upper Ebro	42.495421, - 2.565839	X	X		Hap_39		MK787629	
102913	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_39	Allele_22 and allele_9	MK787613	MK787945
102914	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X		Hap_39		MK787612	
102915	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_39	Allele_23 and allele_9	MK787611	MK787944
102916	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_40	Allele_9 and allele_9	MK787610	MK787943
102917	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_39	Allele_9 and allele_9	MK787609	MK787942
102918	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_40	Allele_21 and allele_21	MK787608	MK787941
102919	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, - 3.362377	X	X	X	Hap_39	Allele_21 and allele_21	MK787607	MK787940

102920	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_21 and allele_21	MK787606	MK787939
102921	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_21 and allele_21	MK787605	MK787938
102922	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_21 and allele_21	MK787604	MK787937
102923	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_23 and allele_9	MK787603	MK787936
102924	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_23 and allele_23	MK787602	MK787935
102925	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_22 and allele_9	MK787601	MK787934
102926	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_9 and allele_9	MK787600	MK787933
102927	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X		Hap_39		MK787599	
102928	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_23 and allele_9	MK787598	MK787932
102929	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X		Hap_39		MK787597	
102930	<i>P. bigerri</i>	Pedroso	Pinilla de los Moros	Duero	Duero	42.049332, -3.362377	X	X	Hap_39	Allele_23 and allele_9	MK787596	MK787931
102931	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_27	Allele_2 and allele_3	MK787564	MK787916
102932	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_27	Allele_4 and allele_4	MK787563	MK787915
102933	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_28	Allele_2 and allele_5	MK787562	MK787914
102934	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_30	Allele_6 and allele_6	MK787561	MK787913
102935	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_28	Allele_2 and allele_2	MK787560	MK787912
102936	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, -2.484336	X	X	Hap_27	Allele_2 and allele_2	MK787559	MK787911

102937	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_2 and allele_2	MK787558	MK787910
102938	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_30	Allele_2 and allele_3	MK787557	MK787909
102939	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_2 and allele_2	MK787556	MK787908
102940	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_3 and allele_7	MK787555	MK787907
102941	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_27	Allele_4 and allele_4	MK787554	MK787906
102942	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_2 and allele_2	MK787553	MK787905
102943	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_4 and allele_4	MK787552	MK787904
102944	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_29	Allele_2 and allele_3	MK787551	MK787903
102945	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X		Hap_27		MK787550	
102946	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_28	Allele_2 and allele_2	MK787549	MK787902
102947	<i>P. bigerri</i>	Artibai	Amelloa	Artibai	Eastern Cantabria	43.293983, - 2.484336	X	X	Hap_27	Allele_2 and allele_2	MK787548	MK787901
102948	<i>P. bigerri</i>	Baixas	Zuazo de Cuartango	Ebro	Upper Ebro	42.923299, - 2.901789	X	X	Hap_33		MK787628	
102949	<i>P. bigerri</i>	Baixas	Zuazo de Cuartango	Ebro	Upper Ebro	42.923299, - 2.901789	X	X	Hap_41	Allele_1 and allele_1	MK787627	MK787950
102950	<i>P. bigerri</i>	Baixas	Zuazo de Cuartango	Ebro	Upper Ebro	42.923299, - 2.901789	X	X	Hap_41		MK787626	
102951	<i>P. bigerri</i>	Baixas	Zuazo de Cuartango	Ebro	Upper Ebro	42.923299, - 2.901789	X	X	Hap_41		MK787625	
102952	<i>P. bigerri</i>	Baixas	Zuazo de Cuartango	Ebro	Upper Ebro	42.923299, - 2.901789	X	X	Hap_41	Allele_1 and allele_32	MK787624	MK787949

102953	<i>P. bigerri</i>	Tirón	Herramelluri	Ebro	Upper Ebro	42.505554, - 3.020071	X		Hap_40		MK787623	
102954	<i>P. bigerri</i>	Tirón	Herramelluri	Ebro	Upper Ebro	42.505554, - 3.020071	X		Hap_33		MK787622	
102955	<i>P. bigerri</i>	Tirón	Herramelluri	Ebro	Upper Ebro	42.505554, - 3.020071	X		Hap_33		MK787621	
102956	<i>P. bigerri</i>	Tirón	Herramelluri	Ebro	Upper Ebro	42.505554, - 3.020071	X		Hap_33		MK787620	
102957	<i>P. bigerri</i>	Tirón	Herramelluri	Ebro	Upper Ebro	42.505554, - 3.020071	X		Hap_33		MK787619	
102958	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X		Hap_68		MK787715	
102959	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X		Hap_52		MK787714	
102960	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X		Hap_52		MK787713	
102961	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_67	Allele_78 and allele_79	MK787712	MK788015
102962	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_66	Allele_12 and allele_48	MK787711	MK788014
102963	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_65	Allele_68 and allele_80	MK787710	MK788013
102964	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_52	Allele_11 and allele_81	MK787709	MK788012
102965	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X		Hap_52		MK787708	
102966	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X		Hap_52		MK787707	
102967	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_53	Allele_82 and allele_83	MK787706	MK788011
102968	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_54	Allele_84 and allele_84	MK787705	MK788010
102969	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_52	Allele_85 and allele_86	MK787704	MK788009
102970	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	Hap_56	Allele_87 and allele_87	MK787703	MK788008

102971	<i>P. septimaniae</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	X	Hap_64	Allele_6 and allele_83	MK787702	MK788007
102972	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	X	Hap_63	Allele_88 and allele_89	MK787701	MK788006
102973	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	X	Hap_56	Allele_48 and allele_82	MK787700	MK788005
102974	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	X	Hap_56	Allele_85 and allele_90	MK787699	MK788004
102975	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X			Hap_62		MK787698	
102976	<i>Phoxinus sp.</i>	Segre	Alás	Ebro	Lower Ebro	42.357702, 1.505714	X	X	X	Hap_61	Allele_91 and allele_92	MK787697	MK788003
102977	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X			Hap_69		MK787723	
102978	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_68	Allele_53 and allele_53	MK787722	MK788021
102979	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_56	Allele_54 and allele_55	MK787833	MK788117
102980	<i>P. septimaniae</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X			Hap_52		MK787721	
102981	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_56	Allele_48 and allele_54	MK787720	MK788020
102982	<i>P. septimaniae</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_52	Allele_12 and allele_14	MK787719	MK788019
102983	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_56	Allele_53 and allele_53	MK787718	MK788018
102984	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_56	Allele_53 and allele_56	MK787717	MK788017
102985	<i>Phoxinus sp.</i>	Llobrega t	Guardiola de Berguedà	Llobregat	Catalonia	42.215892, 1.869982	X	X	X	Hap_56	Allele_48 and allele_48	MK787716	MK788016
102986	<i>Phoxinus sp.</i>	Noguera de Tor	Castelló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	X	Hap_48	Allele_68 and allele_73	MK787675	MK787986
102987	<i>Phoxinus sp.</i>	Noguera de Tor	Castelló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	X	Hap_48	Allele_70 and allele_72	MK787674	MK787985
102988	<i>Phoxinus sp.</i>	Noguera de Tor	Castelló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	X	Hap_49	Allele_48 and allele_6	MK787673	MK787984

102989	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_48	Allele_14 and allele_48	MK787672	MK787983
102990	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_48	Allele_48 and allele_74	MK787671	MK787982
102991	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_47	Allele_14 and allele_70	MK787670	MK787981
102992	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_46	Allele_48 and allele_70	MK787669	MK787980
102993	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_45	Allele_14 and allele_48	MK787668	MK787979
102994	<i>Phoxinus sp.</i>	Noguera de Tor	Castilló de Tor	Ebro	Lower Ebro	42.432648, 0.743125	X	X	Hap_44	Allele_48 and allele_74	MK787667	MK787978
102995	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X		Hap_45		MK787682	
102996	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X	X	Hap_45	Allele_68 and allele_69	MK787681	MK787991
102997	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X	X	Hap_50	Allele_70 and allele_70	MK787680	MK787990
102998	<i>P. septimanie</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X	X	Hap_52	Allele_48 and allele_71	MK787679	MK787989
102999	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X		Hap_51		MK787678	
103000	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X	X	Hap_50	Allele_14 and allele_14	MK787677	MK787988
103001	<i>Phoxinus sp.</i>	Noguera Pallaresa	Sossís	Ebro	Lower Ebro	42.270737, 1.028744	X	X	Hap_50	Allele_48 and allele_72	MK787676	MK787987
103002	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X		Hap_57		MK787696	
103003	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	Hap_56	Allele_48 and allele_48	MK787695	MK788002
103004	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	Hap_59	Allele_48 and allele_68	MK787694	MK788001
103005	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	Hap_60	Allele_34 and allele_93	MK787693	MK788000
103006	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	Hap_59	Allele_68 and allele_94	MK787692	MK787999

103007	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	X	Hap_58	Allele_12 and allele_14	MK787691	MK787998
103008	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	X	Hap_57	Allele_12 and allele_15	MK787690	MK787997
103009	<i>Phoxinus sp.</i>	Segre	Bellver de Cerdanya	Ebro	Lower Ebro	42.365294, 1.74702	X	X	X	Hap_55	Allele_48 and allele_68	MK787689	MK787996
103010	<i>P. septimaniae</i>	Segre	Organyà	Ebro	Lower Ebro	42.202986, 1.332979	X	X	X	Hap_52	Allele_12 and allele_55	MK787688	MK787995
103011	<i>Phoxinus sp.</i>	Segre	Organyà	Ebro	Lower Ebro	42.202986, 1.332979	X	X	X	Hap_55	Allele_55 and allele_58	MK787687	MK787994
103012	<i>Phoxinus sp.</i>	Segre	Organyà	Ebro	Lower Ebro	42.202986, 1.332979	X	X	X	Hap_54	Allele_48 and allele_95	MK787686	MK787993
103013	<i>Phoxinus sp.</i>	Segre	Organyà	Ebro	Lower Ebro	42.202986, 1.332979	X			Hap_54		MK787685	
103014	<i>Phoxinus sp.</i>	Segre	Organyà	Ebro	Lower Ebro	42.202986, 1.332979	X	X	X	Hap_53	Allele_11 and allele_53	MK787684	MK787992
103015	<i>P. septimaniae</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X	X	X	Hap_52	Allele_48 and allele_55	MK787767	MK788055
103016	<i>Phoxinus sp.</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X	X	X	Hap_56	Allele_14 and allele_72	MK787766	MK788054
103017	<i>P. septimaniae</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X	X	X	Hap_72	Allele_12 and allele_53	MK787765	MK788053
103018	<i>P. septimaniae</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X	X	X	Hap_52	Allele_48 and allele_54	MK787764	MK788052
103019	<i>P. septimaniae</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X	X	X	Hap_52	Allele_11 and allele_12	MK787763	MK788051
103020	<i>Phoxinus sp.</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X			Hap_62		MK787762	
103021	<i>Phoxinus sp.</i>	Gurri	Vich	Ter	Catalonia	41.983731, 2.289501	X			Hap_62		MK787761	
103022	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	X	Hap_52	Allele_54 and allele_68	MK787760	MK788050
103023	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	X	Hap_52	Allele_11 and allele_12	MK787759	MK788049
103024	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	X	Hap_71	Allele_12 and allele_79	MK787758	MK788048

103025	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X		Hap_71		MK787757	
103026	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	Hap_38	Allele_48 and allele_95	MK787756	MK788047
103027	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	Hap_52	Allele_11 and allele_12	MK787755	MK788046
103028	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	Hap_52	Allele_48 and allele_54	MK787754	MK788045
103029	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	Hap_38	Allele_11 and allele_72	MK787753	MK788044
103030	<i>P. septimaniae</i>	Ter	Ripoll	Ter	Catalonia	42.222724, 2.241401	X	X	Hap_52	Allele_11 and allele_11	MK787752	MK788043
103031	<i>P. septimaniae</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_38	Allele_48 and allele_48	MK787751	MK788042
103032	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_62	Allele_10 and allele_10	MK787750	MK788041
103033	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_70	Allele_48 and allele_48	MK787749	MK788040
103034	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_62	Allele_10 and allele_48	MK787748	MK788039
103035	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_70	Allele_112 and allele_72	MK787747	MK788038
103036	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_70	Allele_113 and allele_48	MK787746	MK788037
103037	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_70	Allele_10 and allele_48	MK787745	MK788036
103038	<i>Phoxinus sp.</i>	Vallfogona	Sant Bernabé de les Tenes	Ter	Catalonia	42.18738, 2.243628	X	X	Hap_70	Allele_10 and allele_48	MK787744	MK788035
103039	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787806	MK788093
103040	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787805	MK788092
103041	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787834	MK788118
103042	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787804	MK788091

103043	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787803	MK788090
103044	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787802	MK788089
103045	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_10	MK787801	MK788088
103046	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_11	MK787835	MK788119
103047	<i>P. septimaniae</i>	Cànoves	Cànoves	Besòs	Catalonia	41.655723, 2.35599	X	X	Hap_38	Allele_10 and allele_11	MK787836	MK788120
103048	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_11	MK787800	MK788087
103049	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_10	MK787799	MK788086
103050	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_10	MK787798	MK788085
103051	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_10	MK787837	MK788121
103052	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_10	MK787797	MK788084
103053	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_10	MK787796	MK788083
103054	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_11 and allele_11	MK787795	MK788082
103055	<i>P. septimaniae</i>	Tenes	Lliça d'Amunt	Besòs	Catalonia	41.624926, 2.243432	X	X	Hap_38	Allele_10 and allele_11	MK787794	MK788081
103056	<i>Phoxinus sp.</i>	Avençó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_12	MK787793	MK788080
103057	<i>Phoxinus sp.</i>	Avençó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_13	MK787792	MK788079
103058	<i>Phoxinus sp.</i>	Avençó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_12	MK787791	MK788078
103059	<i>Phoxinus sp.</i>	Avençó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_13	MK787790	MK788077
103060	<i>Phoxinus sp.</i>	Avençó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_13	MK787789	MK788076

103061	<i>Phoxinus sp.</i>	Avencó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_14	MK787788	MK788075
103062	<i>Phoxinus sp.</i>	Avencó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_15	MK787787	MK788074
103063	<i>Phoxinus sp.</i>	Avencó	Aiguafreda	Besòs	Catalonia	41.765248, 2.263007	X	X	Hap_62	Allele_12 and allele_13	MK787786	MK788073
103064	<i>P. septimaniae</i>	Muga	Albanyà	Muga	Catalonia	42.309618, 2.704398	X	X	Hap_38	Allele_10 and allele_10	MK787824	MK788108
103065	<i>P. septimaniae</i>	Muga	Albanyà	Muga	Catalonia	42.309618, 2.704398	X	X	Hap_38	Allele_10 and allele_11	MK787825	MK788109
103066	<i>P. septimaniae</i>	Muga	Albanyà	Muga	Catalonia	42.309618, 2.704398	X	X	Hap_38	Allele_10 and allele_11	MK787826	MK788110
103067	<i>P. septimaniae</i>	Muga	Albanyà	Muga	Catalonia	42.309618, 2.704398	X	X	Hap_38	Allele_10 and allele_61	MK787827	MK788111
103068	<i>P. septimaniae</i>	Muga	Albanyà	Muga	Catalonia	42.309618, 2.704398	X	X	Hap_38	Allele_10 and allele_11	MK787828	MK788112
103069	<i>P. septimaniae</i>	Arnera	Maçanet de Cabrenys	Muga	Catalonia	42.37554, 2.748278	X	X	Hap_38	Allele_11 and allele_62	MK787829	MK788113
103070	<i>P. septimaniae</i>	Arnera	Maçanet de Cabrenys	Muga	Catalonia	42.37554, 2.748278	X	X	Hap_38	Allele_10 and allele_62	MK787830	MK788114
103071	<i>P. septimaniae</i>	Arnera	Maçanet de Cabrenys	Muga	Catalonia	42.37554, 2.748278	X	X	Hap_38	Allele_62 and allele_62	MK787831	MK788115
103072	<i>P. septimaniae</i>	Arnera	Maçanet de Cabrenys	Muga	Catalonia	42.37554, 2.748278	X	X	Hap_38	Allele_10 and allele_11	MK787832	MK788116
103073	<i>Phoxinus sp.</i>	Cardener	Cardona	Llobregat	Catalonia	41.931773, 1.641066	X	X	Hap_67	Allele_13 and allele_48	MK787814	MK788100
103074	<i>Phoxinus sp.</i>	Cardener	Cardona	Llobregat	Catalonia	41.931773, 1.641066	X	X	Hap_67	Allele_13 and allele_57	MK787815	MK788101
103075	<i>Phoxinus sp.</i>	Cardener	Cardona	Llobregat	Catalonia	41.931773, 1.641066	X		Hap_57		MK787817	
103076	<i>Phoxinus sp.</i>	Cardener	Cardona	Llobregat	Catalonia	41.931773, 1.641066	X	X	Hap_67	Allele_12 and allele_54	MK787816	MK788102
103077	<i>Phoxinus sp.</i>	Aigua d'Ora	Llinàs del Vallès	Llobregat	Catalonia	42.113902, 1.709104	X	X	Hap_68	Allele_10 and allele_53	MK787818	MK788103
103078	<i>Phoxinus sp.</i>	Aigua d'Ora	Llinàs del Vallès	Llobregat	Catalonia	42.113902, 1.709104	X	X	Hap_53	Allele_53 and allele_58	MK787819	MK788104

103079	<i>P. septimaniae</i>	Aigua d'Ora	Llinás del Vallés	Llobregat	Catalonia	42.113902, 1.709104	X		Hap_38		MK787821	
103080	<i>Phoxinus sp.</i>	Aigua d'Ora	Llinás del Vallés	Llobregat	Catalonia	42.113902, 1.709104	X	X	Hap_53	Allele_48 and allele_59	MK787820	MK788105
103081	<i>P. septimaniae</i>	Aigua d'Ora	Llinás del Vallés	Llobregat	Catalonia	42.113902, 1.709104	X	X	Hap_52	Allele_10 and allele_60	MK787822	MK788106
103082	<i>P. septimaniae</i>	Aigua d'Ora	Llinás del Vallés	Llobregat	Catalonia	42.113902, 1.709104	X	X	Hap_38	Allele_12 and allele_53	MK787823	MK788107
103083	<i>P. septimaniae</i>	Gualba	Gualba de Dalt	Tordera	Catalonia	41.721804, 2.518209	X		Hap_38		MK787807	
103084	<i>P. septimaniae</i>	Gualba	Gualba de Dalt	Tordera	Catalonia	41.721804, 2.518209	X	X	Hap_38	Allele_10 and allele_10	MK787595	MK787930

Table S2. *Phoxinus* samples available from GenBank used in this study. Different references for each sample is also included in the table. The following information is reported in the table: the number of the sample, the clade to which it belongs *sensu* Palandačić *et al.* (2017) and the study from which it has been taken.

GenBank accession numbers	Clade <i>sensu</i> Palandačić <i>et al.</i> (2017)	Reference
KT166566	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166569	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166579	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166580	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166583	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166607	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166636	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166722	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166723	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166724	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166736	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166739	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166771	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166825	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166827	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166828	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166830	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166831	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166832	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166833	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166834	Clade 1a: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166595	Clade 1b: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166596	Clade 1b: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015
KT166597	Clade 1b: <i>Phoxinus lumaireul sensu</i> Palandačić <i>et al.</i> (2017)	Palandacic <i>et al.</i> 2015

KT166682	Clade 8: <i>Phoxinus</i> sp. 5 sensu Palandačić et al. (2017)	Palandacic et al. 2015
KT166683	Clade 8: <i>Phoxinus</i> sp. 5 sensu Palandačić et al. (2017)	Palandacic et al. 2015
KX673483	Clade 9a: <i>Phoxinus marsilii</i> sensu Palandačić et al. (2017)	Ramler et al. 2017
MF408190	Clade 9a: <i>Phoxinus marsilii</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408191	Clade 9a: <i>Phoxinus marsilii</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408192	Clade 9a: <i>Phoxinus marsilii</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408197	Clade 9a: <i>Phoxinus marsilii</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408215	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408216	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408217	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408218	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408219	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408220	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408221	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408222	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408223	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
Y10448	Clade 10: <i>Phoxinus phoxinus</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408209	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Briolay et al. 1998
MF408210	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408211	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408212	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408213	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
MF408214	Clade 10: <i>Phoxinus septimaniae</i> sensu Palandačić et al. (2017)	Palandacic et al. 2017
HM560122	Clade 13: <i>Phoxinus bigerri</i> sensu Palandačić et al. (2017)	Perea et al. 2010

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 Hap_68

Hap_57	G	C	C	G	T	T	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_58	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_59	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_60	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_61	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_62	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_63	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_64	.	C	C	G	T	C	C	A	.	.	.	A	T	.	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T
Hap_65	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_66	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_67	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_68	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_69	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_70	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_71	.	C	C	G	T	C	.	.	C	A	C	.	A	T	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T
Hap_72	.	C	C	G	T	C	.	.	C	A	C	.	A	T	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T
Hap_73	.	C	C	G	T	C	.	.	C	A	C	.	A	T	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T
Hap_74	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_75	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_76	G	C	C	G	T	.	.	.	C	.	.	A	T	.	.	.	.	C	.	.	A	C	.	G	.	G	.	T	.	.	A	G	.	.
Hap_77	.	C	C	G	T	C	.	.	C	A	C	.	A	T	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T
Hap_78	.	C	C	G	T	.	.	.	C	A	C	.	A	T	.	.	.	G	.	.	T	A	.	T	.	.	.	.	.	C	.	.	.	T

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Haplotype	T	G	A	A	C	G	A	T	T	A	C	G	A	C	C	T	A	G	G	A	C	T	G	C	A	C	G	A	C	G	A	C	G	A	C
Hap_1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Hap_6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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Hap_44	C	.	.	T	T	A	.	C	C	.	A	A	T	G	.	A	G	.	A	.	.	.	G
Hap_45	C	.	.	T	T	A	.	C	C	.	T	A	.	G	.	A	G	.	A	.	.	.	G
Hap_46	C	.	.	T	T	A	.	C	C	.	T	A	.	.	G	.	A	G	.	A	T	.	G
Hap_47	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	T	.	G
Hap_48	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_49	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_50	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_51	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_52	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_53	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_54	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_55	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_56	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_57	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_58	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	.	G	.	A	.	.	G
Hap_59	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_60	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_61	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_62	C	.	.	T	T	A	.	C	C	.	T	A	.	.	G	.	A	G	.	A	.	.	G
Hap_63	C	.	.	T	T	A	.	C	C	.	T	A	.	.	G	.	A	G	.	A	.	.	G
Hap_64	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_65	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	.	G	.	.	.	G
Hap_66	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	.
Hap_67	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_68	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	.	G	.	A	.	.	G
Hap_69	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_70	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	.	G	.	A	.	.	G
Hap_71	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_72	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_73	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_74	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_75	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_76	C	.	.	T	T	A	.	C	C	.	T	A	.	.	.	G	A	G	.	A	.	.	G
Hap_77	.	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.
Hap_78	C	.	.	.	.	A	.	C	G	.	A	A	.	T	.	.	A	.	.	A	.	A	.

Table S4. Polymorphisms in the nuclear gene (*RAG1*) in *Phoxinus* populations from the Iberian Peninsula and southern France.

[illegible]

Table S5. Uncorrected *p*-distances between the phylogenetic clades are represented below the diagonal. Standard errors of the uncorrected *p*-distances are indicated above the diagonal.

	<i>P. bigerri</i> from western Cantabrian basins	<i>P. bigerri</i> from eastern Cantabrian basins	<i>P. bigerri</i> from Artibai Basin	<i>P. septimaniae</i> from Iberian Peninsula	<i>Phoxinus sp.</i>	<i>P. septimaniae</i> from East-Rhône	<i>P. phoxinus</i>
<i>P. bigerri</i> from western Cantabrian basins	-	0.004	0.004	0.009	0.009	0.009	0.009
<i>P. bigerri</i> from western Cantabrian basins	0.022	-	0.003	0.009	0.008	0.008	0.009
<i>P. bigerri</i> from Artibai Basin	0.02	0.013	-	0.009	0.008	0.008	0.009
<i>P. septimaniae</i> from Iberian Peninsula	0.078	0.077	0.072	-	0.009	0.003	0.009
<i>Phoxinus sp.</i>	0.077	0.069	0.067	0.092	-	0.009	0.009
<i>P. septimaniae</i> from East-Rhône	0.074	0.07	0.068	0.018	0.09	-	0.009
<i>P. phoxinus</i>	0.081	0.076	0.075	0.076	0.079	0.074	-

***P. bigerri* from western Cantabrian basins:** Esva, Narcea, Sella, Deva, Nansa and Pas basins.

***P. bigerri* from eastern Cantabrian basins:** Asón, Agüera, Barbadún, Butrón, Oka, Nivelles, Bidasoa, Nervión, Duero and upper Ebro basins.

***P. bigerri* from Artibai Basin:** Artibai Basin.

***P. septimaniae* from Iberian Peninsula:** Lower Ebro (except Noguera de Tor), Hérault and Catalanian basins.

***Phoxinus sp.*:** Lower Ebro, Garonne and Catalanian (except Muga and Tordera) basins.

***P. septimaniae* from East-Rhône:** Rhône, Rhine, Po and Bévère basins.

***P. phoxinus*:** Agger-Rhine River

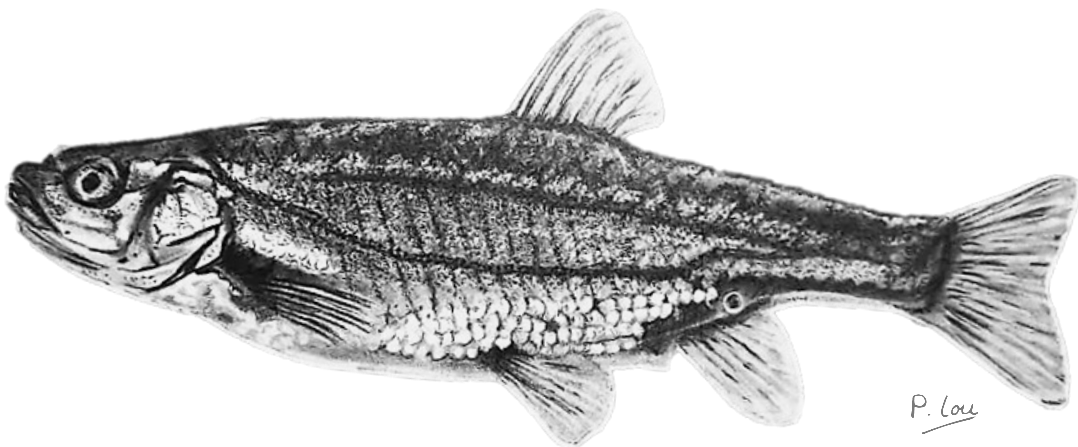
Table S6. Values of Φ_{st} between localities. Only significant values after Bonferroni correction are shown. $\Phi_{st} < 0.3$ in red, $\Phi_{st} > 0.3$ but < 0.6 in orange, $\Phi_{st} > 0.6$ but < 0.8 in yellow and $\Phi_{st} > 0.80$ in green.

	Esv	Nar	Sel	Car	Nan	Pas	Aso	Agu	Bar	Ner	Oka	Art	Uga	Bid	But	UpEbr	Not	Nop	Seg	Due	Llo	Ter	Bes	Tor	Mug
Esva																									
Narcea																									
Sella																									
Cares																									
Nansa	0.53																								
Pas	0.58	0.58	0.54	0.57	0.66																				
Asón					0.94	0.86																			
Agüera					0.95	0.86																			
Barbadún					0.95	0.86																			
Nervión					0.93	0.84																			
Oka					0.93	0.84																			
Artibai	0.92	0.92	0.92	0.92	0.92	0.87	0.88	0.91	0.9	0.88	0.87														
Ugarana	0.87	0.87	0.86	0.87	0.89	0.83	0.69	0.81	0.79	0.76	0.75	0.79													
Bidasoa					0.88	0.78						0.78													
Butrón					0.93	0.84						0.88	0.77												
Upper Ebro	0.89	0.89	0.88	0.89	0.89	0.87	0.74	0.81	0.79	0.77	0.77	0.8	0.46		0.75										
Noguera de Tor	0.95	0.95		0.94	0.96	0.94	0.94	0.94	0.94	0.93	0.93	0.96	0.94	0.91	0.93	0.96									
Noguera Pallaresa		0.78			0.89	0.86	0.76	0.77	0.77		0.76	0.88	0.84		0.75	0.91									
Segre	0.6	0.6	0.57	0.59	0.69	0.67	0.58	0.59	0.58	0.57	0.58	0.66	0.63	0.54	0.57	0.74									
Duero	0.98	0.99	0.99	0.99	0.96	0.91	0.94	0.98	0.98	0.96	0.94	0.91	0.63	0.78	0.93	0.17	0.97	0.9	0.68						
Llobregat	0.53	0.53	0.5	0.52	0.62	0.6	0.51	0.52	0.51	0.5	0.51	0.59	0.56	0.47	0.5	0.68				0.61					
Ter	0.57	0.57	0.55	0.56	0.65	0.64	0.57	0.58	0.57	0.56	0.57	0.63	0.61	0.54	0.56	0.71	0.53		0.26	0.66					
Besos	0.6	0.6	0.57	0.58	0.7	0.68	0.59	0.6	0.59	0.57	0.59	0.68	0.66		0.58	0.77	0.55			0.71					
Tordera																0.97			0.49						
Muga	1	1		1	0.99	0.96	0.99	1	1		0.99	0.98	0.97	0.97	0.99	0.97	0.97	0.84	0.59	1	0.38				
Garonne					0.98	0.95						0.97	0.96			0.97				0.99		0.49		1	

II

CAPÍTULO

Alta diferenciación genética en *Achondrostoma salmantinum* Doadrio y Elvira, 2007, un pez de agua dulce endémico y en peligro de extinción de España, revelado por un marcador mitocondrial y SNPs



Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585-600.



High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers

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Received: 1 May 2020 / Accepted: 25 May 2021 / Published online: 3 June 2021
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Abstract

Achondrostoma salmantinum is a small, endangered freshwater fish endemic to Spain. Although the species is a member of the widely distributed family Leuciscidae, its geographic range is restricted to a few rivers in the southwestern Duero Basin. Its populations are in decline, with some now extirpated from rivers within its historical distribution. A captive breeding program has already been implemented for the species; however, there are no population genetic studies on *A. salmantinum*, despite the importance of information on genetic variability and variation as fundamental tools for management and conservation efforts. Here, we assessed the genetic diversity of *A. salmantinum* and defined its Operational Conservation Units (OCUs). We sampled throughout the entire known distribution area of *A. salmantinum*, and analysed both nuclear and mitochondrial genes and 4123 single nucleotide polymorphisms (SNPs). Contrary to expectations, due to its small distribution area within the same basin, *A. salmantinum* showed signs of marked genetic erosion and a strong population structure that is not associated with the current hydrogeographic configuration of the region, but rather with historical geomorphological processes. On the basis of our genetic analyses, we identified four OCUs for the species: (I) Águeda Sub-basin; (II) Uces Sub-basin, Huebra River and the right-bank tributaries of Huebra Sub-basin; (III) left-bank tributaries of Huebra Sub-basin and (IV) Camaces River, a small left-bank tributary of the Huebra River. Overall, our findings provide insight on the population genetic structure of *A. salmantinum* and on some intrinsic and extrinsic factors that threaten the viability of the species.

Keywords Conservation · Cyprinid fishes · *Achondrostoma* · Iberian Peninsula · Conservation units · Genetic structure

Introduction

The identification of conservation units for a given species is a key element in conservation biology. These units help to guide management and conservation efforts when resources are limited (Fraser and Bernatchez 2001). Genetic data are used to establish differences among populations within species, which can be used to define conservation units (Doadrio et al. 1996; Knapen et al. 2003; Mandimbihasina et al. 2019). As a result, the genetic structure of

freshwater species has been widely studied for conservation purposes, since freshwater ecosystems are among the most threatened systems (Murphy et al. 2013; Faulks et al. 2017; Dudgeon 2019; Shao et al. 2019; Klunzinger et al. 2020). In this context, though, the degree of population differentiation in several freshwater species with restricted distributions has been underestimated, as these species present a stronger genetic structure than initially expected (e.g. Lima et al. 2017; Fluker et al. 2019; Alcaraz and Gholami 2020). In many cases, this differentiation can be linked to ancient isolation processes, highlighting the importance of the evolution of the aquatic system as a promoter of the current genetic structure of populations (Ramos-Fregonezi et al. 2017; Huey et al. 2017; Bartáková et al. 2018; Fluker et al. 2019). Knowing the population genetic structure of species with a restricted distribution area is important due to their vulnerability to anthropogenic and/or environmental changes, given their limited dispersal capacity and higher extinction risk compared with other freshwater species.

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These features make the study of endemic species a key element in conservation biology (Filipe et al. 2012).

Within the Iberian Peninsula, 80% of the native freshwater ichthyofauna is endemic (Doadrio et al. 2011), making it the most unique fish fauna in the Mediterranean region (Kottelat and Freyhof 2007; Doadrio et al. 2011). The high proportion of Iberian endemisms is associated with both the isolation of Iberian aquatic systems from those of Europe and the massive and complex hydrographic network of the region (Zardoya and Doadrio 1998; Levy et al. 2009). Two main biogeographic events have been identified as the most important for the structuring of the primary freshwater fauna in the Iberian Peninsula: the formation of endorheic lagoons during the Miocene and Pliocene, and the transformation of these endorheic lagoons into exorheic basins during the Plio-Pleistocene (Doadrio 1988; Doadrio and Carmona 2006; Robalo et al. 2006; Perea et al. 2016; Sousa-Santos et al. 2019). These events, along with the culmination of the formation of the current Iberian basins during the Upper Pliocene–Pleistocene, have led to the current diversity and high endemism of the Iberian ichthyofauna (Alonso et al. 2007; Perea et al. 2010; Pais et al. 2012; Perea et al. 2016).

This complex biogeographic history and the low dispersal capacity of many fishes have given rise to the existence of endemic species restricted to small regions within the Iberian Peninsula. For some of these species, their distribution is limited to a single hydrographic basin or even to a few rivers within a hydrographic basin. A notable case of an Iberian endemic with such a distribution is the species *Achondrostoma salmantinum* (Doadrio and Elvira 2007), which inhabits a small area of the Duero Basin within the province of Salamanca in western Spain (Doadrio and Elvira 2007). Speciation of *A. salmantinum* has been associated with the isolation of the ancient endorheic basin of Ciudad Rodrigo prior to the formation of the current Iberian hydrographic basins in which the species occurs, including the Huebra-Yeltes, Águeda and Uces sub-basins (Carmona et al. 2000; Doadrio and Elvira 2007). The distribution area of *A. salmantinum* encompasses less than 500 km², and a population decline has been detected in some rivers (Doadrio et al. 2011; Velasco et al. 2019). This negative trajectory is a consequence of several anthropogenic activities (Doadrio and Elvira 2007; Sousa-Santos et al. 2014). Moreover, due to the high level of water stress in the Mediterranean during the summer, the majority of the rivers inhabited by *A. salmantinum* become isolated pools during this season. In addition, it is likely that the course of these rivers are affected by activities related to water harvesting and agriculture. Indeed, this situation has already been observed in the Turones River in which the species has not been found since the end of the last century (Carmona et al. 2000; Doadrio et al. 2011). Consequently, *A. salmantinum* is currently in decline and is considered Endangered, under criteria B1ab(ii,v) + 2ab(ii,v),

on the IUCN Red List (<https://www.iucnredlist.org/species/184445/8277728>).

To alleviate the population decline, a captive breeding strategy has been developed for *A. salmantinum* (Velasco et al. 2019). However, plans for the release of captive-bred individuals have not considered the population structure and the genetic variability of the species. Rather all localities within the species distribution area are treated as a single population. However, a lack of population structure has not been demonstrated in *A. salmantinum*, despite its small distribution area. This situation highlights the importance of defining conservation units in order to plan the management of the species. Knowledge of the population genetic structure of a species is highly relevant for generating efficient management strategies. If these genetic factors are disregarded, it can lead to inappropriate recovery strategies and the potential loss of genetic distinctiveness within populations (Frankham et al. 2002; Flucker et al. 2010).

The main aim of this study is to describe the genetic structure of *A. salmantinum*, which is currently considered as a single homogeneous unit for management purposes, in order to identify and establish conservation units and to estimate parameters of genetic variability for the conservation units identified. To delimit conservation units, the mitochondrial marker cytochrome b (*MT-CYB*), the nuclear marker recombination activating gene 1 (*RAG1*) and single nucleotide polymorphism (SNP) markers obtained through next-generation sequencing were analysed. Knowledge of the genetic structure and diversity of populations of *A. salmantinum* will provide key information for the effective management and conservation of the species along its entire distribution area, and contribute to greater knowledge about diversification processes in freshwater microendemics.

Materials and methods

Sampling, DNA extraction, amplification and sequencing

A total of 171 individuals of *A. salmantinum* from 11 localities, covering the entire current distribution range of the species, was analysed in this study (Fig. 1; Table 1). In addition, we included samples collected in 1994 of the now extinct Turones river population (locality 10; Fig. 1; Table 1). Given that natural hybrids between *A. salmantinum* and *Pseudochondrostoma duriense* (Coelho 1985) are known for some populations, 15 individuals of *P. duriense* were also included in the study (Elvira et al. 1990; Table 1). Some specimens of *A. salmantinum* were collected by electrofishing, with permission from the relevant authorities. Tissue samples from the other specimens were obtained from the DNA and Tissue

Fig. 1 Map showing the sampling localities for *A. salmantinum* in the Iberian Peninsula. Numbers correspond to those used in Table 1. The purple shading delimits the Duero Basin, and the thick blue line represents the Duero River and red outline delimits the ancient endorheic basin of Ciudad Rodrigo. The sub-basins Uces, Huebra and Águeda are also indicated (see legend)

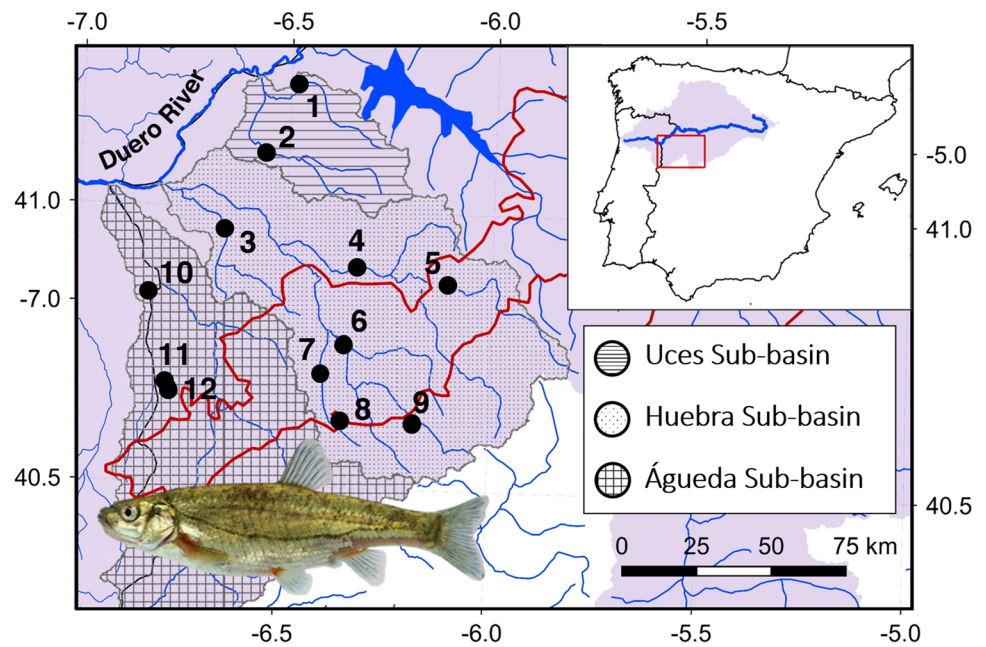


Table 1 Information about the sampling localities included in the present study

No.	Species	River	Basin	Sub-basin	Locality	MT-CYB	RAG1	SNPs
1	<i>A. salmantinum</i>	Cabeza de Iruelos	Duero	Uces	Cabeza de Framontanos (Salamanca)	15	8	10
2	<i>A. salmantinum</i>	Uces	Duero	Uces	Cabezas del Caballo (Salamanca)	14	14	9
3	<i>A. salmantinum</i>	Camaces	Duero	Huebra	Lumbrals (Salamanca)	21	21	5
4	<i>A. salmantinum</i>	Huebra	Duero	Huebra	El Cubo de Don Sancho (Salamanca)	19	17	9
5	<i>A. salmantinum</i>	Arganza	Duero	Huebra	Garcirrey (Salamanca)	21	20	–
6	<i>A. salmantinum</i>	Yeltes	Duero	Huebra	San Martín de Yeltes (Salamanca)	10	9	10
7	<i>A. salmantinum</i>	Gavilanes	Duero	Huebra	Sancti-Spiritus (Salamanca)	21	21	5
8	<i>A. salmantinum</i>	Tenebrilla	Duero	Huebra	Tenebrón (Salamanca)	5	5	–
9	<i>A. salmantinum</i>	Yeltes	Duero	Huebra	Puebla de Yeltes (Salamanca)	7	6	10
10	<i>A. salmantinum</i>	Turones	Duero	Águeda	La Bouza (Salamanca)	17	13	–
11	<i>A. salmantinum</i>	De dos Casas	Duero	Águeda	Alameda de Gardón (Salamanca)	20	20	16
12	<i>A. salmantinum</i>	De dos Casas	Duero	Águeda	Alameda de Gardón (Salamanca)	1	–	–
	<i>P. duriense</i>	Yeltes	Duero	Huebra	Martín de Yeltes (Salamanca)	11	7	–
	<i>P. duriense</i>	Águeda	Duero	Águeda	Castillejo de Martin Viejo (Salamanca)	1	–	–
	<i>P. duriense</i>	Mayas	Duero	Águeda	El Sahugo (Salamanca)	3	–	–

The number of sequenced individuals for the mitochondrial marker (*MT-CYB*), the nuclear marker (*RAG1*) and single nucleotide polymorphism markers (SNPs) is also indicated

Collection at the National Museum of Natural Sciences of Madrid (MNCN–CSIC).

For each individual, DNA was extracted from ventral fin tissue using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's protocol. Polymerase chain reaction (PCR) was used to amplify mitochondrial cytochrome b (*MT-CYB*) from 186 individuals (171 of *A. salmantinum* and 15 of *P. duriense*) and nuclear recombination activating gene 1 (*RAG1*) from 161 individuals (154 of *A. salmantinum*

and 7 of *P. duriense*). The protocol by Corral-Lou et al. (2019) was used to amplify both genes. PCR products were purified with ExoSAP-IT (USB Cleveland, OH, USA) and then sequenced on a 3730xl DNA Analyzer by MacroGen Europe Inc. (<http://www.macrogen.com>).

All sequences were aligned using MAFFT (Katoh and Standley 2013) implemented in Geneious 10.1.3 (<http://www.geneious.com>; Kearse et al. 2012) and then manually examined. *RAG1* alleles were separated using the PHASE algorithm (Stephens and Donnelly 2001; Stephens et al.

2003) implemented in DnaSP v. 6.10.01 (Rozas et al. 2017).

Genotyping and SNP filtering

For the SNPs study, all populations were included except for those from the Arganza, Tenebrilla and Turones rivers, due to the low DNA quality of these samples (Table 1). A total of 74 individuals identified on the basis of the *MT-CYB* sequences as *A. salmantinum* were used to prepare libraries for double digest restriction site-associated DNA sequencing (ddRAD-seq), following the protocol described by Kess et al. (2015). Individuals identified by both genes as *P. duriense* or hybrids were excluded from this data set.

Some of the samples were sequenced in two lanes on an Illumina HiSeq 2500 PE100 sequencer in Rapid-Run mode. The rest of the samples were run later in a fraction of a NovaSeq 6000 PE150 lane. Trimmomatic 0.36 (Bolger et al. 2014) was used to remove adapters (ILLUMINACLIP option). Through the *process-radtags* program in STACKS 2.4 (Catchen et al. 2013), all reads were truncated to the same length (95 bp). Low quality reads were removed using the -q parameter. The enzyme cut site was checked by using the options -e pstI and -renz-2 bgIII.

A de novo analysis was performed in STACKS 2.4 in which different programs were run to (1) assemble loci in each individual (*ustacks*), (2) build the catalogue (*cstacks*), (3) match all generic samples against the catalogue (*sstacks*) and (4) reconstruct loci using R2 reads and identify SNPs using the metapopulation information (*gstacks*). Main stack parameters including the minimum coverage to form a stack (*m*), the minimum distance to form loci (*M*) and the distance between loci across populations (*n*) were explored (Catchen et al. 2017). Finally, the combination that maximised the number of SNPs obtained in 80% of the individuals was selected (*m*=5, *M*=3 and *n*=4). The *populations* program in STACKS 2.4 (Catchen et al. 2013) was used to filter the SNPs using the following parameters: -R 0.80, -min-maf=0.05, -max-obs-het=0.75 and -write-random-snp.

Evolutionary relationships and population structure (mtDNA and nDNA)

Haplotype networks for both the mitochondrial (*MT-CYB*) and the nuclear (*RAG1*) genes were constructed to infer the population structure of *A. salmantinum*. The *MT-CYB* analysis included all of the individuals belonging to *A. salmantinum* (171) and *P. duriense* (15). For *RAG1*, 154 individuals of *A. salmantinum* and seven of *P. duriense* were included. To reconstruct the network, we used the median-joining algorithm (Bandelt et al. 1999) implemented in PopART (Leigh and Bryant 2015). For *MT-CYB*, differences among identified genetic groups were assessed through uncorrected

genetic p-distances and corresponding p-values (1000 pseudo-replicates) using MEGA v.6.0 (Tamura et al. 2013).

To infer evolutionary relationships among haplotypes, phylogenetic analyses were performed using only the *MT-CYB* dataset due to the low signal of *RAG1*. Also, due to the presence of hybrids (see below), only 168 of the 171 individuals of *A. salmantinum* were included in the following analyses. In order to establish these relationships, we used the collapsed haplotypes matrix obtained in DnaSP with 27 haplotypes. The phylogenetic reconstruction was performed using a Bayesian inference (BI) approach. The substitution model GTR+G was used as it produced the best likelihood values in jModelTest 2.1.10 (Darriba et al. 2012) using the Akaike information criterion (AIC; Akaike 1973). The analysis, implemented in MrBayes v3.2 (Ronquist et al. 2012), consisted of two simultaneous independent runs, each with four Markov chain Monte Carlo (MCMC), which were run for 10^6 generations. The first 25% of generations were removed as burn-in. Convergence between the two runs was assessed using Tracer v1.6 (Rambaut et al. 2014). *Protochondrostoma genei* (Bonaparte 1839) (GenBank Accession Number: AY568622) was used as the outgroup (Tan and Armbruster 2018).

Population genetic differentiation of *MT-CYB* was estimated through an analysis of molecular variance (AMOVA) in Arlequin v.3.11 (Excoffier and Lischer 2010). Different hierarchical levels including species, river, sub-basin and haplogroup were evaluated. Significance values were calculated with 10,000 permutations for the variance components and F-statistic values. In addition, genetic differentiation among haplogroups of *MT-CYB* was assessed through Φ_{ST} pairwise comparisons (Hudson et al. 1992).

Mitochondrial diversity and demography (mtDNA)

Genetic diversity of *MT-CYB* and *RAG1* was characterised in DnaSP v. 6.10.01. The mitochondrial DNA sequences were used to test the hypothesis of neutral evolution through Fu's *F_s* (Fu 1997) and Tajima's D (Tajima 1989) with 10,000 permutations, as implemented in Arlequin v.3.11. The same hierarchical levels as in the AMOVA analyses were studied.

Population structure, genetic differentiation and diversity (SNPs)

The genetic structure of the 4123 SNPs genotyped was assessed in STRUCTURE 2.3.4 (Pritchard et al. 2000). Two analyses were performed: one that included all populations and another with all populations excluding Yeltes and Gavilanes. We ran 10 independent simulations for each K value with a burn-in length of 50,000 and 50,000 repetitions after the burn-in period, and selected the admixture model. The K-value tested for each matrix

was based on the number of populations that formed the matrix + 1 ($K = 8$ and $K = 6$, respectively). Complementary information gathered from the analysis of neutral and adaptive loci can then be used to evaluate conservation units (Funk et al. 2012). For this reason, we evaluated the presence of loci under selection with BayeScan v.2.0 (Foll and Gaggiotti 2008), using the default parameters except for the prior odds (prior odds = 100; Lotterhos and Whitlock 2014). Likewise, the most probable number of subpopulations was estimated from the ΔK statistic implemented in CLUMPAK (Kopelman et al. 2015). A principal component analysis (PCA) was performed to examine patterns of genetic covariance using the R package SNPRelate version 1.12.0 (Zheng et al. 2012). Genetic differentiation among populations was estimated with the unbiased fixation index (F_{ST}) and 10,000 permutations in GenoDive (Meirmans and Van Tienderen 2004).

The standard set of population genetic statistics (i.e. number of private alleles, loci, variable loci and polymorphic sites; percentage of polymorphic sites; observed and expected heterozygosity; nucleotide diversity and the inbreeding coefficient) for each population was generated using the *populations* program in STACKS 2.4. The significance of the inbreeding coefficient was assessed by a permutation test with 10,000 permutations in Arlequin v.3.11.

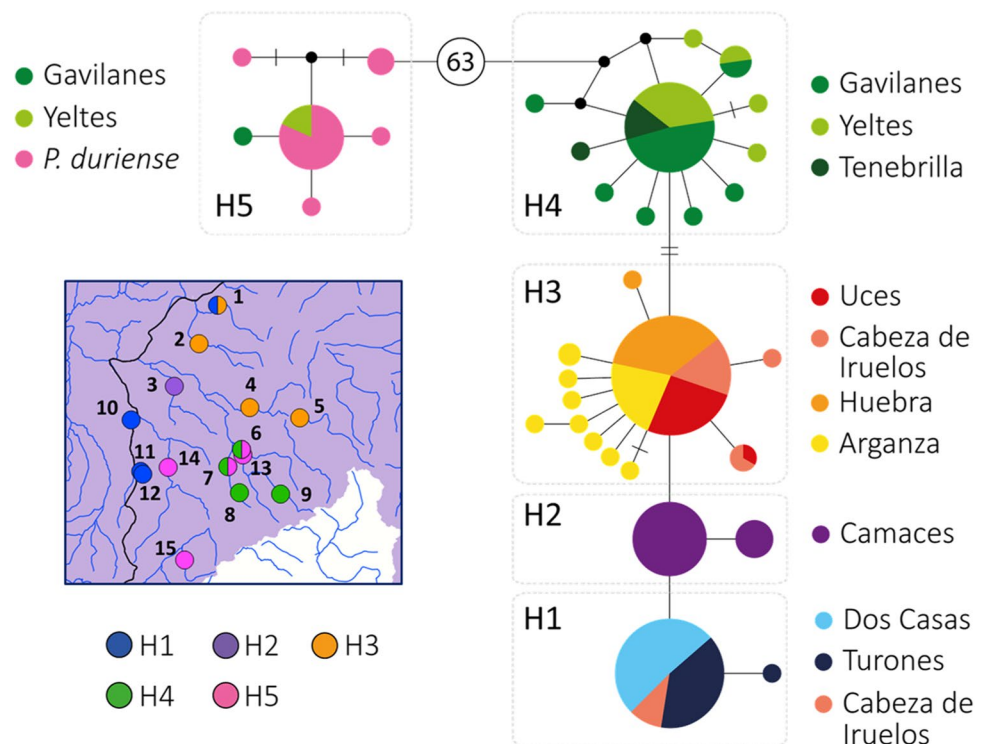
Results

Evolutionary relationships and population structure (mtDNA and nDNA)

A total of 29 haplotypes for *MT-CYB* were identified (Table S1). However, two individuals considered as *A. salmantinum* grouped with those identified as *P. duriense* (see below); therefore, the total number of haplotypes for the species *A. salmantinum* was 27 (Table S1). The *RAG1* analyses identified 23 nuclear alleles and 24 polymorphic sites (Table S1).

The *MT-CYB* haplotype network recovered five groups (Fig. 2; see Fig. 1 for locations). The first group (H1) was composed of haplotypes specific to the Águeda sub-basin (Dos Casas and Turones rivers) and to some individuals from Cabeza de Iruelos River within the Uces sub-basin. The second group (H2) comprised haplotypes found in the Camaces River, a left-bank tributary of the Huebra sub-basin located near the mouth of the Huebra River that enters the Duero River. The third group (H3) clustered all the haplotypes specific to Uces River and those of some individuals from Cabeza de Iruelos River (both rivers are within the Uces sub-basin) as well as those from the main channel of the Huebra River and its right-bank tributary, the Arganza River. Therefore, the Cabeza de Iruelos (Uces sub-basin) population shares mitochondrial haplotypes with both groups H1 and H3. The fourth group (H4) was composed

Fig. 2 Haplotype network for the mitochondrial marker *MT-CYB*. No line, one line and two lines, respectively, indicate one, two and three mutational steps. Mutational steps greater than three are indicated by a number. The colour of the circles on the map indicates the genetic group to which the different locations belong based on the mitochondrial marker. The number adjacent to each circle corresponds to numbers used in Table 1 for the different sampling localities for *A. salmantinum* and *P. duriense*



of haplotypes from the Gavilanes, Yeltes and Tenebrilla rivers, which are all left-bank tributaries of the Huebra sub-basin. The uncorrected p-distances among these four groups ranged between 0.2 and 0.6%, with H4 presenting the highest genetic distances (Table S2). Only one (H1–H2 and H2–H3) or three mutational steps (H3–H4) differentiated the haplogroups. The fifth group (H5) was composed of haplotypes of *P. duriense* and of some individuals from the Gavilanes and Yeltes rivers ascribed to *A. salmantinum*. In contrast with the other groups, 63 mutational steps differentiated this group from H4. No significant differences in pairwise Φ_{ST} values were found between the following population pairs: Yeltes–Tenebrilla, Gavilanes–Tenebrilla, Yeltes–Gavilanes, Uces–Huebra, Uces–Arganza, Cabeza de Iruelos–Huebra and Turones–Dos Casas (Table S3). Despite this, lower Φ_{ST} values were observed between the populations within the same lineage than between those from different lineages. Large significant differences in Φ_{ST} values were observed for the rest of the pairwise comparisons with the exception of Arganza–Cabeza de Iruelos and Camaces–Cabeza de Iruelos, which had small differences.

The *RAG1* haplotype network, which had a less robust signal than the *MT-CYB* one, showed the presence of four abundant alleles and four differentiated groups (Fig. 3). All populations were represented in both the A2 and A3 groups, except Gavilanes, Yeltes and Tenebrilla, which only appeared in A3 and A4. Within A4, a shared unique allele was found in the three aforementioned populations (Fig. 3a). The haplotypes of *P. duriense* and one shared with Gavilanes were represented in A1 (Fig. 3b). The presence

of *P. duriense* mitochondrial and nuclear haplotypes in *A. salmantinum* confirms the existence of genetic introgression of this species in the Yeltes River and its tributary, the Gavilanes River.

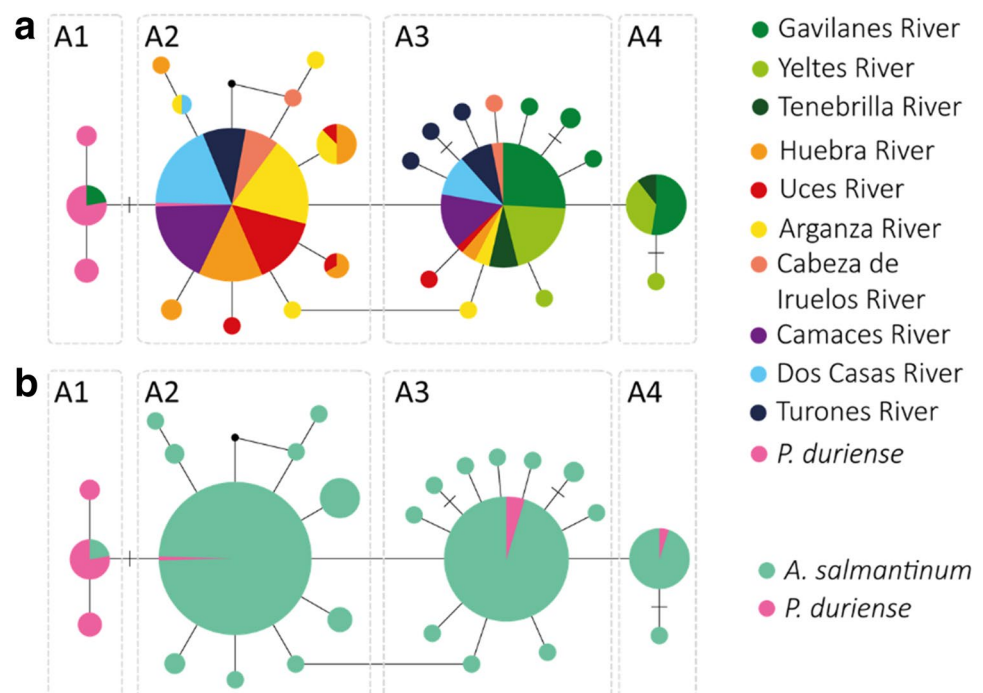
Our phylogenetic analysis revealed a close relationship among haplogroups H1, H2 and H3, which together comprised populations from the Águeda and Uces sub-basins and some populations from the Huebra sub-basin (Camaces, Huebra and Arganza) (Fig. 4). Moreover, within this group, the closest relationship was observed between H1 and H2. The relationships among the haplotypes assigned to H4, comprised of the populations from left-bank tributaries of the Huebra sub-basin (i.e. Yeltes, Gavilanes and Tenebrilla), were unresolved.

The results of the AMOVA showed that variance among groups (accounting for 85.95% of the variance) best explains the data when the groups were defined as those obtained from the mitochondrial network (Table S4). The percentage of the variance within populations was high in all cases (> 13%), regardless of the criteria selected to form the groups (Table S4). When the groups were defined by biogeographic region (sub-basins), the differences were not significant.

Mitochondrial and nuclear diversity (mtDNA and nDNA) and demography (mtDNA)

Overall, genetic diversity parameters based on *MT-CYB* were low (Table S1). The population with the lowest diversity values with only one haplotype was Dos Casas River.

Fig. 3 Haplotype network for the nuclear gene *RAG1*. No line indicates one mutational step and one line indicates two mutational steps. **a** Haplotype network according to rivers and species (see legend). **b** Haplotype network according to species (*A. salmantinum* in green, *P. duriense* in pink)



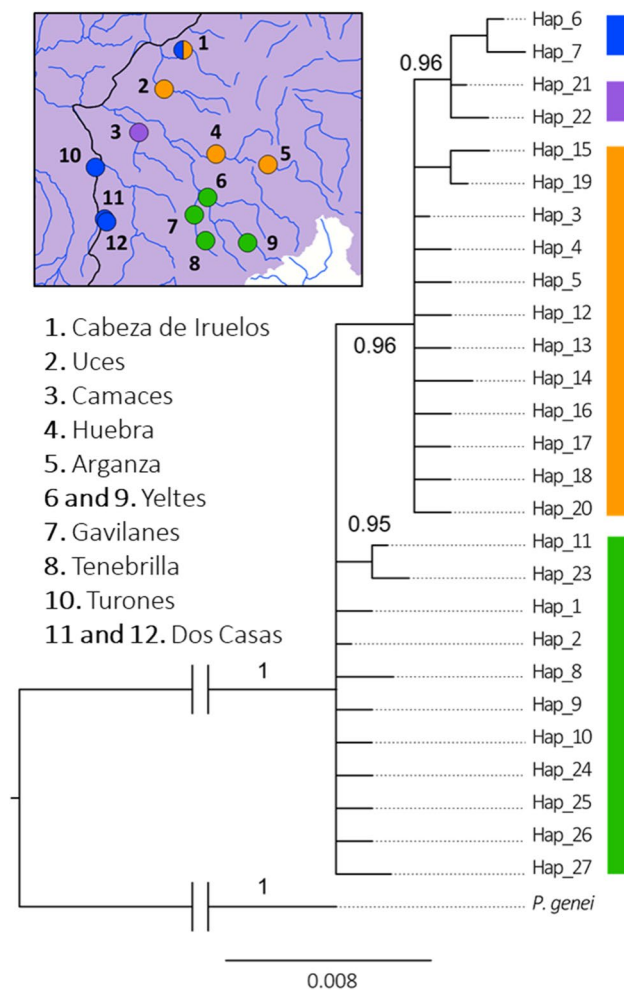


Fig. 4 Bayesian phylogenetic reconstruction based on the mitochondrial marker *MT-CYB*. The colour of the circles on the map indicate the genetic group to which each location belongs according to the results of this analysis

The population from Arganza River had the highest genetic diversity values, with the exception of that for nucleotide diversity (π), which was higher in the Cabeza de Iruelos river population due to the presence of two distant haplogroups at this locality. Likewise, the number of haplotypes found per population was generally higher for the nuclear versus mitochondrial marker (Table S1). The populations with the lowest number of nuclear (*RAG1*) alleles were Camaces, Tenebrilla, Yeltes and Dos Casas, while Uces and Tenebrilla had the lowest values of allelic diversity (Table S1).

When all populations were treated as a single one, the population showed significant negative values in the neutrality tests, indicating a population expansion after a bottleneck (Table S1). Of the four haplogroups analysed, two had significant negative values for Tajima's D (H3 and H4) and three for Fu's FS (H1, H3 and H4). The only group that showed no significant deviation in the neutrality tests was H2 from the Camaces River. Likewise, when the populations were analysed independently, only three had significant negative values (Yeltes, Arganza and Gavilanes rivers). The results of both neutrality tests according to sub-basin grouping were negative and significant for only the Huebra sub-basin.

Single Nucleotide Polymorphism analyses

Of the 4123 SNPs identified, only three were found to be under selection; therefore, we treated the data equally. The results of the structure analyses showed that a subpopulation number of $K = 2$ ($\Delta K = 7498.272$) best explained the data when all analysed populations were included in the analysis (Fig. 5). The populations belonging to the left-bank tributaries of the Huebra sub-basin (Yeltes and Gavilanes) were separated from the rest of the populations. As the results also showed a high ΔK value for $K = 3$ ($\Delta K = 2465.780$), the

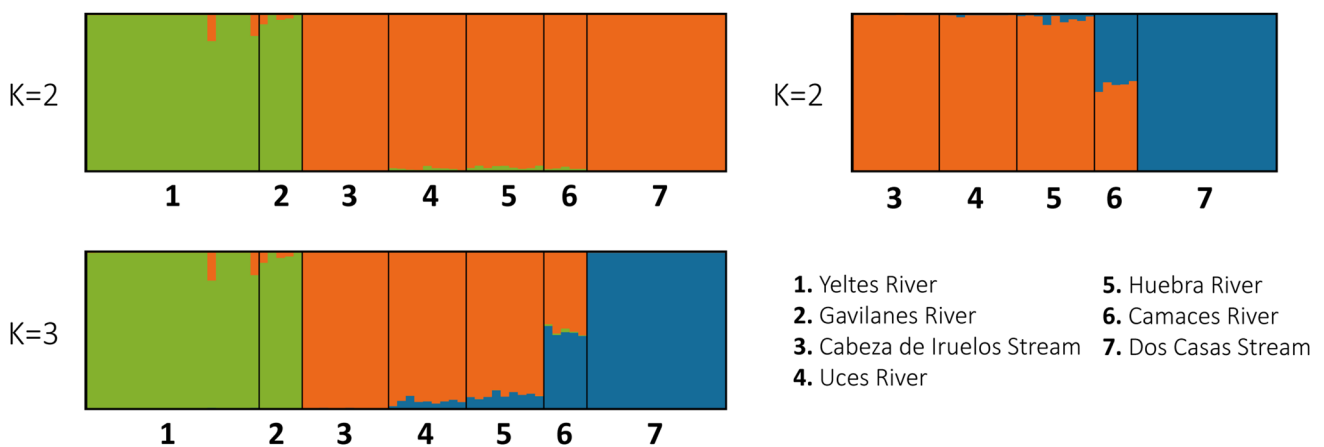


Fig. 5 Genetic structure of the 4123 single nucleotide polymorphisms identified in 74 individuals of *A. salmantinum* across all genotyped populations (left) and all populations except Yeltes and Gavilanes

(right). The K values shown are those selected under the criteria of ΔK (Evanno et al. 2005)

analysis was repeated with all populations except Gavilanes and Yeltes. This analysis showed two groups, revealing greater structuring among the populations (Fig. 5). The first group was composed of the populations from Cabeza de Iruelos, Uces (both within the Uces sub-basin) and Huebra rivers, while the second group included only the Dos Casas population. Camaces was the only population assigned to both groups with high values.

The results of the PCA identified the same four groups detected in the structure analysis (Fig. 6). The first principal component (PC1) explained 32.11% of the variance and differentiated the Yeltes and Gavilanes rivers from the others. These results are consistent with the pairwise comparison of F_{ST} , in which Yeltes and Gavilanes were the most differentiated populations with respect to the rest of the populations, though no significant differences were found between the two (Table S5). The second component (PC2) explained 11.11% of the variance and differentiated three groups of populations: (1) the Huebra, Uces and Cabeza de Iruelos rivers, (2) Camaces and (3) Dos Casas River (Fig. 6).

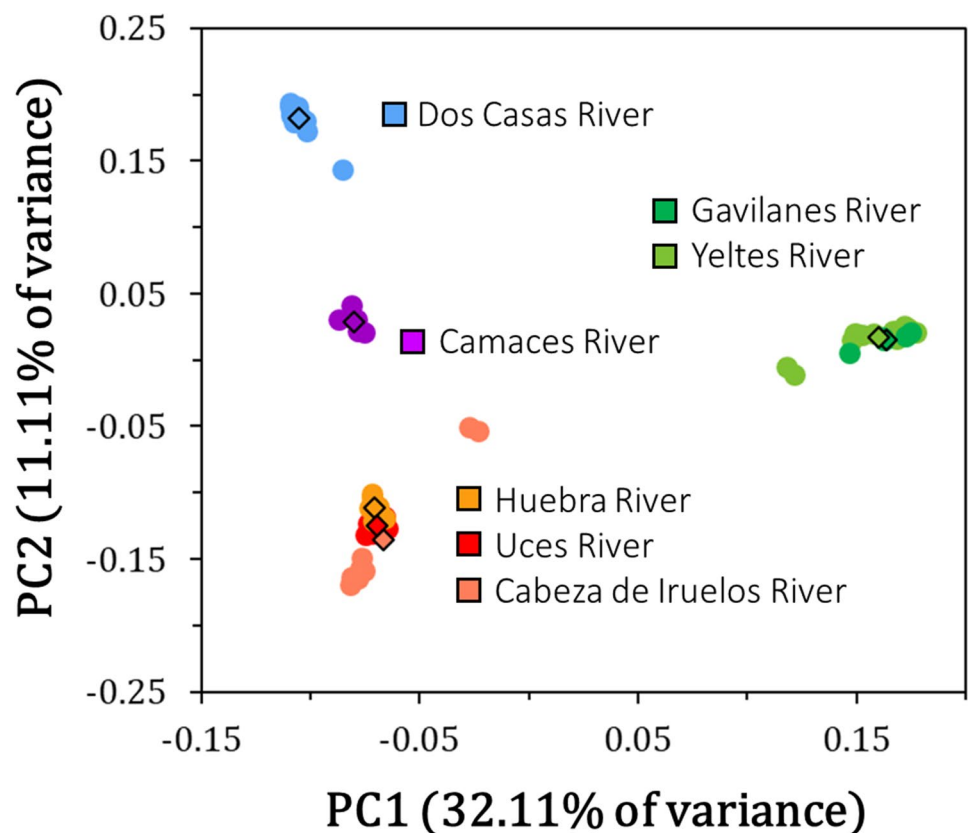
The Yeltes, Cabeza de Iruelos and Dos Casas populations were the only ones with private alleles (Table S6). Observed heterozygosity was slightly higher than expected heterozygosity in all populations except Cabeza de Iruelos,

indicating an excess of heterozygosity. However, F_{IS} values were not significant in any of the populations. Dos Casas had the lowest values of heterozygosity, nucleotide diversity and percentage of polymorphic sites.

Discussion

Species with highly restricted geographic ranges are generally more threatened than those with wide ranges given their greater vulnerability to environmental or anthropogenic factors (Gaston and Fuller 2009; IUCN 2014). In addition, those with a population structure, in combination with a low migration rate and the absence of gene flow, may experience a high rate of extinction, as has been observed in some small freshwater fishes (Frankham et al. 2002; Schönhuth et al. 2003). For this reason, many studies have focused on the distribution, genetic structure and evolutionary history of threatened freshwater fishes. By studying the evolutionary history and phylogeography of *A. salmantinum* in the Iberian Peninsula, we increase our knowledge of not only the evolution of this geographic area but also the processes affecting endemic and endangered freshwater fish species with restricted distributions.

Fig. 6 Principal component analysis of the 4123 single nucleotide polymorphisms genotyped in 74 individuals of *A. salmantinum* for all available populations. Each population is represented by a distinct colour. The delineated diamonds represent the centroid for each population



Population structure and the origin of the genetic groups

The most surprising result of this study was the strong genetic structure observed for *A. salmantinum*, despite its restricted distribution, not only between different sub-basins within the same hydrographic basin but also between small tributaries within the same sub-basin. The usual pattern observed in European freshwater fishes with a wide distribution area is a high level of gene flow within the same hydrographic basin and high genetic differentiation between basins (Seifertová et al. 2011; Takács et al. 2015; Marić et al. 2017; Buj et al. 2017; Corral-Lou et al. 2019; Marić et al. 2019; Cahsan et al. 2020; Wetjen et al. 2020). Likewise, the high level of genetic variation present in *A. salmantinum* is unique and not found in other small cyprinids with similarly sized distribution areas that inhabit only one basin in the Iberian Peninsula, such as *Squalius torgalensis* (Coelho et al. 1998) and *Iberochondrostoma almaiai* (Coelho et al. 2005) (Henriques et al. 2010; Sousa et al. 2010), nor in several *Telestes* and *Cobitis* species that occur in the Balkans, such as *T. ukliwa* (Heckel 1843) and *C. dalmatina* Karaman 1928 (Buj et al. 2015, 2017, 2020). Although there are no population genetic studies of other groups of freshwater organisms within the distribution area of *A. salmantinum*, a survey of the widely distributed freshwater mussel species *Margaritifera margaritifera* (Linnaeus 1758) revealed a high level of differentiation between the Águeda population and the rest of the populations from the western Duero Basin (Stoeckle et al. 2017), reinforcing the idea of the uniqueness of this small region for freshwater organisms.

The genetic groups identified in our analyses do not group according to sub-basin regions; therefore, they do not reflect the current hydrogeographic configuration of the area, suggesting that complex changes that occurred at a small spatial scale in the hydrographic network in the geological past account for the origin of the groups. Unexpected patterns of genetic structure observed in other freshwater fish species with small distribution areas has been associated with events that shaped hydrographic networks during the past, such as piracy (*Etheostoma cyanoprosopum* Near & Kozal 2017 in the USA; Fluker et al. 2019), variations in sea level (*Neoceratodus forsteri* (Krefft 1870) in the USA; Bishop et al. 2018) and the configuration of ancient paleodrainages (*Rhinogobius duospilus* (Herre 1935) in Hong Kong; Wu et al. 2016). Therefore, in order to understand the origin of the strong population structure observed in *A. salmantinum*, it is essential to know about the hydrogeomorphological evolution of its distribution range over time.

The divergence of *A. salmantinum* from other species of *Achondrostoma* early during the evolutionary history of the genus, dated to the transition of the Tortonian to Messinian period (~7.1 MYA) (Berggren et al. 1995; Doadrio

and Elvira 2007), has been associated with the isolation of Miocene endorheic basins from Ciudad Rodrigo (Arenillas and Saenz 1987; López-Martínez 1989; De la Peña 1995; Carmona et al. 2000). Consequently, *A. salmantinum* is considered one of the oldest cyprinid endemics in the Iberian Peninsula (Doadrio and Elvira 2007). However, the genetic distances found among populations of *A. salmantinum* were lower than those observed in other Iberian leuciscid species with wider distribution areas, such as *Squalius pyrenaicus* (Günther 1868) (0.6 to 1.0%; Perea et al. 2016), *Luciobarbus sclateri* (Günther 1968) (0.01 to 1.8%; Casal-López et al. 2017) and *Phoxinus phoxinus* Kottelat 2007 (1.3 to 2.2%; Corral-Lou et al. 2019). The very low genetic distances in *A. salmantinum* indicate that, despite being an ancient endemic, the fragmentation of its populations occurred in recent historical periods, likely in association with the drastic changes that took place in the Iberian hydrographic network during the Pleistocene (Doadrio and Carmona 2006; Robalo et al. 2006; Perea et al. 2016; Sousa-Santos et al. 2019), which resulted in the formation of strong barriers to gene flow within the small region encompassing the distribution of the species.

The hydrographic network of the study area, in its current configuration, is located in a high plain area around 800 m.a.s.l. within the Iberian Massif, and consists of ancient hard rocks such as granite and slate, which are resistant to erosion (Salazar and Portero 2010). The main rivers of the network, which have high flow rates, such as the Duero, Águeda and the mouth of the Huebra River, cause deep valleys within the plain. By contrast, most of the tributaries, which have lower flow rates, form hanging valleys. This geomorphological configuration produces substantial unevenness at the mouth of the tributaries, leading some to have high waterfalls that isolated them from the main courses into which they flow, such as occurs in the Camaces and Uces rivers (Fig. 7). Both rivers have a high waterfall at the mouth that prevents connections between fish populations located upstream and downstream of it, leading to the isolation of these populations (Fig. 7; Durán 2010). In fact, the presence of waterfalls is considered a driver of population structure in several European and North American freshwater fish species (e.g. Domínguez-Domínguez et al. 2006; Van Leeuwen et al. 2017; Beltrán-López et al. 2020).

In the case of the Camaces population, which is located upstream of the Cachón del Camaces waterfall, a unique mitochondrial group was observed, indicating its ancient isolation. However, the results of the SNP analyses showed evidence of recent mixing between this population and those from the Águeda and Huebra sub-basins, highlighting the permeability of this barrier in the recent past. On the other hand, genetic homogeneity in all markers was observed between the Uces sub-basin populations, which are also located upstream of a waterfall (Pozo de los Humos),

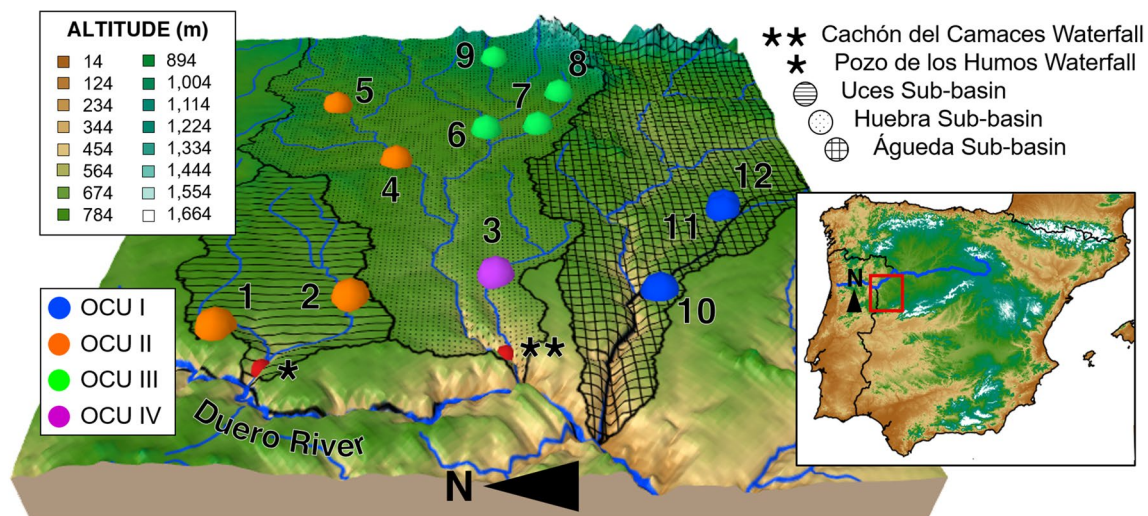


Fig. 7 3D map showing the four OCUs established for *A. salmantinum* in the Duero Basin and the relief of the area. Numbers correspond to the ones used in Table 1 for sampling locality. Each sam-

pling point is coloured according to its OCU. The red dots (and asterisks) indicate the two waterfalls mentioned in the study

and populations downstream of the Huebra River and its right-bank tributary, the Arganza. This finding can only be explained by stream piracy of the headwaters of these rivers during the recent past, a common phenomenon associated with the population structure found in other Iberian cyprinids (Mesquita et al. 2005; Perea et al. 2016; Casal-López et al. 2017; Corral-Lou et al. 2019), as well as in other freshwater fish species worldwide (e.g. Domínguez-Domínguez et al. 2006; Aquino and Colli 2017; Fluker et al. 2019). Within the Uces sub-basin, the Cabeza de Iruelos population formed its own mitochondrial subgroup but also shared one haplotype with downstream Águeda sub-basin populations; however, in the nuclear analysis, the populations from the two sub-basins were distinct. This result indicates the presence of ancestral polymorphisms in the Cabeza de Iruelos population and gene flow among localities prior to the establishment of the Uces waterfall as a barrier. Finally, given the lack of any evident geographic barrier isolating the left-bank tributaries of the Huebra sub-basin (Yeltes River and its tributaries Gavilanes and Tenebrilla), we expected to observe homogeneity in all of the Huebra sub-basin populations; however, these left-bank tributaries were the most differentiated of the four obtained mitochondrial lineages. We hypothesise that the canyon located at the mouth of the Yeltes is an unsuitable habitat for the species, effectively preventing it from spreading to other areas of the Huebra sub-basin.

