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Géneros basales endémicos de lumbrícidos de la Península Ibérica y Sur de Francia

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

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“Géneros basales endémicos de lumbrícidos de la Península Ibérica y Sur de Francia”

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Índice

RESUMEN:

La Península Ibérica y el Sur de Francia parecen ser un centro de diversificación de Lumbrícidos, ya que en este área geográfica se encuentra una gran diversidad tanto de especies como de géneros, algunos de ellos endémicos de esta zona. Esta tesis doctoral pretende ahondar en el conocimiento de algunos de estos géneros endémicos, concretamente *Zophoscolex*, *Prosellodrilus*, *Iberoscolex* y *Cataladrilus*, géneros que apenas han sido incluídos en trabajos filogenéticos anteriores y cuya especie tipo no ha sido nunca incluída en dichos trabajos.

Tras realizar campañas de muestreo para capturar ejemplares de estos géneros y realizar estudios filogenéticos amplificando los marcadores moleculares COI, 16S, 28S, ND1 y 12S amplificados mediante PCR, se obtuvieron resultados muy esclarecedores:

El género *Zophoscolex,* situado a ambos lados de los Pirineos no es un género monofilético, y queda dividido en dos géneros diferentes: *Zophoscolex*, que contiene a la especie tipo y a las especies francesas del género, *Castellodrilus*, un nuevo género endémico de la Península Ibérica que contiene a las especies de distribución ibérica.

El género *Iberoscolex*, endémico de la Península Ibérica, forma un clado monofilético con las especies del género *Orodrilus* con una distribución limitada a zonas montañosas (Género Franco-Ibérico), encontrándose las especies tipo de ambos géneros en dicho clado, por lo que se ha procedido a la sinonimización de *Iberoscolex*, ya que *Orodrilus* es el nombre más antiguo. De esta forma desaparece el género endémico de la Península Ibérica *Iberoscolex* y se pone de manifiesto que el género *Orodrilus* tiene una distribución mucho mayor de la esperada, no solo en las montañas sino también en otros tipos de terreno.

El género Franco-Ibérico *Prosellodrilus*, del que se han incluido en este estudio más del 70% de sus especies, incluída la especie tipo *Prosellodrilus idealis*, forma un clado monofilético por lo que sí parece ser un género válido, cuya distribución de especies y relaciones interespecíficas se han visto muy influenciadas por el proceso de formación de los Pirineos.

El género *Cataladrilus*, del que se conoce muy poco, también parece formar un clado monofilético, por lo que también sería un género válido.

Además, actualmente se está trabajando en la descripción de un nuevo género de posición muy basal en la filogenia de los lumbrícidos.

ABSTRACT:

The Iberian Peninsula and the south of France appear to be a centre of Lumbricidae diversification, given the high diversity of species and genera in this geographical area, including some endemic to the region. This doctoral thesis aims to deepen our understanding of some of these endemic genera, specifically the genera Zophoscolex, Prosellodrilus, Iberoscolex and Cataladrilus. These genera have barely been included in previous phylogenetic works, and their type species have never been included in said works.

After carrying out sampling campaigns to capture specimens of these genera and carrying out phylogenetic studies using the molecular markers COI, 16S, 28S, ND1 and 12S amplified by PCR, very important results were obtained:

The genus *Zophoscolex*, located on both sides of the Pyrenees, is not a monophyletic genus and is divided into two different genera: The genus *Zophoscolex*, which contains the type species and the French species of the genus, and the genus *Castellodrilus*, a new genus endemic to the Iberian Peninsula that contains species with an Iberian distribution.

The genus *Iberoscolex*, endemic to the Iberian Peninsula, forms a monophyletic clade with the species of the mountains limited distribution genus *Orodrilus* (Franco-Iberian Genus), with the type species of both genera being found in said clade, which is why we have proceeded to the synonymization of the genus, being called *Orodrilus* because this genus is the oldest. In this way, the endemic genus *Iberoscolex* of the Iberian Peninsula disappears and it becomes clear that the genus *Orodrilus* has a much greater distribution than expected, not only in the mountains.

The Franco-Iberian genus *Prosellodrilus*, of which more than 70% of its species have been included in this study, including the type species, forms a monophyletic clade so it does seem to be a valid genus, whose species distribution and interspecific relationships have been greatly influenced by the formation process of the Pyrenees.

The genus *Cataladrilus*, about which very little is known, also appears to form a monophyletic clade, so it would also be a valid genus.

Furthermore, a study is currently being done on the description of a new genus with a very basal position in the lumbricids phylogeny.

INTRODUCCIÓN GENERAL

Los oligoquetos se han considerado habitualmente como macroorganismos pertenecientes al filo Annelida, clase Clitellata y subclase Oligochaeta. Existen actualmente más de 6000 especies, que se distribuyen prácticamente por todo nuestro planeta, y que pueden ser terrestres (la mayor parte), dulceacuícolas y marinas (Martin *et al*., 2008). Dentro de los oligoquetos, las lombrices de tierra son los organismos más numerosos y mejor conocidos. Estos organismos edáficos viven en el suelo excavando galerías y alimentándose de la materia orgánica existente en el medio y en diferentes estados de descomposición. Según Misirlioğlu *et al* (2023) hay 5.738 especies/subespecies de lombrices de tierra pertenecientes a 23 familias pero potencialmente se podría llegar a las 8000 especies, y según Decäens et al (2024) podrían existir unas 30.000 especies que al ritmo actual de trabajo en este grupo tardarían en describirse unos 120 años.

De acuerdo a sus características morfológicas y a cómo éstas pueden predecir el modo de vida de las lombrices, Bouché (1972) las dividió en categorías ecológicas. Las tres categorías ecológicas principales son:

Epigeas: Son de tamaño pequeño, con pigmentación rojiza. Viven en las capas más superficiales del suelo, y se alimentan de restos orgánicos en diferentes grados de descomposición.

Endogeas: Su tamaño puede variar de pequeño a mediano (ocasionalmente grande) y suelen carecer de pigmentación. Viven en las capas más profundas del suelo, alimentándose de la materia orgánica del suelo, que se encuentra muy acomplejada en los coloides del suelo.

Anécicas: Son de tamaño mediano a grande y presentan pigmentación oscura (a veces sólo en su parte anterior o dorsal). Viven en las capas más profundas del suelo, pero excavan grandes galerías verticales por las que suben a la superficie para alimentarse de hojarasca y restos de materia orgánica en descomposición.

Existen otras categorías ecológicas que aparecen menos citadas en la literatura y con características intermedias (epiendogeas, epianécicas, endoanécicas y epi-endo-anécicas -Botinelli et al. 2020). Además, recientemente se han definido grupos funcionales para las lombrices de tierra basándose en su comportamiento de alimentación y excavación; estos grupos se corresponden aproximadamente con las categorías anteriores: tuneladoras intensas (relacionadas con las anécicas), cavadoras (relacionadas con las epianécicas), bioturbadoras superficiales y profundas (relacionadas con las endogeas), habitantes de la hojarasca (relacionadas con las epigeas) e intermedias (Capowiez et al. 2024).

Las lombrices de tierra son consideradas unos de los organismos más importantes para los ecosistemas, y tiene, además, una relativa importancia para el ser humano:

1- Constituyen la principal biomasa animal del medio edáfico.
2- Mediante la excavación de galerías y la conversión de materia orgánica en inorgánica contribuyen de forma sustancial a la formación de suelo.
3- Contribuyen en los ciclos biogeoquímicos del nitrógeno y el carbono, consumiendo estos elementos en su forma orgánica y devolviéndola al suelo en su forma mineral, lo que hace que estos elementos estén disponibles para las plantas.
4- Promueven la actividad microbiana.
5- Participan en las cadenas tróficas como alimento principal de muchas otras especies animales.

La importancia de estos organismos hace que sea relevante conocer su diversidad, la distribución de sus especies y las características tanto ecológicas como biológicas que necesitan las diferentes especies para poder llevar a cabo su ciclo biológico.

Dentro de las lombrices de tierra, una de las familias más numerosas y más estudiadas es la familia Lumbricidae, compuesta por más de 40 géneros y más de 600 especies. Uno de los principales centros de diversificación de esta familia se cree que puede estar en la Península Ibérica y el Sur de Francia, ya que esta zona cuenta con una gran diversidad tanto de especies como géneros, siendo muchos de estos géneros y especies endémicos de esta zona (Omodeo & Rota, 2008; Novo *et al.*, 2015; Marchán *et al.,* 2021a), lo cual puede estar favorecido por la compleja historia geológica y el gran número de ambientes diversos que presenta esta zona geográfica (Hewitt, 1996). En el trabajo "Fauna Ibérica" que está llevando a cabo el grupo de oligoquetos d ela UCM, se han listado más de 190 especies y subespecies de las que unas 90 son endémicas

Dentro de los géneros endémicos de este área geográfica, existen algunos que se localizan por todo el territorio peninsular, como *Iberoscolex* (Qiu & Bouché, 1998b), género bastante problemático que sería de aparición más reciente en la evolución, y por el contrario existen otros géneros que se encuentran limitados a una pequeña zona de esta área geográfica, principalmente al noreste de España y el sur de Francia (a ambos lados de la cordillera de los Pirineos).

Estos géneros de distribución más limitada, además, parecen ser géneros bastante antiguos y basales en la filogenia del grupo. Estos géneros son: *Zophoscolex* (Qiu & Bouché, 1998a), *Cataladrilus* (Qiu & Bouché, 1998b) y *Prosellodrilus* (Bouché, 1972).

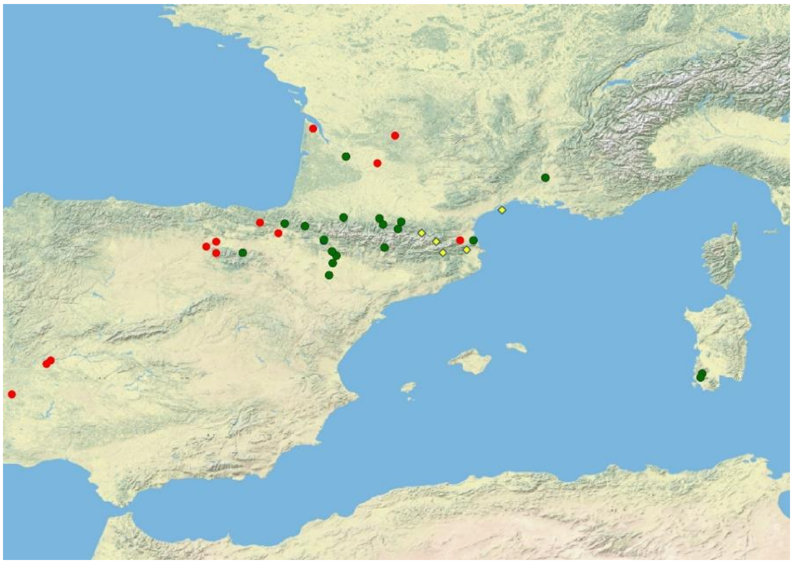

Figura 1: *Mapa de distribución aproximada de las especies de Prosellodrilus, Zophoscolex y Cataldrilus. Puntos naranjas: Espacies de Zophoscolex. Puntos Verdes: Especies de Prosellodrilus. Rombos amarillos: especies de Cataladrilus.*

La mayor parte de los estudios realizados con lombrices de tierra que cuentan con al menos una especie de cada uno de estos géneros, han sido solamente trabajos morfológicos, los cuales tienen grandes limitaciones ya que en el caso de las lombrices de tierra, los caracteres utilizados no suelen ser muy numerosos debido a las limitaciones que el propio suelo impone (Jones *et al.,* 1992) y suelen ser muy variables a nivel de especie (Briones *et al*., 2009), lo que da lugar a solapamientos de caracteres entre especies (Pérez-Losada *et al.,* 2009) y, por tanto, a la aparición de homoplasias, es decir, caracteres que han convergido evolutivamente en distintas especies . Además la especiación críptica es algo muy común en lombrices de tierra, sobre todo en especies con poblaciones aisladas (King *et al*., 2008, Shekhovtsov *et al.,* 2013; Taheri *et al.,* 2018), por lo que los estudios morfológicos presentan serias limitaciones a la hora de intentar reconstruir las relaciones evolutivas entre especies de lombrices de tierra, que deben ser abordadas mediante estudios integrados basados en fuentes complementarias como la morfología y la filogenia molecular.

En los últimos años, la aparición de nuevos y cada vez más potentes métodos de filogenia molecular han hecho posible obtener nueva y más fiable información sobre las relaciones evolutivas entre las distintas especies y géneros de lumbrícidos, permitiendo la creación de nuevos géneros (Marchán *et al*., 2021a), la división de géneros previamente definidos mediante morfología (Marchan *et al*., 2021b), o el descubrimiento de nuevas especies (Novo *et al.,*2012; Marchán *et al.,* 2020). Estos métodos moleculares utilizan diversos genes para inferir las relaciones filogenéticas entre diversos grupos, minimizando así el problema de la convergencia adaptativa o de las mutaciones al azar que pudieran crear unas relaciones evolutivas falsas y enmascarar las verdaderas (Novo *et al*., 2011; Pérez-Losada *et al*., 2012).

OBJETIVOS GENERALES:

A lo largo de esta memoria se pretende:

1- ahondar en el conocimiento de las relaciones evolutivas de los géneros endémicos basales de Lumbrícidos del noreste de la Península Ibérica y Sur de Francia.
2- Estudiar tanto la posición filogenética que ocupan estos géneros en el árbol de los lumbrícidos como las relaciones evolutivas entre las especies de cada género.
3- Validar o no su carácter de buenos géneros.

Los tres géneros que se pretenden abordar en esta tesis son: *Zophoscolex*, *Cataladrilus* y *Prosellodrilus*, por ser los tres géneros endémicos basales conocidos de esta zona geográfica, sin embargo, durante la realización de la tesis surgió la posibilidad de estudiar también el género *Iberoscolex*, un género endémico de la Península Ibérica que presenta distribución más amplia que los otros mencionados, es mucho más reciente en términos evolutivos y puede aportar aspectos muy interesantes para conocer la evolución de las lombrices de tierra en las zonas estudiadas.

A continuación se hará una breve introducción a cada uno de estos géneros que serán el objeto de estudio de esta tesis.

Género *Zophoscolex*

Este género es tratado con profundidad en el Capítulo 1 de la tesis: "Sorry atlanticus, you are not my type: molecular assessment splits Zophoscolex (Lumbricidae: Crassiclitellata) into French and Iberian genera."

El género *Zophoscolex* (Qiu & Bouché, 1998a) es un género endémico de la Península Ibérica y el sur de Francia que cuenta con 22 especies divididas en cuatro subgéneros (*Zophoscolex*, *Aquiloniberícus*, *Castellodrilus* y *Euibericus*).

La mayoría de sus especies se localizan en el noreste de España y en el sur de Francia, sin embargo, existen dos especies, ambas pertenecientes al subgénero *Castellodrilus*, que tienen una distribución mucho más amplia: *Zophoscolex ibericus* (Trigo *et al*., 1988), que se extiende por Extremadura, Madrid y Portugal y *Zophoscolex chitae* (Díaz Cosín *et al*., 1988), que se extiende por Extremadura, Burgos y Portugal.

La mayor parte de los estudios realizados que incluyen a este género, son estudios meramente morfológicos que se llevaron a cabo cuando se describieron las diferentes especies, pero no hay apenas estudios moleculares que lo incluyan.

El único estudio filogenético que incluye a algún representante de este género fue llevado a cabo en 2012 por James y Davidson, y parece situar al género *Zophoscolex* junto a otros géneros basales endémicos del noreste de España y el sur de Francia, como *Prosellodrilus*, pero este estudio tiene muchas limitaciones ya que solo incluye a una especie de este género: *Zophoscolex zhongi* (Qiu & Bouché, 1998a), pero no incluye a la especie tipo: *Zophoscolex atlanticus* (Bouché, 1972), que fue designada como tal por los descubridores del género.

Género *Iberoscolex*

Este género es tratado en profundidad en el segundo capítulo de la tesis: "Comparative phylogeography and integrative systematic revision of Iberian endemic earthworms (Crassiclitellata, Lumbricidae)."

El género *Iberoscolex* (Qiu & Bouché, 1998b) incluye exclusivamente a especies presentes en la Península Ibérica y su especie tipo es *Iberoscolex microepigeus* (Qiu & Bouché, 1998b). Este género incluye algunas especies que se habían incluído anteriormente en el género cosmopolita *Eiseniona* Omodeo, 1956, especies como *Iberoscolex albolineatus* Díaz Cosín *et al.,* 1989, *I. microepigeus*, *Iberoscolex carpetanus* Álvarez, 1970 y *Iberoscolex pseudorroseus* Moreno *et al.,* 1981. A pesar de que este nuevo género ha sido aceptado por algunos autores (Rota, 2013), otros siguen negando su existencia y consideran que las especies de *Iberoscolex* deberían incluirse en el género *Eiseniona* (Blakemore, 2008). Además, algunos estudios moleculares recientes (Díaz-Cosín *et al.,* 2014; Domínguez *et al.,* 2015) han validado, no sólo el género *Iberoscolex* sino que han incluído a algunas especies como *Aporrectodea oliveirae* Rosa 1894 y *Eiseniona gerardoi* (Díaz Cosín *et al.,* 2014) dentro de este género. Sin embargo, no existen estudios moleculares que incluyan a todas las especies de género y no se han realizado estudios moleculares que incluyan a la especie tipo, algo imprescindible para poder validar o no el género.

Hay que destacar también la existencia de un pequeño género endémico de una zona reducida del Sur de Francia y el noreste de España (cerca de los Pirineos), el género *Orodrilus* (Bouché, 1972), del cual no existen estudios moleculares y que ha sido incluido dentro del género *Eiseniona* por muchos autores.