Genetic diversity

A low level of genetic diversity disadvantages the adaptation of populations to environmental changes, leaving these

populations highly susceptible to stochastic and potentially catastrophic events. For this reason, maintaining or increasing genetic diversity is a priority area in conservation biology (McNeely et al. 1990; Frankham et al. 2002). The study of the genetic diversity of populations of freshwater species of, for instance, mussels or fishes is extensively used as a tool to define and establish adequate management plans and to focus conservation efforts on the most threatened populations (Geist 2010; Kattawar and Piller, 2020). Therefore, the results of our genetic analysis should be taken into consideration in the conservation planning of the endangered *A. salmantinum*.

In the case of *A. salmantinum*, the overall genetic diversity of *MT-CYB* was high ($H_d=0.82$; $h=27$), in line with results found in other Mediterranean leuciscid species with similarly sized distribution ranges, such as *Telestes croaticus* (Steindachner 1866) ($H_d=0.88$, $h=22$; Buj et al. 2020), *T. ukliva* ($H_d=0.98$, $h=13$; Buj et al. 2020), *Iberochondrostoma almaiai* ($H_d=0.81$, $h=8$; calculated through the used sequences from Sousa et al. 2010), *I. lusitanicum* (Collares-Pereira 1980) ($H_d=0.9$; $h=20$; Sousa et al. 2008) and *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues & Collares-Pereira 1998) ($H_d=0.84$, $h=23$; calculated through the used sequences Mesquita et al. 2005). High genetic diversity values may reflect not only the development of adaptations and an increase in genetic diversity but also a strong population structure. In this context, when the populations of *A. salmantinum* were considered independently, they showed generally low levels of genetic diversity ($H_d=0-0.72$, $h=1-9$), similar to the results found in the above mentioned endangered species *I. lusitanicum* ($H_d=0.00-0.88$, $h=1-10$; Sousa et al. 2008) and *S. aradensis* ($H_d=0.00-0.76$; $h=1-5$;

Mesquita et al. 2005), which both showed high levels of population differentiation. The low number of haplotypes in the majority of the populations indicates that these populations have suffered recent bottlenecks from which they have not yet recovered, with the exception of Yeltes, Gavilanes and Arganza, which all showed evidence of subsequent population expansion. Moreover, with the exception of Cabeza de Iruelos, all of the populations included in the SNP analysis showed an excess of heterozygotes, indicating that these populations are going through a bottleneck.

When the populations were analysed in terms of the obtained mitochondrial lineages, the highest genetic diversity values were observed for H3 (Uces sub-basin, the main channel of the Huebra River and its right-bank tributary, the Arganza River) and H4 (the left-bank tributaries of the Huebra sub-basin, i.e. the Yeltes, Gavilanes and Tenebrilla rivers). For H4, genetic diversity values were similar among its populations. By contrast, for H3, the population from the Arganza River had a higher level of genetic diversity compared with the one from the Huebra, the river into which the Arganza flows, for which only two mitochondrial haplotypes were found. Unlike the Huebra River, the Arganza has a large shoal that generates pools, suggesting that populations within this river should have low levels of genetic diversity. While sampling in the Arganza River, however, we observed the presence of only a single invasive alien fish species, the leuciscid *Alburnus alburnus* (Linnaeus 1758), indicating the lack of strong competition for *A. salmantinum* in this river, which may, in part, account for this population's high level of genetic diversity. Although, the hypothesis of a recent bottleneck followed by population expansion cannot be rejected for this population. The H1 (Águeda sub-basin) and H2 (Camaces River within the Huebra sub-basin) lineages, comprised of only two haplotypes each, showed the lowest levels of genetic diversity, particular for the Águeda sub-basin populations (Turones and Dos Casas; H1), at least according to the mitochondrial data. Moreover, Dos Casas, the only persistent population in the sub-basin, presented a unique haplotype and the lowest diversity values in the SNP analysis.

A pattern of strong genetic differentiation and low population genetic diversity has been described for several widely distributed freshwater species, including the Holarctic freshwater mussel *Margaritifera margaritifera* (Bouza et al. 2007; Stoeckle et al. 2017) and the East African catfish *Bagrus docmak* (Forsskål 1775) (Basiita et al. 2017). However, in species with a restricted distribution range such as *A. salmantinum*, a low level of genetic diversity may have important consequences for their long-term conservation. Genetic drift, favoured by the isolation of populations, combined with bottleneck events in some of the lineages, could be responsible for the low level of genetic diversity currently found in most of the populations of *A. salmantinum*.

Implication for conservation

The use of molecular approaches has allowed us to characterise populations of *A. salmantinum* throughout its entire distribution area. Although it provides a good starting point, ecological and biological studies of this highly endangered species are still necessary in order to develop an optimal recovery plan, which can then be used to guide further conservation efforts and the proper management of the species. On the basis of the results obtained with the mitochondrial data indicating a high level of differentiation among populations of *A. salmantinum* and those obtained with the genomic SNPs, we establish four Operational Conservation Units (OCUs; Doadrio et al. 1996), following the protocol used by Funk et al. (2012) (Fig. 7). The four OCUs for *A. salmantinum* are as follows: (I) rivers within the Águeda sub-basin (Turones and Dos Casas); (II) rivers within the Uces sub-basin (Uces and Cabeza de Iruelos) and the Huebra River and its right-bank tributary, the Arganza River; (III) left-bank tributaries within the Huebra sub-basin (Yeltes, Tenebrilla and Gavilanes) and (IV) Camaces River, a small left-bank tributary within the Huebra sub-basin that is geographically isolated due to a waterfall near its mouth.

The viability of *A. salmantinum* is highly threatened due to both intrinsic and extrinsic factors. On the one hand, the low level of genetic variability shown by most populations of this species and the strong population structure caused by their isolation make *A. salmantinum* more sensitive to extrinsic changes compared with other species and, therefore, at greater risk of extinction (Hunter 1996; Frankham et al. 2002). Empirical data have demonstrated that genetic diversity rapidly deteriorates in small isolated populations due to the effect of genetic drift, leading to a reduction in adaptive potential and reproductive fitness (Eizaguirre and Baltazar-Soares 2014; Hoffman et al. 2017 and references therein). Furthermore, low levels of intra-population genetic diversity indicate small effective population sizes in the past, which would be consistent with the reduced distribution of those populations. A reduction in genetic diversity may also result in increased inbreeding (Reed and Frankham 2003), although in the case of *A. salmantinum*, none of the F_{IS} values estimated for its populations were significant, implying inbreeding is not a problem for the species. On the other hand, the habitats of *A. salmantinum* have been and continue to be threatened by the main causes of biodiversity loss (e.g. overexploitation, water pollution, flow modification, habitat destruction and degradation and the introduction of invasive species) (Dudgeon et al. 2005; Doadrio et al. 2011; Morán-Tejeda et al. 2012). Ongoing global warming coupled with the increased demand for water resources by humans is exacerbating these negative impacts in the distribution range of *A. salmantinum* as a consequence of changes in the fluvial

dynamics of the rivers located in the southwest sector of the Duero Basin (Ceballos-Barbancho et al. 2008).

With regards to their conservation, although all four OCUs of *A. salmantinum* are characterised by generally low levels of genetic diversity that may compromise the viability of many of the populations, those established for the Huebra and Uces sub-basins (OCU II, III and IV) are already included in a protected area (ZEC ES4150064 “Riverbanks of the Huebra, Yeltes, Uces and their tributaries” and “The Arribes del Duero National Park”). For this reason, we do not suggest any intervention for these populations unless conservation conditions deteriorate. On the contrary, the situation in the Águeda sub-basin (OCU I) is of particular concern: one of the two sub-basin populations is extinct and the other showed the lowest level of genetic diversity of all the studied populations, and the region inhabited by this population is not included in a protected area. Moreover, rivers in the Águeda region are characterised by strong droughts during the summer, with some areas often reduced to small pools of water or becoming completely dry. Also, the rivers that hang over the high plain depend on underground freshwater reservoirs, which have been over-exploited in the Iberian Peninsula (Leduc et al. 2017). These factors, together with anthropic activities that have affected river channels in this region (Velasco et al. 1997; Campos Sánchez-Bordona et al. 2013), threaten the long-term survival of the only persistent population of *A. salmantinum* in the Águeda sub-basin, as evidenced by the disappearance of the species in the Turones River. The loss of this population was caused by a change in land use (reparcelling), which has led to an increase in farming, water pollution and the presence of invasive species, the elimination of riverbank vegetation and the complete desiccation of habitats (Doadrio et al. 2011; Freyhof and Brooks 2017).

For the above reasons and for the recovery and habitat protection of the species in the Águeda sub-basin, we first recommend a restriction on water extraction for agriculture and farming, the control of invasive species and the inclusion of the sub-basin in the conservation area “LIC Riverbanks of the Águeda” (ES4150087). Second, we recommend the implementation of a captive breeding program for the Águeda sub-basin, using specimens only from Dos Casas River in order to reinforce its population and also to repopulate the Turones River once habitat conditions there have improved. The strong genetic structure of *A. salmantinum*, as revealed by this study, makes it indispensable to consider genetic variation in the development of any captive breeding program for the species, in order to preserve the genetic composition of the four identified OCUs. If in situ conservation strategies fail to restore the endangered populations, ex situ ones may have to be implemented (Maceda-Veiga 2013). Even though the genetic diversity of the populations in OCU

I may be further reduced through the actions of captive breeding programs, these kinds of measures should still be planned and implemented before the only surviving population of this OCU becomes extinct, like the Turones population. Given the success of recent captive breeding programs for several highly endangered freshwater fish species (e.g. *Achondrostoma occidentale* (Robalo et al. 2005), Mameri et al. 2018; *Cottus hispaniolensis* Băcescu and Băcescu-Mester, 1964, Manubens et al. 2020; Stoessel et al. 2020), this strategy is a promising tool for the long-term preservation for the most endangered populations of *A. salmantinum*.

In conclusion, this study provides information about the current genetic structure of populations of *A. salmantinum*, which is associated with the complex evolution of the hydrogeological architecture of the region. We hypothesise that other cyprinids and aquatic species whose distribution area overlaps with that of *A. salmantinum* will show similar biogeographic patterns. Now that OCUs have been established for the species, we highlight the importance of respecting these units in the implementation of management plans. Otherwise, populations of *A. salmantinum* may become homogenized to the extent that variation among conservation units would likely be lost. To avoid this, the captive breeding of individuals, and their subsequent release into the wild, must be done taking into consideration the genetic divergence among OCUs. Overall, our work highlights the importance of genetic studies on species with highly restricted distribution areas whose populations are inferred to lack structure, and are at greater risk of extinction.

Supplementary Information The online version of this article (<https://doi.org/10.1007/s10592-021-01381-y>).

Acknowledgements Samples used in this study are stored in the DNA and Tissue Collection at the National Museum of Natural Sciences of Madrid (MNCN-CSIC). However, we specifically want to thank P. Garzón, I. Doadrio Jr, J.L. González and G. González for collecting almost every sample from 2009 to 2010 under the project “Basis for the monitoring of Spanish Freshwater Fishes”, supported by the Ministerio de Medio Ambiente y Medio Rural y Marino (MARM), and C. Marcos, J.C Velasco and G. González for their help in the project Life13 nat/es/000772. We greatly thank L. Alcaraz for laboratory assistance. We are grateful for the valuable suggestions and English editing performed by M. Modrell. This study was also supported by a management commission funded by the Duero Hydrographic Confederation in Spain (Análisis y estudios filogeográficos y de variabilidad genética de endemismos ibéricos de ciprínidos: Life13 nat/es/000772 “Actuaciones para la protección y conservación de ciprínidos ibéricos de interés comunitario”) and by grant IND2017/AMB-7699 to AC-L, funded by the Community of Madrid (Spain) through its Industrial Doctorate programme. We acknowledge the Santander Supercomputation support group at the University of Cantabria for providing access to the supercomputer Altamira at the Institute of Physics of Cantabria (IFCA-CSIC), a member of the Spanish Supercomputing Network, which was used to perform simulations/analyses.

Funding (1) Industrial Doctorate of Community of Madrid. Grant Number: IND2017/AMB-7699. (2) Duero Hydrographic Confederation. Grant Number: Life13 nat/es/000772

Data availability The new sequences of the mitochondrial (*MT-CYTB*) and nuclear (*RAG1*) markers obtained from this study are available in GenBank (MW174250–MW174757). The SNP data have been deposited in VCF format in Figshare (https://figshare.com/articles/dataset/populations_snps_vcf/13136069).

Declarations

Conflicts of interest The authors declare no conflict of interest.

Ethical approval This study does not include experimentation with animals.

Consent to participate (Ethics) No consent to participate was required for this study.

Consent to publish (Ethics) All authors consent to the publication of this manuscript

References

- Akaike H (1974) A new look at the statistical model identification. Springer Series in Statistics Selected Papers of Hirotugu Akaike 215–222. https://doi.org/10.1007/978-1-4612-1694-0_16
- Alcaraz C, Gholami Z (2020) Diversity and structure of fragmented populations of a threatened endemic cyprinodontid (*Aphanius sophiae*) inferred from genetics and otolith morphology: implications for conservation and management. *J Zool Syst Evol Res* 58:341–355. <https://doi.org/10.1111/jzs.12333>
- Alonso JL, Pulgar FJA, Pedreira D (2007) El relieve de la Cordillera Cantábrica. Enseñanza de las Ciencias de la Tierra 15:151–163
- Aquino PDP, Colli GR (2017) Headwater captures and the phylogenetic structure of freshwater fish assemblages: a case study in central Brazil. *J Biogeogr* 44(1):207–216. <https://doi.org/10.1111/jbi.12870>
- Arenillas M, Sáenz C (1987) Los ríos. Guía física de España. Alianza, Madrid
- Bandelt HJ, Forster P, Rohl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 16:37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Bartáková V, Bryja J, Šanda R et al (2018) High cryptic diversity of bitterling fish in the southern West Palearctic. *Mol Phylogenet Evol* 133:1–11. <https://doi.org/10.1016/j.ympev.2018.12.025>
- Basiita RK, Zenger KR, Jerry DR (2017) Populations genetically rifted within a complex geological system: the case of strong structure and low genetic diversity in the migratory freshwater catfish, *Bagrus docmak*, East Africa. *Ecol Evol* 7(16):6172–6187. <https://doi.org/10.1002/ece3.3153>
- Beltrán-López RG, Domínguez-Domínguez O, Piller KR, Mejía-Mojica H, Mar-Silva FA, Doadrio I (2020) Genetic differentiation among populations of the blackfin goodea *Goodea atripinnis* (Cyprinodontiformes: Goodeidae): implications for its evolutionary history. *J Fish Biol Early Online View*. <https://doi.org/10.1111/jfb.14654>
- Berggren WA, Kent DV, Swisher CC, Aubry M-P (1995) A revised cenozoic geochronology and chronostratigraphy. *Geochronol Time Scales Global Stratigr Correl*. <https://doi.org/10.2110/pec.95.04.0129>
- Bishop CR, Hughes JM, Schmidt DJ (2018) Mitogenomic analysis of the Australian lungfish (*Neoceratodus forsteri*) reveals structuring of indigenous riverine populations and late Pleistocene movement between drainage basins. *Conserv Genet* 19:587–597. <https://doi.org/10.1007/s10592-017-1034-7>
- Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Bouza C, Castro J, Martínez P, Amaro R, Fernández C, Ondina P, Outeiro A, San Miguel E (2007) Threatened freshwater pearl mussel *Margaritifera margaritifera* L. in NW Spain: low and very structured genetic variation in southern peripheral populations assessed using microsatellite markers. *Conserv Genet* 8:937–948. <https://doi.org/10.1007/s10592-006-9248-0>
- Buj I, Čaleta M, Marčić Z et al (2015) Different histories, different destinies-impact of evolutionary history and population genetic structure on extinction risk of the Adriatic spined loaches (Genus *Cobitis*; Cypriniformes, Actinopterygii). *PLoS ONE* 10(7): <https://doi.org/10.1371/journal.pone.0131580>
- Buj I, Marčić Z, Čaleta M et al (2017) Ancient connections among the European rivers and watersheds revealed from the evolutionary history of the genus *Telestes* (Actinopterygii; Cypriniformes). *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0187366>
- Buj I, Marčić Z, Flauder E et al (2020) Population genetic structure of endemic fish species facilitating their survival in changing environments: a case study on the genus *Telestes* in Croatia. *Authoria*. <https://doi.org/10.22541/au.159294147.70101085>
- Cahsan BD, Nagel R, Schedina IM et al (2020) Phylogeography of the European brook lamprey (*Lampetra planeri*) and the European river lamprey (*Lampetra fluviatilis*) species pair based on mitochondrial data. *J Fish Biol* 96:905–912. <https://doi.org/10.1111/jfb.14279>
- Campos Sánchez-Bordona F, Sánchez González S, García Sánchez A, et al (2013) Cuenca del río Águeda: un territorio para dos países. Universidad Europea Miguel de Cervantes
- Carmona JA, Domínguez J, Doadrio I (2000) Congruence between allozyme and cytochrome b gene sequence data in assessing genetic differentiation within the Iberian endemic *Chondrostoma lemmingii* (Pisces: Cyprinidae). *Heredity* 84:721–732. <https://doi.org/10.1046/j.1365-2540.2000.00720.x>
- Casal-López M, Perea S, Sousa-Santos C et al (2017) Paleobiogeography of an Iberian endemic species, *Luciobarbus sclateri* (Günther, 1868) (Actinopterygii, Cyprinidae), inferred from mitochondrial and nuclear markers. *J Zool Syst Evol Res* 56:127–147. <https://doi.org/10.1111/jzs.12192>
- Catchen J, Hohenlohe PA, Bassham S et al (2013) Stacks: an analysis tool set for population genomics. *Mol Ecol* 22:3124–3140. <https://doi.org/10.1111/mec.12354>
- Catchen JM, Hohenlohe PA, Bernatchez L et al (2017) Unbroken: RADseq remains a powerful tool for understanding the genetics of adaptation in natural populations. *Mol Ecol Resour* 17:362–365. <https://doi.org/10.1111/1755-0998.12669>
- Ceballos-Barbancho A, Morán-Teheda E, Luengo-Ugidos MA et al (2008) Water resources and environmental change in a Mediterranean environment: the south-west sector of the Duero river basin (Spain). *J Hydrol* 351(1–2):126–138. <https://doi.org/10.1016/j.jhydrol.2007.12.004>
- Corral-Lou A, Perea S, Aparicio E, Doadrio I (2019) Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *J Zool Syst Evol Res* 57:926–941. <https://doi.org/10.1111/jzs.12320>
- Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: more models, new heuristics and parallel computing. *Nat Methods* 9:772. <https://doi.org/10.1038/nmeth.2109>
- De la Peña A (1995) Tertiary fishes from the Iberian continental basins: history and fossil record. *Coloquios de Paleontología* 47:25–47

- Doadrio I (1988) Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. *Bonner Zool Beitr* 39:113–128
- Doadrio I, Carmona JA (2006) Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species. *Cybum* 30:199–214
- Doadrio I, Elvira B (2007) A new species of the genus *Achondrostoma* Robalo, Almada, Levy and Doadrio, 2007 (Actinopterygii, Cyprinidae) from western Spain. *Graellsia* 63:295–304. <https://doi.org/10.3989/graelisia.2007.v63.i2.96>
- Doadrio I, Perdices A, Machordom A (1996) Allozymic variation of the endangered killifish *Aphanius iberus* and its application to conservation. *Environ Biol Fish* 45:259–271. <https://doi.org/10.1007/bf00003094>
- Doadrio I, Perea S, Garzón-Heydt P, González JL (2011) Ictiofauna continental española: bases para su seguimiento. Ministerio de Medio Ambiente y Medio Rural y Marino, Centro de Publicaciones, Madrid
- Domínguez-Domínguez O, Doadrio I, Pérez-Ponce de León G (2006) Historical biogeography of some river basins in central Mexico evidenced by their goodeine freshwater fishes: a preliminary hypothesis using secondary Brooks parsimony analysis. *J Biogeogr* 33(8):1437–1447. <https://doi.org/10.1111/j.1365-2699.2006.01526.x>
- Dudgeon D (2019) Multiple threats imperil freshwater biodiversity in the Anthropocene. *Curr Biol*. <https://doi.org/10.1016/j.cub.2019.08.002>
- Dudgeon D, Arthington AH, Gessner MO et al (2005) Freshwater biodiversity: importance, threats, status and conservation challenges. *Biol Rev* 81:163. <https://doi.org/10.1017/s1464793105006950>
- Durán JJ (2010) Patrimonio geológico: los ríos en roca de la Península Ibérica. Instituto Tecnológico GeoMinero de España, Madrid
- Eizaguirre C, Baltazar-Soares M (2014) Evolutionary conservation: evaluating the adaptive potential of species. *Evol Appl* 7(9):963–967. <https://doi.org/10.1111/eva.12227>
- Elvira B, Rincon PA, Velasco JC (1990) *Chondrostoma polylepis* Steindachner X *Rutilus lemmingii* (Steindachner) (Osteichthyes, Cyprinidae), a new natural hybrid from the Duero River basin, Spain. *J Fish Biol* 37:745–754. <https://doi.org/10.1111/j.1095-8649.1990.tb02538.x>
- Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* 14(8):2611–2620. <https://doi.org/10.1111/j.1365-294x.2005.02553.x>
- Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Mol Ecol Resour* 10:564–567. <https://doi.org/10.1111/j.1755-0998.2010.02847.x>
- Faulks LK, Kerezy A, Unmack PJ et al (2017) Going, going, gone? Loss of genetic diversity in two critically endangered Australian freshwater fishes, *Scaturiginichthys vermeilipinnis* and *Chlamydogobius squamigenus*, from Great Artesian Basin springs at Edgbaston, Queensland, Australia. *Aquatic Conserv* 27:39–50. <https://doi.org/10.1002/aqc.2684>
- Filipe AF, Lawrence JE, Bonada N (2012) Vulnerability of stream biota to climate change in mediterranean climate regions: a synthesis of ecological responses and conservation challenges. *Hydrobiologia*. <https://doi.org/10.1007/s10750-012-1244-4>
- Fluker BL, Kuhajda BR, Lang NJ, Harris PM (2010) Low genetic diversity and small long-term population sizes in the spring endemic watercress darter, *Etheostoma nuchale*. *Conserv Genet* 11:2267–2279. <https://doi.org/10.1007/s10592-010-0111-y>
- Fluker B, Jones K, Kuhajda B (2019) Genetic structure and diversity of the blueface darter *Etheostoma cyanoprosopum*, a microendemic freshwater fish in the southeastern USA. *Endanger Species Res* 40:133–147. <https://doi.org/10.3354/esr00986>
- Foll M, Gaggiotti O (2008) A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. *Genetics* 180:977–993. <https://doi.org/10.1534/genetics.108.092221>
- Frankham R, Ballou JD, Briscoe DA (2002) Introduction to conservation genetics. Cambridge University Press, Cambridge
- Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Mol Ecol* 10:2741–2752. <https://doi.org/10.1046/j.1365-294x.2001.t01-1-01411.x>
- Freyhof J, Brooks E (2017). European red list of freshwater fishes. IUCN
- Fu YX (1997) Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* 147:915–925
- Funk WC, McKay JK, Hohenlohe PA, Allendorf FW (2012) Harnessing genomics for delineating conservation units. *Trends Ecol Evol* 27:489–496. <https://doi.org/10.1016/j.tree.2012.05.012>
- Gaston KJ, Fuller RA (2009) The sizes of species' geographic ranges. *J Appl Ecol* 46:1–9. <https://doi.org/10.1111/j.1365-2664.2008.01596.x>
- Geist J (2010) Strategies for the conservation of endangered freshwater pearl mussels (*Margaritifera margaritifera* L.): a synthesis of Conservation Genetics and Ecology. *Hydrobiologia* 644:69–88. <https://doi.org/10.1007/s10750-010-0190-2>
- Henriques R, Sousa V, Coelho MM (2010) Migration patterns counteract seasonal isolation of *Squalius torgalensis*, a critically endangered freshwater fish inhabiting a typical Circum-Mediterranean small drainage. *Conserv Genet* 11:1859–1870. <https://doi.org/10.1007/s10592-010-0078-8>
- Hoffmann AA, Sgrò CM, Kristensen TN (2017) Revisiting adaptive potential, population size, and conservation. *Trends Ecol Evol* 32:506–517. <https://doi.org/10.1016/j.tree.2017.03.012>
- Hudson RR, Boss DD, Kaplan NL (1992) A statistical test for detecting geographic subdivision. *Mol Biol Evol*. <https://doi.org/10.1093/oxfordjournals.molbev.a040703>
- Huey JA, Balcombe SR, Real KM et al (2017) Genetic structure and effective population size of the most northern population of the Australian River Blackfish, *Gadopsis marmoratus* (Richardson 1848): implications for long-term population viability. *Freshw Sci* 36:113–123. <https://doi.org/10.1086/690557>
- Hunter M (1996) Fundamentals of conservation biology. Blackwell Science, Inc, Oxford, p 7
- Katoh K, Standley DM (2013) MAFFT multiple sequence alignment Software Version 7: improvements in performance and usability. *Mol Biol Evol* 30:772–780. <https://doi.org/10.1093/molbev/mst010>
- Kattawar J, Piller KR (2020) Comparative population genetics of the federally endangered Relict Darter, and its sister taxon the Clarks Darter (Teleostei: Percidae). *Conserv Genet* 21:957–970. <https://doi.org/10.1007/s10592-020-01300-7>
- Kearse M, Moir R, Wilson A et al (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28:1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>
- Kess T, Gross J, Harper F, Boulding EG (2015) Low-cost ddRAD method of SNP discovery and genotyping applied to the periwinkle *Littorina saxatilis*. *J Molluscan Stud*. <https://doi.org/10.1093/mollus/eyv042>
- Klunzinger MW, Lopes-Lima M, Gomes-Dos-Santos A et al (2020) Phylogeographic study of the West Australian freshwater mussel, *Westralunio carteri*, uncovers evolutionarily significant units that raise new conservation concerns. *Hydrobiologia*. <https://doi.org/10.1007/s10750-020-04200-6>
- Knapen G, Bervoets L, Verheyen E, Eens M (2003) Relationship between population size and genetic diversity in endangered

- populations of the European bullhead (*Cottus gobio*): implications for conservation. *Biol Cons* 115:403–410. [https://doi.org/10.1016/s0006-3207\(03\)00156-3](https://doi.org/10.1016/s0006-3207(03)00156-3)
- Kopelman NM, Mayzel J, Jakobsson M et al (2015) Clumpak: a program for identifying clustering modes and packaging population structure inferences across K. *Mol Ecol Resour* 15:1179–1191. <https://doi.org/10.1111/1755-0998.12387>
- Kottelat M, Freyhof J (2007) Handbook of European freshwater fishes. Kottelat, Berlin
- Leduc C, Pulido-Bosch A, Remini B (2017) Anthropization of groundwater resources in the Mediterranean region: processes and challenges. *Hydrogeol J* 25:1529–1547. <https://doi.org/10.1007/s10040-017-1572-6>
- Leigh JW, Bryant D (2015) Popart: full-feature software for haplotype network construction. *Methods Ecol Evol* 6:1110–1116. <https://doi.org/10.1111/2041-210x.12410>
- Levy A, Doadrio I, Almada VC (2009) Historical biogeography of European leuciscins (Cyprinidae): evaluating the Lago Mare dispersal hypothesis. *J Biogeogr* 36:55–65. <https://doi.org/10.1111/j.1365-2699.2008.01969.x>
- Lima SMQ, Berbel-Filho WM, Araújo TFP et al (2017) Headwater capture evidenced by paleo-rivers reconstruction and population genetic structure of the Armored Catfish (*Pareiorhaphis garbei*) in the Serra do Mar Mountains of Southeastern Brazil. *Front Genet*. <https://doi.org/10.3389/fgene.2017.00199>
- López-Martínez N (1989) Tendencias en Paleobiogeografía: El futuro de la biogeografía del pasado. *Paleontología* 10:271
- Lotterhos KE, Whitlock MC (2014) Evaluation of demographic history and neutral parameterization on the performance of FST outlier tests. *Mol Ecol* 23:2178–2192. <https://doi.org/10.1111/mec.12725>
- Maceda-Veiga A (2013) Towards the conservation of freshwater fish: Iberian Rivers as an example of threats and management practices. *Rev Fish Biol Fish* 23:1–22. <https://doi.org/10.1007/s1160-012-9275-5>
- Mameri D, Sousa-Santos C, Robalo JI, Magalhães MF (2018) Growth and age structure in captive and wild stocks of the endangered western ruivaco *Achondrostoma occidentale* (Cyprinidae). *Limnética* 37(1):105–115
- Mandimbahasina AR, Frasier CL, Hagenson RA et al (2019) Conservation genetics of Madagascar's critically endangered ploughshare tortoise (*Astrochelys yniphora*). *Conserv Genet* 21:109–121. <https://doi.org/10.1007/s10592-019-01236-7>
- Manubens J, Comas O, Valls N, Benjam L (2020) First captive breeding program for the endangered Pyrenean Sculpin (*Cottus hispaniolensis* Bacescu-Master, 1964). *Water* 12(11):2986. <https://doi.org/10.3390/w12112986>
- Marić S, Stanković D, Wanzenböck J et al (2017) Phylogeography and population genetics of the European mudminnow (*Umbra krameri*) with a time-calibrated phylogeny for the family Umbriidae. *Hydrobiologia* 792:151–168. <https://doi.org/10.1007/s10750-016-3051-9>
- Marić S, Stanković D, Šanda R et al (2019) Genetic characterisation of European mudminnow (*Umbra krameri*) populations from the Sava River system. *Knowl Manag Aquatic Ecosyst*. <https://doi.org/10.1051/kmae/2019035>
- McNeely JA, Miller KR, Reid WV et al (1990) Conserving the world's biological diversity. IUCN, Washington, DC
- Meirmans PG, Tienderen PHV (2004) Genotype and genodive: two programs for the analysis of genetic diversity of asexual organisms. *Mol Ecol Notes* 4:792–794. <https://doi.org/10.1111/j.1471-8286.2004.00770.x>
- Mesquita N, Hänfling B, Carvalho GR, Coelho MM (2005) Phylogeography of the cyprinid *Squalius aradensis* and implications for conservation of the endemic freshwater fauna of southern Portugal. *Mol Ecol* 14:1939–1954. <https://doi.org/10.1111/j.1365-294x.2005.02569.x>
- Morán-Tejeda E, López-Moreno JI, Vicente-Serrano SM et al (2012) The contrasted evolution of high and low flows and precipitation indices in the Duero basin (Spain). *Hydrol Sci J* 57:591–611. <https://doi.org/10.1080/02626667.2012.673722>
- Murphy N, Adams M, Guzik M, Austin A (2013) Extraordinary micro-endemism in Australian desert spring amphipods. *Mol Phylogenet Evol* 66:645–653. <https://doi.org/10.1016/j.ympev.2012.10.013>
- Pais J, Cunha PP, Pereira D, et al (2012) The Paleogene and Neogene of western Iberia (Portugal): a Cenozoic record in the European Atlantic domain. The Paleogene and Neogene of western Iberia (Portugal) Springer Briefs in Earth Sciences pp 1–138. https://doi.org/10.1007/978-3-642-22401-0_1
- Perea S, Böhme M, Zupančič P et al (2010) Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evol Biol* 10:265. <https://doi.org/10.1186/1471-2148-10-265>
- Perea S, Cobo-Simon M, Doadrio I (2016) Cenozoic tectonic and climatic events in southern Iberian Peninsula: implications for the evolutionary history of freshwater fish of the genus *Squalius* (Actinopterygii, Cyprinidae). *Mol Phylogenet Evol* 97:155–169. <https://doi.org/10.1016/j.ympev.2016.01.007>
- Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure from multilocus genotype data. *Genetics* 155:945–959. <https://doi.org/10.1093/genetics/155.2.945>
- Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) Tracer v1.6. <http://beast.community/tracer>
- Ramos-Fregonezi AMC, Malabarba LR, Fagundes NJR (2017) Population genetic structure of *Cnesterodon decemmaculatus* (Poeciliidae): a freshwater look at the Pampa Biome in Southern South America. *Front Genet*. <https://doi.org/10.3389/fgene.2017.00214>
- Reed DH, Frankham R (2003) Correlation between fitness and genetic diversity. *Conserv Biol* 17(1):230–237. <https://doi.org/10.1046/j.1523-1739.2003.01236.x>
- Robalo JI, Santos CS, Almada VC, Doadrio I (2006) Paleobiogeography of two Iberian endemic cyprinid fishes (*Chondrostoma arcasii*-*Chondrostoma macrolepidotus*) Inferred from mitochondrial DNA sequence data. *J Hered* 97:143–149. <https://doi.org/10.1093/jhered/esj025>
- Ronquist F, Teslenko M, Mark PVD et al (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst Biol* 61:539–542. <https://doi.org/10.1093/sysbio/sys029>
- Rozas J, Ferrer-Mata A, Sánchez-Delbarrio JC et al (2017) DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Mol Biol Evol* 34:3299–3302. <https://doi.org/10.1093/molbev/msx248>
- Salazar AR, Portero GG (2010) Los Arribes del Duero. Patrimonio geológico: Los ríos en roca de la Península Ibérica. *Serie Geología y Geofísica* 4:337–404
- Schönhuth S, Luikart G, Doadrio I (2003) Effects of a founder event and supplementary introductions on genetic variation in a captive breeding population of the endangered Spanish killifish. *J Fish Biol* 63:1538–1551. <https://doi.org/10.1111/j.1095-8649.2003.00265.x>
- Seifertová M, Bryja J, Vyskočilová M et al (2011) Multiple Pleistocene refugia and post-glacial colonization in the European chub (*Squalius cephalus*) revealed by combined use of nuclear and mitochondrial markers. *J Biogeogr* 39:1024–1040. <https://doi.org/10.1111/j.1365-2699.2011.02661.x>
- Shao J, Xiong D, Chu Z et al (2019) Population differentiation and genetic diversity of endangered *Brachymystax tsinlingensis* Li between Yangtze River and Yellow River in China based on

- mtDNA. Mitochondrial DNA Part A 30:695–701. <https://doi.org/10.1080/24701394.2019.1572130>
- Sousa V, Penha F, Collares-Pereira MJ et al (2008) Genetic structure and signature of population decrease in the critically endangered freshwater cyprinid *Chondrostoma lusitanicum*. *Conserv Genet* 9:791–805. <https://doi.org/10.1007/s10592-007-9399-7>
- Sousa V, Penha F, Pala I et al (2010) Conservation genetics of a critically endangered Iberian minnow: evidence of population decline and extirpations. *Anim Conserv* 13:162–171. <https://doi.org/10.1111/j.1469-1795.2009.00317.x>
- Sousa-Santos C, Robalo JJ, Pereira A, Doadrio I (2014) Threatened fishes of the world: *Achondrostoma salmantinum* Doadrio and Elvira, 2007 (Cyprinidae). *Croat J Fish* 72:128–129
- Sousa-Santos C, Jesus T, Fernandes C et al (2019) Fish diversification at the pace of geomorphological changes: evolutionary history of western Iberian Leuciscinae (Teleostei: Leuciscidae) inferred from multilocus sequence data. *Mol Phylogenet Evol* 133:263–285. <https://doi.org/10.1016/j.ympev.2018.12.020>
- Stephens M, Donnelly P (2003) A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *Am J Hum Genet* 73:1162–1169. <https://doi.org/10.1086/379378>
- Stephens M, Smith NJ, Donnelly P (2001) A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* 68:978–989. <https://doi.org/10.1086/319501>
- Stoeckle BC, Araujo R, Geist J et al (2017) Strong genetic differentiation and low genetic diversity of the freshwater pearl mussel (*Margaritifera margaritifera* L.) in the southwestern European distribution range. *Conserv Genet* 18:147–157. <https://doi.org/10.1007/s10592-016-0889-3>
- Stoessel DJ, Raadik TA, Nicol MD et al (2020) Captive breeding of two rare non-migratory galaxiids (Teleostei: Galaxiidae) for species conservation. *Proc R Soc Vict* 132:42. <https://doi.org/10.1071/rs20003>
- Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* 123:585–595. <https://doi.org/10.1093/genetics/123.3.585>
- Takács P, Erős T, Specziár A et al (2015) Population genetic patterns of threatened European Mudminnow (*Umbra krameri* Walbaum, 1792) in a fragmented landscape: implications for conservation management. *PLoS ONE*. <https://doi.org/10.1371/journal.pone.0138640>
- Tamura K, Stecher G, Peterson D et al (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* 30:2725–2729. <https://doi.org/10.1093/molbev/mst197>
- Tan M, Armbruster JW (2018) Phylogenetic classification of extant genera of fishes of the order Cypriniformes (Teleostei: Ostariophysi). *Zootaxa*. <https://doi.org/10.11646/zootaxa.4476.1>
- Van Leeuwen CHA, Dalen K, Museth J, Junge C, Vollestad LA (2017) Habitat fragmentation has interactive effects on the population genetic diversity and individual behaviour of a freshwater salmonid fish. *River Res Appl* 34(1):60–68
- Velasco JC, Peris SJ, Pollo CJ, González N (1997) Los peces de la Provincia de Salamanca: atlas de distribución. Universidad de Salamanca, Salamanca
- Velasco JC, Mateos LA, Mateos JM (2019) Manual técnico sobre la cría en cautividad de peces endémicos de interés comunitario. Junta de Castilla y León. Confederación Hidrográfica del Duero, España
- Wetjen M, Hübner D, Seehausen O, Schulz R (2020) Genetic diversity of endangered *Chondrostoma nasus* in the River Rhine system: conservation genetics considerations on stocking and reintroduction. *Knowl Manag Aquat Ecosyst*. <https://doi.org/10.1051/kmae/2020016>
- Wu TH, Tsang LM, Chen I-S, Chu KH (2016) Multilocus approach reveals cryptic lineages in the goby *Rhinogobius duospilus* in Hong Kong streams: role of paleodrainage systems in shaping marked population differentiation in a city. *Mol Phylogenet Evol* 104:112–122. <https://doi.org/10.1016/j.ympev.2016.07.014>
- Zardoya R, Doadrio I (1998) Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. *Proc R Soc Lond B* 265:1365–1372. <https://doi.org/10.1098/rspb.1998.0443>
- Zheng X, Levine D, Shen J et al (2012) A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics* 28:3326–3328. <https://doi.org/10.1093/bioinformatics/bts606>

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Material Suplementario

Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585-600.

High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio & Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers

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Table S1 Genetic diversity of the mitochondrial (*MT-CYB*) and nuclear (*RAG1*) genes. The following parameters are included in the table: N: number of analysed samples; h: haplotype number; Hd: haplotype diversity; π : nucleotide diversity; S: number of polymorphic sites. Results of the neutrality tests Tajima's D and Fu's FS are also included for *MT-CYB* (*p < 0.05; **p < 0.01; ***p < 0.001)

GENERAL	<i>MT-CYB</i>							<i>RAG1</i>				
	N	h	Hd	π	S	Tajima's D	Fu's FS	N	h	Hd	π	S
	168	27	0.82	22.9 10 ⁻⁴	30	-1.56*	-14.1***	302	23	0.64	6.3 10 ⁻⁴	24
RIVER	N	h	Hd	π	S	Tajima's D	Fu's FS	N	h	Hd	π	S
Cabeza de Iruelos River	15	4	0.67	11.6 10 ⁻⁴	4	-0.03	0.11	16	4	0.52	4.6 10 ⁻⁴	3
Uces River	14	2	0.14	1.4 10 ⁻⁴	1	-1.16	-0.59	28	6	0.39	3.5 10 ⁻⁴	5
Camaces River	21	2	0.38	3.6 10 ⁻⁴	1	0.66	0.94	42	2	0.47	3.4 10 ⁻⁴	1
Huebra River	19	2	0.11	1 10 ⁻⁴	1	-1.17	-0.84	34	6	0.6	5.5 10 ⁻⁴	6
Arganza River	21	9	0.72	10.5 10 ⁻⁴	9	-1.89*	-5.99***	40	7	0.47	4.4 10 ⁻⁴	6
Yeltes River	15	5	0.56	7.4 10 ⁻⁴	5	-1.66*	-2.17*	26	3	0.48	5.3 10 ⁻⁴	3
Gavilanes River	20	7	0.58	8.3 10 ⁻⁴	7	-1.84*	-4.09***	42	6	0.54	5.7 10 ⁻⁴	7
Tenebrilla River	5	2	0.4	3.8 10 ⁻⁴	1	-0.82	0.09	10	2	0.36	2.5 10 ⁻⁴	1
Turones River	17	2	0.01	1.1 10 ⁻⁴	1	-1.16	-0.75	26	5	0.6	5.9 10 ⁻⁴	5
Dos Casas River	21	1	0	0	0	0	0	40	3	0.45	3.3 10 ⁻⁴	2
SUB-BASIN	N	h	Hd	π	S	Tajima's D	Fu's FS	N	h	Hd	π	S
Uces Sub-basin	29	4	0.46	7.2 10 ⁻⁴	4	-0.67	-0.3	44	8	0.43	3.9 10 ⁻⁴	7
Huebra Sub-basin	101	23	0.82	23.3 10 ⁻⁴	26	-1.53*	-11.7***	192	16	0.68	7.1 10 ⁻⁴	17
Águeda Sub-basin	38	2	0.05	0.5 10 ⁻⁴	1	-1.13	-1.41*	42	7	0.58	5.5 10 ⁻⁴	7
LINEAGE	N	h	Hd	π	S	Tajima's D	Fu's FS	N	h	Hd	π	S
H1	42	2	0.05	0.5 10 ⁻⁴	1	-1.12	-1.49*	72	7	0.21	4.4 10 ⁻⁴	7
H2	21	2	0.38	3.6 10 ⁻⁴	1	0.66	0.94	42	2	0.47	3.4 10 ⁻⁴	1
H3	65	12	0.41	4.9 10 ⁻⁴	12	-2.25***	-13***	112	13	0.19	4.5 10 ⁻⁴	12
H4	40	11	0.54	7.4 10 ⁻⁴	12	-2.24***	-9.39***	76	7	0.5	5.2 10 ⁻⁴	9

Table S2 Uncorrected p-distances between the genetic groups found with the mitochondrial marker *MT-CYB* (below the diagonal). The standard deviation is shown in the upper diagonal. See Fig. 2 for the assignation of the groups

	H1	H2	H3	H4	H5
H1	-	0.001	0.001	0.002	0.008
H2	0.002	-	0.001	0.002	0.008
H3	0.003	0.002	-	0.002	0.008
H4	0.006	0.005	0.005	-	0.007
H5	0.073	0.071	0.071	0.070	-

Table S3 Pairwise Φ_{ST} values of the *A. salmantinum* populations. Only significant values after Bonferroni correction are shown ($p < 0.001$). In green, $0 < \Phi_{ST} < 0.25$; in yellow, $0.25 < \Phi_{ST} < 0.5$; in orange, $0.5 < \Phi_{ST} < 0.75$; in red, $\Phi_{ST} > 0.75$

	Tenebrilla	Uces	Cabeza de Iruelos	Turones	Yeltes	Huebra	Arganza	Camaces	Dos Casas	Gavilanes
Tenebrilla										
Uces	0.94									
Cabeza de Iruelos	0.75									
Turones	0.97	0.94	0.63							
Yeltes		0.86	0.76	0.92						
Huebra	0.95			0.95	0.88					
Arganza	0.75		0.11	0.75	0.76					
Camaces	0.91	0.78	0.47	0.80	0.88	0.81	0.59			
Dos Casas	0.99	0.97	0.68		0.94	0.98	0.79	0.85		
Gavilanes		0.84	0.76	0.91		0.86	0.75	0.87	0.92	

Table S4 Results of the AMOVA analyses for groups established according to three different levels (see text for details). Significance: * $p < 0.05$ ** $p < 0.01$; *** $p < 0.001$; df = degrees of freedom

	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices		
						FST	FSC	FCT
Group 1: Sub-basins	Among groups	2	66.94	0.42	28.58			
	Among populations within groups	7	93.08	0.79	53.85***			
	Within populations	158	40.72	0.26	17.57***	0.82	0.75	0.28
	Total	167	200.74	1.47				
Group 2: Lineages	Among groups	3	164.33	1.36	85.95***			
	Among populations within groups	7	1.67	0.001	0.08			
	Within populations	157	34.74	0.22	13.97***	0.86	0.006	0.86
	Total	167	200.74	1.58				
Group 3: Rivers	Among populations	9	160.01	1.05	80.32***			
	Within populations	158	40.72	0.26	19.68	0.80		
	Total	167	200.74	1.31				

Table S5 Pairwise *FST* values between populations. Only significant values are shown ($p < 0.05$). In green, $0 < FST < 0.25$; in yellow, $0.25 < FST < 0.5$; in orange, $0.5 < FST < 0.75$

	Camaces	Cabeza de Iruelos	Dos Casas	Gavilanes	Huebra	Uces	Yeltes
Camaces							
Cabeza de Iruelos	0.205						
Dos Casas	0.268	0.393					
Gavilanes	0.509	0.482	0.640				
Huebra	0.144	0.079	0.333	0.440			
Uces	0.157	0.058	0.346	0.441	0.020		
Yeltes	0.545	0.528	0.631	-	0.492	0.494	

Table S6 Parameters of genetic variability for the SNP data. Values are given for both variant positions (VP) and all positions (variant and fixed; AP) as indicated in the Type column. Private: number of private alleles; Sites: number of loci; Var Sites: number of variable loci; P: number of polymorphic sites; %P: percentage of polymorphic sites based on total sites; Ho: mean observed heterozygosity; He: mean expected heterozygosity; π : mean of nucleotide diversity; FIS: mean of the inbreeding coefficient. The columns with the letter “V” before each parameter indicates the variance

Locality	Type	Private	Sites	Var Sites	P	%P	Ho	VHo	He	VHe	π	V π	FIS	VFIS
Yeltes River	VP	21	4123	4123	2244	54.43	0.157	0.041	0.157	0.035	0.162	0.037	0.017	0.038
	AP	21	1269089	4123	2244	0.18	0.0005	0.0002	0.0005	0.0002	0.0005	0.0002	0.0001	0.0001
Gavilanes River	VP	0	4121	4121	1816	44.07	0.167	0.056	0.154	0.037	0.172	0.046	0.012	0.058
	AP	0	1267884	4121	1816	0.14	0.0005	0.0003	0.0005	0.0002	0.0006	0.0002	0.00003	0.0002
Cabeza de Iruelos River	VP	3	4123	4123	2575	62.45	0.214	0.05	0.218	0.039	0.232	0.045	0.046	0.073
	AP	3	1269089	4123	2575	0.20	0.0007	0.0003	0.0007	0.0003	0.0008	0.0003	0.00015	0.0002
Uces River	VP	0	4123	4123	2895	70.21	0.245	0.051	0.235	0.036	0.25	0.041	0.013	0.067
	AP	0	1269089	4123	2895	0.23	0.0008	0.0004	0.0008	0.0003	0.0008	0.0003	0.00006	0.0002
Huebra River	VP	0	4123	4123	2942	71.35	0.246	0.05	0.235	0.035	0.25	0.04	0.011	0.065
	AP	0	1269089	4123	2942	0.23	0.0008	0.0004	0.0008	0.0003	0.0008	0.0003	0.00004	0.0002
Camaces River	VP	0	4122	4122	2192	53.18	0.208	0.064	0.188	0.039	0.21	0.049	0.006	0.069
	AP	0	1267762	4122	2192	0.17	0.0007	0.0004	0.0006	0.0002	0.0007	0.0003	0.00001	0.0002
Dos Casas River	VP	38	4123	4123	1553	37.67	0.132	0.042	0.129	0.035	0.133	0.037	0.003	0.027
	AP	38	1269089	4123	1553	0.12	0.0004	0.0002	0.0004	0.0002	0.0004	0.0002	0.00001	0.0001

III

CAPÍTULO

Cambios geomorfológicos y climáticos cuaternarios asociados a la diversificación de los peces ibéricos de agua dulce: el caso del género *Cobitis* (Cypriniformes, Cobitidae)



Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12(3), e8635.

RESEARCH ARTICLE

Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae)

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Funding information

Industrial Doctorate of Community of Madrid, Grant/Award Number: IND2017/AMB-7699; Duero Hydrographic Confederation, Grant/Award Number: Life13 nat/es/000772

Abstract

We studied the population genetic structure of *Cobitis vettonica*, an endangered freshwater fish species endemic to the Iberian Peninsula, in order to propose a biogeographic model of the responses of species to the multiple changes that occurred in the Iberian hydrological system during the Quaternary period. We also deciphered the relationship of *C. vettonica* with its sister species *C. paludica*, particularly in sympatric areas, and provide genetic information for conservation purposes. To achieve this goal, we analyzed both mitochondrial and nuclear data (the *cytochrome b* and the *nuclear recombination activating 1* genes) and a battery of single-nucleotide polymorphisms (SNPs) of 248 individuals of *C. vettonica* or *C. paludica* from 38 localities, including some sympatric ones, covering the entire distribution area of *C. vettonica*. We highlight the important role played by the hydrogeomorphological processes and climatic changes that occurred in the Iberian Peninsula during the Quaternary on both the population structure of *C. vettonica* and its relationship with its sister species *C. paludica*. Our results support the genetic introgression of populations at the eastern limit of the distribution of *C. vettonica*. Furthermore, we postulate genetic introgression in sympatric areas. Finally, we propose the establishment or expansion of four Operational Conservation Units (OCUs) for *C. vettonica*, and highlight the threat faced by its populations due to the low level of genetic diversity detected for some of its populations and genetic introgression with *C. paludica*, which could eventually displace *C. vettonica*, resulting in a loss of diversity in this species.

KEYWORDS

Cobitis, hybridization, Iberian Peninsula, population structure and conservation, quaternary

TAXONOMY CLASSIFICATION

Population genetics

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1 | INTRODUCTION

The Quaternary is a geological period characterized by glacial-interglacial cycles that have dominated the global climate since 2.58 Mya to the present era (Gibbard et al., 2010; Pillans & Naish, 2004), which have had drastic consequences on the evolution of the biota of many regions. Due to advances in the field of phylogeography, we have a better understanding of the responses of organisms to Pleistocene events (Hewitt, 2004; Weiss & Ferrand, 2007). Classical studies support the role of Mediterranean peninsulas as refuges for fauna during the Quaternary, which provided the stock for recolonizations of northern and central Europe (Hewitt, 1996; Taberlet & Bouvet, 1994). The Iberian Peninsula is considered one of the most important refuges during this period, as shown by several studies, mainly of terrestrial fauna (Querejeta et al., 2017; Valdiosera et al., 2008).

Primary freshwater fishes (i.e., fishes strictly intolerant of salt water; Myers, 1966) have limited dispersal abilities and are often restricted to specific hydrographic basins. Dispersal between basins during the Quaternary was possible mainly through downstream connections caused by the decrease in sea level during cold periods or by upstream piracy (Corral-Lou et al., 2019; Mesquita et al., 2005; Perea & Doadrio, 2015). The formation of the Iberian fluvial network also culminated during this period, which affected the region's hydrogeomorphology and therefore the current evolutionary patterns found in primary freshwater fauna (Alonso et al., 2007; Corral-Lou et al., 2021; Pais et al., 2012; Perea et al., 2016). Likewise, the current population structure of many Iberian freshwater fishes has been attributed to the interaction of various natural factors that occurred during the Quaternary such as the drying up of bodies of water, sea-level fluctuations, tsunamis, stream piracy, isolation of basins, hydrogeomorphological changes, and secondary contact of two different basins (Casal-López et al., 2017; Corral-Lou et al., 2019; Gante et al., 2009; Perea et al., 2016).

Despite efforts made in the last several decades, there is still much to be learned about the impact of Quaternary changes on the evolutionary processes and patterns of diversification of Iberian freshwater fish populations. Especially those species with a restricted distribution range since they were probably more affected by climatic and geological changes during the Quaternary than species with larger ranges. In order to address some open evolutionary questions, such as the role of hydrogeomorphologic changes and genetic introgression in species evolution, we analyzed populations of *Cobitis vettonica* as a case study. This species is an endangered Iberian freshwater fish whose distribution is restricted to a few rivers in the Tagus and Duero basins in the mid-western Iberian Peninsula (Figure 1). It inhabits rivers with low pH and water hardness levels, and gravel and rocky bottoms, and is more commonly found in the headwaters of these rivers (Carmona et al., 1999; Collares-Pereira et al., 2021; Doadrio et al., 2011; Perdices & Coelho, 2020). In contrast, the sister species of *C. vettonica*, *Cobitis paludica* (Doadrio & Perdices, 2005; Perdices & Doadrio, 2001), is a generalist that inhabits most of the Iberian drainages including Tagus and

Douro, with a preference for streams close to the main channel with high suspended solids, high water hardness, low transparency, low current velocity and muddy bottoms (Carmona et al., 1999; Doadrio et al., 2011). Sympatric zones of *C. vettonica* and *C. paludica* have been reported in the western limits of the distribution area of *C. vettonica* but genetic introgression between them has not been reported (Perdices & Coelho, 2020). However, in some eastern populations there has been mention of genetic introgression between *C. vettonica* and *C. paludica*, but no more information has been detailed (Doadrio et al., 2011, 2021). Sister species of Iberian freshwater fishes generally have an allopatric distribution that was established mainly before the Quaternary (Doadrio, 1988; Sousa-Santos et al., 2019). Some species now show patterns of sympatry in certain areas as a consequence of secondary contacts during the Quaternary, which has led to genetic introgression, as is the case of some species within the genera of *Luciobarbus* and *Phoxinus* (Corral-Lou et al., 2019; Denys et al., 2013; Machordom et al., 1990). In the case of *C. vettonica* and *C. paludica*, phylogenetic studies have shown they diverged during the Plio-Pleistocene (Doadrio & Perdices, 2005; Sousa-Santos et al., 2014). However, both the origin of the sympatric zones between *C. vettonica* and *C. paludica* and the plausible genetic introgression between them in the western distribution area of *C. vettonica* are unknown. All these points make fishes with restricted distribution areas as excellent models to decipher the Quaternary effects on the diversification of freshwater-restricted taxa within a glacial refuge as the Iberian Peninsula (Gante et al., 2009).

Phylogeographic studies of Iberian primary freshwater fishes have mainly used mitochondrial genes combined with nuclear markers such as microsatellites or introns (Casal-López & Doadrio, 2018; Corral-Lou et al., 2019; Gonzalez et al., 2014, 2018; Perea & Doadrio, 2015). In recent years, phylogeographic and population genetics studies have taken advantage of next-generation sequencing (NGS) technologies to broadly screen the genome at high resolution, yet some challenges remain in the analysis of NGS

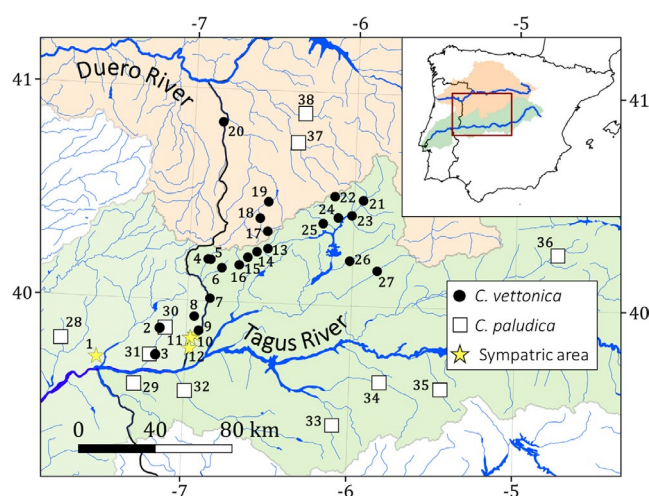


FIGURE 1 Sampling localities included in this study. The orange shading delimits the Duero Basin, and the green shading delimits the Tagus Basin. Numbers correspond to those listed in Table 1

data such as the handling of large-scale and complex data, the upstream process of pipeline, and the limited number of available reference genomes (McCormack et al., 2013; Tan et al., 2019). Combined analyses of single-nucleotide polymorphisms (SNPs) with mitochondrial and nuclear markers have proven useful to provide more complete and more detailed phylogeographic and biogeographic models of the past and present relationships of populations of various species (Corral-Lou et al., 2021; Mendes et al., 2019; Zarraonaindia et al., 2012).

The main aim of this study is to provide a robust biogeographic model for the species *C. vettonica* throughout its entire distribution as a witness of the evolution of the Iberian Peninsula throughout the Quaternary. We also decipher the relationship of *C. vettonica* with its sister species *C. paludica* at the limits of its distribution area. In addition, we assess the population structure and genetic diversity of the species across its distribution to revise the Operational Conservation Units (OCUs; Doadrio et al., 1996) previously established for *C. vettonica* (Doadrio et al., 2021) or to establish new ones. More detailed and accurate OCUs and deciphering the relationship with its sister species throughout its entire range are key for the effective management and conservation of the endangered *C. vettonica*. To achieve these goals, we analyzed the mitochondrial marker *cytochrome b* (MT-CYB), the nuclear marker *recombination activating gene 1* (RAG1), and a set of SNPs obtained through next-generation sequencing of populations throughout the entire distribution range of *C. vettonica* and some of *C. paludica* from adjacent sub-basins.

2 | MATERIALS AND METHODS

2.1 | Sampling, DNA extraction, amplification and sequencing

We sampled 204 individuals of *C. vettonica* and 60 of *C. paludica* from a total of 38 localities (Figure 1; Table 1). The 27 localities (23 in the Tagus and 4 in the Duero) in which *C. vettonica* was found cover the entire known distribution area of the species, and includes its type locality (Árrago River, Tagus Basin). The samples of *C. paludica* were collected from 13 localities, including its type locality (Tiétar River, Tagus Basin): of these, 11 were either adjacent to those of *C. vettonica* or in sympatric localities in the Tagus Basin, and two were in the Duero Basin (Figure 1; Table 1). Tissue samples were obtained from the DNA and Tissue Collection at the National Museum of Natural Sciences of Madrid (MNCN-CSIC; Table S1). Sequences of MT-CYB of *C. vettonica* and *C. paludica* available in GenBank were also included in the study (33 of *C. vettonica* and 7 of *C. paludica*; Table S1).

For each individual, DNA was extracted from ventral fin tissue using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA), following the manufacturer's protocol. Polymerase chain reaction (PCR) was used to amplify 1140 bp of MT-CYB from 208 individuals (164 of *C. vettonica* and 44 of *C. paludica*),

and 1500 bp of RAG1 from 90 individuals (66 of *C. vettonica* and 24 of *C. paludica*) (Table S1). Amplifications were performed following the protocol described by Doadrio and Perdices (2005) for MT-CYB, and Corral-Lou et al. (2021) for RAG1.

All sequences (the new ones and the ones downloaded from GenBank; Table S1) for MT-CYB (248 sequences, 240 from the present study and 40 from GenBank; Table S1) and RAG1 (90 sequences from the present study; Table S1) were aligned using MAFFT (Katoh & Standley, 2013), as implemented in Geneious 10.1.3 (<https://www.geneious.com>; Kearse et al., 2012), and then manually examined. Alleles of RAG1 were separated using the PHASE algorithm (Stephens & Donnelly, 2003), as implemented in DnaSP v. 6.10.01 (Rozas et al., 2017).

2.2 | Genotyping and SNP filtering

For the SNP study, we selected eight populations of each *C. vettonica* and *C. paludica* (Table 1) based on the structure observed in a previous study using mitochondrial data and available DNA (Doadrio et al., 2021). A total of 106 individuals (60 of *C. vettonica* and 46 of *C. paludica*; Table S1) were used to prepare libraries for double digest restriction site-associated DNA sequencing (ddRAD-seq) following the protocol described by Kess et al. (2016). All DNA samples were used as an input for a custom library preparation protocol. Libraries were dual-indexed for postsequencing demultiplexing. The samples were run in a NovaSeq 6000 PE150 lane. Trimmomatic 0.36 (Bolger et al., 2014) was used to remove adapters (ILLUMINACLIP option). Using the *process_radtags* program in STACKS 2.4 (Catchen et al., 2013), all reads were truncated to the same length of 95 bp, and low-quality reads were removed using the *-q* parameter according to *phred33* system. A *de novo* map analysis was also performed in STACKS, in which different programs were run to assemble loci in each individual (*ustacks*), build a catalogue (*cstacks*), match all generic samples against the catalogue (*sstacks*), and reconstruct loci using R2 reads and identify SNPs using the metapopulation information (*gstacks*). Prior to running the STACKS modules, several tests were used to identify which parameters maximized the number of SNPs obtained in at least 80% of the individuals (*r80* rule; Paris et al., 2017). Since SNPs were obtained for both species, we explored parameters in a conservative way in the *de novo* maps module of stacks. The parameter *m* (i.e., the minimum depth of coverage required to create a stack) was set to 5 due to the depth of coverage values obtained. The *M* parameter (i.e., number of mismatches allowed between stacks within individuals) has to be carefully set. If it is set too high, paralogous or nonhomologous loci can be incorrectly merged into the same locus and if it is set too low, homologous loci can be lost. For this reason, the parameter *M* was explored between 3 and 5. Following the indications of Paris et al. (2017), the values of *n* (i.e., the number of mismatches allowed between stacks between individuals) were explored from 2 to 4 (Table S2). Finally, the selected parameters were *m* = 5, *M* = 3, and *n* = 2. The

TABLE 1 Information on the sampling localities included in the present study

No	Species	River	Basin	Sub-basin	Locality	Province	mtDNA	nDNA	SNPs
1	Sympatric area	Alfrividas	Tagus	Ponsul	Alfrividas	Portugal	4/1	–	–
2	<i>C. vettonica</i>	Aravil	Tagus	Aravil	Alcafozes	Portugal	1	–	–
3	<i>C. vettonica</i>	Aravil	Tagus	Aravil	Cegonhas Novas	Portugal	4	–	–
4	<i>C. vettonica</i>	Erjas	Tagus	Upper Erjas	Valverde del Fresno	Spain	12	9?	5
5	<i>C. vettonica</i>	San Martin	Tagus	Upper Erjas	San Martín de Trevejo	Spain	6	1	–
6	<i>C. vettonica</i>	Trevejana	Tagus	Upper Erjas	Cilleros	Spain	4	4	–
7	<i>C. vettonica</i>	Erjas	Tagus	Middle Erjas	Termas de Monfortinho	Portugal	6	–	–
8	<i>C. vettonica</i>	Arades	Tagus	Middle Erjas	Salvaterra do Extremo	Portugal	2	–	–
9	<i>C. vettonica</i>	Erjas	Tagus	Lower Erjas	Salvaterra do Extremo	Portugal	2	–	–
10	Sympatric area	Erjas	Tagus	Lower Erjas	Cabeça Queimado	Portugal	1/1	–	–
11	Sympatric area	Erjas	Tagus	Lower Erjas	Azenha do Roque	Portugal	4/1	–	–
12	Sympatric area	Erjas	Tagus	Lower Erjas	Serrinha	Portugal	2/1	–	–
13	<i>C. vettonica</i>	Árrago	Tagus	Western Alagón	Cadalso de Gata	Spain	15	3?	–
14	<i>C. vettonica</i>	Gata	Tagus	Western Alagón	Gata	Spain	19	5	5
15	<i>C. vettonica</i>	San Blas	Tagus	Western Alagón	Gata	Spain	1	–	–
16	<i>C. vettonica</i>	Acebo	Tagus	Western Alagón	Hoyos	Spain	3	1	–
17	<i>C. vettonica</i>	Mayas	Duero	Águeda	Descargamaría	Spain	2	–	–
18	<i>C. vettonica</i>	Mayas	Duero	Águeda	El Sahugo	Spain	20	4	10
19	<i>C. vettonica</i>	Águeda	Duero	Águeda	La Herguijuela	Spain	1	–	–
20	<i>C. vettonica</i>	Turones	Duero	Águeda	La Bouza	Spain	1	1	–
21	<i>C. vettonica</i>	Alagón	Tagus	Eastern Alagón	Santi Ibañez de la Sierra	Spain	14	9	4
22	<i>C. vettonica</i>	Francia	Tagus	Eastern Alagón	Nava de Francia	Spain	14	5	8
23	<i>C. vettonica</i>	Cuerpo de Hombre	Tagus	Eastern Alagón	Sotoserrano	Spain	19	9	10
24	<i>C. vettonica</i>	Ladrillar	Tagus	Eastern Alagón	La Rebollosa	Spain	1	–	–
25	<i>C. vettonica</i>	Hurdano	Tagus	Eastern Alagón	Vegas de Coria	Spain	1	1	–
26	<i>C. vettonica</i>	Caparro	Tagus	Eastern Alagón	Zarza de Granadilla	Spain	23	7	8
27	<i>C. vettonica</i>	Jerte	Tagus	Eastern Alagón	Navaconcejo	Spain	15	5	10
28	<i>C. paludica</i>	Alvito	Tagus	Ocreza	Monte Gordo	Portugal	1	–	–
29	<i>C. paludica</i>	Aurela	Tagus	Salor	Santiago de Alcántara	Spain	5	5	9
30	<i>C. paludica</i>	Toula	Tagus	Aravil	Alcafozes	Portugal	1	–	–
31	<i>C. paludica</i>	Aravil	Tagus	Aravil	Cegonhas Novas	Portugal	1	–	–
32	<i>C. paludica</i>	Salor	Tagus	Salor	Membrío	Spain	2	–	2
33	<i>C. paludica</i>	Tamuja	Tagus	Almonte	Trujillo	Spain	4	–	4

TABLE 1 (Continued)

No	Species	River	Basin	Sub-basin	Locality	Province	mtDNA	nDNA	SNPs
34	<i>C. paludica</i>	Almonte	Tagus	Almonte	Jaraicejo	Spain	5	5	5
35	<i>C. paludica</i>	Ibor	Tagus	Lower Tagus	Castañar de Ibor	Spain	3	–	3
36	<i>C. paludica</i>	Tiétar	Tagus	Tiétar	La Iglesuela del Tiétar	Spain	11	5	6
37	<i>C. paludica</i>	Yeltes	Duero	Huebra-Yeltes	San Martín de Yeltes	Spain	5	5	8
38	<i>C. paludica</i>	Huebra	Duero	Huebra-Yeltes	El cubo de don Sancho	Spain	9	4	9

Notes: Also detailed are the number of individuals studied for each of the marker types: mtDNA (MT-CYB), nDNA (RAG1), and SNPs. Under the mtDNA column, for each sympatric area, the two numbers indicate the number of individuals analyzed for *C. vettonica* and *C. paludica*, respectively.

program *populations*, also in STACKS, was used to filter the SNPs using the following parameters: $-p\ 16$ $-r\ 0.80$, $-min_maf = 0.05$, $-max_obs_het = 0.75$, and $-write_random_snp$.

Two matrices for the SNPs were built. The allowed percentage of missing data per locus and per individual was 30% (Table S3). The first matrix included the populations of *C. vettonica* and data from 60 individuals from 8 localities and 4000 polymorphic loci. The second included populations of both *C. vettonica* and *C. paludica*, referred to as *C. vettonica* + *C. paludica*, and data from 106 individuals from 16 localities and 4538 polymorphic loci.

2.3 | Phylogeny

A phylogenetic tree was constructed to assess relationships based on the collapsed MT-CYB haplotypes matrix. The selected substitution models were SYM, HKY + I, and GTR + G for the first, second, and third position, respectively, based on the results obtained in PartitionFinder2 (Lanfear et al., 2017) using the Akaike information criterion (AIC; Akaike, 1974). The analysis was implemented in MrBayes v3.2 (Ronquist et al., 2012), with two simultaneous independent runs each with four Markov chain Monte Carlo (MCMC), which were run for 5×10^7 generations. The first 25% of generations were removed as burn-in. Posterior probability (pp) values were used to assess the reliability of the phylogenetic hypothesis. Two sequences of other species of *Cobitis* were used as outgroups: *C. bilineata* (EF605321.1) and *C. zanandreae* (AF263089.1). The genetic divergence between the lineages obtained in the MT-CYB analysis was evaluated through uncorrected *p*-distances with 1000 permutations using MEGA v.6.0 (Tamura et al., 2013). For the SNP data, phylogenetic relationships were evaluated using the complete SNP matrix (*C. vettonica* + *C. paludica*) through the Maximum Likelihood (ML) method in the RaxML program implemented in CIPRES Science Gateway v3.3. The evolutionary model selected was ASC_GTRGAMMA, as recommended in the program manual for data that contain only variable sites, and the Lewis ascertainment bias correction was used (Stamatakis, 2016). The robustness of the tree was evaluated with 1000 bootstrap (b) replicates.

2.4 | Genetic structure

To examine the genetic structure of all studied populations of *C. vettonica* and *C. paludica*, haplotype networks for the two genes were constructed using the median-joining algorithm (Bandelt et al., 1999) implemented in PopART (Leigh & Bryant, 2015). Analysis of molecular variance (AMOVA) was used to determine the source of the genetic variation in MT-CYB for the populations identified as *C. vettonica* (excluding V4 subset, see below) using different groupings based on basin, sub-basin, and evolutionary lineage. These analyses were implemented in Arlequin v.3.11 (Excoffier & Lischer, 2010) with 10,000 permutations. Population differentiation in terms of Φ_{st} (Hudson et al., 1992) between all population pairs (except those represented by only one individual) for the complete dataset (*C. vettonica* and *C. paludica*) was also calculated in Arlequin v.3.11. For the localities in which mitochondrial haplotypes of both *C. vettonica* and *C. paludica* were found, the Φ_{st} was calculated by treating the individuals of each species as separate populations.

For the SNPs data, only neutral loci were taken into account to study the population structure in order to avoid the bias that could be caused by candidate loci for selection. For this reason, we evaluated the presence of loci under selection with BayeScan v.2.0 (Foll & Gaggiotti, 2008), using the default parameters except for the prior odds (prior odds = 100; Lotterhos & Whitlock, 2014). We removed the loci under selection and constructed two new matrices for the neutral loci, keeping 3998 loci in the *C. vettonica* matrix and 4500 loci in the *C. vettonica* + *C. paludica* matrix. STRUCTURE (Pritchard et al., 2000) was used to assess the genetic structure based on the two SNP matrices. The most probable number of subpopulations (*K*) for each analysis was equal to the number of populations studied + 1 (*K* = 9 and *K* = 17, respectively). We performed 10 independent simulations for each *K* with a burn-in length of 50,000 and 50,000 MCMC repetitions after the burn-in. The most probable number of subpopulations for each matrix was estimated by taking into account the results of both the ΔK (Evanno et al., 2005) and the Puechmaille method (Puechmaille, 2016). The web server StructureSelector (Li & Liu, 2018) was used to make these estimations. We selected *K* based on both methods because a greater probability of *K* = 2 exists when the structure is analyzed with the ΔK

method (Janes et al., 2017), and similar results for different methods indicate a clear signal (Puechmaille, 2016).

2.5 | Genetic diversity and demography

Different genetic diversity parameters were estimated for both *MT-CYB* and *RAG1* in DnaSP for each river, sub-basin, species, evolutionary lineage, and OCUs. The studied genetic diversity parameters were haplotype or nuclear alleles number (h or a for *MT-CYB* and *RAG1*, respectively), haplotype or allelic diversity (H_d or a_d for *MT-CYB* and *RAG1*, respectively), nucleotide diversity (π), and number of polymorphic sites (S). For *MT-CYB*, the populations with shared haplotypes between *C. vettonica* and *C. paludica* were analyzed considering only the individuals identified as *C. vettonica*. For the SNP data, a different set of genetic diversity parameters was estimated using the *populations* program in STACKS 2.4 (Catchen et al., 2013). These parameters were evaluated for the two matrices independently (*C. vettonica* and *C. vettonica* + *C. paludica*); however, only the results for *C. vettonica* are shown. In addition, a third matrix was studied taking into account the populations of both species, except the populations of the Aurela and Salor rivers of *C. paludica*, due to the close relationship that these localities showed with the localities of the Erjas sub-basin of *C. vettonica*.

To evaluate the demography of populations of *C. vettonica*, deviations from a model of mutation-drift equilibrium for *MT-CYB* were tested using Fu's F_s (Fu, 1997) and Tajima's D (Tajima, 1989), as implemented in Arlequin v.3.11.

2.6 | Divergence times and niche modeling

Divergence times among the populations of *C. vettonica* were estimated using a relaxed lognormal clock and a coalescent model on the collapsed *MT-CYB* haplotypes matrix in BEAST v 1.8.4 (Drummond et al., 2012). The molecular clock was calibrated using, as a normal prior, an evolutionary rate of 0.34% divergence per lineage per million years (Doadrio & Perdices, 2005), and the substitution model used was GTR + I.

To identify the potential niche of *C. vettonica* in both past and present scenarios, ecological niche models, as applied in the software MaxEnt v.3.4.1 (Phillips et al., 2017), were used to predict a mean habitat suitability value for each locality. To select the most appropriate of the 19 accessible bioclimatic variables downloaded from the WorldClim dataset (Fick & Hijmans, 2017) for the final analysis, correlation and collinearity analyses and preliminary analyses were first performed. Three variables based on the ecological characteristics of the species were chosen for the final analysis: Isothermality (BIO3), Precipitation of Driest Month (BIO14), and Precipitation Seasonality (BIO15). The data matrix on the presence of the species included only the studied populations of *C. vettonica* that do not have any putative genetic introgression with *C. paludica*. The niche modeling was made for three periods of time: Present (the

years 1970–2000), Last Glacial Maximum (LGM; ~22,000 years before present, BP), and Last Interglacial (LIG; ~120,000–140,000 BP). The quality of the model was evaluated by the Area Under the Curve (AUC) derived from the Receiver Operating Characteristic (ROC).

2.7 | Ancestral area reconstructions

The ancestral areas of *C. vettonica* were reconstructed using both the S-DIVA (Yu et al., 2010) and S-DEC (Beaulieu et al., 2013; Ree & Smith, 2008) methods implemented in RASP v.4.2 (Yu et al., 2015, 2020). The established areas were based on the current hydrographic subdivision of the Iberian Peninsula. Due to the genetic differentiation detected for *MT-CYB* in the Alagón sub-basin, this area was divided into an eastern and a western part. The code for the areas was as follows: A: Águeda, B: Western Alagón, C: Eastern Alagón, D: Aravil, E: Erjas, F: Ponsul, G: Salor, H: Ocreza, I: Aurela, J: Huebra and Yeltes, K: Tamuja and Almonte, L: Ibor, and M: Tiétar. We used the BEAST-derived trees as the input files for RASP. We eliminated 50% of the total initial trees, and used 100 random trees for the analysis. Ancestral ranges were limited to include no more than three adjacent areas. Only the reconstructed areas for *C. vettonica* are shown as this lineage is the focus of the present study.

3 | RESULTS

3.1 | Phylogeny

The phylogenetic analysis of *MT-CYB* recovered two main lineages corresponding to the species *C. vettonica* (S1) and *C. paludica* (S2) with high support ($pp = 1$; Figure 2). The species *C. vettonica* (S1) was not monophyletic since many individuals identified morphologically as *C. vettonica* from the Alagón sub-basin populations were found in both lineages (i.e., Jerte, Francia, Alagón, Caparro, Cuerpo de Hombre [Cdh], and Gata rivers). The lineage of *C. paludica* (S2) was also not a monophyletic group since one individual identified morphologically as *C. paludica* from the Salor sub-basin was included in the *C. vettonica* lineage (S1). Within *C. vettonica* (S1), there were two well-differentiated lineages (Figure 2). The first grouped populations from the Alagón sub-basin in the Tagus Basin (Árrago, Gata, San Blas, Acebo, Alagón, Francia Cdh, Ladrillar, Hurdano, Caparro, and Jerte rivers) and those from the Águeda sub-basin in the Duero Basin (Turones, Mayas, and Águeda rivers). Within this lineage, these populations grouped into two distinct lineages, V1 and V2. The populations from the eastern Alagón sub-basin (Alagón, Francia, Cdh, Ladrillar, Hurdano, Caparro, and Jerte rivers) formed the V2 lineage ($pp = 0.99$), while those from the western Alagón (Árrago, Gata, San Blas, and Acebo rivers) and Águeda sub-basins (Turones, Mayas, and Águeda rivers), and one individual from Jerte River (from eastern Alagón sub-basin) that was not included in V2, formed the V1 lineage. The phylogenetic relationships of the sequences included in V1 were not resolved. However, within V2, we detected another highly

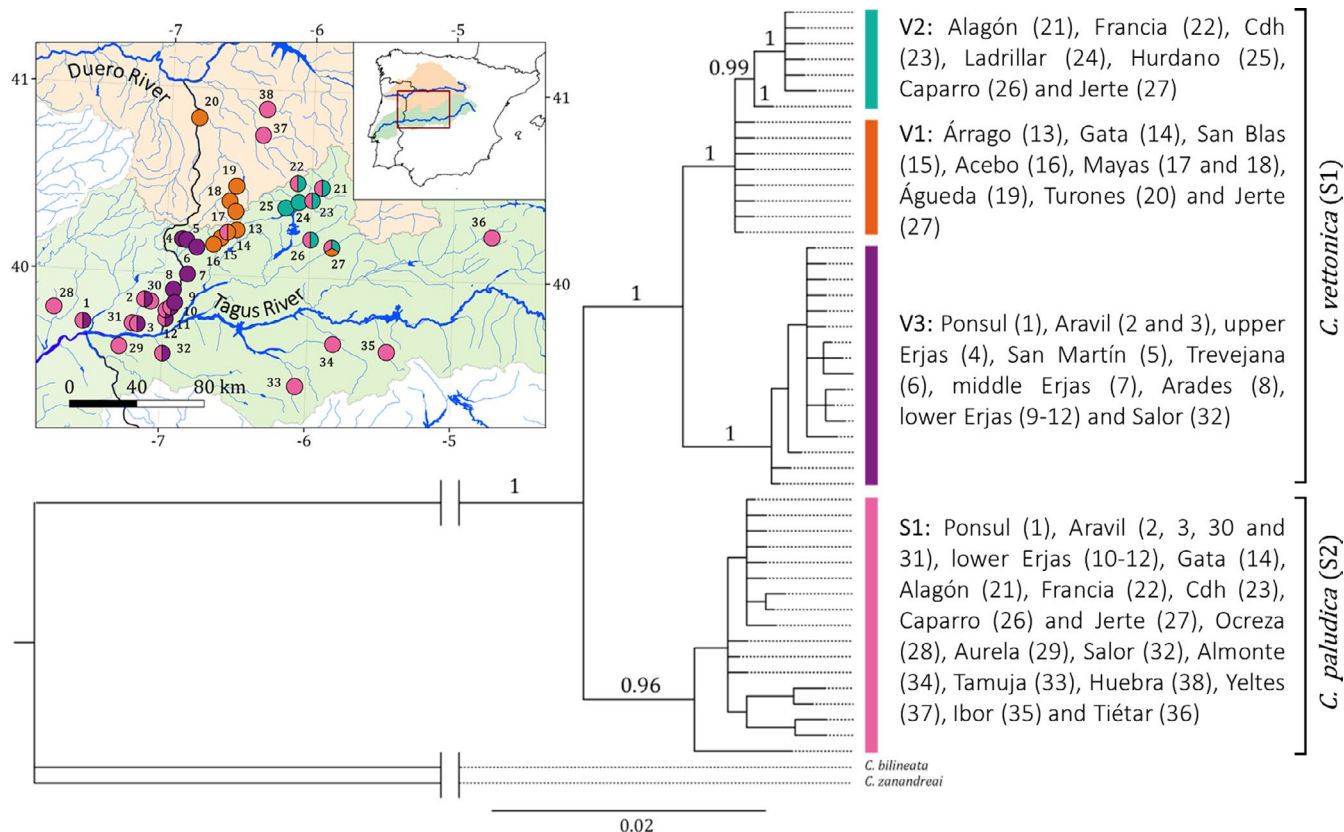


FIGURE 2 Phylogenetic tree based on Bayesian inference for the mitochondrial marker *MT-CYB*. Posterior probability values are indicated above branches. S1: *Cobitis vettonica*. The vertical colored bars represent the three main lineages (V1–V3) of the species and their localities (see legend). S2: *Cobitis paludica*. Also, the map is shown indicating the sampling locations referenced in Table 1. The color of the sampling point indicates the detected lineage in each population based on the results of the phylogenetic tree

TABLE 2 Uncorrected absolute genetic distances between the main lineages of *C. vettonica* (V1, V2, and V3) and *C. paludica* (S1). The standard deviation is shown on the upper diagonal

	V1	V2	V3	S2
V1		0.002	0.003	0.005
V2	0.003		0.003	0.004
V3	0.011	0.012		0.005
S2	0.022	0.025	0.026	

supported monophyletic grouping ($pp = 1$) in which only the single individual studied from Ladrillar River was excluded. The second well-differentiated lineage within *C. vettonica* (V3; $pp = 1$) included populations from the Ponsul, Aravil, and Erjas sub-basins located along the western limit of the species' distribution range, and one individual from Salor River (Figure 2).

Genetic distances among the three lineages detected in *C. vettonica* (V1, V2, and V3) ranged from 0.3% to 1.2% (Table 2).

The unrooted phylogenetic tree of *C. vettonica* and *C. paludica* based on the 4538 SNPs was largely congruent with the results obtained in the phylogenetic reconstruction for *MT-CYB*, although with some slight differences (Figure 3). The individuals of the populations analyzed for SNPs belonging to the V1 lineage (i.e., Mayas from

Águeda sub-basin, Duero Basin and Gata from western Alagón sub-basin, Tagus Basin) were well differentiated not only from the rest of the individuals analyzed ($b > 90$) but also from each other ($b > 90$ and $75 < b < 90$, respectively). Those belonging to the V3 lineage (Erjas River), which were separated from the rest of the populations of *C. vettonica*, were more related to individuals of *C. paludica* from Aurela River, a tributary of the Tagus Basin that is geographically close to the Erjas sub-basin. Likewise, individuals of *C. vettonica* from the Caparro river population (belonging to V2 and S2) were more related to those of *C. paludica* from the populations in the Salor, Tamuja, Ibor, Huebra, and Yeltes rivers. The remaining populations of *C. vettonica* included in the SNP analysis belonging to the V2 and S2 lineages (Alagón, Cdh, Jerte, and Francia rivers) did not show a clear phylogenetic structure with several intermediate individuals.

3.2 | Genetic structure

The haplotype network for *MT-CYB* was consistent with the phylogenetic reconstruction for *MT-CYB*. The network showed two main groups separated by 20 mutational steps corresponding to the species *C. vettonica* and *C. paludica* (S1 and S2, respectively; Figure 4). For *C. vettonica* (S1), the network was more informative

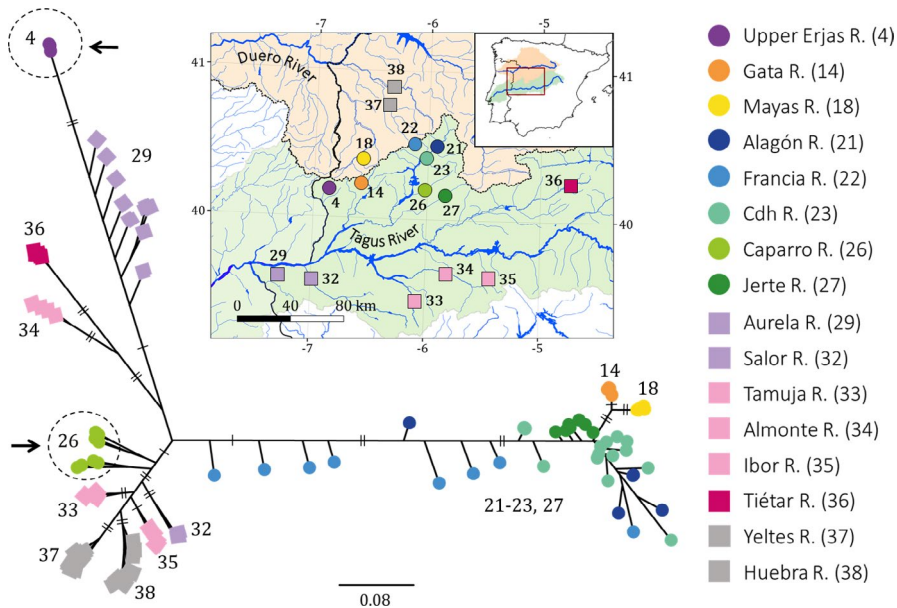


FIGURE 3 Unrooted ML phylogenetic tree based on the 4538 SNP loci. Circles and squares indicate individuals morphologically identified as *C. vettonica* and *C. paludica*, respectively. The numbers refer to those assigned to the populations in Table 1. The bootstrap values (b) are represented with a line perpendicular to the branch for $75 < b > 90$, and two lines for $b > 90$. The arrows highlight the populations of *C. vettonica* from the Caparro (→) and upper Erjas (←) rivers. Abbreviation: R. for river. Also, the map is shown indicating the sampling locations referenced in Table 1

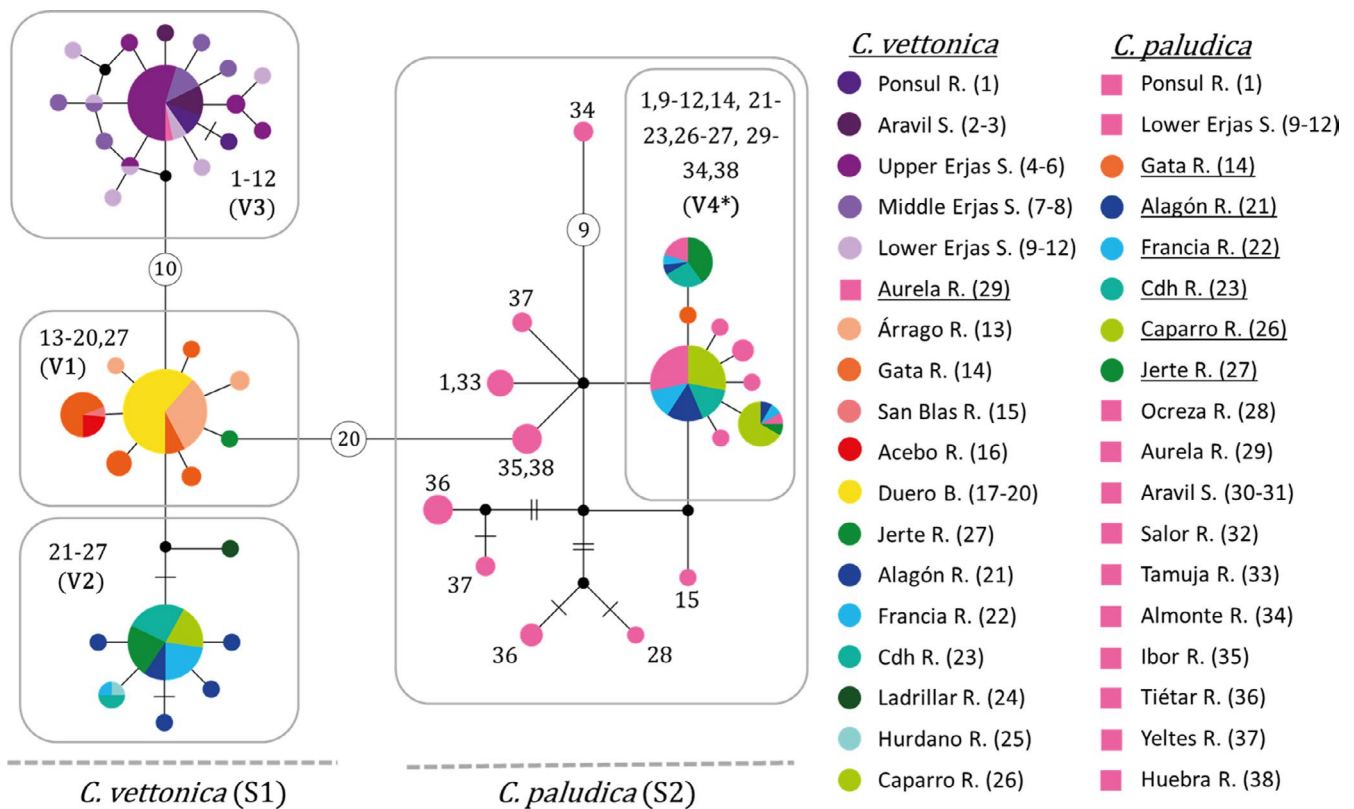


FIGURE 4 Haplotype network for the mitochondrial marker MT-CYB. The localities are indicated by different colors and numbers (see Table 1). Mutational steps are represented as follows: one short line for two steps, two short lines for three steps, or a circle with the number of steps indicated for four or more steps. V4* refers only to those individuals identified as *C. vettonica* and found in the S2 lineage (individuals indicated by pink are not included in V4*). In the legend, populations identified as *C. vettonica* are represented by circles, and those identified as *C. paludica*, by squares. Populations that contain individuals with contrasting morphological and genetic identifications are underlined. Abbreviations: R. for river; S. for sub-basin and B. for basin

than the phylogenetic reconstruction since there were three well-differentiated haplogroups (V1, V2, and V3). The first haplogroup (V1) clustered populations from the Águeda sub-basin in the Duero

Basin and in the Tagus Basin, those from the western part of Alagón sub-basin, plus one individual belonging to Jerte River (eastern Alagón sub-basin) (Figure 4). These populations corresponded to the

nondifferentiated lineage obtained in the phylogenetic reconstruction (i.e., V1 in Figure 4). The second haplogroup (V2) was composed of all the eastern Alagón sub-basin populations in the Tagus Basin, corresponding to the V2 lineage obtained in the phylogenetic reconstruction. Likewise, the third haplogroup (V3), corresponding to the V3 lineage in the phylogenetic reconstruction, included populations from the western limit of the distribution range of *C. vettonica* in the Tagus Basin, namely those from the Ponsul, Aravil, and Erjas sub-basins, plus one individual from Salor River. The individual from Salor River (identified as *C. paludica*) presented the most common haplotype of the haplogroup. Haplogroups V1 and V2 were separated by three/four mutational steps, and V3 was separated from V1 by 10 mutational steps. In the group comprising haplotypes found mainly in *C. paludica* (S2), a subset was composed of individuals identified as *C. vettonica* from the Alagón and Erjas sub-basins (V4; referring to only those individuals identified morphologically as *C. vettonica* but that are genetically closer to *C. paludica* according to the mitochondrial data) along with other individuals of *C. paludica* from other Tagus and Duero tributaries. However, no evidence of a geographic structure was found within S2. The haplotype network for the nuclear marker (*RAG1*) showed a single group in which all studied populations of both species were represented and therefore was of low resolution (Figure 5).

According to the AMOVAs with the MT-CYB data, the highest percentage of variance among groups was when the groupings were comprised of the lineages found in the previous analysis (V1, V2, V3, and V4; Table 3). The percentage of variance explained between basins (Duero and Tagus) was not significant, with most of the variation explained by differences between and within populations. Population differentiation between localities within each lineage of *C. vettonica* for MT-CYB was low ($\Phi_{st} < 0.34$), and no significant differences were found, except for the Acebo River, which showed a high level of differentiation with the Árrago river ($\Phi_{st} > 0.74$; Table S4). High values of Φ_{st} were detected between populations from different lineages ($\Phi_{st} > 0.75$).

The results of the SNP structure analyses showed that the most probable number of subpopulations (genetic groups) for *C. vettonica* matrix was $K = 4$ and $K = 2$ for Puechmaille and ΔK methods, respectively (Table S5). For $K = 4$, only three populations showed very little admixture: Mayas River in the Duero Basin (first group, in

yellow) and Caparro and Erjas rivers in the Tagus Basin (second and third groups, in blue and purple, respectively) (Figure 6). The other populations (Alagón, Cdh, Jerte, and Francia) showed different degrees of admixture of the second (blue) and fourth (orange) groups. Gata River had a similar level of admixture of the first (yellow) and fourth (orange) groups with a small contribution from the third (purple). The results for $K = 2$ were consistent with those obtained for $K = 4$. Mayas and Gata belonged to the first group (orange), Caparro to the second group (blue), and the rest of the populations presented a mixture of both groups. When we analyzed the populations of both species, the most probable number of subpopulations was $K = 6$ and $K = 2$ for Puechmaille and ΔK methods, respectively (Table S5). For $K = 6$, Mayas and Gata comprised the first group (orange), with Gata having a small contribution from the third group (purple) in all individuals (Figure 6). Alagón, Cdh, Jerte, Francia, and Caparro showed different degrees of admixture of the first (orange) and second (blue) groups, with Cdh and Francia also having a small proportion of the third group (purple) and Jerte having a small proportion of the fourth group (pink). The second group (blue) was present in all populations of *C. paludica*. The upper Erjas River was assigned to a single genetic group (Group 3; purple), which also comprised approximately 29% and 15% of the total composition of the Aurela and Salor rivers, respectively, with very small contributions from the second (blue) and fourth (pink) groups in some individuals. The results for $K = 2$ were consistent with those obtained for $K = 6$. Mayas and Gata belonged to the first group (orange), Ibor, Tamuja, Huebra, and Yeltes to the second group (blue), and the rest of the populations presented a mixture of both groups.

3.3 | Genetic diversity and demography

Overall genetic diversity parameters for all individuals identified as *C. vettonica* based on MT-CYB were high (Table 4). Excluding populations of *C. vettonica* in which only one individual was studied, Hd ranged from 0 to 1, and h ranged from 1 to 7. The populations with the lowest values of genetic diversity were Mayas, Acebo, Arades, and Caparro with only one haplotype each (Table 4). The most variable populations for all of the diversity parameters were the lower and middle Erjas, Alagón, and Gata. The sub-basins with the highest



FIGURE 5 Haplotype network for the nuclear marker *RAG1*. The colors correspond to the different localities, except pink, which is used for all localities of *C. paludica*. Mutational steps are represented as follows: no line for one step and one short line for two steps. In the legend, the populations identified as *C. vettonica* are represented by circles and those identified as *C. paludica* are represented by squares. Numbers correspond to those used in Table 1

TABLE 3 Results of the AMOVA for MT-CYB for groups established according to basin, sub-basin and lineage (see text for more details)

	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices		
						FST	FSC	FCT
Group 1: Basin	Among groups	1	49.26	-0.08	-2.29	0.91	0.91	-0.02
	Among populations within groups	21	386.16	3.12	92.92***			
	Within populations	127	39.9	0.31	9.37***			
	Total	149	491.13	3.54				
Group 2: Sub-basin	Among groups	4	363	3.54	78.49***	0.93	0.68	0.78
	Among populations within groups	18	72.42	0.66	14.55***			
	Within populations	127	39.90	0.31	6.96***			
	Total	149	475.31	4.51				
Group 3: Lineages	Among groups	2	419.46	4.24	91.46***	0.93	0.2	0.91
	Among populations within groups	20	15.96	0.08	1.77***			
	Within populations	127	39.9	0.31	6.77***			
	Total	149	475.31	4.63				

Notes: Groupings refer to the categories shown in Table 1. Significance: * $p < .05$; ** $p < .01$; *** $p < .001$; df = degrees of freedom.

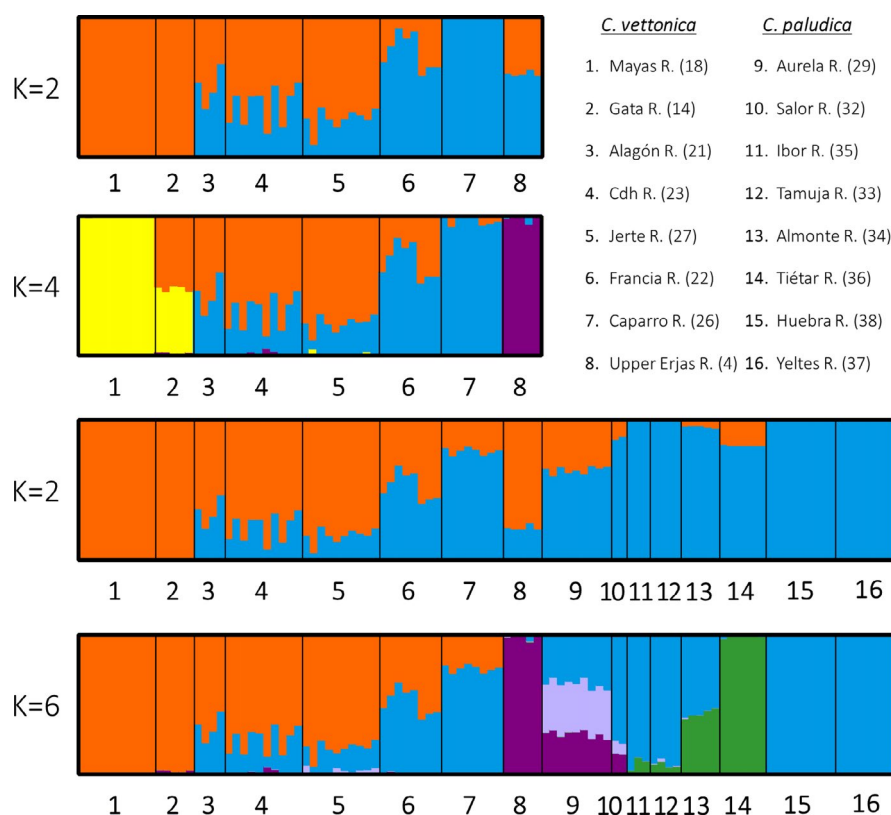


FIGURE 6 Results of the STRUCTURE analyses. The first graph shows the results obtained for $K = 2$ and $K = 4$ when only the populations of *C. vettonica* were analyzed (1–8). The third and fourth graphs show the results obtained when $K = 2$ and $K = 6$ when populations of both *C. vettonica* (1–8) and *C. paludica* (9–16) were analyzed. Abbreviation: R. for river

genetic diversity values were Erjas and Alagón, specifically the western Alagón sub-basin. The one with the lowest values was Águeda with only one haplotype.

The highest number of private alleles for *C. vettonica*, based on the SNP data, was found in the upper Erjas (227 alleles) and Caparro (76 alleles) rivers (Table 5). However, when the complete matrix

including the populations of *C. paludica* was taken into account, the number of private alleles decreased drastically for all rivers, except Mayas (41 vs. 40) and Gata (1 vs. 0), whose numbers remained nearly the same as when only *C. vettonica* was considered. In the upper Erjas and Caparro rivers, the number of private alleles decreased to eight and two, respectively (Table 5). Notably, when Aurela and Salor

TABLE 4 Genetic variability parameters for MT-CYB and RAG1 and the results of the neutrality tests of Tajima and Fu for the different groups (see text for details). The * indicate significance values

General	MT-CYB							RAG1				
	N	h	H _d	π	S	Tajima's D	Fu's FS	N	a	a _d	π	S
<i>C. vettonica</i>	150	31	0.85	5.86E-03	38	-0.18	-4.8	45	4	0.09	8.00E-05	3
By locality	N	h	H _d	π	S	Tajima's D	Fu's FS	N	a	a _d	π	S
Alfrividas R.	4	2	0.5	9.20E-04	2	-0.70	1.09	-	-	-	-	-
Aravil R.	5	2	0.4	3.70E-04	1	-0.81	0.09	-	-	-	-	-
Upper Erjas R.	12	4	0.46	4.60E-04	3	-1.62*	-2.12*	9	3	0.21	2.30E-04	2
San Martin R.	6	2	0.33	6.10E-04	2	-1.13	0.95	1	1	0	0	0
Trevejana R.	4	2	0.5	4.60E-04	1	-0.61	0.17	4	2	0.43	3.00E-04	1
Middle Erjas R.	6	6	1	1.78E-03	5	-0.65	-4.45*	-	-	-	-	-
Lower Erjas S.	9	7	0.92	1.94E-03	8	-1.28	-3.61*	-	-	-	-	-
Arades R.	2	1	0	0	0	-	-	-	-	-	-	-
Árrago R.	15	3	0.36	3.50E-04	2	-1.00	-0.91	3	1	0	0	0
Gata R.	18	5	0.72	1.05E-03	4	-0.04	-0.91	5	1	0	0	0
San Blas R.	1	1	-	-	-	-	-	-	-	-	-	-
Acebo R.	3	1	0	0	0	-	-	1	1	0	0	0
Mayas R.	22	1	0	0	0	-	-	4	1	0	0	0
Águeda R.	1	1	-	-	-	-	-	-	-	-	-	-
Turones R.	1	1	-	-	-	-	-	1	1	0	0	0
Alagón R.	7	5	0.86	1.31E-03	5	-1.48*	-2.31*	6	1	0	0	0
Francia R.	8	2	0.25	2.30E-04	1	-1.05	-0.18	1	1	0	0	0
Cdh R.	10	2	0.36	3.30E-04	1	0.01	0.41	4	1	0	0	0
Ladrillar R.	1	1	-	-	-	-	-	-	-	-	-	-
Hurdano R.	1	1	-	-	-	-	-	1	1	0	0	0
Caparro R.	6	1	0	0	0	-	-	2	1	0	0	0
Jerte R.	8	2	0.25	9.20E-04	4	-1.53*	1.94	3	1	0	0	0
By sub-basin	N	h	H _d	π	S	Tajima's D	Fu's FS	N	h	H _d	π	S
Ponsul S.	4	2	0.5	9.20E-04	2	-0.71	1.09	-	-	-	-	-
Aravil S.	5	2	0.4	3.70E-04	1	-0.81	0.09	-	-	-	-	-
Erjas S.	39	14	0.66	1.03E-03	12	-1.87*	-12.04*	14	4	0.29	2.60E-04	3
Águeda S.	24	1	0	0	0	0	0	5	1	0	0	0
Alagón S.	78	15	0.781	2.12E-03	17	-0.96	-4.34*	26	1	0	0	0
Western Alagón S.	37	7	0.71	8.60E-04	6	-0.94	-2.62*	-	-	-	-	-
Eastern Alagón S.	41	8	0.43	7.00E-04	11	-2.12*	-4.56*	-	-	-	-	-
By lineage (MT-CYB)	N	h	H _d	π	S	Tajima's D	Fu's FS	N	h	H _d	π	S
V1	62	8	0.56	6.00E-04	7	-1.42	-4.54*	15	1	0	0	0
V2	40	7	0.4	5.40E-04	9	-2.11*	-4.36*	16	1	0	0	0
V3	48	16	0.61	9.60E-04	15	-2.14*	-15.53*	14	4	0.29	2.60E-04	3
By OCUs (MT-CYB)	N	h	H _d	π	S	Tajima's D	Fu's FS	N	h	H _d	π	S
OCU I	24	1	0	0	0	-	-	5	1	0	0	0
OCU II	37	7	0.72	8.60E-04	6	-0.94	-2.63	9	1	0	0	0
OCU III	41	8	0.42	7.00E-04	11	-2.13*	-4.57*	17	1	0	0	0
OCU IV	48	16	0.61	9.60E-04	15	-2.14*	-15.53*	14	4	0.29	2.60E-04	3

Notes: The following parameters are included in the table for MT-CYB: N: number of analyzed samples; h: haplotype number; H_d: haplotype diversity; π: nucleotide diversity; S: number of polymorphic sites. The following parameters are included in the table for RAG1: N: number of analyzed samples; a: number of nuclear alleles; a_d: allelic diversity; π: nucleotide diversity; S: number of polymorphic sites. Abbreviations: R. for river and S. for sub-basin.

TABLE 5 Genetic diversity parameters for the SNP data

Loc	Matrix	Pri	Pri*	V _s	P	%P	H _o	H _e	π	F _{IS}
Mayas R. (18)	V	41	-	3992	193	4.83	0.02 ± 0.011	0.017 ± 0.007	0.018 ± 0.007	-0.006 ± 0.005
	VP	40	40	4528	193	4.26	0.018 ± 0.01	0.015 ± 0.006	0.016 ± 0.007	-0.005 ± 0.004
Gata R. (14)	V	1	-	3998	502	12.56	0.047 ± 0.022	0.041 ± 0.014	0.046 ± 0.017	-0.00008 ± 0.017
	VP	0	0	4535	502	11.07	0.041 ± 0.02	0.036 ± 0.012	0.041 ± 0.015	-0.00007 ± 0.015
Alagón R. (21)	V	14	-	3999	2493	62.34	0.257 ± 0.07	0.227 ± 0.038	0.26 ± 0.05	0.003 ± 0.082
	VP	1	2	4537	2493	54.95	0.227 ± 0.068	0.2 ± 0.039	0.229 ± 0.051	0.003 ± 0.072
Cdh R. (23)	V	21	-	4000	2918	72.95	0.198 ± 0.033	0.204 ± 0.028	0.216 ± 0.031	0.046 ± 0.064
	VP	1	2	4538	2918	64.30	0.174 ± 0.033	0.18 ± 0.029	0.19 ± 0.032	0.041 ± 0.057
Jerte R. (27)	V	39	-	4000	2664	66.6	0.182 ± 0.036	0.172 ± 0.026	0.182 ± 0.029	0.0005 ± 0.048
	VP	2	2	4538	2664	58.70	0.16 ± 0.035	0.152 ± 0.026	0.16 ± 0.029	0.0004 ± 0.043
Francia R. (22)	V	28	-	4000	3042	76.05	0.309 ± 0.063	0.285 ± 0.038	0.306 ± 0.044	-0.006 ± 0.084
	VP	1	1	4538	3042	67.03	0.272 ± 0.066	0.251 ± 0.042	0.269 ± 0.048	-0.005 ± 0.074
Caparro R. (26)	V	76	-	4000	3053	76.33	0.282 ± 0.053	0.261 ± 0.033	0.279 ± 0.038	-0.007 ± 0.074
	VP	2	3	4538	3053	67.28	0.249 ± 0.055	0.23 ± 0.036	0.246 ± 0.041	-0.006 ± 0.066
Upper Erjas R. (4)	V	227	-	3960	496	12.53	0.039 ± 0.016	0.035 ± 0.011	0.039 ± 0.014	0.001 ± 0.01
	VP	8	141	4482	496	11.07	0.034 ± 0.014	0.031 ± 0.01	0.035 ± 0.012	0.001 ± 0.009
Aurela R. (29)	V	15	-	4538	3145	69.3	0.24 ± 0.05	0.24 ± 0.04	0.25 ± 0.04	0.0265 ± 0.073
	VP	0	-	4515	1665	36.88	0.2 ± 0.1	0.15 ± 0.04	0.21 ± 0.08	0.0125 ± 0.062
Ibor R. (35)	V	10	13	4534	1470	32.42	0.15 ± 0.07	0.12 ± 0.03	0.15 ± 0.06	0.0114 ± 0.051
	VP	2	5	4532	1582	34.91	0.14 ± 0.05	0.12 ± 0.03	0.15 ± 0.05	0.0163 ± 0.053
Almonte R. (34)	V	25	40	4516	1298	28.74	0.11 ± 0.04	0.09 ± 0.03	0.11 ± 0.03	-0.0028 ± 0.038
	VP	146	175	4484	743	16.57	0.05 ± 0.02	0.05 ± 0.02	0.06 ± 0.02	0.0104 ± 0.023
Tíetar R. (36)	V	8	8	4538	1926	42.44	0.14 ± 0.04	0.14 ± 0.03	0.15 ± 0.04	0.0159 ± 0.042
	VP	7	9	4534	1042	22.98	0.09 ± 0.04	0.08 ± 0.03	0.09 ± 0.03	0.0033 ± 0.027

Notes: Two matrices were analyzed: one including only the populations of *C. vettonica* (V) and the other, the populations of *C. paludica* together (VP), as indicated in the Matrix column. Pri: number of private alleles; Pri*: number of private alleles when Aurela and Salor were excluded from the analysis (only shown for VP grouping); V_s: number of variant sites; P: number of polymorphic sites; %P: percentage of polymorphic sites; H_o: observed heterozygosity; H_e: expected heterozygosity; π : nucleotide diversity; π : inbreeding coefficient. Abbreviation: R. for river.

rivers were removed from the analysis, the number of private alleles in the Erjas population rose to 141. The lowest nucleotide diversity values were found in Mayas, Gata, and the upper Erjas rivers, and the highest, in Alagón, Cdh, Jerte, Francia, and Caparro rivers (Table 5).

Significant negative values for the neutrality tests indicate a population expansion following a bottleneck. All three of the detected lineages were significantly negative for Fu's FS; V2 and V3 were also significantly negative for Tajima's *D* (Table 4).

3.4 | Divergence time and niche modeling

The topology of the tree obtained by calibrating the molecular clock was identical to that of the phylogenetic Bayesian tree (Figure 7) but showed higher support for the V2 lineage ($pp = 0.99$ vs. $pp = 0.87$). The V3 lineage diverged approximately 250 Ka (HPD 95%: 30 Ka–2.76 Ma), while V1 and V2 diverged from each other approximately 80,000 years ago (HPD 95%: 10–910 Ka).

All niche distribution models (Figure 8) had AUC values greater than 0.97. Values above 0.75 are considered useful, while those above 0.90 are very good (Elith et al., 2006; Swets, 1988). Mean habitat suitability values above 0.7 were found for the present-day period for all localities where *C. vettonica* is currently present, except some areas of the eastern Alagón sub-basin, including Jerte, Francia, Caparro, Alagón, and Cdh rivers, and the lower and middle courses of the Ponsul and Aravil sub-basins. In addition, a potentially habitable zone outside the known current distribution area for *C. vettonica* was observed upstream of Aurela River, a tributary of the left margin of the Tagus Basin, and also upstream of Côa River, a tributary of the Duero Basin that is adjacent to the Águeda sub-basin (Figure 8A,B). For the LIG period (Figure 8C), the area with the optimal conditions for the species generally overlapped with that found for the present, although the highest probability values were concentrated in the central part of the species' current distribution (i.e.,

the western Alagón and Águeda sub-basins in the Tagus and Duero basins, respectively). For the LGM period (Figure 8D), optimal conditions were located in the southwestern area of the species current distribution (the Erjas and lower Árrago sub-basins and Salor, Aravil, and Ponsul sub-basins). Some rivers outside the current distribution of the species in Portugal also showed optimal conditions.

3.5 | Ancestral area reconstructions

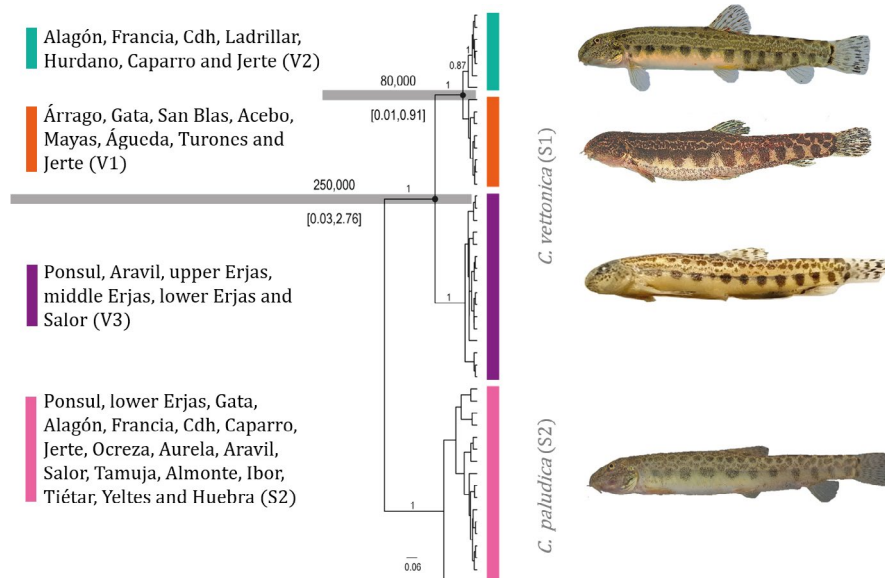
In general, the results of the ancestral area reconstructions using either the S-DIVA or S-DEC method were congruent (Figure 9). Both the vicariant events between lineages and the ancestral area with the highest marginal probability were consistent between the two methods. The most probable ancestral areas were as follows: Alagón–Erjas sub-basins for the junction node of V1–V2–V3, Alagón sub-basin for V1–V2, and Erjas sub-basin for V3. Both analyses supported the diversification of the main evolutionary lineages due to vicariant events. Several dispersal and vicariant events were also detected within V1 and V3 (Figure 9).

4 | DISCUSSION

4.1 | Genetic structure

A strong population genetic structure, based on mitochondrial (MT-CYB) and SNP data, was detected for *C. vettonica*, in line with previous molecular studies of MT-CYB (Doadrio et al., 2021; Perdiges & Coelho, 2020). No differences were found for nuclear RAG1, neither among the populations of *C. vettonica* nor between *C. vettonica* and *C. paludica*. Similar findings have also been reported for this gene in other Iberian species, probably as a consequence of incomplete lineage sorting (Corral-Lou et al., 2019; Perea et al., 2021).

FIGURE 7 Phylogenetic relationships and divergence time estimates based on the analysis of the mitochondrial marker MT-CYB in BEAST. The horizontal bars represent the 95% HPD intervals. Posterior probabilities of the main nodes are indicated above branches. S1: *Cobitis vettonica*. The vertical colored bars represent the three main lineages (V1–V3) of the species and their localities (see legend). S2: *Cobitis paludica*. On the right, images of representative specimens belonging to one of the localities of each lineage: (from top to bottom): Caparro, Gata, middle Erjas, and Tiétar rivers



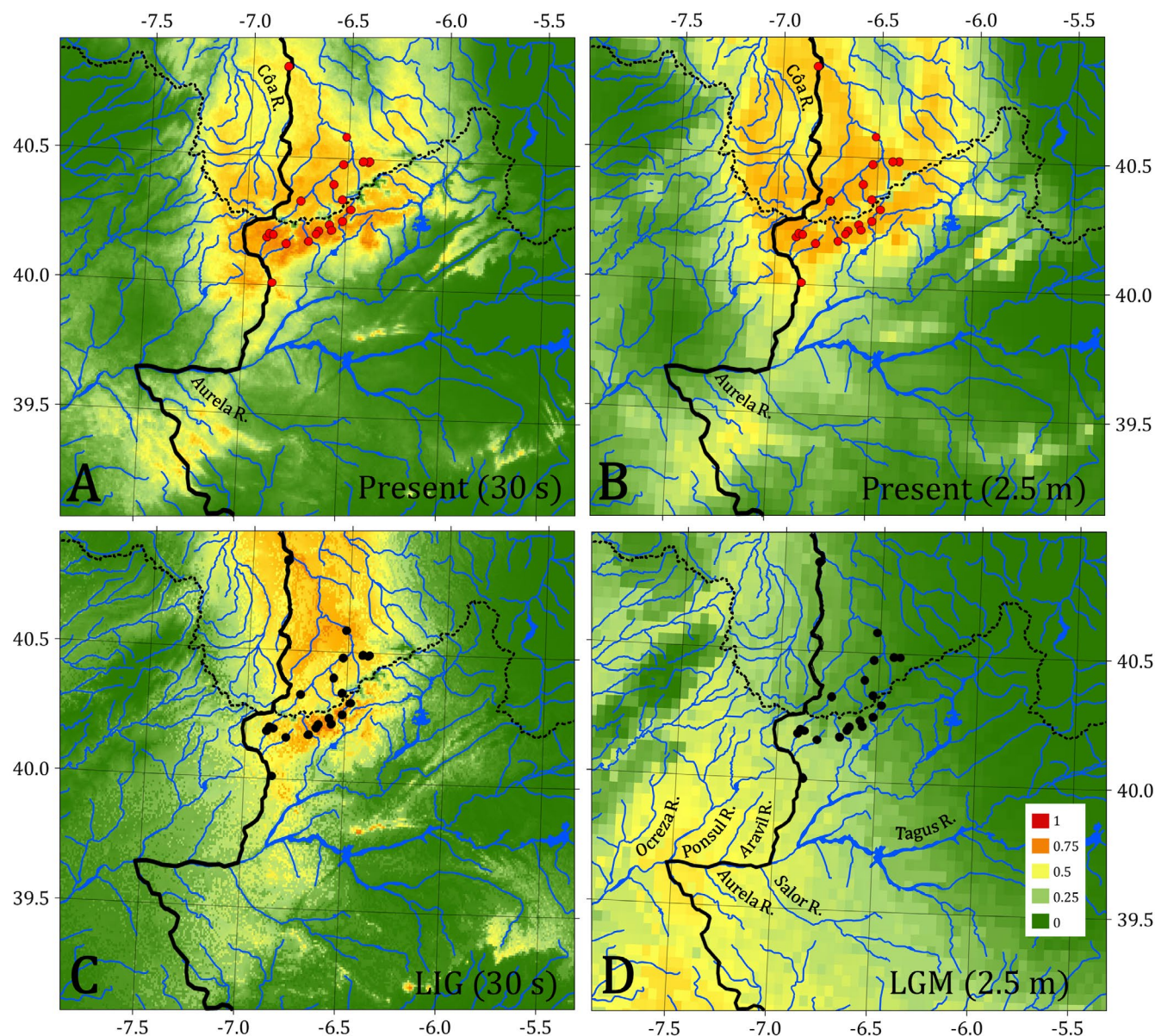


FIGURE 8 Modeling of the potential niche of *C. vettonica* for (A) and (B) the present (1970–2000) at a resolution of 30 s (s) and 2.5 min (m), respectively, (C) Last Interglacial (LIG; 130,000 years ago) and (D) Last Glacial Maximum (LGM; 22,000 years ago). The colors indicate the probability of the presence of the species (see legend). Some of the rivers (R.) mentioned in the text are labeled

The population structure of *C. vettonica* based on the mitochondrial data differed from the main pattern observed in other European freshwater fishes, which is that gene flow within the same basin is greater than among different basins (e.g., Buj et al., 2017; Corral-Lou et al., 2019; Marić et al., 2017, 2019; Seifertová et al., 2012; Wetjen et al., 2020). Specifically, for *C. vettonica*, genetic differentiation between populations from the same basin (Tagus Basin) is greater than between populations from the two main basins (Duero and Tagus basins). Similarly, greater differentiation within the same sub-basins than between different sub-basins has been found for *Achondrostoma salmantinum*, which has a restricted distribution area that partially overlaps with that of *C. vettonica* (Corral-Lou et al., 2021). Iberian freshwater fishes with a wider distribution range that

partially or completely overlaps with that of *C. vettonica*, such as *Squalius carolitertii*, *S. pyrenaicus*, *S. alburnoides*, or *Luciobarbus bocagei*, do not show a population structure pattern similar to that of *C. vettonica* (Cunha et al., 2004; Doadrio et al., 2002; Perea et al., 2021; Sousa-Santos et al., 2007). Differences in the genetic pattern of the various species may be due to differences in size and dispersal abilities. Species of *Cobitis* are smaller benthic organisms with lower dispersal abilities (like *A. salmantinum*) than species of *Luciobarbus* and *Squalius* (Doadrio et al., 2011). This difference in the biology of freshwater fish species, along with different colonization times, could account for the population structure found in *C. vettonica* and *A. salmantinum* and the lack of structure in the species of *Luciobarbus* and *Squalius* (see Biogeography section).

FIGURE 9 Reconstruction of the ancestral area using either S-DIVA or S-DEC. Only the lineage of *C. vettonica* is shown. The color(s) of the circles indicates the most probable ancestral area for each node (see legend for color code). Vicariant events are indicated with an *, and dispersals with a <. Abbreviations: S. for sub-basin and B. for basin

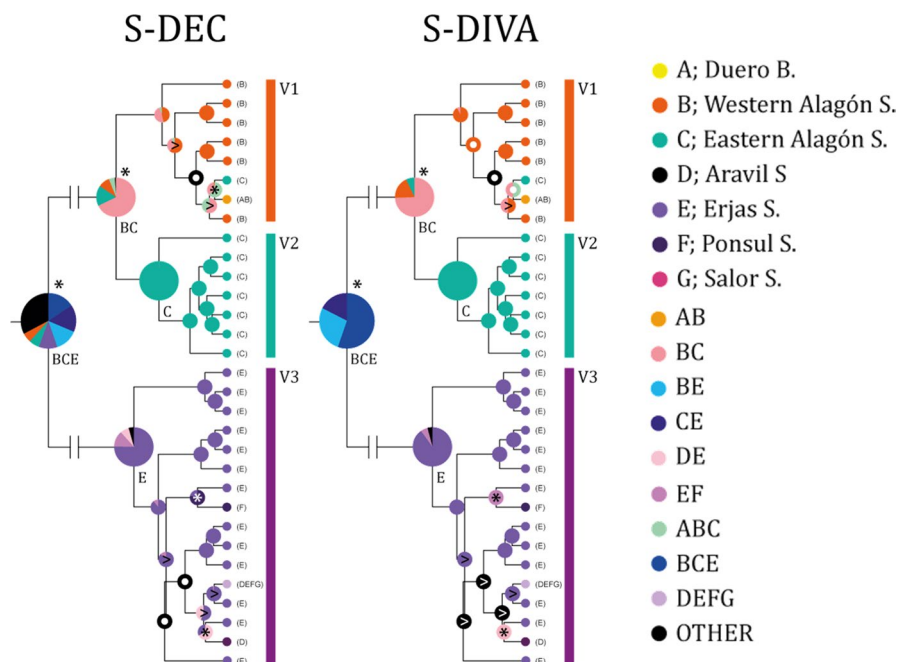
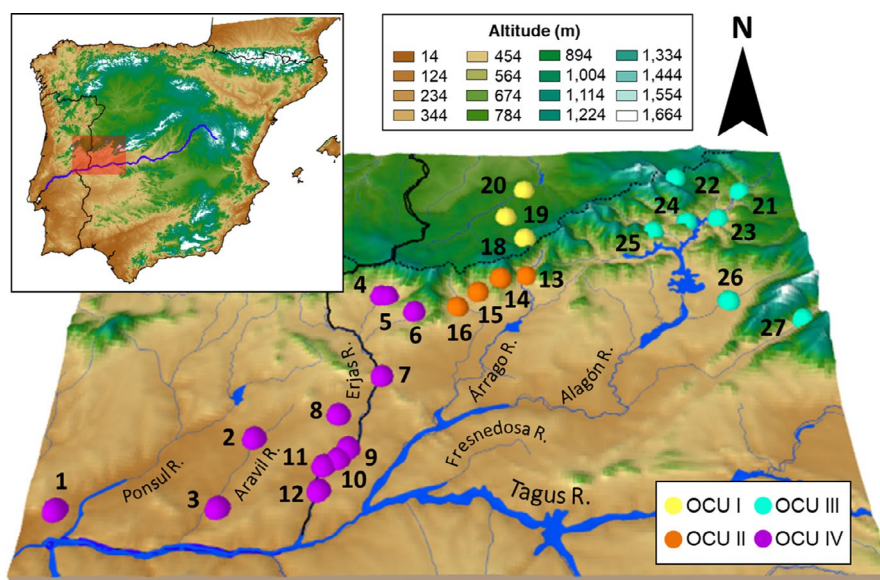


FIGURE 10 3D map indicating the sampling locations referenced in Table 1. The color of the sampling point indicates the Operational Conservation Units (OCU I to IV) to which it is assigned. Some of the main rivers sampled in the study are also indicated on the map



4.1.1 | (V1) Western Alagón sub-basin and Duero Basin

This mitochondrial lineage included the populations from the headwaters of the rivers in the western Alagón sub-basin in the Tagus Basin, and those in the Duero Basin. Only one individual from Jerte River in the eastern Alagón sub-basin had a haplotype close to the western lineage, however, it was not shared by any other locality in the western Alagón sub-basin or the Duero Basin. Therefore, it is possible that the close relationship between this and the rest of the haplotypes is a consequence of an ancestral polymorphism; hence, we did not consider Jerte River within this lineage. The results of the SNP analyses showed slight differentiation between the Duero

and Tagus populations, indicating that these isolated populations were joined in the past. These results suggest that the Duero basin and western Alagón sub-basin populations of *C. vettonica*, which are currently assigned to single OCU (Doadrio et al., 2021), should be assigned to two separate OCUs (OCU I and OCU II, respectively; Figure 10).

The Duero populations of *C. vettonica* (OCU I) showed the lowest diversity values of all the populations. Two possible explanations may account for this low diversity: in the first case, these populations may have not yet recovered from a recent bottleneck that occurred following their separation from the western Alagón sub-basin, and in the second, they may have not yet diversified following a founder event that occurred after the estimated dispersal of a

small number of individuals from the rivers of the western Alagón sub-basin (as suggested in our ancestral area reconstructions).

4.1.2 | (V2) Eastern Alagón sub-basin

This group, composed of the populations from the eastern Alagón sub-basin, was not monophyletic based on *MT-CYB*, and in the SNP analysis, presented a high level of admixture with *C. paludica*. These results suggest extensive genetic introgression of *C. vettonica* with its sister species *C. paludica*, except in Hurdano and Ladrillar rivers, where only one individual from each was studied. Therefore, regardless of the different levels of genetic introgression, we propose that these populations all be assigned to the OCU that was recently established by Doadrio et al. (2021) (OCU III; Figure 10).

4.1.3 | (V3) Ponsul, Aravil, and Erjas sub-basins

According to the *MT-CYB* analyses, the Ponsul, Aravil, and Erjas sub-basin populations constituted a well-differentiated independent lineage of *C. vettonica*, supporting evidence from other studies that suggest the uniqueness of the Erjas populations (Doadrio et al., 2011, 2021). The upper Erjas was also an independent group, according to the SNP analyses, indicating its present-day isolation with respect to the rest of the populations in the Alagón sub-basin and Duero Basin. For these reasons, we include the upper Erjas, Ponsul, and Aravil sub-basins in the OCU previously established for only the Erjas sub-basin (Doadrio et al., 2021), thereby expanding this OCU's area of coverage (OCU IV; Figure 10).

4.2 | Hybridization

Hybridization due to introgression as a result of both anthropic and natural factors has been detected for other Mediterranean cyprinid and cobitid species (Almodóvar et al., 2012; Bohlen & Ráb, 2001; Choleva et al., 2014; Corral-Lou et al., 2019; Cunha et al., 2004; Denys et al., 2013; Janko et al., 2005; Perea et al., 2016; Tancioni et al., 2013). Within the Iberian Peninsula, the majority of the natural hybrid populations originated during the Quaternary period as a consequence of hydrographical rearrangements and/or Pleistocene glaciation–deglaciation cycles (Almodóvar et al., 2012; Gante, 2009). Consistent with this, we present strong evidence confirming the introgression previously reported in the eastern Alagón populations of *C. vettonica* by Doadrio et al. (2011), Doadrio et al. (2021), and also postulate the genetic introgression of populations in sympatric areas (Ponsul, Aravil, and low Erjas; see below). Supporting this hypothesis is the location of these populations outside the potential range of the species based on the ecological niche models. The introgression of even a few loci due to hybridization can lead to adaptive divergence if the loci are better adapted to other niches and persist in the genome (Abbott et al., 2013).

4.2.1 | (V1) Western Alagón sub-basin and Duero Basin

The Gata population within the western Alagón sub-basin was the only one that presented a mitochondrial haplotype of *C. paludica*. However, in the SNP analysis, none of the individuals showed admixture with *C. paludica*, and they also resolved as a phylogenetically isolated group. Therefore, at present, there is no evidence to support genetic introgression with *C. paludica* in this area.

4.2.2 | (V2) Eastern Alagón sub-basin

We found strong evidence of genetic introgression between *C. paludica* and *C. vettonica* in the eastern Alagón sub-basin, in line with previous studies (Doadrio et al., 2011, 2021). These populations not only showed a high level of admixture with *C. paludica* but also high values of genetic diversity in the SNP analyses. Indeed, they had the highest values of all the populations, as would be expected for hybrids populations. The high diversity values obtained for the SNP data were not congruent with the low ones obtained for *MT-CYB*. However, this observation is not surprising as hybrids typically cannot be detected by mitochondrial markers due to their maternal inheritance.

The case of the population from Caparro River is particularly interesting. It was the only eastern Alagón sub-basin population that showed a closer phylogenetic relationship with populations of *C. paludica* than with those of *C. vettonica* and a higher degree of genetic introgression. This fact could be explained by two hypotheses. The first is that the source population was *C. paludica* and, through a founder effect, a few individuals of *C. vettonica* established themselves in this locality, giving rise to a population of *C. paludica* introgressed by *C. vettonica*. The second hypothesis is that the source population was *C. vettonica*, but it was displaced by *C. paludica* after this species' arrival due to its better adapted genome (Abbott et al., 2013).

4.2.3 | (V3) Ponsul, Aravil, and Erjas sub-basins

According to the mitochondrial analyses, the Ponsul, Aravil, and Erjas sub-basin populations of *C. vettonica* were related to a population of *C. paludica* from Salor River whose most common haplotype was also shared by all of these populations of *C. vettonica*. In addition, the results of the SNP analysis revealed a strong relationship between the Erjas population (upper Erjas) with those in the Salor and Aurela sub-basins, particularly the latter with which it was most closely related phylogenetically. The relationship between these populations was more evident when the Aurela and Salor populations were removed from the SNP genetic diversity analysis, which resulted in an increase in the number of private alleles from eight to 141. However, in the SNP structure analysis, only one individual belonging to the Erjas population had a very low contribution of the genetic group

assigned to *C. paludica*. Altogether, the results do not support the presence of genetic introgression in the upper Erjas, where no mitochondrial haplotypes of *C. paludica* were found. Moreover, higher values of genetic diversity would be expected for the SNP data if there were introgressions, as occurs in the eastern Alagón sub-basin populations.

However, we postulate genetic introgression in the Ponsul, Aravil, and lower Erjas sub-basin, where both species are present (Perdices & Coelho, 2020). This postulation is based on their location outside the area indicated by the ecological niche models and the fact that introgression by hybridization is a common phenomenon that has been detected for not only *C. vettonica* and *C. paludica* but also other species of European *Cobitis* (Bohlen & Ráb, 2001; Janko et al., 2007; Ráb & Slavík, 1996). Furthermore, this general pattern has often been observed in the Iberian Peninsula for two sympatric species as a consequence of secondary contact after diversification (Corral-Lou et al., 2019; Doadrio et al., 2021; Perea et al., 2016, 2021).

Within this group, hybrid localities seem to be restricted to the lower reaches of rivers, while the upper reaches are inhabited by only *C. vettonica* (Perdices & Coelho, 2020). The habitat preferences of the two species, *C. paludica* in the middle and lower reaches, and *C. vettonica* in the upper reaches (Doadrio et al., 2011), likely explains the restricted distribution of the hybrids.

4.3 | Biogeography

According to our divergence time estimations, the timing of these divergences agrees with a common period of population differentiation for many Iberian freshwater fish species, which in turn coincides with the culmination of the formation of the Iberian drainage network and the Pleistocene glacial cycles (Casal-López et al., 2017; Corral-Lou et al., 2019, 2021; Pais et al., 2012; Perea et al., 2016). Although geological information for the study area is scarce, several tectonic and climatic events are known to have occurred during the Quaternary near the study area, which influenced the hydrogeomorphology of the aquatic network such as the fluvial capture phenomena, the presence of paleoglaciers during the last glacial cycle, and changes in the trajectory of some rivers (Benito et al., 2003; Carrasco et al., 2013, 2015; Goy et al., 2020). Therefore, we propose several hypotheses based on the biogeography of the species, which may reveal the hydrogeomorphological evolution of the study area within the Iberian Peninsula.

The earliest divergence in *C. vettonica* was the Ponsul-Aravil-Erjas lineage (V3), which split from the two Alagón sub-basin lineages (V1-V2) during the Pleistocene (Calabrian period, ~250,000 Mya). Based on the ancestral area reconstructions, the diversification of these lineages occurred in the area currently comprising both the Erjas and Alagón sub-basins. This implies that the Erjas acquired its current configuration during this period of time, interrupting any gene flow that may have been established by connections between tributaries on the right bank of Erjas River and the left bank of Árrago River.

Subsequently, following the isolation and diversification of the Erjas sub-basin population from the rest of the populations, individuals from this sub-basin dispersed to downstream areas of the Tagus Basin (reaching Ponsul, Aravil and Salor sub-basins). These dispersal events could have taken place as a consequence of climatic changes associated with the LGM, as supported by our niche models which indicated the Ocreza, Nisa, Sever, Ponsul, Aravil, and Salor sub-basins as more suitable habitats than the rest of the rivers in the current distribution range of the species. These events gave rise to both the colonization of new areas to the west (Ponsul and Aravil sub-basins) and the sympatry with *C. paludica*, which led to introgression events.

The divergence of the two other lineages comprising the western and the eastern Alagón sub-basin populations (V1 and V2) also occurred during the Pleistocene (Chibanian period, ~80,000 Mya). The complex geology of the Alagón sub-basin is evident by the anomalous current trajectory of some of its rivers (Carrasco & Pedraza, 1991; Díez Herrero, 2003; Goy et al., 2020; Jiménez, 1994; Schnabel & Gutiérrez, 2014). For instance, the two banks of the Alagón sub-basin, represented by the two mitochondrial lineages, are currently joined by the mouth of the Árrago River in the Alagón River, that is, the main river of the western and the eastern Alagón sub-basin, respectively. The union between the two banks occurred relatively recently as a consequence of the upriver action of the Alagón River, which captured the Caparro River that eventually flowed into the Tagus through the Fresnedosa riverbank (Schnabel & Gutiérrez, 2014). Given this context, we propose two scenarios for the divergence of V1 and V2. In the first, the two lineages diverged prior to the union of the two banks and later genetic flow between populations was not possible due to the poor habitat conditions for *C. vettonica* in the lower Alagón sub-basin. In the second, they diverged after the union of the banks due to climatic changes during the LGM. According to our niche models, the optimal ecological conditions for *C. vettonica* were located further downstream and, as predicted by phylogeography theory, the latitudinal distribution of organisms retracted southwards (Ehrich et al., 2007; Hewitt, 1996; Rodríguez et al., 2011). Once optimal conditions returned to the river headwaters, the species went back upstream toward both the east and west margins of the Alagón sub-basin, and subsequently diversified.

After the divergence of the two banks of the Alagón, the populations in the Duero and western Alagón sub-basin diverged. Several studies have reported on the genetic structure of freshwater fauna associated with connections between the Tagus Basin and adjacent basins (e.g., Alagón, Alberche and Lozoya rivers; Carmona et al., 2000; Casas-Sainz & De Vicente, 2009; Doadrio, 1988; Pérez-González, 1980; Sousa-Santos et al., 2007). Although recent connections may be explained as a consequence of the tectonic activity in the region (Goy et al., 2020), there are no geological studies that support a recent connection between the western Alagón sub-basin (Tagus Basin) and the Águeda sub-basin (Duero Basin), despite the close proximity of some of their river headwaters (in some cases, as little as 150 m of linear distance).

A connection, however, has been hypothesized to explain the co-occurrence of individuals with the mitochondrial genome of *Squalius carolitertii* (distributed in the Duero Basin) and those of *S. pyrenaicus* (distributed in the Tagus Basin) in the Árrago River (Perea et al., 2021). The close proximity of Mayas (Duero Basin) and Árrago (Tagus Basin) rivers, whose courses are only separated by ~150 m, is particularly notable. Piracy events that occurred between these two rivers in the Pleistocene may explain the close mitochondrial relationship of the populations inhabiting them, as suggested by the dispersal event estimated in our ancestral area reconstruction analysis. Later isolation of the Duero and Tagus hydrological basins then led to the divergence of these populations, as corroborated by the SNP analysis.

4.4 | Conservation

The habitats of *C. vettonica* have been and continue to be threatened by the main causes of biodiversity loss (e.g., overexploitation, water pollution, flow modification, habitat destruction and degradation, and the introduction of invasive species) (Doadrio et al., 2011; Dudgeon, 2019; Sousa-Santos et al., 2014). Given these threats, the populations in OCU I (Águeda sub-basin) are particularly vulnerable as they had the lowest genetic diversity values for both MT-CYB and the SNPs, and only one MT-CYB haplotype shared by all the populations. This low level of genetic diversity makes them more sensitive to extrinsic changes and therefore at greater risk of extinction (Frankham et al., 2002). In addition, populations in OCU I are shrinking as a result of the formation of dams along the Águeda sub-basin, and in some localities (i.e., Turones), they have even disappeared. The species' distribution in this area is now restricted to a few tributaries of Mayas River. Although the genetic diversity values of populations in OCU II were higher than those of OCU I, the possibility of a bottleneck followed by a population expansion cannot be rejected for these populations, despite a decrease in the number of individuals observed in recent years (Doadrio et al., 2011). Exotic species, such as *Lepomis gibbosus* and *Micropterus salmoides*, have colonized the upper parts of the rivers covered by OCU II, which may be one of the main causes for the decline in the number of *C. vettonica* in these rivers. Other potential causes of this decline remain unknown.

For OCU IV, there was no genetic distinction between the non-introgressed (upper Erjas) and putatively introgressed (Ponsul, Aravil, and lower Erjas) populations. Despite this, any management plan must take into consideration this potential introgression as the indiscriminate mixing of individuals from these two groups could lead to genetic introgression with *C. paludica* throughout the entire area of OCU IV. *Cobitis paludica*, which is more of a generalist than *C. vettonica*, is widely distributed throughout the Iberian Peninsula, occupying a great variety of ecological niches (Doadrio et al., 2011). Thus, genetic introgression by *C. paludica* in sympatric localities would likely prove disadvantageous for *C. vettonica* in general, as two of the four OCUs (III and IV) would be affected by this phenomenon. It could lead to the extinction of *C. vettonica* in these areas or

to adaptive variations, resulting in a major loss of the genetic diversity of this species.

ACKNOWLEDGMENTS

We thank P. Garzón, I. Doadrio Jr, J.L. González, and G. González for collecting almost every sample from 2009 to 2010 under the project "Basis for the monitoring of Spanish Freshwater Fishes", supported by the Ministerio de Medio Ambiente y Medio Rural y Marino (MARM), and C. Marcos, J.C Velaco, and G. González for their help in the project Life13 nat/es/000772. We greatly thank L. Alcaraz for laboratory assistance. We are grateful for the valuable suggestions and English editing performed by M. Modrell. This study was also supported by a management commission funded by the Duero Hydrographic Confederation in Spain (Análisis y estudios filogeográficos y de variabilidad genética de endemismos ibéricos de ciprínidos: Life13 nat/es/000772 "Actuaciones para la protección y conservación de ciprínidos ibéricos de interés comunitario") and by grant IND2017/AMB-7699 to AC-L, funded by the Community of Madrid (Spain) through its Industrial Doctorate program. We acknowledge the Santander Supercomputacion support group at the University of Cantabria for providing access to the supercomputer Altamira at the Institute of Physics of Cantabria (IFCA-CSIC), a member of the Spanish Supercomputing Network, which was used to perform simulations/analyses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Andrea Corral-Lou: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Methodology (equal); Resources (lead); Software (lead); Validation (lead); Visualization (lead); Writing – original draft (lead); Writing – review & editing (lead). **Silvia Perea:** Conceptualization (equal); Data curation (equal); Formal analysis (supporting); Methodology (supporting); Supervision (supporting); Writing – review & editing (equal). **Anabel Perdices:** Resources (supporting); Supervision (supporting); Writing – review & editing (supporting). **Ignacio Doadrio:** Conceptualization (lead); Data curation (equal); Funding acquisition (lead); Investigation (lead); Methodology (lead); Resources (equal); Supervision (lead); Validation (lead); Visualization (equal); Writing – review & editing (equal).

DATA AVAILABILITY STATEMENT

The new sequences of the mitochondrial (MT-CYTB; OM234794-OM235001) and nuclear (RAG1; OM235002-OM235091) markers obtained from this study are available in GenBank. The SNP data have been deposited in VCF format in Figshare (<https://doi.org/10.6084/m9.figshare.18778148.v1>).

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REFERENCES

- Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., Boughman, J., Brelsford, A., Buerkle, C. A., Buggs, R., Butlin, R. K., Dieckmann, U., Eroukmanoff, F., Grill, A., Cahan, S. H., Hermansen, J. S., Hewitt, G., Hudson, A. G., Jiggins, C., ... Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, 26(2), 229–246. <https://doi.org/10.1111/j.1420-9101.2012.02599.x>
- Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19(6), 716–723. <https://doi.org/10.1109/TAC.1974.1100705>
- Almodóvar, A., Nicola, G. G., Leal, S., Torralva, M., & Elvira, B. (2012). Natural hybridization with invasive bleak *Alburnus alburnus* threatens the survival of Iberian endemic calandino *Squalius alburnoides* complex and Southern Iberian chub *Squalius pyrenaicus*. *Biological Invasions*, 14(11), 2237–2242. <https://doi.org/10.1109/TAC.1974.1100705>
- Alonso, J. L., Pulgar, F. J. Á., & Pedreira, D. (2007). The relief of the Cantabrian Mountains. *Teaching Earth Sciences [In Spanish]*, 15(2), 151–163. <https://doi.org/10.1109/TAC.1974.1100705>
- Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16(1), 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Beaulieu, J. M., Tank, D. C., & Donoghue, M. J. (2013). A Southern Hemisphere origin for campanulid angiosperms, with traces of the break-up of Gondwana. *BMC Evolutionary Biology*, 13(1), 1–17. <https://doi.org/10.1186/1471-2148-13-80>
- Benito, G., Sopeña, A., Sánchez-Moya, Y., Machado, M. J., & Pérez-González, A. (2003). Palaeoflood record of the Tagus River (central Spain) during the Late Pleistocene and Holocene. *Quaternary Science Reviews*, 22(15–17), 1737–1756. [https://doi.org/10.1016/S0277-3791\(03\)00133-1](https://doi.org/10.1016/S0277-3791(03)00133-1)
- Bohlen, J., & Ráb, P. (2001). Species and hybrid richness in spined loaches of the genus *Cobitis* (Teleostei: Cobitidae), with a checklist of European forms and suggestions for conservation. *Journal of Fish Biology*, 59, 75–89. <https://doi.org/10.1111/j.1095-8649.2001.tb01380.x>
- Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>
- Buj, I., Marčić, Z., Čaleta, M., Šanda, R., Geiger, M. F., Freyhof, J., Machordom, A., & Vukić, J. (2017). Ancient connections among the European rivers and watersheds revealed from the evolutionary history of the genus *Telestes* (Actinopterygii: Cypriniformes). *PLoS One*, 12(12), e0187366. <https://doi.org/10.1371/journal.pone.0187366>
- Carmona, J. A., Doadrio, I., Márquez, A. L., Real, R., Huguely, B., & Vargas, J. M. (1999). Distribution patterns of indigenous freshwater fishes in the Tagus River basin, Spain. *Environmental Biology of Fishes*, 54(4), 371–387. <https://doi.org/10.1023/A:1007535200837>
- Carmona, J. A., Domínguez, J., & Doadrio, I. (2000). Congruence between allozyme and cytochrome b gene sequence data in assessing genetic differentiation within the Iberian endemic *Chondrostoma lemmingii* (Pisces: Cyprinidae). *Heredity*, 84(6), 721–732. <https://doi.org/10.1046/j.1365-2540.2000.00720.x>
- Carrasco, R. M., & Pedraza, J. (1991). In the Jerte valley [In Spanish]. *Cuaternario Y Geomorfología*, 5, 15–25. <https://doi.org/10.1046/j.1365-2540.2000.00720.x>
- Carrasco, R. M., Pedraza, J., Domínguez-Villar, D., Villa, J., & Willenbring, J. K. (2013). The plateau glacier in the Sierra de Béjar (Iberian Central System) during its maximum extent. Reconstruction and chronology. *Geomorphology*, 196, 83–93. <https://doi.org/10.1109/TAC.1974.1100705>
- Carrasco, R. M., Pedraza, J., Domínguez-Villar, D., Willenbring, J. K., & Villa, J. (2015). Sequence and chronology of the Cuerpo de Hombre paleoglaciar (Iberian Central System) during the last glacial cycle. *Quaternary Science Reviews*, 129, 163–177. <https://doi.org/10.1016/j.quascirev.2015.09.021>
- Casal-López, M., & Doadrio, I. (2018). The Messinian imprint on the evolution of freshwater fishes of the genus *Luciobarbus* Heckel, 1843 (Teleostei, Cyprinidae) in the western Mediterranean. *Journal of Biogeography*, 45(7), 1593–1603. <https://doi.org/10.1111/jbi.13244>
- Casal-López, M., Perea, S., Sousa-Santos, C., Robalo, J. I., Torralva, M., Oliva-Paterna, F. J., & Doadrio, I. (2017). Paleobiogeography of an Iberian endemic species, *Luciobarbus sclateri* (Günther, 1868) (Actinopterygii, Cyprinidae), inferred from mitochondrial and nuclear markers. *Journal of Zoological Systematics and Evolutionary Research*, 56(2), 127–147. <https://doi.org/10.1111/jzs.12192>
- Casas-Sainz, A. M., & De Vicente, G. (2009). On the tectonic origin of Iberian topography. *Tectonophysics*, 474(1–2), 214–235. <https://doi.org/10.1016/j.tecto.2009.01.030>
- Catchen, J. M., Hohenlohe, P. A., Bernatchez, L., Funk, W. C., Andrews, K. R., & Allendorf, F. W. (2013). Stacks: An analysis tool set for population genomics. *Molecular Ecology*, 22(11), 3124–3140. <https://doi.org/10.1111/mec.12354>
- Choleva, L., Musilova, Z., Kohoutova-Sediva, A., Paces, J., Rab, P., & Janko, K. (2014). Distinguishing between incomplete lineage sorting and genomic introgressions: complete fixation of allospecific mitochondrial DNA in a sexually reproducing fish (Cobitis; Teleostei), despite clonal reproduction of hybrids. *PLoS One*, 9(6), e80641. <https://doi.org/10.1371/journal.pone.0080641>
- Collares-Pereira, M. J., Alves, M. J., Ribeiro, F., Domingos, I., Almeida, P. R., Da Costa, L., Gante, H., Filipe, A. F., Aboim, A., Rodrigues, P. M., & Magalhães, M. F. (2021). *Guide to freshwater fish and migrants from mainland Portugal [In Portuguese]* (p. 292). Edições Afrontamento.
- Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926–941. <https://doi.org/10.1111/jzs.12320>
- Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585–600. <https://doi.org/10.1007/s10592-021-01381-y>
- Cunha, C., Coelho, M. M., Carmona, J. A., & Doadrio, I. (2004). Phylogeographical insights into the origins of the *Squalius alburnoides* complex via multiple hybridization events. *Molecular Ecology*, 13(9), 2807–2817. <https://doi.org/10.1111/j.1365-294X.2004.02283.x>
- Denys, G. P. J., Dettai, A., Persat, H., Doadrio, I., Cruaud, C., & Keith, P. (2013). Status of the Catalan chub *Squalius laietanus* (Actinopterygii, Cyprinidae) in France: Input from morphological and molecular data. *Knowledge and Management of Aquatic Ecosystems*, 408, 04. <https://doi.org/10.1051/kmae/2013039>
- Díez Herrero, A. (2003). Geomorfología e Hidrología fluvial del río Alberche. Modelos y SIG para la gestión de riberas. Serie Tesis Doctorales 2. Publicaciones del Instituto Geológico y Minero de España, Madrid. 587 pp.
- Doadrio, I. (1988). Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. *Bonner Zoologische Beiträge*, 39, 113–128.
- Doadrio, I., Carmona, J. A., & Machordom, A. (2002). Haplotype diversity and phylogenetic relationships among the Iberian barbels (*Barbus*, Cyprinidae) reveal two evolutionary lineages. *Journal of Heredity*, 93(2), 140–147. <https://doi.org/10.1093/jhered/93.2.140>
- Doadrio, I., Perdices, A., & Machordom, A. (1996). Allozymic variation of the endangered killifish *Aphanius iberus* and its application to conservation. *Environmental Biology of Fishes*, 45(3), 259–271.
- Doadrio, I., & Perdices, A. (2005). Phylogenetic relationships among the Ibero-African cobitids (Cobitis, Cobitidae) based on cytochrome b

- sequence data. *Molecular Phylogenetics and Evolution*, 37(2), 484–493. <https://doi.org/10.1016/j.ympev.2005.07.009>
- Doadrio, I., Perea, S., & Corral-Lou, A. (2021). *Genetic characterization and identification of Conservation Units in endemic fish of community interest* [In Spanish]. Retrieved from <http://hdl.handle.net/10261/241564>
- Doadrio, I., Perea, S., Paloma, G. H., & González, J. L. (2011). *Ictiofauna continental española: bases para su seguimiento*, Ministerio de Medio Ambiente y Medio Rural y Marino. Centro de Publicaciones. DG Medio Natural y Política Forestal, MARM.
- Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*, 29(8), 1969–1973. <https://doi.org/10.1093/molbev/mss075>
- Dudgeon, D. (2019). Multiple threats imperil freshwater biodiversity in the Anthropocene. *Current Biology*, 29(19), R960–R967. <https://doi.org/10.1016/j.cub.2019.08.002>
- Ehrich, D., Gaudeul, M., Assefa, A., Koch, M. A., Mummenhoff, K., Nemomissa, S., Consortium, I., & Brochmann, C. (2007). Genetic consequences of Pleistocene range shifts: Contrast between the Arctic, the Alps and the East African mountains. *Molecular Ecology*, 16(12), 2542–2559. <https://doi.org/10.1111/j.1365-294X.2007.03299.x>
- Elith, J., H. Graham, C., P. Anderson, R., Dudík, M., Ferrier, S., Guisan, A., J. Hijmans, R., Huettmann, F., R. Leathwick, J., Lehmann, A., Li, J., G. Lohmann, L., A. Loiselle, B., Manion, G., Moritz, C., Nakamura, M., Nakazawa, Y., McC. M. Overton, J., Townsend Peterson, A., ... E. Zimmermann, N. (2006). Novel methods improve prediction of species' distributions from occurrence data. *Ecography*, 29(2), 129–151. <https://doi.org/10.1111/j.2006.0906-7590.04596.x>
- Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14(8), 2611–2620. <https://doi.org/10.1111/j.1365-294X.2005.02553.x>
- Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, 10(3), 564–567. <https://doi.org/10.1111/j.1755-0998.2010.02847.x>
- Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: New 1-km spatial resolution climate surfaces for global land areas. *International Journal of Climatology*, 37(12), 4302–4315. <https://doi.org/10.1002/joc.5086>
- Foll, M., & Gaggiotti, O. (2008). A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: A Bayesian perspective. *Genetics*, 180(2), 977–993. <https://doi.org/10.1534/genetics.108.092221>
- Frankham, R., Ballou, S. E. J. D., Briscoe, D. A., & Ballou, J. D. (2002). *Introduction to conservation genetics*. Cambridge University Press.
- Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147(2), 915–925. <https://doi.org/10.1093/genetics/147.2.915>
- Gante, H. M. F. (2009). *A role for introgressive hybridization in the evolution of freshwater fishes: The case-study of Iberian Barbus (Teleostei, Cyprinidae)*. Arizona State University.
- Gante, H. F., Micael, J., Oliva-Paterna, F. J., Doadrio, I., Dowling, T. E., & Alves, M. J. (2009). Diversification within glacial refugia: tempo and mode of evolution of the polytypic fish *Barbus sclateri*. *Molecular Ecology*, 18(15), 3240–3255. <https://doi.org/10.1111/j.1365-294X.2009.04264.x>
- Gibbard, P. L., Head, M. J., Walker, M. J., & Subcommission on Quaternary Stratigraphy (2010). Formal ratification of the Quaternary System/Period and the Pleistocene Series/Epoch with a base at 2.58 Ma. *Journal of Quaternary Science*, 25(2), 96–102. <https://doi.org/10.1002/jqs.1338>
- Gonzalez, E. G., Cunha, C., Ghanavi, H. R., Oliva-Paterna, F. J., Torralva, M., & Doadrio, I. (2018). Phylogeography and population genetic analyses in the Iberian toothcarp (*Aphanius iberus* Valenciennes, 1846) at different time scales. *Journal of Heredity*, 109(3), 253–263. <https://doi.org/10.1093/jhered/esx076>
- Gonzalez, E. G., Pedraza-Lara, C., & Doadrio, I. (2014). Genetic diversity and population history of the endangered killifish *Aphanius baeticus*. *Journal of Heredity*, 105(5), 597–610. <https://doi.org/10.1093/jhered/esu034>
- Goy, J. L., Cruz, R., Martínez-Graña, A., Valdés, V., & Yenes, M. (2020). Geomorphological map and quaternary landscape evolution of the Monfragüe Park (Cáceres, Spain). *Sustainability*, 12(23), 10099. <https://doi.org/10.3390/su122310099>
- Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, 58(3), 247–276. <https://doi.org/10.1111/j.1095-8312.1996.tb01434.x>
- Hewitt, G. M. (2004). Genetic consequences of climatic oscillations in the Quaternary. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 359(1442), 183–195. <https://doi.org/10.1098/rstb.2003.1388>
- Hudson, R. R., Boos, D. D., & Kaplan, N. L. (1992). A statistical test for detecting geographic subdivision. *Molecular Biology and Evolution*, 9(1), 138–151. <https://doi.org/10.1093/oxfordjournals.molbev.a040703>
- Janes, J. K., Miller, J. M., Dupuis, J. R., Malenfant, R. M., Gorrell, J. C., Cullingham, C. I., & Andrew, R. L. (2017). The K = 2 conundrum. *Molecular Ecology*, 26(14), 3594–3602. <https://doi.org/10.1111/mec.14187>
- Janko, K., Culling, M. A., Rab, P., & Kotlik, P. (2005). Ice age cloning-comparison of the Quaternary evolutionary histories of sexual and clonal forms of spiny loaches (Cobitis; Teleostei) using the analysis of mitochondrial DNA variation. *Molecular Ecology*, 14(10), 2991–3004. <https://doi.org/10.1111/j.1365-294X.2005.02583.x>
- Janko, K., Flajšhans, M., Choleva, L., Bohlen, J., Šlechtová, V., Rábová, M., Lajbner, Z., Šlechtá, V., Ivanova, P., Dobrovolov, I., Culling, M., Persat, H., Kotusz, J., & Ráb, P. (2007). Diversity of European spined loaches (genus *Cobitis* L.): an update of the geographic distribution of the *Cobitis taenia* hybrid complex with a description of new molecular tools for species and hybrid determination. *Journal of Fish Biology*, 71, 387–408. <https://doi.org/10.1111/j.1095-8649.2007.01663.x>
- Jiménez, A. S. D. C. (1994). The mountain-lower surface contact in the southern slope of the Sierra de Gata (Spanish Central System, Cáceres) [In Spanish]. *Cuaternario Y Geomorfología*, 8(1–2), 45–55.
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, 30(4), 772–780. <https://doi.org/10.1093/molbev/mst010>
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., & Drummond, A. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647–1649. <https://doi.org/10.1093/bioinformatics/bts199>
- Kess, T., Gross, J., Harper, F., & Boulding, E. G. (2016). Low-cost ddRAD method of SNP discovery and genotyping applied to the periwinkle *Littorina saxatilis*. *Journal of Molluscan Studies*, 82(1), 104–109. <https://doi.org/10.1093/mollus/eyv042>
- Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., & Calcott, B. (2017). PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution*, 34(3), 772–773. <https://doi.org/10.1093/molbev/msw260>
- Leigh, J. W., & Bryant, D. (2015). popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9), 1110–1116. <https://doi.org/10.1111/2041-210X.12410>
- Li, Y. L., & Liu, J. X. (2018). StructureSelector: A web-based software to select and visualize the optimal number of clusters using multiple methods. *Molecular Ecology Resources*, 18(1), 176–177. <https://doi.org/10.1111/1755-0998.12719>