Algunas especies de *Iberoscolex* (*I. carpetanus*, *I. oliveirae* e *I. albolineatus*), presentan un área de distribución que coincide, en parte, con el de las dos especies del género *Zophoscolex* que presentan una distribución más amplia, las ya mencionadas *Z. chitae* y *Z. ibericus* que capturamos juntas en los muestreos de campo, por lo que además de aclarar en la medida de lo posible la filogenia de este género, se nos abrió la posibilidad de comparar patrones filogeográficos (incluyendo genética de poblaciones) de las especies que comparten área de distribución de estos dos géneros. Por estas razones nos planteamos la posibilidad de estudiar no solo los géneros basales de lumbrícidos endémicos de esta zona, sino también del género endémico *Iberoscolex*, aunque sea más reciente en términos evolutivos.

Géneros *Prosellodrilus* y *Cataladrilus*

Estos géneros son tratados en profundidad en el tercer capítulo de la tesis: “Tightly linked evolution of Franco-Iberian earthworms (Prosellodrilini, Lumbricidae) and Pyrenean Mountain range supported by molecular phylogenetics”

El género *Prosellodrilus* (Bouché, 1972) comprende 27 especies y algunas subespecies, y su distribución se encuentra restringida al noreste de la Península Ibérica y el sur de Francia (a ambos lados de los Pirineos).

Es un género que solo presenta el clitelo y los tubérculos pubertarios bien desarrollados (una de las características morfológicas principales para identificar especies) durante unos 10-15 días al año, y, además, muchas especies presentan características morfológicas muy similares, lo que hace que los estudios morfológicos no sean muy fiables y que las diferentes especies no sean nada fáciles de identificar. Además, muchas de las especies que conforman este género tienen una distribución muy restringida o se conocen muy pocos puntos de su distribución, lo que complica aún más su estudio.

No existen apenas estudios filogenéticos que incluyan especies de este género; los únicos estudios publicados (James & Davidson, 2012; Domínguez *et al.,* 2018) sitúan a este género como un género muy relacionado con otros géneros basales endémicos de esta zona como *Zophoscolex* o *Cataladrilus*, sin embargo, estos estudios no han incorporado a la especie tipo: *Prosellodrilus idealis* (Bouché, 1972).

Este género tiene varias especies bastante controvertidas que han cambiado varias veces de nombre o género a medida que iban surgiendo nuevos estudios, como *Prosellodrilus amplisetosus* (Bouché, 1972) descrito originalmente sobre material francés. Souto *et a*l. (1991) describieron ejemplares gallegos asignables a esta especie, pero estos mismos ejemplares fueron interpretados por Qiu y Buché (1998b) como una especie diferente a la que nominaron *Prosellodrilus occidentalis*. Blakemore (2012) eleva el subgénero *Kenleenus* a género (con quetas separadas), describe una nueva especie *Kenleenus armadas* procedente de Irlanda, a la que atribuye los ejemplares de Souto *et al.* (1991) y sitúa, con alguna reserva, *P. occidentalis* de Qiu y Bouché en su nuevo género *Cadanera.* Hay además varias especies muy poco estudiadas que no tienen clara su limitación especie-subespecie (*Prosellodrilus psammophilus* y *Prosellodrilus psammophilus magnus* o *Prosellodrilus pyrenaicus* (Cognetti, 1904).

El género *Cataladrilus* (Qiu & Bouché, 1998b), es otro género endémico del noreste de España y el sur de Francia que cuenta solo con 9 especies. Es un género muy poco estudiado y la mayoría de sus especies sólo se han encontrado una vez y en un lugar concreto, lo que dificulta mucho su estudio.

El único estudio filogenético que se ha llevado a cabo con algún ejemplar de este género, lo sitúa junto a otros géneros basales de la Península Ibérica y del sur de Francia como *Prosellodrilus*, con el que parece estar muy relacionado, o con *Zophoscolex*, y aunque en este caso sí que se ha incluido en el estudio la especie tipo *Cataladrilus monticola* (Qiu & Bouché, 1998b). La no inclusión de otras especies y el hecho de que parece que podría formar un grupo parafilético con *Prosellodrilus* (Marchán *et al.*, 2020), hacen que sea necesario un estudio en mayor profundidad para aclarar estos puntos.

Esta tesis doctoral se compone de tres capítulos correspondientes a los diferentes géneros ya mencionados, además de un cuarto capítulo correspondiente a un trabajo en preparación que abre la posibilidad de estudiar más en profundidad en futuros trabajos la existencia de otro género endémico de la Península Ibérica de separación muy temprana en la filogenia.

Capítulo 1: **“Sorry atlanticus, you are not my type: molecular assessment splits Zophoscolex (Lumbricidae: Crassiclitellata) into French and Iberian genera.”**

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Capítulo 2: **“Comparative phylogeography and integrative systematic revision of Iberian endemic earthworms (Crassiclitellata, Lumbricidae).”**

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Capítulo 3: “**Tightly linked evolution of Franco-Iberian earthworms (Prosellodrilini, Lumbricidae) and Pyrenean Mountain range supported by molecular phylogenetics.”**

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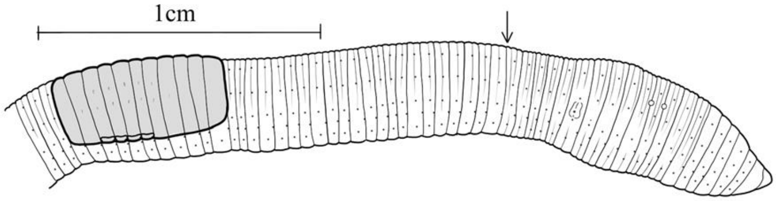
Capítulo 4: **“Un nuevo género de separación temprana endémico de la Península Ibérica”**

Este trabajo se encuentra actualmente en preparación, por lo que presentamos aquí una primera versión.

Capítulo 1

"Sorry atlanticus, you are not my type: molecular assessment splits *Zophoscolex* (Lumbricidae: Crassiclitellata) into French and Iberian genera."



La filogenética molecular contribuye a lograr importantes avances en el desafiante campo de la taxonomía de las lombrices de tierra. El uso de este tipo de análisis ha permitido aclarar las relaciones filogenéticas entre algunos géneros basales de lumbrícidos de la Península Ibérica y Sur de Francia. Sin embargo, los estudios filogenéticos moleculares del género *Zophoscolex* son escasos y han generado incertidumbre taxonómica debido a un muestreo insuficiente y la ausencia de la especie tipo, *Z. atlanticus*, en dichos estudios. El presente estudio incluyó 11 especies de *Zophoscolex* (incluido *Z. atlanticus*), y las relaciones filogenéticas se dedujeron a partir de siete marcadores moleculares (COI, COII, 16S, tRNA, ND1, 12S, 28S) mediante inferencia bayesiana y de máxima verosimilitud. Los hallazgos muestran que las especies de *Zophoscolex* no pertenecen a un solo clado. *Zophoscolex atlanticu*s se encuentra en un clado con *Z. micellus*, *Z. graffi* y *Ethnodrilus zajonci*. Pero la mayoría de las especies ibéricas forman un clado distinto, que se describió formalmente como el género revisado *Castellodrilus*. Según estos hallazgos, *Zophoscolex* está restringido a los representantes franceses. Estos resultados resaltan la importancia de incorporar especies tipo en los análisis filogenéticos moleculares para conciliar taxonomía y sistemática.

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Sorry *atlanticus*, you are not my type: molecular assessment splits *Zophoscolex* (Lumbricidae: Crassiclitellata) into French and Iberian genera

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Molecular phylogenetics contributes to making important advances in the challenging field of earthworm taxonomy. Use of this type of analysis has enabled clarification of the phylogenetic relationships between early-branching genera of Lumbricidae within the highly diverse Franco-Iberian realm. However, molecular phylogenetic studies of the genus *Zophoscolex* are scarce and have led to taxonomic uncertainty due to insufficient sampling and the absence of the type species, *Z. atlanticus*, from such studies. The present study investigated 11 species of *Zophoscolex* (including *Z. atlanticus*), and the phylogenetic relationships were deduced from seven molecular markers (*COI*, *COII*, 16S, tRN*As*, *ND1*, 12S, 28S) by Bayesian and maximum likelihood inference. The findings show that species of *Zophoscolex* did not belong to a single clade. *Zophoscolex atlanticus* was placed in a clade with *Z. micellus*, *Z. graffi* and *Ethnodrilus zajonci*. Other species of the genus were found to belong to the genera *Cataladrilus* and *Compostelandrilus*. Finally, most of the Iberian species form a distinct clade, which was formally described as the revised genus ***Castellodrilus***. Based on these findings, *Zophoscolex* is restricted to French representatives. These results highlight the importance of incorporating type species in molecular phylogenetic analyses in order to reconcile taxonomy and systematics.

ADITIONAL KEYWORDS: earthworms – molecular phylogenetics – systematics – taxonomy.

INTRODUCTION

Traditional morphology-based taxonomy of the earthworm (Crassiclitellata: Annelida) faces several difficulties. Earthworms have few main diagnostic characters and the ones they have are usually displayed only during short periods of the year when the earthworms are sexually mature. The variability in these characters appears to be constrained due to the limiting nature of the soil habitat (morphological stasis; Jones *et al.,* 1992), leading to the overlapping of diagnostic characters between species (Pérez- Losada *et al.,* 2009) and the widespread existence of cryptic species (King *et al.,* 2008). In addition, high intraspecific variability can be observed in the same characters (Briones et al., 2009).

For all these reasons, multigene molecular phylogenetic methods are contributing greatly to clarify earthworm relationships, allowing the description of new species (Novo *et al*., 2012; Marchán *et al*., 2020), new genera (Domínguez *et al.,* 2018; Marchán *et al.,* 2018), the placing species in their correct genera (Csuzdi *et al.,* 2017) and providing the first robust genus-level phylogeny of the family Lumbricidae (Domínguez *et al*., 2015). This approach is increasingly being applied to the native earthworm fauna of the Iberian Peninsula and southern France (Hormogastridae and Lumbricidae). These are areas of great evolutionary interest, because they are the centre of diversification for these families (Omodeo & Rota, 2008; Novo *et al*., 2015) due to their high diversity, endemicity and presence of putatively early-branching genera.

For instance, multigene phylogenetic analyses focusing on lumbricid genera have provided key information on the phylogenetic relationships between *Prosellodrilus* Bouché, 1972, *Cataladrilus* Qiu & Bouché, 1998b, *Postandrilus* Qiu & Bouché, 1998b and the new genera *Galiciandrilus* Domínguez *et al*., 2018 and *Compostelandrilus*

Domínguez *et al.,* 2018 (Pérez-Losada *et al*., 2011; Domínguez *et al.,* 2018). The phylogenetic analysis carried out in the latter study revealed a close phylogenetic affinity between *Zophoscolex cyaneus* (Briones & Díaz Cosín, 1993) and *Postandrilus*, *Galiciandrilus* and *Compostelandrilus*. *Zophoscolex cyaneus* was initially described as *Eophila cyanea* and its assignment *to Zophoscolex* Qiu & Bouché, 1998a is not firmly supported.

Zophoscolex is a Franco-Iberian genus that has received little attention, despite its high diversity (22 species in four subgenera: *Aquilonibericus, Castillodrilus, Euibericus* and *Zophoscolex*). The only previous molecular phylogenetic analysis to include a representative of *Zophoscolex*, *Z. zhongi* Qiu & Bouché, 1998a (James & Davidson, 2012), was limited to three molecular markers (18S, 16S and 28S) and six representatives of the Lumbricidae.

Furthermore, no currently published phylogenetic work has included the species *Zophoscolex atlanticus* (Bouché, 1972), which was designated by Qiu & Bouché (1998) as the type species of the genus *Zophoscolex* (thus being the name-bearer according to ICZN). Thus, only its inclusion in a phylogenetic context will enable the name *Zophoscolex* to be assigned to a clade. The inclusion of type species for genera of interest in molecular phylogenetics studies is of the utmost importance for a reconciliation between taxonomy and evolutionary relationships, and is slowly being adopted in earthworm systematics (de Sosa *et al.,* 2019). By including *Z. atlanticus* and ten additional species of the genus in a phylogenetic analysis, the aims of the present study were as follows:

1. To resolve the phylogenetic relationships between the genus *Zophoscolex* and the other members of the early-branching genera of Lumbricidae.

2. To check the validity of the genus *Zophoscolex* as monophyletic or to demonstrate its artificial status as a non-monophyletic taxon encompassing phylogenetically unrelated species.

3. To analyse the phylogenetic relationship of the type species of *Zophoscolex* to other species of the genus.

MATERIAL AND METHODS

Sampling and morphological Study

Two sampling surveys were carried out, in April and November 2019, throughout the northern Iberian Peninsula (Castilla y León and the Basque Country) and south-western France, with the aim of capturing species of the genus *Zophoscolex*. The locations where the type species were originally found were also visited (Qiu & Bouché, 1998a) (Table 1). Individuals were collected by digging and hand-sorting, and were washed with distilled water, fixed in 96% ethanol and maintained at –20 °C in the laboratory. Ethanol- fixed specimens of *Zophoscolex* from the collection of the Faculty of Biological Sciences of the Complutense University of Madrid (UCMLT Collection) were also included in the study.

The morphology of the specimens was examined under a binocular stereomicroscope following Qiu & Bouché (1998a). The external characters considered were weight, length, number of segments, position of the clitellum, position of the pubertal tubercles, position of the first dorsal pore, position of spermathecal pores and distance between chaetae. In order to ensure reliable identification, several specimens of each species were dissected and their main internal characters, such as the shape of nephridial vesicles, shape and position of calciferous (Morren's) glands, number and position of seminal vesicles and spermathecae, were studied following Qiu & Bouché (1998a).

Molecular Analyses

Samples were obtained from the body wall of two individual specimens of each of the following species:

Z. alavanensis Qiu & Bouché, 1998, *Z. atlanticus* (Bouché, 1969), *Z. chitae* (Díaz Cosín et al., 1988), *Z. graffi* Bouché, 1972, *Z. hongae* Qiu & Bouché, 1998, *Z. micellus* (Bouché, 1972), *Z. opisthoporus* Qiu & Bouché, 1998 and *Z. zhongi* Qiu y Bouché, 1998, and from one specimen (no more were available) each of *Z. ibericus* (Trigo *et al.,* 1988), *Z. eurytrichos* Qiu & Bouché, 1998 and *Z. pulvinus* Qiu & Bouché, 1998. In total, 23 representatives of 11 species of the four subgenera of *Zophoscolex* were included, providing a comprehensive representation of the diversity of the genus. Unpublished sequences for *Ethnodrilus zajonci* Bouché, 1972 (a putative close relative of the genus) were provided by the authors.

Genomic DNA was extracted and isolated using the DNeasy Blood & Tissue Kit (QIAGEN). Different regions of the mitochondrial genes 16S rRNA, tRNAs for Leu and Ala, 12S rRNA, NADH dehydrogenase subunit 1 (ND1), cytochrome c oxidase subunit II (COII) and cytochrome c oxidase subunit I (COI) and the nuclear gene 28S rRNA were amplified by polymerase chain reaction (PCR) with the primers described by Pérez-Losada *et al.* (2005, 2009), and under the same conditions outlined in Pérez-Losada *et al.* (2011) and Domínguez *et al.* (2015). The amplified PCR products were purified using the Multiscreen PCRµ96 purification kit (Millipore) and bidirectionally sequenced using an Applied Biosystems (ABI) 377XL type automatic sequencer in STAB Vida (Portugal). Sequences obtained in this work are available on Genbank with accession numbers 394357-411241.

Sequences were aligned with MAFFT v.7 (Katoh & Standley, 2013) by using the default parameters. The sequences of the seven molecular markers were then concatenated in BioEdit v.7.0.9 (Hall, 1999), to obtain a single sequence of 4739 base pairs for each of the specimens.

Reference sequences belonging to the families Lumbricidae, Hormogastridae and Criodrilidae (the latter two were chosen as outgroups) and determined by Domínguez *et al.* (2015, 2018), Pérez-Losada *et al.* (2015), Csuzdi *et al.* (2017), Bozorgi *et al.* (2019) and de Sosa *et al.* (2019) were retrieved from GenBank. Bayesian inference and maximum likelihood phylogenetic approaches were implemented with MrBayes v.3.2.6 (Ronquist *et al.,* 2012) and RAxML-NG (Kozlov *et al.,* 2019), respectively, both through the CIPRES Science Gateway v.3.3 (Miller *et al.,* 2010).

The optimal evolutionary model for each gene was selected using JModelTest v.2.1.3 (Darriba *et al.,* 2012), following the Akaike information criterion (AIC; Akaike, 1973) and the Bayesian information criterion (BIC; Schwarz, 1978) (which agreed in all cases). GTR+I+G was chosen as the optimal evolutionary model for the COI, 28S and ND1 markers; GTR+G was chosen for 12S; and the HKY+G+I model was selected for COII, 16S and tRNAs. These models were implemented in both the Bayesian and maximum likelihood analyses.

For Bayesian inference, the number of generations was set at 50 000 000. Two independent parallel analyses were initiated and 20% of the trees obtained were discarded. The other trees were combined to find the maximum posterior probability and to estimate the phylogeny.

The maximum likelihood analysis was performed with ten different starting trees, and support of resulting topology was estimated using 1000 rapid bootstrap replicates.

A time-calibrated phylogenetic inference was estimated using relative dating. To generate a suitable starting tree, the maximum likelihood tree was converted into an ultrametric tree by non-parametric rate smoothing (NPRS) using the function chronopl in the R package ape v5.2.