- Lotterhos, K. E., & Whitlock, M. C. (2014). Evaluation of demographic history and neutral parameterization on the performance of FST outlier tests. *Molecular Ecology*, 23(9), 2178–2192. <https://doi.org/10.1111/mec.12725>
- Machordom, A., Berrebi, P., & Doadrio, I. (1990). Spanish barbel hybridization detected using enzymatic markers: *Barbus meridionalis* Risso $\times$ *Barbus haasi* Mertens (Osteichthyes, Cyprinidae). *Aquatic Living Resources*, 3(4), 295–303. <https://doi.org/10.1051/alr:1990030>
- Marić, S., Stanković, D., Šanda, R., Čaleta, M., Čolić, S., Šukalo, G., & Snoj, A. (2019). Genetic characterisation of European mudminnow (*Umbra krameri*) populations from the Sava River system. *Knowledge & Management of Aquatic Ecosystems*, 420, 46. <https://doi.org/10.1051/kmae/2019035>
- Marić, S., Stanković, D., Wanzenböck, J., Šanda, R., Erős, T., Takács, P., Specziár, A., Sekulić, N., Bănăduc, D., Čaleta, M., Trombitsky, I., Galambos, L., Sipos, S., & Snoj, A. (2017). Phylogeography and population genetics of the European mudminnow (*Umbra krameri*) with a time-calibrated phylogeny for the family Umbridae. *Hydrobiologia*, 792(1), 151–168. <https://doi.org/10.1007/s10750-016-3051-9>
- McCormack, J. E., Hird, S. M., Zellmer, A. J., Carstens, B. C., & Brumfield, R. T. (2013). Applications of next-generation sequencing to phylogeography and phylogenetics. *Molecular Phylogenetics and Evolution*, 66(2), 526–538. <https://doi.org/10.1016/j.ympev.2011.12.007>
- Mendes, S. L., Coelho, M. M., & Sousa, V. C. (2019). Population genomics data supports introgression between Western Iberian *Squalius* freshwater fish species from different drainages. *bioRxiv*, 585687.
- Mesquita, N., Hänfling, B., Carvalho, G. R., & Coelho, M. M. (2005). Phylogeography of the cyprinid *Squalius aradensis* and implications for conservation of the endemic freshwater fauna of southern Portugal. *Molecular Ecology*, 14(7), 1939–1954. <https://doi.org/10.1111/j.1365-294X.2005.02569.x>
- Myers, G. S. (1966). Derivation of the freshwater fish fauna of Central America. *Copeia*, 766–773. <https://doi.org/10.2307/1441405>
- Pais, J., Cunha, P. P., Pereira, D., Legoinha, P., Dias, R., Moura, D., González-Delgado, J. A. (2012). The Paleogene and Neogene of western Iberia (Portugal): a Cenozoic record in the European Atlantic domain. In *The Paleogene and Neogene of Western Iberia (Portugal)* (pp. 1–138). Springer Briefs in Earth Sciences, Springer, Series ID: 8897, 158 pp. https://doi.org/10.1007/978-3-642-22401-0_1
- Paris, J. R., Stevens, J. R., & Catchen, J. M. (2017). Lost in parameter space: A road map for stacks. *Methods in Ecology and Evolution*, 8(10), 1360–1373. <https://doi.org/10.1111/2041-210X.12775>
- Perdices, A., & Coelho, M. M. (2020). First records of the Vettonian spined loach *Cobitis vettonica* in Portugal with update on its Iberian distribution. *Fishes in Mediterranean Environments*, 2020(002), 15. <https://doi.org/10.29094/FISHMED.2020.002>
- Perdices, A., & Doadrio, I. (2001). The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 19(3), 468–478. <https://doi.org/10.1006/mpev.2000.0900>
- Perea, S., & Doadrio, I. (2015). Phylogeography, historical demography and habitat suitability modelling of freshwater fishes inhabiting seasonally fluctuating Mediterranean river systems: a case study using the Iberian cyprinid *Squalius valentinus*. *Molecular Ecology*, 24(14), 3706–3722.
- Perea, S., Cobo-Simon, M., & Doadrio, I. (2016). Cenozoic tectonic and climatic events in southern Iberian Peninsula: Implications for the evolutionary history of freshwater fish of the genus *Squalius* (Actinopterygii, Cyprinidae). *Molecular Phylogenetics and Evolution*, 97, 155–169. <https://doi.org/10.1016/j.ympev.2016.01.007>
- Perea, S., Sousa-Santos, C., Robalo, J., & Doadrio, I. (2021). Historical biogeography of the Iberian Peninsula: Multilocus phylogeny and ancestral area reconstruction for the freshwater fish genus *Squalius* (Actinopterygii, Leuciscidae). *Journal of Zoological Systematics and Evolutionary Research*, 59(4), 858–886. <https://doi.org/10.1111/jzs.12464>
- Pérez-González, A. (1980). Geology and stratigraphy of the Aridos deposits in the alluvial plain of Arganda (Madrid) [In Spanish]. In M. Santonja, N. López Martínez, & A. Pérez-González (Eds.), *Ocupaciones achelenses en el valle del Jarama (Arganda-Madrid)* (pp. 49–61). Publicaciones de la Excelentísima Diputación Provincial de Madrid.
- Phillips, S. J., Anderson, R. P., Dudík, M., Schapire, R. E., & Blair, M. E. (2017). Opening the black box: An open-source release of Maxent. *Ecography*, 40(7), 887–893. <https://doi.org/10.1111/ecog.03049>
- Pillans, B., & Naish, T. (2004). Defining the quaternary. *Quaternary Science Reviews*, 23(23–24), 2271–2282. <https://doi.org/10.1016/j.quascirev.2004.07.006>
- Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155(2), 945–959. <https://doi.org/10.1093/genetics/155.2.945>
- Puechmaile, S. J. (2016). The program structure does not reliably recover the correct population structure when sampling is uneven: Subsampling and new estimators alleviate the problem. *Molecular Ecology Resources*, 16(3), 608–627. <https://doi.org/10.1111/1755-0998.12512>
- Querejeta, M., Fernández-González, A., Romero, R., & Castresana, J. (2017). Postglacial dispersal patterns and mitochondrial genetic structure of the Pyrenean desman (*Galemys pyrenaicus*) in the northwestern region of the Iberian Peninsula. *Ecology and Evolution*, 7(12), 4486–4495. <https://doi.org/10.1002/ece3.3034>
- Ráb, P., & Slavík, O. (1996). Diploid-triploid-tetraploid complex of the spined loach, genus *Cobitis* in Pšovka Creek: The first evidence of new species of Cobitis in the ichthyofauna of the Czech Republic. *Acta Universitatis Carolinae. Biologica*, 39, 201–214.
- Ree, R. H., & Smith, S. A. (2008). Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic Biology*, 57(1), 4–14. <https://doi.org/10.1080/10635150701883881>
- Rodríguez, R., Ramirez, O., Valdiosera, C. E., García, N., Alda, F., Madurell-Malapeira, J., & Dalen, L. (2011). 50,000 years of genetic uniformity in the critically endangered Iberian lynx. *Molecular Ecology*, 20(18), 3785–3795. <https://doi.org/10.1111/j.1365-294X.2011.05231.x>
- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A., & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61(3), 539–542. <https://doi.org/10.1093/sysbio/sys029>
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*, 34(12), 3299–3302. <https://doi.org/10.1093/molbev/msx248>
- Schnabel, S., & Gutiérrez, Á. G. (2014). Advances in Geomorphology in Spain 2012–2014. In S. Schnabel & Á. G. Gutiérrez (Eds.), *Actas de la XIII Reunión Nacional de Geomorfología. Cáceres, Universidad de Extremadura-Sociedad Española de Geomorfología*.
- Seifertová, M., Bryja, J., Vyskočilová, M., Martínková, N., & Šimková, A. (2012). Multiple Pleistocene refugia and post-glacial colonization in the European chub (*Squalius cephalus*) revealed by combined use of nuclear and mitochondrial markers. *Journal of Biogeography*, 39(6), 1024–1040. <https://doi.org/10.1111/j.1365-2699.2011.02661.x>
- Sousa-Santos, C., Collares-Pereira, M. J., & Almada, V. (2007). Reading the history of a hybrid fish complex from its molecular record. *Molecular Phylogenetics and Evolution*, 45(3), 981–996. <https://doi.org/10.1016/j.ympev.2007.05.011>
- Sousa-Santos, C., Jesus, T. F., Fernandes, C., Robalo, J. I., & Coelho, M. M. (2019). Fish diversification at the pace of geomorphological changes: Evolutionary history of western Iberian Leuciscinae

- (Teleostei: Leuciscidae) inferred from multilocus sequence data. *Molecular Phylogenetics and Evolution*, 133, 263–285. <https://doi.org/10.1016/j.ympev.2018.12.020>
- Sousa-Santos, C., Robalo, J. I., & Pereira, A. (2014). Threatened fishes of the world: Cobitis vettonica Doadrio & Perdices, 1997 (Cobitidae). *Croatian Journal of Fisheries*, 72(4), 174–175. <https://doi.org/10.1109/TAC.1974.1100705>
- Stamatakis, A. (2016). *The RAxML v8. 2. X Manual*. Heidelberg Institute for Theoretical Studies. Retrieved from <https://cmeh-itsorg/exelixis/resource/download/NewManualpdf> <https://doi.org/10.1109/TAC.1974.1100705>
- Stephens, M., & Donnelly, P. (2003). A comparison of bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, 73(5), 1162–1169. <https://doi.org/10.1086/379378>
- Swets, J. A. (1988). Measuring the accuracy of diagnostic systems. *Science*, 240(4857), 1285–1293. <https://doi.org/10.1109/TAC.1974.1100705>
- Taberlet, P., & Bouvet, J. (1994). Mitochondrial DNA polymorphism, phylogeography, and conservation genetics of the brown bear *Ursus arctos* in Europe. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 255(1344), 195–200. <https://doi.org/10.1098/rspb.1994.0028>
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585–595. <https://doi.org/10.1093/genetics/123.3.585>
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, 30(12), 2725–2729. <https://doi.org/10.1093/molbev/mst197>
- Tan, M. P., Wong, L. L., Razali, S. A., Afiqah-Aleng, N., Mohd Nor, S. A., Sung, Y. Y., Van de Peer, Y., Sorgeloos, P., & Danish-Daniel, M. (2019). Applications of next-generation sequencing technologies and computational tools in molecular evolution and aquatic animals conservation studies: A short review. *Evolutionary Bioinformatics*, 15, 1176934319892284. <https://doi.org/10.1177/1176934319892284>
- Tancioni, L., Russo, T., Cataudella, S., Milana, V., Hett, A. K., Corsi, E., & Rossi, A. R. (2013). Testing species delimitations in four Italian sympatric leuciscine fishes in the Tiber River: A combined morphological and molecular approach. *PLoS One*, 8(4), e60392. <https://doi.org/10.1371/journal.pone.0060392>
- Valdiosera, C. E., García-Garitaigotia, J. L., García, N., Doadrio, I., Thomas, M. G., Hänni, C., & Götherström, A. (2008). Surprising migration and population size dynamics in ancient Iberian brown bears (*Ursus arctos*). *Proceedings of the National Academy of Sciences of the United States of America*, 105(13), 5123–5128. <https://doi.org/10.1073/pnas.0712223105>
- Weiss, S., & Ferrand, N. (2007). *Phylogeography of southern European refugia* (pp. 341–357). Springer.
- Wetjen, M., Hübner, D., Seehausen, O., & Schulz, R. (2020). Genetic diversity of endangered *Chondrostoma nasus* in the River Rhine system: Conservation genetics considerations on stocking and reintroduction. *Knowledge & Management of Aquatic Ecosystems*, 421, 25. <https://doi.org/10.1051/kmae/2020016>
- Yu, Y., Blair, C., & He, X. (2020). RASP 4: ancestral state reconstruction tool for multiple genes and characters. *Molecular Biology and Evolution*, 37(2), 604–606. <https://doi.org/10.1093/molbev/msz257>
- Yu, Y., Harris, A. J., Blair, C., & He, X. (2015). RASP (Reconstruct Ancestral State in Phylogenies): a tool for historical biogeography. *Molecular Phylogenetics and Evolution*, 87, 46–49. <https://doi.org/10.1016/j.ympev.2015.03.008>
- Yu, Y., Harris, A. J., & He, X. (2010). S-DIVA (Statistical Dispersal-Vicariance Analysis): A tool for inferring biogeographic histories. *Molecular Phylogenetics and Evolution*, 56(2), 848–850. <https://doi.org/10.1016/j.ympev.2010.04.011>
- Zarraonaindia, I., Iriondo, M., Albaina, A., Pardo, M. A., Manzano, C., Grant, W. S., Irigoien, X., & Estonba, A. (2012). Multiple SNP markers reveal fine-scale population and deep phylogeographic structure in European anchovy (*Engraulis encrasicolus* L.). *PLoS One*, 7(7), e42201. <https://doi.org/10.1371/journal.pone.0042201>

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12, e8635. <https://doi.org/10.1002/ece3.8635>

Material Suplementario

Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12(3), e8635.

Supplementary Information for:

Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: the case of the genus *Cobitis* (Cypriniformes, Cobitidae)

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Supporting information

Table S1. Information on the individuals included in the present study. The following parameters are included in the table: Identification code used in this study (ID), species, the origin of the genetic information used (Source), identification number of the DNA and Tissue Collection at the National Museum of Natural Sciences of Madrid (MNCN/DNA), the date when the sample was taken (Date), the genetic marker studied indicated with an X (for SNP, MT-CYB and RAG1), the GenBank access number for MT-CYB and RAG1 (GenBank_ID RAG1 and GenBank_ID MT-CYB respectively), the locality (Locality) and the number of the locality referenced in Table 1 (Number of the locality).

ID	Species	Source	MNCN/ADN	Date	SNPs	MT-CYB	RAG1	GenBank_ID (MT-CYB)	GenBank_ID (RAG1)	Locality	Number of the locality
AT15755	<i>Cobitis paludica</i>	This study	119350	04/05/2010	X	X		OM234965		Spain: Castañar de Ibor, Tagus Basin, lower Tagus Sub-basin, Ibor River	38
AT15756	<i>Cobitis paludica</i>	This study	119351	04/05/2010	X	X		OM234966		Spain: Castañar de Ibor, Tagus Basin, lower Tagus Sub-basin, Ibor River	38
AT15757	<i>Cobitis paludica</i>	This study	119352	04/05/2010	X	X		OM234967		Spain: Castañar de Ibor, Tagus Basin, lower Tagus Sub-basin, Ibor River	38
AT15826	<i>Cobitis vettonica</i>	This study	119394	05/05/2010		X	X	OM234930	OM235035	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	38
AT15827	<i>Cobitis vettonica</i>	This study	119395	05/05/2010		X		OM234931		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	34
AT15828	<i>Cobitis vettonica</i>	This study	119396	05/05/2010		X		OM234932		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	34
AT15829	<i>Cobitis vettonica</i>	This study	119397	05/05/2010		X	X	OM234933	OM235036	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	34
AT15831	<i>Cobitis vettonica</i>	This study	119398	05/05/2010		X	X	OM234934	OM235037	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	34
AT15832	<i>Cobitis vettonica</i>	This study	119399	05/05/2010		X	X	OM234935	OM235038	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	34
AT15833	<i>Cobitis vettonica</i>	This study	119400	05/05/2010		X	X	OM234936	OM235039	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	37
AT15834	<i>Cobitis vettonica</i>	This study	119401	05/05/2010		X		OM234937		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	37
AT15835	<i>Cobitis vettonica</i>	This study	119402	05/05/2010		X		OM234938		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	37
AT15836	<i>Cobitis vettonica</i>	This study	119403	05/05/2010		X		OM234939		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	37
AT15837	<i>Cobitis vettonica</i>	This study	119404	05/05/2010		X		OM234940		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	37
AT15838	<i>Cobitis vettonica</i>	This study	119405	05/05/2010		X		OM234941		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	29

AT15839	<i>Cobitis vettonica</i>	This study	119406	05/05/2010		X		OM234942		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	29
AT15866	<i>Cobitis vettonica</i>	This study	119407	05/05/2010		X	X	OM234919	OM235044	Spain: Vegas de Coria, Tagus Basin, eastern Alagón Sub-basin, Hurdano River	29
AT15905	<i>Cobitis vettonica</i>	This study	119408	06/05/2010		X	X	OM234860	OM235058	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	29
AT15906	<i>Cobitis vettonica</i>	This study	119409	06/05/2010		X	X	OM234861	OM235059	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	29
AT15907	<i>Cobitis vettonica</i>	This study	119410	06/05/2010		X	X	OM234862	OM235060	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	36
AT15908	<i>Cobitis vettonica</i>	This study	119411	06/05/2010		X	X	OM234863	OM235061	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	36
AT15909	<i>Cobitis vettonica</i>	This study	119412	06/05/2010		X		OM234864		Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	36
AT15910	<i>Cobitis vettonica</i>	This study	119413	06/05/2010		X		OM234865		Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	36
AT15911	<i>Cobitis vettonica</i>	This study	119414	06/05/2010		X		OM234850		Spain: Hoyos, Tagus Basin, western Alagón Sub-basin, Acebo River	36
AT15919	<i>Cobitis vettonica</i>	This study	119415	06/05/2010		X		OM234847		Spain: Gata, Tagus Basin, western Alagón Sub-basin, San Blas River	26
AT15930	<i>Cobitis vettonica</i>	This study	119416	06/05/2010		X		OM234825		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	26
AT15931	<i>Cobitis vettonica</i>	This study	119417	06/05/2010		X		OM234826		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	26
AT15932	<i>Cobitis vettonica</i>	This study	119418	06/05/2010		X		OM234827		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	26
AT15933	<i>Cobitis vettonica</i>	This study	119419	06/05/2010		X		OM234828		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	26
AT15934	<i>Cobitis vettonica</i>	This study	119420	06/05/2010		X		OM234829		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	25
AT15935	<i>Cobitis vettonica</i>	This study	119421	06/05/2010	X	X	X	OM234830	OM235007	Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	4
AT15936	<i>Cobitis vettonica</i>	This study	119422	06/05/2010	X	X	X	OM234831	OM235008	Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	4
AT15937	<i>Cobitis vettonica</i>	This study	119423	06/05/2010	X	X	X	OM234832	OM235009	Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	4
AT15938	<i>Cobitis vettonica</i>	This study	119424	06/05/2010	X	X	X	OM234833	OM235010	Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	4
AT15939	<i>Cobitis vettonica</i>	This study	119425	06/05/2010	X	X	X	OM234834	OM235011	Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	14

AT15985	<i>Cobitis paludica</i>	This study		119353	13/05/2010	X	X			OM234968		Spain: Trujillo, Tagus Basin, Almonte Sub-basin, Tamuja River	14
AT15987	<i>Cobitis paludica</i>	This study		119354	13/05/2010	X	X			OM234969		Spain: Trujillo, Tagus Basin, Almonte Sub-basin, Tamuja River	14
AT16031	<i>Cobitis paludica</i>	This study		119355	13/05/2010	X	X			OM234964		Spain: Membri6, Tagus Basin, Salor Sub-basin, Salor River	14
AT20195	<i>Cobitis vettonica</i>	This study		119426	23/09/2010	X	X			OM234950		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	14
AT20196	<i>Cobitis vettonica</i>	This study		119427	23/09/2010	X	X			OM234951		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	6
AT20197	<i>Cobitis vettonica</i>	This study		119428	23/09/2010	X	X			OM234952		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	6
AT20198	<i>Cobitis vettonica</i>	This study		119572	23/09/2010	X						Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	6
AT20199	<i>Cobitis vettonica</i>	This study		119429	23/09/2010	X	X			OM234953		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	6
AT20201	<i>Cobitis vettonica</i>	This study		119430	23/09/2010	X	X			OM234954		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	5
AT20202	<i>Cobitis vettonica</i>	This study		119431	23/09/2010	X	X			OM234955		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	13
AT20203	<i>Cobitis vettonica</i>	This study		119432	23/09/2010	X	X			OM234956		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	13
AT20204	<i>Cobitis vettonica</i>	This study		119433	23/09/2010	X	X			OM234957		Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	23
AT20206	<i>Cobitis vettonica</i>	This study		119573	23/09/2010	X						Spain: Navaconcejo, Tagus Basin, eastern Alag6n Sub-basin, Jerte River	23
AT20372	<i>Cobitis paludica</i>	This study		119356	24/09/2010	X	X			OM234977		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT20373	<i>Cobitis paludica</i>	This study		119357	24/09/2010	X	X			OM234978		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT20374	<i>Cobitis paludica</i>	This study		119358	24/09/2010	X	X			OM234979		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT20375	<i>Cobitis paludica</i>	This study		119359	24/09/2010	X	X			OM234980		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT20376	<i>Cobitis paludica</i>	This study		119360	24/09/2010	X	X			OM234981		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT20377	<i>Cobitis paludica</i>	This study		119361	24/09/2010	X	X			OM234982		Spain: La Iglesia del Ti6tar, Tagus Basin, Ti6tar Sub-basin, Ti6tar River	23
AT2313	<i>Cobitis vettonica</i>	This study		119434	04/06/2004		X	X		OM234851	OM235054	Spain: Cilleros, Tagus Basin, upper Erjas Sub-basin, Trevejana River	23

AT2314	<i>Cobitis vettonica</i>	This study		119435	04/06/2004		X	X	OM235055	Spain: Cilleros, Tagus Basin, upper Erjas Sub-basin, Trevejana River	18
AT2315	<i>Cobitis vettonica</i>	This study		119436	04/06/2004		X	X	OM235056	Spain: Cilleros, Tagus Basin, upper Erjas Sub-basin, Trevejana River	18
AT2316	<i>Cobitis vettonica</i>	This study		119437	04/06/2004		X	X	OM235057	Spain: Cilleros, Tagus Basin, upper Erjas Sub-basin, Trevejana River	18
AT2321	<i>Cobitis vettonica</i>	This study		119438	04/06/2004		X	X	OM235067	Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	18
AT2322	<i>Cobitis vettonica</i>	This study		119439	04/06/2004		X			Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	22
AT2323	<i>Cobitis vettonica</i>	This study		119440	04/06/2004		X			Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	22
AT2324	<i>Cobitis vettonica</i>	This study		119441	04/06/2004		X			Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	22
AT2325	<i>Cobitis vettonica</i>	This study		119442	04/06/2004		X			Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	22
AT2326	<i>Cobitis vettonica</i>	This study		119443	04/06/2004		X			Spain: San Martín de Trevejo, Tagus Basin, upper Erjas Sub-basin, San Martín River	22
AT2355	<i>Cobitis vettonica</i>	This study		119444	04/06/2004		X			Spain: Hoyos, Tagus Basin, western Alagón Sub-basin, Acebo River	21
AT2359	<i>Cobitis vettonica</i>	This study		119445	04/06/2004		X			Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	21
AT2360	<i>Cobitis vettonica</i>	This study		119446	04/06/2004		X			Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	21
AT2361	<i>Cobitis vettonica</i>	This study		119447	04/06/2004		X			Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	21
AT2362	<i>Cobitis vettonica</i>	This study		119448	04/06/2004		X			Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	21
AT2372	<i>Cobitis vettonica</i>	This study		119449	04/06/2004		X	X	OM235012	Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	21
AT2373	<i>Cobitis vettonica</i>	This study		119450	04/06/2004		X			Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	21
AT2374	<i>Cobitis vettonica</i>	This study		119451	04/06/2004		X			Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	21
AT2375	<i>Cobitis vettonica</i>	This study		119452	04/06/2004		X	X	OM235013	Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	21
AT2376	<i>Cobitis vettonica</i>	This study		119453	04/06/2004		X			Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	4
AT27309	<i>Cobitis vettonica</i>	This study	X	119454	20/11/2015		X	X	OM235045	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	4

AT27310	<i>Cobitis vettonica</i>	This study		119455	20/11/2015	X	X	X	OM234901	OM235046	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	4
AT27311	<i>Cobitis vettonica</i>	This study		119456	20/11/2015	X	X	X	OM234902	OM235047	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	4
AT27312	<i>Cobitis vettonica</i>	This study		119457	20/11/2015	X	X	X	OM234903	OM235048	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	4
AT27313	<i>Cobitis vettonica</i>	This study		119458	20/11/2015	X	X	X	OM234904	OM235049	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	26
AT27314	<i>Cobitis vettonica</i>	This study		119459	20/11/2015	X	X		OM234905		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	26
AT27315	<i>Cobitis vettonica</i>	This study		119460	20/11/2015	X	X		OM234906		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	27
AT27316	<i>Cobitis vettonica</i>	This study		119461	20/11/2015	X	X		OM234907		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	27
AT27317	<i>Cobitis vettonica</i>	This study		119462	20/11/2015	X	X		OM234908		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	27
AT27318	<i>Cobitis vettonica</i>	This study		119463	20/11/2015	X	X		OM234909		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	27
AT27319	<i>Cobitis vettonica</i>	This study		119464	20/11/2015		X	X	OM234910	OM235050	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	27
AT27320	<i>Cobitis vettonica</i>	This study		119465	20/11/2015		X		OM234911		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	26
AT27321	<i>Cobitis vettonica</i>	This study		119466	20/11/2015		X		OM234912		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	26
AT27322	<i>Cobitis vettonica</i>	This study		119467	20/11/2015		X	X	OM234913	OM235051	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	16
AT27323	<i>Cobitis vettonica</i>	This study		119468	20/11/2015		X	X	OM234914	OM235052	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	13
AT27324	<i>Cobitis vettonica</i>	This study		119469	20/11/2015		X	X	OM234915	OM235053	Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	20
AT27325	<i>Cobitis vettonica</i>	This study		119470	20/11/2015		X		OM234916		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	13
AT27326	<i>Cobitis vettonica</i>	This study		119471	20/11/2015		X		OM234917		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	13
AT27327	<i>Cobitis vettonica</i>	This study		119472	20/11/2015		X		OM234918		Spain: Sotoserrano, Tagus Basin, eastern Alagón Sub-basin, Cuerpo de Hombre River	19
AT27342	<i>Cobitis vettonica</i>	This study		119473	19/11/2015		X		OM234795		Spain: El Sahujo, Duero Basin, Águeda Sub-basin, Mayas River	24
AT27343	<i>Cobitis vettonica</i>	This study		119474	19/11/2015		X		OM234796		Spain: El Sahujo, Duero Basin, Águeda Sub-basin, Mayas River	4

AT27344	<i>Cobitis vettonica</i>	This study		119475	19/11/2015		X		OM234797		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	13
AT27345	<i>Cobitis vettonica</i>	This study		119476	19/11/2015		X		OM234798		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	17
AT27346	<i>Cobitis vettonica</i>	This study		119477	19/11/2015		X		OM234799		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	3
AT27347	<i>Cobitis vettonica</i>	This study		119478	19/11/2015		X		OM234800		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	1
AT27348	<i>Cobitis vettonica</i>	This study		119479	19/11/2015		X		OM234801		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	1
AT27349	<i>Cobitis vettonica</i>	This study		119480	19/11/2015		X		OM234802		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	1
AT27350	<i>Cobitis vettonica</i>	This study		119481	19/11/2015		X		OM234803		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	1
AT27351	<i>Cobitis vettonica</i>	This study		119482	19/11/2015		X		OM234804		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27365	<i>Cobitis vettonica</i>	This study		119483	19/11/2015	X	X		OM234805		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27366	<i>Cobitis vettonica</i>	This study		119484	19/11/2015	X	X		OM234806		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27367	<i>Cobitis vettonica</i>	This study		119485	19/11/2015	X	X		OM234807		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27368	<i>Cobitis vettonica</i>	This study		119486	19/11/2015	X	X		OM234808		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27369	<i>Cobitis vettonica</i>	This study		119487	19/11/2015	X	X		OM234809		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	7
AT27370	<i>Cobitis vettonica</i>	This study		119488	19/11/2015	X	X	X	OM234810	OM235003	Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	11
AT27371	<i>Cobitis vettonica</i>	This study		119489	19/11/2015	X	X	X	OM234811	OM235004	Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	11
AT27372	<i>Cobitis vettonica</i>	This study		119490	19/11/2015	X	X	X	OM234812	OM235005	Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	11
AT27373	<i>Cobitis vettonica</i>	This study		119491	19/11/2015	X	X	X	OM234813	OM235006	Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	11
AT27374	<i>Cobitis vettonica</i>	This study		119492	19/11/2015	X	X		OM234814		Spain: El Sahuco, Duero Basin, Águeda Sub-basin, Mayas River	12
AT27375	<i>Cobitis paludica</i>	This study		119562	19/11/2015	X					Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	12
AT27376	<i>Cobitis paludica</i>	This study		119563	19/11/2015	X					Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	10

AT27377	<i>Cobitis paludica</i>	This study		119564	19/11/2015	X											Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	8
AT27378	<i>Cobitis paludica</i>	This study		119565	19/11/2015	X											Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	8
AT27379	<i>Cobitis paludica</i>	This study		119566	19/11/2015	X											Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	9
AT27553	<i>Cobitis vettonica</i>	This study		119493	20/11/2015	X	X	X								OM235021	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	9
AT27554	<i>Cobitis vettonica</i>	This study		119494	20/11/2015			X	X							OM235022	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	2
AT27555	<i>Cobitis vettonica</i>	This study		119495	20/11/2015	X		X	X							OM235023	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	3
AT27556	<i>Cobitis vettonica</i>	This study		119496	20/11/2015	X		X	X							OM235024	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	3
AT27557	<i>Cobitis vettonica</i>	This study		119497	20/11/2015	X		X	X							OM235025	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	3
AT27558	<i>Cobitis vettonica</i>	This study		119498	20/11/2015	X		X								OM234891	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	11
AT27559	<i>Cobitis vettonica</i>	This study		119499	20/11/2015				X							OM234892	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	12
AT27560	<i>Cobitis vettonica</i>	This study		119500	20/11/2015	X		X								OM234893	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	10
AT27561	<i>Cobitis vettonica</i>	This study		119501	20/11/2015	X		X								OM234894	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	30
AT27562	<i>Cobitis vettonica</i>	This study		119502	20/11/2015	X		X								OM234895	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	31
AT27563	<i>Cobitis vettonica</i>	This study		119503	20/11/2015				X							OM234896	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	1
AT27564	<i>Cobitis vettonica</i>	This study		119504	20/11/2015				X							OM234897	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	28
AT27565	<i>Cobitis vettonica</i>	This study		119505	20/11/2015				X							OM234898	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	35
AT27566	<i>Cobitis vettonica</i>	This study		119506	20/11/2015				X							OM234899	Spain: Nava de Francia, Tagus Basin, eastern Alagón Sub-basin, Francia River	35
AT27631	<i>Cobitis paludica</i>	This study		119362	22/10/2014	X		X								OM234988	Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	35
AT27632	<i>Cobitis paludica</i>	This study		119363	22/10/2014	X		X								OM234989	Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	33
AT27633	<i>Cobitis paludica</i>	This study		119364	22/10/2014	X		X								OM234990	Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	33

AT27634	<i>Cobitis paludica</i>	This study		119365	22/10/2014	X	X	X	OM234991	OM235080	Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	32
AT27635	<i>Cobitis paludica</i>	This study		119366	22/10/2014	X	X	X	OM234992	OM235081	Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	36
AT27636	<i>Cobitis paludica</i>	This study		119367	22/10/2014	X	X		OM234993		Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	36
AT27637	<i>Cobitis paludica</i>	This study		119368	22/10/2014	X	X		OM234994		Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	36
AT27638	<i>Cobitis paludica</i>	This study		119369	22/10/2014	X	X		OM234995		Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	36
AT27640	<i>Cobitis paludica</i>	This study		119370	22/10/2014	X	X		OM234996		Spain: El cubo de don Sancho, Duero Basin, Huebra-Yeltes Sub-basin, Huebra River	36
AT27661	<i>Cobitis vettonica</i>	This study		119507	22/10/2014		X	X	OM234872	OM235026	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	36
AT27662	<i>Cobitis vettonica</i>	This study		119508	22/10/2014		X	X	OM234873	OM235027	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	38
AT27663	<i>Cobitis vettonica</i>	This study		119509	22/10/2014		X	X	OM234874	OM235028	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	38
AT27664	<i>Cobitis vettonica</i>	This study		119510	22/10/2014		X	X	OM234875	OM235029	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	38
AT27665	<i>Cobitis vettonica</i>	This study		119511	22/10/2014		X	X	OM234876	OM235030	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	38
AT27666	<i>Cobitis vettonica</i>	This study		119512	22/10/2014	X	X		OM234877		Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	38
AT27667	<i>Cobitis vettonica</i>	This study		119513	22/10/2014	X	X		OM234878		Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	33
AT27668	<i>Cobitis vettonica</i>	This study		119514	22/10/2014	X	X		OM234879		Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	33
AT27669	<i>Cobitis vettonica</i>	This study		119515	22/10/2014	X	X		OM234880		Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	32

AT27670	<i>Cobitis vettonica</i>	This study	119516	22/10/2014			X	X	OM234881	OM235031	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	26
AT27671	<i>Cobitis vettonica</i>	This study	119517	22/10/2014			X		OM234882		Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	26
AT27672	<i>Cobitis vettonica</i>	This study	119518	22/10/2014			X	X	OM234883	OM235032	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	26
AT27673	<i>Cobitis vettonica</i>	This study	119519	22/10/2014			X	X	OM234884	OM235033	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	26
AT27674	<i>Cobitis vettonica</i>	This study	119520	22/10/2014			X	X	OM234885	OM235034	Spain: Santi Ibañez de la Sierra, Tagus Basin, eastern Alagón Sub-basin, Alagón River	26
AT2874	<i>Cobitis vettonica</i>	This study	119521	26/06/2005			X		OM234815		Spain: Descargamaria, Duero Basin, Águeda Sub-basin, Malena River	26
AT6689	<i>Cobitis vettonica</i>	This study	119522	20/03/2009	X		X	X	OM234855	OM235062	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	26
AT6690	<i>Cobitis vettonica</i>	This study	119523	20/03/2009	X		X	X	OM234856	OM235063	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	26
AT6691	<i>Cobitis vettonica</i>	This study	119524	20/03/2009	X		X	X	OM234857	OM235064	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	4
AT6692	<i>Cobitis vettonica</i>	This study	119525	20/03/2009	X		X	X	OM234858	OM235065	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	4
AT6699	<i>Cobitis vettonica</i>	This study	119526	20/03/2009	X		X	X	OM234859	OM235066	Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	16
AT6734	<i>Cobitis vettonica</i>	This study	119527	20/03/2009			X		OM234920		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	15
AT6736	<i>Cobitis vettonica</i>	This study	119528	20/03/2009			X		OM234921		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6737	<i>Cobitis vettonica</i>	This study	119529	20/03/2009			X	X	OM234922	OM235040	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6738	<i>Cobitis vettonica</i>	This study	119530	20/03/2009			X		OM234923		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6739	<i>Cobitis vettonica</i>	This study	119531	20/03/2009			X		OM234924		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6740	<i>Cobitis vettonica</i>	This study	119532	20/03/2009			X	X	OM234925	OM235041	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14

AT6741	<i>Cobitis vettonica</i>	This study		119533	20/03/2009		X		OM234943		Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6743	<i>Cobitis vettonica</i>	This study		119534	20/03/2009		X		OM234944		Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6746	<i>Cobitis vettonica</i>	This study		119535	20/03/2009		X	X	OM234945	OM235016	Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6747	<i>Cobitis vettonica</i>	This study		119536	20/03/2009		X	X	OM234946	OM235017	Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6748	<i>Cobitis vettonica</i>	This study		119537	20/03/2009		X	X	OM234947	OM235018	Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6749	<i>Cobitis vettonica</i>	This study		119538	20/03/2009		X	X	OM234948	OM235019	Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6750	<i>Cobitis vettonica</i>	This study		119539	20/03/2009		X	X	OM234949	OM235020	Spain: Navaconcejo, Tagus Basin, eastern Alagón Sub-basin, Jerte River	27
AT6834	<i>Cobitis vettonica</i>	This study	X	119567	21/03/2009	X					Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	27
AT6835	<i>Cobitis vettonica</i>	This study	X	119540	21/03/2009	X	X	X	OM234926	OM235043	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	5
AT6836	<i>Cobitis vettonica</i>	This study	X	119568	21/03/2009	X					Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	5
AT6837	<i>Cobitis vettonica</i>	This study	X	119541	21/03/2009	X	X	X	OM234927	OM235042	Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	5
AT6838	<i>Cobitis vettonica</i>	This study	X	119542	21/03/2009	X	X		OM234928		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	5
AT6839	<i>Cobitis vettonica</i>	This study	X	119569	21/03/2009	X					Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	5
AT6840	<i>Cobitis vettonica</i>	This study		119543	21/03/2009		X		OM234929		Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	16
AT6841	<i>Cobitis vettonica</i>	This study	X	119570	21/03/2009	X					Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6843	<i>Cobitis vettonica</i>	This study	X	119571	21/03/2009	X					Spain: Zarza de Granadilla, Tagus Basin, eastern Alagón Sub-basin, Caparro River	14
AT6865	<i>Cobitis vettonica</i>	This study		119544	22/03/2009		X		OM234820		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	14
AT6866	<i>Cobitis vettonica</i>	This study		119545	22/03/2009		X		OM234821		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	14
AT6867	<i>Cobitis vettonica</i>	This study		119546	22/03/2009		X		OM234822		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	13
AT6868	<i>Cobitis vettonica</i>	This study		119547	22/03/2009		X		OM234823		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	13

AT6869	<i>Cobitis vettonica</i>	This study	119548	22/03/2009		X		OM234824		Spain: Gata, Tagus Basin, western Alagón Sub-basin, Gata River	13
AT6892	<i>Cobitis vettonica</i>	This study	119549	22/03/2009		X	X	OM234848	OM235015	Spain: Hoyos, Tagus Basin, western Alagón Sub-basin, Acebo River	23
AT6922	<i>Cobitis vettonica</i>	This study	119550	22/03/2009		X		OM234840		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT6923	<i>Cobitis vettonica</i>	This study	119551	22/03/2009		X		OM234841		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT6924	<i>Cobitis vettonica</i>	This study	119552	22/03/2009		X		OM234842		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT6925	<i>Cobitis vettonica</i>	This study	119553	22/03/2009		X	X	OM234843	OM235014	Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT6926	<i>Cobitis vettonica</i>	This study	119554	22/03/2009		X		OM234844		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT6927	<i>Cobitis vettonica</i>	This study	119555	22/03/2009		X		OM234845		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Árrago River	23
AT7308	<i>Cobitis paludica</i>	This study	119371	18/04/2009	X	X	X	OM234972	OM235073	Spain: Jaraicejo, Tagus Basin, Almonte Sub-basin, Almonte River	23
AT7309	<i>Cobitis paludica</i>	This study	119372	18/04/2009	X	X	X	OM234973	OM235074	Spain: Jaraicejo, Tagus Basin, Almonte Sub-basin, Almonte River	23
AT7310	<i>Cobitis paludica</i>	This study	119373	18/04/2009	X	X	X	OM234974	OM235075	Spain: Jaraicejo, Tagus Basin, Almonte Sub-basin, Almonte River	23
AT7311	<i>Cobitis paludica</i>	This study	119374	18/04/2009	X	X	X	OM234975	OM235076	Spain: Jaraicejo, Tagus Basin, Almonte Sub-basin, Almonte River	18
AT7312	<i>Cobitis paludica</i>	This study	119375	18/04/2009	X	X	X	OM234976	OM235077	Spain: Jaraicejo, Tagus Basin, Almonte Sub-basin, Almonte River	18
AT8964	<i>Cobitis paludica</i>	This study	119376	31/05/2009	X	X	X	OM234997	OM235082	Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	18
AT8965	<i>Cobitis paludica</i>	This study	119377	31/05/2009		X	X	OM234998	OM235083	Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	18
AT8966	<i>Cobitis paludica</i>	This study	119378	31/05/2009	X	X	X	OM234999	OM235084	Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	18
AT8967	<i>Cobitis paludica</i>	This study	119379	31/05/2009		X	X	OM235000	OM235085	Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	18
AT8968	<i>Cobitis paludica</i>	This study	119380	31/05/2009	X	X	X	OM235001	OM235086	Spain: San Martín de Yeltes, Duero Basin, Huebra-Yeltes Sub-basin, Yeltes River	18
AT9091	<i>Cobitis vettonica</i>	This study	119556	01/06/2009		X	X	OM234794	OM235002	Spain: La Bouza, Duero Basin, Águeda Sub-basin, Turones River	18
AT9296	<i>Cobitis paludica</i>	This study	119381	11/06/2009	X	X	X	OM234970		Spain: Trujillo, Tagus Basin, Almonte Sub-basin, Tamiña River	18

AT9297	<i>Cobitis paludica</i>	This study	119382	11/06/2009	X	X			OM234971		Spain: Trujillo, Tagus Basin, Almonte Sub-basin, Tamuja River	18
AT9346	<i>Cobitis paludica</i>	This study	119383	11/06/2009	X	X	X		OM234958	OM235068	Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9347	<i>Cobitis paludica</i>	This study	119384	11/06/2009	X	X	X		OM234959	OM235069	Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9348	<i>Cobitis paludica</i>	This study	119385	11/06/2009	X	X	X		OM234960	OM235070	Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9349	<i>Cobitis paludica</i>	This study	119386	11/06/2009	X	X	X		OM234961	OM235071	Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9350	<i>Cobitis paludica</i>	This study	119387	11/06/2009	X	X	X		OM234962	OM235072	Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9351	<i>Cobitis paludica</i>	This study	119558	11/06/2009	X						Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	18
AT9352	<i>Cobitis paludica</i>	This study	119559	11/06/2009	X						Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	22
AT9354	<i>Cobitis paludica</i>	This study	119560	11/06/2009	X						Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	22
AT9355	<i>Cobitis paludica</i>	This study	119561	11/06/2009	X						Spain: Santiago de Alcántara, Tagus Basin, Salor Sub-basin, Aurela River	22
AT9375	<i>Cobitis paludica</i>	This study	119388	11/06/2009	X	X			OM234963		Spain: Membrión, Tagus Basin, Salor Sub-basin, Salor River	22
CM6CC	<i>Cobitis vettonica</i>	This study	119557	07/05/1995		X			OM234846		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	22
CP591	<i>Cobitis paludica</i>	This study	119389	07/05/1995		X	X		OM234983	OM235087	Spain: La Iglesuela del Tiétar, Tagus Basin, Tiétar Sub-basin, Tiétar River	22
CP592	<i>Cobitis paludica</i>	This study	119390	07/05/1995		X	X		OM234984	OM235088	Spain: La Iglesuela del Tiétar, Tagus Basin, Tiétar Sub-basin, Tiétar River	22
CP593	<i>Cobitis paludica</i>	This study	119391	07/05/1995		X	X		OM234985	OM235089	Spain: La Iglesuela del Tiétar, Tagus Basin, Tiétar Sub-basin, Tiétar River	22
CP594	<i>Cobitis paludica</i>	This study	119392	07/05/1995		X	X		OM234986	OM235090	Spain: La Iglesuela del Tiétar, Tagus Basin, Tiétar Sub-basin, Tiétar River	22
CP595	<i>Cobitis paludica</i>	This study	119393	07/05/1995		X	X		OM234987	OM235091	Spain: La Iglesuela del Tiétar, Tagus Basin, Tiétar Sub-basin, Tiétar River	21
2284PT	<i>Cobitis vettonica</i>	Perdices et al., 2018				X			MH842916.1		Portugal: Cegonhas Novas, Tagus Basin, Aravil Sub-basin, Aravil River	21
158CP	<i>Cobitis vettonica</i>	Perdices et al., 2016				X			KP161123.1		Spain: Descargamaria, Duero Basin, Águeda Sub-basin, Malena River	21
1ARR	<i>Cobitis vettonica</i>	Perdices & Doadrio, 2001				X			AF263072.1		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	21

2279PT	<i>Cobitis paludica</i>	Perdices & Coelho, 2020						X	MN583213.1		Portugal: Serrinha, Tagus Basin, lower Erjas Sub-basin, Erjas River	13
2285PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583208.1		Portugal: Cegonhas Novas, Tagus Basin, Aravil Sub-basin, Aravil River	26
2286PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583209.1		Portugal: Cegonhas Novas, Tagus Basin, Aravil Sub-basin, Aravil River	26
2287PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583210.1		Portugal: Cegonhas Novas, Tagus Basin, Aravil Sub-basin, Aravil River	26
2291PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583183.1		Portugal: Alfrividas, Tagus Basin, Ponsul Sub-basin, Alfrividas River	26
2292PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583184.1		Portugal: Alfrividas, Tagus Basin, Ponsul Sub-basin, Alfrividas River	26
2293PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583185.1		Portugal: Alfrividas, Tagus Basin, Ponsul Sub-basin, Alfrividas River	29
2294PT	<i>Cobitis paludica</i>	Perdices & Coelho, 2020						X	MN583219.1		Portugal: Alfrividas, Tagus Basin, Ponsul Sub-basin, Alfrividas River	29
2295PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583186.1		Portugal: Alfrividas, Tagus Basin, Ponsul Sub-basin, Alfrividas River	29
2304PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583194.1		Portugal: Azenha do Roque, Tagus Basin, lower Erjas Sub-basin, Erjas River	29
2305PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583195.1		Portugal: Azenha do Roque, Tagus Basin, lower Erjas Sub-basin, Erjas River	27
2306PT	<i>Cobitis vettonica</i>	Perdices & Coelho, 2020						X	MN583196.1		Portugal: Azenha do Roque, Tagus Basin, lower Erjas Sub-basin, Erjas River	27
3187PT	<i>Cobitis paludica</i>	Perdices & Coelho, 2020						X	MN583220.1		Portugal: Monte Gordo, Tagus Basin, Ocreza Sub-basin, Alvito River	37
168CP	<i>Cobitis vettonica</i>	Doadrio & Perdices, 2005						X	AY860181.1		Spain: La Herguijuela, Duero Basin, Águeda Sub-basin, Águeda River	37
268CP	<i>Cobitis vettonica</i>	Doadrio & Perdices, 2005						X	AY860183.1		Spain: Valverde del Fresno, Tagus Basin, upper Erjas Sub-basin, Erjas River	37
575A	<i>Cobitis vettonica</i>	Doadrio & Perdices, 2005						X	AY860182.1		Spain: La Rebollosa, Tagus Basin, eastern Alagón Sub-basin, Ladrillar River	37
5ARR	<i>Cobitis vettonica</i>	Doadrio & Perdices, 2005						X	KP161122.1		Spain: Cadalso de Gata, Tagus Basin, western Alagón Sub-basin, Arrago River	37

Table S2. Stacks results for each analysis group.

Ustacks parameters	Mean loci	Mean coverage	Cstacks parameters	Catalog loci	Populations loci	Variable SNPs	Fixed SNPs
$m=5, M=3$	15668	28.31	n=2	108550	6684	4696	49
			n=3	103401	6691	4683	37
			n=4	100072	6695	4677	51

Table S3. Percentage of missing data by locus and by individual for SNPs data.

<i>Cobitis vettonica</i>										<i>Cobitis vettonica</i> + <i>Cobitis paludica</i>									
Locus	% of missing data	Locus	% of missing data	Locus	% of missing data	Individual	% of missing data	Locus	% of missing data	Locus	% of missing data	Locus	% of missing data	Locus	% of missing data	Individual	% of missing data	Locus	% of missing data
2_105	0.00	4791_89	8.33	9747_68	1.67	AT27666	1.90	2_105	0.00	3564_112	0.94	7407_15	6.60	10820_43	2.83	AT7308	3.15		
6_12	0.00	4794_9	0.00	9749_150	6.67	AT27667	0.92	4_103	3.77	3566_7	2.83	7408_28	0.00	10823_14	1.89	AT7309	4.30		
7_164	0.00	4795_138	3.33	9753_36	6.67	AT27668	0.65	6_12	0.00	3567_19	0.94	7412_111	1.89	10824_44	15.09	AT7310	2.95		
8_97	0.00	4796_17	16.67	9754_53	3.33	AT27669	1.08	7_164	1.89	3569_70	1.89	7413_65	0.94	10826_49	6.60	AT7311	6.48		
9_43	5.00	4815_12	6.67	9755_5	0.00	AT6834	1.20	8_97	3.77	3574_123	0.00	7414_102	12.26	10827_78	3.77	AT7312	2.84		
10_133	1.67	4819_145	1.67	9756_23	0.00	AT6835	5.68	9_43	10.38	3577_52	5.66	7422_65	2.83	10830_74	3.77	AT9346	1.04		
11_109	3.33	4820_157	0.00	9764_137	3.33	AT6836	3.40	10_133	0.94	3578_93	2.83	7428_16	0.94	10832_128	5.66	AT9347	1.10		
15_48	0.00	4821_25	0.00	9766_9	5.00	AT6837	7.85	11_109	8.49	3580_78	5.66	7434_184	0.00	10834_60	3.77	AT9348	1.17		
20_46	8.33	4825_152	1.67	9767_43	5.00	AT6838	1.90	13_6	3.77	3582_71	2.83	7437_79	1.89	10838_91	9.43	AT9349	1.39		
21_111	1.67	4826_11	3.33	9770_145	5.00	AT6839	1.20	15_48	0.00	3587_132	0.94	7439_54	4.72	10844_84	1.89	AT9350	2.93		
31_157	1.67	4829_97	6.67	9772_175	0.00	AT6841	10.68	20_46	8.49	3589_105	8.49	7441_32	0.94	10845_105	0.94	AT9351	4.28		
32_24	5.00	4835_151	1.67	9774_28	1.67	AT6843	1.43	21_111	0.94	3595_103	7.55	7446_50	1.89	10848_74	8.49	AT9352	8.40		
33_54	0.00	4836_39	3.33	9780_29	0.00	AT72317	4.58	31_157	3.77	3598_158	0.94	7451_14	0.00	10849_65	13.21	AT9354	6.48		
39_17	0.00	4840_57	1.67	9784_102	1.67	AT72318	1.05	32_24	5.66	3600_111	11.32	7452_130	6.60	10850_44	0.94	AT9355	3.68		
44_105	0.00	4853_39	0.00	9785_100	21.67	AT72309	0.67	33_54	4.72	3602_146	5.66	7457_7	7.55	10851_59	0.94	AT72631	1.92		
53_103	0.00	4861_85	0.00	9786_106	0.00	AT72310	1.18	39_17	0.00	3605_97	9.43	7461_119	12.26	10852_170	9.43	AT72632	1.50		
57_47	3.33	4872_152	13.33	9795_8	10.00	AT72311	0.85	44_105	0.00	3611_83	3.77	7462_103	4.72	10853_9	0.00	AT72633	1.04		
60_149	0.00	4875_51	0.00	9798_49	3.33	AT72312	3.30	53_103	0.00	3613_133	0.00	7465_45	5.66	10854_7	5.66	AT72634	1.41		
62_109	1.67	4877_121	1.67	9802_29	1.67	AT72313	22.00	57_47	3.77	3614_37	0.00	7468_161	0.00	10857_19	0.94	AT72635	1.67		
64_20	3.33	4878_87	1.67	9807_157	3.33	AT72314	0.72	60_149	0.00	3619_51	1.89	7473_36	0.94	10858_5	0.00	AT72636	2.18		
66_84	3.33	4879_137	0.00	9812_72	0.00	AT72315	28.53	62_109	0.94	3622_145	2.83	7474_165	7.55	10859_33	11.32	AT72637	1.21		
69_67	6.67	4880_24	8.33	9814_57	0.00	AT72316	3.60	64_20	2.83	3626_156	6.60	7476_160	7.55	10860_156	16.98	AT72639	6.13		
79_123	10.00	4881_30	0.00	9815_148	6.67	AT6689	2.43	66_84	7.55	3627_90	0.00	7478_72	16.04	10861_95	4.72	AT72640	1.17		
86_65	0.00	4890_38	0.00	9816_10	0.00	AT6690	2.15	69_67	5.66	3632_62	13.21	7479_128	5.66	10865_26	0.00	AT15755	10.67		
87_6	1.67	4895_82	1.67	9829_155	0.00	AT6691	2.47	79_123	12.26	3634_82	1.89	7487_41	0.94	10870_11	2.83	AT15756	7.82		
101_75	8.33	4897_65	3.33	9834_139	3.33	AT6692	2.70	81_184	2.83	3636_69	13.21	7496_43	0.94	10875_134	6.60	AT15757	2.20		
102_14	8.33	4898_74	6.67	9836_8	8.33	AT6699	2.63	86_65	0.00	3637_138	1.89	7500_159	6.60	10879_129	2.83	AT9375	2.86		
107_113	8.33	4902_113	1.67	9837_96	0.00	AT72553	9.43	87_6	2.83	3641_10	11.32	7505_23	2.83	10880_156	13.21	AT16031	3.48		
108_26	8.33	4903_92	10.00	9840_82	5.00	AT72555	1.45	101_75	5.66	3648_14	2.83	7506_139	0.00	10896_73	0.94	AT9296	3.00		
109_9	0.00	4904_61	3.33	9847_44	0.00	AT72556	2.47	102_14	7.55	3650_130	4.72	7507_28	2.83	10898_123	1.89	AT9297	7.65		
111_114	6.67	4909_42	10.00	9848_42	0.00	AT72557	1.38	107_113	11.32	3655_42	0.00	7508_25	0.94	10899_43	0.00	AT15985	14.43		
113_61	0.00	4910_85	0.00	9849_47	8.33	AT72558	14.08	108_26	6.60	3656_43	3.77	7512_131	0.94	10900_27	1.89	AT15987	9.10		
121_170	8.33	4914_30	1.67	9855_36	0.00	AT72560	5.50	109_9	0.94	3657_115	9.43	7514_49	4.72	10901_90	6.60	AT20372	17.72		
124_111	5.00	4915_33	3.33	9857_82	0.00	AT72561	3.08	111_114	12.26	3659_167	3.77	7515_23	5.66	10909_43	0.94	AT20373	5.53		
125_31	0.00	4917_75	1.67	9859_67	6.67	AT72562	1.90	113_61	0.00	3660_36	1.89	7519_58	0.00	10915_7	1.89	AT20374	7.98		
130_149	3.33	4922_129	1.67	9861_183	0.00	AT20195	3.13	121_170	8.49	3661_86	4.72	7524_87	0.94	10916_58	0.94	AT20375	7.14		
131_110	11.67	4925_60	0.00	9864_73	11.67	AT20196	0.95	124_111	4.72	3662_124	4.72	7526_134	1.89	10921_195	1.89	AT20376	11.15		
133_80	3.33	4932_175	13.33	9865_61	0.00	AT20197	1.75	125_31	1.89	3669_47	5.66	7527_113	3.77	10929_84	0.00	AT20377	3.75		
134_90	3.33	4936_45	8.33	9868_153	0.00	AT20198	1.15	130_149	2.83	3670_156	0.94	7528_145	0.00	10933_91	4.72	AT8964	3.02		
140_121	0.00	4938_140	3.33	9869_59	0.00	AT20199	0.88	131_110	10.38	3671_71	0.00	7530_158	8.49	10934_84	1.89	AT8966	16.66		

142_89	0.00	4941_122	1.67	9873_60	0.00	AT20201	15.13	133_80	3.77	3674_30	8.49	7532_107	0.00	10936_158	2.83	AT8968	3.24
146_151	10.00	4948_116	3.33	9875_73	18.33	AT20202	0.92	134_90	2.83	3678_56	6.60	7533_49	2.83	10937_51	4.72	AT27375	7.12
147_5	0.00	4949_71	0.00	9886_113	0.00	AT20203	1.50	140_121	0.94	3680_34	5.66	7543_186	9.43	10946_93	5.66	AT27376	4.39
149_54	6.67	4951_83	0.00	9888_106	5.00	AT20204	0.85	142_89	0.94	3681_95	1.89	7545_137	3.77	10959_125	2.83	AT27377	1.19
154_57	0.00	4952_82	8.33	9889_149	0.00	AT20206	3.68	146_151	8.49	3682_107	7.55	7546_48	5.66	10960_68	13.21	AT27378	1.26
156_18	1.67	4956_39	3.33	9891_122	18.33	AT27365	0.80	147_5	1.89	3686_102	0.00	7553_139	0.94	10962_109	0.94	AT27379	2.67
161_97	0.00	4957_25	0.00	9894_119	0.00	AT27366	0.75	149_54	9.43	3687_91	12.26	7554_91	1.89	10963_117	1.89	AT27666	1.87
166_102	1.67	4961_139	1.67	9896_46	0.00	AT27367	2.60	154_57	0.94	3688_8	14.15	7557_62	11.32	10964_42	2.83	AT27667	0.84
168_11	3.33	4964_14	18.33	9898_45	1.67	AT27368	1.65	156_18	0.94	3692_91	14.15	7560_59	0.00	10971_73	0.00	AT27668	0.59
184_8	0.00	4965_148	15.00	9904_71	1.67	AT27369	0.72	161_97	0.94	3693_166	0.94	7565_121	0.94	10972_96	1.89	AT27669	1.06
186_23	0.00	4976_99	1.67	9906_43	0.00	AT27370	2.53	166_102	1.89	3695_66	0.94	7572_174	11.32	10989_59	0.94	AT6834	1.17
187_105	6.67	4978_8	0.00	9911_95	1.67	AT27371	1.63	168_11	6.60	3696_70	0.94	7573_13	9.43	10992_98	0.94	AT6835	5.44
194_95	1.67	4980_185	0.00	9915_122	1.67	AT27372	2.80	175_70	1.89	3699_40	0.94	7574_64	0.94	10994_43	7.55	AT6836	3.33
200_8	0.00	4984_55	6.67	9916_148	10.00	AT27373	1.55	183_126	9.43	3700_84	1.89	7577_116	2.83	10997_43	0.00	AT6837	7.82
201_75	3.33	4985_129	5.00	9924_105	0.00	AT27374	1.13	184_8	3.77	3701_80	0.94	7581_144	5.66	10998_169	2.83	AT6838	1.76
206_58	0.00	4986_70	0.00	9925_24	1.67	AT15935	9.30	186_23	0.00	3702_179	4.72	7582_119	4.72	11001_100	1.89	AT6839	1.12
210_87	0.00	4995_83	1.67	9937_137	6.67	AT15936	1.05	187_105	8.49	3704_86	10.38	7583_127	1.89	11002_55	2.83	AT6841	10.64
213_109	1.67	5000_16	0.00	9938_98	21.67	AT15937	2.13	190_126	0.00	3706_78	1.89	7584_148	0.94	11004_91	0.00	AT6843	1.32
215_29	0.00	5002_157	1.67	9944_71	5.00	AT15938	3.00	194_95	0.94	3712_45	1.89	7589_105	3.77	11007_157	13.21	AT27317	4.54
216_110	16.67	5005_51	1.67	9947_101	0.00	AT15939	0.78	200_8	0.94	3713_7	8.49	7594_176	8.49	11013_31	0.94	AT27318	0.97
217_25	0.00	5006_26	0.00	9952_5	3.33			201_75	4.72	3717_95	4.72	7596_50	0.00	11015_165	3.77	AT27309	0.64
218_96	0.00	5007_73	3.33	9954_44	0.00			206_58	0.00	3721_111	0.00	7597_139	0.00	11018_5	4.72	AT27310	1.08
220_17	5.00	5009_94	1.67	9962_158	0.00			210_87	0.00	3722_9	1.89	7599_7	0.94	11025_55	2.83	AT27311	0.77
221_158	6.67	5012_112	15.00	9969_35	15.00			213_109	0.94	3727_163	0.94	7600_6	10.38	11028_114	8.49	AT27312	3.11
224_60	0.00	5014_19	0.00	9973_88	8.33			215_29	0.00	3728_71	0.00	7603_81	0.94	11031_24	3.77	AT27313	22.32
237_94	1.67	5015_65	1.67	9975_117	0.00			216_110	15.09	3735_15	6.60	7605_234	5.66	11038_138	2.83	AT27314	0.68
240_79	3.33	5024_113	8.33	9976_20	1.67			217_25	0.00	3736_38	2.83	7607_41	0.00	11046_36	1.89	AT27315	27.88
241_90	0.00	5026_133	3.33	9977_134	0.00			218_96	3.77	3737_34	2.83	7608_36	11.32	11053_53	0.00	AT27316	3.31
246_53	6.67	5031_41	13.33	9980_80	0.00			220_17	7.55	3745_9	2.83	7609_159	1.89	11057_160	0.00	AT6689	2.64
248_29	10.00	5032_21	0.00	9988_117	16.67			221_158	7.55	3749_45	2.83	7611_106	0.00	11061_57	9.43	AT6690	2.38
249_79	5.00	5045_67	0.00	9990_85	0.00			224_60	0.00	3754_53	1.89	7615_190	6.60	11064_29	0.00	AT6691	2.67
253_5	5.00	5047_132	3.33	9991_96	13.33			227_102	5.66	3757_150	0.94	7618_90	10.38	11072_95	0.94	AT6692	2.89
256_29	8.33	5049_32	5.00	9993_109	13.33			237_94	1.89	3766_114	0.94	7622_158	0.00	11077_27	13.21	AT6699	2.86
257_151	5.00	5056_66	0.00	9994_155	1.67			240_79	1.89	3769_153	1.89	7624_52	5.66	11078_21	3.77	AT27553	9.26
258_16	0.00	5058_38	0.00	9997_40	1.67			241_90	1.89	3770_98	3.77	7625_51	0.00	11085_104	1.89	AT27555	1.32
260_72	0.00	5060_24	0.00	10002_65	3.33			246_53	7.55	3775_50	5.66	7631_28	0.94	11086_18	0.94	AT27556	2.34
261_120	0.00	5062_114	0.00	10006_165	1.67			248_29	11.32	3778_126	2.83	7633_88	4.72	11089_96	3.77	AT27557	1.28
262_163	10.00	5063_14	1.67	10008_105	5.00			249_79	4.72	3779_131	0.94	7635_108	11.32	11090_69	0.00	AT27558	13.99
264_116	3.33	5064_51	1.67	10014_159	0.00			253_5	5.66	3782_106	6.60	7636_68	7.55	11100_164	0.94	AT27560	5.33
268_14	1.67	5069_58	13.33	10015_125	8.33			254_82	0.00	3784_122	4.72	7638_86	11.32	11104_13	6.60	AT27561	2.93
275_110	1.67	5072_61	1.67	10016_50	3.33			256_29	10.38	3785_98	0.00	7642_24	2.83	11109_55	3.77	AT27562	1.85
276_166	8.33	5073_5	0.00	10021_74	10.00			257_151	11.32	3786_153	4.72	7646_143	7.55	11113_187	4.72	AT20195	3.00
278_35	0.00	5077_57	0.00	10022_63	5.00			258_16	0.00	3790_92	3.77	7649_143	7.55	11118_159	2.83	AT20196	0.88
283_40	0.00	5078_183	10.00	10023_46	1.67			260_72	0.00	3792_84	4.72	7650_155	3.77	11121_45	0.00	AT20197	1.61
296_85	5.00	5079_117	8.33	10025_172	1.67			261_120	3.77	3793_84	0.00	7651_28	8.49	11122_29	0.94	AT20198	1.06
300_47	11.67	5082_63	0.00	10029_132	0.00			262_163	15.09	3795_98	1.89	7653_81	3.77	11123_27	11.32	AT20199	0.82

311_104	0.00	5085_19	3.33	10030_36	0.00					264_116	2.83	3799_75	1.89	7654_180	5.66	11125_58	0.00	AT20201	14.92
312_120	1.67	5090_95	0.00	10036_70	0.00					268_14	0.94	3803_44	13.21	7660_123	10.38	11126_27	0.94	AT20202	0.84
316_74	1.67	5096_194	5.00	10037_35	0.00					272_62	7.55	3807_135	1.89	7663_17	6.60	11137_152	0.00	AT20203	1.45
323_20	5.00	5098_178	1.67	10051_34	10.00					275_110	5.66	3808_71	4.72	7664_130	5.66	11140_13	0.00	AT20204	0.82
327_167	11.67	5099_48	1.67	10056_153	3.33					276_166	6.60	3809_60	10.38	7666_156	8.49	11141_13	6.60	AT20206	3.46
328_143	1.67	5108_79	15.00	10058_121	1.67					278_35	0.00	3820_28	3.77	7668_87	2.83	11142_103	1.89	AT27365	0.88
329_44	6.67	5115_37	3.33	10059_151	0.00					283_40	0.94	3826_95	0.00	7669_54	8.49	11149_124	8.49	AT27366	0.77
332_100	6.67	5116_17	3.33	10060_31	0.00					292_42	2.83	3830_26	5.66	7672_139	10.38	11150_67	2.83	AT27367	2.60
336_25	0.00	5117_145	0.00	10063_9	8.33					296_85	2.83	3837_61	3.77	7674_69	1.89	11154_92	0.94	AT27368	1.72
337_106	0.00	5130_36	3.33	10064_82	1.67					300_47	9.43	3838_106	11.32	7682_89	1.89	11156_106	0.00	AT27369	0.75
343_59	1.67	5135_47	0.00	10067_37	11.67					311_104	0.00	3843_110	7.55	7685_23	9.43	11159_91	11.32	AT27370	2.67
344_120	5.00	5138_135	3.33	10072_152	11.67					312_120	2.83	3845_152	12.26	7686_60	0.00	11162_94	14.15	AT27371	1.59
346_26	1.67	5139_51	1.67	10081_9	1.67					316_74	2.83	3854_7	0.94	7692_40	0.94	11168_95	0.00	AT27372	2.91
355_111	5.00	5141_194	8.33	10085_51	16.67					322_14	0.94	3860_120	0.00	7693_152	3.77	11172_135	10.38	AT27373	1.52
359_90	6.67	5146_98	0.00	10087_77	3.33					323_20	13.21	3861_23	3.77	7701_39	0.00	11176_50	0.00	AT27374	1.15
366_174	5.00	5149_70	10.00	10089_158	0.00					326_56	0.94	3865_122	0.94	7705_83	5.66	11181_113	0.00	AT15935	9.21
369_149	3.33	5152_45	10.00	10094_73	3.33					327_167	11.32	3867_138	15.09	7707_125	0.00	11185_72	14.15	AT15936	1.17
371_12	0.00	5157_7	1.67	10095_145	5.00					328_143	3.77	3871_10	12.26	7709_148	0.00	11189_78	0.94	AT15937	2.01
379_49	8.33	5161_91	5.00	10107_174	5.00					329_44	5.66	3877_17	0.94	7710_40	0.94	11190_37	1.89	AT15938	3.02
382_33	5.00	5182_42	1.67	10109_97	0.00					332_100	6.60	3882_162	8.49	7712_106	0.00	11195_53	0.94	AT15939	0.75
385_58	0.00	5185_150	8.33	10112_17	0.00					336_25	0.94	3883_168	6.60	7722_106	8.49	11197_144	10.38	107	7.82
392_111	10.00	5189_127	0.00	10117_49	3.33					337_106	0.00	3892_136	0.94	7724_83	2.83	11204_62	0.00	108	7.82
396_47	0.00	5190_40	1.67	10119_45	3.33					343_59	2.83	3894_24	11.32	7726_85	7.55	11206_45	0.94	109	1.76
397_67	0.00	5191_44	8.33	10120_65	0.00					344_120	2.83	3897_30	5.66	7729_96	0.94	11207_75	14.15	110	1.76
399_83	11.67	5203_9	1.67	10128_50	0.00					346_26	4.72	3902_28	5.66	7730_66	6.60	11208_85	6.60	111	1.12
401_90	0.00	5224_41	1.67	10131_123	1.67					355_111	8.49	3904_48	0.00	7733_73	0.00	11211_127	0.00	112	1.12
407_110	0.00	5257_155	1.67	10135_136	0.00					359_90	5.66	3905_149	4.72	7737_109	4.72	11213_47	1.89	113	10.64
415_83	1.67	5263_59	6.67	10138_58	8.33					360_93	9.43	3909_19	0.00	7738_73	2.83	11220_17	0.00	114	10.64
417_94	8.33	5273_174	13.33	10147_113	0.00					366_174	3.77	3913_31	10.38	7743_23	0.00	11225_125	0.00	115	1.32
418_76	3.33	5307_54	3.33	10148_9	3.33					369_149	1.89	3915_93	12.26	7746_81	6.60	11227_40	6.60	116	1.32
420_103	1.67	5308_74	0.00	10150_36	5.00					371_12	0.00	3916_26	9.43	7752_124	3.77	11234_35	1.89	117	4.54
423_78	0.00	5341_50	3.33	10154_107	3.33					379_49	11.32	3917_51	0.00	7756_111	7.55	11242_120	0.00	118	4.54
424_166	6.67	5353_91	1.67	10156_116	5.00					382_33	2.83	3928_117	0.94	7758_15	7.55	11243_114	0.94	119	0.97
425_85	0.00	5355_92	0.00	10157_55	0.00					385_58	0.00	3937_179	2.83	7761_179	12.26	11250_87	4.72	120	0.97
429_109	0.00	5358_50	6.67	10159_52	0.00					392_111	10.38	3939_20	2.83	7763_12	7.55	11251_31	0.94	121	0.64
431_127	0.00	5360_69	3.33	10165_53	1.67					396_47	0.00	3945_75	2.83	7768_66	3.77	11252_114	0.94	122	0.64
432_86	1.67	5362_64	6.67	10166_139	1.67					397_67	0.94	3948_7	8.49	7772_97	1.89	11254_57	8.49	123	1.08
439_113	8.33	5367_154	13.33	10175_43	1.67					399_83	9.43	3949_75	0.00	7776_7	7.55	11255_70	3.77	124	1.08
443_125	1.67	5377_103	1.67	10178_12	6.67					401_90	0.00	3951_82	0.94	7780_94	0.00	11256_7	8.49	125	0.77
444_84	6.67	5382_5	1.67	10182_65	1.67					407_110	0.00	3953_148	7.55	7784_51	0.00	11257_99	0.94	126	0.77
449_79	1.67	5394_34	5.00	10190_8	5.00					409_55	10.38	3955_10	3.77	7785_27	6.60	11259_120	0.00	127	3.11
459_91	6.67	5400_65	1.67	10193_21	1.67					410_78	0.00	3969_16	2.83	7787_108	1.89	11260_161	0.94	128	3.11
466_113	3.33	5402_52	0.00	10194_87	0.00					415_83	3.77	3971_127	0.00	7788_183	0.00	11271_73	1.89	129	22.32
471_54	5.00	5407_27	3.33	10196_131	6.67					417_94	5.66	3972_139	0.94	7797_56	3.77	11274_194	0.00	130	22.32
476_12	0.00	5418_26	10.00	10198_151	0.00					418_76	3.77	3973_80	0.94	7799_12	0.00	11278_100	2.83	131	0.68
477_178	6.67	5424_122	5.00	10199_88	6.67					420_103	1.89	3974_22	4.72	7804_75	0.94	11284_41	3.77	132	0.68

479_157	3.33	5428_120	1.67	10201_171	3.33				423_78	0.00	3976_155	5.66	7808_15	13.21	11286_91	3.77	133	27.88
485_93	3.33	5429_53	0.00	10206_12	1.67				424_166	8.49	3979_87	8.49	7815_139	5.66	11292_127	8.49	134	27.88
490_161	6.67	5430_70	0.00	10208_28	18.33				425_85	5.66	3980_159	0.94	7824_10	6.60	11293_111	0.00	135	3.31
494_123	6.67	5436_50	0.00	10214_98	3.33				429_109	0.00	3984_69	2.83	7825_86	2.83	11294_55	0.00	136	3.31
499_125	1.67	5437_73	1.67	10220_13	3.33				431_127	0.00	3986_59	0.94	7828_99	0.00	11295_9	1.89	137	2.64
503_94	6.67	5442_44	5.00	10223_38	0.00				432_86	8.49	3988_117	13.21	7830_57	12.26	11297_27	9.43	138	2.64
507_134	5.00	5447_13	0.00	10226_84	0.00				439_113	7.55	3990_68	0.94	7836_151	0.00	11298_127	2.83	139	2.38
516_83	1.67	5450_10	0.00	10227_131	11.67				440_161	5.66	3991_65	6.60	7837_157	2.83	11302_92	0.00	140	2.38
518_78	1.67	5455_85	0.00	10230_120	6.67				443_125	2.83	3992_61	10.38	7838_11	3.77	11304_10	1.89	141	2.67
520_108	10.00	5468_117	1.67	10232_95	1.67				444_84	5.66	3993_140	1.89	7839_134	2.83	11305_78	1.89	142	2.67
525_94	1.67	5478_92	0.00	10233_112	0.00				449_79	1.89	4004_14	10.38	7840_149	12.26	11307_54	2.83	143	2.89
526_42	8.33	5480_79	6.67	10237_34	1.67				457_44	2.83	4005_52	13.21	7844_93	0.94	11309_49	0.00	144	2.89
527_61	0.00	5484_63	3.33	10240_62	1.67				459_91	3.77	4006_50	7.55	7849_26	2.83	11310_67	1.89	145	2.86
532_131	1.67	5485_14	0.00	10243_141	1.67				466_113	1.89	4007_77	5.66	7852_67	0.94	11314_53	2.83	146	2.86
542_52	5.00	5489_94	0.00	10244_87	0.00				471_54	5.66	4009_93	13.21	7853_73	1.89	11316_15	3.77	147	9.26
547_23	1.67	5496_55	0.00	10245_80	0.00				476_12	0.94	4012_96	9.43	7854_32	0.00	11317_31	9.43	148	9.26
549_80	0.00	5497_6	1.67	10247_52	5.00				477_178	4.72	4013_6	0.00	7856_85	8.49	11320_142	10.38	149	1.32
551_153	1.67	5498_122	1.67	10248_19	0.00				478_70	0.00	4016_30	0.94	7861_35	0.00	11322_117	11.32	150	1.32
553_83	1.67	5501_26	8.33	10249_123	5.00				479_157	2.83	4025_11	6.60	7863_129	10.38	11329_20	0.00	151	2.34
554_75	0.00	5504_81	1.67	10259_8	1.67				480_83	2.83	4027_76	12.26	7864_41	0.94	11330_114	1.89	152	2.34
565_53	15.00	5506_134	8.33	10264_121	1.67				485_93	2.83	4030_8	0.94	7871_139	0.94	11331_35	8.49	153	1.28
566_36	1.67	5510_51	8.33	10265_97	0.00				490_161	4.72	4031_62	3.77	7872_14	0.00	11334_59	8.49	154	1.28
567_186	15.00	5514_70	0.00	10270_107	1.67				491_35	1.89	4040_27	2.83	7873_37	11.32	11335_156	3.77	155	13.99
568_82	3.33	5520_20	5.00	10272_125	10.00				494_123	4.72	4041_78	3.77	7876_40	7.55	11336_130	5.66	156	13.99
570_72	1.67	5528_119	1.67	10273_24	1.67				499_125	0.94	4049_64	0.00	7878_80	1.89	11339_120	13.21	157	5.33
571_82	0.00	5529_100	0.00	10277_70	0.00				503_94	7.55	4051_57	1.89	7879_75	0.00	11341_53	6.60	158	5.33
575_46	1.67	5530_182	3.33	10278_93	0.00				507_134	12.26	4052_93	0.94	7881_133	5.66	11342_146	0.94	159	2.93
576_79	0.00	5533_91	3.33	10280_75	0.00				512_104	1.89	4056_132	3.77	7883_129	1.89	11343_48	0.94	160	2.93
579_41	0.00	5536_78	1.67	10281_29	0.00				516_83	2.83	4059_141	3.77	7884_186	2.83	11346_47	0.00	161	1.85
580_32	3.33	5537_47	5.00	10283_32	0.00				518_78	0.94	4060_78	4.72	7890_89	4.72	11348_38	3.77	162	1.85
584_110	0.00	5541_111	0.00	10286_86	0.00				520_108	5.66	4064_123	0.00	7892_6	1.89	11350_130	0.00	163	3.00
586_74	3.33	5546_51	10.00	10288_103	5.00				525_94	3.77	4065_76	0.94	7895_145	0.00	11352_10	1.89	164	3.00
590_42	0.00	5555_24	6.67	10289_46	1.67				526_42	4.72	4066_44	0.00	7896_23	10.38	11354_56	2.83	165	0.88
591_120	1.67	5560_81	0.00	10292_161	5.00				527_61	0.94	4068_191	5.66	7901_27	0.00	11355_37	10.38	166	0.88
592_9	1.67	5564_40	0.00	10296_75	0.00				532_131	1.89	4073_16	1.89	7902_74	0.94	11362_132	0.00	167	1.61
597_129	1.67	5566_126	6.67	10297_38	1.67				542_52	7.55	4075_68	0.00	7905_111	2.83	11363_36	2.83	168	1.61
598_17	0.00	5572_62	1.67	10298_116	0.00				547_23	1.89	4078_143	0.94	7907_97	0.94	11369_38	3.77	169	1.06
599_103	0.00	5577_32	0.00	10299_111	1.67				549_80	0.00	4079_64	0.00	7912_93	0.94	11372_80	5.66	170	1.06
603_66	21.67	5579_60	0.00	10302_15	0.00				551_153	2.83	4080_141	10.38	7916_79	0.94	11374_103	0.00	171	0.82
607_75	1.67	5583_75	6.67	10304_24	6.67				553_83	6.60	4085_28	0.00	7919_89	0.00	11378_153	1.89	172	0.82
608_13	1.67	5584_26	1.67	10308_48	1.67				554_75	5.66	4086_72	4.72	7925_140	0.00	11379_39	0.00	173	14.92
617_129	0.00	5587_33	8.33	10309_69	1.67				559_122	8.49	4088_64	0.94	7926_43	0.94	11381_17	2.83	174	14.92
619_187	13.33	5591_29	10.00	10317_68	0.00				563_35	0.00	4090_70	6.60	7934_76	3.77	11385_32	8.49	175	0.84
623_141	1.67	5595_90	0.00	10318_57	0.00				565_53	15.09	4091_87	0.00	7935_101	0.00	11387_147	2.83	176	0.84
624_7	1.67	5604_21	0.00	10320_148	8.33				566_36	1.89	4092_126	3.77	7936_190	11.32	11388_120	9.43	177	1.45
633_5	16.67	5606_23	0.00	10322_44	0.00				567_186	12.26	4101_29	1.89	7938_79	0.00	11399_28	0.94	178	1.45

634_55	0.00	5612_119	5.00	10323_57	0.00			568_82	4.72	4104_100	12.26	7940_36	1.89	11400_58	9.43	179	0.82
636_26	6.67	5618_45	0.00	10324_138	3.33			570_72	1.89	4105_90	1.89	7947_132	0.00	11401_140	0.94	180	0.82
645_84	11.67	5619_155	1.67	10325_86	1.67			571_82	0.00	4106_21	3.77	7950_26	0.00	11402_13	3.77	181	3.46
654_161	10.00	5621_41	3.33	10326_53	0.00			573_18	0.00	4110_145	0.00	7952_117	1.89	11405_29	0.00	182	3.46
659_40	11.67	5622_6	0.00	10327_146	1.67			575_46	1.89	4111_144	6.60	7956_125	1.89	11407_72	3.77	183	0.88
666_52	1.67	5623_147	0.00	10337_158	10.00			576_79	0.00	4113_53	9.43	7957_104	2.83	11410_54	11.32	184	0.88
670_133	0.00	5625_72	1.67	10339_128	8.33			577_35	0.94	4116_88	6.60	7960_123	4.72	11416_33	1.89	185	0.77
671_118	1.67	5631_128	11.67	10341_132	5.00			578_42	2.83	4117_5	1.89	7972_29	0.94	11418_110	0.94	186	0.77
672_65	0.00	5632_76	25.00	10343_76	3.33			579_41	0.00	4120_85	0.00	7974_55	0.00	11421_89	6.60	187	2.60
674_119	10.00	5634_164	3.33	10344_7	5.00			580_32	7.55	4124_8	0.00	7977_85	0.00	11426_15	5.66	188	2.60
675_55	15.00	5636_44	0.00	10346_104	0.00			582_39	0.00	4128_107	6.60	7979_47	9.43	11432_24	1.89	189	1.72
676_130	1.67	5652_25	0.00	10349_48	3.33			584_110	0.00	4129_30	2.83	7987_70	0.00	11434_75	3.77	190	1.72
678_41	8.33	5653_16	1.67	10356_149	6.67			586_74	6.60	4132_109	10.38	7988_46	3.77	11440_149	2.83	191	0.75
683_143	1.67	5654_13	0.00	10360_119	3.33			590_42	0.94	4133_86	4.72	7989_49	0.00	11444_164	2.83	192	0.75
684_63	3.33	5655_87	1.67	10362_76	0.00			591_120	5.66	4135_27	0.00	7991_34	1.89	11445_81	0.00	193	2.67
701_68	1.67	5664_55	3.33	10367_82	11.67			592_9	0.94	4141_129	4.72	7992_84	9.43	11447_36	0.00	194	2.67
702_95	6.67	5667_87	6.67	10373_38	1.67			597_129	4.72	4145_23	7.55	7995_101	4.72	11451_119	14.15	195	1.59
707_121	1.67	5669_43	1.67	10379_92	1.67			598_17	0.00	4148_11	0.94	7997_9	10.38	11456_25	4.72	196	1.59
708_82	0.00	5670_128	8.33	10385_26	10.00			599_103	2.83	4150_62	0.00	7998_183	9.43	11461_193	8.49	197	2.91
712_127	3.33	5672_122	13.33	10386_80	1.67			603_66	15.09	4151_116	8.49	7999_48	5.66	11465_161	3.77	198	2.91
713_109	3.33	5676_77	5.00	10393_34	0.00			604_61	1.89	4154_153	0.00	8000_144	3.77	11467_5	2.83	199	1.52
714_38	0.00	5681_106	10.00	10394_65	0.00			607_75	0.94	4157_156	5.66	8001_93	8.49	11472_81	0.94	200	1.52
719_67	3.33	5686_74	3.33	10402_172	10.00			608_13	8.49	4165_67	11.32	8003_71	1.89	11480_11	2.83	201	1.15
722_183	3.33	5688_72	3.33	10407_92	0.00			617_129	0.00	4169_40	0.94	8005_131	9.43	11481_152	0.94	202	1.15
731_5	6.67	5689_93	5.00	10410_136	8.33			619_187	11.32	4170_96	8.49	8007_188	14.15	11484_71	0.00	203	9.21
734_74	3.33	5700_107	6.67	10412_182	8.33			623_141	1.89	4173_157	0.00	8009_31	1.89	11487_112	5.66	204	9.21
738_6	1.67	5708_149	1.67	10414_68	3.33			624_7	0.94	4174_153	0.94	8013_109	1.89	11488_150	2.83	205	1.17
743_87	0.00	5709_88	8.33	10416_76	1.67			629_62	0.00	4175_55	6.60	8015_53	0.00	11490_92	0.00	206	1.17
744_118	1.67	5710_8	6.67	10418_8	8.33			633_5	10.38	4180_172	10.38	8026_114	2.83	11493_45	6.60	207	2.01
747_106	3.33	5713_18	11.67	10419_13	0.00			634_55	0.00	4187_63	6.60	8030_82	0.00	11497_63	0.00	208	2.01
750_71	5.00	5714_152	8.33	10421_177	8.33			636_26	11.32	4189_123	3.77	8040_118	6.60	11498_10	0.94	209	3.02
751_195	11.67	5718_103	0.00	10425_78	0.00			645_84	13.21	4190_67	8.49	8044_26	0.94	11501_64	0.00	210	3.02
752_78	0.00	5719_169	0.00	10437_160	5.00			654_161	9.43	4195_138	0.00	8048_5	0.00	11505_136	1.89	211	0.75
753_22	0.00	5730_5	6.67	10439_56	1.67			659_40	10.38	4201_25	7.55	8055_96	0.94	11511_164	3.77	212	0.75
754_105	1.67	5742_88	8.33	10444_84	0.00			666_52	2.83	4204_28	0.00	8056_30	0.00	11515_8	0.00		
758_93	6.67	5750_57	5.00	10445_82	5.00			670_133	0.94	4206_73	1.89	8059_82	1.89	11516_76	1.89		
762_57	3.33	5754_46	1.67	10448_131	6.67			671_118	2.83	4209_11	5.66	8061_117	0.94	11518_70	4.72		
765_13	6.67	5757_144	1.67	10449_112	0.00			672_65	0.94	4210_87	0.00	8065_19	0.94	11520_39	11.32		
766_195	1.67	5758_111	0.00	10451_127	0.00			673_100	0.00	4214_37	0.00	8066_51	4.72	11522_138	7.55		
771_74	0.00	5762_111	0.00	10452_126	1.67			674_119	10.38	4216_151	3.77	8067_94	4.72	11536_169	7.55		
776_110	1.67	5772_55	10.00	10453_64	6.67			675_55	8.49	4221_30	10.38	8068_30	2.83	11540_44	0.00		
780_160	6.67	5780_27	5.00	10454_172	6.67			676_130	3.77	4222_17	0.94	8069_130	0.00	11542_126	16.04		
783_90	0.00	5788_69	0.00	10455_52	0.00			677_6	0.94	4225_51	10.38	8073_158	4.72	11548_131	2.83		
784_56	0.00	5789_21	11.67	10456_67	8.33			678_41	6.60	4232_118	0.94	8074_21	0.94	11551_121	13.21		
789_10	0.00	5790_87	10.00	10459_78	3.33			681_10	7.55	4234_94	8.49	8076_76	5.66	11557_25	1.89		
792_182	0.00	5796_88	5.00	10460_129	6.67			683_143	0.94	4235_104	4.72	8077_29	11.32	11561_14	3.77		

794_121	1.67	5798_110	1.67	10463_24	0.00			684_63	1.89	4239_99	2.83	8078_106	0.00	11562_157	0.00		
806_162	0.00	5802_79	1.67	10470_153	0.00			697_50	0.00	4243_82	1.89	8081_86	4.72	11564_113	0.94		
808_15	0.00	5811_30	1.67	10474_37	1.67			701_68	0.94	4244_100	11.32	8083_46	0.00	11566_26	0.00		
818_98	3.33	5815_101	0.00	10485_41	0.00			702_95	5.66	4246_53	3.77	8084_67	9.43	11569_35	1.89		
837_83	0.00	5819_104	1.67	10487_124	0.00			707_121	1.89	4247_40	0.00	8085_129	2.83	11570_129	12.26		
838_71	3.33	5826_161	10.00	10502_19	0.00			708_82	0.00	4248_111	6.60	8090_82	1.89	11573_26	0.00		
839_62	1.67	5830_40	1.67	10503_57	1.67			712_127	1.89	4252_81	0.94	8091_30	4.72	11574_99	1.89		
841_105	0.00	5833_11	10.00	10505_39	8.33			713_109	2.83	4255_155	5.66	8096_86	0.00	11579_54	6.60		
844_24	5.00	5841_70	0.00	10506_83	0.00			714_38	0.00	4257_131	0.94	8097_193	3.77	11590_143	4.72		
852_35	1.67	5842_121	0.00	10514_29	3.33			719_67	2.83	4258_65	6.60	8101_146	4.72	11593_150	0.94		
853_113	5.00	5843_65	0.00	10518_155	1.67			722_183	4.72	4266_177	6.60	8103_69	0.00	11595_86	0.94		
855_75	8.33	5844_37	3.33	10521_52	3.33			728_192	0.00	4269_149	0.94	8104_67	6.60	11603_98	4.72		
860_32	3.33	5848_48	0.00	10531_38	3.33			731_5	5.66	4271_107	1.89	8107_39	10.38	11606_73	2.83		
871_74	5.00	5858_61	1.67	10533_74	5.00			734_74	6.60	4272_39	5.66	8111_77	0.00	11609_177	4.72		
874_46	0.00	5860_30	3.33	10535_50	6.67			738_6	1.89	4274_186	10.38	8119_160	1.89	11611_10	0.94		
891_34	0.00	5866_98	0.00	10536_121	1.67			743_87	0.94	4278_43	9.43	8120_79	0.94	11629_13	1.89		
899_78	3.33	5871_113	10.00	10537_140	3.33			744_118	0.94	4280_46	1.89	8121_54	1.89	11633_110	0.94		
900_94	6.67	5874_33	3.33	10538_32	0.00			747_106	1.89	4282_57	0.94	8124_49	7.55	11634_120	0.00		
902_162	0.00	5880_116	1.67	10546_128	5.00			750_71	7.55	4285_99	7.55	8130_29	8.49	11636_124	0.00		
910_85	0.00	5886_85	0.00	10554_85	1.67			751_195	11.32	4288_144	6.60	8133_82	10.38	11641_7	10.38		
914_29	1.67	5891_9	3.33	10558_107	0.00			752_78	0.00	4298_160	0.94	8136_13	5.66	11643_61	6.60		
923_30	1.67	5898_90	5.00	10559_66	8.33			753_22	2.83	4299_136	0.00	8139_111	8.49	11646_10	14.15		
930_78	1.67	5899_33	3.33	10561_151	0.00			754_105	0.94	4301_130	10.38	8140_22	6.60	11657_149	0.94		
939_173	1.67	5900_86	0.00	10564_151	13.33			758_93	10.38	4302_67	13.21	8143_190	4.72	11667_40	0.00		
943_134	0.00	5901_48	0.00	10565_143	6.67			759_124	10.38	4306_41	11.32	8145_52	7.55	11669_27	4.72		
952_27	11.67	5903_67	6.67	10575_114	0.00			762_57	1.89	4308_73	0.94	8148_56	0.00	11670_29	3.77		
954_65	5.00	5904_72	10.00	10577_108	10.00			765_13	8.49	4314_84	4.72	8149_14	1.89	11686_120	0.94		
956_98	0.00	5911_62	1.67	10582_166	8.33			766_195	0.94	4322_60	5.66	8150_188	6.60	11700_73	4.72		
957_98	0.00	5913_122	3.33	10587_92	1.67			768_29	0.00	4326_67	0.94	8155_32	14.15	11712_110	5.66		
961_59	5.00	5923_62	6.67	10590_134	5.00			771_74	0.00	4327_144	0.00	8158_79	0.94	11717_80	0.00		
964_66	6.67	5927_149	0.00	10597_43	1.67			772_28	2.83	4331_73	5.66	8165_110	3.77	11718_145	10.38		
972_158	3.33	5930_142	5.00	10616_100	0.00			776_110	1.89	4333_99	1.89	8166_56	5.66	11738_7	1.89		
973_56	1.67	5931_24	0.00	10622_151	10.00			780_160	5.66	4334_8	0.00	8168_61	3.77	11742_116	6.60		
978_46	0.00	5932_91	16.67	10628_32	0.00			782_187	1.89	4344_86	0.94	8170_148	0.00	11756_27	1.89		
979_23	5.00	5936_132	6.67	10630_62	5.00			783_90	0.00	4346_164	5.66	8171_41	4.72	11768_147	4.72		
981_51	1.67	5938_80	1.67	10640_132	6.67			784_56	0.00	4348_144	2.83	8175_74	2.83	11770_36	0.00		
982_115	0.00	5941_110	3.33	10641_96	18.33			787_28	0.00	4349_43	0.00	8176_130	0.94	11773_72	2.83		
983_92	15.00	5944_64	5.00	10643_20	6.67			789_10	2.83	4352_53	2.83	8177_86	0.00	11776_168	6.60		
985_84	3.33	5948_61	1.67	10646_75	5.00			792_182	2.83	4357_6	0.94	8179_23	1.89	11783_135	0.94		
992_124	0.00	5949_88	6.67	10647_43	1.67			794_121	1.89	4370_104	9.43	8182_24	4.72	11796_49	2.83		
999_79	0.00	5970_21	15.00	10654_115	8.33			806_162	0.00	4372_8	6.60	8188_95	5.66	11798_62	0.94		
1003_62	0.00	5973_74	0.00	10663_93	0.00			808_15	1.89	4373_117	1.89	8190_47	9.43	11806_56	12.26		
1004_65	0.00	5983_44	8.33	10665_41	0.00			818_98	2.83	4384_97	3.77	8193_62	4.72	11819_135	10.38		
1006_180	3.33	5986_86	3.33	10666_48	1.67			837_83	0.00	4389_155	12.26	8196_123	1.89	11822_72	5.66		
1007_77	6.67	5988_66	1.67	10669_32	0.00			838_71	3.77	4390_154	0.00	8197_103	1.89	11832_40	1.89		
1010_83	0.00	5990_17	0.00	10671_38	0.00			839_62	7.55	4392_89	0.94	8198_157	0.00	11837_120	2.83		

1028_125	1.67	5991_30	8.33	10676_38	3.33			841_105	0.00	4394_83	0.00	8201_92	0.00	11839_114	11.32	
1038_37	3.33	5992_151	5.00	10681_59	1.67			844_24	6.60	4398_5	2.83	8206_115	4.72	11842_121	0.00	
1039_21	0.00	5994_56	0.00	10682_20	0.00			852_35	1.89	4405_106	2.83	8210_42	2.83	11845_121	0.94	
1041_87	0.00	5995_67	3.33	10683_49	1.67			853_113	2.83	4407_64	4.72	8212_69	0.00	11849_7	4.72	
1047_70	5.00	5998_33	0.00	10684_127	0.00			855_75	5.66	4408_13	0.00	8213_16	0.94	11855_82	6.60	
1050_10	16.67	6003_18	1.67	10685_110	3.33			859_83	0.94	4418_35	3.77	8214_139	7.55	11857_125	1.89	
1058_92	11.67	6010_162	1.67	10689_90	1.67			860_32	12.26	4425_122	4.72	8215_37	2.83	11859_77	5.66	
1060_103	0.00	6011_103	0.00	10692_50	0.00			871_74	6.60	4426_13	2.83	8218_140	1.89	11864_64	1.89	
1063_174	3.33	6012_37	0.00	10695_56	5.00			874_46	0.00	4427_137	2.83	8219_64	11.32	11865_30	10.38	
1064_134	0.00	6014_47	3.33	10697_61	0.00			891_34	0.94	4428_100	0.94	8220_37	0.00	11866_119	1.89	
1065_169	0.00	6017_90	1.67	10707_70	0.00			894_191	0.00	4431_102	1.89	8227_57	12.26	11867_90	0.94	
1074_34	8.33	6019_14	0.00	10709_133	0.00			899_78	2.83	4432_133	1.89	8229_26	3.77	11873_51	7.55	
1086_116	0.00	6021_6	5.00	10710_96	1.67			900_94	8.49	4434_167	4.72	8231_89	5.66	11882_150	13.21	
1090_32	0.00	6023_62	6.67	10711_133	0.00			902_162	2.83	4437_53	0.00	8235_169	2.83	11883_90	5.66	
1091_7	1.67	6032_70	3.33	10712_126	5.00			910_85	0.00	4439_48	0.94	8238_13	0.00	11886_194	13.21	
1095_110	1.67	6033_57	16.67	10714_21	6.67			914_29	2.83	4441_65	6.60	8239_20	1.89	11896_137	9.43	
1100_85	1.67	6038_9	0.00	10717_22	0.00			923_30	0.94	4443_24	3.77	8240_41	0.00	11899_31	0.00	
1104_66	6.67	6039_68	0.00	10719_119	8.33			930_78	0.94	4450_67	2.83	8242_99	0.94	11910_32	4.72	
1112_71	0.00	6042_75	5.00	10720_72	1.67			935_42	9.43	4452_97	1.89	8244_148	2.83	11921_112	4.72	
1113_75	0.00	6045_47	3.33	10726_78	5.00			939_173	1.89	4454_102	8.49	8246_72	0.00	11923_112	3.77	
1119_28	0.00	6046_46	3.33	10729_145	0.00			943_134	0.00	4456_95	1.89	8247_74	0.00	11927_135	0.94	
1122_87	6.67	6049_74	6.67	10740_76	0.00			948_143	10.38	4457_30	4.72	8253_18	4.72	11929_40	1.89	
1127_110	6.67	6051_107	3.33	10743_76	1.67			952_27	10.38	4467_99	0.94	8255_132	3.77	11930_125	4.72	
1130_64	25.00	6055_168	1.67	10744_91	8.33			954_65	8.49	4471_20	6.60	8259_17	0.94	11944_149	4.72	
1137_166	8.33	6056_142	0.00	10745_86	1.67			956_98	0.00	4474_6	0.94	8260_99	3.77	11952_157	6.60	
1139_7	5.00	6062_6	0.00	10752_100	1.67			957_98	0.94	4475_11	0.00	8261_141	4.72	11968_190	1.89	
1140_103	0.00	6069_131	1.67	10753_117	1.67			961_59	4.72	4477_73	0.00	8264_100	0.00	11969_85	0.00	
1142_125	10.00	6075_39	3.33	10754_100	1.67			964_66	11.32	4479_68	0.00	8265_56	5.66	11975_21	0.00	
1149_20	20.00	6081_17	0.00	10755_42	0.00			972_158	1.89	4486_9	0.00	8267_179	7.55	11978_89	10.38	
1150_184	13.33	6085_187	5.00	10758_74	8.33			973_56	0.94	4488_60	6.60	8273_51	0.00	11990_187	10.38	
1151_136	0.00	6087_72	5.00	10765_71	8.33			978_46	0.94	4490_185	5.66	8276_35	2.83	11998_76	3.77	
1154_33	6.67	6094_83	8.33	10766_149	10.00			979_23	8.49	4491_71	0.00	8277_83	0.00	12017_107	5.66	
1159_26	20.00	6096_28	6.67	10770_62	1.67			981_51	3.77	4501_72	9.43	8279_43	8.49	12030_9	14.15	
1161_63	1.67	6097_106	0.00	10771_128	8.33			982_115	0.00	4502_18	0.00	8281_37	0.00	12042_191	5.66	
1164_84	5.00	6102_93	1.67	10773_13	8.33			983_92	12.26	4503_79	9.43	8288_10	3.77	12056_23	0.94	
1174_6	1.67	6103_98	1.67	10775_52	0.00			984_101	1.89	4506_22	1.89	8290_159	14.15	12061_166	6.60	
1176_107	6.67	6104_129	3.33	10778_15	1.67			985_84	3.77	4507_109	0.00	8291_125	0.00	12065_79	8.49	
1182_140	0.00	6108_108	5.00	10780_99	3.33			992_124	0.94	4509_72	0.00	8296_96	0.94	12073_154	6.60	
1184_112	0.00	6112_91	1.67	10783_165	3.33			999_79	4.72	4511_95	5.66	8297_102	0.00	12080_34	0.00	
1185_60	3.33	6113_46	3.33	10793_67	0.00			1003_62	0.94	4515_39	12.26	8301_187	5.66	12082_122	1.89	
1188_19	1.67	6117_18	1.67	10804_49	0.00			1004_65	0.00	4520_44	14.15	8303_54	0.00	12083_104	0.94	
1190_152	0.00	6118_33	1.67	10805_17	5.00			1006_180	5.66	4521_183	9.43	8312_36	2.83	12092_152	5.66	
1191_91	21.67	6120_143	0.00	10811_55	3.33			1007_77	5.66	4528_171	1.89	8313_111	0.94	12098_49	4.72	
1193_24	5.00	6125_18	3.33	10814_166	0.00			1010_83	0.00	4534_92	0.00	8317_116	7.55	12102_37	7.55	
1197_121	5.00	6127_41	1.67	10820_43	1.67			1020_59	0.94	4536_176	0.94	8318_55	1.89	12116_36	3.77	
1199_92	0.00	6128_86	8.33	10823_14	0.00			1028_125	0.94	4539_50	0.94	8322_119	2.83	12117_190	6.60	

1200_129	1.67	6129_111	10.00	10824_44	26.67					1038_37	1.89	4540_158	3.77	8324_64	11.32	12119_23	2.83
1201_20	5.00	6150_27	8.33	10826_49	3.33					1039_21	0.00	4541_137	2.83	8329_177	5.66	12123_23	0.00
1206_164	1.67	6151_111	1.67	10827_78	5.00					1041_87	0.00	4544_91	0.00	8335_6	0.94	12126_36	7.55
1219_17	1.67	6155_114	0.00	10832_128	1.67					1046_11	4.72	4545_128	11.32	8337_54	4.72	12129_130	1.89
1224_91	3.33	6158_46	0.00	10834_60	5.00					1047_70	2.83	4548_120	4.72	8338_37	3.77	12131_40	0.00
1225_73	3.33	6159_160	5.00	10838_91	11.67					1050_10	12.26	4559_160	6.60	8339_98	0.00	12132_154	0.94
1229_21	1.67	6160_121	13.33	10844_84	1.67					1056_62	7.55	4563_55	1.89	8344_42	2.83	12140_17	4.72
1230_72	3.33	6162_116	1.67	10845_105	1.67					1058_92	10.38	4566_85	0.00	8345_120	3.77	12144_20	0.94
1231_30	1.67	6166_23	0.00	10848_74	5.00					1060_103	0.00	4574_129	0.00	8348_136	0.00	12147_93	12.26
1233_183	16.67	6168_139	6.67	10849_65	8.33					1063_174	2.83	4581_60	0.00	8352_107	1.89	12151_11	0.00
1234_71	6.67	6170_75	0.00	10851_59	1.67					1064_134	0.00	4583_109	0.00	8353_119	1.89	12162_35	1.89
1239_142	1.67	6171_65	5.00	10852_170	11.67					1065_169	0.00	4585_158	9.43	8354_110	0.00	12163_12	2.83
1241_129	1.67	6172_178	1.67	10853_9	0.00					1074_34	9.43	4596_177	6.60	8357_72	0.00	12164_82	4.72
1243_46	0.00	6177_35	6.67	10854_7	6.67					1080_51	2.83	4598_35	0.00	8358_67	0.00	12173_127	2.83
1245_191	0.00	6179_12	0.00	10858_5	0.00					1086_116	0.00	4600_37	5.66	8365_30	0.00	12180_99	2.83
1248_17	11.67	6180_128	0.00	10859_33	11.67					1090_32	0.00	4603_122	1.89	8366_68	2.83	12181_86	3.77
1250_42	6.67	6187_92	1.67	10860_156	5.00					1091_7	4.72	4604_10	3.77	8377_54	0.00	12183_11	3.77
1254_131	11.67	6191_190	8.33	10861_95	1.67					1095_110	0.94	4611_31	6.60	8380_107	0.00	12199_134	0.00
1258_77	11.67	6202_38	0.00	10865_26	0.00					1099_10	0.00	4613_104	7.55	8382_22	3.77	12200_58	2.83
1262_60	0.00	6209_48	16.67	10870_11	1.67					1100_85	0.94	4622_128	0.00	8383_94	0.00	12204_107	11.32
1263_174	3.33	6217_87	6.67	10875_134	6.67					1104_66	5.66	4623_56	0.94	8384_108	4.72	12207_142	7.55
1264_57	1.67	6220_25	0.00	10879_129	3.33					1112_71	0.00	4624_34	10.38	8385_64	0.00	12209_125	10.38
1267_33	1.67	6221_55	10.00	10880_156	13.33					1113_75	0.00	4627_186	5.66	8392_31	7.55	12214_69	0.00
1268_183	8.33	6225_133	0.00	10896_73	1.67					1119_28	0.94	4629_101	0.94	8394_26	0.00	12230_75	0.00
1270_19	0.00	6230_32	5.00	10898_123	3.33					1122_87	12.26	4631_152	6.60	8396_28	0.00	12232_33	0.94
1272_70	3.33	6240_103	0.00	10899_43	0.00					1127_110	6.60	4633_207	12.26	8399_44	4.72	12239_121	3.77
1281_93	1.67	6242_46	1.67	10900_27	0.00					1130_64	14.15	4639_130	2.83	8401_83	0.00	12242_188	6.60
1284_154	8.33	6245_82	1.67	10901_90	5.00					1137_166	11.32	4642_83	12.26	8402_5	1.89	12246_74	0.94
1288_106	8.33	6246_127	3.33	10909_43	1.67					1139_7	3.77	4646_79	12.26	8403_51	1.89	12247_38	0.94
1296_129	1.67	6247_40	6.67	10915_7	0.00					1140_103	0.00	4650_134	3.77	8408_139	11.32	12251_109	8.49
1305_101	0.00	6251_164	5.00	10916_58	1.67					1142_125	11.32	4655_25	4.72	8412_152	2.83	12257_112	5.66
1308_134	1.67	6260_72	6.67	10921_195	3.33					1143_82	0.00	4657_15	1.89	8417_47	9.43	12258_33	5.66
1313_122	8.33	6261_15	3.33	10929_84	0.00					1149_20	12.26	4668_88	0.94	8418_102	4.72	12272_79	0.94
1314_91	1.67	6276_109	1.67	10934_84	3.33					1150_184	10.38	4669_46	4.72	8427_95	1.89	12273_194	12.26
1319_192	1.67	6280_72	3.33	10936_158	3.33					1151_136	0.00	4677_96	10.38	8429_63	8.49	12276_128	3.77
1321_86	0.00	6295_108	1.67	10937_51	0.00					1154_33	9.43	4681_112	0.94	8430_37	2.83	12278_87	1.89
1323_58	1.67	6300_134	0.00	10946_93	10.00					1159_26	13.21	4682_51	0.00	8435_8	3.77	12287_198	8.49
1326_18	0.00	6303_76	0.00	10959_125	3.33					1161_63	3.77	4683_77	0.00	8440_136	8.49	12294_56	6.60
1328_44	1.67	6311_5	0.00	10960_68	10.00					1164_84	5.66	4690_98	10.38	8452_172	0.94	12295_31	8.49
1337_125	0.00	6312_25	0.00	10962_109	1.67					1174_6	1.89	4693_5	0.94	8460_13	4.72	12296_61	0.94
1339_52	0.00	6314_63	0.00	10964_42	1.67					1176_107	4.72	4701_46	0.00	8461_52	2.83	12297_175	5.66
1340_66	1.67	6316_48	6.67	10971_73	0.00					1177_9	0.94	4705_41	5.66	8462_42	0.94	12300_5	1.89
1348_115	10.00	6318_107	8.33	10992_98	0.00					1180_55	1.89	4711_95	2.83	8467_9	0.00	12303_64	12.26
1357_147	0.00	6330_5	5.00	10994_43	6.67					1182_140	2.83	4717_38	2.83	8468_116	0.94	12312_124	0.00
1361_121	8.33	6350_105	1.67	10997_43	0.00					1184_112	0.00	4723_36	2.83	8470_105	0.00	12325_147	0.00
1365_96	3.33	6351_20	5.00	10998_169	5.00					1185_60	3.77	4728_114	7.55	8479_139	0.00	12333_112	3.77

1368_59	3.33	6353_13	10.00	11001_100	3.33					1188_19	3.77	4729_170	0.94	8483_105	11.32	12346_92	10.38		
1370_134	5.00	6355_44	5.00	11002_55	5.00					1190_152	0.00	4730_45	11.32	8485_37	6.60	12352_36	0.94		
1371_90	6.67	6359_17	6.67	11004_91	0.00					1191_91	14.15	4732_98	1.89	8486_78	14.15	12355_48	5.66		
1374_62	1.67	6362_50	1.67	11007_157	18.33					1193_24	7.55	4733_66	10.38	8488_85	0.00	12365_121	7.55		
1378_155	0.00	6367_11	11.67	11013_31	1.67					1197_121	4.72	4735_20	0.00	8490_96	2.83	12369_165	11.32		
1379_85	0.00	6368_53	0.00	11015_165	5.00					1199_92	0.00	4737_10	11.32	8493_142	5.66	12372_42	3.77		
1380_118	5.00	6369_14	10.00	11018_5	6.67					1200_129	1.89	4741_74	0.00	8495_20	7.55	12377_121	0.00		
1381_116	1.67	6371_78	3.33	11025_55	3.33					1201_20	4.72	4746_96	1.89	8499_168	2.83	12378_189	11.32		
1382_160	3.33	6373_74	1.67	11028_114	3.33					1206_164	3.77	4750_57	0.94	8501_63	3.77	12382_83	7.55		
1384_78	3.33	6375_32	0.00	11031_24	5.00					1212_45	2.83	4752_89	0.00	8504_38	1.89	12384_10	10.38		
1386_55	1.67	6376_59	3.33	11038_138	3.33					1219_17	0.94	4755_86	5.66	8505_56	3.77	12389_49	4.72		
1387_54	0.00	6378_5	0.00	11053_53	0.00					1224_91	1.89	4758_190	2.83	8507_73	0.00	12394_93	0.94		
1392_129	0.00	6380_175	8.33	11064_29	0.00					1225_73	2.83	4764_170	10.38	8511_135	0.94	12403_128	0.00		
1393_41	0.00	6382_68	1.67	11072_95	1.67					1229_21	1.89	4765_102	0.00	8512_71	2.83	12410_98	11.32		
1394_32	6.67	6384_67	1.67	11077_27	15.00					1230_72	3.77	4766_102	4.72	8517_34	5.66	12411_146	4.72		
1399_50	0.00	6386_44	5.00	11078_21	5.00					1231_30	0.94	4768_14	12.26	8520_22	5.66	12412_57	0.00		
1405_92	0.00	6387_114	8.33	11085_104	1.67					1233_183	10.38	4770_62	0.94	8522_8	1.89	12415_14	0.00		
1407_36	1.67	6389_103	10.00	11086_18	1.67					1234_71	4.72	4771_79	3.77	8523_99	0.00	12418_84	4.72		
1409_24	8.33	6393_130	5.00	11089_96	3.33					1239_142	0.94	4772_35	11.32	8524_12	9.43	12420_58	11.32		
1414_133	0.00	6397_119	1.67	11100_164	0.00					1241_129	1.89	4773_56	0.94	8525_139	7.55	12424_106	11.32		
1416_66	15.00	6398_143	1.67	11104_13	3.33					1243_46	0.94	4774_42	0.00	8529_75	1.89	12426_103	0.00		
1419_162	1.67	6406_171	11.67	11109_55	3.33					1245_191	0.00	4778_84	0.00	8531_32	11.32	12428_169	8.49		
1420_83	8.33	6407_154	5.00	11118_159	0.00					1246_32	6.60	4779_63	2.83	8533_31	0.00	12429_130	10.38		
1423_69	11.67	6408_52	0.00	11121_45	0.00					1248_17	15.09	4786_103	0.94	8534_98	1.89	12430_85	7.55		
1424_30	0.00	6412_22	0.00	11122_29	1.67					1250_42	3.77	4787_12	3.77	8538_173	9.43	12431_87	6.60		
1426_71	8.33	6413_116	0.00	11123_27	8.33					1254_131	11.32	4789_32	0.00	8541_62	0.00	12436_110	0.94		
1431_82	1.67	6415_120	0.00	11125_58	0.00					1258_77	10.38	4791_89	6.60	8542_177	2.83	12437_58	6.60		
1440_100	0.00	6417_103	15.00	11126_27	0.00					1262_60	0.00	4794_9	0.00	8547_92	1.89	12438_72	2.83		
1443_72	5.00	6423_20	10.00	11137_152	0.00					1263_174	2.83	4795_138	2.83	8556_78	0.94	12447_82	0.00		
1444_102	1.67	6431_95	0.00	11140_13	0.00					1264_57	4.72	4796_17	11.32	8561_66	0.00	12461_69	4.72		
1445_144	11.67	6433_35	0.00	11141_13	6.67					1267_33	0.94	4812_83	10.38	8563_41	0.00	12473_118	6.60		
1449_23	0.00	6435_74	1.67	11142_103	3.33					1268_183	14.15	4813_142	5.66	8567_30	0.00	12475_107	0.94		
1453_122	0.00	6436_57	1.67	11149_124	5.00					1270_19	0.00	4815_12	6.60	8570_19	5.66	12493_104	7.55		
1456_84	0.00	6444_60	5.00	11150_67	3.33					1272_70	12.26	4817_82	7.55	8572_9	2.83	12504_81	11.32		
1458_43	1.67	6446_13	5.00	11154_92	1.67					1281_93	0.94	4819_145	0.94	8574_44	0.94	12508_49	0.94		
1468_82	3.33	6452_40	0.00	11156_106	0.00					1284_154	8.49	4820_157	0.00	8575_68	6.60	12515_84	7.55		
1470_154	0.00	6455_62	3.33	11159_91	8.33					1288_106	9.43	4821_25	0.94	8580_12	0.00	12521_75	0.00		
1481_90	1.67	6456_7	1.67	11162_94	25.00					1296_129	2.83	4825_152	1.89	8581_32	0.00	12525_104	0.00		
1484_36	3.33	6457_90	10.00	11168_95	0.00					1305_101	0.00	4826_11	1.89	8585_75	0.00	12528_93	7.55		
1487_27	0.00	6463_90	1.67	11172_135	11.67					1308_134	1.89	4829_97	9.43	8592_89	14.15	12538_71	0.94		
1493_107	6.67	6467_103	0.00	11176_50	0.00					1313_122	4.72	4835_151	1.89	8593_15	6.60	12541_84	0.00		
1494_10	3.33	6470_51	13.33	11181_113	0.00					1314_91	1.89	4836_39	4.72	8597_158	8.49	12543_50	4.72		
1495_91	0.00	6477_74	1.67	11185_72	8.33					1319_192	0.94	4840_57	0.94	8602_38	0.00	12544_35	6.60		
1496_110	0.00	6480_20	3.33	11189_78	0.00					1321_86	0.00	4853_39	0.94	8606_70	0.00	12547_89	2.83		
1501_103	5.00	6482_72	1.67	11190_37	3.33					1323_58	0.94	4861_85	0.00	8611_115	0.00	12553_24	0.00		
1502_27	1.67	6485_86	5.00	11195_53	0.00					1326_18	0.00	4872_152	8.49	8616_48	0.94	12557_35	0.94		

1503_16	3.33	6489_79	3.33	11197_144	5.00		1328_44	0.94	4874_8	13.21	8618_140	0.00	12568_61	1.89
1507_69	0.00	6491_29	11.67	11204_62	0.00		1330_13	0.00	4875_51	0.00	8619_96	0.94	12575_245	16.04
1517_47	0.00	6492_6	0.00	11206_45	1.67		1337_125	0.00	4877_121	2.83	8621_33	6.60	12577_89	7.55
1521_111	1.67	6496_7	3.33	11207_75	5.00		1339_52	0.00	4878_87	0.94	8624_15	1.89	12578_15	12.26
1523_39	0.00	6500_22	3.33	11208_85	5.00		1340_66	1.89	4879_137	0.00	8626_13	0.00	12580_13	12.26
1524_124	0.00	6501_63	10.00	11211_127	0.00		1348_115	10.38	4880_24	8.49	8628_98	0.00	12581_26	2.83
1526_83	0.00	6508_96	10.00	11213_47	1.67		1351_8	3.77	4881_30	0.00	8633_74	6.60	12584_29	0.94
1527_102	0.00	6512_107	3.33	11220_17	0.00		1357_147	0.00	4890_38	0.00	8637_72	0.00	12597_21	5.66
1528_41	0.00	6518_33	5.00	11225_125	0.00		1361_121	6.60	4895_82	1.89	8639_18	0.00	12601_48	4.72
1535_68	0.00	6521_31	0.00	11227_40	5.00		1362_7	0.00	4897_65	1.89	8640_151	0.00	12605_118	0.94
1538_25	0.00	6528_98	0.00	11234_35	0.00		1365_96	2.83	4898_74	6.60	8641_101	1.89	12610_157	1.89
1540_84	0.00	6533_90	6.67	11250_87	6.67		1368_59	10.38	4902_113	4.72	8642_86	0.94	12611_16	6.60
1542_42	0.00	6534_44	0.00	11251_31	0.00		1370_134	3.77	4903_92	9.43	8643_83	8.49	12612_31	0.00
1547_53	0.00	6536_86	0.00	11252_114	1.67		1371_90	6.60	4904_61	6.60	8648_78	0.94	12616_12	4.72
1551_13	0.00	6538_18	0.00	11254_57	8.33		1374_62	1.89	4909_42	8.49	8651_88	5.66	12617_19	7.55
1557_90	3.33	6543_117	0.00	11255_70	3.33		1378_155	4.72	4910_85	0.00	8652_35	0.94	12618_175	0.94
1570_101	5.00	6548_8	10.00	11256_7	11.67		1379_85	3.77	4911_110	1.89	8654_81	0.00	12624_172	5.66
1573_89	0.00	6550_194	10.00	11257_99	1.67		1380_118	2.83	4914_30	0.94	8663_72	0.00	12644_171	10.38
1574_96	3.33	6555_121	5.00	11259_120	0.00		1381_116	0.94	4915_33	1.89	8674_151	1.89	12650_60	8.49
1579_30	1.67	6565_57	1.67	11260_161	0.00		1382_160	1.89	4917_75	2.83	8679_49	0.94	12654_90	0.94
1582_78	15.00	6567_63	8.33	11271_73	3.33		1384_78	3.77	4922_129	0.94	8680_136	12.26	12658_185	8.49
1584_44	0.00	6568_55	0.00	11274_194	0.00		1386_55	5.66	4925_60	0.00	8686_90	10.38	12659_102	5.66
1586_98	0.00	6581_29	1.67	11278_100	1.67		1387_54	0.00	4932_175	14.15	8688_28	0.00	12670_166	4.72
1589_51	5.00	6582_5	5.00	11284_41	5.00		1392_129	0.00	4934_45	7.55	8694_150	0.00	12673_29	0.94
1590_136	0.00	6589_63	1.67	11286_91	1.67		1393_41	0.00	4936_45	10.38	8697_7	0.00	12674_91	0.00
1593_19	1.67	6595_136	8.33	11292_127	5.00		1394_32	8.49	4938_140	1.89	8702_128	0.00	12678_126	0.94
1595_150	1.67	6598_118	0.00	11293_111	0.00		1399_50	0.00	4941_122	1.89	8704_18	10.38	12680_32	14.15
1596_141	1.67	6603_123	18.33	11294_55	0.00		1403_8	0.94	4948_116	1.89	8706_22	1.89	12685_106	4.72
1600_24	0.00	6620_45	3.33	11295_9	1.67		1405_92	0.00	4949_71	1.89	8715_88	0.00	12692_104	1.89
1603_37	0.00	6621_90	3.33	11297_27	15.00		1407_36	0.94	4951_83	10.38	8719_97	1.89	12695_9	13.21
1606_97	6.67	6623_62	13.33	11298_127	0.00		1409_24	10.38	4952_82	11.32	8721_57	12.26	12700_84	1.89
1609_53	11.67	6625_120	1.67	11302_92	0.00		1414_133	0.00	4956_39	1.89	8723_133	10.38	12703_86	0.00
1610_51	3.33	6626_33	3.33	11304_10	0.00		1416_66	12.26	4957_25	0.00	8727_182	2.83	12721_13	10.38
1612_6	10.00	6628_104	0.00	11305_78	3.33		1419_162	0.94	4958_152	2.83	8729_65	0.00	12722_62	0.94
1615_155	0.00	6629_85	0.00	11307_54	3.33		1420_83	8.49	4961_139	1.89	8730_67	10.38	12724_83	10.38
1616_165	5.00	6636_160	0.00	11309_49	0.00		1423_69	11.32	4963_27	7.55	8731_77	0.00	12727_102	2.83
1618_120	0.00	6637_73	10.00	11310_67	1.67		1424_30	4.72	4964_14	15.09	8739_177	4.72	12731_57	6.60
1623_133	6.67	6639_46	1.67	11314_53	5.00		1426_71	10.38	4965_148	10.38	8746_40	3.77	12732_123	5.66
1625_66	0.00	6641_78	0.00	11320_142	11.67		1431_82	2.83	4976_99	0.94	8751_127	2.83	12743_93	0.00
1637_30	1.67	6642_39	8.33	11322_117	5.00		1438_92	3.77	4978_8	0.00	8753_33	0.94	12747_59	0.00
1642_16	3.33	6643_73	5.00	11329_20	0.00		1440_100	0.00	4980_185	0.00	8757_175	0.94	12748_130	1.89
1643_59	0.00	6654_73	1.67	11330_114	1.67		1441_54	9.43	4984_55	3.77	8759_177	3.77	12751_134	0.94
1644_53	0.00	6661_151	5.00	11331_35	6.67		1443_72	3.77	4985_129	2.83	8763_173	8.49	12753_133	10.38
1646_45	3.33	6662_130	0.00	11334_59	8.33		1444_102	0.94	4986_70	0.00	8767_81	0.00	12757_136	1.89
1649_26	0.00	6663_52	1.67	11335_156	5.00		1445_144	9.43	4990_77	7.55	8769_102	0.00	12763_69	3.77
1664_72	13.33	6664_83	0.00	11336_130	5.00		1449_23	0.00	4995_83	3.77	8770_11	0.00	12765_82	0.94

1668_46	0.00	6665_42	8.33	11339_120	18.33			1453_122	0.00	5000_16	1.89	8772_134	6.60	12769_14	6.60
1669_31	0.00	6666_151	8.33	11341_53	5.00			1456_84	0.00	5002_157	4.72	8777_29	5.66	12772_166	16.04
1673_57	0.00	6669_192	3.33	11342_146	1.67			1457_109	1.89	5005_51	0.94	8782_79	1.89	12774_138	2.83
1676_123	1.67	6684_104	0.00	11346_47	0.00			1458_43	0.94	5006_26	0.00	8785_5	0.94	12777_108	4.72
1680_122	1.67	6699_185	11.67	11348_38	3.33			1465_63	2.83	5007_73	4.72	8786_59	0.94	12784_44	6.60
1684_40	6.67	6701_10	0.00	11350_130	0.00			1468_82	2.83	5009_94	1.89	8790_46	6.60	12785_150	4.72
1688_93	0.00	6702_128	0.00	11352_10	1.67			1470_154	0.00	5012_112	12.26	8791_52	2.83	12791_104	5.66
1691_164	5.00	6704_16	0.00	11354_56	5.00			1481_90	0.94	5014_19	0.00	8803_46	8.49	12793_155	1.89
1692_31	10.00	6707_14	1.67	11355_37	5.00			1484_36	5.66	5015_65	1.89	8805_153	2.83	12796_5	1.89
1694_102	0.00	6708_76	1.67	11362_132	0.00			1487_27	0.00	5024_113	8.49	8809_38	1.89	12798_109	7.55
1697_92	0.00	6710_19	1.67	11363_36	1.67			1488_121	1.89	5026_133	4.72	8810_29	9.43	12804_19	0.94
1700_151	11.67	6713_132	0.00	11369_38	3.33			1493_107	6.60	5032_21	7.55	8812_38	15.09	12810_23	2.83
1701_165	1.67	6715_9	0.00	11372_80	5.00			1494_10	6.60	5032_21	2.83	8813_107	0.00	12811_156	0.00
1702_195	0.00	6717_35	1.67	11374_103	0.00			1495_91	0.00	5033_175	0.94	8814_101	3.77	12812_127	0.94
1706_23	0.00	6721_193	16.67	11378_153	3.33			1496_110	0.00	5037_121	3.77	8816_79	0.00	12815_72	10.38
1713_13	1.67	6722_6	0.00	11379_39	0.00			1501_103	8.49	5042_47	4.72	8817_7	2.83	12819_132	8.49
1714_56	0.00	6730_101	3.33	11381_17	1.67			1502_27	2.83	5045_67	0.00	8820_106	7.55	12833_175	0.94
1719_64	0.00	6733_17	10.00	11385_32	5.00			1503_16	1.89	5047_132	1.89	8821_33	0.00	12835_56	11.32
1721_140	1.67	6736_143	0.00	11387_147	0.00			1504_125	5.66	5049_32	7.55	8826_37	0.00	12846_60	8.49
1725_90	1.67	6742_106	1.67	11388_120	6.67			1507_69	0.00	5056_66	0.00	8829_46	0.00	12852_169	0.00
1727_99	0.00	6743_119	10.00	11399_28	0.00			1517_47	0.00	5057_63	14.15	8830_123	0.00	12856_59	0.94
1730_84	3.33	6749_87	13.33	11400_58	3.33			1521_111	0.94	5058_38	5.66	8832_116	0.00	12861_133	4.72
1734_82	0.00	6753_28	0.00	11401_140	1.67			1523_39	0.00	5060_24	4.72	8837_180	5.66	12863_147	3.77
1736_120	0.00	6754_23	1.67	11402_13	3.33			1524_124	2.83	5062_114	0.00	8838_125	10.38	12873_150	0.00
1742_113	6.67	6756_26	8.33	11405_29	0.00			1526_83	1.89	5063_14	0.94	8842_121	2.83	12885_65	5.66
1744_84	0.00	6767_79	0.00	11407_72	3.33			1527_102	0.00	5064_51	0.94	8848_67	0.00	12889_60	0.00
1749_101	10.00	6772_86	0.00	11410_54	10.00			1528_41	4.72	5069_58	14.15	8855_40	0.00	12892_64	3.77
1755_110	1.67	6775_53	10.00	11416_33	1.67			1535_68	0.00	5072_61	1.89	8857_42	1.89	12900_121	0.00
1759_72	3.33	6778_121	0.00	11418_110	1.67			1538_25	0.00	5073_5	0.00	8858_164	4.72	12908_22	5.66
1760_9	1.67	6779_70	5.00	11421_89	5.00			1540_84	0.00	5077_57	0.00	8868_44	7.55	12913_52	0.00
1767_47	3.33	6780_168	1.67	11426_15	10.00			1542_42	0.94	5078_183	9.43	8871_93	0.00	12918_26	3.77
1768_44	1.67	6790_62	0.00	11432_24	1.67			1547_53	0.00	5079_117	8.49	8872_82	0.00	12924_134	3.77
1776_68	0.00	6793_22	3.33	11434_75	3.33			1551_13	0.00	5082_63	0.00	8883_81	6.60	12927_192	8.49
1779_18	5.00	6798_109	0.00	11440_149	0.00			1556_141	4.72	5083_84	2.83	8887_101	0.00	12930_69	12.26
1780_59	1.67	6801_114	3.33	11444_164	3.33			1557_90	1.89	5085_19	2.83	8889_74	5.66	12935_76	0.00
1785_39	3.33	6809_44	1.67	11445_81	0.00			1559_38	0.00	5090_95	0.00	8890_66	0.00	12938_65	2.83
1786_158	6.67	6810_130	0.00	11447_36	0.00			1570_101	14.15	5096_194	4.72	8893_73	0.00	12941_118	0.94
1793_59	0.00	6811_100	1.67	11451_119	10.00			1573_89	0.00	5098_178	6.60	8894_138	0.00	12946_81	8.49
1794_8	10.00	6817_30	0.00	11456_25	5.00			1574_96	5.66	5099_48	4.72	8897_117	0.00	12947_142	0.94
1796_132	1.67	6818_13	0.00	11461_193	15.00			1579_30	0.94	5105_52	0.00	8898_22	9.43	12955_120	6.60
1797_28	0.00	6823_80	1.67	11465_161	3.33			1582_78	10.38	5108_79	12.26	8900_192	9.43	12969_122	6.60
1802_160	6.67	6825_60	3.33	11467_5	1.67			1583_138	0.00	5115_37	2.83	8901_40	0.00	12973_31	0.94
1806_92	5.00	6829_72	5.00	11472_81	0.00			1584_44	0.00	5116_17	1.89	8907_148	1.89	12981_5	0.94
1808_87	0.00	6832_120	1.67	11480_11	3.33			1586_98	0.00	5117_145	1.89	8908_169	12.26	12983_124	0.00
1809_38	3.33	6837_68	0.00	11484_71	0.00			1589_51	2.83	5130_36	1.89	8912_60	3.77	12988_71	8.49
1810_152	3.33	6839_89	0.00	11487_112	1.67			1590_136	0.00	5135_47	0.94	8915_90	0.94	12992_175	10.38

1813_59	0.00	6841_45	0.00	11488_150	1.67				1593_19	1.89	5138_135	5.66	8919_79	11.32	12994_15	0.00
1817_11	0.00	6844_41	0.00	11490_92	0.00				1595_150	1.89	5139_51	6.60	8923_57	0.94	12995_58	14.15
1822_97	1.67	6848_154	3.33	11493_45	1.67				1596_141	1.89	5141_194	8.49	8924_21	0.94	12999_124	0.94
1825_107	13.33	6866_75	5.00	11498_10	0.00				1599_105	8.49	5146_98	0.00	8925_106	7.55	13002_30	6.60
1827_54	0.00	6867_123	5.00	11501_64	0.00				1600_24	0.00	5149_70	6.60	8926_38	0.00	13004_8	6.60
1834_136	5.00	6877_17	8.33	11505_136	1.67				1603_37	0.00	5152_45	10.38	8929_103	5.66	13012_173	10.38
1846_75	5.00	6884_54	3.33	11511_164	5.00				1605_16	1.89	5153_43	0.00	8931_109	0.94	13018_75	0.00
1857_153	3.33	6885_178	5.00	11515_8	0.00				1606_97	11.32	5157_7	0.94	8932_168	1.89	13020_177	11.32
1858_60	5.00	6889_43	0.00	11516_76	0.00				1609_53	10.38	5161_91	8.49	8933_66	0.94	13024_115	4.72
1861_38	6.67	6897_84	3.33	11518_70	0.00				1610_51	5.66	5164_23	4.72	8936_65	9.43	13028_95	2.83
1862_8	5.00	6901_142	3.33	11520_39	10.00				1612_6	6.60	5182_42	0.94	8938_150	0.00	13034_36	0.00
1863_100	0.00	6908_115	11.67	11522_138	1.67				1615_155	0.00	5185_150	7.55	8943_80	0.94	13037_130	13.21
1865_90	6.67	6909_122	3.33	11536_169	8.33				1616_165	7.55	5188_13	3.77	8945_140	10.38	13039_142	13.21
1868_129	0.00	6914_153	13.33	11540_44	0.00				1618_120	0.94	5189_127	0.00	8946_48	6.60	13041_84	9.43
1870_89	0.00	6915_63	10.00	11542_126	20.00				1620_72	5.66	5190_40	2.83	8948_110	0.94	13044_43	5.66
1875_49	8.33	6920_187	1.67	11548_131	1.67				1623_133	3.77	5191_44	4.72	8952_109	9.43	13055_30	0.00
1878_51	8.33	6921_28	1.67	11551_121	10.00				1625_66	0.00	5203_9	5.66	8953_97	3.77	13057_145	2.83
1882_19	0.00	6923_46	0.00	11557_25	3.33				1637_30	1.89	5224_41	3.77	8955_116	1.89	13059_98	6.60
1883_70	6.67	6927_112	1.67	11561_14	6.67				1638_116	0.00	5257_155	6.60	8956_76	7.55	13060_30	12.26
1884_59	8.33	6936_6	0.00	11562_157	0.00				1642_16	1.89	5263_59	4.72	8959_39	7.55	13066_67	7.55
1887_67	8.33	6940_33	0.00	11564_113	1.67				1643_59	0.94	5273_174	7.55	8962_50	0.00	13068_194	10.38
1888_149	13.33	6942_80	0.00	11566_26	0.00				1644_53	0.94	5276_146	7.55	8967_42	12.26	13072_34	0.94
1892_85	10.00	6943_105	0.00	11569_35	1.67				1646_45	9.43	5307_54	2.83	8968_67	1.89	13085_192	2.83
1898_25	1.67	6944_35	1.67	11570_129	1.67				1649_26	0.00	5308_74	1.89	8970_16	9.43	13094_105	9.43
1900_78	0.00	6947_85	0.00	11573_26	0.00				1664_72	9.43	5341_50	1.89	8973_172	5.66	13096_126	0.00
1901_140	0.00	6951_65	1.67	11574_99	1.67				1668_46	0.00	5353_91	0.94	8976_18	0.00	13099_40	0.00
1902_103	0.00	6955_23	5.00	11579_54	3.33				1669_31	0.00	5355_92	0.00	8977_110	2.83	13101_41	10.38
1908_81	0.00	6956_36	1.67	11590_143	8.33				1673_57	0.00	5358_50	7.55	8978_159	10.38	13105_40	11.32
1909_38	0.00	6958_133	0.00	11593_150	1.67				1676_123	0.94	5360_69	4.72	8979_130	0.94	13106_128	11.32
1913_131	1.67	6961_73	0.00	11595_86	1.67				1678_44	13.21	5362_64	4.72	8982_139	6.60	13110_126	4.72
1924_10	11.67	6965_25	0.00	11603_98	3.33				1680_122	0.94	5367_154	12.26	8985_51	0.00	13111_107	4.72
1936_106	5.00	6967_124	1.67	11606_73	1.67				1684_40	7.55	5377_103	6.60	8987_103	1.89	13116_192	0.00
1939_85	0.00	6972_136	3.33	11611_10	1.67				1685_142	1.89	5382_5	0.94	8992_106	0.00	13119_46	2.83
1941_8	3.33	6981_95	0.00	11629_13	3.33				1688_93	1.89	5394_34	5.66	8995_110	10.38	13120_75	0.94
1942_168	8.33	6984_18	5.00	11633_110	1.67				1691_164	2.83	5400_65	6.60	8996_14	5.66	13128_19	0.00
1945_91	3.33	6990_142	1.67	11634_120	0.00				1692_31	11.32	5402_52	0.00	8998_130	0.00	13129_47	5.66
1951_157	1.67	6994_24	8.33	11636_124	0.00				1694_102	0.00	5407_27	4.72	9000_22	5.66	13133_131	6.60
1954_193	8.33	6995_152	0.00	11641_7	8.33				1697_92	0.00	5418_26	8.49	9003_150	1.89	13138_131	3.77
1960_15	10.00	6999_36	1.67	11643_61	1.67				1700_151	13.21	5424_122	5.66	9006_105	0.00	13144_78	4.72
1968_192	5.00	7000_56	5.00	11646_10	1.67				1701_165	7.55	5428_120	0.94	9008_10	0.00	13151_160	0.00
1976_101	0.00	7002_163	1.67	11657_149	0.00				1702_195	0.00	5429_53	0.00	9010_65	3.77	13152_7	0.00
1981_16	1.67	7012_98	1.67	11667_40	0.00				1706_23	0.00	5430_70	0.00	9013_22	9.43	13153_15	3.77
1983_110	0.00	7015_24	0.00	11669_27	3.33				1708_59	4.72	5436_50	0.00	9015_115	1.89	13159_101	4.72
1988_93	0.00	7016_163	0.00	11670_29	3.33				1713_13	0.94	5437_73	0.94	9016_22	7.55	13161_36	0.00
1990_50	3.33	7018_56	0.00	11686_120	1.67				1714_56	1.89	5442_44	7.55	9024_73	0.94	13162_133	0.94
1991_77	1.67	7019_59	1.67	11712_110	6.67				1719_64	0.00	5447_13	2.83	9033_83	0.94	13169_15	0.00

1993_36	0.00	7027_66	8.33	11717_80	0.00			1721_140	0.94	5450_10	0.00	9037_100	15.09	13170_22	0.00
1994_106	1.67	7028_16	6.67	11718_145	11.67			1725_99	1.89	5455_85	0.00	9040_127	4.72	13171_97	8.49
2002_146	5.00	7029_65	6.67	11738_7	3.33			1727_90	0.00	5468_117	0.94	9041_21	12.26	13178_5	0.00
2004_135	5.00	7033_97	6.67	11742_116	3.33			1730_84	5.66	5469_141	3.77	9043_41	0.00	13185_64	0.94
2006_72	1.67	7036_80	0.00	11768_147	1.67			1734_82	0.00	5478_92	0.94	9049_62	0.00	13188_109	0.00
2009_31	5.00	7037_105	3.33	11770_36	0.00			1736_120	0.00	5480_79	4.72	9058_81	1.89	13194_15	2.83
2014_97	5.00	7040_30	0.00	11773_72	1.67			1742_113	14.15	5484_63	1.89	9059_82	0.94	13195_25	0.00
2016_94	3.33	7043_58	1.67	11776_168	3.33			1744_84	4.72	5485_14	1.89	9061_95	0.00	13198_142	2.83
2019_7	0.00	7045_92	6.67	11783_135	1.67			1749_101	10.38	5489_94	0.00	9062_29	3.77	13202_73	4.72
2021_46	10.00	7052_68	1.67	11798_62	1.67			1755_110	0.94	5494_123	0.00	9065_115	0.00	13203_47	2.83
2026_14	3.33	7055_26	5.00	11806_56	6.67			1759_72	7.55	5496_55	0.94	9071_51	0.94	13204_97	0.94
2028_58	0.00	7058_75	1.67	11819_135	13.33			1760_9	0.94	5497_6	0.94	9072_126	11.32	13206_120	0.00
2029_141	5.00	7062_180	0.00	11822_72	1.67			1766_71	0.94	5498_122	0.94	9080_37	7.55	13221_92	0.94
2031_38	1.67	7063_18	1.67	11832_40	3.33			1767_47	2.83	5501_26	8.49	9083_132	3.77	13223_46	1.89
2035_33	3.33	7070_50	10.00	11837_120	5.00			1768_44	0.94	5504_81	3.77	9084_146	0.00	13224_89	0.00
2036_9	0.00	7072_172	0.00	11839_114	1.67			1776_68	0.00	5506_134	6.60	9091_69	0.00	13229_113	0.00
2041_37	6.67	7077_111	0.00	11842_121	0.00			1779_18	4.72	5510_51	13.21	9094_9	4.72	13248_46	12.26
2045_105	1.67	7080_92	6.67	11845_121	1.67			1780_59	1.89	5514_70	0.00	9095_54	0.00	13249_62	2.83
2047_19	0.00	7090_75	0.00	11849_7	0.00			1786_158	3.77	5528_119	0.94	9108_125	0.00	13259_177	2.83
2051_39	0.00	7093_73	1.67	11855_82	0.00			1793_59	7.55	5529_100	0.00	9109_18	0.00	13265_103	0.94
2053_128	0.00	7096_163	1.67	11857_125	1.67			1794_8	6.60	5530_182	8.49	9116_76	0.94	13271_33	2.83
2056_73	0.00	7100_149	1.67	11859_77	5.00			1796_132	6.60	5533_91	2.83	9119_96	2.83	13281_193	4.72
2057_10	0.00	7103_67	1.67	11864_64	1.67			1797_28	5.66	5536_78	4.72	9121_117	1.89	13282_116	3.77
2061_70	5.00	7110_148	0.00	11865_30	10.00			1802_160	4.72	5537_47	7.55	9124_180	19.81	13284_20	0.00
2064_166	5.00	7112_78	0.00	11866_119	3.33			1806_92	3.77	5541_111	0.00	9131_46	0.94	13287_101	1.89
2065_61	1.67	7116_116	0.00	11867_90	1.67			1808_87	0.00	5546_51	11.32	9134_69	7.55	13292_87	4.72
2070_122	1.67	7124_132	0.00	11873_51	3.33			1809_38	2.83	5555_24	8.49	9136_5	2.83	13295_164	5.66
2078_190	6.67	7126_23	8.33	11882_150	16.67			1810_152	1.89	5556_90	1.89	9139_47	5.66	13296_152	4.72
2081_114	0.00	7127_6	1.67	11883_90	3.33			1813_59	0.00	5560_81	0.00	9147_10	0.94	13303_113	3.77
2082_185	1.67	7139_59	3.33	11886_194	18.33			1815_14	0.94	5563_91	0.00	9150_22	0.00	13308_34	15.09
2086_127	11.67	7142_100	1.67	11896_137	10.00			1817_11	0.00	5564_40	0.94	9151_139	0.00	13312_112	1.89
2087_70	0.00	7145_141	3.33	11899_31	0.00			1819_184	6.60	5566_126	10.38	9152_8	0.00	13320_93	5.66
2090_22	0.00	7148_112	0.00	11910_32	5.00			1822_97	2.83	5572_62	2.83	9154_44	0.94	13322_172	2.83
2097_139	6.67	7149_29	11.67	11921_112	0.00			1825_107	9.43	5577_32	0.94	9158_115	0.94	13323_193	4.72
2099_101	1.67	7150_117	1.67	11923_112	6.67			1827_54	0.00	5579_60	3.77	9163_100	0.00	13328_140	0.94
2111_102	0.00	7151_60	0.00	11927_135	1.67			1833_106	0.94	5583_75	6.60	9169_111	12.26	13329_177	5.66
2116_131	0.00	7160_27	6.67	11929_40	1.67			1834_136	2.83	5584_26	0.94	9174_168	4.72	13336_59	1.89
2117_26	1.67	7163_168	0.00	11944_149	3.33			1842_25	0.00	5587_33	11.32	9176_13	5.66	13337_99	2.83
2124_55	1.67	7169_80	0.00	11952_157	6.67			1846_75	9.43	5591_29	9.43	9177_59	4.72	13342_141	3.77
2127_113	6.67	7170_28	1.67	11968_190	0.00			1855_67	0.94	5595_90	1.89	9179_68	7.55	13346_163	5.66
2131_84	0.00	7171_71	0.00	11969_85	0.00			1857_153	10.38	5604_21	0.00	9180_16	0.94	13348_60	0.00
2132_154	1.67	7173_102	0.00	11975_21	0.00			1858_60	5.66	5606_23	0.94	9183_22	1.89	13350_27	1.89
2139_67	0.00	7175_27	0.00	11978_89	8.33			1861_38	9.43	5612_119	5.66	9189_127	15.09	13352_57	6.60
2142_168	6.67	7188_106	1.67	11998_76	1.67			1862_8	4.72	5613_149	0.94	9194_122	1.89	13353_44	5.66
2144_80	23.33	7190_79	0.00	12017_107	1.67			1863_100	1.89	5618_45	0.00	9200_11	2.83	13363_64	9.43

2145_85	0.00	7194_54	3.33	12030_9	8.33					1865_90	9.43	5619_155	0.94	9201_41	0.94	13380_25	4.72	
2148_10	1.67	7199_139	1.67	12056_23	1.67					1868_129	0.00	5621_41	5.66	9202_107	0.00	13382_13	5.66	
2151_17	0.00	7204_43	0.00	12061_166	5.00					1870_89	7.55	5622_6	0.00	9204_133	3.77	13384_46	4.72	
2153_67	0.00	7206_144	0.00	12065_79	11.67					1875_49	6.60	5623_147	3.77	9207_158	0.94	13386_34	4.72	
2159_71	1.67	7215_150	0.00	12073_154	3.33					1878_51	9.43	5625_72	15.09	9210_78	11.32	13387_98	4.72	
2163_67	0.00	7218_73	1.67	12080_34	0.00					1881_56	0.00	5631_128	11.32	9212_122	0.94	13388_121	5.66	
2169_116	0.00	7221_157	3.33	12082_122	1.67					1882_19	0.00	5632_76	14.15	9216_120	0.00	13389_135	0.94	
2170_123	0.00	7224_11	0.00	12092_152	3.33					1883_70	5.66	5634_164	8.49	9219_113	0.94	13395_5	12.26	
2172_51	5.00	7226_83	0.00	12098_49	3.33					1884_59	10.38	5635_35	0.94	9222_19	0.94	13405_61	3.77	
2181_19	1.67	7227_137	0.00	12102_37	6.67					1886_116	13.21	5636_44	0.00	9224_64	10.38	13409_6	7.55	
2188_63	1.67	7229_82	3.33	12116_36	1.67					1887_67	8.49	5642_112	0.00	9225_95	5.66	13418_97	1.89	
2192_166	3.33	7231_43	1.67	12117_190	8.33					1888_149	13.21	5648_142	4.72	9226_57	4.72	13419_92	1.89	
2194_38	1.67	7233_166	11.67	12123_23	0.00					1892_85	6.60	5652_25	0.94	9228_145	2.83	13422_46	0.00	
2195_59	5.00	7234_56	1.67	12129_130	1.67					1898_25	1.89	5653_16	5.66	9237_66	0.00	13430_8	12.26	
2197_59	0.00	7236_71	1.67	12131_40	0.00					1900_78	2.83	5654_13	0.00	9241_76	2.83	13432_55	0.00	
2198_185	6.67	7241_17	8.33	12132_154	1.67					1901_140	0.00	5655_87	2.83	9242_26	4.72	13435_75	0.00	
2199_26	13.33	7243_107	0.00	12140_17	3.33					1902_103	0.94	5658_131	1.89	9253_48	0.94	13437_39	8.49	
2201_166	15.00	7251_167	6.67	12144_20	1.67					1907_102	0.00	5664_55	4.72	9264_103	0.00	13442_150	13.21	
2205_109	1.67	7253_115	0.00	12151_11	0.00					1908_81	0.00	5667_87	9.43	9265_15	16.98	13444_44	5.66	
2206_37	10.00	7255_145	3.33	12162_35	3.33					1909_38	0.00	5669_43	1.89	9266_38	0.94	13452_110	6.60	
2209_99	0.00	7260_32	1.67	12163_12	5.00					1913_131	0.94	5670_128	11.32	9267_95	9.43	13453_137	0.94	
2211_17	0.00	7261_60	1.67	12164_82	0.00					1924_10	12.26	5672_122	12.26	9269_105	3.77	13465_47	5.66	
2214_22	3.33	7265_58	0.00	12173_127	3.33					1934_122	0.00	5676_77	4.72	9276_162	7.55	13478_63	6.60	
2217_62	11.67	7271_138	0.00	12180_99	3.33					1936_106	6.60	5681_106	9.43	9277_11	2.83	13479_18	10.38	
2219_41	1.67	7275_109	6.67	12181_86	3.33					1937_81	9.43	5686_74	1.89	9278_17	10.38	13481_93	11.32	
2223_84	8.33	7281_103	3.33	12183_11	5.00					1939_85	0.00	5688_72	4.72	9279_112	0.00	13490_123	5.66	
2224_56	16.67	7292_128	0.00	12200_58	3.33					1941_8	1.89	5689_93	8.49	9288_64	1.89	13495_37	0.00	
2232_14	3.33	7293_88	0.00	12204_107	10.00					1942_168	7.55	5700_107	5.66	9289_80	5.66	13496_141	2.83	
2233_37	0.00	7294_192	1.67	12207_142	6.67					1945_91	3.77	5703_24	0.94	9290_62	0.00	13502_127	1.89	
2236_53	1.67	7297_60	0.00	12209_125	11.67					1951_157	0.94	5708_149	0.94	9291_68	4.72	13506_10	4.72	
2245_80	10.00	7302_47	1.67	12214_69	0.00					1954_193	7.55	5709_88	7.55	9293_113	15.09	13512_5	6.60	
2247_74	1.67	7309_21	1.67	12230_75	0.00					1960_15	12.26	5710_8	10.38	9296_136	0.00	13513_44	1.89	
2253_6	1.67	7310_162	0.00	12232_33	0.00					1968_192	7.55	5713_18	10.38	9298_8	6.60	13516_54	5.66	
2254_114	0.00	7318_81	1.67	12239_121	5.00					1969_117	6.60	5714_152	11.32	9300_93	0.94	13520_161	0.94	
2257_123	3.33	7320_28	0.00	12242_188	8.33					1976_101	0.00	5718_103	0.00	9301_125	0.00	13534_11	11.32	
2259_41	13.33	7322_116	11.67	12246_74	1.67					1981_16	0.94	5719_169	0.00	9305_25	7.55	13537_18	5.66	
2260_68	0.00	7325_50	0.00	12247_38	1.67					1983_110	0.00	5730_5	3.77	9308_95	0.00	13541_36	9.43	
2263_181	6.67	7331_32	5.00	12251_109	1.67					1988_93	11.32	5739_104	2.83	9309_170	9.43	13549_7	1.89	
2264_86	11.67	7338_157	1.67	12257_112	6.67					1990_50	8.49	5742_88	7.55	9310_18	12.26	13561_129	10.38	
2266_33	6.67	7342_77	1.67	12258_33	5.00					1991_77	1.89	5748_24	1.89	9319_148	0.00	13563_82	0.00	
2267_62	3.33	7343_79	5.00	12272_79	1.67					1993_36	0.00	5750_57	3.77	9324_62	3.77	13564_110	0.00	
2270_83	0.00	7344_10	3.33	12273_194	6.67					1994_106	0.94	5754_46	3.77	9325_109	0.94	13573_161	1.89	
2272_128	0.00	7345_124	1.67	12276_128	1.67					1996_129	0.00	5757_144	0.94	9326_125	2.83	13581_108	6.60	
2274_80	0.00	7351_52	0.00	12278_87	1.67					2002_146	8.49	5758_111	0.00	9328_128	0.00	13584_100	7.55	
2275_79	3.33	7356_75	10.00	12287_198	10.00					2004_135	4.72	5762_111	0.00	9331_59	0.00	13591_10	10.38	
2277_126	0.00	7359_9	1.67	12294_56	5.00					2006_72	2.83	5770_105	7.55	9336_56	0.00	13592_32	10.38	

2279_167	1.67	7360_143	1.67	12295_31	6.67					2009_31	4.72	5772_55	8.49	9338_14	6.60	13593_86	1.89
2281_78	5.00	7365_41	5.00	12297_175	8.33					2014_97	4.72	5777_21	3.77	9340_172	1.89	13596_41	0.94
2286_15	5.00	7366_135	0.00	12300_5	1.67					2016_94	2.83	5780_27	7.55	9342_46	19.81	13601_103	5.66
2295_61	1.67	7370_9	0.00	12303_64	18.33					2019_7	0.94	5788_69	0.00	9352_37	5.66	13602_151	10.38
2302_152	0.00	7372_79	0.00	12312_124	0.00					2021_7	0.00	5789_21	8.49	9357_99	3.77	13607_10	0.00
2303_14	3.33	7379_67	15.00	12325_147	0.00					2023_46	12.26	5790_87	6.60	9359_28	3.77	13608_63	0.00
2316_100	0.00	7384_33	0.00	12333_112	1.67					2026_14	2.83	5795_46	0.94	9361_75	0.00	13612_115	1.89
2319_79	1.67	7385_145	5.00	12346_92	13.33					2028_58	0.00	5796_88	8.49	9362_121	5.66	13618_60	4.72
2322_61	5.00	7386_31	0.00	12352_36	1.67					2029_141	3.77	5798_110	0.94	9363_145	8.49	13626_95	0.94
2323_102	1.67	7390_14	1.67	12355_48	3.33					2031_38	1.89	5802_79	0.94	9365_75	0.94	13627_85	3.77
2328_76	1.67	7396_44	0.00	12365_121	5.00					2035_33	1.89	5811_30	0.94	9372_9	0.94	13630_52	0.94
2331_47	1.67	7397_94	10.00	12369_165	6.67					2036_9	5.66	5815_101	0.00	9377_15	0.00	13635_78	3.77
2338_109	3.33	7398_68	0.00	12372_42	3.33					2038_97	1.89	5819_104	1.89	9382_23	0.94	13637_154	11.32
2341_150	5.00	7403_58	1.67	12377_121	0.00					2041_37	5.66	5826_161	7.55	9383_51	8.49	13646_41	4.72
2342_138	1.67	7406_107	3.33	12378_189	6.67					2045_105	2.83	5830_40	0.94	9392_114	1.89	13650_143	0.00
2347_189	8.33	7407_15	0.00	12382_83	5.00					2047_19	0.00	5833_11	10.38	9400_97	11.32	13653_135	1.89
2360_38	3.33	7408_28	0.00	12389_49	5.00					2051_39	3.77	5841_70	6.60	9404_177	6.60	13673_176	5.66
2364_126	11.67	7412_111	3.33	12394_93	1.67					2053_128	6.60	5842_121	0.94	9405_77	4.72	13677_26	11.32
2368_61	5.00	7413_65	1.67	12410_98	8.33					2056_73	0.00	5843_65	0.94	9409_74	3.77	13680_22	1.89
2377_137	8.33	7414_102	18.33	12411_146	6.67					2057_10	0.94	5844_37	2.83	9410_7	9.43	13681_100	0.94
2378_38	1.67	7428_16	1.67	12412_57	0.00					2061_70	4.72	5848_48	0.00	9414_57	7.55	13683_99	0.00
2380_100	0.00	7434_184	0.00	12415_14	0.00					2064_166	9.43	5858_61	2.83	9418_51	0.94	13684_64	9.43
2382_90	0.00	7437_79	0.00	12418_84	5.00					2065_61	0.94	5860_30	5.66	9419_180	9.43	13690_18	0.00
2385_14	0.00	7439_54	6.67	12420_58	10.00					2070_122	0.94	5866_98	0.00	9422_114	3.77	13696_48	2.83
2388_38	8.33	7441_32	0.00	12424_106	15.00					2072_49	8.49	5871_113	5.66	9425_7	4.72	13700_130	9.43
2400_156	1.67	7452_130	8.33	12428_169	1.67					2077_139	9.43	5873_90	0.00	9427_57	10.38	13702_109	4.72
2401_35	1.67	7457_7	8.33	12429_130	10.00					2078_190	4.72	5874_33	2.83	9430_10	0.00	13705_33	0.00
2402_68	0.00	7462_103	5.00	12430_85	8.33					2081_114	5.66	5880_116	3.77	9438_88	9.43	13708_14	0.00
2409_139	3.33	7465_45	8.33	12431_87	5.00					2082_185	0.94	5886_85	0.00	9442_86	3.77	13713_140	4.72
2416_46	0.00	7473_36	0.00	12436_110	0.00					2086_127	16.98	5891_9	2.83	9453_24	1.89	13714_129	1.89
2418_164	1.67	7474_165	5.00	12437_58	5.00					2087_70	0.00	5898_90	6.60	9456_50	2.83	13715_55	4.72
2422_78	0.00	7476_160	10.00	12438_72	3.33					2090_22	0.00	5899_33	2.83	9460_19	0.00	13718_116	6.60
2424_168	3.33	7478_72	15.00	12447_82	0.00					2097_139	4.72	5900_86	1.89	9461_41	1.89	13722_92	11.32
2425_176	13.33	7479_128	3.33	12461_69	1.67					2099_101	0.94	5901_48	0.94	9462_124	5.66	13726_21	10.38
2428_109	3.33	7487_41	0.00	12473_118	8.33					2111_102	0.94	5903_67	7.55	9470_146	2.83	13730_104	7.55
2429_30	0.00	7496_43	1.67	12475_107	1.67					2116_131	0.00	5904_72	11.32	9471_8	1.89	13735_89	1.89
2439_11	18.33	7500_159	8.33	12493_104	1.67					2117_26	1.89	5906_131	5.66	9472_35	7.55	13736_54	4.72
2445_74	0.00	7505_23	3.33	12504_81	11.67					2119_27	2.83	5907_54	1.89	9473_57	4.72	13737_84	5.66
2449_138	1.67	7506_139	0.00	12508_49	1.67					2124_55	0.94	5908_149	9.43	9476_10	0.94	13740_106	0.94
2453_61	15.00	7508_25	0.00	12515_84	6.67					2125_81	0.00	5911_62	0.94	9479_66	2.83	13745_110	4.72
2455_89	0.00	7512_131	1.67	12521_75	0.00					2127_113	8.49	5913_122	1.89	9481_115	1.89	13746_36	1.89
2458_12	3.33	7514_49	3.33	12525_104	0.00					2131_84	0.00	5923_62	6.60	9484_54	9.43	13748_73	0.00
2459_8	0.00	7515_23	3.33	12528_93	5.00					2132_154	0.94	5927_149	1.89	9489_165	1.89	13756_106	5.66
2465_104	3.33	7519_58	0.00	12538_71	1.67					2138_115	3.77	5930_142	8.49	9495_31	5.66	13757_54	10.38
2466_30	1.67	7524_87	1.67	12541_84	0.00					2139_67	0.94	5931_24	0.00	9496_43	0.94	13770_82	6.60
2470_41	0.00	7526_134	3.33	12544_35	6.67					2142_168	9.43	5932_91	13.21	9498_57	10.38	13784_202	6.60