The final ultrametric tree was generated with BEAST v.1.10 (Suchard et al., 2018). Each partition was trimmed with GBlocks (Castresana, 2000) under the less stringent parameters, and the best-fitting evolutionary models (shown above) were specified. A normal distribution of mean = 1 and SD = 0.05 was implemented for the root of the tree as a relative calibration. A Yule diversification model and an uncorrelated lognormal relaxed clock were specified. Three parallel runs were performed, each of which included 50 million generations, sampling every 5000th generation. Tree and log files were combined in LogCombiner v.1.10 (Suchard et al., 2018) by resampling at lower frequency (15 000) and results were visualized in TRACER v. 1.7.1 (Rambaut et al., 2018). The final tree was generated by TreeAnnotator v.1.10 (Suchard et al., 2018) with a burn-in of 2000.

RESULTS

Species	Sampling location	Coordinates
Z. ibericus	Quinta do Reconço (Portugal)	41° 50' 19,96" N 6° 45' 0,67" W
Z. chitae	Guijo de Granadilla (Cáceres, Spain)	40° 11' 37.51" N 6° 9' 47.94" W
Z. alavanensis	Azazeta (Álava, Spain)	42° 47' 0" N 2° 30' 0" W
Z.atlanticus	Dax (Landes, France)	43° 42' 25" N 1° 3' 5" W
Z. pulvinus	Los Altos (Burgos, Spain)	42° 48' 31" N 3° 37' 54" W
Z. opisthoporus	Hortigüela (Burgos, Spain)	42°04'05"N 3°25'31" W
Z. eurytrichos	Covarrubias (Burgos, Spain)	42° 3' 33" N 3° 31' 12" W
Z. hongae	San Martín de Gomacín (Navarra, Spain)	42°40'18"N 1°48'56"W
Z. micellus	Anterrieux (Cantal, France)	44°50'8" N 3°2'38" E
Z. graffi	Treban (Allier, France)	46°24'16" N 3°10'29" E

Z. zhongi	Sant Joan de les Abadesses (Gerona, Spain)	42°14'10" N 2°17'12" E

Table 1. *Species of the genus Zophoscolex studied, sampling locations and Genbank accession numbers for the sequenced molecular markers*

Morphological characters

All studied specimens matched the original descriptions by Bouché (1972) and Qiu & Bouché (1998a).

Phylogenetic inferences

Bayesian inference (Fig. 1; Supporting Information, Fig. S1) and maximum likelihood inference of the phylogenetic tree provided consistent topologies. The genus *Zophoscolex* was not recovered as monophyletic. Instead, the studied representatives were scattered within a larger clade comprising *Cataladrilus, Compostelandrilus, Ethnodrilus, Postandrilus* and *Prosellodrilus*.

Zophoscolex atlanticus (the type species of the genus) was recovered within a strongly supported clade including *Z. micellus* and *Z. graffi* but also *Ethnodrilus zajonci* as its closest relative. Representatives of *Prosellodrilus* and *Cataladrilus* were recovered as a sister-clade.

Zophoscolex zhongi was recovered within *Cataladrilus* , with *Ca. edwarsi* as a sister-taxon. *Zophoscolex cyaneus* was included within an independent clade comprising *Postandrilus, Galiciandrilus* and *Compostelandrilus*, with the latter as a sister-taxon.

The other Iberian *Zophoscolex* representatives (*Z. alavanensis, Z. chitae, Z. eurytrichos, Z. hongae, Z. ibericus, Z. opisthoporus* and *Z. pulvinus*) resolved in a well-supported clade (monophyly, sister-taxa of equal rank; Lin et al., 2013). Internal relationships were well resolved, with *Z. opisthoporus* and *Z. eurytrichos* as a sister-clade to (*Z. chitae–Z. ibericus–Z. pulvinus*) and (*Z. hongae–Z. alavanensis*).

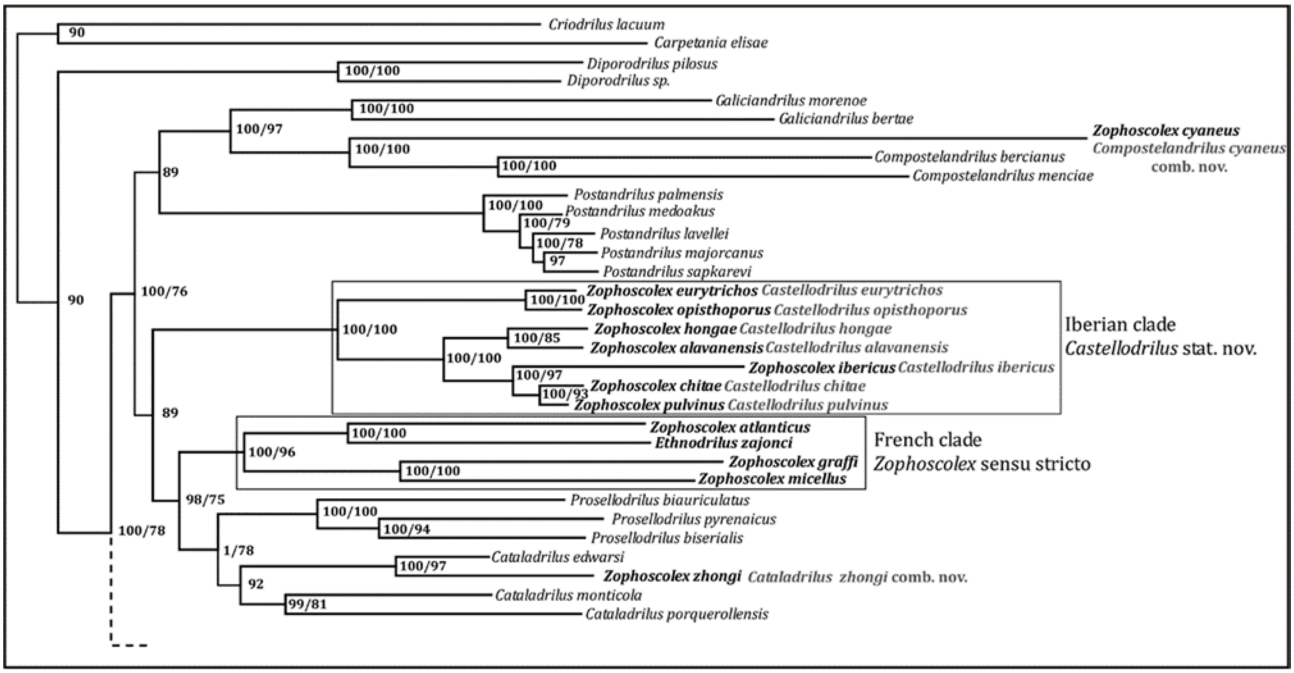

Figure 1. *Detail of the clades including the Zophoscolex species under study by Bayesian phylogenetic analysis of the concatenated sequence of molecular markers COI–COII–16S–tRNAs–ND1–12S–28S. Focus species of are shown in bold. Proposed taxonomic changes are shown in grey. Posterior probability support values and bootstrap support values (from maximum likelihood analysis) higher than 70 are shown beside the corresponding nodes. The complete phylogenetic tree is shown in Supporting Information, Figure S1.*

The time-calibrated phylogenetic tree (Supporting Information, Fig. S2) estimated a relative root age for the Iberian *Zophoscolex* clade that was similar to other well-established genera, while the clade including *Z. atlanticus*, *Z. micellus*, *Z. graffi* and *Ethnodrilus* showed a relative age significantly older than other lumbricid genus-level clades. The estimated root age for the clade including *Zophoscolex cyaneus* and *Compostelandrilus* was within the interval of genus- level clade root ages.

Systematics

The genus-level clade of Iberian earthworms formerly assigned to *Zophoscolex*, but unrelated to *Zophoscolex atlanticus*, is proposed as a revised genus, together with a diagnosis and list of the species included. An updated list of the species included within a re-defined *Zophoscolex* is also provided.

Genus ***Castellodrilus*** Jiménez-Pinadero *et al.,* 2021

PHYLUM ANNELIDA LAMARCK, 1802

CLASS OLIGOCHAETA GRUBE, 1850/CLITELLATA MICHAELSEN, 1919

ORDER MEGADRILI BENHAM, 1890/HAPLOTAXIDA MICHAELSEN, 1900

FAMILY LUMBRICIDAE RAFINESQUE-SCHMALTZ, 1815

GENUS ***CASTELLODRILUS*** (QIU & BOUCHÉ, 1998), **STAT NOV**

ZOPHOSCOLEX (CASTELLODRILUS) QIU & BOUCHÉ, 1998: 189.

ZOPHOSCOLEX (AQUILONIBERICUS) QIU & BOUCHÉ, 1998: 189. SYNON. NOV.

ZOPHOSCOLEX (EUIBERICUS) QIU & BOUCHÉ, 1998: 189. SYNON. NOV.

Type species:

Zophoscolex (Castellodrilus) opisthoporus Qiu & Bouché, 1998 by original designation.

Zoobank registration: urn:lsid:zoobank.org:act:C2661B3C-B353-4285-A0AF-E460722378B2.

Species included*:*

Castellodrilus alavanensis (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus anamariae***? (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus chitae*** (Diaz Cosín, Mato

& Trigo, 1988) **comb. nov.**, ***Castellodrilus eurythrichos*** (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus hongae*** (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus ibericus*** (Trigo *et al.*, 1988) **comb. nov.** , ***Castellodrilus joffrei***? (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus lopezi***? (Bouché, 1979) **comb. nov.**, ***Castellodrilus navarrensis***? (Qiu & Bouché, 1998) **comb. nov.**, ***Castellodrilus opisthoporus*** (Qiu & Bouché, 1998) **comb. nov., *Castellodrilus pulvinus*** (Qiu & Bouché, 1998) **comb. nov.** and ***Castellodrilus vasconensis***? (Bouché, 1979) **comb. nov.**

***Diagnosis**:* Small- to intermediate-sized (38– 138 mm) Lumbricidae, with fewer than 260 segments. Prostomium epylobous. Closely paired (separate in two species) setae. First dorsal pore in segments 6/7–12/13 (rarely in 19/20, 20/21). Nephridial pores aligned. Male pores in segments ½ 15 with poorly or well-developed porophore. Spermathecal pores simple, in 9/10, 10/11. Clitellum starts in a relatively posterior to significantly posterior position (between segments 32 and 43). Tubercula pubertatis start in a relatively posterior to significantly posterior position (usually between segments 34 and 55). Calciferous gland in segments 11–15 with dilations in 11 (rarely in 13). Typhlosole bifid or multifid. Two or three pairs of seminal vesicles in segments (9), 11 and 12. Nephridial vesicles inverted U or J-shape in anterior segments (generally before 10), usually inverted V-shape between 11 and 14, digitoid with ampulla from segment 15. Strongly developed *ab* chaetophores between segments 10 and 14, sometimes forming a glandular area in segments 10–13.

Differential diagnosis: Species of *Castellodrilus* stat. nov. can be distinguished from the closely related genera *Cataladrilus*, *Ethnodrilus*, *Prosellodrilus* and *Zophoscolex* by the backward displaced clitellum (starting in segments 32–43 vs. 22–29, 21–22, 19–21 and 22–24, respectively) and tubercula pubertatis (starting in segments 34–55 vs. 27–33, 23–30, 19–25 and 30–34, respectively). The shape of the nephridial vesicles (inverted U- or J-shape in anterior segments, generally before 10, usually inverted V-shape between 11 and 14, digitoid with ampulla from segment) differentiate *Castellodrilus* from *Cataladrilus* and several species of *Prosellodrilus.* Type and position of spermathecae (simple, in 9/10, 10/11) separate *Castellodrilus* from some species of *Zophoscolex* (*Z. micellus*: 9/10, 10/11 double; *Z. graffi*: 9/10, 10/11 double or multiple; *Z. aragonensis*: 9/10, 10/11 double or multiple; *Z. albacetensis*: 9/10, 10/11 simple or double) and from all species of *Prosellodrilus* [(12/13), 13/14, 14/15].

Remarks: *Zophoscolex* (*Castellodrilus*) is here elevated to genus status as it was found to be phylogenetically unrelated to the type species of *Zophoscolex*, *Z. atlanticus*. The same reasoning was applied to *Zophoscolex* (*Aquiloniberícus*) and *Zophoscolex* (*Euibericus*), which were considered synonymous to the former. None of the three names has objective priority due to being published simultaneously, thus as first revisers we chose *Castellodrilus* as the senior synonym. This is justified because *Zophoscolex* (*Castellodrilus*) *opisthoporus* (the type species of this subgenus) was included in the molecular phylogenetic analyses, unlike *Zophoscolex* (*Aquiloniberícus*) *navarrensis* and *Zophoscolex* (*Euibericus*) *joffrei.*

Several species placed in *Zophoscolex* by Qiu & Bouché (1998a, b) could not be included in this work. Thus, the species included in the same subgenera (*Aquiloniberícus*, *Castellodrilus* and *Euibericus*) as the studied representatives, are provisionally included within *Castellodrilus* at the genus level until further molecular phylogenetic analyses featuring these can be performed. *Zophoscolex* (*Aquiloniberícus*) *microprodromos* and *Zophoscolex* (*Aquiloniberícus*) *aragonensis* have been provisionally transferred to *Zophoscolex* due to the closer morphological affinity.

Genus ***Zophoscolex* Qiu & Bouché, 1998**

Type species: ***Zophoscolex atlanticus* Bouché, 1972.**

*Species included**:***

Zophoscolex albacetensis? Perez Onteniente & Rodriguez Babio, 2010, *Zophoscolex andorranensis*? Qiu & Bouché, 1998, *Zophoscolex aragonensis*? (Qiu & Bouché, 1998) comb. nov., *Zophoscolex atlanticus* Bouché, 1972, *Zophoscolex byanensis*? Qiu & Bouché, 1998, *Zophoscolex diazi*? Qiu & Bouché, 1998, *Zophoscolex graffi* Bouché, 1972, *Zophoscolex micellus* Bouché, 1972, *Zophoscolex microprodromos*? (Qiu & Bouché, 1998) comb. nov., *Zophoscolex zicsianus*? Szederjesi & Csuzdi, 2016.

Remarks:

Species included by Qiu & Bouché (1998a) within the subgenus *Zophoscolex* (*Zophoscolex*) are retained within *Zophoscolex*, except *Zophoscolex zhongi*, which was recovered in a different clade by phylogenetic analyses. *Zophoscolex andorranensis*, *Z. byanensis* and *Z. diazi* were not included in the phylogenetic analyses, hence their inclusion is provisional. *Zophoscolex aragonensis* and *Z. microprodromos* are transferred from *Zophoscolex* (*Aquilonibericus*) to *Zophoscolex* pending molecular phylogenetic assessment. The species *Z. albacetensis* and *Z. zicsianus* are also included as they were not originally assigned to any subgenera; their systematic placement must be confirmed by phylogenetic analyses.

DISCUSSION

Type species in integrative systematics

It is now widely acknowledged that taxonomy must reflect the evolutionary relationships of an animal group (Wiley & Lieberman, 2011). Thus, taxa higher than species level (e.g. genus) must comply with the requirement of monophyly. While some earthworm genera are highly homogeneous and do not appear to suffer from this problem (*Eisenia* Michaelsen, 1900, *Lumbricus* Linnaeus, 1758 and *Octodrilus* Omodeo, 1956), others are more complex, including several (more or less) related evolutionary lineages. For example, Csuzdi *et al.* (2017) clarified the phylogenetic relationships within the 'systematic wastebasket' of *Bimastos* Moore, 1893 and allegedly related genera *Allolobophoridella* Mrsic, 1990, *Dendrobaena* Eisen, 1873, *Dendrodrilus* Omodeo, 1956, *Eisenoides* Gates, 1969, *Healyella* Omodeo & Rota, 1989 and *Spermophorodrilus* Bouché, 1975, by including their type species in a comprehensive molecular analysis. This enabled *Allolobophoridella* and *Dendrodrilus* to be established as junior synonyms of *Bimastos*, and ruled out a close relationship between the former and *Healyella* and *Spermophorodrilus*. A more explicit effort to include the type species of an earthworm genus in order to solve its conflictive status was performed by de Sosa *et al.* (2019). *Eophila* Rosa, 1893 is another example of a 'catch-all' genus that included phylogenetically unrelated species based on a lax morphological diagnosis. The inclusion of the type species *Eophila tellinii* (Rosa, 1888) enabled a more taxonomically and biogeographically restricted genus to be established.

In this study, the inclusion of the type species *Zophoscolex atlanticus* revealed a taxonomic paradox: most of the species originally assigned to *Zophoscolex* belong to at least three other genera.

Systematic implications

According to the molecular phylogenetic analysis, *Zophoscolex* should be restricted to the species recovered in the same clade as *Z. atlanticus*. This would include *Z. graffi* and *Z. micellus* and, surprisingly, also *Ethnodrilus zajonci* (type species of *Ethnodrilus*). This species (as well as other representatives of the genus) closely resembles *Z. atlanticus* (in the clitellum position, position of first dorsal pore, number and position of spermathecae and shape of nephridial bladders), but there are some morphological differences (chaetal arrangement, calciferous glands and number of seminal vesicles). Thus, it would be premature to suggest synonymizing both genera until the other species of *Ethnodrilus* (*Et. aveli* Bouché, 1972, *Et. gatesi* Bouché, 1972 and *Et. lydiae* Bouché, 1972) are included in molecular phylogenetic analyses. Interestingly, an eventual synonymizing of *Zophoscolex* and *Ethnodrilus* would result in the disappearance of *Zophoscolex*, because *Ethnodrilus* has taxonomic priority.