2472_85	1.67	7528_145	0.00	12547_89	0.00			2144_80	14.15	5936_132	10.38	9501_107	0.94	13785_26	0.94
2475_132	11.67	7530_158	5.00	12553_24	0.00			2145_85	0.00	5938_80	1.89	9505_126	7.55	13786_27	4.72
2476_32	0.00	7532_107	0.00	12557_35	1.67			2148_10	2.83	5941_110	2.83	9506_55	5.66	13788_90	7.55
2477_47	13.33	7533_49	0.00	12568_61	1.67			2151_17	0.00	5944_64	7.55	9507_23	1.89	13789_71	3.77
2479_143	3.33	7545_137	5.00	12575_245	10.00			2153_67	0.00	5948_61	0.94	9508_133	0.94	13793_10	9.43
2482_56	0.00	7546_48	1.67	12577_89	6.67			2159_71	0.94	5949_88	4.72	9509_105	0.00	13797_153	1.89
2483_85	1.67	7553_139	0.00	12578_15	8.33			2162_108	4.72	5970_21	12.26	9510_87	4.72	13801_163	1.89
2485_46	5.00	7554_91	1.67	12580_13	15.00			2163_67	0.94	5973_74	4.72	9511_68	3.77	13802_42	0.00
2486_137	0.00	7557_62	15.00	12581_26	5.00			2169_116	0.00	5983_44	12.26	9512_126	2.83	13812_21	1.89
2487_106	0.00	7560_59	0.00	12584_29	0.00			2170_123	3.77	5986_86	13.21	9517_30	2.83	13815_91	6.60
2488_113	0.00	7565_121	1.67	12597_21	8.33			2172_51	7.55	5988_66	1.89	9519_75	0.00	13817_125	8.49
2490_59	8.33	7572_174	10.00	12601_48	6.67			2181_19	2.83	5990_17	0.00	9525_45	0.00	13823_83	1.89
2491_26	0.00	7573_13	8.33	12610_157	0.00			2188_63	3.77	5991_30	10.38	9526_105	2.83	13826_94	0.94
2493_20	0.00	7577_116	1.67	12611_16	8.33			2192_166	3.77	5992_151	5.66	9531_53	0.94	13828_111	4.72
2494_37	10.00	7581_144	3.33	12612_31	0.00			2194_38	2.83	5994_56	0.94	9536_95	0.94	13835_131	0.00
2502_20	11.67	7582_119	1.67	12617_19	6.67			2195_59	4.72	5995_67	1.89	9539_142	0.00	13837_110	12.26
2508_89	6.67	7583_127	3.33	12618_175	1.67			2197_59	0.00	5998_33	0.00	9541_11	1.89	13842_149	2.83
2515_47	0.00	7584_148	1.67	12624_172	6.67			2198_185	6.60	5999_26	3.77	9542_131	0.94	13849_8	2.83
2518_102	8.33	7589_105	1.67	12644_171	6.67			2199_26	10.38	6003_18	1.89	9544_7	7.55	13853_136	8.49
2523_97	0.00	7594_176	8.33	12650_60	8.33			2201_166	8.49	6010_162	0.94	9547_112	8.49	13855_87	0.00
2524_44	0.00	7596_50	0.00	12654_90	1.67			2205_109	1.89	6011_103	0.00	9550_163	10.38	13857_153	4.72
2525_64	6.67	7597_139	0.00	12658_185	3.33			2206_37	11.32	6012_37	0.00	9552_55	0.94	13871_63	4.72
2527_184	1.67	7599_7	1.67	12659_102	3.33			2207_54	0.00	6014_47	6.60	9555_169	0.94	13872_116	0.94
2534_107	1.67	7600_6	15.00	12670_166	6.67			2209_99	0.00	6017_90	3.77	9567_71	0.00	13877_114	0.94
2537_109	1.67	7603_81	0.00	12673_29	1.67			2211_17	0.00	6019_14	0.00	9568_95	3.77	13881_117	0.94
2542_58	1.67	7605_234	3.33	12674_91	0.00			2214_22	1.89	6021_6	4.72	9573_83	2.83	13884_96	1.89
2551_98	3.33	7607_41	0.00	12678_126	0.00			2217_62	6.60	6023_62	5.66	9576_12	10.38	13888_52	1.89
2553_63	0.00	7608_36	10.00	12680_32	25.00			2219_41	1.89	6025_33	3.77	9578_100	0.94	13892_42	9.43
2558_114	6.67	7609_159	1.67	12685_106	5.00			2223_84	4.72	6032_70	7.55	9581_138	8.49	13893_118	2.83
2560_108	1.67	7611_106	0.00	12692_104	0.00			2224_56	13.21	6033_57	9.43	9584_43	0.00	13918_19	0.00
2561_96	5.00	7615_190	1.67	12695_9	11.67			2231_84	2.83	6038_9	0.00	9585_28	6.60	13922_13	6.60
2563_18	1.67	7618_90	15.00	12700_84	1.67			2232_14	6.60	6039_68	0.00	9597_31	2.83	13930_59	1.89
2564_59	10.00	7622_158	0.00	12703_86	0.00			2233_37	0.94	6042_75	4.72	9598_99	0.00	13933_168	9.43
2569_132	3.33	7624_52	3.33	12721_13	10.00			2236_53	1.89	6043_25	0.00	9599_23	8.49	13936_43	3.77
2570_179	8.33	7625_51	0.00	12722_62	1.67			2237_68	2.83	6045_47	6.60	9600_66	0.00	13941_92	11.32
2572_47	1.67	7631_28	1.67	12727_102	3.33			2243_7	0.00	6046_46	7.55	9604_107	3.77	13944_48	0.00
2573_99	0.00	7633_88	1.67	12731_57	6.67			2245_80	8.49	6047_87	6.60	9609_70	3.77	13955_13	2.83
2574_129	0.00	7635_108	15.00	12732_123	5.00			2247_74	2.83	6049_74	5.66	9611_59	1.89	13959_111	0.00
2575_76	11.67	7636_68	10.00	12743_93	0.00			2253_6	0.94	6051_107	2.83	9615_63	8.49	13960_152	2.83
2577_125	0.00	7638_86	11.67	12747_59	0.00			2254_114	0.00	6055_168	0.94	9617_73	5.66	13965_34	14.15
2578_156	0.00	7642_24	1.67	12751_134	1.67			2257_123	3.77	6056_142	0.00	9619_71	5.66	13970_71	12.26
2580_81	0.00	7649_143	3.33	12757_136	1.67			2259_41	10.38	6058_83	5.66	9621_66	2.83	13973_50	0.00
2581_39	6.67	7650_155	5.00	12763_69	3.33			2260_68	0.00	6062_6	0.94	9623_61	2.83	13981_184	5.66
2584_122	1.67	7651_28	8.33	12772_166	16.67			2263_181	5.66	6069_131	0.94	9624_195	7.55	13988_14	11.32
2585_66	1.67	7653_81	1.67	12774_138	1.67			2264_86	13.21	6075_39	2.83	9625_14	0.00	13992_10	8.49
2590_73	6.67	7654_180	5.00	12777_108	0.00			2266_33	6.60	6081_17	0.00	9633_95	7.55	13993_27	0.94

2977_38	10.00	7992_84	5.00	13248_46	11.67					2551_98	3.77	6397_119	1.89	9894_119	0.00	14452_35	0.94	
2987_102	5.00	7995_101	6.67	13249_62	3.33					2553_63	0.00	6398_143	0.94	9896_46	0.00	14467_106	0.94	
2995_102	1.67	7997_9	5.00	13254_76	3.33					2556_73	3.77	6406_171	12.26	9898_45	0.94	14470_73	5.66	
3001_139	1.67	7998_183	8.33	13265_103	1.67					2558_114	3.77	6407_154	2.83	9900_24	2.83	14471_72	1.89	
3004_8	0.00	7999_48	5.00	13271_33	3.33					2560_108	0.94	6408_52	0.00	9904_71	1.89	14483_133	2.83	
3005_55	1.67	8000_144	0.00	13282_116	1.67					2561_96	12.26	6412_22	0.00	9906_43	0.94	14484_117	12.26	
3008_92	0.00	8001_93	6.67	13284_20	0.00					2563_18	0.94	6413_116	3.77	9911_95	2.83	14503_38	0.94	
3009_61	3.33	8003_71	1.67	13287_101	3.33					2564_59	6.60	6415_120	0.00	9915_122	9.43	14512_104	1.89	
3011_61	8.33	8007_188	8.33	13292_87	3.33					2565_185	0.94	6417_103	14.15	9916_148	9.43	14516_171	12.26	
3012_18	5.00	8013_109	1.67	13295_164	8.33					2567_87	3.77	6423_20	9.43	9924_105	0.00	14525_77	5.66	
3022_90	5.00	8015_53	0.00	13303_113	5.00					2569_132	6.60	6426_27	4.72	9925_24	0.94	14530_58	0.00	
3024_43	0.00	8026_114	0.00	13308_34	15.00					2570_179	6.60	6431_95	0.00	9937_137	6.60	14544_41	0.00	
3029_6	1.67	8030_82	0.00	13312_112	3.33					2572_47	1.89	6433_35	0.00	9938_98	15.09	14548_10	2.83	
3037_86	11.67	8040_118	0.00	13320_93	3.33					2573_99	3.77	6435_74	0.94	9944_71	6.60	14552_29	7.55	
3038_70	11.67	8044_26	1.67	13322_172	1.67					2574_129	0.00	6436_57	2.83	9947_101	2.83	14555_33	0.94	
3046_86	13.33	8048_5	0.00	13323_193	1.67					2575_76	9.43	6444_60	2.83	9951_173	5.66	14556_29	2.83	
3047_181	6.67	8055_96	0.00	13328_140	1.67					2577_125	0.94	6446_13	6.60	9952_5	5.66	14558_43	9.43	
3051_117	5.00	8056_30	0.00	13329_177	5.00					2578_156	0.00	6447_106	0.00	9954_44	1.89	14563_181	5.66	
3053_36	1.67	8059_82	3.33	13336_59	1.67					2580_81	0.94	6452_40	5.66	9962_158	0.00	14566_42	7.55	
3055_121	0.00	8061_117	1.67	13337_99	3.33					2581_39	12.26	6455_62	2.83	9963_125	1.89	14573_92	5.66	
3057_80	3.33	8065_19	1.67	13342_141	0.00					2584_122	0.94	6456_7	0.94	9967_68	2.83	14587_55	13.21	
3058_117	6.67	8066_51	8.33	13346_163	5.00					2585_66	7.55	6457_90	6.60	9969_35	13.21	14593_12	12.26	
3072_84	0.00	8067_94	6.67	13350_27	3.33					2590_73	14.15	6463_90	0.94	9972_85	4.72	14596_38	5.66	
3075_93	0.00	8068_30	5.00	13352_57	6.67					2591_87	8.49	6467_103	0.00	9973_88	5.66	14598_126	0.00	
3077_124	5.00	8069_130	0.00	13353_44	6.67					2593_134	5.66	6470_51	10.38	9975_117	0.00	14599_179	6.60	
3085_159	1.67	8073_158	5.00	13363_64	8.33					2596_169	9.43	6477_74	0.94	9976_20	0.94	14603_100	0.94	
3088_50	6.67	8074_21	1.67	13380_25	3.33					2599_17	0.00	6480_20	2.83	9977_134	0.00	14607_90	0.94	
3093_51	1.67	8076_76	5.00	13382_13	0.00					2604_98	0.94	6482_72	0.94	9980_80	0.00	14610_62	0.00	
3100_52	5.00	8077_29	10.00	13384_46	5.00					2608_85	0.00	6485_86	3.77	9988_117	11.32	14613_152	0.94	
3102_40	0.00	8078_106	0.00	13386_34	3.33					2612_38	0.00	6489_79	4.72	9990_85	0.00	14619_112	11.32	
3109_23	1.67	8081_86	6.67	13388_121	5.00					2616_23	4.72	6491_29	13.21	9991_96	13.21	14621_118	7.55	
3110_79	5.00	8083_46	0.00	13389_135	0.00					2621_18	0.94	6492_6	0.00	9992_151	0.00	14624_52	12.26	
3111_29	5.00	8084_67	10.00	13395_5	13.33					2623_78	0.94	6494_117	0.94	9993_109	10.38	14628_103	6.60	
3116_128	5.00	8085_129	5.00	13405_61	3.33					2626_56	0.00	6496_7	10.38	9994_155	0.94	14633_138	0.94	
3118_152	11.67	8090_82	0.00	13409_6	1.67					2631_90	0.00	6500_22	2.83	9997_40	2.83	14639_143	9.43	
3121_128	8.33	8091_30	6.67	13418_97	1.67					2641_88	2.83	6501_63	10.38	10002_65	2.83	14640_175	0.00	
3127_91	3.33	8096_86	0.00	13419_92	1.67					2645_53	0.00	6508_96	10.38	10006_165	2.83	14641_109	4.72	
3128_53	8.33	8097_193	3.33	13422_46	0.00					2664_15	0.00	6510_120	5.66	10008_105	4.72	14648_140	4.72	
3129_39	11.67	8101_146	1.67	13432_55	0.00					2666_15	13.21	6512_107	4.72	10012_72	3.77	14653_90	9.43	
3130_67	0.00	8103_69	0.00	13435_75	0.00					2678_50	6.60	6518_33	7.55	10014_159	0.00	14658_112	7.55	
3131_134	8.33	8107_39	13.33	13442_150	15.00					2680_58	0.00	6521_31	0.00	10015_125	12.26	14659_108	6.60	
3137_74	3.33	8111_77	0.00	13444_44	6.67					2681_73	0.94	6528_98	0.00	10016_50	1.89	14663_90	2.83	
3138_91	1.67	8119_160	3.33	13452_110	5.00					2683_90	10.38	6533_90	4.72	10021_74	11.32	14667_107	0.00	
3140_88	0.00	8120_79	1.67	13453_137	1.67					2684_103	0.94	6534_44	0.00	10022_63	2.83	14676_32	3.77	
3141_59	1.67	8121_54	3.33	13465_47	0.00					2685_49	1.89	6536_86	0.00	10023_46	7.55	14681_101	4.72	
3145_60	1.67	8130_29	5.00	13478_63	6.67					2689_17	0.94	6538_18	0.00	10025_172	2.83	14688_43	4.72	

3146_113	8.33	8.33	8133_82	6.67	13479_18	6.67				2690_79	0.94	6543_117	1.89	10027_127	4.72	14693_88	0.00
3147_143	1.67	8136_13	3.33	13481_93	8.33					2691_88	9.43	6548_8	5.66	10029_132	4.72	14695_151	4.72
3149_125	1.67	8139_111	8.33	13490_123	5.00					2692_58	7.55	6550_194	9.43	10030_36	5.66	14698_16	8.49
3150_58	5.00	8140_22	3.33	13495_37	0.00					2693_120	0.00	6552_137	0.94	10036_70	0.00	14701_88	4.72
3152_86	1.67	8143_190	1.67	13496_141	1.67					2695_38	0.94	6555_121	3.77	10037_35	0.00	14703_132	0.00
3153_62	1.67	8145_52	0.00	13502_127	3.33					2696_87	6.60	6564_105	0.00	10038_29	11.32	14704_39	10.38
3157_140	8.33	8149_14	3.33	13506_10	0.00					2697_164	0.00	6565_57	0.94	10051_34	11.32	14713_64	0.00
3159_146	6.67	8150_188	3.33	13512_5	5.00					2698_169	11.32	6567_63	9.43	10056_153	2.83	14720_55	8.49
3168_75	1.67	8155_32	15.00	13513_44	1.67					2699_113	0.00	6568_55	7.55	10058_121	0.94	14722_129	1.89
3174_18	10.00	8158_79	1.67	13516_54	6.67					2703_89	3.77	6572_117	0.00	10059_151	0.00	14729_61	1.89
3175_144	0.00	8165_110	3.33	13520_161	1.67					2704_86	0.94	6581_29	6.60	10060_31	2.83	14734_159	9.43
3177_51	8.33	8166_56	10.00	13534_11	10.00					2707_118	0.00	6582_5	9.43	10063_9	10.38	14738_43	9.43
3181_103	0.00	8168_61	1.67	13537_18	1.67					2718_62	13.21	6589_63	0.94	10064_82	2.83	14739_76	3.77
3182_111	6.67	8170_148	0.00	13541_36	8.33					2723_168	6.60	6591_36	6.60	10067_37	8.49	14747_69	6.60
3186_101	8.33	8171_41	1.67	13549_7	0.00					2724_134	5.66	6595_136	7.55	10070_36	5.66	14756_85	4.72
3187_100	0.00	8175_74	3.33	13561_129	6.67					2727_37	3.77	6598_118	0.00	10072_152	11.32	14757_131	2.83
3188_138	5.00	8176_130	1.67	13563_82	0.00					2730_104	2.83	6599_76	2.83	10075_111	0.00	14769_67	10.38
3193_53	1.67	8177_86	0.00	13564_110	0.00					2731_183	12.26	6603_123	12.26	10081_9	0.94	14773_113	4.72
3194_118	6.67	8179_23	1.67	13581_108	5.00					2732_7	0.94	6620_45	5.66	10085_51	10.38	14776_75	0.00
3200_13	0.00	8182_24	3.33	13584_100	5.00					2744_91	3.77	6621_90	2.83	10087_77	5.66	14783_107	0.94
3201_172	1.67	8188_95	3.33	13591_10	16.67					2748_25	2.83	6623_62	7.55	10089_158	0.94	14787_36	2.83
3203_86	1.67	8190_47	11.67	13592_32	3.33					2750_63	6.60	6625_120	3.77	10094_73	2.83	14795_64	3.77
3209_143	10.00	8193_62	1.67	13593_86	1.67					2754_36	10.38	6626_33	3.77	10095_145	5.66	14797_99	0.94
3212_67	0.00	8196_123	1.67	13601_103	5.00					2756_15	12.26	6628_104	0.94	10101_23	6.60	14798_88	0.94
3214_20	0.00	8197_103	3.33	13602_151	11.67					2759_106	9.43	6629_85	0.00	10107_174	3.77	14799_82	0.94
3216_73	0.00	8198_157	0.00	13607_10	0.00					2760_16	5.66	6635_41	3.77	10109_97	0.00	14800_124	10.38
3228_108	0.00	8201_92	0.00	13608_63	0.00					2766_164	0.94	6636_160	0.00	10112_17	0.00	14808_120	0.00
3231_10	11.67	8206_115	1.67	13612_115	3.33					2767_47	0.94	6637_73	12.26	10117_49	4.72	14809_71	9.43
3233_76	0.00	8212_69	0.00	13618_60	5.00					2769_76	2.83	6639_46	0.94	10119_45	5.66	14812_112	12.26
3238_81	10.00	8213_16	0.00	13626_95	1.67					2771_115	8.49	6640_9	12.26	10120_65	0.00	14813_170	7.55
3239_21	1.67	8214_139	1.67	13627_85	1.67					2772_125	10.38	6641_78	0.00	10128_50	3.77	14826_115	0.00
3241_54	1.67	8215_37	3.33	13630_52	1.67					2777_21	1.89	6642_39	7.55	10131_123	0.94	14828_112	0.00
3243_110	6.67	8218_140	0.00	13635_78	3.33					2786_14	8.49	6643_73	4.72	10134_52	6.60	14838_143	1.89
3248_9	0.00	8219_64	5.00	13637_154	13.33					2790_86	5.66	6654_73	2.83	10135_136	6.60	14840_118	2.83
3251_112	0.00	8220_37	0.00	13646_41	5.00					2792_11	14.15	6661_151	4.72	10138_58	5.66	14841_92	3.77
3252_166	0.00	8227_57	8.33	13653_135	0.00					2796_51	4.72	6662_130	0.00	10144_61	4.72	14844_158	4.72
3254_151	8.33	8229_26	5.00	13673_176	5.00					2798_91	0.94	6663_52	1.89	10147_113	0.00	14845_101	6.60
3259_81	5.00	8238_13	0.00	13677_26	16.67					2807_18	4.72	6664_83	0.00	10148_9	0.00	14853_51	0.94
3263_9	0.00	8239_20	1.67	13680_22	0.00					2813_13	4.72	6665_42	6.60	10150_36	5.66	14856_81	2.83
3265_49	0.00	8240_41	0.00	13681_100	1.67					2815_74	1.89	6666_151	11.32	10153_71	0.94	14864_113	2.83
3266_172	3.33	8242_99	1.67	13683_99	0.00					2817_30	6.60	6669_192	7.55	10154_107	7.55	14866_28	0.94
3273_50	5.00	8244_148	1.67	13684_64	6.67					2818_22	3.77	6677_93	0.00	10156_116	4.72	14872_115	6.60
3275_14	3.33	8246_72	0.00	13690_18	0.00					2819_53	4.72	6684_104	0.00	10157_55	0.00	14876_64	3.77
3280_85	1.67	8247_74	0.00	13696_48	0.00					2821_64	0.00	6691_98	6.60	10159_52	0.00	14878_138	10.38
3285_109	3.33	8255_132	3.33	13700_130	8.33					2822_133	0.00	6695_82	4.72	10165_53	1.89	14886_49	4.72
3287_63	1.67	8259_17	1.67	13702_109	6.67					2826_194	1.89	6699_185	11.32	10166_139	1.89	14897_127	7.55

3291_13	5.00	8260_99	5.00	13705_33	0.00			2828_123	0.00	6701_10	9.43	10175_43	1.89	14898_60	0.00
3299_18	6.67	8261_141	6.67	13708_14	0.00			2831_56	0.94	6702_128	1.89	10178_12	6.60	14905_27	0.00
3304_98	1.67	8264_100	0.00	13713_140	1.67			2834_117	0.00	6704_16	0.00	10182_65	5.66	14909_89	11.32
3307_58	5.00	8267_179	3.33	13714_129	3.33			2839_138	1.89	6705_112	4.72	10190_8	2.83	14921_190	8.49
3308_52	3.33	8273_51	0.00	13715_55	1.67			2841_15	0.00	6707_14	6.60	10193_21	0.94	14928_10	2.83
3311_86	10.00	8276_35	1.67	13718_116	8.33			2854_93	4.72	6708_76	0.94	10194_87	0.00	14929_10	3.77
3314_116	5.00	8277_83	0.00	13722_92	8.33			2859_113	1.89	6710_19	1.89	10196_131	6.60	14939_141	0.94
3321_25	6.67	8279_43	0.00	13726_21	5.00			2866_45	0.00	6713_132	0.00	10198_151	0.00	14941_116	7.55
3324_171	3.33	8288_10	3.33	13730_104	3.33			2873_95	9.43	6715_9	0.94	10199_88	8.49	14958_158	7.55
3325_132	1.67	8290_159	13.33	13735_89	3.33			2882_115	5.66	6717_35	0.94	10201_171	4.72	14972_5	1.89
3329_24	0.00	8291_125	0.00	13736_54	5.00			2884_172	1.89	6721_193	14.15	10206_12	0.94	14979_156	10.38
3331_12	0.00	8296_96	1.67	13737_84	1.67			2887_109	3.77	6722_6	0.00	10208_28	13.21	14984_9	2.83
3336_18	0.00	8297_102	0.00	13740_106	1.67			2888_14	0.94	6729_107	1.89	10214_98	4.72	14985_111	0.00
3339_40	3.33	8301_187	0.00	13745_110	3.33			2889_53	3.77	6730_101	5.66	10215_10	6.60	14989_117	9.43
3340_58	3.33	8303_54	0.00	13746_36	3.33			2908_68	0.00	6733_17	12.26	10220_13	7.55	14991_46	6.60
3341_38	6.67	8312_36	1.67	13756_106	5.00			2911_151	3.77	6736_143	0.00	10223_38	0.00	15001_133	0.00
3342_55	1.67	8313_111	0.00	13770_82	6.67			2913_128	1.89	6742_106	1.89	10226_84	0.94	15006_10	5.66
3350_102	3.33	8317_116	6.67	13785_26	1.67			2917_145	7.55	6743_119	6.60	10227_131	11.32	15008_50	0.00
3357_17	3.33	8318_55	1.67	13786_27	1.67			2918_148	0.94	6749_87	8.49	10229_59	7.55	15021_5	0.94
3362_85	0.00	8322_119	3.33	13788_90	8.33			2922_50	0.94	6753_28	0.94	10230_120	9.43	15025_28	11.32
3365_39	1.67	8324_64	11.67	13789_71	6.67			2924_128	0.00	6754_23	5.66	10231_72	7.55	15029_99	9.43
3366_130	3.33	8329_177	5.00	13793_10	3.33			2925_8	0.00	6756_26	7.55	10232_95	0.94	15040_142	9.43
3369_128	0.00	8335_6	0.00	13797_153	0.00			2928_31	0.00	6767_79	0.00	10233_112	0.00	15046_103	3.77
3370_117	3.33	8338_37	1.67	13801_163	0.00			2940_125	4.72	6772_86	0.00	10237_34	0.94	15052_119	1.89
3371_88	0.00	8339_98	0.00	13802_42	0.00			2943_5	0.94	6774_158	3.77	10240_62	0.94	15053_38	1.89
3372_183	11.67	8344_42	0.00	13812_21	3.33			2944_103	0.00	6775_53	12.26	10243_141	0.94	15060_58	0.00
3381_91	10.00	8345_120	1.67	13815_91	8.33			2948_164	4.72	6778_121	0.00	10244_87	0.00	15068_153	5.66
3382_159	0.00	8348_136	0.00	13823_83	3.33			2951_57	4.72	6779_70	10.38	10245_80	0.00	15075_33	11.32
3383_110	5.00	8353_119	0.00	13826_94	0.00			2953_99	0.94	6780_168	1.89	10247_52	2.83	15086_18	5.66
3385_37	1.67	8354_110	0.00	13828_111	1.67			2957_76	2.83	6790_62	0.00	10248_19	5.66	15090_186	5.66
3389_34	0.00	8366_68	3.33	13835_131	0.00			2959_122	13.21	6793_22	1.89	10249_123	3.77	15095_49	1.89
3401_89	0.00	8377_54	0.00	13837_110	11.67			2963_22	7.55	6795_144	0.00	10251_155	7.55	15114_56	6.60
3405_21	0.00	8380_107	0.00	13842_149	3.33			2965_85	0.00	6796_112	0.00	10257_46	0.00	15115_137	0.94
3409_86	1.67	8382_22	3.33	13849_8	0.00			2970_143	9.43	6798_109	0.00	10259_8	0.94	15120_7	3.77
3412_159	13.33	8383_94	0.00	13853_136	8.33			2971_81	1.89	6801_114	1.89	10264_121	1.89	15123_61	0.00
3413_10	0.00	8384_108	8.33	13855_87	0.00			2972_60	7.55	6806_96	9.43	10265_97	0.00	15135_178	4.72
3415_52	3.33	8385_64	0.00	13857_153	6.67			2973_20	0.00	6809_44	0.94	10270_107	0.94	15158_87	0.94
3416_27	6.67	8392_31	8.33	13871_63	1.67			2977_38	5.66	6810_130	2.83	10272_125	8.49	15160_133	4.72
3417_40	0.00	8394_26	0.00	13872_116	1.67			2983_132	11.32	6811_100	0.94	10273_24	1.89	15166_34	0.00
3420_21	0.00	8396_28	0.00	13881_117	1.67			2987_122	8.49	6817_30	0.94	10277_70	1.89	15170_90	5.66
3427_14	0.00	8399_44	5.00	13884_96	3.33			2995_102	0.94	6818_13	0.00	10278_93	0.00	15172_142	3.77
3432_85	10.00	8401_83	0.00	13888_52	1.67			3001_139	7.55	6823_80	3.77	10280_75	0.00	15174_80	7.55
3439_66	3.33	8402_5	0.00	13892_42	11.67			3004_8	0.00	6825_60	4.72	10281_29	0.94	15179_91	6.60
3446_38	3.33	8403_51	3.33	13893_118	5.00			3005_55	0.94	6829_72	2.83	10283_32	0.00	15180_107	3.77
3447_55	6.67	8408_139	6.67	13918_19	0.00			3008_92	0.00	6830_76	0.00	10286_86	0.00	15185_107	0.00
3449_15	3.33	8412_152	1.67	13922_13	5.00			3009_61	6.60	6832_120	1.89	10288_103	4.72	15206_48	1.89

3453_61	5.00	8417_47	10.00	13930_59	0.00			3011_61	4.72	6834_168	2.83	10289_46	2.83	15207_31	0.00
3456_82	3.33	8427_95	1.67	13933_168	1.67			3012_18	14.15	6837_68	0.00	10292_161	3.77	15209_157	5.66
3459_129	16.67	8430_8	5.00	13936_43	1.67			3022_90	14.15	6839_89	0.00	10296_75	1.89	15212_95	0.94
3460_156	3.33	8440_136	8.33	13941_92	13.33			3023_77	11.32	6841_45	1.89	10297_38	12.26	15217_137	7.55
3465_53	0.00	8452_172	1.67	13944_48	0.00			3024_43	0.00	6844_41	0.00	10298_116	0.00	15219_149	7.55
3468_45	1.67	8460_13	3.33	13955_13	3.33			3025_41	7.55	6848_154	7.55	10299_111	10.38	15222_62	2.83
3486_133	0.00	8461_52	1.67	13959_111	0.00			3029_6	0.94	6866_75	5.66	10302_15	0.00	15226_101	3.77
3498_126	0.00	8462_42	0.00	13960_152	1.67			3032_64	5.66	6867_123	7.55	10304_24	5.66	15228_164	7.55
3504_100	5.00	8467_9	0.00	13965_34	5.00			3037_86	10.38	6877_17	6.60	10308_48	2.83	15229_186	7.55
3510_66	0.00	8468_116	1.67	13973_50	0.00			3038_70	10.38	6884_54	1.89	10309_69	1.89	15231_95	4.72
3512_155	13.33	8470_105	0.00	13981_184	8.33			3046_86	9.43	6885_178	2.83	10317_68	0.00	15233_66	2.83
3522_16	0.00	8479_139	0.00	13988_14	8.33			3047_181	10.38	6889_43	2.83	10318_57	0.00	15259_163	4.72
3523_98	16.67	8483_105	5.00	13992_10	0.00			3051_117	7.55	6897_84	3.77	10320_148	9.43	15261_146	5.66
3525_82	5.00	8486_78	18.33	13993_27	1.67			3052_45	9.43	6900_65	0.00	10322_44	0.00	15262_23	12.26
3530_61	11.67	8488_85	0.00	13995_19	1.67			3053_36	0.94	6901_142	3.77	10323_57	0.00	15266_130	10.38
3534_91	8.33	8490_96	0.00	14003_10	0.00			3055_121	0.00	6908_115	7.55	10324_138	7.55	15275_29	0.00
3536_72	3.33	8493_142	6.67	14018_97	8.33			3057_80	1.89	6909_122	3.77	10325_86	0.94	15276_22	8.49
3543_46	1.67	8495_20	1.67	14023_100	5.00			3058_117	4.72	6914_153	9.43	10326_53	0.00	15278_63	1.89
3544_22	1.67	8499_168	0.00	14030_65	5.00			3072_84	1.89	6915_63	15.09	10327_146	0.94	15282_146	1.89
3545_55	3.33	8501_63	0.00	14033_129	3.33			3074_74	0.94	6920_187	4.72	10329_89	0.00	15288_66	0.94
3546_71	3.33	8504_38	0.00	14042_34	0.00			3075_93	0.94	6921_28	1.89	10337_158	13.21	15296_117	0.00
3550_174	5.00	8505_56	6.67	14046_84	6.67			3077_124	2.83	6923_46	0.94	10339_128	7.55	15299_35	6.60
3554_88	13.33	8507_73	0.00	14054_146	13.33			3083_18	0.94	6927_112	0.94	10341_132	6.60	15302_66	0.00
3555_31	0.00	8511_135	0.00	14059_89	11.67			3085_159	1.89	6936_6	0.00	10343_76	1.89	15306_41	8.49
3560_151	6.67	8512_71	3.33	14060_104	8.33			3088_50	4.72	6940_33	0.00	10344_7	5.66	15313_71	12.26
3562_110	0.00	8517_34	10.00	14061_193	11.67			3093_51	4.72	6942_80	0.00	10346_104	0.00	15319_34	7.55
3564_112	1.67	8520_22	8.33	14066_100	8.33			3100_52	7.55	6943_105	1.89	10349_48	12.26	15322_109	0.00
3566_7	1.67	8523_99	0.00	14068_34	0.00			3102_40	0.00	6944_55	0.94	10356_149	5.66	15323_10	12.26
3567_19	0.00	8524_12	11.67	14076_143	0.00			3109_23	8.49	6947_85	0.00	10360_119	3.77	15324_19	0.00
3569_70	0.00	8525_139	8.33	14077_57	10.00			3110_79	0.94	6951_65	1.89	10362_76	2.83	15335_21	3.77
3574_123	0.00	8533_31	0.00	14080_113	0.00			3111_29	10.38	6955_23	6.60	10367_82	12.26	15340_105	4.72
3577_52	3.33	8538_173	6.67	14082_24	3.33			3114_24	0.00	6956_56	2.83	10370_111	1.89	15341_124	0.94
3578_93	5.00	8541_62	0.00	14098_14	6.67			3116_128	4.72	6958_133	0.00	10373_38	1.89	15344_108	3.77
3582_71	1.67	8542_177	0.00	14101_36	5.00			3118_152	11.32	6961_73	0.00	10379_92	6.60	15349_185	2.83
3587_132	1.67	8547_92	1.67	14105_19	3.33			3121_128	8.49	6964_134	1.89	10383_72	0.00	15358_33	2.83
3595_103	6.67	8556_78	1.67	14113_42	3.33			3127_91	1.89	6965_25	3.77	10385_26	11.32	15361_38	12.26
3598_158	1.67	8563_41	0.00	14118_120	1.67			3128_53	8.49	6967_124	0.94	10386_80	3.77	15362_53	0.00
3600_111	11.67	8567_30	0.00	14122_180	10.00			3129_39	8.49	6972_136	5.66	10388_106	0.00	15369_100	0.94
3605_97	8.33	8570_19	6.67	14123_74	3.33			3130_67	4.72	6981_95	0.00	10390_122	0.00	15382_43	0.94
3611_83	1.67	8572_9	1.67	14128_151	0.00			3131_134	6.60	6984_18	4.72	10393_34	12.26	15383_102	1.89
3613_133	0.00	8574_44	0.00	14133_121	0.00			3137_74	1.89	6990_142	4.72	10394_65	0.00	15384_170	0.00
3614_37	0.00	8575_68	8.33	14138_10	8.33			3138_91	1.89	6994_24	8.49	10402_172	10.38	15386_124	3.77
3619_51	1.67	8580_12	0.00	14140_160	1.67			3140_88	0.94	6995_152	1.89	10407_92	5.66	15389_45	4.72
3622_145	3.33	8585_75	0.00	14144_175	1.67			3141_59	0.94	6999_36	0.94	10410_136	12.26	15392_185	8.49
3626_156	3.33	8592_89	8.33	14146_138	0.00			3145_60	0.94	7000_56	8.49	10412_182	7.55	15393_85	10.38
3627_90	0.00	8597_158	11.67	14150_109	0.00			3146_133	10.38	7002_163	4.72	10414_68	1.89	15407_159	8.49

3632_62	8.33	8602_38	0.00	14152_88	3.33			3147_143	0.94	7012_98	1.89	10416_76	0.94	15409_65	2.83	
3634_82	1.67	8611_115	0.00	14165_192	6.67			3149_125	0.94	7015_24	1.89	10418_8	8.49	15419_97	5.66	
3636_69	3.33	8616_48	0.00	14174_58	6.67			3150_58	4.72	7016_163	0.00	10419_13	2.83	15434_168	11.32	
3637_138	3.33	8619_96	1.67	14175_165	0.00			3152_86	0.94	7018_56	0.00	10421_177	8.49	15436_136	14.15	
3641_10	10.00	8621_33	1.67	14188_108	13.33			3153_62	3.77	7019_59	1.89	10425_78	0.00	15438_25	9.43	
3648_14	3.33	8624_15	1.67	14209_69	1.67			3157_140	11.32	7023_16	5.66	10428_30	1.89	15447_97	0.94	
3650_130	6.67	8628_98	0.00	14210_123	0.00			3159_146	5.66	7027_66	8.49	10437_160	10.38	15449_53	4.72	
3655_42	0.00	8638_74	5.00	14216_29	13.33			3168_75	0.94	7028_16	10.38	10439_56	2.83	15457_60	0.94	
3656_43	5.00	8639_18	0.00	14224_172	0.00			3174_18	9.43	7029_65	5.66	10444_84	0.00	15465_154	8.49	
3657_115	10.00	8641_101	1.67	14229_59	1.67			3175_144	3.77	7033_97	8.49	10445_82	3.77	15475_96	7.55	
3659_167	5.00	8642_86	1.67	14234_18	1.67			3177_51	6.60	7036_80	0.00	10448_131	6.60	15481_74	15.09	
3660_36	3.33	8643_83	6.67	14238_41	11.67			3181_103	0.00	7037_105	3.77	10449_112	0.00	15497_47	3.77	
3661_86	0.00	8648_78	1.67	14241_58	5.00			3182_111	8.49	7040_30	1.89	10451_127	0.00	15507_127	0.00	
3662_124	1.67	8651_88	3.33	14244_36	11.67			3186_101	5.66	7043_58	2.83	10452_126	1.89	15516_123	8.49	
3669_47	3.33	8652_35	1.67	14257_24	8.33			3187_100	0.00	7045_92	9.43	10453_64	5.66	15519_116	0.94	
3670_156	0.00	8654_81	0.00	14260_94	11.67			3188_138	9.43	7047_56	0.94	10454_172	8.49	15523_112	0.00	
3671_71	0.00	8663_72	0.00	14264_29	5.00			3193_53	6.60	7050_81	2.83	10455_52	0.94	15525_49	0.94	
3680_34	5.00	8674_151	3.33	14268_78	15.00			3194_118	3.77	7052_68	1.89	10456_67	8.49	15526_114	2.83	
3681_95	3.33	8679_49	0.00	14269_86	5.00			3200_13	1.89	7055_26	6.60	10459_78	2.83	15532_11	4.72	
3686_102	0.00	8680_136	11.67	14270_8	0.00			3201_172	1.89	7058_75	7.55	10460_129	9.43	15545_40	15.09	
3687_91	11.67	8686_90	11.67	14279_45	0.00			3203_86	1.89	7062_180	1.89	10463_24	5.66	15548_109	0.94	
3688_8	11.67	8688_28	0.00	14288_79	6.67			3209_143	11.32	7063_18	1.89	10469_188	10.38	15551_76	0.00	
3692_91	6.67	8694_150	0.00	14289_124	0.00			3212_67	0.00	7064_30	0.00	10470_153	0.00	15557_58	1.89	
3693_166	0.00	8697_7	0.00	14295_187	6.67			3214_20	0.00	7070_50	6.60	10474_37	7.55	15562_94	0.94	
3696_70	0.00	8702_128	0.00	14297_59	0.00			3216_73	0.94	7071_111	0.94	10476_137	1.89	15563_71	0.94	
3699_40	0.00	8704_18	6.67	14307_53	10.00			3228_108	0.00	7072_172	0.00	10479_30	6.60	15565_70	0.00	
3700_84	1.67	8706_22	0.00	14320_17	0.00			3231_10	11.32	7074_159	0.00	10484_15	6.60	15568_175	6.60	
3701_80	1.67	8715_88	0.00	14337_126	0.00			3233_76	0.00	7077_111	0.00	10485_41	0.00	15569_44	0.94	
3702_179	5.00	8719_97	3.33	14338_94	0.00			3238_81	6.60	7080_92	6.60	10487_124	0.00	15575_48	4.72	
3704_86	5.00	8721_57	13.33	14340_122	1.67			3239_21	3.77	7085_50	0.00	10496_89	1.89	15583_108	0.00	
3706_78	1.67	8723_133	8.33	14346_100	0.00			3241_54	2.83	7090_75	0.00	10502_19	1.89	15585_43	4.72	
3712_45	1.67	8730_67	10.00	14350_62	11.67			3243_110	3.77	7093_73	1.89	10503_57	1.89	15588_149	0.94	
3717_95	3.33	8731_77	0.00	14354_57	0.00			3244_75	13.21	7096_163	5.66	10505_39	10.38	15595_133	14.15	
3721_111	0.00	8739_177	3.33	14355_92	1.67			3246_118	1.89	7099_76	6.60	10506_83	0.00	15601_144	6.60	
3727_163	1.67	8746_40	3.33	14356_162	5.00			3248_9	0.94	7100_149	0.94	10511_83	4.72	15602_74	4.72	
3728_71	0.00	8751_127	1.67	14359_122	1.67			3251_112	0.00	7103_67	13.21	10514_29	1.89	15603_73	0.00	
3735_15	5.00	8753_33	0.00	14362_127	0.00			3252_166	0.00	7110_148	0.00	10518_155	0.94	15613_184	0.00	
3736_38	1.67	8757_175	0.00	14363_97	3.33			3254_151	11.32	7112_78	0.00	10519_41	2.83	15617_184	14.15	
3737_34	1.67	8763_173	8.33	14366_65	1.67			3258_64	0.00	7116_116	0.00	10521_52	2.83	15622_63	0.94	
3745_9	1.67	8767_81	0.00	14372_10	0.00			3259_81	3.77	7124_132	0.00	10531_38	7.55	15624_37	0.00	
3754_53	0.00	8769_102	0.00	14373_80	3.33			3263_9	0.00	7126_23	6.60	10533_74	4.72	15628_126	0.00	
3757_150	0.00	8770_11	0.00	14379_117	0.00			3265_49	2.83	7127_6	4.72	10535_50	9.43	15637_94	0.00	
3766_114	0.00	8772_134	10.00	14390_36	5.00			3266_172	2.83	7139_59	5.66	10536_121	6.60	15639_171	0.00	
3769_153	0.00	8777_29	6.67	14393_83	0.00			3268_40	0.94	7142_100	0.94	10537_140	4.72	15640_83	9.43	
3770_98	3.33	8782_79	1.67	14400_80	5.00			3273_50	2.83	7145_141	2.83	10538_32	0.00	15666_12	6.60	
3775_50	3.33	8785_5	1.67	14413_103	0.00			3275_14	4.72	7148_112	0.94	10546_128	8.49	15690_72	8.49	

3778_126	1.67	8786_59	1.67	14421_39	1.67			3280_85	3.77	7149_29	10.38	10548_37	2.83	15771_32	11.32		
3779_131	1.67	8791_52	3.33	14443_24	8.33			3285_109	4.72	7150_117	4.72	10551_30	0.94	15779_14	13.21		
3782_106	0.00	8805_153	1.67	14447_76	10.00			3287_63	2.83	7151_60	0.00	10554_85	0.94	15790_86	3.77		
3784_122	0.00	8809_38	1.67	14451_9	1.67			3291_13	6.60	7160_27	5.66	10556_14	0.00	15838_88	8.49		
3785_98	0.00	8810_29	11.67	14467_106	1.67			3299_18	7.55	7163_168	0.00	10558_107	0.00	15855_95	14.15		
3786_153	5.00	8812_38	11.67	14470_73	5.00			3304_98	5.66	7169_80	0.94	10559_66	5.66	16005_99	10.38		
3790_92	1.67	8813_107	0.00	14471_72	1.67			3307_58	11.32	7170_28	0.94	10561_151	4.72	16109_98	9.43		
3792_84	1.67	8816_79	0.00	14483_133	3.33			3308_52	9.43	7171_71	0.00	10564_151	12.26	16180_160	6.60		
3793_84	0.00	8817_7	1.67	14484_117	10.00			3311_86	5.66	7173_102	0.00	10565_143	10.38	16199_80	9.43		
3795_98	1.67	8820_106	6.67	14503_38	1.67			3314_116	5.66	7175_27	0.00	10568_108	6.60	16224_17	12.26		
3799_75	1.67	8826_37	0.00	14512_104	0.00			3321_25	5.66	7181_27	0.94	10575_114	0.00	16233_81	6.60		
3803_44	15.00	8829_46	0.00	14516_171	10.00			3324_171	2.83	7188_106	0.94	10577_108	9.43	16252_70	9.43		
3807_135	1.67	8830_123	0.00	14525_77	6.67			3325_132	0.94	7190_79	0.00	10579_170	2.83	16304_88	8.49		
3808_71	5.00	8832_116	0.00	14530_58	0.00			3329_24	0.00	7194_54	1.89	10582_166	5.66	16359_148	12.26		
3809_60	5.00	8837_180	5.00	14544_41	0.00			3331_12	0.94	7199_139	0.94	10587_92	0.94	16361_145	12.26		
3820_28	0.00	8838_125	6.67	14548_10	1.67			3336_18	0.00	7202_72	0.00	10590_134	5.66	16430_7	8.49		
3826_95	0.00	8842_121	5.00	14555_33	1.67			3339_40	1.89	7204_43	0.00	10597_43	1.89	16459_59	11.32		
3830_26	1.67	8848_67	0.00	14556_29	3.33			3340_58	6.60	7206_144	0.00	10609_34	0.00	16505_99	9.43		
3838_106	13.33	8855_40	0.00	14558_43	8.33			3341_38	7.55	7215_150	0.00	10611_73	10.38	16515_65	14.15		
3843_110	3.33	8857_42	1.67	14563_181	3.33			3342_55	0.94	7218_73	3.77	10616_100	0.00	16617_36	11.32		
3854_7	1.67	8858_164	3.33	14566_42	6.67			3350_102	7.55	7221_157	1.89	10621_115	0.00	16702_18	11.32		
3860_120	0.00	8868_44	10.00	14573_92	5.00			3357_17	1.89	7223_92	0.00	10622_151	9.43	16731_118	5.66		
3861_23	1.67	8871_93	0.00	14587_55	13.33			3362_85	0.00	7224_11	1.89	10628_32	0.94	16814_120	14.15		
3865_122	0.00	8872_82	0.00	14593_12	10.00			3363_7	0.94	7226_83	0.00	10630_62	2.83	16884_13	13.21		
3867_138	11.67	8887_101	0.00	14598_126	0.00			3365_39	1.89	7227_137	0.00	10640_132	7.55	16900_70	11.32		
3871_10	21.67	8889_74	5.00	14599_179	5.00			3366_130	1.89	7229_82	6.60	10641_96	13.21	16915_31	8.49		
3877_17	1.67	8890_66	0.00	14603_100	1.67			3369_128	1.89	7231_43	5.66	10643_20	10.38	16988_64	15.09		
3892_136	1.67	8893_73	0.00	14607_90	0.00			3370_117	1.89	7233_166	15.09	10646_75	4.72	17020_46	13.21		
3897_30	6.67	8894_138	0.00	14613_152	0.00			3371_88	0.00	7234_56	1.89	10647_43	1.89	17035_125	1.89		
3902_28	3.33	8897_117	0.00	14619_112	10.00			3372_183	12.26	7236_71	2.83	10654_115	9.43	17041_111	7.55		
3904_48	0.00	8898_22	8.33	14621_118	3.33			3379_69	10.38	7241_17	8.49	10663_93	0.94	17052_106	10.38		
3905_149	8.33	8900_192	11.67	14624_52	6.67			3381_91	11.32	7243_107	0.00	10665_41	2.83	17063_39	11.32		
3909_19	0.00	8901_40	0.00	14628_103	6.67			3382_159	0.00	7251_167	5.66	10666_48	3.77	17074_72	5.66		
3913_31	11.67	8907_148	1.67	14633_138	0.00			3383_110	5.66	7253_115	3.77	10669_32	0.00	17083_54	14.15		
3915_93	3.33	8908_169	8.33	14639_143	8.33			3385_37	0.94	7255_145	7.55	10671_38	0.00	17085_63	2.83		
3916_26	1.67	8915_90	1.67	14640_175	0.00			3389_34	0.00	7260_32	1.89	10676_38	4.72	17088_59	13.21		
3917_51	0.00	8919_79	13.33	14641_109	0.00			3392_162	5.66	7261_60	0.94	10681_59	1.89	17131_67	11.32		
3928_117	1.67	8923_57	1.67	14648_140	5.00			3401_89	0.00	7265_58	0.00	10682_20	0.00	17204_106	9.43		
3937_179	1.67	8924_21	1.67	14653_90	6.67			3405_21	1.89	7271_138	0.00	10683_49	4.72	17282_67	14.15		
3939_20	0.00	8925_106	10.00	14658_112	10.00			3409_86	0.94	7275_109	8.49	10684_127	2.83	17358_192	11.32		
3945_75	0.00	8926_38	0.00	14659_108	3.33			3412_159	11.32	7279_87	2.83	10685_110	5.66	17440_85	11.32		
3949_75	0.00	8929_103	10.00	14663_90	0.00			3413_10	1.89	7281_103	1.89	10689_90	4.72	17458_50	2.83		
3951_82	0.00	8931_109	1.67	14667_107	0.00			3415_52	1.89	7290_139	0.94	10692_50	0.94	17529_43	12.26		
3953_148	3.33	8932_168	1.67	14676_32	3.33			3416_27	9.43	7292_128	0.00	10695_56	3.77	17586_77	13.21		
3955_10	1.67	8936_65	3.33	14688_43	0.00			3417_40	0.00	7293_88	0.00	10697_61	0.00	17623_112	12.26		
3969_16	0.00	8938_150	0.00	14693_88	0.00			3420_21	0.94	7294_192	0.94	10702_15	0.00	17638_119	8.49		

3971_127	0.00	8945_140	13.33	14695_151	1.67					3427_14	0.00	7297_60	0.00	10706_203	6.60	17659_147	8.49	
3972_139	0.00	8946_48	3.33	14698_16	6.67					3432_85	13.21	7302_47	0.94	10707_70	0.94	17734_50	8.49	
3973_80	0.00	8948_110	1.67	14701_88	5.00					3439_66	2.83	7307_122	0.94	10709_133	0.00	17789_64	10.38	
3974_22	5.00	8952_109	3.33	14704_39	8.33					3440_96	3.77	7309_21	1.89	10710_96	2.83	17795_136	15.09	
3976_155	3.33	8953_97	0.00	14713_64	0.00					3446_38	2.83	7310_162	0.00	10711_133	0.00	17799_89	13.21	
3979_87	8.33	8955_116	1.67	14720_55	10.00					3447_55	7.55	7318_81	3.77	10712_126	7.55	17808_119	12.26	
3980_159	1.67	8956_76	3.33	14722_129	3.33					3449_15	3.77	7320_28	0.00	10714_21	9.43	17855_85	4.72	
3984_69	3.33	8959_39	8.33	14729_61	0.00					3453_61	5.66	7322_116	10.38	10717_22	5.66	17868_108	13.21	
3986_59	1.67	8962_50	0.00	14734_159	6.67					3456_82	1.89	7325_50	0.94	10719_119	6.60	17870_91	11.32	
3988_117	21.67	8967_42	13.33	14738_43	3.33					3459_129	13.21	7331_32	3.77	10720_72	0.94	17873_113	11.32	
3992_61	13.33	8968_67	3.33	14739_76	3.33					3460_156	4.72	7338_157	1.89	10726_78	5.66	17908_9	15.09	
3993_140	1.67	8970_16	1.67	14747_69	5.00					3465_53	0.94	7342_77	1.89	10729_145	0.94	18002_205	14.15	
4004_14	8.33	8973_172	5.00	14756_85	3.33					3468_45	1.89	7343_79	5.66	10740_76	0.00	18461_90	10.38	
4005_52	6.67	8976_18	0.00	14757_131	1.67					3486_133	0.00	7344_10	2.83	10743_76	0.94	18785_110	9.43	
4006_50	13.33	8977_110	0.00	14769_67	6.67					3498_126	0.00	7345_124	3.77	10744_91	7.55	19004_30	12.26	
4009_93	16.67	8978_159	10.00	14773_113	1.67					3504_100	7.55	7351_52	0.00	10745_86	3.77	27864_114	0.00	
4012_96	8.33	8979_130	1.67	14776_75	0.00					3510_66	0.94	7356_75	8.49	10752_100	1.89	46788_60	3.77	
4013_6	0.00	8982_139	5.00	14783_107	1.67					3512_155	9.43	7359_9	0.94	10753_117	1.89	47019_76	3.77	
4016_30	0.00	8985_51	0.00	14787_36	1.67					3522_16	0.00	7360_143	6.60	10754_100	10.38	48066_67	1.89	
4025_11	6.67	8987_103	1.67	14795_64	3.33					3523_98	9.43	7365_41	4.72	10755_42	0.00	48165_156	8.49	
4027_76	6.67	8992_106	0.00	14797_99	1.67					3524_78	8.49	7366_135	0.00	10758_74	9.43	52488_83	7.55	
4030_8	1.67	8995_110	5.00	14798_88	0.00					3525_82	5.66	7367_89	0.94	10765_71	4.72	52584_92	2.83	
4031_62	1.67	8996_14	8.33	14799_82	1.67					3530_61	6.60	7370_9	2.83	10766_149	10.38	56414_95	16.04	
4040_27	1.67	8998_130	0.00	14800_124	5.00					3534_91	10.38	7372_79	0.00	10770_62	0.94	57688_42	6.60	
4041_78	5.00	9000_22	3.33	14809_71	6.67					3535_45	0.00	7376_88	0.00	10771_128	10.38	59595_15	12.26	
4049_64	0.00	9003_150	1.67	14812_112	6.67					3536_72	1.89	7379_67	13.21	10773_13	6.60	59857_36	6.60	
4051_57	0.00	9006_105	0.00	14813_170	6.67					3539_77	2.83	7382_35	0.00	10775_52	0.00	64248_101	0.94	
4052_93	0.00	9008_10	0.00	14826_115	0.00					3540_8	4.72	7384_33	0.00	10777_42	10.38	65447_157	0.00	
4056_132	5.00	9010_65	1.67	14838_143	3.33					3543_46	0.94	7385_145	2.83	10778_15	0.94	67800_109	9.43	
4059_141	3.33	9015_115	1.67	14840_118	1.67					3544_22	7.55	7386_31	0.00	10780_99	8.49	72819_229	5.66	
4060_78	1.67	9016_22	6.67	14841_92	1.67					3545_55	2.83	7390_14	0.94	10783_165	1.89	83869_25	3.77	
4064_123	0.00	9024_73	1.67	14844_158	3.33					3546_71	2.83	7396_44	0.00	10789_31	1.89	85489_48	12.26	
4065_76	0.00	9033_83	1.67	14845_101	8.33					3550_174	8.49	7397_94	12.26	10793_67	0.00	97241_126	8.49	
4066_44	0.00	9037_100	15.00	14853_51	0.00					3554_88	12.26	7398_68	0.00	10804_49	0.00	100159_34	12.26	
4068_191	6.67	9040_127	6.67	14864_113	3.33					3555_31	1.89	7403_58	3.77	10805_17	8.49	104333_82	4.72	
4073_16	1.67	9041_21	5.00	14872_115	10.00					3560_151	4.72	7405_95	1.89	10811_55	3.77			
4075_68	0.00	9049_62	0.00	14876_64	5.00					3562_110	0.00	7406_107	1.89	10814_166	0.00			
4078_143	0.00	9058_81	3.33	14878_138	5.00													
4079_64	0.00	9059_82	1.67	14886_49	0.00													
4080_141	6.67	9061_95	0.00	14897_127	5.00													
4085_28	0.00	9062_29	5.00	14898_60	0.00													
4086_72	5.00	9071_51	1.67	14905_27	0.00													
4088_64	1.67	9072_126	10.00	14909_89	11.67													
4090_70	5.00	9080_37	6.67	14921_190	8.33													
4091_87	0.00	9083_132	3.33	14928_10	1.67													
4101_29	3.33	9084_146	0.00	14929_10	5.00													

4774_42	0.00	9734_119	1.67	72819_229	10.00															
4778_84	0.00	9736_138	3.33	83869_25	3.33															
4779_63	1.67	9744_18	0.00	85489_48	16.67															
4786_103	1.67	9745_55	10.00	97241_126	3.33															
4789_32	0.00	9746_119	6.67	100159_34	10.00															
				104333_82	3.33															

Table S4. Pairwise values of Φ_{st} for *MT-CYB* between the different localities of *C. paludica* and *C. vettonica*. Only significant values are shown (p -value < 0.05). Red cells: $\Phi_{st} > 0.75$; Orange: $0.5 > \Phi_{st} < 0.75$; Yellow: $0.25 > \Phi_{st} < 0.5$; Green: $\Phi_{st} < 0.25$. The localities where mitochondrial haplotypes of *C. vettonica* and *C. paludica* were detected, they were studied for each species separately (_V and _P respectively).

	Ponsul	Aravil V	High Erjas	SanMartin	Trevejuna	Middle Erjas	Lower Erjas_V	Arades	Arrago	Gata	Acebo	Mayas	Alagon V	Alagon P	Francia_V
Ponsul															
Aravil V															
High Erjas															
San Martin															
Trevejuna															
Middle Erjas			0.2												
Lower Erjas_V															
Arades															
Arrago	0.96	0.97	0.96	0.96	0.96	0.93	0.92	0.97							
Gata	0.91	0.92	0.93	0.92	0.91	0.9	0.88	0.91	0.25						
Acebo	0.95	0.98	0.97	0.96	0.98	0.9	0.87		0.74						
Mayas	0.99	0.99	0.98	0.99	0.99	0.97	0.95	1		0.34	1				
Alagon_V	0.9	0.92	0.94	0.92	0.91	0.88	0.87	0.91	0.81	0.73	0.78	0.91			
Alagon_P	0.97	0.98	0.98	0.97	0.97	0.95	0.94	0.97	0.98	0.96	0.97	0.99	0.96		
Francia_V	0.96	0.98	0.97	0.97	0.97	0.93	0.9	0.98	0.9	0.79	0.95	0.98		0.98	
Francia_P	0.96	0.97	0.98	0.97	0.97	0.95	0.94	0.97	0.98	0.95	0.97	0.99	0.95		0.98
Cdh_V	0.96	0.97	0.96	0.96	0.97	0.93	0.91	0.97	0.89	0.79	0.93	0.97		0.98	
Cdh_P	0.96	0.97	0.97	0.97	0.97	0.95	0.94	0.96	0.97	0.95	0.96	0.99	0.95		0.97
Caparro_V	0.97	0.99	0.97	0.97	0.98	0.93	0.9	1	0.91	0.78	1	1		0.98	
Caparro_P	0.98	0.98	0.98	0.98	0.98	0.97	0.96	0.98	0.98	0.97	0.98	0.99	0.97		0.98
Jerte_V	0.92	0.94	0.94	0.93	0.93	0.89	0.88	0.93	0.8	0.69	0.8	0.9		0.96	
Jerte_P	0.97	0.98	0.98	0.97	0.97	0.95	0.95	0.97	0.98	0.96	0.98	0.99	0.96		0.98
Lower Erjas_P	0.95	0.97	0.97	0.96	0.96	0.93	0.93		0.98	0.95		0.99	0.94		0.98
Aurela	0.94	0.96	0.97	0.96	0.95	0.93	0.93	0.94	0.97	0.95	0.95	0.99	0.94		0.97
Aravil_P		0.97	0.98			0.93	0.93		0.98	0.95	1	0.94			0.98
Salor									0.82	0.79		0.89	0.68		0.75
Tamuja	0.94	0.95	0.97	0.95	0.95	0.93	0.92		0.97	0.95	0.94	0.99	0.94		0.97
Almonte	0.85	0.88	0.92	0.88	0.86	0.86	0.87	0.82	0.93	0.91	0.84	0.96	0.87	0.32	0.9
Ibor	0.91	0.94	0.96	0.94	0.93	0.91	0.91		0.96	0.94		0.98	0.92	0.65	0.95
Tietar	0.88	0.89	0.92	0.9	0.89	0.89	0.89	0.87	0.92	0.91	0.86	0.94	0.89	0.55	0.9
Yeltes	0.98	0.99	0.99	0.99	0.99	0.96	0.95	1	0.99	0.96	1	1	0.97	0.79	0.99
Huebra	0.96	0.97	0.97	0.96	0.96	0.95	0.94	0.96	0.97	0.95	0.96	0.99	0.95	0.35	0.97

	Francia P	Cdh V	Cdh P	Caparro V	Caparro P	Jerte V	Jerte P	Lower Erjas P	Aurela	Aravil P	Salor	Tamuja	Almonte	Ibor	Tietar	Yeltes
Ponsul																
Aravil V																
High Erjas																
San Martin																
Trevejana																
Middle Erjas																
Lower Erjas V																
Arades																
Árrago																
Gata																
Acebo																
Mayas																
Alagon V																
Alagon P																
Francia V																
Francia P																
Cdh V	0.98															
Cdh P		0.97														
Caparro V	0.98		0.97													
Caparro P		0.98	0.43	0.98												
Jerte V	0.96		0.96		0.97											
Jerte P	0.47	0.98		0.98	0.71	0.97										
Lower Erjas P		0.98		0.98	0.38	0.95										
Aurela		0.97		0.97	0.28	0.95										
Aravil P		0.98		0.99	0.47	0.96										
Salor		0.79		0.7		0.71										
Tamuja		0.97	0.34	0.97	0.37	0.95	0.57									
Almonte	0.29	0.91	0.39	0.89	0.53	0.89	0.48									
Ibor	0.62	0.96	0.67	0.95	0.79	0.93	0.73		0.5			0.43				
Tietar	0.54	0.91	0.59	0.9	0.68	0.89	0.63	0.47	0.48	0.55	0.44	0.48				
Yeltes	0.78	0.99	0.75	1	0.84	0.98	0.87	0.76	0.67	0.86	0.52	0.58	0.43	0.68	0.54	
Huebra	0.34	0.97	0.44	0.97	0.52	0.96	0.66		0.3			0.36	0.58	0.53	0.65	

Table S5. Results of the Puechmaille method and the ΔK method for different values of K , considering the populations of both *C. paludica* and *C. vettonica* and only those of *C. vettonica*.

Puechmaille method										ΔK method			
K	Reps	MedMed	MedMean	MaxMed	MaxMean	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	Ln''(K)	ΔK			
1	10	1	1	1	1	-417450.72	9.30266	NA	NA	NA			
2	10	2	2	2	2	-298911.31	16.96797	118539.41	87652.43	5165.75725			
3	10	3	3	3	3	-268024.33	753.95264	30886.98	9514.24	12.61915			
4	10	4	4	4	4	-246651.59	37.93636	21372.74	15762.43	415.4966			
5	10	5	5	5	5	-241041.28	2477.0614	5610.31	2311.93	0.93334			
6	10	5	5	6	6	-237742.9	4678.81323	3298.38	1109158.87	237.05987			
7	10	5	5	6	6	-1343603.39	3506856.566	-1105860.49	173280.61	0.04941			
8	10	5	5	6	6	-2622744.49	4154430.413	-1279141.1	3656018.8	0.88003			
9	10	5	5	6	6	-245866.79	19857.88545	2376877.7	3896356.19	196.21204			
10	10	5	5	5	5	-1765345.28	3516514.633	-1519478.49	1506164.7	0.42831			
11	10	5	5	6	6	-1778659.07	3585999.984	-13313.79	128319.03	0.03578			
12	10	5	5	6	6	-1663653.83	3155816.949	115005.24	1970965.35	0.62455			
13	10	5	5	6	6	-3519613.94	5374467.812	-1855960.11	794401.5	0.14781			
14	10	5	5	6	6	-4581172.55	6299608.254	-1061558.61	2964726.8	0.47062			
15	10	6	6	6	6	-2678004.36	4373326.803	1903168.19	2501014.89	0.57188			
16	10	6	6	6	6	-3275851.06	4781295.799	-597846.7	207757.56	0.04345			
17	10	5	5	6	6	-3665940.2	4641233.514	-390089.14	NA	NA			
		MedMedK=6		MedMeanK=6		MaxMedK=6		MaxMeaK=6					
1	10	1	1	1	1	-196871.89	12.34706	NA	NA	NA			
2	10	2	2	2	2	-156750.04	12.51623	40121.85	17995.29	1437.75648			
3	10	3	3	3	3	-134623.48	11.38194	22126.56	14563.76	1279.55045			
4	10	4	4	4	4	-127060.68	26.12401	7562.8	22852.53	874.77107			
5	10	4	4	4	4	-142350.41	50523.35434	-15289.73	15132.98	0.29952			
6	10	4	4	4	4	-142507.16	35675.63309	-156.75	27144.86	0.76088			
7	10	4	4	4	4	-169808.77	69819.26493	-27301.61	71075.97	1.018			
8	10	4	4	4	4	-126034.41	448.90798	43774.36	66656.78	148.48651			
9	10	4	4	4	4	-148916.83	44654.20503	-22882.42	NA	NA			
		MedMedK=4		MedMeanK=4		MaxMedK=4		MaxMeaK=4					

C. vettonica

C. vettonica + C. paludica

C. vettonica + C. paludica

IV

CAPÍTULO

El uso de la herramienta de *metabarcoding* de ADN ambiental de muestras de agua para el seguimiento de los peces ibéricos de agua dulce



Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Environmental DNA metabarcoding of water samples as a tool for monitoring Iberian freshwater fish composition. *Under Review*.

Environmental DNA metabarcoding of water samples as a tool for monitoring Iberian freshwater fish composition

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Abstract

Environmental DNA (eDNA) metabarcoding has been increasingly used to monitor the community assemblages of a wide variety of organisms. Here, we test the efficacy of eDNA metabarcoding to assess the composition of Iberian freshwater fishes, one of the most endangered groups of vertebrates in Spain. For this purpose, we sampled 12 localities throughout one of Spain's largest basins, the Duero, which is home to approximately 70% of the genera and 30% of the primary freshwater fish in Spain. We sampled these localities in the summer by using electrofishing, a traditional sampling method, and eDNA metabarcoding of river water samples using the mitochondrial 12S rRNA gene (*12S*) as a marker. We also resampled four of these localities in autumn by eDNA. We compared the results obtained through eDNA metabarcoding with those of electrofishing surveys (ones conducted for the present study and past ones), and assessed the suitability of *12S* as an eDNA metabarcoding marker for this group of freshwater fishes. We found that the *12S* fragment, analyzed for 25 Iberian species, showed sufficient taxonomic resolution to be useful for eDNA approaches, and even showed population-level differences for *Achondrostoma arcasii*. In most cases, a greater number of species was detected through eDNA metabarcoding than through electrofishing. Based on our results, eDNA metabarcoding is a powerful tool to study the freshwater fish composition in the Iberian Peninsula and to unmask cryptic diversity. However, we highlight the need to generate a local database for such studies and to interpret the results with caution when studying only mitochondrial DNA. Finally, the high detection sensitivity of eDNA metabarcoding allows it to also act as an early detection system for invasive species, a key aspect for conservation management.

Keywords: Environmental DNA, metabarcoding, Iberian Peninsula, freshwater fishes, monitoring, conservation

Introduction

Approximately 9.5% of the planet's animal biodiversity is found in freshwater ecosystems, with vertebrates representing one-third of this percentage (Balian et al., 2007; Strayer & Dudgeon, 2010). Of the freshwater vertebrates, fishes represent one of the most abundant groups, contributing to a large part of the biodiversity found in rivers (Geist et al., 2011). The monitoring of this group is, therefore, fundamental for ecosystem conservation and the sustainability of biological resources (Common & Norton, 1994; Lepetz et al., 2009; Goodwin et al., 2018). Freshwater fish monitoring has traditionally been conducted by using direct capture approaches such as electrofishing, netting and trapping (Bonar et al., 2009). Sometimes, these traditional methods are hampered by the geomorphological characteristics of a river, unfavorable weather and water conditions and study design (e.g., the need for legal permits to capture specimens or for experts to make the morphological identifications). They are also invasive techniques: in most cases, the species of interest must be handled or captured to obtain a positive identification, which can compromise the viability of the species, especially if they are endangered (Snyder, 2003). Traditional (capture) methods also tend to be inefficient when dealing with the detection of species present at a low density, such as those suffering a population decline or recently introduced species. Their exclusive use may result in an underestimation of freshwater fish diversity or necessitate extremely laborious sampling efforts.

Over the last decade, an alternative method for detecting the presence of freshwater fishes has been developed based on the use of environmental DNA (eDNA), i.e., extra-organismal genetic material suspended in environmental samples such as water or sediment (Taberlet et al., 2012). In recent years, eDNA approaches utilizing water samples have been broadly used to monitor the biodiversity of fish communities or specific species in freshwater ecosystems (Minamoto et al., 2012; Goldberg et al., 2015; Thomsen & Willerslev, 2015; Valentini et al., 2016; Roy et al., 2018; Cilleros et al., 2019; Coutant et al., 2021; Sales et al., 2021). This technique has proven to be, among other factors, more time and cost effective than traditional methods, and has shown greater sensitivity in detecting low-abundance species (Rees et al., 2014; Hinlo et al., 2017; Sigsgaard et al., 2020). Moreover, the difficulties associated with directly sampling fishes (e.g., river conditions or depth) are overcome using eDNA as only a water sample is needed. However, eDNA approaches have

limitations related to making inferences across space and time, the presence versus viability of populations, the PCR artifacts, or the ability of eDNA as a quantitative tool (Goldberg et al., 2016; Sigsgaard et al., 2020).

One of the most important points to consider when using eDNA metabarcoding for freshwater fishes is the choice of the genetic marker used to amplify target taxa (Collins et al., 2018). The use of mitochondrial markers for eDNA metabarcoding has been shown to be successful for some groups, and more advantageous compared with nuclear ones, likely due to the higher copy number per cell and less susceptibility to environmental degradation of mitochondrial DNA (mtDNA) (Foran, 2006; Rees et al., 2014). In particular, mitochondrial 12S rRNA (*12S*) has been the most widely used gene marker for eDNA metabarcoding of bony fishes (McElroy et al., 2020). The strength of *12S* markers for eDNA metabarcoding lies primarily in its fish-specific amplification (Deagle et al., 2014; Collins et al., 2019; McElroy et al., 2020). Despite this, the use of this fragment for eDNA metabarcoding of freshwater fishes has some drawbacks. Its variability, although moderate, is lower than that of other mitochondrial markers such as cytochrome c oxidase I (*COI*) and cytochrome b (*MT-CYB*), and sometimes, it shows a low level of taxonomic resolution and cannot be used to distinguish among some fish taxa (Civade et al., 2016; Stoeckle et al., 2017; Cilleros et al., 2019; Takeuchi et al., 2019). Moreover, the reference database for *12S* is not as extensive as those for *MT-CYTB* or *COI*, markers widely used in phylogenetic and phylogeographic studies, making the taxonomic assignment of freshwater fishes more difficult (Collins et al., 2019).

Primary freshwater fishes of the Iberian Peninsula have been widely studied in a phylogenetic and phylogeographic context (e.g., Perdices & Doadrio, 2005; Perea et al., 2015; Casal-Lopez et al., 2018; Corral-Lou et al., 2021). As with many other of these types of studies, *MT-CYTB* and *COI* are the markers of choice to study this group of fishes, with *12S* only being analyzed in a handful of cases (Casal-Lopez et al., 2015; Perea et al., 2016; Gonzalez et al., 2018; Corral-Lou et al., 2019; Doadrio et al., 2021; Corral-Lou et al., 2022). In fact, unlike for the other markers, there is no *12S* database for native Iberian freshwater fishes. Compared with other vertebrate groups in the Iberian Peninsula, the freshwater fishes in this area consist of a greater proportion of endemic species, and represent one of the most endemic fish faunas in the Mediterranean region (Doadrio et al., 2011).

The Duero Basin, with a surface area of 97,290 km², is the largest basin in the Iberian Peninsula (81% of it is in Spain and 19% is in Portugal). The Duero Basin currently hosts 70% of the genera and 30% of the species of Spain's autochthonous freshwater fishes approximately, including one of the most endangered and restricted of the Iberian endemic fish species, *Achondrostoma salmantinum* Elvira and Doadrio, 2007. The distribution area of this species is restricted to a few rivers in the southwestern Duero Basin. Several species within this basin are also distributed in other Iberian basins, however, population genetics studies of many of these have revealed that Duero basin populations behave as an independent evolutionary lineage (Doadrio et al., 2021). For this reason, the conservation of freshwater fish populations in the Duero helps to protect not only the species richness of the Iberian Peninsula but also intraspecific richness. Of the autochthonous primary freshwater species present in the Duero, 64% are included within one of the three most critical categories of the IUCN red list (IUCN, 2020). The main threats against the viability of these at-risk species are water extraction, the presence of exotic species, pollution, habitat alteration and changes in land use (Doadrio et al., 2011). For several of the species, these threats have also led to an evident decline in population (Doadrio et al., 2011).

Due to the large number of genera and the high percentage of both native and exotic species inhabiting the Duero Basin, and the strong threat to their populations, accurate knowledge of the composition of this basin's ichthyofauna is of critical importance. The main objective of this study is to assess eDNA metabarcoding as a tool for monitoring this fauna. For this purpose, we sequenced a fragment of *12S*, built a local *12S* reference database and tested the taxonomic resolution of this fragment for some Iberian freshwater fishes and also its ability to distinguish local variation.

Material & Methods

Sampling sites

A total of 12 localities were analyzed in this study. These localities were distributed throughout the entire Spanish Duero Basin (Figure 1; Table 1). The selection of the sampling sites was based on some ichthyological samplings made during 2009 and 2010 from previous projects attempting to obtain a representative sample of all the fish that inhabit the Duero Basin (mainly "Basis for the monitoring of Spanish Freshwater Fishes", supported by the Ministry of Environmental 2009–2010, Spain; Doadrio et al., 2011). The samples for eDNA

metabarcoding were collected from all localities prior to electrofishing in order to avoid DNA contamination from the electrofishing gear between sampling points. In the locality Juarros de Voltoya (11; Table 1 and Figure 1), electrofishing is prohibited within the reservoir itself, therefore we sampled the area just below the mouth of the reservoir.

Environmental DNA sampling and extraction

Sampling was mainly carried out during July 2020 (12 sampling sites; M1; Table 1). We also resampled four of the 12 sites in October 2020 (4 sampling sites; M2; Table 1). Filtration was performed in situ to avoid cross contamination and DNA degradation. All the material used was first disinfected with 20% bleach, and all water samples were filtered through Nalgene™ Reusable Filter Holders with Receivers fitted with Supor®-200 Membrane Filters (Pall Corporation, Life Sciences, Ann Arbor, MI, USA) with a pore size of either 0.45 µm or 0.2 µm. For each locality, 1 L of distilled water was first filtered as a negative control (blank, BL) from the laboratory and then two 1-L samples of the river water (L1 and L2) were collected and filtered. Depending on the turbidity of the water, we had to use between one and three 0.45 µm-filters per liter. The filtered water samples were then refiltered through a 0.2-µm filter. Each filter was stored directly in 1 ml of ATL buffer. Within 24 hours, 35 µl of proteinase K was added to each sample and it was digested for 15 hours at 56 °C.

DNA was extracted using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol, except for the first step. After digestion for 15 hours, 1 ml of the digestion product of all the filters of the same liter was placed in a single 15 ml falcon tube, and 1 ml of AL buffer and 1 ml of ethanol were added for each milliliter of digestion product. This solution was then passed through a spin column. After this step, the manufacturer's protocol was followed.

Traditional sampling (electrofishing)

The traditional sampling approach of electrofishing was also carried out at all the localities in order to compare the species detected by the two methods. All scientific fishing permits were obtained from the relevant authorities. At each sampling site, a river section of approximately 100 meters of distance was electrofished from downstream to upstream. The same transect was surveyed twice, with a total time investment of between 40 and 50 minutes. To account for habitat heterogeneity, all accessible mesohabitats

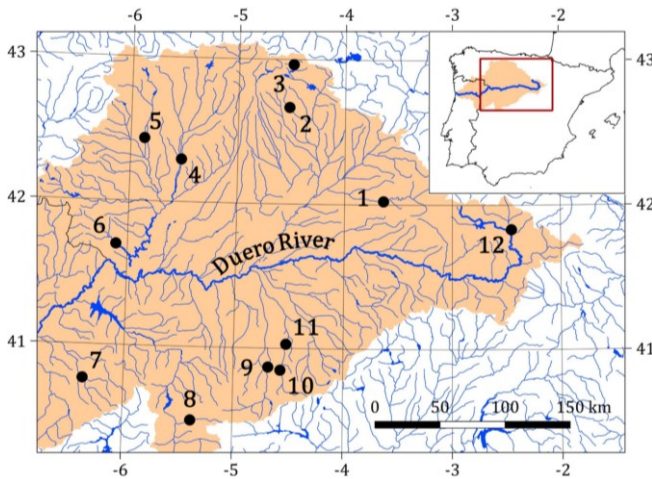


FIGURE 1. Sampling localities for this study. Numbers correspond to those used in Table 1. The orange shading delimits the Duero Basin.

were surveyed. For some specimens, a fin tissue sample was taken before they were returned to the river. Fin samples were preserved in 95% ethanol and stored at 4°C until further processing.

Inventory of the ichthyofaunal composition

An inventory of the ichthyofaunal composition made at each sampling site was based on the electrofishing data collected for this study and from previous projects led by the second author (mainly “Basis for the monitoring of Spanish Freshwater Fishes”, supported by the Ministry of Environmental 2009–2010, Spain; Doadrio et al., 2011). The species record compiled for Juarros de Voltoya Reservoir was based on sightings of species within the reservoir provided by local fishermen and on data gathered in previous years for the area just downstream of the reservoir.

Library preparation and sequencing

A total of 48 samples for eDNA metabarcoding was analyzed for the 12 sampling sites (plus the 4 re-sampled sites). Three samples, 1 blank (distilled water) and 2 eDNA (river water), were analyzed for each site. For the library preparation, an approximately 250-base pair (bp) fragment of *12S* was amplified using the primers MiFish_U_F (5' GTCGGTAAACTCGTGCCAGC 3') and MiFish_U_R (5' CATAGTGGGGTATCTAATCCCAGTTTG 3') (Miya et al., 2015). Illumina sequencing primers were attached to these primers at the 5' ends.

The summer (M1) and autumn (M2) samples were treated independently but equally as explained below. Polymerase chain reactions (PCRs) were carried out in a final volume of 25 µL containing 2.5 µL of

TABLE 1. Sampling localities include in the present study.

Nº	ID_M1	ID_M2	River	Locality	Sub-basin
1	AT5138		Arlanza	Quintanilla del Agua, Burgos	Pisuerga
2	AT5141		Boedo	Báscones de Ojeda, Palencia	Pisuerga
3	AT5140		Pisuerga	San Salvador de Cantamuda, Palencia	Pisuerga
4	AT5144	AT5166	Esla	Valecia de Don Juan, León	Esla
5	AT5145	AT5167	Órbigo	Barrio de Buenos Aires, León	Esla
6	AT5143		Aliste	Vegalatrave, Zamora	Esla
7	AT5152		Yeltes	Martín de Yeltes, Salamanca	Huebra
8	AT5153		Comeja	Bartolomé de Comeja, Ávila	Tormes
9	AT5155	AT5168	Adaja	Blascosanco, Ávila	Adaja
10	AT5156		Voltoya	Pinar de Puente Viejo, Ávila	Adaja
11	AT5154		Voltoya	Juarros de Voltoya, Segovia	Adaja
12	AT5142	AT5169	Tera	Tardesillas, Soria	Tera

template DNA, 0.5 µM of the primers, 12.5 µL of Supreme NZYTaQ 2x Green Master Mix (NZYTech) and ultrapure water up to 25 µL. The thermocycling conditions were as follows: an initial denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 45 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The oligonucleotide indices required for multiplexing different libraries in the same sequencing pool were attached in a second round of PCR using same thermocycling conditions except only 5 cycles were performed and the annealing temperature was 60 °C. A negative control containing no DNA (BL M1 and BL M2) was included in every PCR round to check for contamination during the library preparation. The libraries were run on 2% agarose gels stained with GreenSafe (NZYTech) and imaged under UV light to verify the library size. Libraries were purified using Mag-Bind RXNPure Plus magnetic beads (Omega Biotek), following the manufacturer's instructions. Then, libraries and DNA extraction blanks that yielded a band in the agarose gel were pooled in equimolar amounts according to the quantification data provided by the Qubit dsDNA HS Assay (Thermo Fisher Scientific). This pool also contained a recommended amount (1 µL) of the DNA extraction

blanks that did not yield a band and of the PCR blank (BL). The pool was sequenced using the Illumina MiSeq PE300 platform.