The close phylogenetic relationship between *Zophoscolex cyaneus* and *Compostelandrilus* supports its inclusion in this north-western Iberian genus as

Compostelandrilus cyaneus (Briones & Diaz Cosín, 1993) comb. nov. Alternatively, as already suggested by Domínguez *et al.* (2018), the species may constitute a genus by itself if new species with a closer affinity to *Compostelandrilus cyaneus* than to the other *Compostelandrilus* were discovered. Until further sampling efforts are focused on the area located between the ranges of these species, the more conservative option is preferred.

The inclusion of *Z. zhongi* within *Cataladrilus* as *Cataladrilus zhongi* (Qiu & Bouché, 1998) comb. nov., close to *Cat.edwarsi* Qiu & Bouché, 1998, appears surprising due to the morphological differences (number and position of spermathecae, shape of nephridial bladders). Nevertheless, *Cat. zhongi* resembles species of *Cataladrilus* in the approximate position and extent of clitellum and tubercula pubertatis, structure of calciferous glands, general body shape and mode of life. Some species of *Zophoscolex* not included in this study and morphologically close to *Z. zhongi* (*Z. andorranensis*, *Z. byanensis* and *Z. diazi*) share overlapping ranges with the known distribution of *Cataladrilus*. A more comprehensive study incorporating these species and more representatives of *Cataladrilus* is necessary to elucidate the phylogenetic relationships in this north- eastern Iberian complex.

The revised genus *Castellodrilus* includes seven of the species studied here (*Castellodrilus alavanensis*, *Cas. chitae*, *Cas. eurythricos*, *Cas. hongae*, *Cas. ibericus*, *Cas. opisthoporus* and *Cas. pulvinus*) and tentatively includes another five species according to their close morphological affinity. These species were divided by Qiu & Bouché (1998b) into three different subgenera: *Euibericus*, *Aquilonibericus* and *Castillodrilus*. While the internal relationships within *Castellodrilus* revealed by the phylogenetic analyses mirrored this division, the validity of each subgenus cannot be confirmed in the absence of a wider sampling of their representatives. Thus, the three subgenera were synonymized until further evidence is explored.

Biogeographic and Evolutionary implications

The genus *Zophoscolex*, as defined by Qiu & Bouché (1998a, 1998b), was characterized by a relatively wide distribution, extending across southern France through the Iberian Peninsula reaching Portugal. The division supported by the present findings shows a different biogeographic scenario: *Zophoscolex* proper is restricted to France, while *Castellodrilus* appears to be endemic to the Iberian Peninsula. Remarkably, *Zophoscolex* appears to be more closely related to *Prosellodrilus* and *Cataladrilus* (which may include some species formerly described as *Zophoscolex*) than to *Castellodrilus*.

Prosellodrilus and *Cataladrilus* share a north- eastern Iberian and southern French distribution, overlapping with that of *Zophoscolex*. Hence, a primitive Pyrenean chain (emergent land since around 55 million years ago; Vacherat *et al.*, 2017) is a possible centre of origin of this clade. According to the topology revealed by the phylogenetic trees, *Castellodrilus* would be an earlier-branching clade, implying that the origin of the clade, including the aforementioned genera, may be located in the western half of the Iberian Peninsula. This area corresponds to the Iberian Massif, an ancient terrain that originated in the Variscan Orogeny and with a stable geological history since the Palaeozoic (Ribeiro *et al.*, 1990). The terrain was isolated from the eastern terrains of the Iberian Peninsula during different stages of the Mesozoic (Gil *et al.*, 2006), finally becoming connected by emerged land in the Late Cretaceous. This hypothesis could be supported by the current range of the sister clade, comprising *Compostelandrilus*, *Galiciandrilus* and *Postandrilus*. The former two clades inhabit the north-western Iberian Peninsula, while the latter is restricted to the Balearic Islands. This is suggestive of an origin in the Iberian Massif and a later colonization of the eastern Iberian Peninsula. However, a putatively early-branching Lumbricidae genus, *Kritodrilus* Dumnicka, 1983, remains to be examined by molecular phylogenetics. Although this genus includes species endemic to south-eastern France (including its type species, *Kritodrilus calarensis* Tetry, 1944), some south-eastern Iberian species have been tentatively assigned to *Kritodrilus* (Omodeo & Rota, 1989) and, surprisingly, also a Czechoslovakian species (*Kritodrilus mrazeki* Cernosvitov, 1935). The phylogenetic placement of these species will add an important piece to the biogeographic and evolutionary puzzle of early-branching Lumbricidae in the Western Mediterranean.

CONCLUSIONS

Inclusion of the type species of the genus *Zophoscolex* (*Z. atlanticus*) in molecular phylogenetic analysis, together with ten other species belonging to the genus, allows delimitation of a monophyletic *Zophoscolex* restricted to France and erection of the Iberian genus *Castellodrilus.* Several species formerly assigned to *Zophoscolex* were provisionally assigned to *Castellodrilus* on the basis of morphological similarity: further sampling and molecular analysis are required to confirm their phylogenetic affinities. Inclusion of these early-branching lumbricids in an explicit phylogenetic context provides insights into the origin and diversification of this family in the Franco-Iberian domain.

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Author notes

Supplementary data

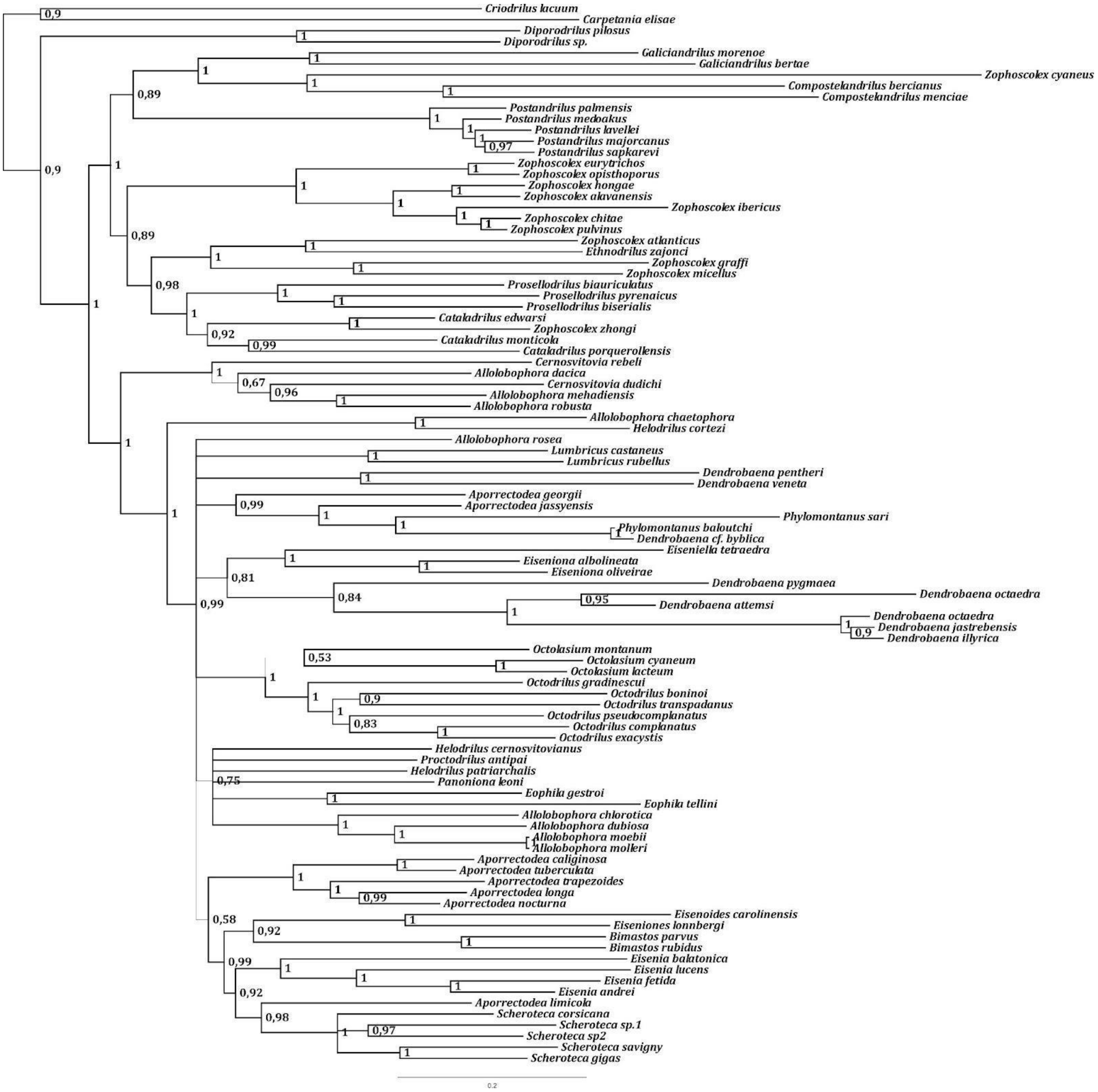


Figure S1.Complete phylogenetic tree obtained from the Bayesian phylogenetic analysis of the concatenated sequence of molecular markers COI–COII-16S-tRNAs–ND1–12S-28S. The posterior probability values are shown besides the corresponding nodes.

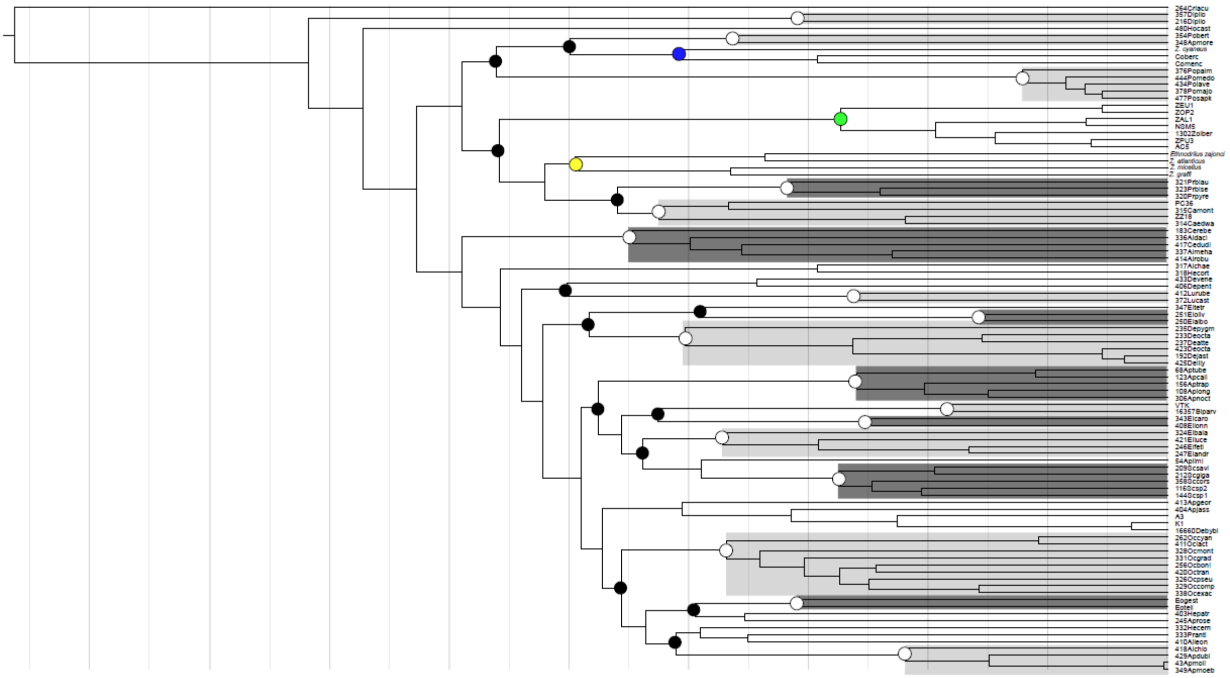
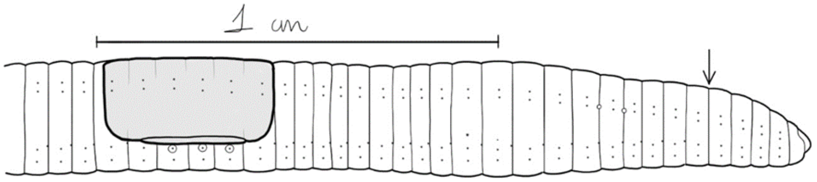


Figure S2. Ultrametric tree obtained by relative dating (root age = 1) through a BEAST phylogenetic analysis of the concatenated sequence of molecular markers COI–COII-16S-tRNAs–ND1–12S-28S. Singleton species and poorly resolved genera were not represented.

Capítulo 2

"Comparative phylogeography and integrative systematic revision of Iberian endemic earthworms (Crassiclitellata, Lumbricidae)."



La Península Ibérica y el suroeste de Francia han sido postulados como centros de diversificación de una amplia variedad de especies y géneros endémicos de lombrices dentro de la familia Lumbricidae. Sin embargo, la posición filogenética y la identidad de algunos de estos endemismos aún son inciertas y sus patrones filogeográficos son totalmente desconocidos. En el presente estudio se realizaron análisis filogenéticos con los marcadores moleculares COI, 16S, 28S, ND1 y 12S para aclarar la posición filogenética del endemismo ibérico *Iberoscolex*. Además, se estudiaron los patrones filogeográficos de tres de sus especies *(I. albolineatus, I. carpetanus* e *I. gerardoi*) basándose en el marcador COI y se compararon con patrones de especies que muestran distribuciones similares dentro de *Castellodrilus* (*C. ibericus* y *C. chitae*), otro endemismo ibérico. Los árboles filogenéticos recuperaron todas las especies del género I*beroscolex* (a excepción de *I. pseudorroseus*) dentro de un clado diferenciado de *Eiseniona* donde algunos autores habían colocado antes especies de *Iberoscolex*. Además, se agruparon con algunas especies (incluido el tipo) de *Orodrilus*, un endemismo íbero-francés también secuenciado para este estudio. Debido a la prioridad de *Orodrilus*, *Iberoscolex* debe considerarse un sinónimo posterior, con todas las especies anteriormente incluidas en él asignadas a un *Orodrilus* redefinido. Se observó una fuerte estructura poblacional para las especies estudiadas de *Iberoscolex* y *Castellodrilus*, junto con un patrón de aislamiento por distancia; sin embargo, estos géneros mostraron diferentes patrones de variabilidad genética: las especies de

Castellodrilus en estudio exhiben una menor diversidad genética, con menos haplotipos por población que las especies de *Iberoscolex*.

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ORIGINAL ARTICLE

Comparative phylogeography and integrative systematic revision of Iberian endemic earthworms (Crassiclitellata, Lumbricidae)

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Abstract

The Iberian Peninsula and south-western France have been postulated as cen- tres of diversification of a wide variety of endemic earthworm species and genera within the Lumbricidae family. However, the phylogenetic position and identity of some of these endemisms is still uncertain and their phylogeographic patterns totally unknown. In the present study, phylogenetic analyses were carried out with the molecular markers COI, 16S, 28S, ND1 and 12S to clarify the phyloge- netic position of the Iberian endemism *Iberoscolex* in the lumbricid tree. In addi- tion, phylogeographic patterns of three of its species (*I. albolineatus*, *I. carpetanus* and *I. gerardoi)* were studied based on the COI marker and compared with pat- terns of species showing similar distributions within *Castellodrilus* (*C. ibericus* and *C. chitae*), another Iberian endemism. Phylogenetic trees recovered all spe- cies of the genus *Iberoscolex* (with the exception of *I. pseudorroseus)* within a clade differentiated from *Eiseniona* where some authors had placed *Iberoscolex* species before. Moreover, they were clustered with some species (including the type) of *Orodrilus*, an Iberian-French endemism also sequenced for this study. Owing to the priority of *Orodrilus*, *Iberoscolex* should be considered a junior synonym, with all of the species formerly included within it assigned to a redefined *Orodrilus*. Strong population structure was observed for *Iberoscolex* and *Castellodrilus* spe- cies under study, together with a pattern of isolation by distance; however, these genera showed different patterns of genetic variability: the *Castellodrilus* species under study exhibit lower genetic diversity, with fewer haplotypes per population than the *Iberoscolex* species.

KEYWORDS

earthworms, Iberian endemism, phylogeography, soil organisms

1. INTRODUCTION

Traditionally, earthworm evolutionary relationships have been inferred from morphological characters, with most being either highly variable at species level or homoplasious (convergently evolved) (Jones *et al.,* 1992; Briones *et al.,* 2009). This has limited the number of systematically useful characters and resulted in unstable, unresolved classifications.

Molecular phylogenetics includes valuable methods for obtaining information about the evolutionary relationships between different lumbricid species and genera. These multigene molecular methods have helped to clarify many relationships between lumbricids, allowing the creation of new genera (Marchán *et al,.* 2021a), the division of morphologically-defined genera into monophyletic groups such as the separation of *Gatesona* Qiu and Bouché, 1998 from *Allolobophora* Eisen, 1873 (Marchán *et al.,* 2021b) and the discovery of new species (Novo *et al.,*2012; Marchán *et al.,* 2020a).

The Iberian peninsula and south-western France have a complex geological history and a large number of different environments (Hewitt, 1996) and appear to be the main centres of diversification of the earthworm family Lumbricidae, as they are home to several early-branching genera and the highest diversity in Europe with many endemic species and genera (Omodeo & Rota, 2008; Novo *et al.*, 2015; Marchán *et al.,* 2021a). Molecular phylogenetic studies have supported and strengthened that view, by establishing the endemic genera *Galiciandrilus* Domínguez *et al.*, 2018, *Compostelandrilus* Domínguez *et al.*, 2018, *Postandrilus* Qiu and Bouché, 1998 and *Castellodrilus* Qiu and Bouché, 1998 (split from a Franco-Iberian *Zophoscolex* Qiu and Bouché, 1998) (Pérez-Losada *et al,.* 2011, Domínguez *et al.,* 2018, Jiménez Pinadero *et al.,* 2021). These facts make Iberian Peninsula and South-western France very interesting areas for studying phylogenetic patterns in different species and genera.