Preparation of the reference database for 12S

The selection of species for the 12S reference database was based on previous knowledge of the ichthyofauna inhabiting the Duero Basin, genetic population structure studies (Doadrio et al., 2021) and unpublished data. The following 26 species (25 species present in Spain and one from northern Morocco) were included in the database: *Achondrostoma arcasii* (Steindachner, 1866); *A. salmantinum* Doadrio & Elvira, 2007; *Alburnus alburnus* Linnaeus, 1758; *Ameiurus melas* Rafinesque, 1820; *Barbatula barbatula* (Linnaeus, 1758); *Carassius auratus* (Linnaeus, 1758); *Cobitis calderoni* Bacescu, 1962; *C. paludica* (de Buen, 1930); *C. vettonica* Doadrio & Perdices, 1997; *Cyprinus carpio* (Linnaeus, 1758); *Esox lucius* Linnaeus, 1758; *Gambusia holbrooki* Girard, 1859; *Gobio lozanoi* Doadrio & Madeira, 2004; *Leuciscus aspius* (Linnaeus, 1758); *Lepomis gibbosus* Linnaeus 1758; *Luciobarbus bocagei* (Steindachner, 1864); *L. rifensis* Doadrio, Casal-Lopez & Yahyaoui, 2015; *Micropterus salmoides* Lacépède, 1802; *Oncorhynchus mykiss* (Walbaum, 1792); *Phoxinus phoxinus* Rafinesque, 1820; *Pseudochondrostoma duriense* (Coelho, 1985); *P. polylepis* (Steindachner, 1864); *Salmo trutta* Linnaeus, 1758; *Squalius alburnoides* (Steindachner, 1866); *S. carolitertti* (Doadrio, 1988) and *Tinca tinca* (Linnaeus, 1758). First, we downloaded all available 12S sequences of the selected species from GenBank, which totaled 149. To these, we added the 12S sequences of 78 specimens belonging to 19 species whose tissue had been previously sampled and stored in the DNA and Tissue Collection at the National Museum of Natural Sciences of Madrid (MNCN-CSIC) (Table S1). When possible, the specimens selected for sequencing came from Duero basin rivers.

DNA of these specimens was extracted using the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA), following the manufacturer's protocol. PCR was used to amplify between 217 and 229 bp of mitochondrial 12S using the same MiFish primers used to make the libraries (Miya et al., 2015). All reactions consisted of a final volume of 12.5 µl containing 6.25 µl of 2X DreamTaq Green PCR Master Mix (Thermo Scientific), 0.25 µl of each primer (10 µM) and 1 µl of template DNA (final concentration 80–200 ng/µl). The following thermocycling conditions were used: initial

denaturation at 95 °C (5 min) followed by 35 cycles of denaturation at 95 °C (20 s), annealing at 65 °C (15 s) and extension at 72 °C (15 s); and a final extension step at 72 °C (7 min). PCR products were purified with ExoSAP-IT (USB Cleveland, OH, USA), and then both strands were sequenced on a 3730xl DNA Analyzer by Macrogen Europe Inc. (<http://www.macrogen.com>).

All sequences were aligned using MAFFT (Katoh & Standley, 2013), as implemented in Geneious 10.1.3 (<http://www.geneious.com>; Kearse et al., 2012), then manually examined and collapsed into haplotypes. Finally, in order to cover a wider spectrum of species, all the sequences were linked to an already existing reference database for 12S (Sato et al., 2018; Teresita, 2020).

Genetic distance

Genetic differentiation for 12S between and within species was based on the percentage of similarity calculated in Geneious 10.1.3. The species *A. arcasii* was divided into three independent groups based on the level of genetic differentiation found for its populations in the Duero Basin. They were named as *A. arcasii* (1), *A. arcasii* (2) and *A. arcasii* (3).

Metabarcoding pipeline

The overall quality of the raw sequencing reads was visualized using FastQC (Andrews, 2010). To merge forward and reverse sequences, we used the program FLASH2 v2.2.00 (Magoč & Salzberg, 2011) and the parameter *-m 30 -M 150*. The primer sequences were trimmed using CUTADAPT version 1.2.1 (Martin, 2011), allowing for a mismatch of up to 3 bp per primer. Reads without primer sequences were removed. CUTADAPT was also used to eliminate sequences with sizes <150 bp or >200 bp since the mean size of the 12S gene is around 170 bp (Miya et al., 2015). We used the *multiple_split_libraries_fastq.py* script from QIIME (Caporaso et al., 2010) to remove low quality reads (score <20), those with 3 consecutive bp of low quality and those with less than 85% of consecutive bp of high quality. We also used this script for demultiplexing the libraries. Retained sequences were clustered into operational taxonomic units (OTUs) and chimeras were filtered out by using VSEARCH (Rognes et al., 2016). The taxonomic assignment was performed using the *assign_taxonomy.py* script from QIIME against our reference database. We performed two assignments, one with a minimum percentage of similarity threshold of 99% and the other, of 97%. Subsequently, unassigned OTUs and those with less than 10 sequences within the same sample were

removed. Unassigned OTU sequences were mapped, using the Blastntool, to sequences in the GenBank nucleotide database from the National Center for Biotechnology Information (NCBI). In addition, to ensure the taxonomic assignment of the unassigned reads, we only kept reads that aligned 100% with the assigned sequence and had a percentage of similarity greater than 97%.

Results

Genetic differentiation

We analyzed 227 sequences of *12S* (149 from GenBank and 78 new sequences) of 26 freshwater fish species. These sequences represent a total of 46 different haplotypes. The percentage of intraspecific similarity ranged from 98.3% to 100% for the autochthonous species, and from 99.7% to 100% for the allochthonous species (Table 2; Table 2; Table S2). Likewise, the percentage of similarity between species varied, but it was never higher than 99%, except for the species pair *P. duriense* and *P. polylepis* (99.4%). Among the three groups of *A. arcasii*, similarity ranged from 94.3% to 97.1%. Species groups showing high interspecific similarity (97–99%) were the following: (*A. salmantinum*, *A. arcasii*, *P. duriense* and *P. polylepis*); (*S. alburnoides* and *S. carolitertii*); (*C. auratus* and *C. carpio*); (*C. paludica*, *C. vettonica* and *C. calderoni*); (*L. bocagei* and *L. rifensis*) and (*A. alburnus* and *L. aspius*) (Table 2 and Table S2).

Metabarcoding pipeline

Excluding the blank samples, a mean of 39,509 and 46,086 raw reads for each sampling site was obtained for M1 and M2, respectively. Samples with the lowest number of reads were L1 and L2 of Voltoya River (AT5156; M1); those with the highest values were L1 from Arlanza River (AT5138; M1) and L2 from Tera

TABLE 2. Summary of the percentage of similarity detected for the *12S* gene within each species. Species pairs/groups showing an interspecific percentage of similarity >97% are also indicated. Allochthonous species are marked with an asterisk.

Species	Intraspecific	Interspecific
<i>S. alburnoides</i>	98.9-100%	98.3%
<i>S. carolitertii</i>	100%	
<i>A. arcasii</i> (2)	100%	94-98%
<i>A. arcasii</i> (1)	100%	99.4%
<i>A. salmantinum</i>	99.4-100%	(between <i>P. duriense</i> and <i>P. polylepis</i> species)
<i>P. duriense</i>	100%	
<i>P. polylepis</i>	100%	
<i>A. arcasii</i> (3)	100%	
<i>A. alburnus</i> *	100%	97.7%
<i>L. aspius</i> *	100%	
<i>P. bigerri</i> *	100%	
<i>G. lozanoi</i>	100%	
<i>T. tinca</i>	100%	
<i>C. auratus</i> *	98.3-100%	96.5-98.3%
<i>C. carpio</i> *	97.7-100%	
<i>L. bocagei</i>	100%	98.3%
<i>L. rifensis</i> *	100%	
<i>B. barbatula</i>	99.4-100%	
<i>C. calderoni</i>	99.4-100%	
<i>C. paludica</i>	100%	96.6-98.3%
<i>C. vettonica</i>	98.8-100%	
<i>A. melas</i> *	100%	
<i>L. gibbosus</i> *	99-100%	
<i>M. salmoides</i> *	98.2-100%	
<i>E. lucius</i> *	100%	
<i>O. mykiss</i> *	97.7-100%	
<i>S. trutta</i> *	99.4-100%	
<i>G. holbrooki</i> *	100%	

River (AT5169; M2) (Figure 2). After merging and filtration steps, 47% (sd±16%) of the raw reads comprised the final data set; of these, a mean of 85% (sd±17%) of reads for each sampling site were assigned to fish species present in our database (Figure 2). Unassigned reads (20.03% and 12.65% for

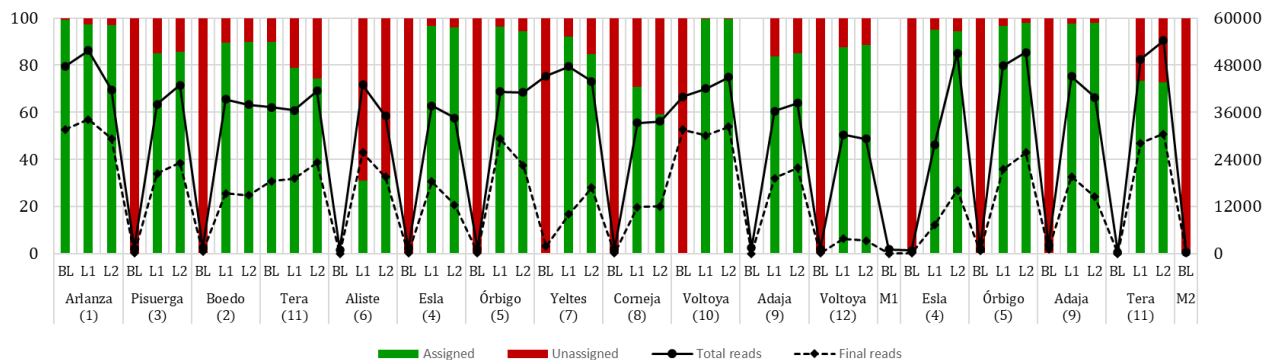


FIGURE 2. Percentage of total reads assigned to some sequence from our taxonomy database. The solid line indicates the number of total obtained reads and while the dashed line indicates the number of the total reads after the clean-up steps for each sampling point. The red and green bars indicate the percentage of the unassigned and assigned final reads to our taxonomy database, respectively.

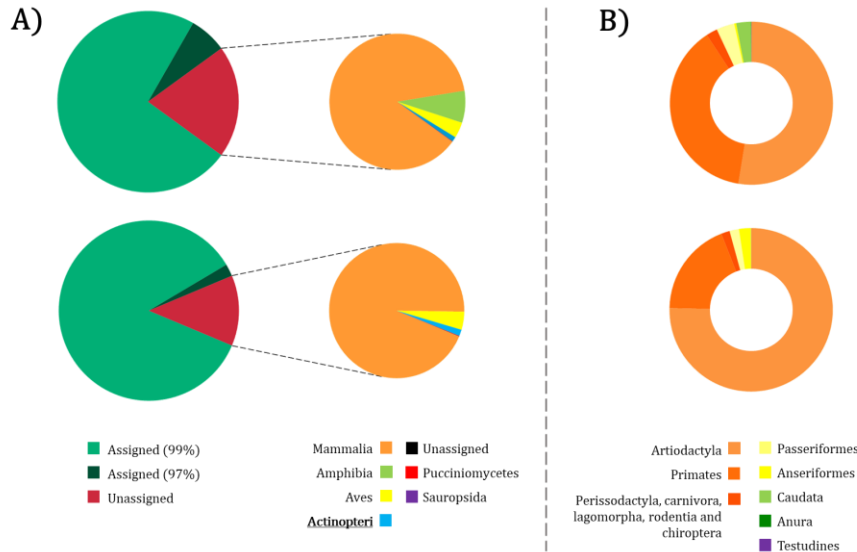


FIGURE 3. (A) Percentage of total assigned and unassigned reads from the samplings carried out in either summer (M1, top) or autumn (M2, bottom). Also shown is the percentage of the unassigned read (to the species level) that were assigned to different classes. See legend for details. (B) Percentage of the unassigned reads by order after read quality filtering.

M1 and M2, respectively) were blasted against the GenBank nucleotide database and, of these, 1.13% and 1.54% were assigned to Actinopterygii (Figure 3). However, the reads assigned to Actinopterygii and others assigned to other vertebrate classes were not considered further because the alignment coverage with the matched sequence was less than 100% and the percentage of similarity was less than 97%. After these considerations, we found that 92.5% and 96.8% of the unassigned reads for M1 and M2, respectively, were reliably assigned: the vast majority of reads were assigned to mammals (93% and 96% for M1 and M2, respectively), followed by birds (4% for both M1 and M2), amphibians (3% and 0.05% for M1 and M2, respectively) and testudines (0.04% for M1) (Figure 3).

The reads assigned to fishes in our database with a percent identity of 99% or higher resulted in a taxonomic assignment of 1270 and 486 OTUs for M1 and M2, respectively; these values rose to 1525 and 533 OTUs, respectively, when percent identity of 97% was considered. The final matrix was constructed by combining the two matrices, but maintaining the 99% assignment level when the OTU had been assigned in both cases. As some species could not be differentiated at the 97% percent identity limit (Table 2), the taxonomic assignment of the OTUs obtained at this percentage was done at the genus level, resulting in a taxonomic assignment of 1270 and 486 OTUs at the species level, and 255 and 47 OTUs at the genus level for M1 and M2, respectively.

A total of 31 freshwater fish species and 3 genera were detected across all sampling localities. Nearly all of the autochthonous and allochthonous fish

species detected here by eDNA had been previously detected in the Duero Basin. The exceptions are detailed below. It is also worth noting that, although marine fish species were not considered in this study, they were detected in the Adaja, Tera, Esla and Órbigo rivers in the summer sampling (M1), and in the Esla and Órbigo rivers in the autumn one (M2) (Table S3). These species included the European seabass (*Dicentrarchus labrax*), yellowfin sole (*Limanda aspera*), gilthead seabream (*Sparus aurata*), turbot (*Scophthalmus maxima*), European pilchard (*Sardina pilchardus*), albacore (*Thunnus alalunga*), Japanese jack mackerel (*Trachurus japonicus*), Atlantic horse mackerel (*Trachurus trauchurus*), European conger (*Conger conger*) and halibut (*Hippoglossus* sp.). As for the exceptions, *L. rifensis*, which is endemic to northern Morocco (Casal-Lopez et al., 2015), was detected at a low proportion ($\bar{x} = 0.03\%$) in the Arlanza River; *L. bocagei*, a related species, was detected at a higher proportion ($\bar{x} = 3.6\%$) (Table S3). Given that *L. rifensis* does not occur in the Iberian Peninsula and its sequences are highly similar to those of *L. bocagei* (98.3%), it was excluded from the study. Also, the genus *Leuciscus* was detected in Esla River in autumn. The only species of this genus present in the Iberian Peninsula is *L. aspius* (Merciai et al., 2018), however, it is not known to occur in the Duero Basin. Low genetic differentiation for *12S* was observed between *L. aspius* and *Alburnus alburnus* (97.7%), a species known to be present in that locality. Therefore, *Leuciscus* genus it was excluded from the study. In the Adaja River, *P. polylepis* was detected in both summer and autumn. This species has not been previously reported in the Duero Basin; however, its current distribution includes the Tajo

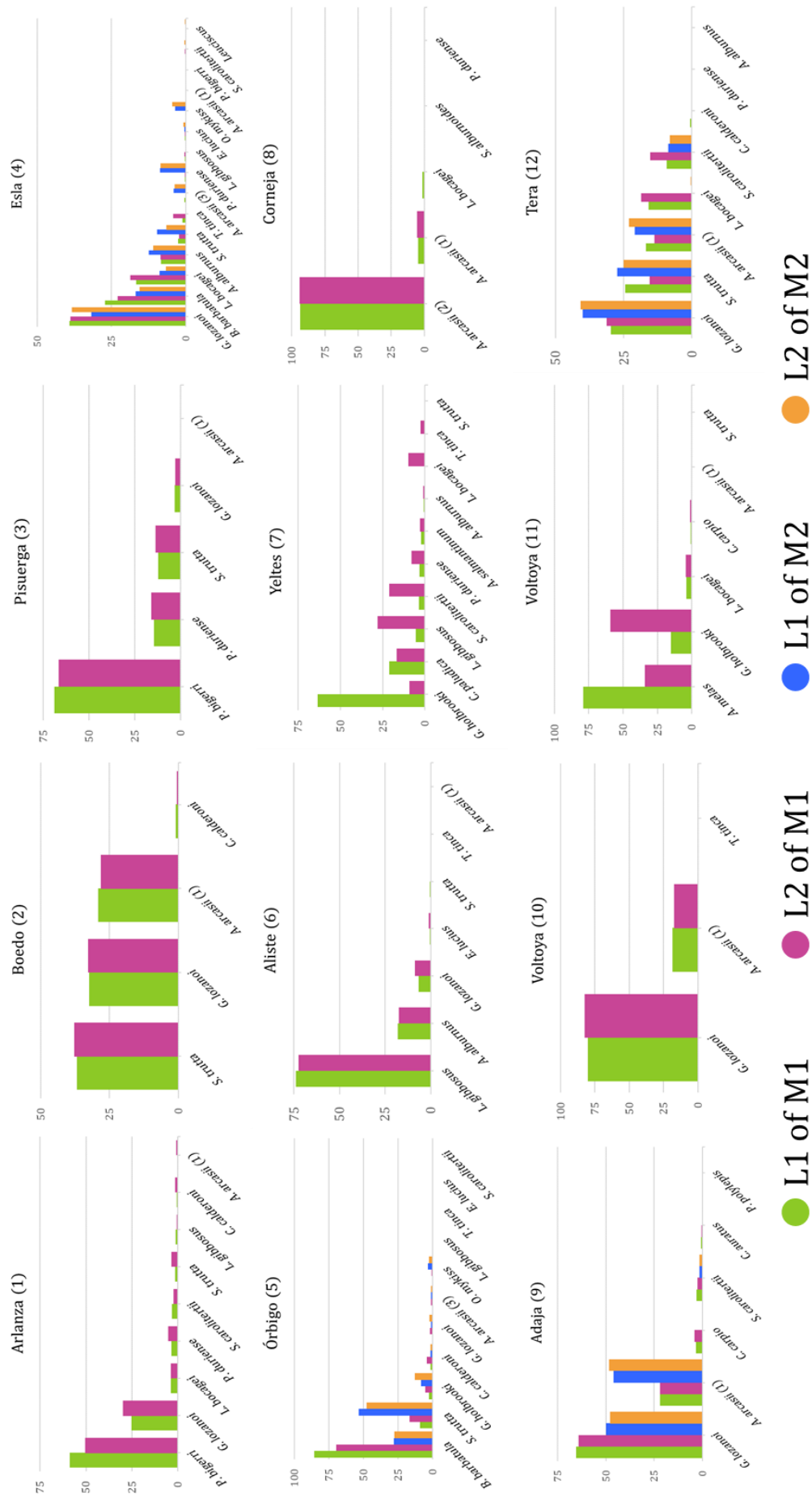


FIGURE 1. Percentage of species detected for each liter (L) of water sampled at each locality. For localities sampled in summer (M1), L1 is in green and L2, in pink; and for those in autumn (M2), L1 is in blue and L2, in orange.

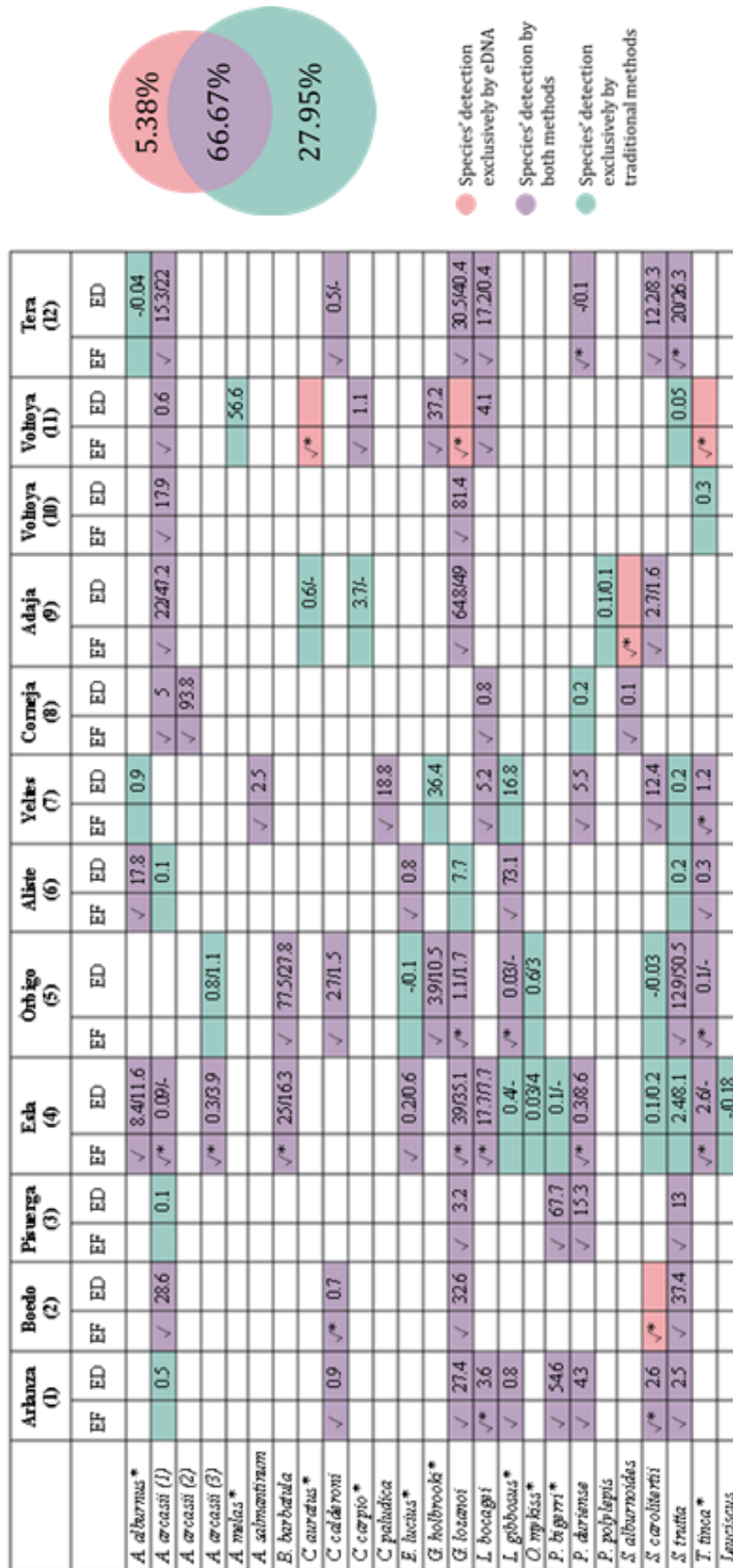


TABLE 3. Summary of the freshwater fish species detected using electrofishing (EF) and eDNA metabarcoding (ED). The check mark (✓) indicates that the species was detected by electrofishing in the sampling campaign conducted for the present study; The check mark with an asterisk (✓*) in the EF section indicates that the species was detected in the samples from 2009-2010 but not in those of the present study. The numbers in the ED section refer to the mean relative abundance of readings obtained from L1 and L2 for each sampling point. For locations that were sampled in summer (M1) and fall (M2), the mean relative abundance of readings for each season is indicated as M1/M2. The pink color indicates that the species has only been detected by EF. The blue color indicates that the species has only been detected by ED. Purple indicates that the species has been detected by both methods. Allochthonous species are marked with an asterisk.

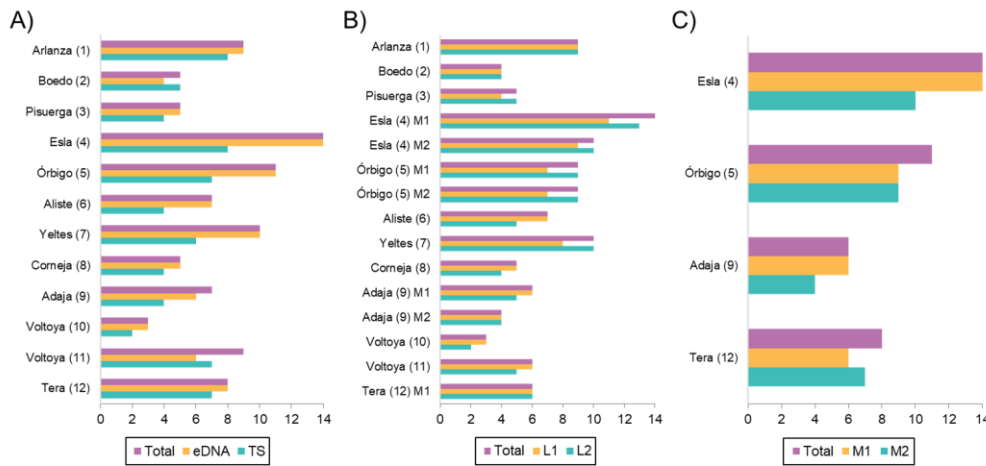


FIGURE 4. Comparison of the number of species detected for each detection method (eDNA and traditional methods; TS; A), for each liter of water sampled at each locality (L1 and L2; B) and for each station (M1 and M2; C).

Basin, which is adjacent to the southern part of the Duero Basin. Therefore, excluding marine species, the *Leuciscus* genus and the species *L. rifensis*, a total of 22 species were detected across all sampling localities.

After the reads were filtered for quality and taxonomically assigned, only the blanks collected from the Arlanza and Tera rivers during summer had reads assigned to fish (Figure 2). For these two sites, the number of the final assigned reads were 31,321 and 16,526, respectively. In both cases, the final all these reads were associated with a single species or genus: *Phoxinus* for Arlanza and *Salmo* and *S. trutta* for Tera. These species were recently detected, or have been detected in the past, in the corresponding river by electrofishing. In addition, *S. trutta* was detected in the Tera River in autumn by eDNA metabarcoding. Therefore, these species were not excluded from the L1 and L2 samples of each site.

The blanks were likely somehow contaminated while at these localities.

Detailed information on the composition of the ichthyofauna at each sampling locality based on past and recent electrofishing of the sites or on eDNA metabarcoding are provided in Table 3 and Figure 4. A total of 22 freshwater fish species was detected by eDNA, and 19 by electrofishing. Considering all cases, 27.95% were exclusively by eDNA, 5.37% were exclusively by traditional methods and 66.66% were by both methods (Table 3). However, all the species detected by only the traditional method had been detected in the past but not for the present study. Also, in all cases, except Boedo (locality 2) and Voltoya (locality 11), a higher number of species was detected by eDNA metabarcoding than by electrofishing (Figure 5A).

For 75% of the sampling localities, the number of species detected per liter varied by a maximum of two species (Figure 5B). The percentage of relative reads of the cases where a species was detected in only one of the two liters of the same sample point was always lower than 0.55% except for two cases (1.29% and 2.48%) (Table S3). For the four localities that were sampled in both summer and autumn, the number of species detected between stations varied by 1, 2 or 4 species for Tera, Adaja and Esla, respectively (Figure 5C). Likewise, the mean relative abundance of the reads of all cases in which a species was detected in only one of the two seasons (M1 or M2) for the same locality ranged between 0.03% and 0.91%, except for two cases that presented higher values (2.6% and 3.70%) (Table 3).

Allochthonous species

Without considering the two species of undetermined origin, *T. tinca* and *G. lozanoi*, all localities had a high number of allochthonous species except Boedo, Corneja and Voltoya rivers, which only presented

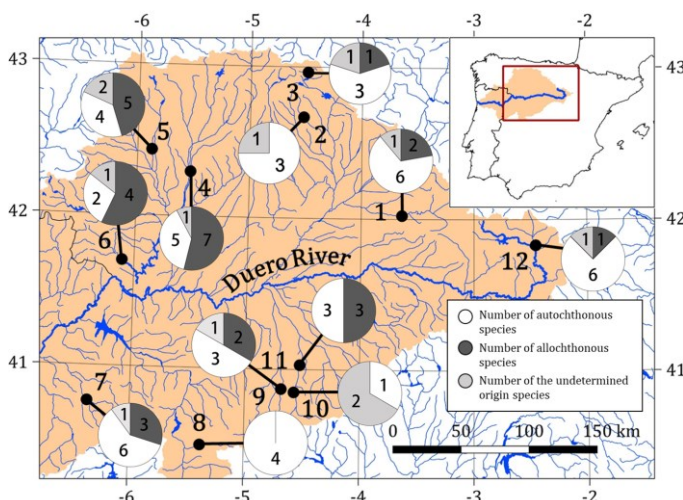


FIGURE 5. Number of autochthonous (white) and allochthonous (dark grey) species present in the different sampling localities. Species of undetermined origin (*G. lozanoi* and *T. tinca*) are indicated in light grey.

autochthonous species. More allochthonous than autochthonous species were detected in the northwestern Duero Basin, specifically in the Esla, Órbigo and Aliste rivers (Figure 6). Moreover, the presence of the allochthonous species has not been previously reported in many of the rivers studied (Table 3).

Discussion

In recent years, advances in next generation sequencing (NGS) technologies have provided very useful tools for a variety of approaches in biodiversity conservation (Tan et al., 2019). Specifically, eDNA metabarcoding has proven to be a powerful non-invasive tool for monitoring the biodiversity of freshwater fishes without the need for morphological identification (Roy et al., 2018; Bylemans et al., 2019; Goutte et al., 2020).

We tested the efficiency of using eDNA metabarcoding with the *12S* marker to describe the composition of the freshwater fishes in the Duero Basin, the largest river basin in Spain. Our survey included all the autochthonous ichthyofauna of this basin in Spain except for the species *C. vettonica*. Recent eDNA metabarcoding studies have been performed for a variety of fauna in Spain (Fernández et al., 2019; Suarez-Mendez et al., 2020; Bellisario et al., 2021), however, to our knowledge, our study is the first to focus on determining the ichthyofaunal composition on such a large geographic scale and to include a large number of freshwater fish species.

Traditional methods versus eDNA metabarcoding

The eDNA metabarcoding approach has a higher capacity to detect the presence of species compared with traditional methods (Shaw et al., 2016; Yamamoto et al., 2017; Cilleros et al., 2019; Goutte et al., 2020; Czeglédi et al., 2021). It is also more efficient (Civade et al., 2016) and able to detect more species in freshwater systems with fewer than 100 species (McElroy et al., 2020). Consistent with these previous studies, we detected more species through eDNA metabarcoding than through traditional methods alone (83.33%). However, as we are detecting DNA that is present in the river and not individuals, these results must be interpreted with caution. Sometimes, DNA detected in one place may have originated from individuals in another due to indirect transport, giving rise false positives and a mischaracterization of the species composition of a given river (Ficetola et al., 2015; Valentini et al., 2015; Pont et al., 2018). In this context, false positives may arise mainly in two ways. First, the DNA detected could come from organisms located

upstream. It has been shown that DNA can persist in water for several kilometers (Deiner & Altermatt, 2014; Civade et al., 2016; Pont et al., 2018). In our study, this phenomenon could explain the presence of the DNA of *A. arcasii* in the Arlanza or the Órbigo rivers. Although this species has never been recorded for either of these sampled localities, there are records of its presence in stretches upstream of these rivers (Doadrio et al., 2011; Doadrio et al., 2021). Second, false positives may be a consequence of DNA transported by other vectors such as bird excrements or human waste (Deiner & Altermatt, 2014; Merkes et al., 2014; Shaw et al., 2016). This explanation evidently accounts for the presence of the DNA of the marine species (i.e., *D. labrax*, *L. aspera*, *S. aurata*, *S. maxima*, *S. pilchardus*, *T. alalunga*, *T. japonicus*, *T. trachurus*, *C. conger* and *Hippoglossus* sp.) in the Esla, Órbigo, Adaja and Tera rivers as all of these species are commonly consumed as food in Spain. This explanation is also plausible, though less evident, for the presence of the DNA of the rainbow trout, *O. mykiss*, an exotic species in Spanish rivers that is also used as food, in the Esla and Órbigo rivers. The DNA of this species may be present due to both the presence of the species and the indirect transport of its DNA to these localities. Notably, these rivers are near large urban centers, increasing the probability of finding eDNA derived from human waste. It is likely eDNA of species consumed as food could lead to false negatives (non-detection of taxa) in an analysis if they are much more prevalent than those of a species inhabiting the studied locality, especially if it is present at a low density.

Only 5.38% of the total detections were made exclusively by traditional methods. Even though a higher ratio of species is often detected by eDNA metabarcoding than by traditional methods, false negatives do occur under this approach (Valentini et al., 2016; Sigsgaard et al., 2020). False negatives may be associated with various factors such as the life cycle of a species (Dostine et al., 2013; de Souza et al., 2016), the abundance of a species (Valentini et al., 2016; Clusa et al., 2017; Cilleros et al., 2019; Lawson Handley et al., 2019), river system dynamics (Lawson Handley et al., 2019), inappropriate markers (Shaw et al., 2016; Collins et al., 2019) or biases in amplification (Ficetola et al., 2015; Sigsgaard et al., 2020). In our case, the non-detection of taxa could be due to the absence of the species in the system since all the non-detections had records from the past but were not captured in the present work. The majority of the detections made exclusively through traditional methods were for Juarros del Voltoya Reservoir

(accounting for 60% of the total). This result is not unexpected as electric fishing was carried out in the vicinity of the reservoir but not in it. Detection via eDNA metabarcoding has been shown to be, in some cases, less efficient in lakes and reservoirs than in river systems (Civade et al., 2016). A relatively rapid decline in eDNA concentrations beyond its source has been observed in some lotic systems (Shogren et al., 2017; Deutschmann et al., 2019; Wood et al., 2020), as has a greater spatial structure of eDNA in summer compared with winter due to differences in the degree of water mixing between seasons (Lawson Handley et al., 2019).

Sample collection and rare species

The total volume of water collected for eDNA metabarcoding samples varies from a few milliliters up to 50 liters (Evans et al., 2016; Cilleros et al., 2019). In a recent review on different optimal methodologies, it was noted that one to two liters of water was enough to obtain a representative sample of biodiversity (Shu et al., 2020). In our case, we observed that 75% of the samples showed a difference in the number of species detected per liter for the same site, suggesting that one liter may be insufficient to fully capture the biodiversity of a site. We therefore recommend that a minimum sample volume of two liters of water. With respect to the samples taken at two different seasons (i.e., Esla, Órbigo, Adaja and Tera), we also observed a difference in the number of species detected in 75% of the samples. Differences in species detected at the same locality but in different seasons have been related to the seasonal movements of species (De Souza et al., 2016; Stoeckle et al., 2017). Therefore, we recommend sampling in multiple seasons in order to obtain a more complete representation of the biodiversity of a locality.

Although eDNA reads cannot be used as a general measure of abundance, recent evidence suggests that eDNA concentration could be an indicator of abundance and/or biomass for fish stock assessments (Stoeckle et al., 2017; Rourke et al., 2021). Consistent with this, it has been shown that less sampling effort is required to detect abundant species compared with less abundant ones (Thomsen et al., 2012; Kelly et al., 2014), and that species present at a low abundance have greater stochasticity in detection through eDNA metabarcoding (Shaw et al., 2016). All the species that were detected in either only one of the two liters (L1 or L2) or one of the two seasons (M1 or M2) were represented by a very low percentage of the relative reads. These species generally coincide with the ones that were not detected through traditional methods. In our case, the stochasticity in the detection of some

species through eDNA is likely explained by the low abundance of these species in the sampled rivers. Although we observed a correlation between species abundance and eDNA read number, multiple biotic and abiotic factors influence this relationship (Rourke et al., 2021). Therefore, an in-depth study of these rivers and species would be necessary to identify the associated factors.

The 12S rRNA gene as a marker for eDNA metabarcoding studies

The 12S rRNA gene fragment amplified with the MiFish primers (Miya et al., 2015) had sufficient taxonomic resolution to distinguish between the Iberian freshwater fish species studied in the present work. The percentage of similarity both within and between species was highly variable. Analysis of this marker revealed a higher interpopulation genetic variability than expected within *A. arcasii*. This species has a strong population genetic structure in the Duero Basin: three OTUs based on the *MT-CYB* marker had been previously identified for the species (Robalo et al., 2006; Doadrio et al., 2021), all three of which were recovered with the 12S fragment in our analysis. These three groups presented a relatively low percentage of intraspecific genetic similarity (96%–97%). As the threshold percentage of similarity used in this study was 99% and 97%, the assignment of these species could have been confusing if the population structure of the species had not been known or considered. Therefore, we emphasize the importance of incorporating previous genetic studies when generating a reference database, to consider intraspecific variation and reduce taxonomic assignment errors. Finally, 1.13% and 1.54% of the reads for M1 and M2, respectively, were assigned to Actinopterygii but not to a particular species. One advantage of eDNA metabarcoding over traditional methods is the possibility of reanalyzing archived samples. Therefore, the development of a broader reference database in the future may reveal species that were not detected by previous studies (Singer et al., 2019; Schenekar et al., 2020).

A surprising finding was the detection of the DNA of *P. polylepis* in the Adaja River, where *A. arcasii* was also detected by both eDNA metabarcoding and electrofishing. Within Spain, *P. polylepis* is naturally distributed in the Tagus basin (Doadrio et al., 2011), and was introduced into the Jucar and Segura basins as a result of the Tajo–Segura transfer (Doadrio et al., 2011). However, there are no records of the presence of *P. polylepis* in the Duero Basin. Stream captures between rivers in the southern part of the Duero Basin and those in the northern part of the Tajo Basin have

been previously reported (Arenillas, 1994; Casas-Sainz & De Vicente, 2009), leading to a movement of the ichthyofauna in these regions (e.g., *Squalius carolitertii* or *Cobitis calderoni*; Perdices & Doadrio, 2000; Perea et al., 2021). Concretely, the current morphology of the Adaja River has been related to a possible stream piracy (Antón et al., 2014). The expansion of *P. polylepis* towards the Duero Basin could also have been promoted by its possible use as live bait. Therefore, it is probable that the species is or was present in the river but that it has not yet been detected by traditional methods as a consequence of stream capture in the past or human translocation. In some rivers, *P. duriense* (in the Arlanza and Pisuerga rivers) and *A. arcasii* (in Corneja River) were detected by electrofishing, however, the eDNA analysis revealed the presence of the mtDNA of *A. arcasii* also in the Arlanza and Pisuerga rivers, and that of *P. duriense* in the Corneja River. Evidence of hybridization between some species of the genera *Achondrostoma* and *Pseudochondrostoma* has been found for some populations within the Iberian Peninsula (Elvira et al., 1990; Corral-Lou et al., 2021). Given that mitochondrial DNA is inherited maternally in most cases (Giles et al., 1980), it is not possible to distinguish hybrids from their maternal species when using eDNA approaches with a mitochondrial marker (Valentini et al., 2015). The same situation arises for the species *C. carpio* and *C. auratus*: they are well known to hybridize (Haynes et al., 2012) and both their DNAs were found in the Adaja River. Therefore, it is likely that an hybridization zones exist for these species in these rivers.

In another interesting finding, we detected the mtDNA of *Leuciscus* in the Esla River. The only species of the genus *Leuciscus* present in the Iberian Peninsula is *L. aspius*, which was introduced to Muga Basin, NE Spain in 2017 (Merciai et al., 2018). This species has also been widely introduced in other rivers in Europe (Schweyer et al., 1991; Dönni & Freyhof, 2002; Kottelat & Freyhof, 2007; Nocita & Zerunian, 2007). Therefore, it is possible that this species is now present in the Esla River in the Duero Basin. However, a low level of genetic differentiation was observed between *L. aspius* and *A. alburnus* (97.7%), a species known to be present in the Esla River, and the reads identified as *Leuciscus* may instead be *A. alburnus*.

Conservation in Duero Basin

The presence of invasive species is one of the main threats to native freshwater fish fauna (Dudgeon et al.,

2006; Dudgeon, 2019). Early detection of invasive species is, therefore, essential for the conservation of biological diversity (Chornesky & Randall, 2003; Rice et al., 2012; Essl et al., 2020; Larson et al., 2020). However, this task can sometimes be hampered by the great effort required to detect a small number of individuals in large aquatic systems (Jerde et al., 2011). The high sensitivity of eDNA metabarcoding to detect species at a low abundance makes it an effective tool for locating potentially invasive rare aquatic or semi-aquatic species (Jerde et al., 2011; Goldberg et al., 2015; McKelvey et al., 2016; Suarez-Menendez et al., 2020; Pukk et al., 2021). Through eDNA metabarcoding, we detected the first presence of different invasive exotic species at six of the sampling localities (i.e., Esla, Órbigo, Yeltes, Adaja, Voltoya and Tera). This finding indicates the expansion of these species within the basin, and, in some of these cases (Esla, Órbigo and Aliste), the ratio of invasive to native species was higher. Therefore, the use of eDNA metabarcoding for monitoring the freshwater fish composition within the Iberian Peninsula appears to be a useful tool not only for the early detection of invasive species, particularly those that are rare or elusive, but also for assessing the geographic distribution of the invasive species. Thus, an analysis and development of conservation and management strategies can be carried out considering these data, which will, in turn, influence the decision-making process and policy.

Acknowledgments

We want to thank P. Garzón, I. Doadrio Jr, J.L. González and G. González for collecting almost every sample from 2009 to 2010 under the project “Basis for the monitoring of Spanish Freshwater Fishes”, supported by the Ministerio de Medio Ambiente y Medio Rural y Marino (MARM), and C. Marcos, J.C Velaco and G. González for their help in the project Life13 nat/es/000772. We greatly thank L. Alcaraz and M. Casal for laboratory and field assistance. We are grateful for the valuable suggestions and English editing performed by M. Modrell. This study was also supported by a management commission funded by the Duero Hydrographic Confederation in Spain (Análisis y estudios filogeográficos y de variabilidad genética de endemismos ibéricos de ciprínidos: Life13 nat/es/000772 “Actuaciones para la protección y conservación de ciprínidos ibéricos de interés comunitario”) and by grant IND2017/AMB-7699 to AC-L, funded by the Community of Madrid (Spain) through its Industrial Doctorate programme.

References

- Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data.
- Antón, L., De Vicente, G., Muñoz-Martín, A., & Stokes, M. (2014). Using river long profiles and geomorphic indices to evaluate the geomorphological signature of continental scale drainage capture, Duero basin (NW Iberia). *Geomorphology*, 206, 250-261.
- Arenillas, M. P. (1994). Los ríos de España. *Op. Rev. Col. Ingenieros Caminos, Canales y Puertos de Cataluña, Comunidad Valenciana, Extremadura*, 28, 4-11.
- Balian, E. V., Segers, H., Martens, K., & Lévêque, C. (2007). The freshwater animal diversity assessment: an overview of the results. *Freshwater animal diversity assessment*, 627-637.
- Bellisario, B., Fais, M., Duarte, S., Vieira, P. E., Canchaya, C., & Costa, F. O. (2021). The network structure of intertidal meiofaunal communities from environmental DNA metabarcoding surveys in Northwest Iberia. *Aquatic Sciences*, 83(4), 1-14.
- Bonar, S. A., Hubert, W. A., & Willis, D. W. (2009). Standard methods for sampling North American freshwater fishes.
- Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M., & Furlan, E. M. (2019). A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA*, 1(4), 402-414.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, 7(5), 335-336.
- Casal-Lopez, M., Perea, S., Yahyaoui, A., & Doadrio, I. (2015). Taxonomic review of the genus *Luciobarbus* Heckel, 1843 (Actinopterygii, Cyprinidae) from Northwestern Morocco with the description of three new species.
- Casal-López, M., & Doadrio, I. (2018). The Messinian imprint on the evolution of freshwater fishes of the genus *Luciobarbus* Heckel, 1843 (Teleostei, Cyprinidae) in the western Mediterranean. *Journal of biogeography*, 45(7), 1593-1603.
- Casas-Sainz, A. M., & De Vicente, G. (2009). On the tectonic origin of Iberian topography. *Tectonophysics*, 474(1-2), 214-235.
- Chornesky, E. A., & Randall, J. M. (2003). The threat of invasive alien species to biological diversity: setting a future course. *Annals of the Missouri Botanical Garden*, 67-76.
- Cilleros, K., Valentini, A., Allard, L., Dejean, T., Etienne, R., Grenouillet, G., ... & Brosse, S. (2019). Unlocking biodiversity and conservation studies in high-diversity environments using environmental DNA (eDNA): A test with Guianese freshwater fishes. *Molecular ecology resources*, 19(1), 27-46.
- Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J. C., Bonin, A., ... & Pont, D. (2016). Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. *PloS one*, 11(6), e0157366.
- Clusa, L., Miralles, L., Basanta, A., Escot, C., & Garcia-Vazquez, E. (2017). eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula. *PloS one*, 12(11), e0188126.
- Collins, R. A., Bakker, J., Wangenstein, O. S., Soto, A. Z., Corrigan, L., Sims, D. W., ... & Mariani, S. (2019). Non-specific amplification compromises environmental DNA metabarcoding with COI. *Methods in Ecology and Evolution*, 10(11), 1985-2001.
- Common, M. S., & Norton, T. W. (1994). Biodiversity, natural resource accounting and ecological monitoring. In *Biodiversity Conservation* (pp. 82-105). Springer, Dordrecht.
- Corral-Lou, A., Perea, S., Aparicio, E., & Doadrio, I. (2019). Phylogeography and species delineation of the genus *Phoxinus* Rafinesque, 1820 (Actinopterygii: Leuciscidae) in the Iberian Peninsula. *Journal of Zoological Systematics and Evolutionary Research*, 57(4), 926-941.
- Corral-Lou, A., Perea, S., & Doadrio, I. (2021). High genetic differentiation in the endemic and endangered freshwater fish *Achondrostoma salmantinum* Doadrio and Elvira, 2007 from Spain, as revealed by mitochondrial and SNP markers. *Conservation Genetics*, 22(4), 585-600.
- Corral-Lou, A., Perea, S., Perdices, A., & Doadrio, I. (2022). Quaternary geomorphological and climatic changes associated with the diversification of Iberian freshwater fishes: The case of the genus *Cobitis* (Cypriniformes, Cobitidae). *Ecology and Evolution*, 12(3), e8635.
- Coutant, O., Cantera, I., Cilleros, K., Dejean, T., Valentini, A., Muriene, J., & Brosse, S. (2021). Detecting fish assemblages with environmental DNA: Does protocol matter? Testing eDNA metabarcoding method robustness. *Environmental DNA*, 3(3), 619-630.
- Czeglédi, I., Sály, P., Specziár, A., Preiszner, B., Szalóky, Z., Maroda, A., ... & Erős, T. (2021).

- Congruency between two traditional and eDNA-based sampling methods in characterising taxonomic and trait-based structure of fish communities and community-environment relationships in lentic environment. *Ecological Indicators*, 129, 107952.
- De Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PloS one*, 11(10), e0165273.
- Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. *Biology letters*, 10(9), 20140562.
- Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PloS one*, 9(2), e88786.
- Deutschmann, B., Müller, A. K., Hollert, H., & Brinkmann, M. (2019). Assessing the fate of brown trout (*Salmo trutta*) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. *Science of the Total Environment*, 655, 321-327.
- Doadrio, I., & Perdices, A. (2005). Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, *Cobitidae*) based on cytochrome b sequence data. *Molecular Phylogenetics and Evolution*, 37(2), 484-493.
- Doadrio, I., Perea, S., Garzón-Heydt, P., & González, J. L. (2011). Ictiofauna continental española. *Bases para su seguimiento. DG Medio Natural y Política Forestal, MARM, Madrid, Spain*.
- Doadrio, I., Perea, S., & Corral-Lou, A. (2021). Caracterización genética e identificación de Unidades de Conservación en peces endémicos de interés comunitario.
- Dönni, W., Freyhof, J., Friedl, C., & Suisse. Division protection des eaux et pêche. (2002). *Einwanderung von Fischarten in die Schweiz: Rheineinzugsgebiet*. Bundesamt für Umwelt Wald und Landschaft (BUWAL).
- Dostine, P. L., Reynolds, S. J., Griffiths, A. D., & Gillespie, G. R. (2013). Factors influencing detection probabilities of frogs in the monsoonal tropics of northern Australia: implications for the design of monitoring studies. *Wildlife Research*, 40(5), 393-402.
- Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., ... & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological reviews*, 81(2), 163-182.
- Dudgeon, D. (2019). Multiple threats imperil freshwater biodiversity in the Anthropocene. *Current Biology*, 29(19), R960-R967.
- Elvira, B., Rincón, P. A., & Velasco, J. C. (1990). *Chondrostoma polylepis* Steindachner x *Rutilus lemmingii* (Steindachner)(Osteichthyes, Cyprinidae), a new natural hybrid from the Duero River basin, Spain. *Journal of Fish Biology*, 37(5), 745-754.
- Essl, F., Latombe, G., Lenzner, B., Pagad, S., Seebens, H., Smith, K., ... & Genovesi, P. (2020). The Convention on Biological Diversity (CBD)'s Post-2020 target on invasive alien species—what should it include and how should it be monitored?. *NeoBiota*, 62, 99.
- Evans, N. T., Olds, B. P., Renshaw, M. A., Turner, C. R., Li, Y., Jerde, C. L., ... & Lodge, D. M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. *Molecular ecology resources*, 16(1), 29-41.
- Fernández, S., Rodríguez-Martínez, S., Martínez, J. L., García-Vazquez, E., & Ardura, A. (2019). How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). *Environmental DNA*, 1(4), 385-401.
- Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguët-Covex, C., De Barba, M., ... & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular ecology resources*, 15(3), 543-556.
- Foran, D. R. (2006). Relative degradation of nuclear and mitochondrial DNA: an experimental approach. *Journal of forensic sciences*, 51(4), 766-770.
- Geist, J. (2011). Integrative freshwater ecology and biodiversity conservation. *Ecological Indicators*, 11(6), 1507-1516.
- Giles, R. E., Blanc, H., Cann, H. M., & Wallace, D. C. (1980). Maternal inheritance of human mitochondrial DNA. *Proceedings of the National academy of Sciences*, 77(11), 6715-6719.
- Goldberg, C. S., Strickler, K. M., & Pilliod, D. S. (2015). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation*, 183, 1-3.
- Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., ... & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic

- p>species.
- Methods in ecology and evolution*
- , 7(11), 1299-1307.
- Gonzalez, E. G., Cunha, C., Ghanavi, H. R., Oliva-Paterna, F. J., Torralva, M., & Doadrio, I. (2018). Phylogeography and population genetic analyses in the Iberian toothcarp (*Aphanius iberus* Valenciennes, 1846) at different time scales. *Journal of Heredity*, 109(3), 253-263.
- Goodwin, C. E., Hodgson, D. J., Bailey, S., Bennie, J., & McDonald, R. A. (2018). Habitat preferences of hazel dormice *Muscardinus avellanarius* and the effects of tree-felling on their movement. *Forest ecology and management*, 427, 190-199.
- Goutte, A., Molbert, N., Guérin, S., Richoux, R., & Rocher, V. (2020). Monitoring freshwater fish communities in large rivers using environmental DNA metabarcoding and a long-term electrofishing survey. *Journal of Fish Biology*, 97(2), 444-452.
- Haynes, G. D., Gongora, J., Gilligan, D. M., Grewe, P., Moran, C., & Nicholas, F. W. (2012). Cryptic hybridization and introgression between invasive *Cyprinid* species *Cyprinus carpio* and *Cyprinus auratus* in Australia: implications for invasive species management. *Animal Conservation*, 15(1), 83-94.
- Hinlo, R., Furlan, E., Sutor, L., & Gleeson, D. (2017). Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. *Management of Biological Invasions*, 8(1), 89.
- Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation letters*, 4(2), 150-157.
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30(4), 772-780.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647-1649.
- Kelly, R. P., Port, J. A., Yamahara, K. M., Martone, R. G., Lowell, N., Thomsen, P. F., ... & Crowder, L. B. (2014). Harnessing DNA to improve environmental management. *Science*, 344(6191), 1455-1456.
- Kottelat, M., & Freyhof, J. (2007). Handbook of European Freshwater Fishes. Cornol (Switzerland) and Berlin (Germany). *Published by the authors*.
- Larson, E. R., Graham, B. M., Achury, R., Coon, J. J., Daniels, M. K., Gambrell, D. K., ... & Suarez, A. V. (2020). From eDNA to citizen science: emerging tools for the early detection of invasive species. *Frontiers in Ecology and the Environment*, 18(4), 194-202.
- Lawson Handley, L., Read, D. S., Winfield, I. J., Kimbell, H., Johnson, H., Li, J., ... & Hänfling, B. (2019). Temporal and spatial variation in distribution of fish environmental DNA in England's largest lake. *Environmental DNA*, 1(1), 26-39.
- Lepetz, V., Massot, M., Schmeller, D. S., & Clobert, J. (2009). Biodiversity monitoring: some proposals to adequately study species' responses to climate change. *Biodiversity and Conservation*, 18(12), 3185-3203.
- Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21), 2957-2963.
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal*, 17(1), 10-12.
- McElroy, M. E., Dressler, T. L., Titcomb, G. C., Wilson, E. A., Deiner, K., Dudley, T. L., ... & Jerde, C. L. (2020). Calibrating environmental DNA metabarcoding to conventional surveys for measuring fish species richness. *Frontiers in Ecology and Evolution*, 276.
- McKelvey, K. S., Young, M. K., Knotek, L., Carim, K. J., Wilcox, T. M., Padgett-Stewart, T. M., & Schwartz, M. K. (2016). Sampling large geographic areas for rare species using environmental DNA (eDNA): a study of bull trout occupancy in western Montana. *J Fish Biol*, 88(3), 1215-1222.
- Merciai, R., Almeida Real, D., Aparicio, E., Cruset, E., Fuentes, M. Á., Pou i Rovira, Q., ... & García-Berthou, E. (2018). First record of the asp *Leuciscus aspius* introduced into the Iberian Peninsula. © *Limnetica*, 2018, vol. 37, núm. 2, p. 341-344.
- Merkes, C. M., McCalla, S. G., Jensen, N. R., Gaikowski, M. P., & Amberg, J. J. (2014). Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. *PLoS One*, 9(11), e113346.
- Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., & Kawabata, Z. I. (2012). Surveillance of fish species composition using environmental DNA. *Limnology*, 13(2), 193-197.
- Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., ... & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society open science*, 2(7), 150088.

- Nocita, A., & Zerunian, S. (2007). L'ittiofauna aliena nei fiumi e nei laghi d'Italia. *Biologia ambientale*, 21(2), 93-96.
- Perdices, A., & Doadrio, I. (2000). Diversification patterns in *Cobitis calderoni* (Osteichthyes: Cobitidae) and relationships with some *Cobitis* lineages. *FOLIA ZOOLOGICA-PRAHA*, 49(SUPP/1), 45-54.
- Perea, S., & Doadrio, I. (2015). Phylogeography, historical demography and habitat suitability modelling of freshwater fishes inhabiting seasonally fluctuating Mediterranean river systems: a case study using the Iberian cyprinid *Squalius valentinus*. *Molecular Ecology*, 24(14), 3706-3722.
- Perea, S., Cobo-Simon, M., & Doadrio, I. (2016). Cenozoic tectonic and climatic events in southern Iberian Peninsula: Implications for the evolutionary history of freshwater fish of the genus *Squalius* (Actinopterygii, Cyprinidae). *Molecular Phylogenetics and Evolution*, 97, 155-169.
- Perea, S., Sousa-Santos, C., Robalo, J., & Doadrio, I. (2021). Historical biogeography of the Iberian Peninsula: multilocus phylogeny and ancestral area reconstruction for the freshwater fish genus *Squalius* (Actinopterygii, Leuciscidae). *Journal of Zoological Systematics and Evolutionary Research*, 59(4), 858-886.
- Pont, D., Rocle, M., Valentini, A., Civade, R., Jean, P., Maire, A., ... & Dejean, T. (2018). Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation. *Scientific reports*, 8(1), 1-13.
- Pukk, L., Kanefsky, J., Heathman, A. L., Weise, E. M., Nathan, L. R., Herbst, S. J., ... & Robinson, J. D. (2021). eDNA metabarcoding in lakes to quantify influences of landscape features and human activity on aquatic invasive species prevalence and fish community diversity. *Diversity and Distributions*, 27(10), 2016-2031.
- Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA—a review of eDNA as a survey tool in ecology. *Journal of applied ecology*, 51(5), 1450-1459.
- Rice, J., Moksness, E., Attwood, C., Brown, S. K., Dahle, G., Gjerde, K. M., ... & Westlund, L. (2012). The role of MPAs in reconciling fisheries management with conservation of biological diversity. *Ocean & coastal management*, 69, 217-230.
- Robalo, J. I., Santos, C. S., Almada, V. C., & Doadrio, I. (2006). Paleobiogeography of two Iberian endemic cyprinid fishes (*Chondrostoma arcasii*-*Chondrostoma macrolepidotus*) inferred from mitochondrial DNA sequence data. *Journal of Heredity*, 97(2), 143-149.
- Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584.
- Rourke, M. L., Fowler, A. M., Hughes, J. M., Broadhurst, M. K., DiBattista, J. D., Fielder, S., ... & Furlan, E. M. (2021). Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA*.
- Roy, M., Belliveau, V., Mandrak, N. E., & Gagné, N. (2018). Development of environmental DNA (eDNA) methods for detecting high-risk freshwater fishes in live trade in Canada. *Biological Invasions*, 20(2), 299-314.
- Sales, N. G., Wangenstein, O. S., Carvalho, D. C., Deiner, K., Præbel, K., Coscia, I., ... & Mariani, S. (2021). Space-time dynamics in monitoring neotropical fish communities using eDNA metabarcoding. *Science of the Total Environment*, 754, 142096.
- Sato, Y., Miya, M., Fukunaga, T., Sado, T., & Iwasaki, W. (2018). MitoFish and MiFish pipeline: a mitochondrial genome database of fish with an analysis pipeline for environmental DNA metabarcoding. *Molecular biology and evolution*, 35(6), 1553-1555.
- Schenekar, T., Schletterer, M., Lecaudey, L. A., & Weiss, S. J. (2020). Reference databases, primer choice, and assay sensitivity for environmental metabarcoding: Lessons learnt from a re-evaluation of an eDNA fish assessment in the Volga headwaters. *River Research and Applications*, 36(7), 1004-1013.
- Schweyer, J. B., Allardi, J., & Dorson, M. (1991). Note ichtyologique: capture dans le Rhin de représentants des espèces *Aspius aspius* (Linné 1758) et *Vimba vimba* (Linné 1758). *Bulletin Français de la Pêche et de la Pisciculture*, (320), 38-42.
- Shaw, J. L., Clarke, L. J., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., & Cooper, A. (2016). Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, 197, 131-138.
- Shogren, A. J., Tank, J. L., Andruszkiewicz, E., Olds, B., Mahon, A. R., Jerde, C. L., & Bolster, D. (2017). Controls on eDNA movement in streams: Transport, retention, and resuspension. *Scientific reports*, 7(1), 1-11.

- Shu, L., Ludwig, A., & Peng, Z. (2020). Standards for methods utilizing environmental DNA for detection of fish species. *Genes*, 11(3), 296.
- Sigsgaard, E. E., Jensen, M. R., Winkelmann, I. E., Møller, P. R., Hansen, M. M., & Thomsen, P. F. (2020). Population-level inferences from environmental DNA—Current status and future perspectives. *Evolutionary Applications*, 13(2), 245-262.
- Singer, G. A. C., Fahner, N. A., Barnes, J. G., McCarthy, A., & Hajibabaei, M. (2019). Comprehensive biodiversity analysis via ultra-deep patterned flow cell technology: a case study of eDNA metabarcoding seawater. *Scientific reports*, 9(1), 1-12.
- Snyder, D. E. (2003). Invited overview: conclusions from a review of electrofishing and its harmful effects on fish. *Reviews in fish biology and fisheries*, 13(4), 445-453.
- Stoeckle, M. Y., Soboleva, L., & Charlop-Powers, Z. (2017). Aquatic environmental DNA detects seasonal fish abundance and habitat preference in an urban estuary. *PloS one*, 12(4), e0175186.
- Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*, 29(1), 344-358.
- Suarez-Menendez, M., Planes, S., Garcia-Vazquez, E., & Ardura, A. (2020). Early alert of biological risk in a coastal lagoon through eDNA metabarcoding. *Frontiers in Ecology and Evolution*, 8, 9.
- Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental dna. *Molecular ecology*, 21(8), 1789-1793.
- Takeuchi, A., Sado, T., Gotoh, R. O., Watanabe, S., Tsukamoto, K., & Miya, M. (2019). New PCR primers for metabarcoding environmental DNA from freshwater eels, genus *Anguilla*. *Scientific Reports*, 9(1), 1-11.
- Tan, M. P., Wong, L. L., Razali, S. A., Afiqah-Aleng, N., Mohd Nor, S. A., Sung, Y. Y., ... & Danish-Daniel, M. (2019). Applications of next-generation sequencing technologies and computational tools in molecular evolution and aquatic animals conservation studies: a short review. *Evolutionary Bioinformatics*, 15, 1176934319892284.
- Teresita, M.P. (2020). 12S MitoFish Reference Set For The RDP Classifier (Version v1.0.1). Zenodo. <http://doi.org/10.5281/zenodo.4741464>
- Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., ... & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular ecology*, 21(11), 2565-2573.
- Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biological conservation*, 183, 4-18.
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular ecology*, 25(4), 929-942.
- Wood, S. A., Biessy, L., Latchford, J. L., Zaiko, A., von Ammon, U., Audrezet, F., ... & Pochon, X. (2020). Release and degradation of environmental DNA and RNA in a marine system. *Science of the Total Environment*, 704, 135314.
- Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., ... & Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific reports*, 7(1), 1-12.

Material Suplementario

Corral-Lou, A., & Doadrio, I. (2022). Environmental DNA metabarcoding of water samples as a tool for monitoring Iberian freshwater fish composition. *Under Review*.

Supplementary information for:

**Environmental DNA metabarcoding of water samples
as a tool for monitoring Iberian freshwater fish
composition**

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Table S1. Species used in the present study to expand the 12S reference database. The table shows both the species obtained from Genbank (G) and the species sequenced in the present study (P). It also includes the total number of haplotypes obtained for each species (H) and the total number of individuals analyzed for each species (N). The accession number of Genbank of the sequences obtained in this study is marked in bold.

Species	G	P	H	N	Accession number of Genbank sequences	
<i>A. arcasii</i>	0	8	3	8	pending	
<i>A. salmantinum</i>	0	5	2	5	pending	
<i>A. alburnus</i>	2	1	1	3	AB239593; NC_008659; pending	
<i>A. melas</i>	1	0	1	1	KT804702.1	
<i>B. barbatula</i>	2	2	2	4	KP715096; NC027192; pending	
<i>C. auratus</i>	44	2	3	46	AB111951; GU086395; GU086396; GU086397; JN105355; AB379915; AB379916; AB379917; AB379918; AB379919; AB379920; AB379921; AP011236; KF147851; KJ476998; KJ874428; KJ874430; KJ874431; KM657133; KM659025; KT756205; KU146528; KU146530; KY798482; MF443758; MF443759; MF443760; MF443761; MF443762; MF443763; MF443764; MF443765; MF443766; MF443767; MF443768; MF443769; MF443770; MF443771; HQ875340; KM657134; NC_015142; AP011239; KM657132; pending	
<i>C. calderoni</i>	0	4	2	4	pending	
<i>C. paludica</i>	0	4	1	4	pending	
<i>C. vettonica</i>	0	5	2	5	pending	
<i>C. carpio</i>	41	2	4	43	AP009047; MK134852; AP017364; AP017365; JN105352; JN105353; JN105354; JN105357; JX188253; KF856964; KF856965; KF932266; KJ511882; KJ511883; KP013086; KP993136; KP993137; KP993138; KP993139; KU050703; KU146529; KU146530; KU159761; KU301745; KX710076; KY798484; MG570426; MG570427; MG570435; MH202953; MK088487; MK291479; MT780875; NC_018035; NC_018036; NC_018037; NC_018039; NC_018366; JX188254; AP017363; NC_001606; pending	
<i>E. lucius</i>	2	0	1	2	AP004103; NC_004593	
<i>G. holbrooki</i>	4	1	1	5	KP013085; NC_028274; KP013115; KY798480; pending	
<i>G. lozanoi</i>	0	6	1	6	pending	
<i>L. aspius</i>	1	0	1	1	MK574834.2	
<i>L. gibbosus</i>	5	2	2	7	KP013097; NC_028284; MF621724; MF621725; MF621726; pending	
<i>L. bocagei</i>	0	6	1	6	pending	
<i>L. rifensis</i>	1	0	1	1	KX348041.1	
<i>M. salmoides</i>	8	0	3	8	NC_014686; MH301076; MH301075; MH301074; HQ391896; NC_008106; DQ536425; AP014537	
<i>O. mykiss</i>	17	0	5	17	AF113120; AF113121; AF125508; AF125509; L29771; NC_001717; CF752467; HQ167664; DQ288269; DQ288270; DQ288271; KP013084; KY798500; LC050735; DQ288268; KP085590; MF621750	
<i>P. bigerri</i>	0	3	1	3	pending	
<i>P. durienne</i>	0	5	1	5	pending	
<i>P. polylepis</i>	2	0	1	2	AP011282.1; NC_031574.1	
<i>S. trutta</i>	16	6	2	22	AM910409; HQ167666; JQ390057; KT633607; KY798501; LT617630; LT617631; LT617632; MF621760; MF621761; MF621762; MF621763; NC_010007; NC_024032; GU233801; KT634053; pending	

<i>S. alburnoides</i>	0	8	2	8	pending
<i>S. carollierii</i>	0	6	1	6	pending
<i>T. tinca</i>	3	2	1	5	AB218686; KY798486; NC_008648; pending
Total	149	78	46	227	

<i>A.alburnus_1*</i>
<i>L.aspius_1*</i>
<i>A.arcaistica_1</i>
<i>A.arcaisiidur_1</i>
<i>A.salmantinum_1</i>
<i>A.salmantinum_2</i>
<i>P.duriense_1</i>
<i>P.polylepis_1</i>
<i>A.arcaisticom_1</i>
<i>S.alburnoides_1</i>
<i>S.alburnoides_2</i>
<i>S.carolinitii_1</i>
<i>P.bigeri_1*</i>
<i>G.iozanoi_1</i>
<i>T.tinca_1</i>
<i>C.auratus_1*</i>
<i>C.auratus_2*</i>
<i>C.auratus_3*</i>
<i>C.carpio_1*</i>
<i>C.carpio_2*</i>
<i>C.carpio_3*</i>

<i>C.carpio_4*</i>	84.7	86.9	84.7	85.8	86.4	85.8	84.7	84.1	86.4	84.7	84.7	84.7	83.5	88.1	89.8	97.1	96.5	96.5	97.7	98.8	98.3		
<i>L.bocagei_1</i>	86.9	88.1	86.4	86.4	86.9	86.4	88.1	87.5	88.1	88.6	89.2	88.6	85.8	91	90.4	93.7	93.7	93.7	93.7	93.7	93.1	92.5	
<i>L.rifensis_1</i>	86.9	88.1	86.4	86.4	86.9	86.4	88.1	87.5	88.1	88.6	89.2	88.6	86.9	89.8	89.3	93.1	93.1	93.1	93.1	93.1	92.5	92	98.3
<i>B.barbatula_1</i>	81.7	82.9	81.7	81.7	84	83.4	83.4	82.9	82.9	81.1	81.1	81.1	78.3	80.8	81.9	81.6	81	82.8	82.2	82.8	82.8	82.8	83.4
<i>B.barbatula_2</i>	82.3	83.4	82.3	82.3	84.6	84	84	83.4	83.4	81.7	81.7	81.7	78.9	81.4	82.5	81.6	81	82.8	82.2	82.8	82.8	82.8	83.4
<i>C.calderoni_1</i>	84.1	85.2	83.5	83	84.7	84.1	84.7	84.1	83.5	85.8	85.2	85.2	82.4	83.1	82.6	84.1	83.5	85.2	83.5	84.1	83.5	83	84.7
<i>C.calderoni_2</i>	84.1	85.2	83.5	83	84.7	84.1	84.7	84.1	83.5	85.8	85.2	85.2	82.4	83.1	82.6	84.1	83.5	85.2	83.5	84.1	83.5	83	84.7
<i>C.paludica_1</i>	82.4	83.5	82.4	82.4	83.5	83	83	82.4	82.4	84.1	83.5	83.5	81.8	82.6	80.9	83.4	82.9	84	83.4	84	83.4	82.9	84.1
<i>C.vettonica_1</i>	81.8	83	81.8	82.4	83	82.4	82.4	81.8	81.8	83.5	83	83	82.4	83.1	81.5	83.4	82.9	84	83.4	84	83.4	82.9	83.5
<i>C.vettonica_2</i>	81.8	83	81.8	82.4	83	82.4	82.4	81.8	81.8	83.5	83	83	82.4	83.1	81.5	83.4	82.9	84	83.4	84	83.4	82.9	83.5
<i>A.melas_1*</i>	74.4	75.6	75	76.1	76.7	76.1	75.6	75	77.3	77.3	77.3	77.3	75.6	79.2	78.7	81	80.5	80.5	81.6	81.6	82.2	81.6	78.9
<i>E.lucius_1*</i>	68.6	69.1	67.4	67.4	68.6	68	68	67.4	68	68.6	69.1	68.6	69.1	66.7	67.8	67.6	67.6	67.6	67.6	67.1	67.6	66.5	68.4
<i>O.mykiss_1*</i>	73.1	73.7	73.1	72.6	74.3	74.9	73.7	73.1	74.3	74.9	75.4	75.4	74.3	74.6	75.7	74.7	74.7	74.7	77	75.9	75.3	75.9	76
<i>O.mykiss_3*</i>	72.2	72.7	72.2	71.6	73.3	73.9	72.7	72.2	73.3	73.9	74.4	74.4	73.3	73.6	74.7	73.7	73.7	73.7	76	74.9	74.3	74.9	75
<i>O.mykiss_4*</i>	72.7	73.3	72.7	72.2	73.9	74.4	73.3	72.7	73.9	74.4	75	75	73.9	74.2	75.3	74.3	74.3	74.3	76.6	75.4	74.9	75.4	75.6
<i>O.mykiss_5*</i>	72.2	72.7	72.2	71.6	73.3	73.9	72.7	72.2	73.3	73.9	74.4	74.4	73.3	73.6	74.7	73.7	73.7	73.7	76	74.9	74.3	74.9	75
<i>O.mykiss_2*</i>	72.2	72.7	72.2	71.6	73.3	73.9	72.7	72.2	73.3	73.9	74.4	74.4	73.3	73.6	74.7	73.7	73.7	73.7	76	74.9	74.3	74.9	75
<i>S.trutta_1</i>	71	71.6	68.8	69.9	71	71.6	69.3	68.8	71.6	70.5	71	71	70.5	72.5	71.3	72	72	72	73.7	72.6	72	72.6	72.7
<i>S.trutta_2</i>	70.5	71	69.3	70.5	71.6	72.2	69.9	69.3	72.2	71	71.6	71.6	70.5	71.9	71.9	71.4	71.4	71.4	73.1	72	71.4	72	72.2
<i>L.gibbosus_1*</i>	71.4	72	69.7	69.7	70.9	71.4	69.7	69.1	70.9	71.4	72.6	71.4	70.3	71.8	68.9	73	73.6	73	71.8	73	72.4	71.8	69.7
<i>L.gibbosus_2*</i>	72	72.6	70.3	70.3	71.4	72	70.3	69.7	71.4	72	73.1	72	70.9	71.8	68.9	73	73.6	73	71.8	73	72.4	71.8	69.7
<i>M.salmoides_1*</i>	70.9	71.4	69.7	70.3	71.4	70.9	70.9	70.3	72	72	72.6	72	69.1	70.6	70.6	71.3	71.3	71.3	71.3	71.3	71.8	70.1	70.9
<i>M.salmoides_2*</i>	71.4	72	70.3	70.9	72	71.4	71.4	70.9	72.6	72.6	73.1	72.6	69.7	71.2	71.2	71.8	71.8	71.8	71.8	71.8	72.4	70.7	71.4
<i>M.salmoides_3*</i>	71.4	72	70.3	70.9	72	71.4	71.4	70.9	72.6	72.6	73.1	72.6	69.7	71.8	70.6	71.3	71.3	71.3	72.4	71.3	71.8	70.1	72
<i>G.holbrooki_1*</i>	66.5	67.6	68.8	67	67.6	67	67	66.5	65.9	68.8	68.2	67.6	67.6	67.4	67.4	67.4	67.4	67.4	68.6	69.1	68.6	68.6	67.6

Table S3. The relative abundance of obtained reads for each sample (BL, L1 and L2) and locality. The summer and winter samples are indicated as M1 and M2 respectively. The green color highlights cases where a species was only detected by one of the two liters.

M1	Arlanza (1)			Pisuegra (3)			Boedo (2)			Tera (12)			Aliste (6)		Esla (4)		Órbigo (5)		Yeltes (7)		Cormeja (8)		Voltoya (11)		Adaja (9)		Voltoya (10)	
	BL	L1	L2	BL	L1	L2	BL	L1	L2	BL	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2	L1	L2
<i>Conger</i>																												
<i>T. japonicus</i>											0.21																	
<i>T. trachurus</i>												1.29																
											0.19	0.09												0.09	0.12			
<i>L. gibbosus</i>	1.12		0.52										73.93	72.36	0.25	0.48	0.05		5.42	28.09								
<i>S. pitchardus</i>																								1.79	1.73			
<i>C. calderoni</i>		0.43	1.36				0.84	0.46		0.59	0.32						1.43	4.02										
<i>C. paludica</i>																			21.06	16.6								
<i>C. auratus</i>																								0.66	0.44			
<i>C. carpio</i>																						0.73	1.38	3.42	3.97			
<i>Cyprinus</i>																								0.19	0.22			
<i>L. bocagei</i>	3.58	3.67								15.85	18.58				16.72	18.73			0.47	9.93	1.27	0.24	3.95	4.3				
<i>L. rifensis</i>	0.07																											
<i>Luciobarbus</i>	0.07	0.11								0.5	0.54				0.7	0.33				0.13								
<i>G. lozanoi</i>	25.08	29.74	3.42	2.9			32.48	32.74		29.67	31.34		6.62	8.75	39.16	38.88	0.49	1.79							65.37	64.22	80.26	82.58
<i>Gobio</i>	0.65	0.76					0.34	0.41		1.31	1.79				1.88	1.35									1.24	1.74	0.34	
<i>A. arcasii</i> (3)															0.51	0.13	0.39	1.13										
<i>A. arcasii</i> (2)																					93.67	94.01						
<i>A. arcasii</i> (1)	0.15	0.85		0.26			29.1	28.14		16.89	13.67		0.19			0.18					4.54	5.54	0.55	0.68	21.98	22.01	18.45	17.42
<i>A. salmantinum</i>																			2.2	2.89								
<i>A. alburnus</i>													18.08	17.56	8.27	8.56			0.66	1.07								
<i>Alburnus</i>															0.43													
<i>Chondrostoma</i>		0.03	0.14				0.38	0.34		0.52	0.61										0.13				1.32	1.15	0.4	
<i>Leuciscidae</i>										0.15	0.19														0.11	0.09		
<i>Phoxinus</i>	100	1.75	1.59	0.88	0.46																							
<i>P. bigeri</i>		58.89	50.39	68.76	66.59											0.1												
<i>P. durienne</i>		3.54	5.15	14.45	16.05										0.27	0.29			3.12	7.88	0.17	0.21						

M2	Esla (4)		Órbigo (5)		Adaja (9)		Tera (12)	
	L1	L2	L1	L2	L1	L2	L1	L2
<i>C. calderoni</i>			1.59	1.35				
<i>L. bocagei</i>	8.79	6.69					0.32	0.54
<i>Luciobarbus</i>	0.17	0.08						
<i>G. lozanoi</i>	31.73	38.44	1.17	2.15	49.98	47.93	39.99	40.9
<i>Gobio</i>	0.74	0.79			0.99	1.18	0.9	0.79
<i>A. arcasii</i> (3)	3.98	3.77	1.1	1.16				
<i>A. arcasii</i> (1)					46.16	48.33	20.95	23.13
<i>Achondrostoma</i>	0.16				1.06	0.93	0.79	0.7
<i>A. alburnus</i>	12.42	10.87					0.2	0.08
<i>Alburnus</i>	0.35	0.32						
<i>Leuciscus</i>	0.16	0.21						
<i>Leuciscidae</i>					0.06		0.17	0.19
<i>Pseudochondrostoma</i>		0.15						
<i>P. duriense</i>	8.66	8.53						
<i>P. polylepis</i>					0.08	0.13		
<i>Squalius</i>					0.07		0.58	0.52
<i>S. carolitertii</i>		0.39		0.06	1.6	1.5	8.58	7.99
<i>B. barbatula</i>	16.92	15.61	27.88	27.68				
<i>G. holbrooki</i>			8.09	12.84				
<i>E. lucius</i>	0.46	0.76		0.11				
<i>D. labrax</i>	0.17	0.09						
<i>Hippoglossus</i>			1.16	1.91				
<i>L. aspera</i>			0.07					
<i>Salmonidae</i>			0.06	0.11				
<i>O. mykiss</i>	3.51	4.5	3.53	2.56				
<i>Oncorhynchus</i>	1.62	2.14	1.39	1.5				
<i>Salmo</i>	0.43	0.2	0.6	0.84			0.09	0.08
<i>S. trutta</i>	9.7	6.47	53.36	47.75			27.43	25.08

DISCUSIÓN GENERAL

El estudio de la diversificación de las poblaciones, la variabilidad genética y los fenómenos de hibridación en las especies del género *Phoxinus* y en *Achondrostoma salmantinum* y *Cobitis vettonica* nos ha permitido contribuir al conocimiento de su historia evolutiva, evaluando su demografía y potencial evolutivo que son claves para llevar a cabo los planes de conservación y manejo de estas especies. A partir de los resultados obtenidos de los diferentes análisis genéticos también hemos construido modelos biogeográficos, los cuales aportan nueva luz a la paleogeografía ibérica. Además, en ocasiones la conservación de especies amenazadas, tales como *A. salmantinum* y *C. vettonica*, necesita programas continuos de seguimiento, que hasta ahora se vienen desarrollando con métodos invasivos, principalmente a través de la pesca eléctrica. En esta tesis se ha puesto a punto el desarrollo de la técnica de *metabarcoding* de ADN ambiental (eDNA) en la cuenca del Duero, una técnica no invasiva de una alta sensibilidad que aporta nuevas herramientas de monitoreo las cuales pueden ser aplicadas en múltiples aspectos implicados en la conservación de las especies de peces de agua dulce de la Península Ibérica.