Other genera endemic to the Iberian peninsula or south-western France remain to be studied in detail by molecular phylogenetics, e.g. the genus *Iberoscolex* Qiu and Bouché, 1998 (type species *I. microepigeus* Qiu & Bouché, 1998). This genus includes exclusively Iberian species previously included within the Palearctic genus *Eiseniona* Omodeo, 1956: *I. albolineatus* Díaz Cosín *et al.,* 1981, *I. microepigeus*, *I. carpetanus* Álvarez, 1970 and *I. pseudorroseus* Moreno *et al.,* 1981. However, although this genus has been accepted by some authors (Rota, 2013) others deny its existence and include it within the genus *Eiseniona* (Blakemore, 2008). There is also some controversy about the systematic position of two other Iberian endemic species *Aporrectodea oliveirae* Rosa, 1894 and *Eiseniona gerardoi* Díaz Cosín *et al.* 2014, where some molecular phylogenetic analyses have shown them to belong to *Iberoscolex* (Díaz Cosín *et al.,* 2014; Domínguez *et al.,* 2015) and they are treated as such in this work.

Orodrilus Bouché, 1972 is another controversial genus that is endemic to the Iberian Peninsula and south-western France (close to the border with Spain). Some authors had also included this genus in *Eiseniona*. Representative members of *Orodrilus* have not yet been included in molecular phylogenetic analyses.

Various studies of edaphic organisms have revealed a series of common genetic characteristics, including high genetic variability, high intraspecific divergence and strong population structure (Costa *et al.,*2011). However, later studies carried out with earthworms have shown that these characteristics are not always present, as is the case of *Aporrectodea icterica* Savignyi, 1826 (Torres-Leguimazón *et al.,* 2014), some invasive species (Novo *et al.,* 2015b) and cosmopolitan and parthenogenetic species such as *Aporrectodea trapezoides* Duges, 1828 (Fernández *et al.,* 2012) and *Eiseniella tetraedra* Savigny, 1826 (de Sosa *et al.,* 2017). To date, no phylogeographic studies on endemic and non-parthenogenetic lumbricids have been performed in the Iberian peninsula.

This study aims to expand the phylogeographic knowledge of some species of two endemic genera of the Iberian peninsula: *Castellodrilus* and *Iberoscolex*. These genera belong to different tribes (Prosellodrilini and Lumbricini respectively -Marchán *et al.,* 2022b) and their respective ecological categories (epiendogeic vs endogeic) but they are of similar body size and partly share a distribution area. Specifically, the distribution of *Castellodrilus chitae* Díaz Cosín *et al.,* 1988 and *Castellodrilus ibericus* Trigo *et al.,* 1988 (the most wide-ranging species of these genera*)*, partly overlaps with those of several species of the genus *Iberoscolex* (*I. carpetanus, I. albolineatus* and *I. gerardoi*).

The aims of the present study were:

1. To test the validity (monophyly) of *Iberoscolex* by including its generotype *I. microepigeus* and other species putatively assigned to this genus.

2. To clarify the relationships between *Iberoscolex* and other genera of Lumbricidae.

3. To analyze and compare phylogeographic patterns (including population genetics) of representative members of the genus *Iberoscolex* (*I. carpetanus, I. albolineatus* and *I. gerardoi*) and the partially sympatric *Castellodrilus* (*C. ibericus* and *C. chitae*).

2. MATERIAL AND METHODS

2.1 Sampling

Several sampling surveys were carried out, in April 2019, November 2020 and September 2021 throughout the northeastern-central Iberian peninsula (the autonomous regions of Castilla y León and Madrid) in order to capture species of the genera *Castellodrilus* and *Iberoscolex*. Individual worms were collected by digging and hand-sorting. The specimens were then washed with distilled water, fixed in 96% ethanol and stored at –20 °C in the laboratory. Ethanol fixed specimens of these genera from the collection of the Faculty of Biological Sciences of the Complutense University of Madrid (UCMLT Collection) were also included in the study (Table 1; Figure 1).

Species	**Locality**	**Geographic coordinates**	**Code**	**N of specimens**	**Det.**	**Habitat characteristics**	**Sampling date**
C. chitae	Guijo de Granadilla	40°10'23.0"N 6°07'52.9"W	GUIJCHI	5	Sergio Jiménez	wet planting area	May 2015
	Covarrubias	42°03'14.1"N 3°30'55.7"W	COV	7	Sergio Jiménez	wet planting area	April 2019
	Peñahorada	42°30'01.0"N 3°38'44.2"W	PEN	7	Sergio Jiménez	old wet planting area	April 2019
C. ibericus	Guijo de Granadilla	40°10'23.0"N 6°07'52.9"W	GUIJIBER	5	Darío DíazCosín	wet planting area	May 2015
	Romangordo	39°44'46.4"N 5°42'05.4"W	ROM	5	Darío DíazCosín	wet planting area	May 2019
	Colmenarejo	40°32'43.1"N 4°01'09.6"W	COL	5	Darío DíazCosín	semi-urbanized oak grove	March 2018
I. albolineatus	San Martín de Hoyos	42°55'58.0"N 4°09'16.1"W	SMH	5	Daniel Fdez Marchán	Limestone area with abundant leaf litter	November 2020
	Fontioso	41°56'30.0"N 3°44'25.5"W	FON	5	Daniel Fdez Marchán	Limestone area with abundant leaf litter	November 2020
	Valdeprado	43°04'27.5"N 4°29'33.2"W	VAL	5	Daniel Fdez Marchán	Moist mountain oak grove	November 2020
	Arcera	42°52'40.0"N 4°02'53.8"W	ARC	5	Daniel Fdez Marchán	Moist mountain oak grove	November 2020
	Asturias	43°13'02.4"N 5°06'53.6"W	AST	1	Daniel Fdez Marchán	Moist mountain oak grove	April 2018
I. carpetanus	Madrid	40°34'05.9"N 4°09'11.4"W	MAD	6	Daniel Fdez Marchán	high mountain pine forest	May 2018
	Villaquilambre	42°39'31.1"N 5°34'27.5"W	VIQUI	5	Sergio Jiménez	Limestone area of pines and oaks	November 2020

	Brazuelo	42°30'48.1"N 6°09'59.5"W	BRAZ	5	Daniel Fdez Marchán	Limestone area of pines and oaks	November 2020
I. gerardoi	El Bronco	40°12'28.4"N 6°18'38.1"W	PLA	5	Darío DíazCosín	Grazed dehesa with *Quercus ilex*	April 2016

Table 1. *Species and populations included in the study.*

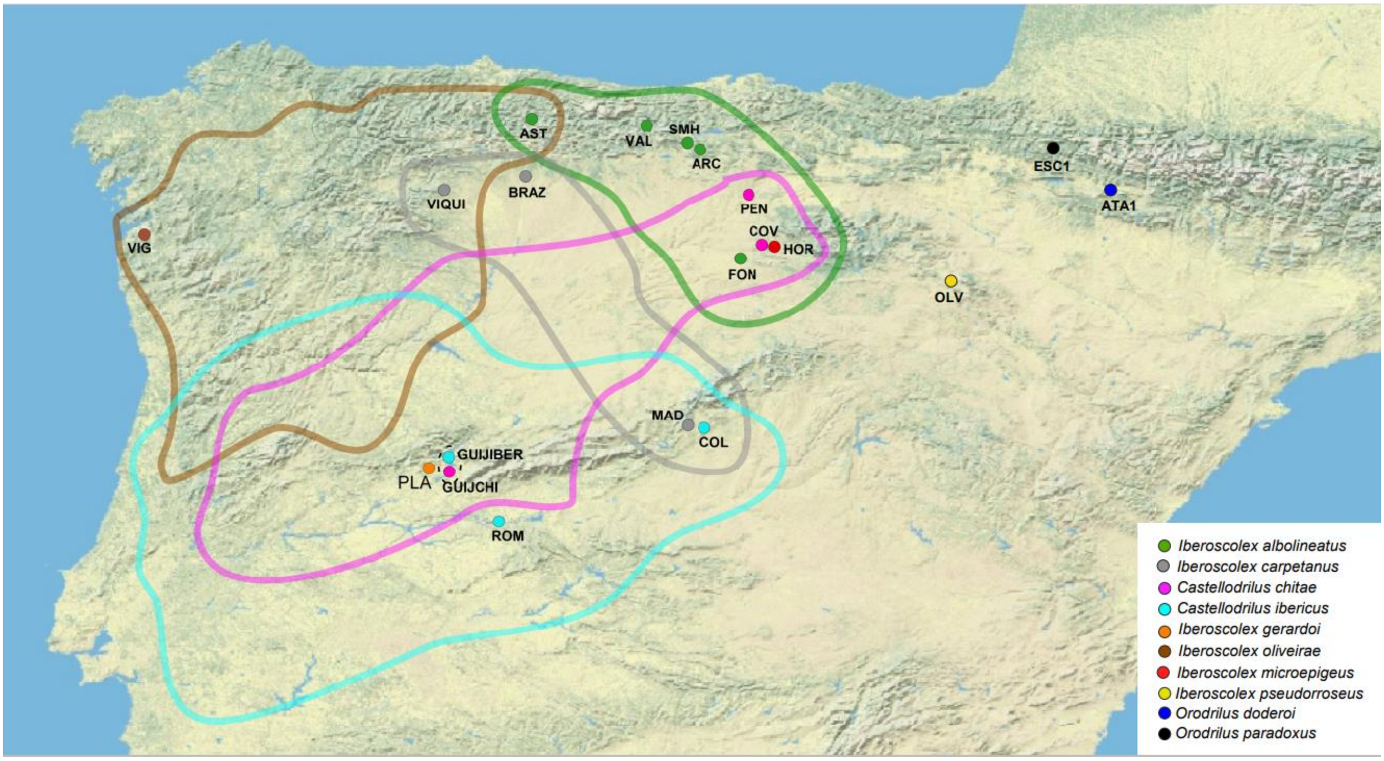

Figure 1*. Map showing the sampling points for all species and populations included in the study and the distribution area of each species: dark blue: I. carpetanus; light blue: C. ibericus; pink: C. chitae; green: I. albolineatus; Brown: I. oliveirae. Locality codes can be consulted in Table 1.*

2.2 DNA isolation and sequencing

Tissue samples were obtained from the body walls of different specimens of each of the species and populations listed in table 1.

Genomic DNA was extracted and isolated using the Speedtools tissue DNA extraction kit (Biotools). Regions of the mitochondrial genes 16S rRNA and COI and the nuclear gene 28S rRNA were amplified by polymerase chain reaction (PCR) with the primers described by Pérez-Losada *et al.* (2005, 2009), and under the same conditions outlined in Pérez-Losada *et al.* (2011) and Domínguez *et al.* (2015). Regions of the mitochondrial genes 12S and ND1 from one specimen of each species used in this study were additionally amplified to be used to estimate a phylogenetic tree. The PCR products were purified using the ExoSAP-IT PCR product cleanup reagent, provided by LIFE TECHNOLOGIES, S.A. and bidirectionally sequenced in an Applied Biosystems (ABI) 377XL DNA sequencer, by MACROGEN (Madrid).

2.3 Phylogenetic analysis and time-calibrated phylogenetic inference

In addition to the specific sequences obtained in this study, COI, 16S, 28S, 12S and ND1 sequences generated for other lumbricid species were used, as reported by Domínguez *et al.* (2015, 2018), Pérez-Losada *et al. (*2015), Csuzdi *et al.* (2017), Bozorgi *et al.* (2019) and de Sosa *et al.* (2019). Sequences from Criodrilidae and Hormogastridae families were included as outgroups.

Sequences were aligned with MAFFT v.7 (Katoh & Standley 2013) with default settings and concatenated with BioEdit (Hall 1999), resulting in a matrix of 3,377 bp. The best fitting evolutionary model for each partition was selected with jModelTest v. 2.1.3 (Darriba *et al.,*2012) by applying the Akaike information criterion (AIC; Akaike, 1973) and the Bayesian information criterion (BIC; Schwarz, 1978) (which agreed in their results). GTR+I+G was selected as the best-fitting evolutionary model for COI, 28S, 12S and ND1, and HKY+I+G was selected for 16S.

Bayesian Inference of the phylogeny was estimated with MrBayes v.3.2.6 (Ronquist *et al.,*2012) as implemented in CIPRES Science Gateway V. 3.3 (Miller *et al.* 2010). The analysis was performed with default parameters, and each of the two independent runs was set to 50 million generations sampling every 5,000th generation (10,000 trees). Twenty-percent of the trees were discarded as burn-in, with remaining trees combined and summarized on a 50% majority-rule consensus tree. Maximum Likelihood phylogenetic inference was performed using RAxML-NG (Kozlov *et al.* 2019) in the CIPRES Science Gateway V. 3.3 platform, from 10 random starting trees and 1,000 rapid bootstrap replicates.

The Maximum Likelihood tree was converted into an ultrametric tree by non-parametric rate smoothing (NPRS) and the chronopl function in the R package *ape* v5.2 was used to generate a suitable starting tree for the time-calibrated phylogenetic inference. The maximum and minimum ages of the clades were the same as those used in the downstream BEAST analysis.

The final ultrametric tree was generated using BEAST v. 1.10 (Suchard *et al.,*2018) with the NPRS tree as the starting tree. Each partition was trimmed using GBlocks (Castresana, 2000) under the less stringent parameters, with the best-fitting evolutionary model (shown above) used as the evolutionary model for each.

The following calibration points were implemented as uniform priors: 87–170 mya (Criodrilidae + Lumbricidae + Hormogastridae clade) and 82–147 mya (Lumbricidae+Hormogastridae clade). These age intervals correspond to the 95% HPD (highest posterior density) intervals reported by Marchán *et al.* (2017). Node ages estimated by Erséus et al. (2020) were not considered as they appear inconsistent with previous results and the historical biogeography of Crassiclitellata.

A Yule diversification model and an uncorrelated lognormal relaxed clock were specified. A run comprising 50 million generations was performed, with sampling every 5,000th generation. Results were visualized in Tracer v. 1.7.1 (Rambaut *et al.,*2018).

The final tree was generated by TreeAnnotator v.1.10 (Suchard *et al.,*2018) with a burn-in of 2,000 generations.

2.4 Population genetics analysis

COI sequences of all individuals of *C. chitae*, *C. ibericus*, *I. albolineatus*, *I. carpetanus* and *I. gerardoi* were used to estimate the main population genetics parameters as described by Chang & James (2011). The haplotype networks of each species were represented using Popart v1.7.

The uncorrected genetic distances within and between populations of each species were calculated using MEGA v7.0, and haplotypic (H) and nucleotidic (π) diversity were calculated using Arlequin v.3.1.

A Mantel test for each species was carried out using Arlequin v.3.1 to compare genetic and geographical matrix distances. Geographical distances were measured as the straight line distance (km) between the different points.

3. RESULTS

3.1 Phylogenetic analysis and time-calibrated phylogenetic inference

The Bayesian (Figure 2) and Maximum Likelihood trees showed the same phylogenetic relationships for the target taxa. Five species included in *Iberoscolex* (*I. microepigeus, I. albolineatus, I. carpetanus, I. oliveirae, I. gerardoi)* -all Iberian endemisms- were grouped in the same clade, while *I. pseudorroseus* appeared more closely related to *Dendrobaena osellai* Zicsi, 1970*.* However, this clade also included two representatives of the south-western France/Iberian endemism *Orodrilus*: *Orodrilus paradoxus* Cognetti 1904 and *Orodrilus doder*oi Cognetti 1904 (the type species of the genus). This clade is separated from the type species of *Eiseniona*, *E. handlirschi* Rosa, 1897.

Bayesian and Maximum likelihood trees for each gene (COI, 16s AND 28S) are showed in the supplementary material in order to clarify that there are no disagreements between mitochondrial and nuclear genes.

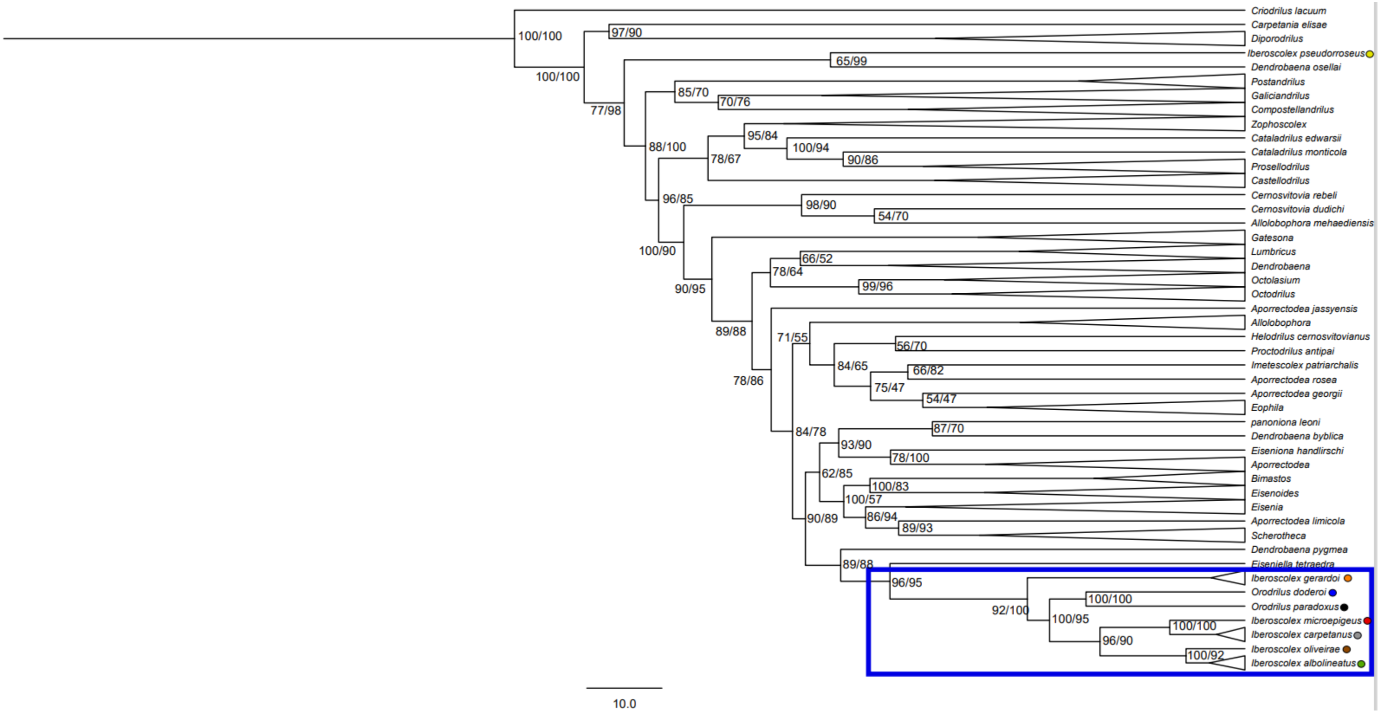

***Figure 2**. Phylogenetic relationships of representatives of Lumbricidae determined by Bayesian inference using the concatenated sequence of molecular markers COI, 16S, 28S, ND1, 12S. Posterior probability values (BI) and bootstrap values (ML) are shown besides corresponding nodes. The blue square indicates the position of the genus Iberoscolex in the tree. A circle next to each Iberoscolex species with the color assigned in the map above is shown.*

The consensus time-calibrated phylogenetic tree (Figure 3) shows that the root of the genus *Castellodrilus* (41.3 mya, 31.1-53.8 95% highest posterior density interval) is older than that of the genus *Iberoscolex* (28.9 mya, 21.6-37.5 95% HPD); however, the estimated age of divergence between the different species and populations within species appears similar in both genera. Divergence between the two focal species of *Castellodrilus* (9.7 mya, 6.7-13.3 95% HPD) is more recent than that of the two focal species of *Iberoscolex* (19.3 mya, 12.9-27 95% HPD). Although the basal divergence of *Castellodrilus* is prior to that of *Iberoscolex*. The estimated time of divergence between the populations of each species was related to geographic location, with recently diverged populations being physically closer.

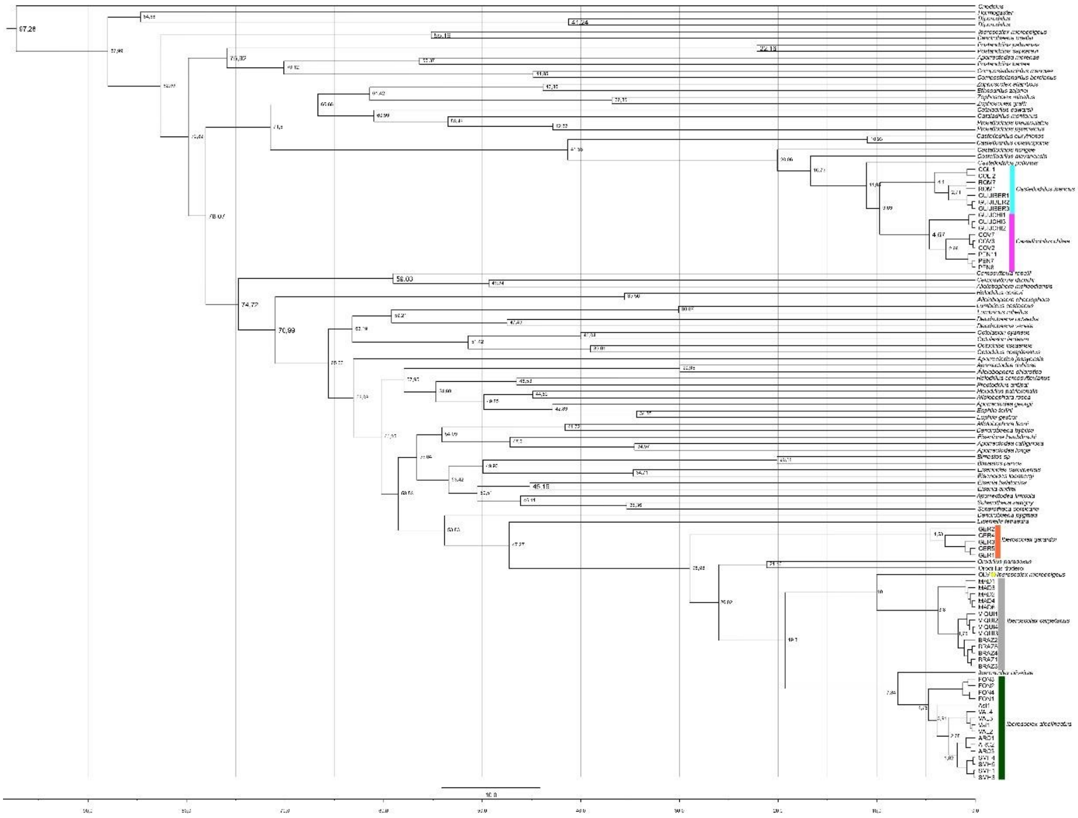

Figure 3. *Time-calibrated phylogenetic tree of Lumbricidae representatives based on molecular markers COI, 16S, 28S, ND1, 12S. Estimated ages are shown in million years. Red square: Castellodrilus genus; Blue square: Iberoscolex genus. Locality codes can be consulted in Table 1. Color bars for each species can be consulted in Figure 1.*

3.2 Population genetics analysis

Population genetic parameters (Table 2) revealed that the populations of species of the genus *Iberoscolex* display very high haplotypic diversity (H) (0.83-1) with a large number of alleles, while the populations of species of the genus *Castellodrilus* display much less haplotypic diversity (0.6-0.71), with many fewer alleles. However, the ranges of nucleotide diversity (π) of both genera overlap, with a higher maximum value for *Iberoscolex* (0.0026-0.0132 vs 0.0022-0.0042).

	Population	**H**	**π**
C. chitae	PEN	0.71 +/- 0.112	0.0042 +/- 0.0033
	COV	0.66 +/- 0.12	0.0039 +/- 0.0027
	GUIJCHI	0.70 +/- 0.21	0.0022 +/- 0.0019
C. ibericus	COL	0.60 +/- 0.17	0.0022 +/- 0.0018
	ROM	0.60 +/- 0.16	0.0033 +/- 0.0027
	GUIJIBER	0.70 +/- 0.21	0.0026 +/- 0.0022
I. carpetanus	VIQUI	0.90 +/- 0.16	0.0046 +/- 0.0035
	BRAZ	1.00 +/- 0.12	0.0053 +/- 0.0038
	MAD	0.93 +/- 0.12	0.0039 +/- 0.0029
I. gerardoi	GER	0.90 +/- 0.16	0.0132 +/- 0.0086
I. albolineatus	FON	0.900+/- 0.16	0.0066 +/- 0.0046
	VAL	1.00 +/- 0.0	0.0060 +/- 0.0046
	ARC	0.83 +/- 0.22	0.0041 +/- 0.0033
	SMH	0.90 +/- 0.16	0.0026 +/- 0.0021

Table 2. *Haplotypic and nucleotide diversity for each population. COL: Colmenarejo; GUIJ: Guijo de Granadilla; VIQUI: Villaquilambre; MAD: Madrid; BRAZ: Brazuelo; COV: Covarrubias; PEN: Peñahorada; FON: Fontioso; VAL: Valdeprado; ARC: Arcera; SMH: San Martín de Hoyos.*

Intraspecific genetic distances between populations for molecular marker COI (Table 3) were higher in *Castellodrilus* (2.7-8.2 %) than in *Iberoscolex* (1.3-3.8 %). The genetic distances follow the same geographical pattern as the estimated times of divergence, with the most geographically distant showing the greater genetic divergence. Geographical distance range includes from 18 to 326 km.

C. ibericus			***C. chitae***		
	ROM	**GUIJIBER**		**COV**	**GUIJCHI**
COL	5.7	5.6	**PEN**	5.1	8.2
ROM		2.7	**COV**		8.1

I. albolineatus				***I. carpetanus***	
	VAL	**ARC**	**SMH**		**BRAZ**
FON	3.8	2.8	3.2	**ViQui**	1.7
VAL		1.3	1.4	**BRAZ**	
ARC			0.8		

Table 3. *COI-based uncorrected genetic distance (%) between populations of C. ibericus, C. chitae, I. albolineatus and I. carpetanus.*

Mantel test result for genetic distance-geographical distance matrix comparison for each species was 0,62- 0,875 and P-value was <0,005 in every case. COI haplotype networks for each species (Figure 4) show that *C. ibericus* and *C. chitae* populations are more isolated from each other, with more mutations separating them and with very few connections between them, while *Iberoscolex* populations display more connections between their haplotypes and are separated by fewer mutational steps (with the exception of the population of *I. albolineatus* from Fontioso) and thus appear to be less isolated.

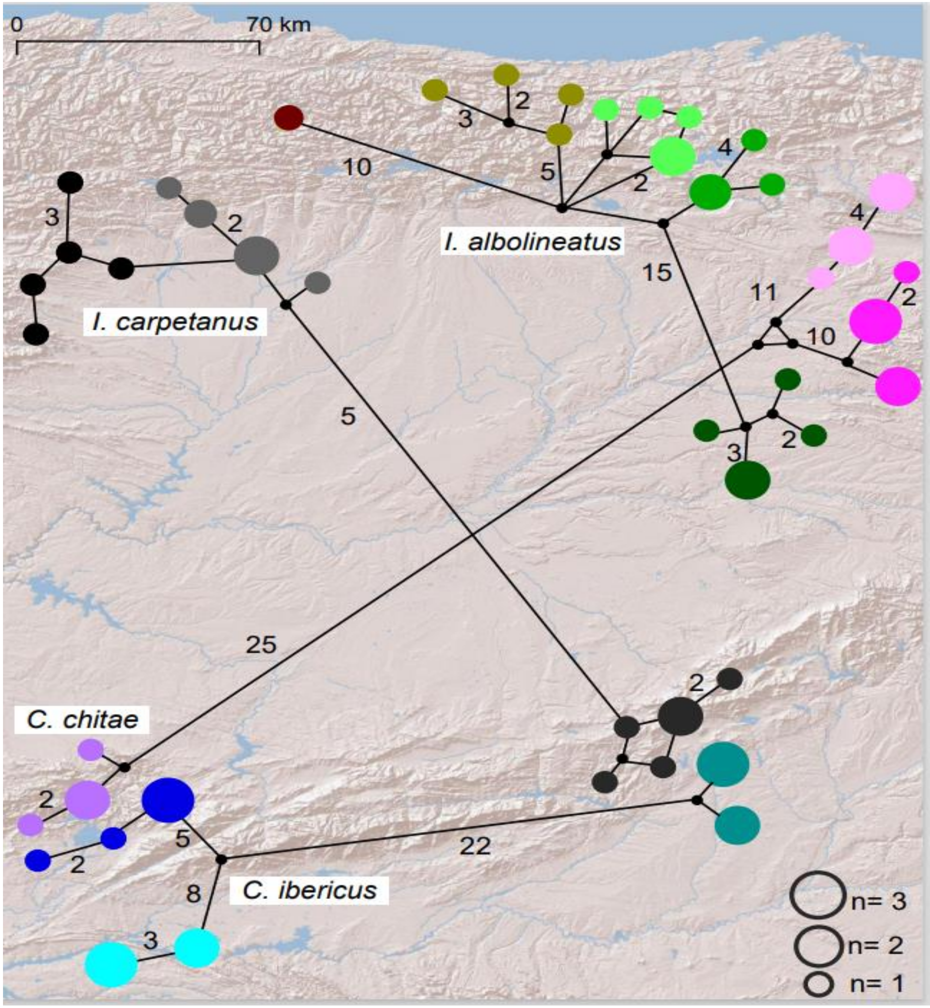

Figure 4. *Haplotype networks based on the COI sequences of I. albolineatus, I. carpetanus, C. chitae and C. ibericus placed within the map. Numbers on the nodes indicate the inferred number of mutational steps (except for 1). Black dots indicate hypothetical intermediate haplotypes.*

4. DISCUSSION

4.1 Systematic implications

Most of the Iberian endemisms included to date in the genus *Iberoscolex* (*I. albolineatus I. oliveirae, I. carpetanus, I. gerardoi* and the generotype *I. microepigeus*) were found to be phylogenetically separate from the Trans-Aegean *Eiseniona* generotype *E. handlirschi.* This suggests that *Iberoscolex* is an Iberian endemic genus, well differentiated from the eastern *Eiseniona.* Surprisingly, the *Iberoscolex* clade also included the two representatives of the Iberian-south-western France endemism *Orodrilus* under study (*O. doderoi* and *O. paradoxus)*, constituting an endemic Franco-Iberian genus. Endemic genera whose range encompasses both Iberian and French territories are not uncommon: *Prosellodrilus* Bouché, 1972, *Cataladrilus* Qiu and Bouché, 1998, *Gatesona* Qiu and Bouché, 1998 and *Scherotheca* Bouché, 1972 share the same distribution pattern. The fact that the type species of the genera *Iberoscolex* and *Orodrilus* (*I. microepigeus* and *O. doderoi*) are both included in this clade indicates that these genera should be considered synonymous. As *Orodrilus* is the senior genus (created in 1972) of the two, according to nomenclature rules (ICZN) the genus formed by most of the *Iberoscolex* species and at least two species of the genus *Orodrilus* should be named *Orodrilus*. The redefined genus *Orodrilus* is phylogenetically closer to the cosmopolitan species *E. tetraedra* Savigny, 1826 than to *Eiseniona handlirschi*.

Another alternative compatible with the topology of the trees is that *Orodrilus* and *Iberoscolex* (with the exclusion of *I.gerardoi*) are monophyletic genera, and that the early branching *I. gerardoi* should be considered an independent, undescribed genus. The absence of significant morphological differences between *I. gerardoi* and other Iberoscolex, as well as the similar clade depth (and estimated age) of the redefined *Orodrilus* and other well established Lumbricidae genera (see de Sosa et al. 2019, Jiménez et al. 2021 for the use of this criterion) suggest that the combination of all these species in a single genus is more robust.

The above findings imply that the genus *Orodrilus* is widely distributed throughout the Iberian peninsula, after previously being thought to have a small distribution in the South of France and to be an anecdotal relict in northern Spain, associated with mountains and occasionally caves (Laínez & Jordana, 1987; Omodeo, 1961; Bouché, 1979; Álvarez, 1971). In addition, although several of the species in this genus are associated with mountains, some are found in different environments, not necessarily at high elevations (all specimens used in this study were found in areas higher than 800 m above sea level except *I. gerardoi,* which was found at about 400-500 m above sea level, in a semiarid area).

Iberoscolex pseudorroseus was recovered as a sister species to *Dendrobaena s.l. osellai,* which had been previously assigned to *Kritodrilus* Bouché, 1972 but shown to be unrelated by Marchán et al. (2021a). Both of them appear to form an independent genus, endemic to the Iberian Peninsula. Pending the addition of other putative members (*Dendrobaena alvaradoi* Moreno, Jesus and Diaz Cosin, 1982*, Dendrobaena ruffoi* Zicsii, 1970*, Dendrobaena lusitana,* Graff, 1957 and *Dendrobaena franzi* Zicsi, 1965), both species should remain as *Dendrobaena s.l. pseudorrosea* and *Dendrobaena s.l. osellai.*

The genus-level clade of Iberian earthworms formerly assigned to *Iberoscolex*, but that contain some species of *Orodrilus* (including the type species of this genus), is proposed as a revised genus, together with a diagnosis and list of the species included.

Phylum: Annelida Lamarck, 1802

Class Clitellata Michaelsen, 1919

Order Crassiclitellata Jamieson, 1988

Family Lumbricidae Rafinesque-Schmaltz, 1815

Tribe Lumbricini Qiu & Bouché, 1998

Genus: *Orodrilus* (Bouché, 1972)

Type species: *Helodrilus doderoi* Cognetti, 1904 by original designation.

Species included:

Orodrilus doderoi (Cognetti, 1904)

Helodrilus doderoi (Cognetti, 1904)

Orodrilus paradoxus (Cognetti, 1904)

Helodrilus paradoxus (Cognetti, 1904)

Eiseniella paradoxa (Petrovic, 2014)

Orodrilus microepigeus (Qiu & Bouché, 1998) ***Comb. Nov.***

Iberoscolex microepigeus (Qiu & Bouché, 1998)

Orodrilus albolineatus (Díaz Cosín *et al.,*1989) ***Comb. Nov.***

Eiseniona albolineata (Díaz-Cosín *et al.,*1989);

Iberoscolex albolineatus (Qiu & Bouché, 1998)

Orodrilus carpetanus (Álvarez, 1970) ***Comb.Nov.***

Eiseniona carpetana (Álvarez, 1970)

Iberoscolex carpetanus (Qiu & Bouché, 1998)

Orodrilus oliveirae (Rosa, 1984) ***Comb. Nov.***

Allolobophora oliveirae (Rosa, 1894)

Iberoscolex oliveirae (Qiu & Bouché, 1998)

Orodrilus gerardoi (Díaz Cosín *et al.,*2014) ***Comb. Nov.***

Eiseniona gerardoi (Díaz-Cosín, 2014)

Diagnosis: Small sized, cylindrical shape sometimes with caudal quadrangular section, pigmentation brown or reddish. Chaetae separated, closely paired or *ab* (ventral pair) separate – *cd* (dorsal pair) variable. Longitudinal wrinkles absent. Prostomium prolobous or epilobous. First dorsal pore in 4/5 or 5/6 (rarely in 12/13). Nephridial pores aligned. Male pores in 1/2 15, porophores absent. Spermathecal pores simple, in 9/10, 10/11 in area C (between chaetae *c* and *d*). Clitellum anterior, ring-shaped occupying 5 to 8 segments. Tubercula pubertatis linear and occupying most of the length of the clitellum. Calciferous glands in 10(11)-15 with diverticula in 10 (rarely absent). Gizzard in 17-19. Typhlosole simple. Nephridial bladders U-shaped (incurvate and reclinate). Male genital organization variable: when holandric, two or four pairs of seminal vesicles appear in (9,10) 11,12; when hemiandric, testicles appear in 11 and seminal vesicles appear in 10 and 12.