A continuación, se sintetizan y discuten algunos aspectos abordados a lo largo de los diferentes capítulos de la tesis doctoral y además abrimos una serie de preguntas para seguir en esta línea de investigación.

La genética aplicada a los estudios filogenéticos y filogeográficos

Los estudios filogenéticos y filogeográficos de los peces de agua dulce ibéricos han sido llevados a cabo principalmente a través del uso de genes mitocondriales, combinados con el uso de marcadores nucleares, tales como microsatélites o intrones (ver por ejemplo Zardoya y Doadrio, 1998; Zardoya y Doadrio, 1999; Machordom y Doadrio, 2001; Salgueiro et al., 2003; Robalo et al., 2007; Robalo et al., 2008; Perea et al., 2010; Pereira et al., 2011; Lopes-Cunha et al., 2012; Perea et al., 2016). El uso de los marcadores mitocondriales frente a los nucleares ha sido preferido algunas veces debido a que poseen una tasa de mutación mayor y poseen una naturaleza no recombinante, siendo capaces de detectar una mayor estructura genética que los intrones nucleares (Aboim et al., 2009;

Perea et al., 2015; Casal-Lopez et al., 2018). Sin embargo, la tasa más alta de mutación del ADN mitocondrial y el menor tiempo de coalescencia hace que el estudio exclusivamente de un marcador mitocondrial sea insuficiente para resolver algunas relaciones entre especies divergentes. Por tanto, la inclusión de marcadores nucleares en los estudios filogenéticos y filogeográficos, ha resultado útil para detectar procesos de especiación, hibridación o para detectar una separación incompleta de linajes, ya que la historia evolutiva de una especie puede estar sesgada con sólo el estudio de marcadores mitocondriales (Perea et al., 2015; Casal-Lopez et al., 2018).

Los estudios filogeográficos y filogenéticos para las especies *A. salmantinum*, *C. vettonica* y para las especies del género *Phoxinus* fueron llevadas a cabo a través del gen mitocondrial del citocromo b (*MT-CYB*) y para el gen activador de recombinación nuclear 1 (*RAG1*). Mientras que el marcador *MT-CYB* resultó ser muy informativo tanto para la delimitación de especies dentro del género *Phoxinus*, como para detectar la estructura genética poblacional de las especies *P. bigerri*, *A. salmantinum* y *C. vettonica*, el marcador nuclear del *RAG1* resultó menos informativo en todos los casos, como ya ha sido señalado en trabajos previos para otros leuciscínidos de la Península Ibérica (Perea et al., 2016; Perea et al., 2021). Sin embargo, gracias al uso de este marcador fueron detectados procesos de hibridación entre las especies *P. septimaniae* y *Phoxinus* sp. (descrita recientemente como *P. dragarum* Denys et al., 2020). También se confirmó la existencia de híbridos entre *A. salmantinum* y *P. duriense*, detectadas previamente por morfología (Elvira et al., 1990). Por otro lado, aunque el estudio filogeográfico llevado a cabo para la especie *C. vettonica* reveló la presencia de introgresión genética con su especie hermana *C. paludica*, en este caso el marcador *RAG1* no resultó informativo ya que tanto las poblaciones de *C. vettonica* como de *C. paludica* presentaban el mismo alelo para este gen.

El avance en las tecnologías de secuenciación de nueva generación (NGS) ha proporcionado una herramienta capaz de obtener una resolución más detallada para estudios de filogeografía y genética de poblaciones gracias a un estudio más amplio del genoma (McCormack et al., 2013; Tan et al., 2019). Particularmente, los polimorfismos de un solo nucleótido (SNP) resultan marcadores exitosos para estudios de ecología y conservación de las poblaciones naturales debido a su capacidad para acceder a la variabilidad a lo largo de todo el genoma. Además, para estudios poblacionales, se obtiene más información sobre la historia evolutiva aumentando el número de loci analizados que

el número de individuos de la muestra, por lo que los tamaños muestrales pueden ser menores, algo relevante para el estudio de especies amenazadas (Rosenberg y Nordborg, 2002).

En esta tesis se han desarrollado los primeros trabajos en la Península Ibérica donde se estudia la variación y la variabilidad genética de especies de peces de agua dulce a un nivel genómico mediante el estudio de SNPs. El estudio de las poblaciones de *A. salmantinum* y *C. vettonica* a través de SNPs nos ofreció una información más detallada sobre la estructura genética, la variabilidad y la relación actual de las poblaciones, que la que nos había ofrecido el marcador mitocondrial. Para la especie *C. vettonica*, el estudio filogenético de los SNPs descubrió nuevas relaciones filogenéticas entre las diferentes poblaciones de *C. vettonica* y *C. paludica*, las cuales no habían sido detectadas a través del marcador mitocondrial. Además, gracias al uso combinado de los SNPs y el *MT-CYB*, se descubrió la existencia y extensión de la introgresión genética entre *C. paludica* y *C. vettonica* en los límites de la distribución conocida de *C. vettonica*. De esta manera, la información obtenida a través de los SNPs, nos permitió llevar a cabo diferentes hipótesis sobre la evolución y la relación entre las poblaciones de las especies *C. vettonica* y *A. salmantinum*. Por estas razones, el uso combinado de SNPs junto con marcadores mitocondriales y nucleares resultan en modelos filogeográficos y biogeográficos más completos y detallados de las relaciones pasadas y presentes como ya ha sido señalado para otras especies (Zarraonaindia et al., 2012; Mendes et al., 2019).

Para las especies del género *Phoxinus* se discute el origen de *P. septimaniae* y *P. dragarum* en España y mientras que para *P. dragarum* se aportan datos sobre su posible origen natural, para *P. septimaniae* se habla de su origen antrópico en la Península Ibérica. Para poder clarificar esta idea, nosotros proponemos un estudio más detallado para estas dos especies incluyendo individuos pertenecientes a las poblaciones de las cuencas mediterráneas de la costa de Francia para *P. septimaniae* y de individuos pertenecientes a la cuenca del Garona de Francia para *P. dragarum*. El estudio de estas poblaciones a través de un marcador mitocondrial junto con un marcador nuclear como los SNPs proporcionarían información suficiente para desentrañar la historia evolutiva de estas especies y por lo tanto clarificar el origen natural o introducido de las poblaciones de *P. dragarum* y *P. septimaniae* en el noreste de España.

El papel del cuaternario en la diversificación de las especies de peces de agua dulce en la Península ibérica

Debido a los múltiples procesos que han ocurrido desde su aislamiento, la ictiofauna de la Península Ibérica se caracteriza por estar formada por pocos géneros pero muy diversificados, siendo el periodo del Cuaternario un periodo clave para la diversificación de estos (Zardoya y Doadrio, 1998; Machordom y Doadrio, 2001; Sanjur et al., 2003; Doadrio y Perdices, 2005; Robalo et al., 2008; Lopes-Cunha et al., 2012; Perea et al., 2016). Durante el Cuaternario la Península Ibérica por un lado se ve afectada por los diferentes ciclos de glaciación-deglaciación y, por otro lado, sufre diversos reordenamientos hidrogeomorfológicos de las cuencas (Gante, 2009; Almodóvar et al., 2012). Todos estos cambios afectan a la fauna que habita en los ríos y, por lo tanto, la estructura genética actual de las especies, son un reflejo de la historia evolutiva de los ríos donde viven, sin tener porque corresponder con la configuración actual de las cuencas hidrográficas. De esta manera, la fuerte estructura genética detectada para las poblaciones de *A. salmantinum* y *C. vettonica*, cuyo patrón difiere del habitual observado en los peces de agua dulce europeos, es decir, un alto nivel de flujo de genes dentro de la misma cuenca hidrográfica y una gran diferenciación genética entre cuencas (Seifertová et al., 2012; Takács et al., 2015; Marić et al., 2017; Buj et al., 2017; Marić et al., 2019; De Cahsan et al., 2020; Wetjen et al., 2020), recalca la compleja evolución de la hidrogeomorfología del área donde se encuentran distribuidas estas especies. Así, conocer los diferentes procesos evolutivos es muy importante para descifrar los patrones que han llevado a la diversificación de las especies ibéricas durante el Cuaternario (Gante et al., 2009; Perea et al., 2016; Perea & Doadrio, 2015).

Por un lado, durante los ciclos de glaciación-desglaciación, diferentes cuencas fluviales se pudieron conectar permitiendo el intercambio de organismos acuáticos, por la confluencia de los tramos bajos de los ríos, como consecuencia de la retracción del nivel del mar, o por capturas fluviales por deglaciación y efecto remontante de algunos ríos (Williams et al., 1998). De esta manera, tanto la diversidad genética dentro de la especie *P. bigerri* a lo largo del norte peninsular como la presencia de *P. dragarum* en la cuenca del Ebro, fueron explicados como consecuencia de estos ciclos de glaciación-deglaciación durante el Cuaternario. Por otro lado, las capturas fluviales son un fenómeno ampliamente reportado en la Península Ibérica que ha dado lugar a la estructura genética tanto de los

leuciscínidos ibéricos (Haywood y Hughes 1998; Mesquita et al., 2005; Craw et al., 2007; Perea et al., 2016; Casal-López et al., 2018), como de otros peces de agua dulce de regiones distribuidas por todo el mundo (por ejemplo, Domínguez-Domínguez et al., 2006; Aquino y Colli 2016; Fluker et al., 2019). Concretamente, múltiples capturas fluviales han sido hipotetizadas entre algunos ríos del norte del Tajo y el sur del Duero para explicar los patrones genéticos actuales detectados en algunas especies de peces (Perea et al., 2021; Perdices y Doadrio, 2000). Aunque algunas de estas hipótesis se ven respaldadas por datos geológicos, normalmente estos son escasos y los datos filogeográficos pueden ser de gran ayuda para la reconstrucción de la historia evolutiva de los diferentes sistemas acuáticos a través de las evidencias faunísticas (Doadrio, 1984; Doadrio, 1988; Callejas y Ochando, 2000; Carmona et al., 2000; Casas-Sainz y De Vicente, 2009; Sousa-Santos et al., 2007). De esta manera nosotros proponemos la existencia de una conexión entre los ríos Árrago y Mayas, de las cuencas del Tajo y Duero respectivamente, para explicar la homogeneidad genética detectada entre las poblaciones de estos dos ríos. Esta misma relación entre ambos ríos en el pasado ya fue postulada anteriormente para explicar la presencia de individuos con el genoma mitocondrial de *Squalius carolitertii* (distribuido en la cuenca del Duero) en el río Árrago (cuenca del Tajo) (Perea et al., 2021).

Aunque una investigación más profunda es necesaria, a través de la técnica del metabarcoding de eDNA se detectó ADN mitocondrial de la especie *P. polylepis* en un río al sur de la cuenca del Duero. Esta especie sólo vive en España de forma natural en la cuenca del Tajo (Doadrio et al., 2011) y nunca había sido citada en la cuenca del Duero. De esta manera, una de las posibilidades de la presencia de la especie *P. polylepis* también se explicó por un posible movimiento de individuos desde los ríos del norte del Tajo a los del sur del Duero como consecuencia de una captura fluvial en el pasado, tal y como ha sido detectado para otras especies como *C. calderoni* (Perdices y Doadrio, 2005). Además de entre las diferentes cuencas fluviales de la Península Ibérica, las capturas también pueden darse como reorganizaciones dentro de una misma cuenca (Schnabel y Gutiérrez, 2014; Rodríguez-Rodríguez et al., 2020). De esta manera, la homogeneidad genética detectada para algunas de las poblaciones *A. salmantinum*, pertenecientes a dos subcuencas distintas y separadas en su desembocadura por una cascada, solo pudo ser explicada como consecuencia de una captura fluvial entre sus cabeceras en un pasado reciente.

Además de los distintos efectos de los ciclos de glaciación-deglaciación y los cambios hidrogemorfológicos que afectaron a la estructura genética actual de las poblaciones de *P. bigerri*, *A. salmantinum* y *C. vettonica*, en la presente tesis también se menciona los requerimientos de hábitat como un factor determinante de la estructura y variabilidad genética actual de algunas de las poblaciones de cada especie. Por un lado, la estructura genética detectada dentro de subcuenca del Huebra donde se encuentra distribuida *A. salmantinum* y la ausencia de barreras geológicas evidentes, dentro de la subcuenca fue explicada por la presencia de un hábitat inapropiado, lo que actuaría como una barrera impidiendo su expansión a otras zonas de la subcuenca (Elvira et al., 1990; Velasco et al., 1997). Para la especie *P. bigerri*, la gran diferencia detectada en los valores de diversidad genética para algunas de sus poblaciones pertenecientes al mismo linaje se achacó a la diferencia en la disponibilidad de hábitat. Sin embargo, estas afirmaciones tanto para *A. salmantinum* como para *P. bigerri* son especulativas ya que, en la presente tesis, no se han llevado a cabo estudios ecológicos para las especies, los cuales ayudarían a una mejor comprensión de los patrones implicados en su diversificación. Por último, para la especie *C. vettonica* se realizó una modelización de nicho ecológico con proyección al pasado, y durante el periodo del LGM se observó una retracción de su área de distribución hacia el sur tal y como la teoría de la filogeografía predice (Hewitt, 1996; Ehrich et al., 2007; Rodríguez et al., 2011). Por lo tanto, esto sirvió para explicar, en primer lugar, la distribución de *C. vettonica* hacia las cuencas portuguesas y, en segundo lugar, sirvió para apoyar una de las hipótesis que se planteó sobre la separación de los dos linajes de la subcuenca del Alagón.

La cuenca del Duero ha sufrido múltiples transformaciones hidrogeomorfológicas a lo largo de la historia desde su origen como lago endorreico en el terciario, lo que ha dado lugar a múltiples eventos de diversificación en los peces de agua dulce de la Península Ibérica (Alonso-Zarza et al., 2002; Martín-González y Heredia, 2011; País et al., 2012; Sousa-Santos et al., 2019; Doadrio et al., 2021; Perea et al., 2021). De hecho, el origen de las especies *A. salmantinum* y *C. vettonica* se asocia a la formación de la paleocuenca de Ciudad Rodrigo, una prolongación de la Cuenca del Duero, que aparece como una unidad geológica bien definida de dirección NE-SO en la provincia de Salamanca (Gavilán y Polo, 1987; Arenillas y Saenz 1987; López-Martínez, 1989; De la Peña, 1995; Carmona et al., 1999; Doadrio et al., 2011), la cual también se asocia al origen de otros leuciscínidos de la Península Ibérica (Sousa-Santos et al., 2019).

La subcuenca del Águeda, localizada en la parte suroccidental de la cuenca del Duero, mostró una compleja evolución hidrogeográfica, la cual se vio reflejada a través de los patrones biogeográficos detectados para *A. salmantinum* y *C. vettonica*. Entre los ríos de la parte suroccidental del Duero el río Águeda es el único que muestra una tipología diferente, con una importante cabecera de origen pluvionival y un profundo valle consecuencia de su mayor caudal, mientras que el resto de los ríos apenas tienen cabecera, dependiendo su caudal principalmente del nivel freático. Así, el río Águeda tiene una aportación media de caudal casi el triple que el río Huebra (<https://www.chduero.es/la-cuenca-del-duero>), el otro gran río de esta región del Duero, y presenta una composición de la ictiofauna algo peculiar. Por un lado, para *A. salmantinum* encontramos que este río se encuentra en medio de su distribución, estando esta especie ausente en su cauce principal y en todos los afluentes que se encuentran dentro de su valle. La única población de *A. salmantinum* que aparece en la subcuenca del río Águeda es en el río Turones (actualmente extinta) y su afluente la rivera de Dos Casas, las cuales fueron genéticamente diferentes al resto. Por otro lado, la especie *C. vettonica* (ausente en las subcuencas del Uces y Huebra, donde es sustituida por *C. paludica*), está presente en la subcuenca del Águeda, cuyo origen ha sido explicado anteriormente como consecuencia de una captura fluvial en el pasado. Gracias a la biogeografía comparada observamos diferentes procesos hidrogeográficos y dada la complejidad de esta área, sería interesante descifrar los diferentes mecanismos que han afectado a la hidrogemorfología y por lo tanto a la diversidad genética actual detectada para las especies de peces.

Aunque no hay estudios filogeográficos previos, y tal y como ocurre con *A. salmantinum*, una fuerte estructura genética dentro de las poblaciones distribuidas a lo largo de la cuenca del Duero ha sido detectada previamente en otras especies como por ejemplo en *C. calderoni*, *P. duriense* o *A. arcasii* (Perdices y Doadrio, 2000; Robalo et al., 2006; Doadrio et al., 2021). Por otro lado, como ocurre con *C. vettonica*, las poblaciones pertenecientes a la cuenca del Duero de las especies *S. alburnoides* y *S. carolitertii* se comportan como linajes independientes (Perea et al., 2021; Cunha et al., 2011; Doadrio et al., 2021). Por lo tanto, debido a la fuerte estructura genética dentro de algunas poblaciones tanto dentro de la cuenca del Duero como entre las diferentes cuencas ibéricas, el Duero se comporta como una unidad biogeográfica imprescindible para la conservación de la diversidad intraespecífica e interespecífica de las especies de peces de la Península Ibérica.

El estudio de la diversidad genética intraespecífica poblacional resulta prometedor a través del estudio de los análisis de eDNA metabarcoding, de hecho, estudios previos han detectado con éxito múltiples haplotipos de ADN mitocondrial de una misma localidad (Parsons et al., 2018; Tsuji et al., 2020; Sigsgaard et al., 2020). De esta manera, se debe destacar el posible uso del metabarcoding como herramienta para el estudio filogeográfico de ciertas especies simultáneamente. En nuestro caso, aunque los resultados obtenidos del metabarcoding de eDNA no fueron analizados bajo el punto de vista de la filogeografía, recientemente ha surgido el concepto de la metafilogeografía (Turon et al., 2020; Antich et al., 2021). Este concepto explica el uso de la herramienta del metabarcoding con muestras de eDNA para el estudio de patrones intraespecíficos, características filogeográficas y genética de poblaciones para múltiples especies simultáneamente (Turon et al., 2020). Sin embargo, aunque el uso del metabarcoding de eDNA para estudios filogeográficos aún se enfrenta a algunos desafíos, éste tiene un gran potencial tanto para la investigación básica como para la aplicada (Sigsgaard et al., 2020). Los avances tanto en métodos analíticos como en tecnología, así como la disminución de los costos de producción de datos aseguran que la metafilogeografía continuará desarrollándose y ampliando su alcance en los estudios evolutivos.

Implicaciones en la conservación

La genética de la conservación se ha mostrado en los últimos años como una herramienta imprescindible para conocer el estado de conservación de los organismos y para implementar programas de conservación (Frankham et al., 2002). Esto es debido a que nos ayuda a comprender tanto la variabilidad intrapoblacional como interpoblacional, de forma que podemos obtener información sobre las unidades de manejo y conservación (Carvalho et al., 1993; Boettcher et al., 2010). De esta manera, hacer el seguimiento de la variabilidad genética permite determinar los impactos a largo plazo y evaluar si las intervenciones en materia de conservación han cumplido los objetivos y han mejorado la biodiversidad (Flanagan et al., 2018). Además, resolver la distribución espacial de la diversidad genética para un grupo particular de organismos es fundamental para comprender los patrones de la biodiversidad y mejorar las decisiones de conservación y gestión.

Los peces primarios de agua dulce representan uno de los grupos de vertebrados más amenazados de la Península Ibérica, con el 80% de sus especies catalogadas dentro de

alguna categoría de amenaza de la IUCN (Doadrio et al., 2011). Sin embargo, esto no se refleja en la legislación española donde menos del 10% de esas especies están en el catálogo de especies amenazadas, debido principalmente a la deficiente información que se tiene de muchas especies ibéricas, especialmente desde la óptica de la genética de la conservación. Los principales factores de amenaza para los peces de agua dulce de la Península Ibérica son la regulación de caudales, las represas, la extracción de agua, la contaminación, el cambio de uso del suelo en las cuencas de los ríos, la sobreexplotación, la transformación de los cauces por la obra civil, la presencia de especies invasoras y el calentamiento global (Doadrio et al., 2011). Los peces, son el grupo de vertebrados más abundante de los ecosistemas de agua dulce y la amenaza de este grupo no afecta únicamente a la pérdida de biodiversidad, sino que debido a que el ecosistema es entendido como una interacción de todos sus elementos, la pérdida de biodiversidad podría desequilibrar el ecosistema afectando a todos los elementos que lo componen (Geist et al., 2011).

Las especies *A. salmantinum* y *C. vettonica* están consideradas bajo la categoría de “En Peligro” por la UICN. Estas especies además están incluidas dentro de la directiva hábitats señalándolas como “Especies de Interés comunitario” (Anexo II de la Directiva 92/43/CEE) y se consideran como especies de “Prioridad de Conservación Regional Alta” en el Plan Director de Red Natura de la Junta de Castilla y León. Sin embargo, estas especies todavía no son contempladas ni por el catálogo de especies amenazadas autonómico ni nacional. Recientemente, la conservación de estas dos especies ha sido uno de los objetivos de un proyecto LIFE dentro del marco “Actuación para la protección y conservación de ciprínidos ibéricos de interés comunitario”, a partir del cual se desarrolló exitosamente un programa de cría en cautividad con el fin de reintroducir y reforzar algunas las poblaciones de *A. salmantinum* y *C. vettonica*. En general, las reintroducciones han resultado eficaces para frenar la pérdida acelerada de biodiversidad (Soorae, 2018, Brichieri-Colombi y Moehrenschlager, 2019). Sin embargo, conocer la diversidad genética de una especie resulta fundamental para este objetivo, ya que contribuye al potencial de adaptación de una especie y promueve la biodiversidad y la integridad del ecosistema. Por lo tanto, en la presente tesis se aportaron las diferentes Unidades operativas de Conservación (OCUs; Doadrio et al., 1996) para *A. salmantinum* y *C. vettonica* a lo largo de toda su área de distribución, los cuales deben de ser tenidos en cuenta en los planes de conservación de estas especies.

En la Lista Roja de la IUCN figuran las especies presentes en España *P. bigerri* y *P. septimaniae* las cuales están catalogadas bajo la categoría de Preocupación Menor (<https://www.iucnredlist.org/species/135542/4141498>; <https://www.iucnredlist.org/es/species/135517/4136425>), sin embargo *P. dragarum* no ha sido todavía evaluada. Para la especie *P. bigerri* se aportan datos genéticos, los cuales deben de ser tenidos en cuenta en posibles planes de manejo y conservación futuros, aunque actualmente presenta poblaciones estables (Doadrio et al., 2011). Sin embargo, la especie *P. bigerri* se comporta como una buena colonizadora en la cuenca del Duero (Doadrio et al., 2011; Garcia-Raventós et al., 2020). Gracias al estudio de eDNA, descubrimos la presencia de esta especie en el tramo medio del río Esla, estando antes su registro restringido a una parte del norte de esta cuenca, apoyando la capacidad colonizadora de la especie (Garcia-Raventós et al., 2020). Aunque las consecuencias ecológicas de la amplia colonización de *P. bigerri* en la cuenca del Duero aún son inciertas, su propagación es de gran preocupación para la conservación debido a la competencia potencial con los peces nativos (Clavero, 2011; Garcia-Raventós et al., 2020)

Bajo el escenario propuesto en la presente tesis sobre el origen natural y artificial para *P. dragarum* y *P. septimaniae* respetivamente, resaltamos, en primer lugar, una especial atención a la especie *P. dragarum* ya que en la mayoría de las poblaciones donde se encuentra distribuida la especie sus individuos presentan introgresión genética con *P. septimaniae*, lo cual podría conllevar una amenaza de la variabilidad genética para *P. dragarum*. En segundo lugar, para la especie *P. septimaniae*, ampliamente distribuida en las cuencas catalanas del noreste peninsular, se recomendaría la inclusión de esta especie en el catálogo de especies exóticas invasoras.

Por último, en la presente tesis se ha desarrollado y testado una herramienta no invasiva de monitoreo para la ictiofauna ibérica presente en la cuenca del Duero. Esta técnica presentó una mayor sensibilidad de detección que los métodos tradicionales y resulta altamente eficaz para detectar especies en baja abundancia, para lo cual los métodos tradicionales pueden resultar insuficientes o muy costosos (Takahara et al., 2013; De Souza et al., 2016; Shaw et al., 2016; Yamamoto et al., 2017; Cilleros et al., 2019; Goutte et al., 2020; Czeglédi et al., 2021). Esto la convierte en una herramienta poderosa para diferentes actuaciones que se pueden llevar a cabo para la protección de la biodiversidad y de los ecosistemas acuáticos (Thomsen et al., 2015; Cilleros et al., 2019). Por un lado,

esta herramienta puede ser empleada para la detección temprana de especies invasoras, las cuales son una de las principales amenazas para la ictiofauna ibérica (Doadrio et al., 2011) y por lo tanto su detección temprana resulta esencial para la conservación de la biodiversidad (Larson et al., 2020) o resultar útil para realizar un seguimiento control después de haber invertido un esfuerzo en la eliminación de una especie dentro de un ecosistema (Miralles et al., 2016; Sepulveda et al., 2020). Por otro lado, el monitoreo no invasivo puede resultar especialmente útil para las especies amenazadas, cuya viabilidad puede verse comprometida por la captura directa (Snyder, 2003; Garlapati et al., 2019). Esta técnica podría resultar también especialmente útil para el monitoreo de especies reintroducidas y por lo tanto comprobar así el éxito de éstas (Hempel et al., 2020; Riaz et al., 2020).

Debido a que los costes de secuenciación masiva se han reducido enormemente en los últimos años y la técnica del metabarcoding de eDNA conlleva un menor esfuerzo de muestreo y un menor coste para el monitoreo de la biodiversidad (Jerde et al., 2011, Biggs et al., 2015, Sigsgaard et al., 2015), ésta podría servir como herramienta rutinaria y complementaria a métodos tradicionales para controlar la introducción y la dispersión tanto de especies exóticas como de especies autóctonas e informar que estrategias de gestión son adecuadas para la conservación de la biodiversidad de nuestros sistemas acuáticos.

CONCLUSIONES GENERALES

1. La reconstrucción filogenética del género *Phoxinus* reveló la presencia de tres especies coexistiendo en la Península Ibérica; la especie *P. bigerri* distribuida en las cuencas cantábricas y en la cuenca del Ebro; *P. dragarum* con su área de distribución restringida a los ríos del Pirineo en la cuenca del Ebro y algunas cuencas catalanas y *P. septimaniae* en las cuencas catalanas.
2. La especie *Phoxinus bigerri* presentó una fuerte estructura genética correspondiente a las cuencas del cantábrico occidental, cantábrico oriental y a la cuenca del río Artibai. Además, postulamos la separación de estos tres grupos genéticos como consecuencia de los ciclos de glaciación-deglaciación que ocurrieron durante el Pleistoceno.
3. Mientras que el origen de *P. dragarum* en la Península Ibérica es atribuida a capturas fluviales en el pasado entre los ríos de la cuenca del Ebro y de la parte alta del Garona en España, para la especie *P. septimaniae* se postuló su traslocación desde el sur de Francia.
4. El uso de tecnologías de secuenciación de nueva generación junto con el uso de marcadores mitocondriales permitió detectar una fuerte estructura genética poblacional para *C. vettonica* y *A. salmantinum*, especies altamente amenazadas y con pequeñas áreas de distribución en las cuencas del Tajo y Duero.
5. La estructura genética poblacional de *A. salmantinum* y *C. vettonica*, no estuvo asociada con la configuración actual de las cuencas hidrográficas, sino a reordenamientos hidrogeomorfológicos que ocurrieron durante el periodo del Cuaternario, para las cuales aportamos diferentes modelos biogeográficos.
6. Se identificaron cuatro OCUs para la especie *A. salmantinum* correspondiente a: (I) Subcuenca Águeda; (II) Subcuenca del Uces, cuenca del Huebra excepto los afluentes de la margen izquierda; (III) El río Yeltes y sus afluentes, (pertenecientes al margen

izquierdo de la subcuenca Huebra) y (IV) el río Camaces (perteneciente al margen izquierdo de la subcuenca del Huebra).

7. El estudio de la diversidad genética de *A. salmantinum* reveló la alta probabilidad de extinción de sus poblaciones, tal como ha ocurrido ya en el río Turones, como consecuencia tanto de factores intrínsecos, tales como la baja variabilidad genética detectada, como de factores extrínsecos, debido principalmente a la pérdida de hábitat.
8. Se identificaron cuatro OCUs para la especie *C. vettonica* correspondiente a: (I) Cuenca del Duero; (II) Subcuenca occidental de Alagón; (III) Subcuenca oriental del Alagón (IV) Subcuencas del Erjas, Ponsul y Aravil.
9. Las poblaciones de *C. vettonica* mostraron una fuerte amenaza debido tanto a la fuerte introgresión genética detectada en la mayoría de sus poblaciones con su especie hermana *C. paludica*, como a la baja variabilidad genética detectada para algunas de sus poblaciones.
10. El *metabarcoding* a través de muestras de ADN ambiental mostró ser una herramienta más poderosa para determinar la composición de peces en la cuenca del Duero que los métodos tradicionales. Además, el fragmento del gen ribosomal del 12S estudiado mostró una resolución taxonómica suficiente para diferenciar entre las diferentes especies e incluso a veces entre diferentes poblaciones.

BIBLIOGRAFÍA

- Aboim, M. A., Cunha, C., & Coelho, M. M. (2009). Redistribution of the geographical ranges of the Iberian cyprinid genus *Pseudochondrostoma* based on a phylogenetic analysis: Implications for the historical rearrangements of the north-western Iberian drainages. *Journal of Fish Biology*, 74(6), 1337-1346.
- Alberti, A. P., Díaz, M. V., & Chao, R. B. (2004). Pleistocene glaciation in Spain. *Quaternary glaciations—extent and chronology. Part I: Europe. Amsterdam, etc., Elsevier. (Developments in Quaternary Science 2.)*
- Alcamo, J. M., Vörösmarty, C. J., Naiman, R. J., Lettenmaier, D. P., & Pahl-Wostl, C. (2008). A grand challenge for freshwater research: understanding the global water system. *Environmental Research Letters*, 3(1), 010202.
- Almodóvar, A., Nicola, G. G., Elvira, B., & García-Marín, J. L. (2006). Introgression variability among Iberian brown trout Evolutionary Significant Units: the influence of local management and environmental features. *Freshwater Biology*, 51(6), 1175-1187.
- Almodóvar, A., Nicola, G. G., Leal, S., Torralva, M., & Elvira, B. (2012). Natural hybridization with invasive bleak *Alburnus alburnus* threatens the survival of Iberian endemic calandino *Squalius alburnoides* complex and Southern Iberian chub *Squalius pyrenaicus*. *Biological Invasions*, 14(11), 2237-2242.
- Alonso, J. L., Pulgar, F. J. Á., & Pedreira, D. (2007). El relieve de la Cordillera Cantábrica. *Enseñanza de las Ciencias de la Tierra*, 15(2), 151-163.
- Alonso-Zarza, A. M., & Wright, V. P. (2010). Palustrine carbonates. *Developments in Sedimentology*, 61, 103-131.
- Antich, A., Palacin, C., Wangensteen, O. S., & Turon, X. (2021). To denoise or to cluster, that is not the question: optimizing pipelines for COI metabarcoding and metaphylogeography. *BMC bioinformatics*, 22(1), 1-24.
- Aquino, P.P.U., & Colli, G.R. (2016). Headwater captures and the phylogenetic structure of freshwater fish assemblages: a case study in central Brazil. *Journal of Biogeography*, 44(1), 207-216.
- Arenillas, M., & Sáenz, C. (1987). *Los ríos. Guía Física de España*. Alianza Editorial.
- Avise, J. C. (1989). A role for molecular genetics in the recognition and conservation of endangered species. *Trends in Ecology & Evolution*, 4(9), 279-281.
- Avise, J. C. (1995). Mitochondrial DNA polymorphism and a connection between genetics and demography of relevance to conservation. *Conservation Biology*, 9(3), 686-690.
- Balian, E. V., Segers, H., Martens, K., & Lévêque, C. (2007). The freshwater animal diversity assessment: an overview of the results. *Freshwater animal diversity assessment*, 627-637.
- Bandelt, H. J., Forster, P., & Röhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular biology and evolution*, 16(1), 37-48.

- Beaulieu, J. M., Tank, D. C., & Donoghue, M. J. (2013). A Southern Hemisphere origin for campanulid angiosperms, with traces of the break-up of Gondwana. *BMC Evolutionary Biology*, 13(1), 1-17.
- Berggren, W. A., Kent, D. V., Swisher, C. C., & Aubry, M. P. (1995). A revised Cenozoic geochronology and chronostratigraphy. *Society of Economic Paleontologists and Mineralogists. Spec. Publ.* 54, 129
- Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R. A., ... & Dunn, F. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*, 183, 19-28.
- Boettcher, P. J., Tixier-Boichard, M., Toro, M. A., Simianer, H., Eding, H., Gandini, G., ... & Globaldiv Consortium. (2010). Objectives, criteria and methods for using molecular genetic data in priority setting for conservation of animal genetic resources. *Animal Genetics*, 41, 64-77.
- Böhme, M., & Ilg, A. (2003). Database of Vertebrates: fossil Fishes. *Amphibians, Reptiles and Birds (fosFAR-base) localities and taxa from the Triassic to the Neogene*. www.wahre-staerke.com. Accessed April, 24, 2015.
- Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30(15), 2114-2120.
- Bonar, S. A., Hubert, W. A., & Willis, D. W. (2009). Standard methods for sampling North American freshwater fishes.
- Brichieri-Colombi, T. A., Lloyd, N. A., McPherson, J. M., & Moehrensclager, A. (2019). Limited contributions of released animals from zoos to North American conservation translocations. *Conservation Biology*, 33(1), 33-39.
- Brooks, D. R., Mayden, R. L., & McLennan, D. A. (1992). Phylogeny and biodiversity: conserving our evolutionary legacy. *Trends in Ecology & Evolution*, 7(2), 55-59.
- Buhay, J. E., Crandall, K. A., & Posada, D. (2009). Nested clade phylogeographic analysis for conservation genetics. *Population genetics for animal conservation*. Cambridge University Press, Cambridge, 80-103.
- Buj, I., Marčić, Z., Čaleta, M., Šanda, R., Geiger, M. F., Freyhof, J., ... & Vukić, J. (2017). Ancient connections among the European rivers and watersheds revealed from the evolutionary history of the genus *Telestes* (Actinopterygii; Cypriniformes). *PLoS One*, 12(12), e0187366.
- Burridge, C. P., Craw, D., Fletcher, D., & Waters, J. M. (2008). Geological dates and molecular rates: fish DNA sheds light on time dependency. *Molecular Biology and Evolution*, 25(4), 624-633.
- Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M., & Furlan, E. M. (2019). A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environmental DNA*, 1(4), 402-414.
- Cabrera, L., & Gaudant, J. (1985). Los Ciprinidos (Pisces) del sistema lacustre Oligocénico-Miocénico de los Monegros (sector SE de la Cuenca del Ebro, provincias de Lleida, Tarragona, Huesca y Zaragoza). *Acta geológica hispánica*, 219-226.

- Callejas, C., & Ochando, M. D. (2000). Recent radiation of Iberian barbel fish (Teleostei, Cyprinidae) inferred from cytochrome b genes. *Journal of Heredity*, 91(4), 283-288.
- Calvet, M., Gunnell, Y., & Laumonier, B. (2021). Denudation history and palaeogeography of the Pyrenees and their peripheral basins: an 84-million-year geomorphological perspective. *Earth-Science Reviews*, 215, 103436.
- Calvo Sorando, J. P., Daams, R., Morales, J., López Martínez, N., Agustí, J., Anadón, P., ... & Mein, P. (1993). Up-to-date Spanish continental Neogene synthesis and paleoclimatic interpretation. *Revista de la Sociedad Geológica Española*, 6, 29 – 40.
- Cambray, J. A., & Bianco, P. G. (1998). Freshwater fish in crisis, a Blue Planet perspective. *Italian Journal of Zoology*, 65(S1), 345-356.
- Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, 7(5), 335-336.
- Carmona, J. A., Doadrio, I., Márquez, A. L., Real, R., Hugueny, B., & Vargas, J. M. (1999). Distribution patterns of indigenous freshwater fishes in the Tagus River basin, Spain. *Environmental Biology of Fishes*, 54(4), 371-387.
- Carmona, J. A., Domínguez, J., & Doadrio, I. (2000). Congruence between allozyme and cytochrome b gene sequence data in assessing genetic differentiation within the Iberian endemic *Chondrostoma lemmingii* (Pisces: Cyprinidae). *Heredity*, 84(6), 721-732.
- Carvalho, G. R. (1993). Evolutionary aspects of fish distribution: genetic variability and adaptation. *Journal of Fish Biology*, 43, 53-73.
- Carvalho, G. R., & Carvalho, G. R. (1998). Molecular ecology: origins and approach. *NATO Asi Series a Life Sciences*, 306, 1-24.
- Casal-López, M., Perea, S., Sousa-Santos, C., Robalo, J. I., Torralva, M., Oliva-Paterna, F. J., & Doadrio, I. (2018). Paleobiogeography of an Iberian endemic species, *Luciobarbus sclateri* (Günther, 1868) (Actinopterygii, Cyprinidae), inferred from mitochondrial and nuclear markers. *Journal of Zoological Systematics and Evolutionary Research*, 56(2), 127-147.
- Casas-Sainz, A. M., & De Vicente, G. (2009). On the tectonic origin of Iberian topography. *Tectonophysics*, 474(1-2), 214-235.
- Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular ecology*, 22(11), 3124-3140.
- Catchen, J. M., Hohenlohe, P. A., Bernatchez, L., Funk, W. C., Andrews, K. R., & Allendorf, F. W. (2017). Unbroken: RADseq remains a powerful tool for understanding the genetics of adaptation in natural populations. *Molecular ecology resources*, 17(3), 362-365.
- Ceballos-Barbancho, A., Morán-Tejeda, E., Luengo-Ugidos, M. Á., & Llorente-Pinto, J. M. (2008). Water resources and environmental change in a Mediterranean environment: the south-west sector of the Duero river basin (Spain). *Journal of Hydrology*, 351(1-2), 126-138.
- Cilleros, K., Valentini, A., Allard, L., Dejean, T., Etienne, R., Grenouillet, G., ... & Brosse, S. (2019). Unlocking biodiversity and conservation studies in high-diversity

- environments using environmental DNA (eDNA): A test with Guianese freshwater fishes. *Molecular ecology resources*, 19(1), 27-46.
- Clavero, M. (2011). Assessing the risk of freshwater fish introductions into the Iberian Peninsula. *Freshwater Biology*, 56(10), 2145-2155.
- Collares-Pereira, M. J., Alves, M. J., Ribeiro, F., Domingos, I., Almeida, P. R., Da Costa, L., Gante, H., Filipe, A. F., Aboim, A., Rodrigues, P. M., & Magalhães, M. F. (2021). *Guide to freshwater fish and migrants from mainland Portugal [In Portuguese]* (p. 292). Edições Afrontamento.
- Craw, D., Burrridge, C., Anderson, L., & Waters, J. M. (2007). Late Quaternary river drainage and fish evolution, Southland, New Zealand. *Geomorphology*, 84(1-2), 98-110.
- Cunha, C., Coelho, M. M., Carmona, J. A., & Doadrio, I. (2004). Phylogeographical insights into the origins of the *Squalius alburnoides* complex via multiple hybridization events. *Molecular Ecology*, 13(9), 2807-2817.
- Cunha, C., Doadrio, I., Abrantes, J., & Coelho, M. M. (2011). The evolutionary history of the allopolyploid *Squalius alburnoides* (Cyprinidae) complex in the northern Iberian Peninsula. *Heredity*, 106(1), 100-112.
- Czeglédi, I., Sály, P., Specziár, A., Preiszner, B., Szalóky, Z., Maroda, Á., ... & Erős, T. (2021). Congruency between two traditional and eDNA-based sampling methods in characterising taxonomic and trait-based structure of fish communities and community-environment relationships in lentic environment. *Ecological Indicators*, 129, 107952.
- Dallmeier, F. (1996). Biodiversity inventories and monitoring: essential elements for integrating conservation principles with resource development projects. *Biodiversity in managed landscapes: theory and practice*, 221-236.
- Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature methods*, 9(8), 772-772.
- De Cahsan, B., Nagel, R., Schedina, I. M., King, J. J., Bianco, P. G., Tiedemann, R., & Ketmaier, V. (2020). Phylogeography of the European brook lamprey (*Lampetra planeri*) and the European river lamprey (*Lampetra fluviatilis*) species pair based on mitochondrial data. *Journal of Fish Biology*, 96(4), 905-912.
- De la Peña, A. (1995). Tertiary fishes from the Iberian continental basins: History and fossil record. *Coloquios de Paleontología*, 47, 25-47.
- De Souza, L. S., Godwin, J. C., Renshaw, M. A., & Larson, E. (2016). Environmental DNA (eDNA) detection probability is influenced by seasonal activity of organisms. *PloS one*, 11(10), e0165273.
- Denys, G. P., Dettai, A., Persat, H., Daszkiewicz, P., Hautecoeur, M., & Keith, P. (2020). Revision of Phoxinus in France with the description of two new species (Teleostei, Leuciscidae). *Cybium: Revue Internationale d'Ichtyologie*, 44(3), 205-237.
- Doadrio, I. (1984). *Relaciones filogenéticas y biogeográficas de los barbos (Barbus, cyprinidae) de la Península Ibérica y aportes corológicos y biogeográficos a su ictiofauna continental*. Universidad Complutense de Madrid. Madrid.

- Doadrio, I., & Garzón, P. (1986). Nuevas localidades de *Phoxinus phoxinus* (L., 1758)(Ostariophysi, Cyprinidae) en la Península Ibérica. *Miscel·lània Zoològica*, 389-390.
- Doadrio, I. (1988). Delimitation of areas in the Iberian Peninsula on the basis of freshwater fishes. *Bonn Zoologischer Beitrage*, 39, 113-128.
- Doadrio, I. (1990). Phylogenetic relationships and classification of western palaearctic species of the genus *Barbus* (Osteichthyes, Cyprinidae). *Aquatic Living Resources*, 3(4), 265-282.
- Doadrio, I., Perdices, A., & Machordom, A. (1996). Allozymic variation of the endangered killifish *Aphanius iberus* and its application to conservation. *Environmental Biology of Fishes*, 45(3), 259-271.
- Doadrio, I., & Perdices, A. (1997). Taxonomic study of the Iberian *Cobitis* (Osteichthyes, Cobitidae), with description of a new species. *Zoological Journal of the Linnean Society*, 119(1), 51-67.
- Doadrio, I. (ed) (2001). Atlas y Libro rojo de los Peces continentales de España. 364 p. Madrid: MNCN-CSIC.
- Doadrio, I., Carmona, J. A., & Machordom, A. (2002). Haplotype diversity and phylogenetic relationships among the Iberian barbels (*Barbus*, Cyprinidae) reveal two evolutionary lineages. *Journal of Heredity*, 93(2), 140-147.
- Doadrio, I., & Carmona, J. A. (2004). Phylogenetic relationships and biogeography of the genus *Chondrostoma* inferred from mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 33(3), 802-815.
- Doadrio, I., & Perdices, A. (2005). Phylogenetic relationships among the Ibero-African cobitids (*Cobitis*, Cobitidae) based on cytochrome b sequence data. *Molecular Phylogenetics and Evolution*, 37(2), 484-493.
- Doadrio, I., & Carmona, J. A. (2006). Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species. *Cybium*, 30(3), 199-214.
- Doadrio, I., & Elvira, B. (2007). A new species of the genus *Achondrostoma* Robalo, Almada, Levy & Doadrio, 2007 (Actynopterigii, Cyprinidae) from western Spain. . *Graellsia*, 63, 295–304.
- Doadrio, I., Perea, S., Garzón-Heydt, P., & González, J. L. (2011). Ictiofauna continental española. Bases para su seguimiento. *DG Medio Natural y Política Forestal, MARM, Madrid, Spain*.
- Doadrio, I., Perea, S., & Corral-Lou, A. (2021). Caracterización genética e identificación de Unidades de Conservación en peces endémicos de interés comunitario. *Ministerio para la Transición Ecológica (España)*.
- Domínguez-Domínguez, O., Doadrio, I., & Pérez-Ponce de León, G. (2006). Historical biogeography of some river basins in central Mexico evidenced by their goodeine freshwater fishes: a preliminary hypothesis using secondary Brooks parsimony analysis. *Journal of Biogeography*, 33(8), 1437-1447.
- Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular biology and evolution*, 29(8), 1969-1973.

- Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z. I., Knowler, D. J., Lévêque, C., ... & Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. *Biological reviews*, 81(2), 163-182.
- Dudgeon, D. (2010). Prospects for sustaining freshwater biodiversity in the 21st century: linking ecosystem structure and function. *Current Opinion in Environmental Sustainability*, 2(5-6), 422-430.
- Dudgeon, D. (2019). Multiple threats imperil freshwater biodiversity in the Anthropocene. *Current Biology*, 29(19), R960-R967.
- Ehrich, D., Gaudeul, M., Assefa, A., Koch, M. A., Mummenhoff, K., Nemomissa, S., ... & Brochmann, C. (2007). Genetic consequences of Pleistocene range shifts: contrast between the Arctic, the Alps and the East African mountains. *Molecular ecology*, 16(12), 2542-2559.
- Eldredge, N. (1999). Cretaceous meteor showers, the human ecological “niche,” and the sixth extinction. In *Extinctions in near time* (pp. 1-15). Springer, Boston, MA.
- Elvira, B., Rincón, P. A., & Velasco, J. C. (1990). *Chondrostoma polylepis* Steindachner x *Rutilus lemmingii* (Steindachner) (Osteichthyes, Cyprinidae), a new natural hybrid from the Duero River basin, Spain. *Journal of Fish Biology*, 37(5), 745-754.
- Elvira, B. (1995). Conservation status of endemic freshwater fish in Spain. *Biological Conservation*, 72(2), 129-136.
- Elvira, B., & Almodóvar, A. (2001). Freshwater fish introductions in Spain: facts and figures at the beginning of the 21st century. *Journal of fish Biology*, 59, 323-331.
- Evanno, G., Regnaut, S., & Goudet, J. (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular ecology*, 14(8), 2611-2620.
- Excoffier, L., & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular ecology resources*, 10(3), 564-567.
- Feio, M. (1952). *A evolução do relevo do Baixo Alentejo e Algarve: estudo de geomorfologia*. Instituto para a Alta Cultura, Centro de Estudos Geográficos, Lisboa, Portugal.
- Fernández, M. H., Sierra, M. Á. Á., & Peláez-Campomanes, P. (2007). Bioclimatic analysis of rodent palaeofaunas reveals severe climatic changes in Southwestern Europe during the Plio-Pleistocene. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 251(3-4), 500-526.
- Fick, S. E., & Hijmans, R. J. (2017). WorldClim 2: new 1-km spatial resolution climate surfaces for global land areas. *International journal of climatology*, 37(12), 4302-4315.
- Filipe, A. F., Araujo, M. B., Doadrio, I., Angermeier, P. L., & Collares-Pereira, M. J. (2009). Biogeography of Iberian freshwater fishes revisited: the roles of historical versus contemporary constraints. *Journal of Biogeography*, 36(11), 2096-2110.
- Flanagan, S. P., Forester, B. R., Latch, E. K., Aitken, S. N., & Hoban, S. (2018). Guidelines for planning genomic assessment and monitoring of locally adaptive variation to inform species conservation. *Evolutionary applications*, 11(7), 1035-1052.

- Fluker, B. L., Jones, K. D., & Kuhajda, B. R. (2019). Genetic structure and diversity of the blueface darter *Etheostoma cyanoprosopum*, a microendemic freshwater fish in the southeastern USA. *Endangered Species Research*, 40, 133-147.
- Foll, M., & Gaggiotti, O. (2008). A genome-scan method to identify selected loci appropriate for both dominant and codominant markers: a Bayesian perspective. *Genetics*, 180(2), 977-993.
- Frankham, R., Ballou, S. E. J. D., Briscoe, D. A., & Ballou, J. D. (2002). *Introduction to conservation genetics*. Cambridge university press.
- Freyhof, J., Ekmekçi, F. G., Ali, A., Khamees, N. R., Özuluğ, M., Hamidan, N., ... & Smith, K. G. (2014). Freshwater fishes. *The Status and Distribution of Freshwater Biodiversity in the Eastern Mediterranean*. Cambridge, UK, Malaga, Spain and Gland, Switzerland: IUCN. Xiv, 132.
- Fu, Y. X. (1997). Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics*, 147(2), 915-925.
- Gante, H. F., Micael, J., Oliva-Paterna, F. J., Doadrio, I., Dowling, T. E., & Alves, M. J. (2009). Diversification within glacial refugia: tempo and mode of evolution of the polytypic fish *Barbus sclateri*. *Molecular ecology*, 18(15), 3240-3255.
- Garcia-Raventós, A., Martins, F., Teixeira, A., Sousa, R., Froufe, E., Varandas, S., ... & Filipe, A. F. (2020). Origin and history of *Phoxinus* (Cyprinidae) introductions in the Douro Basin (Iberian Peninsula): an update inferred from genetic data. *Biological Invasions*, 22(8), 2409-2419.
- Garlapati, D., Charankumar, B., Ramu, K., Madeswaran, P., & Murthy, R. (2019). A review on the applications and recent advances in environmental DNA (eDNA) metagenomics. *Reviews in Environmental Science and Bio/Technology*, 18(3), 389-411.
- Gavilán, G. A., & Polo, M. A. (1987). Evolución tecto-sedimentaria olugo-miocénica del SO de la fosa de Ciudad Rodrigo. Salamanca. *Acta geológica hispánica*, 419-426.
- Geist, J. (2011). Integrative freshwater ecology and biodiversity conservation. *Ecological Indicators*, 11(6), 1507-1516.
- Gibbard, P. L., Head, M. J., Walker, M. J., & Subcommission on Quaternary Stratigraphy. (2010). Formal ratification of the Quaternary System/Period and the Pleistocene Series/Epoch with a base at 2.58 Ma. *Journal of Quaternary Science*, 25(2), 96-102.
- Gleick, P. H. (1993). *Water in crisis: a guide to the world's fresh water resources*: Oxford University Press. New York.
- Gleick, P. H. (1998). *Water in crisis: paths to sustainable water use*. *Ecological applications*, 8(3), 571-579.
- Goldberg, C. S., Strickler, K. M., & Pilliod, D. S. (2015). Moving environmental DNA methods from concept to practice for monitoring aquatic macroorganisms. *Biological Conservation*, 183, 1-3.
- Goutte, A., Molbert, N., Guérin, S., Richoux, R., & Rocher, V. (2020). Monitoring freshwater fish communities in large rivers using environmental DNA metabarcoding and a long-term electrofishing survey. *Journal of Fish Biology*, 97(2), 444-452.

- Haywood, A. M., Dowsett, H. J., & Dolan, A. M. (2016). Integrating geological archives and climate models for the mid-Pliocene warm period. *Nature communications*, 7(1), 1-14.
- Hempel, C. A., Peinert, B., Beermann, A. J., Elbrecht, V., Macher, J. N., Macher, T. H., ... & Leese, F. (2020). Using environmental DNA to monitor the reintroduction success of the Rhine sculpin (*Cottus rhenanus*) in a restored stream. *Frontiers in Ecology and Evolution*, 8, 81.
- Hewitt, G. M. (1996). Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological journal of the Linnean Society*, 58(3), 247-276.
- Hewitt, G. M. (2001). Speciation, hybrid zones and phylogeography—or seeing genes in space and time. *Molecular ecology*, 10(3), 537-549.
- Hinlo, R., Furlan, E., Sutor, L., & Gleeson, D. (2017). Environmental DNA monitoring and management of invasive fish: comparison of eDNA and fyke netting. *Management of Biological Invasions*, 8(1), 89.
- Hudson, R. R., Boos, D. D., & Kaplan, N. L. (1992). A statistical test for detecting geographic subdivision. *Molecular biology and evolution*, 9(1), 138-151.
- Hügueny, B., Oberdorff, T., & Tedesco, P. A. (2010). Community ecology of river fishes: a large-scale perspective. In *American Fisheries Society Symposium* (Vol. 73, pp. 29-62).
- Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation letters*, 4(2), 150-157.
- Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular biology and evolution*, 30(4), 772-780.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28(12), 1647-1649.
- Kess, T., Gross, J., Harper, F., & Boulding, E. G. (2016). Low-cost ddRAD method of SNP discovery and genotyping applied to the periwinkle *Littorina saxatilis*. *Journal of Molluscan Studies*, 82(1), 104-109.
- Kottelat, M. (2007). Three new species of *Phoxinus* from Greece and southern France (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwaters*, 18(2), 145.
- Kottelat, M., & Freyhof, J. (2007). *Handbook of European freshwater fishes*. Publications Kottelat.
- Krijgsman, W., Garcés, M., Agustí, J., Raffi, I., Taberner, C., & Zachariasse, W. J. (2000). The ‘Tortonian salinity crisis’ of the eastern Betics (Spain). *Earth and Planetary Science Letters*, 181(4), 497-511.
- Krijgsman, W., Hilgen, F. J., Raffi, I., Sierro, F. J., & Wilson, D. S. (1999). Chronology, causes and progression of the Messinian salinity crisis. *Nature*, 400(6745), 652-655.
- Kull, T., Sammul, M., Kull, K., Lanno, K., Tali, K., Gruber, B., ... & Henle, K. (2008). Necessity and reality of monitoring threatened European vascular plants. *Biodiversity and Conservation*, 17(14), 3383-3402.

- Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular biology and evolution*, 29(6), 1695-1701.
- Larson, E. R., Graham, B. M., Achury, R., Coon, J. J., Daniels, M. K., Gambrell, D. K., ... & Suarez, A. V. (2020). From eDNA to citizen science: emerging tools for the early detection of invasive species. *Frontiers in Ecology and the Environment*, 18(4), 194-202.
- Leakey, R. E., & Lewin, R. (1995). *The sixth extinction: biodiversity and its survival*. Phoenix.
- Leigh, J. W., & Bryant, D. (2015). POPART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, 6(9), 1110-1116.
- Levy, A., Doadrio, I., & Almada, V. C. (2009). Historical biogeography of European leuciscins (Cyprinidae): evaluating the Lago Mare dispersal hypothesis. *Journal of Biogeography*, 36(1), 55-65.
- Lopes-Cunha, M., Aboim, M. A., Mesquita, N., Alves, M. J., Doadrio, I., & Coelho, M. M. (2012). Population genetic structure in the Iberian cyprinid fish *Iberochondrostoma lemmingii* (Steindachner, 1866): disentangling species fragmentation and colonization processes. *Biological Journal of the Linnean Society*, 105(3), 559-572.
- López-Martínez, N. (1989). Tendencias en Paleobiogeografía. El futuro de la biogeografía del pasado. *Paleontología*, 10, 271.
- Lozano-Rey, L. (1935). Los peces fluviales de España. *Memorias de la Academia de Ciencias Exactas, Físicas y Naturales de Madrid, Serie de Ciencias Naturales*, 5 (p. 387). Madrid, Spain.
- Machordom, A., & Doadrio, I. (2001). Evidence of a Cenozoic Betic–Kabilian connection based on freshwater fish phylogeography (*Luciobarbus*, Cyprinidae). *Molecular Phylogenetics and Evolution*, 18(2), 252-263.
- Magoč, T., & Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics*, 27(21), 2957-2963.
- Marić, S., Stanković, D., Wanzenböck, J., Šanda, R., Erős, T., Takács, P., ... & Snoj, A. (2017). Phylogeography and population genetics of the European mudminnow (*Umbra krameri*) with a time-calibrated phylogeny for the family Umbridae. *Hydrobiologia*, 792(1), 151-168.
- Marić, S., Stanković, D., Šanda, R., Čaleta, M., Čolić, S., Šukalo, G., & Snoj, A. (2019). Genetic characterisation of European mudminnow (*Umbra krameri*) populations from the Sava River system. *Knowledge & Management of Aquatic Ecosystems*, (420), 46.
- Marsh, D. M., & Trenham, P. C. (2008). Current trends in plant and animal population monitoring. *Conservation Biology*, 22(3), 647-655.
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet. journal*, 17(1), 10-12.
- Martinez, A., Vergés, J., Clavell, E., & Kennedy, J. (1989). Stratigraphic framework of the thrust geometry and structural inversion in the southeastern Pyrenees: La Garrotxa area. *Geodinamica Acta*, 3(3), 185-194.

- Martín-González, F., & Heredia, N. (2011). Complex tectonic and tectonostratigraphic evolution of an Alpine foreland basin: The western Duero Basin and the related Tertiary depressions of the NW Iberian Peninsula. *Tectonophysics*, 502(1-2), 75-89.
- McCormack, J. E., Hird, S. M., Zellmer, A. J., Carstens, B. C., & Brumfield, R. T. (2013). Applications of next-generation sequencing to phylogeography and phylogenetics. *Molecular phylogenetics and evolution*, 66(2), 526-538.
- Meirmans, P. G., & Van Tienderen, P. H. (2004). GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular ecology notes*, 4(4), 792-794.
- Mendes, S. L., Coelho, M. M., & Sousa, V. C. (2019). Population genomics data supports introgression between western Iberian *Squalius* freshwater fish species 2. *bioRxiv*, 585687.
- Mesquita, N., Hänfling, B., Carvalho, G. R., & Coelho, M. M. (2005). Phylogeography of the cyprinid *Squalius aradensis* and implications for conservation of the endemic freshwater fauna of southern Portugal. *Molecular Ecology*, 14(7), 1939-1954.
- Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *2010 gateway computing environments workshop (GCE)* (pp. 1-8). Ieee.
- Miralles, L., Dopico, E., Devlo-Delva, F., & Garcia-Vazquez, E. (2016). Controlling populations of invasive pygmy mussel (*Xenostrobus securis*) through citizen science and environmental DNA. *Marine pollution bulletin*, 110(1), 127-132.
- Miró, Alexandre, and Marc Ventura. "Evidence of exotic trout mediated minnow invasion in Pyrenean high mountain lakes." *Biological Invasions* 17.2 (2015): 791-803.
- Miya, M., Sato, Y., Fukunaga, T., Sado, T., Poulsen, J. Y., Sato, K., ... & Iwasaki, W. (2015). MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. *Royal Society open science*, 2(7), 150088.
- Morán-Tejeda, E., Ceballos-Barbancho, A., & Llorente-Pinto, J. M. (2010). Hydrological response of Mediterranean headwaters to climate oscillations and land-cover changes: The mountains of Duero River basin (Central Spain). *Global and Planetary Change*, 72(1-2), 39-49.
- Morán-Tejeda, E., Ceballos-Barbancho, A., Llorente-Pinto, J. M., & López-Moreno, J. I. (2012). Land-cover changes and recent hydrological evolution in the Duero Basin (Spain). *Regional Environmental Change*, 12(1), 17-33.
- Moritz, C. (1994). Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, 3(4), 401-411.
- Moritz, C. (1995). Uses of molecular phylogenies for conservation. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 349(1327), 113-118.
- Moritz, C. (2002). Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic biology*, 51(2), 238-254.
- Muñoz, J. A., Martínez, A., & Vergés, J. (1986). Thrust sequences in the eastern Spanish Pyrenees. *Journal of Structural Geology*, 8(3-4), 399-405.

- Myers, N., Mittermeier, R. A., Mittermeier, C. G., Da Fonseca, G. A., & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, 403(6772), 853-858.
- Nelson, J. S., Grande, T. C., & Wilson, M. V. (2016). *Fishes of the World*. John Wiley & Sons.
- Oliva, M., Palacios, D., Fernández-Fernández, J. M., Rodríguez-Rodríguez, L., García-Ruiz, J. M., Andrés, N., ... & Hughes, P. D. (2019). Late Quaternary glacial phases in the Iberian Peninsula. *Earth-science reviews*, 192, 564-600.
- Ortuño, M., Martí, A., Martín-Closas, C., Jiménez-Moreno, G., Martinetto, E., & Santanach, P. (2013). Palaeoenvironments of the Late Miocene Prüedo Basin: implications for the uplift of the Central Pyrenees. *Journal of the Geological Society*, 170(1), 79-92.
- País, J., Cunha, P. P., Pereira, D., Legoinha, P., Dias, R., Moura, D., ... & González-Delgado, J. A. (2012). The Paleogene and Neogene of western Iberia (Portugal): a Cenozoic record in the European Atlantic domain. In *The Paleogene and Neogene of Western Iberia (Portugal)* (pp. 1-138). Springer, Berlin, Heidelberg.
- Palandačić, A., Naseka, A., Ramler, D., & Ahnelt, H. (2017). Contrasting morphology with molecular data: an approach to revision of species complexes based on the example of European *Phoxinus* (Cyprinidae). *BMC evolutionary biology*, 17(1), 1-17.
- Parsons, K. M., Everett, M., Dahlheim, M., & Park, L. (2018). Water, water everywhere: Environmental DNA can unlock population structure in elusive marine species. *Royal Society open science*, 5(8), 180537.
- Perdices, A., & Doadrio, I. (2000). Diversification patterns in *Cobitis calderoni* (Osteichthyes: Cobitidae) and relationships with some *Cobitis* lineages. *Folia Zoologica*, 49 (1), 45-54.
- Perdices, A., & Doadrio, I. (2001). The molecular systematics and biogeography of the European cobitids based on mitochondrial DNA sequences. *Molecular Phylogenetics and Evolution*, 19(3), 468-478.
- Perdices, A., Doadrio, I., & Bermingham, E. (2005). Evolutionary history of the synbranchid eels (Teleostei: Synbranchidae) in Central America and the Caribbean islands inferred from their molecular phylogeny. *Molecular Phylogenetics and Evolution*, 37(2), 460-473.
- Perdices, A., & Coelho, M. M. (2020). First records of the Vettonian spined loach *Cobitis vettonica* in Portugal with update on its Iberian distribution. *Fishes in Mediterranean Environments*, (002), 15.
- Perea, S., Böhme, M., Zupančič, P., Freyhof, J., Šanda, R., Özuluğ, M., ... & Doadrio, I. (2010). Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evolutionary Biology*, 10(1), 1-27.
- Perea, S., & Doadrio, I. (2015). Phylogeography, historical demography and habitat suitability modelling of freshwater fishes inhabiting seasonally fluctuating Mediterranean river systems: a case study using the Iberian cyprinid *Squalius valentinus*. *Molecular Ecology*, 24(14), 3706-3722.
- Perea, S., Cobo-Simon, M., & Doadrio, I. (2016). Cenozoic tectonic and climatic events in southern Iberian Peninsula: Implications for the evolutionary history of freshwater

- fish of the genus *Squalius* (Actinopterygii, Cyprinidae). *Molecular Phylogenetics and Evolution*, 97, 155-169.
- Perea, S., Sousa-Santos, C., Robalo, J., & Doadrio, I. (2021). Historical biogeography of the Iberian Peninsula: multilocus phylogeny and ancestral area reconstruction for the freshwater fish genus *Squalius* (Actinopterygii, Leuciscidae). *Journal of Zoological Systematics and Evolutionary Research*, 59(4), 858-886.
- Pereira, A. M., Almada, V. C., & Doadrio, I. (2011). Genetic relationships of brook lamprey of the genus *Lampetra* in a Pyrenean stream in Spain. *Ichthyological research*, 58(3), 278-282.
- Phillips, S. J., Anderson, R. P., Dudík, M., Schapire, R. E., & Blair, M. E. (2017). Opening the black box: An open-source release of Maxent. *Ecography*, 40(7), 887-893.
- Pillans, B., & Naish, T. (2004). Defining the quaternary. *Quaternary Science Reviews*, 23(23-24), 2271-2282.
- Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., ... & Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science*, 344(6187), 1246752.
- Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155(2), 945-959.
- Puechmaille, S. J. (2016). The program structure does not reliably recover the correct population structure when sampling is uneven: subsampling and new estimators alleviate the problem. *Molecular ecology resources*, 16(3), 608-627.
- Puigdefàbregas, C., Muñoz, J. A., & Vergés, J. (1992). Thrusting and foreland basin evolution in the southern Pyrenees. In *Thrust tectonics* (pp. 247-254). Springer, Dordrecht.
- Rambaut, A., Suchard, M. A., Xie, D., & Drummond, A. J. (2015). Tracer v1. 6. 2014.
- Raup, D. M. (1995). *Extinction rates* (Vol. 11). J. H. Lawton, & R. M. May (Eds.). Oxford: Oxford University Press.
- Ree, R. H., & Smith, S. A. (2008). Maximum likelihood inference of geographic range evolution by dispersal, local extinction, and cladogenesis. *Systematic biology*, 57(1), 4-14.
- Riaz, M., Kuemmerlen, M., Wittwer, C., Cocchiararo, B., Khaliq, I., Pfenninger, M., & Nowak, C. (2020). Combining environmental DNA and species distribution modeling to evaluate reintroduction success of a freshwater fish. *Ecological Applications*, 30(2), e02034.
- Rincón, P. A., V., J. C., Gonzalez-Sanchez, N., & Pollo, C. (1990). Fish assemblages in small streams in western Spain: the influence of an introduced predator. *Archiv für Hydrobiologie. Stuttgart*, 118(1), 81-91.
- Robalo, J. I., Santos, C. S., Almada, V. C., & Doadrio, I. (2006). Paleobiogeography of two Iberian endemic cyprinid fishes (*Chondrostoma arcasii*-*Chondrostoma macrolepidotus*) inferred from mitochondrial DNA sequence data. *Journal of Heredity*, 97(2), 143-149.
- Robalo, J. I., Almada, V. C., Levy, A., & Doadrio, I. (2007). Re-examination and phylogeny of the genus *Chondrostoma* based on mitochondrial and nuclear data and

- the definition of 5 new genera. *Molecular Phylogenetics and Evolution*, 42(2), 362-372.
- Robalo, J. I., Doadrio, I., Valente, A., & Almada, V. C. (2008). Insights on speciation patterns in the genus *Iberochondrostoma* (Cyprinidae): Evidence from mitochondrial and nuclear data. *Molecular Phylogenetics and Evolution*, 46(1), 155-166.
- Rodríguez Vidal, J., Cáceres, L. M., & Rodríguez Ramírez, A. (1993). Modelo evolutivo de la red fluvial cuaternaria en el suroeste de la Península Iberica. *Actas da 3ª reunião do Quaternário Ibérico, Coimbra*, 93-96.
- Rodriguez, R., Ramirez, O., Valdiosera, C. E., Garcia, N., Alda, F., Madurell-Malapeira, J. O. A. N., ... & Dalen, L. (2011). 50,000 years of genetic uniformity in the critically endangered Iberian lynx. *Molecular Ecology*, 20(18), 3785-3795.
- Rodríguez-Rodríguez, L., Antón, L., Pallàs, R., García-Castellanos, D., Jiménez-Munt, I., & Pastor-Martín, C. (2020). A GIS method to identify flat surfaces and restore relict fluvial long-profiles from terrace remnants gives new clues on how large basins respond to endorheic-exorheic transitions (Duero basin, Iberian Peninsula). *Earth Surface Processes and Landforms*, 45(4), 1013-1027.
- Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584.
- Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., ... & Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic biology*, 61(3), 539-542.
- Rosenberg, N. A., & Nordborg, M. (2002). Genealogical trees, coalescent theory and the analysis of genetic polymorphisms. *Nature Reviews Genetics*, 3(5), 380-390.
- Rosenzweig, M. L. (1995). *Species diversity in space and time* (No. 574.524 R6).
- Roy, M., Belliveau, V., Mandrak, N. E., & Gagné, N. (2018). Development of environmental DNA (eDNA) methods for detecting high-risk freshwater fishes in live trade in Canada. *Biological Invasions*, 20(2), 299-314.
- Rozas, J., Ferrer-Mata, A., Sánchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sánchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular biology and evolution*, 34(12), 3299-3302.
- Sales, N. G., Wangenstein, O. S., Carvalho, D. C., Deiner, K., Præbel, K., Coscia, I., ... & Mariani, S. (2021). Space-time dynamics in monitoring neotropical fish communities using eDNA metabarcoding. *Science of the Total Environment*, 754, 142096.
- Salgueiro, P., Carvalho, G., Collares-Pereira, M. J., & Coelho, M. M. (2003). Microsatellite analysis of genetic population structure of the endangered cyprinid *Anaocypris hispanica* in Portugal: implications for conservation. *Biological Conservation*, 109(1), 47-56.
- Sánchez-Hernández, J., Servia, M. J., Vieira-Lanero, R., & Cobo, F. (2012). New record of translocated *Phoxinus phoxinus* Kottelat, 2007 from a river basin in the North-West Atlantic coast of the Iberian Peninsula. *BioInvasions Records*, 1(1), 37-39.

- Sanjur, O. I., Carmona, J. A., & Doadrio, I. (2003). Evolutionary and biogeographical patterns within Iberian populations of the genus *Squalius* inferred from molecular data. *Molecular Phylogenetics and Evolution*, 29(1), 20-30.
- Santisteban, J. I., & Schulte, L. (2007). Fluvial networks of the Iberian Peninsula: a chronological framework. *Quaternary Science Reviews*, 26(22-24), 2738-2757.
- Schnabel, S., & Gutiérrez, Á. G. (2014). Advances in Geomorphology in Spain 2012-2014. In S. Schnabel & Á. G. Gutiérrez (Eds.), *Actas de la XIII Reunión Nacional de Geomorfología*. Cáceres, Universidad de Extremadura–Sociedad Española de Geomorfología.
- Seifertová, M., Bryja, J., Vyskočilová, M., Martínková, N., & Šimková, A. (2012). Multiple Pleistocene refugia and post-glacial colonization in the European chub (*Squalius cephalus*) revealed by combined use of nuclear and mitochondrial markers. *Journal of Biogeography*, 39(6), 1024-1040.
- Sepulveda, A. J., Nelson, N. M., Jerde, C. L., & Luikart, G. (2020). Are environmental DNA methods ready for aquatic invasive species management?. *Trends in ecology & evolution*, 35(8), 668-678.
- Shaw, J. L., Clarke, L. J., Wedderburn, S. D., Barnes, T. C., Weyrich, L. S., & Cooper, A. (2016). Comparison of environmental DNA metabarcoding and conventional fish survey methods in a river system. *Biological Conservation*, 197, 131-138.
- Shiklomanov, I. A., & Linz, G. (1991). The influence of climate change on hydrology and water management. *Метеорология и гидрология*, (4), 51-66.
- Sigsgaard, E. E., Carl, H., Møller, P. R., & Thomsen, P. F. (2015). Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. *Biological Conservation*, 183, 46-52.
- Sigsgaard, E. E., Jensen, M. R., Winkelmann, I. E., Møller, P. R., Hansen, M. M., & Thomsen, P. F. (2020). Population-level inferences from environmental DNA—Current status and future perspectives. *Evolutionary Applications*, 13(2), 245-262.
- Simberloff, D. (1988). The contribution of population and community biology to conservation science. *Annual review of ecology and systematics*, 19(1), 473-511.
- Smith, K. G., & Darwall, W. R. (Eds.). (2006). *The status and distribution of freshwater fish endemic to the Mediterranean Basin* (Vol. 1). IUCN.
- Snyder, D. E. (2003). Invited overview: conclusions from a review of electrofishing and its harmful effects on fish. *Reviews in fish biology and fisheries*, 13(4), 445-453.
- Soorae, P. S. (Ed.). (2018). *Global reintroduction perspectives, 2018: case studies from around the globe*. IUCN-International union for conservation of nature and natural resources.
- Sousa-Santos, C., Collares-Pereira, M. J., & Almada, V. (2007). Reading the history of a hybrid fish complex from its molecular record. *Molecular phylogenetics and evolution*, 45(3), 981-996.
- Sousa-Santos, C., Jesus, T. F., Fernandes, C., Robalo, J. I., & Coelho, M. M. (2019). Fish diversification at the pace of geomorphological changes: evolutionary history of western Iberian Leuciscinae (Teleostei: Leuciscidae) inferred from multilocus sequence data. *Molecular phylogenetics and evolution*, 133, 263-285.

- Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9), 1312-1313.
- Stange, K. M., Van Balen, R. T., Garcia-Castellanos, D., & Cloetingh, S. (2016). Numerical modelling of Quaternary terrace staircase formation in the Ebro foreland basin, southern Pyrenees, NE Iberia. *Basin Research*, 28(1), 124-146.
- Stephens, M., Smith, N. J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, 68(4), 978-989.
- Stephens, M., & Donnelly, P. (2003). A comparison of bayesian methods for haplotype reconstruction from population genotype data. *The American Journal of Human Genetics*, 73(5), 1162-1169.
- Strayer, D. L., & Dudgeon, D. (2010). Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society*, 29(1), 344-358.
- Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental dna. *Molecular ecology*, 21(8), 1789-1793.
- Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, 123(3), 585-595.
- Takács, P., Erős, T., Specziár, A., Sály, P., Vitál, Z., Ferincz, Á., ... & Csoma, E. (2015). Population genetic patterns of threatened European Mudminnow (*Umbra krameri* Walbaum, 1792) in a fragmented landscape: implications for conservation management. *PLoS One*, 10(9), e0138640.
- Takahara, T., Minamoto, T., & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PloS one*, 8(2), e56584.
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular biology and evolution*, 30(12), 2725-2729.
- Tan, M. P., Wong, L. L., Razali, S. A., Afiqah-Aleng, N., Mohd Nor, S. A., Sung, Y. Y., ... & Danish-Daniel, M. (2019). Applications of next-generation sequencing technologies and computational tools in molecular evolution and aquatic animals conservation studies: a short review. *Evolutionary Bioinformatics*, 15, 1176934319892284.
- The Royal Society (2003). *Measuring biodiversity for conservation*, p. 56. London: The Royal Society.
- Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids research*, 22(22), 4673-4680.
- Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biological conservation*, 183, 4-18.
- Tierno de Figueroa, J. M., López-Rodríguez, M. J., Fenoglio, S., Sánchez-Castillo, P., & Fochetti, R. (2013). Freshwater biodiversity in the rivers of the Mediterranean Basin. *Hydrobiologia*, 719(1), 137-186.

- Tsuji, S., Shibata, N., Sawada, H., & Ushio, M. (2020). Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. *Molecular Ecology Resources*, 20(5), 1323-1332.
- Turon, X., Antich, A., Palacín, C., Præbel, K., & Wangensteen, O. S. (2020). From metabarcoding to metaphylogeography: separating the wheat from the chaff. *Ecological Applications*, 30(2), e02036.
- Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P. F., ... & Dejean, T. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Molecular ecology*, 25(4), 929-942.
- Vargas, J. M., Real, R., & Guerrero, J. C. (1998). Biogeographical regions of the Iberian Peninsula based on freshwater fish and amphibian distributions. *Ecography*, 21(4), 371-382.
- Velasco, J. C., Peris, S. J., Pollo, C. J., & González, N. (1997). Los peces de la Provincia de Salamanca: atlas de distribución. Universidad de Salamanca. *Salamanca*.
- Wetjen, M., Hübner, D., Seehausen, O., & Schulz, R. (2020). Genetic diversity of endangered *Chondrostoma nasus* in the River Rhine system: Conservation genetics considerations on stocking and reintroduction. *Knowledge & Management of Aquatic Ecosystems*, (421), 25.
- Williams, M., Dunkerley, D., De Deckker, P., Kershaw, P., & Chappell, J. (1998). *Quaternary Environments.*, 2nd edn.(Arnold: London.).
- Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., ... & Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. *Scientific reports*, 7(1), 1-12.
- Yu, Y., Harris, A. J., & He, X. (2010). S-DIVA (Statistical Dispersal-Vicariance Analysis): a tool for inferring biogeographic histories. *Molecular phylogenetics and Evolution*, 56(2), 848-850.
- Yu, Y., Harris, A. J., Blair, C., & He, X. (2015). RASP (Reconstruct Ancestral State in Phylogenies): a tool for historical biogeography. *Molecular phylogenetics and evolution*, 87, 46-49.
- Yu, Y., Blair, C., & He, X. (2020). RASP 4: ancestral state reconstruction tool for multiple genes and characters. *Molecular Biology and Evolution*, 37(2), 604-606.
- Zardoya, R., & Doadrio, I. (1998). Phylogenetic relationships of Iberian cyprinids: systematic and biogeographical implications. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 265(1403), 1365-1372.
- Zardoya, R., & Doadrio, I. (1999). Molecular evidence on the evolutionary and biogeographical patterns of European cyprinids. *Journal of molecular evolution*, 49(2), 227-237.
- Zarraonaindia, I., Iriando, M., Albaina, A., Pardo, M. A., Manzano, C., Grant, W. S., ... & Estonba, A. (2012). Multiple SNP markers reveal fine-scale population and deep phylogeographic structure in European anchovy (*Engraulis encrasicolus* L.). *PLoS ONE*, 7, e42201.
- Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics*, 29(22), 2869-2876.

- Zheng, X., Levine, D., Shen, J., Gogarten, S. M., Laurie, C., & Weir, B. S. (2012). A high-performance computing toolset for relatedness and principal component analysis of SNP data. *Bioinformatics*, 28(24), 3326-3328.