Remarks: Due to their morphological resemblance, it is very likely that the two other species previously assigned to *Orodrilus* (*Orodrilus gavarnicus* Cognetti, 1904 and *Orodrilus septumperforatus* Díaz Cosín, 1993) belong to this redefined genus; however, this should be further confirmed by including them in a molecular phylogenetic analysis.

4.2 Phylogeographic patterns

The phylogeographic patterns identified for the representatives of *Castellodrilus* (*C. ibericus* and *C. chitae*) and *Iberoscolex* (*I. carpetanus*, *I. albolineatus)* were similar, with all showing strong population structure (as indicated by the haplotype networks). The isolation of populations seems consistent with previous observations in other edaphic organisms (Caesar *et al.,*2005; Costa *et al.* in 2011) and strongly contrasts with the phylogeographic patterns reported for the cosmopolitan *A. trapezoides* (Fernández *et al.,* 2011), *A. rosea* (Fernández *et al.,* 2016) and *E. tetraedra* (de Sosa *et al.* in press). Although both genera displayed remarkable population structure, differentiation appeared weaker for *Iberoscolex*. The two genetically closest populations separated by the smallest genetic distance are the populations from San Martín de Hoyos and Arcera (*I. albolineatus*): this could be explained by the small geographic distance between those populations (the smallest in the data set 18km-326km) at which gene flow may be possible (or could recently have been interrupted).

Cryptic speciation has been shown to be quite common in earthworms (Shekhovtsov *et al.,* 2013; Taheri *et al.,* 2018), especially in species with isolated populations, as in this case.

Interestingly, despite the remarkable genetic structure and isolation of populations, the species under study did not show any hints of cryptic speciation, as the greatest COI-based distance between populations was 8.2% (i.e. lower than the usual threshold of 9% -Chang & James 2011). Yet, cryptic speciation cannot be established only on the basis of mitochondrial genes (Dupont et al. 2016): nuclear markers must be examined in order to check for the existence (or absence) of gene flow between populations.

For all of the species, genetic distances increased with geographic distance (P-value of Mantel test between genetic and geographical distances < 0.05 for all four species), which is consistent with the pattern known as isolation by distance (Slatkin, 1993), The positive correlation between genetic distance and geographical distance has been observed in several studies with different types of organisms, including invertebrates (Hutchison & Templeton, 1999; Chang & James, 2011; Novo et al*,*. 2010), vertebrates (Cádiz *et al.,* 2018) and plants (Conte et al*.,* 2004). This can be explained by the fact that the gene flow decreases and the degree of isolation increases as the distance between population increases (Sexton et al*.,* 2014).

Regarding the intrapopulation genetic variation, haplotype and nucleotide diversity provide different insights into the population dynamics. Haplotype diversity in the populations of the *Castellodrilus* species under study (*C. chitae* and *C. ibericus*) is clearly lower than in the *Iberoscolex* studied species (which have a high or very high haplotype diversity). The populations of *Iberoscolex* displayed high haplotype variability, possibly indicating that these populations (and species) have not undergone processes of genetic drift (or not to the same extent as *Castellodrilus* populations) or natural selection has not decreased the numbers of haplotypes and populations (and species). The more diverse *Iberoscolex* may be better prepared in regard to adapting to changes in environmental conditions, surviving in more types of environments and being widely distributed. The lower haplotypic diversity observed in the populations of *Castellodrilus* could be explained by genetic drift (common in populations that are isolated as in this case) or by a higher action of natural selection, which would select the most successful haplotypes and thus decrease the possibility of genetic divergence. This species may not therefore be able to adapt as well as *Iberoscolex* species could adapt to environmental changes and could be more endangered if environmental conditions change (Frankhan, 2002; Maki & Horie, 1999). In fact, low genetic diversity has been described as being characteristic of rare and endangered species (Frankham, 2003). The different ways in which natural selection appear to affect both genera could be explained by the different ecological categories to which these belong. Most *Castellodrilus* species are epiendogeous, living in the more organic layers of the soil but not so up as epigeous earthworm, where the environment is relatively stable and homogeneous. Under these conditions, stabilizing selection may be the main mechanism, acting against deleterious mutations and reducing genetic diversity. These species are well adapted to the stable environment where they live, and are expected to display a low degree of resilience to environmental changes. On the other hand, *Iberoscolex* species are epigeous, i.e. they live in the upper layers of the soil, where environmental conditions are more heterogeneous and unstable. Diversifying selection could thus occur in response to multiple adaptive peaks and maintain a higher haplotype diversity. These hypotheses could be tested more robustly by using reduced representation genomic techniques such as RADseq or GBS, which provide a sample of the variability across the whole genome. Such techniques have been used in cosmopolitan lumbricids (Anderson et al*.,* 2017) and endemic hormogastrids (Marchán et al*.,* 2020b), providing insights into the way that selection has shaped the genetic diversity of the populations. In addition, increased sampling both at the regional and local scales could ensure that the observed differences are not due to differences in sample size or other biases.

The epiendogeous - epigeous difference could also explain the stronger population structure of *Castellodrilus*: as these species spend most of their lives below the soil surface, they are expected to be less exposed to being transported passively by animals or humans (passive dispersal) and their active dispersal ability will therefore be lower. On the other hand, the epigeic *Iberoscolex* species are more active and could be easily transported in association with leaf litter or topsoil (Langraf *et al.,* 2021*).* Although comparative phylogeography appears to be suitable for studying the differences in dispersal patterns of different earthworm ecological categories, it has scarcely been used for this purpose.

5. CONCLUSIONS

1. The genus *Iberoscolex* is independent from *Eiseniona* and constitutes an endemic genus from the Iberian Peninsula and south-western France, along with two species of *Orodrilus* (including the type species of the latter). *Iberoscolex* should therefore be considered a junior synonym of *Orodrilus*, and *I. pseudorroseus* as not belonging to the genus *Orodrilus*.

2. The studied species of the genera *Castellodrilus* and *Iberoscolex* possess a strong population structure with no hints of cryptic speciation, and the COI sequence-based genetic distance between populations appears to be correlated with geographic distance
3. *Castellodrilus* species display lower genetic diversity than *Iberoscolex* species, with fewer haplotypes per population, which could be explained by the different ecological categories of these two genera, as *Castellodrilus* is epiendogeus and *Iberoscolex* is epigeous.

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Supporting Information

Filename

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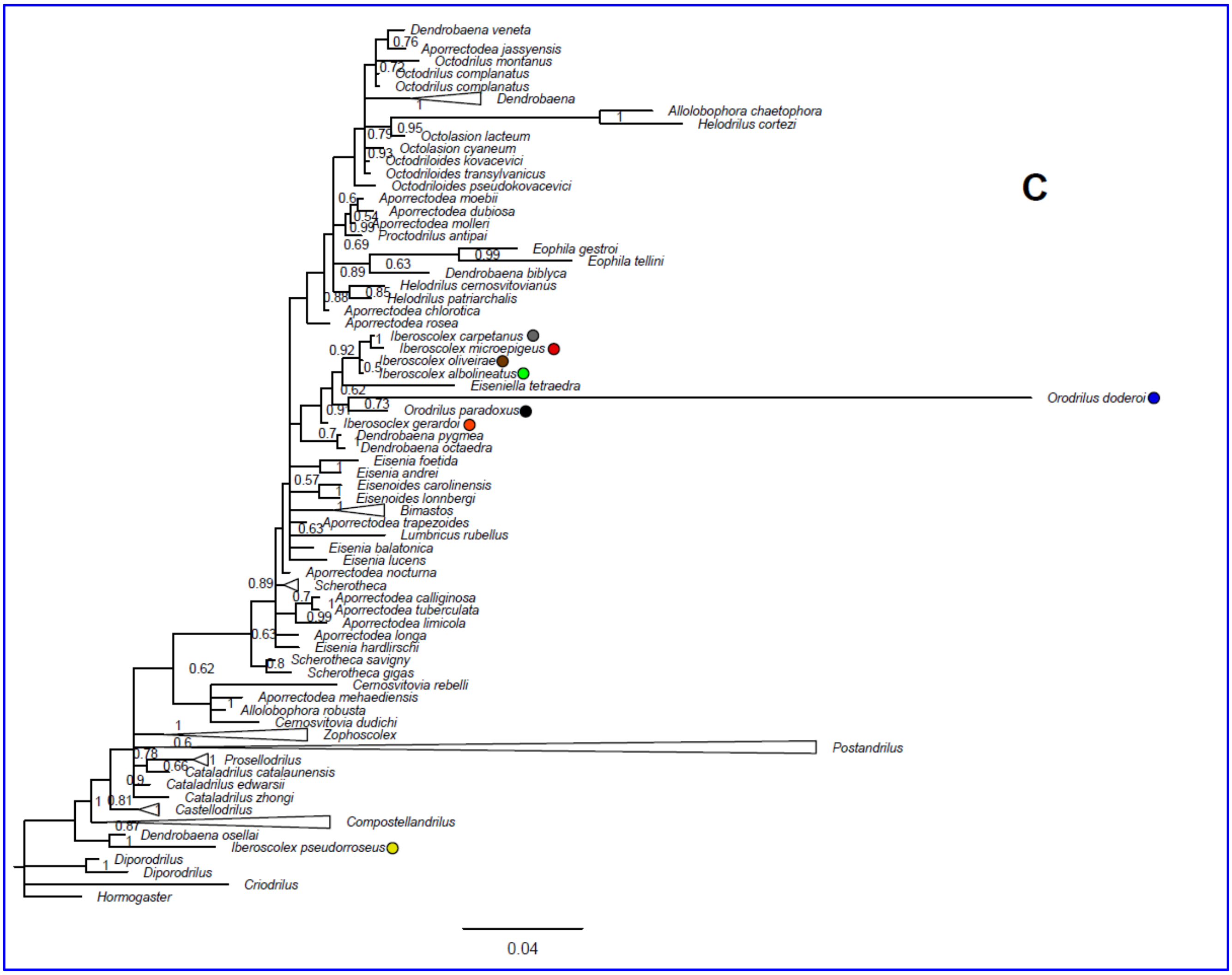


Figure S1. Supplementary Bayes

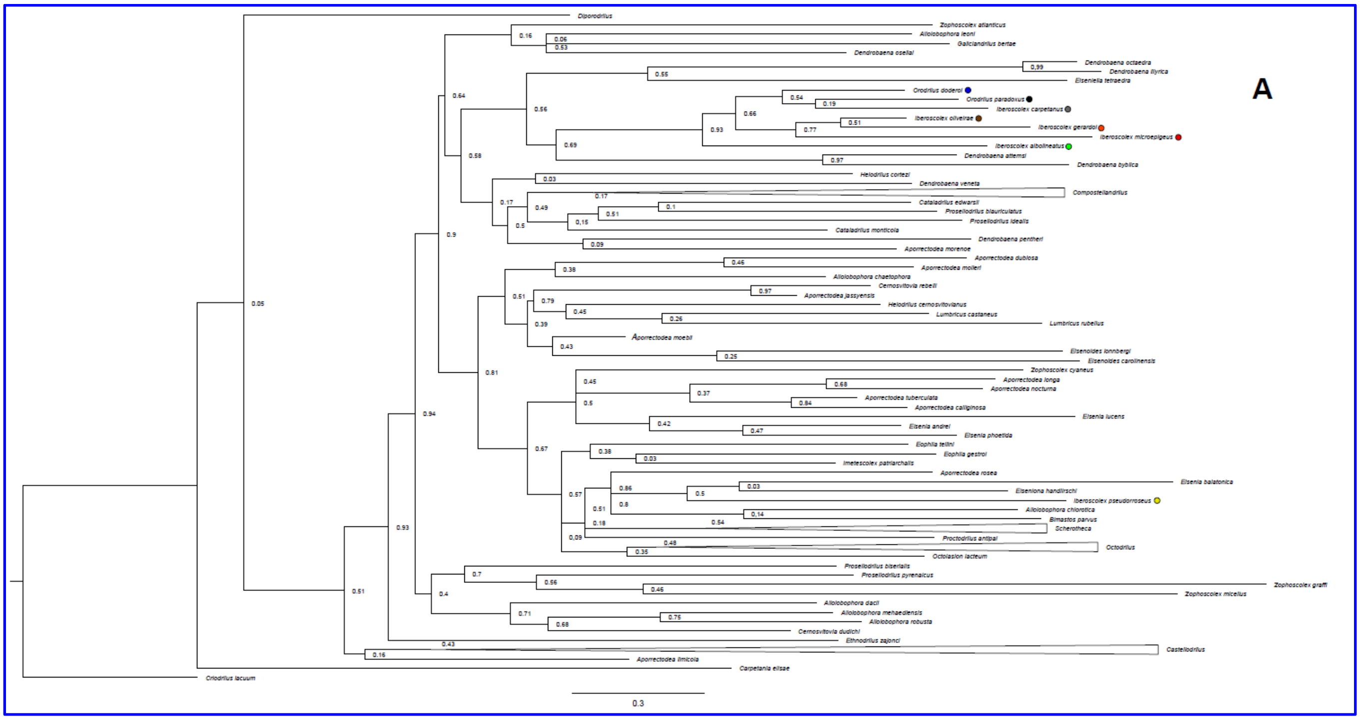
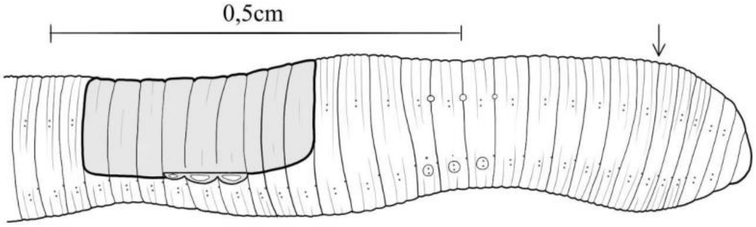


Figure S2. Supplementary ML.

Capítulo 3

"Tightly linked evolution of Franco-Iberian earthworms (Prosellodrilini, Lumbricidae) and Pyrenean Mountain range supported by molecular phylogenetics."



La Península Ibérica y el Sur de Francia son un centro de diversificación de la familia Lumbricidae, que presenta numerosos endemismos y géneros basales en la filogenia de los lumbricidos de esa zona.

Dos géneros basales y endémicos de esta zona son *Cataladrilus* y *Prosellodrilus*. Estos géneros están muy poco estudiados y hay muy pocos estudios filogenéticos en los que se hayan incluido representantes de estos géneros. En el caso de *Prosellodrilus*, la especie tipo nunca ha sido incluida en los análisis filogenéticos. Por ello, en este estudio, tras capturar ejemplares de diferentes especies de *Cataladrilus* y *Prosellodrilus* en sus localidades tipo (incluyendo las especies tipo de ambos géneros), se realizó un análisis filogenético utilizando los marcadores moleculares COI, 16S (mitocondrial) y 28S (nuclear). También se incluyeron secuencias de otros géneros de lumbricidos obtenidas en estudios previos, con el fin de comprobar la monofilia de *Prosellodrilus* y *Cataladrilus* y recuperar sus relaciones filogenéticas con otros géneros basales. Como resultado, se pudo demostrar que ambos géneros son monofiléticos y, por tanto, válidos. Ambos géneros son evolutivamente muy cercanos y se encuentran muy cerca de otros géneros basales como *Castellodrilus* y *Zophoscolex*. Además, se puede observar que la filogenia y distribución de las especies del género *Prosellodrilus* se encuentra muy influenciada por la formación de la cordillera de los Pirineos.

Tightly linked evolution of Franco-Iberian earthworms (Prosellodrilini, Lumbricidae) and Pyrenean Mountains supported by molecular phylogenetics

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ABSTRACT

The Iberian Peninsula and the South of France are a centre of diversification of the Lumbricidae family, which has many endemisms and basal genera in the phylogeny of lumbricids in that area.

Two basal and endemic genera of this area are *Cataladrilus* and *Prosellodrilus*. These genera are very poorly studied and there are very few phylogenetic studies in which representatives of these genera have been included. In the case of *Prosellodrilus,* the type species has never been included in phylogenetic analyses. Therefore, in this study, after capturing specimens of different species of *Cataladrilus* and *Prosellodrilus* in their type localities (including the type species of both genera), a phylogenetic analysis was carried out using the molecular markers COI, 16S (mitochondrial) and 28S (nuclear). Sequences from other lumbricid genera obtained in previous studies were also included, with the purpose of testing the monophyly of *Prosellodrilus* and *Cataladrilus* and recovering their phylogenetic relationships with other basal genera.

As a result, it was possible to demonstrate that both genera are monophyletic and, therefore, valid. Both genera are evolutionarily very close and are found very close to other basal genera such as *Castellodrilus* and *Zophoscolex*.

Furthermore, by calculating the ancestral area of *Prosellodrilus* it was possible to observe that the origin of the genus is located in an area between Sardinia and eastern France, and that the genus predates the separation of Sardinia from France and the formation of the Pyrenees, which could explain the presence of *P. festae* in Sardinia and the presence of this genus on both sides of the Pyrenees. Furthermore, if the geological age of the Pyrenees and its formation process are studied in detail, it can be observed how the different stages of formation of this mountain range largely coincide with the distribution of the different species of *Prosellodrilus.*

KEYWORDS

Earthworms, soil organisms, phylogeography, Iberian endemisms, Lumbricidae, Primitive Genera

INTRODUCTION

The Iberian Peninsula, with a very complex geological history and diverse environments (Hewitt, 1996), seems to be an ideal place for the appearance of highly variable characters between populations of the same species and for speciation phenomena. The north-eastern area of the Iberian Peninsula and southern France are indeed areas with a great diversity of earthworms (Díaz-Cosín *et al.,* 1992). These areas seem to be a center of diversification of these animals and especially of the family Lumbricidae, since a large number of basal genera of this group, as well as a large numbers of early branching and evolutionarily recent endemisms, have been found (Jiménez-Pinadero *et al.*, 2023). Particularly interesting for speciation studies are the Pyrenees. This mountain range is known to act as a barrier for populations and has been studied regarding several speciation events in different taxa (Yi-Shao *et al.*, 2019; García-Jacas *et al.*, 2023).

The case of the endemic earthworm genus *Castellodrilus* Jiménez-Pinadero *et al.,* 2021 perfectly illustrates the role of the Pyrenees as a centre of speciation. This genus was previously included as a subgenus within the Iberian and south French genus *Zophoscolex* Qiu & Bouché, 1998a. However, phylogenetic studies confirmed its genus status (Jimenez-Pinadero *et al.*, 2021). *Zophoscolex* was restricted to the northern area of the Pyrenees (southern France) and *Castellodrilus* to the southern area (Iberian Peninsula), which indicates the role of this mountain range in the speciation of these taxa.

Although phylogenetic analyses have clarified the evolutionary relationships between genera and have helped to create new Lumbricidae genera such as *Compostelandrilus* Domínguez *et al.,* 2018 or the aforementioned *Castellodrilus*, there are still some genera in this area that need to be studied in depth. Phylogenetic analyses combining mitochondrial and nuclear markers have been fundamental tools to increase knowledge about the evolutionary relationships between the different species of earthworms. (Pérez-Losada *et al*., 2011).

The systematics and evolution within the genus *Prosellodrilus* Bouché, 1972 have not yet been studied with a molecular phylogenetic approach. This genus currently comprises 27 species and some subspecies and it is distributed in the southern area of France and in the northern area of Spain (on both sides of the Pyrenees). This genus has been very difficult to study morphologically since most of the characters used to determine the species are sexual characters such as the clitellum, and some of the species only present these characters for 10–15 days of the year (personal observation). In addition, most species are very similar to each other, with characters that overlap between species. This makes a phylogenetic study of this genus essential. There is little literature that places a very limited sampling of *Prosellodrilus* (three species from the French Pyrenees) in a phylogenetic framework (Domínguez et al., 2018; Jiménez-Pinadero et al., 2023), but the type species *Prosellodrilus idealis* Bouché, 1972 was never included in the analyses. Those preliminary suggested a close relationship between those *Prosellodrilus* species and the early-branching genus *Castellodrilus* (Jiménez-Pinadero *et al.,* 2021; Domínguez *et al*., 2018).

This genus has some controversial species like *Prosellodrilus amplisetosus* Bouché, 1972 that have changed genera over the years as new studies have been published (*P. occidentalis*, *Kenleenus armadas*) (Blakemore, 2012), and it contains some species such as *Prosellodrilus pyrenaicus* Cognetti, 1904 and *Prosellodrilus psammophilus* Qiu & Bouché, 1998b with dubious subspecies. For example, while *Prosellodrilus pyrenaicus* (Cognetti, 1904) is a French species with several subspecies described, *Prosellodrilus pyrenaicus aragonicus* (Álvarez, 1971b) was described from the Iberian Peninsula and without including key internal morphological characters such as the shape of the nephridial bladders. . Moreover, most species of this genus have very restricted distribution ranges or knowledge of their distribution is limited, which can complicate studies due to the difficulty of sampling.

Another genus endemic to both sides of the Pyrenees is the genus *Cataladrilus* Qiu & Bouché, 1998, a very understudied genus comprising only nine species; most of them have been found only once and only in a single locality. The aforementioned phylogenetics and molecular studies on *Prosellodrilus* show that *Cataladrilus* would be close to this genus and to *Castellodrilus*, but only two species were included (one of them being its type species *Cataladrilus monticola* Qiu & Bouché, 1998b) until *Cataladrilus porquerollensis* Marchán *et al*., 2020b was described. The addition of this French species resulted in a less resolved topology, with an apparently paraphyletic *Cataladrilus* and *Prosellodrilus* nested within itself (Marchán et al., 2020b). Another recent addition to the phylogenetic background was *Allolobophora s.l. tiginosa* Bouché, 1972, which was putatively assigned to its own genus by Martínez-Navarro *et al.,*2022; this earthworm possesses an intermediate morphology between *Prosellodrilus* and *Cataladrilus* and, like *Ca. porquerollensis*, it is found in south-eastern France, far from the main range of these genera. Phylogenetic analyses were not able to resolve its position in regard to *Cataladrilus* and *Prosellodrilus*, obtaining a polytomy instead.

Since some of the aforementioned studies (such as Jiménez-Pinadero *et al.,* 2021) have shown that the Pyrenees may play a key role in the separation of early-branching lumbricid genera, it would be interesting to test whether the formation of this mountain range is correlated with the evolution and diversification of *Prosellodrilus* and *Cataladrilus*.

The aims of the present study were:

i) To test the status of *Prosellodrilus* and *Cataladrilus* as monophyletic genera and to clarify the evolutionary relationships between these genera and rest of the early-branching genera (*Castellodrilus, Zophoscolex*…)

ii) To clarify the evolutionary relationships between the species within the *Prosellodrilus* genus with an emphasis on the role of the Pyrenees as a potential cause of speciation.

MATERIAL AND METHODS

Sampling

Sampling surveys were carried out in February 2021, February 2022 and November 2023 in northeast Spain and in the South of France to capture species of the genera *Prosellodrilus* and *Cataladrilus*. Individual worms were collected by digging and hand-sorting. The specimens were then washed with distilled water, fixed in 96% ethanol and stored at −20 °C. Ethanol fixed specimens of these genera from the earthworm collection of the Faculty of Biological Sciences of the Complutense University of Madrid (UCMLT Collection) were also included in the study (Table 1, Figure 1).

Species	Locality	Coordinates
P. alatus	Saint-pe-de-Bigorre (Hautes-Pyrénées, France)	43°06'05.4"N 0°09'22.9"W
P. amplisetosus	La Reóle (Nouvelle-Aquitanie, France)	44°35'26.7"N 0°05'44.3"W
P. arenicola	Barbués (Huesca, Spain)	41°58'55.1"N 0°25'10.4"W
P. biauriculatus	Ariège (Occitanie, France)	42°59'38"N 01°15'23''E
P. biserialis	Langedot-Rousillon (Vaucluze, France)	44° 04' 18''N 04° 47' 15''E
P. dactylothecus	Argèles (Occitanie, France)	42°32'13.4"N 3°01'27.2"E
P. festae	Villamassargia (Sardinia, Italy)	39°16'30.8"N 8°37'37.2"E
	Tani (Sardinia, Italy)	39°13'41.2"N 8°33'27.0"E
P. idealis	Aspet (Occitanie, France)	43°00'19.1"N 0°47'45.9"E
P. jamiesoni	Torrecilla en Cameros (La Rioja, Spain)	42°13'59.4"N 2°37'29.1"W
P. ombrophilus	Pervés (Lleida, Spain)	42°21'43.4"N 0°50'52.1"E
P. polythecosus	Leazkue (Navarra, Spain)	42°56'54.8"N 1°35'41.2"W
P. praticola	Saint-Gaudens (Occitanie, France)	43°04'35.2"N 0°43'26.3"E
P. psammophilus	Farlete (Huesca, Spain)	41°41'05.0"N 0°30'33.2"W
P. psammophilus magnus	Loporzano (Huesca, Spain)	42°09'47.4"N 0°19'49.8"W
	Nueno (Huesca, Spain)	42°16'04.1"N 0°26'14.7"W
P. pyrenaicus	Ariège (Occitanie, France)	42° 59' 27''N 01° 13' 26''E
P. pyrenaicus aragonicus	Jaca (Huesca, Spain)	42°33'20.0"N 0°37'57.9"W
	Atarés (Huesca, Spain)	42°32'01.3"N 0°38'12.9"W
P. trigoi	Ezcároz (Navarra, Spain)	42°53'13.6"N 1°06'05.1"W

C. catalaunensis	Mont Louis (Pyrénées-Orientales, France)	42°30'31.4"N 2°06'46.6"E
C. edwarsi	Terrades (Girona, Spain)	42º18'26.2"N 2º51'30.4"E
C. monticola	Sant Julià de Lòria (Andorra)	41°47'23.8"N 2°14'17.1"W

Table 1. *Localities and coordinates where Prosellodrilus and Catalladrilus species used in this study were sampled.*

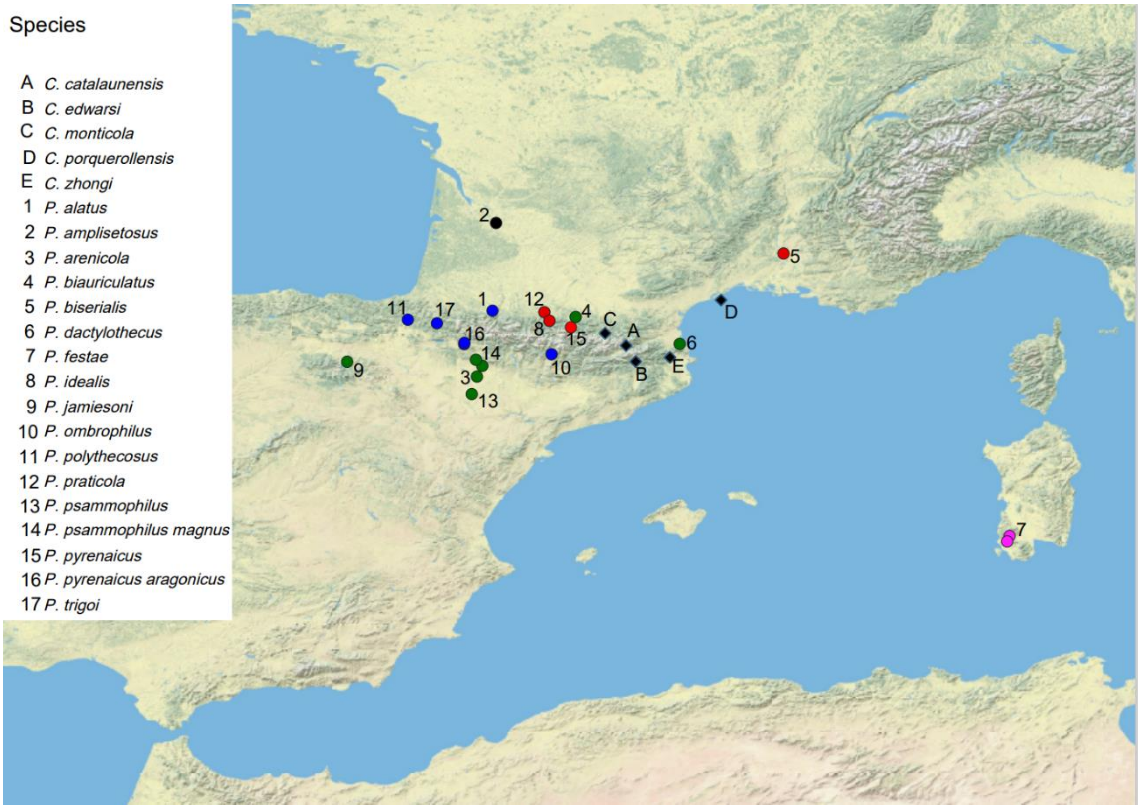


Figure 1. *Map of sampled localities for the different species of Cataladrilus and Prosellodrilus used in this study. Blue dots: Species of Prosellodrilus distributed in areas that were submerged before the formation of the Pyrenees. Red dots: Species of Prosellodrilus that remained on the north face of the Pyrenees during and after their uplift. Green dots: Species of Prosellodrilus that remained on the south face of the*

DNA isolation and sequencing

Tissue samples were obtained from the body walls of different specimens of each of the species and populations listed in Table 1. The piece of wall picked from each specimen was about 0.5 x 0.5 cm.

Genomic DNA was extracted and isolated using the Speedtools Tissue DNA Extraction Kit (Biotools). Regions of the mitochondrial genes 16S rRNA and COI and the nuclear gene 28S rRNA were amplified by polymerase chain reaction (PCR) with the primers described by Pérez-Losada *et al.* (2005, 2009), and under the same conditions outlined in Pérez-Losada *et al.* (2011) and Domínguez *et al.* (2015).The PCR products were purified using the ExoSAP-IT PCR product cleanup reagent, provided by LIFE TECHNOLOGIES, S.A. and bidirectionally sequenced in an Applied Biosystems (ABI) 377XL DNA sequencer by MACROGEN (Madrid).

Phylogenetic analysis and time-calibrated phylogenetic inference

In addition to the specific sequences obtained in this study, COI, 16S and 28S sequences generated for other lumbricid species were used, as reported by Domínguez *et al.* (2015, 2018), Pérez-Losada *et al.* (2015), Csuzdi *et al.* (2017), Bozorgi *et al.* (2019), de Sosa *et al.* (2019), Marchán *et al.* (2020a), Jiménez-Pinadero *et al.* (2021) and Martínez-Navarro *et al.* (2022). Sequences from the Criodrilidae and Hormogastridae families were included as outgroups.

Sequences were aligned with MAFFT v.7 (Katoh & Standley, 2013) with default settings and concatenated with BioEdit (Hall, 1999), resulting in a matrix of 2,552bp. The best fitting evolutionary model for each partition was selected with jModelTest v. 2.1.3 (Darriba *et al.*, 2012) by applying the Akaike information criterion (AIC; Akaike, 1973) and the Bayesian information criterion (BIC; Schwarz, 1978) (which agreed in their results). GTR + I + G was selected as the best-fitting evolutionary model for COI and 28S and HKY + I + G was selected for 16S.

Bayesian Inference of the phylogeny was estimated with MrBayes v.3.2.6 (Ronquist *et al.*, 2012) as implemented in the CIPRES Science Gateway V. 3.3 (Miller *et al.*, 2010). The analysis was performed with default parameters, and each of the two independent runs was set to 50 million generations sampling every 5,000th generation (10,000 trees). Twenty percent of the trees were discarded as burn-in, with the remaining trees combined and summarized on a 50% majority-rule consensus tree. Maximum Likelihood phylogenetic inference was performed using RAxML-NG (Kozlov *et al.*, 2019) in the CIPRES Science Gateway v. 3.3 platform, from 10 random starting trees and 1,000 rapid bootstrap replicates.

The Maximum Likelihood tree was converted into an ultrametric tree by non-parametric rate smoothing (NPRS) and the chronopl function in the R package ape v5.2 was used to generate a suitable starting tree for the time-calibrated phylogenetic inference. The maximum and minimum ages of the clades were the same as those used in the downstream BEAST analysis.

The final ultrametric tree was generated using BEAST v. 1.10 (Suchard *et al.*, 2018) with the NPRS tree as the starting tree. Each partition was trimmed using GBlocks (Castresana, 2000) under the less stringent parameters, with the best-fitting evolutionary model (shown above) used as the evolutionary model for each.

The following calibration points were implemented as uniform priors: 93–180 Ma (Criodrilidae + Lumbricidae + Hormogastridae clade) and 69–128 Ma (Lumbricidae clade). These age intervals correspond to the 95% HPD (highest posterior density) intervals reported by Marchán *et al.,* 2017.

A Yule diversification model and an uncorrelated lognormal relaxed clock were specified. A run comprising 50 million generations was performed, with sampling every 5,000th generation. Results were visualized in Tracer v. 1.7.1 (Rambaut *et al.*,2018). The final tree was generated by TreeAnnotator v. 1.10 (Suchard *et al.*, 2018) with a burn-in of 2,000 generations.

Ancestral area and ancestral character state reconstruction

Ancestral area reconstruction for *Prosellodrilus* was performed in RASP 4 (Yu *& He.*, 2020), and the most suitable model was chosen using BioGeoBEARS (DEC) and allowing a maximum of two areas for each node. The areas considered were coded as follows: A) Sardinia, B) Continental France (excluding the Pyrenees area), C) Western Pyrenees, D) Ebro's basin and E) Eastern Pyrenees. Species were assigned to those categories according to their main range according to Bouché (1972) and Qiu & Bouché (1998a).

Ancestral character states for body weight in *Prosellodrilus* were reconstructed with Mesquite v. 3.0.1 (Maddison & Maddison, 2007) with parsimony and maximum likelihood as optimality criteria. Body weight was coded as < 0.5 gr (state 0), 0.5 gr-1 gr (state 1) and >1 gr (state 2). Values for this trait were taken from the literature (Qiu & Bouché, 1998b), where they are provided as the average of all the collected specimens.

RESULTS

The Bayesian inference and maximum likelihood trees showed no conflicts for the phylogenetic relationships of the early-branching lumbricids *Prosellodrilus*, *Cataladrilus* and *Zophoscolex* (the focus of this study).

Both *Prosellodrilus* and *Cataladrilus* appeared as two well-supported monophyletic clades that included their respective type species (*P. idealis* and *Ca. monticola*) and both genera are evolutionarily close to each other and to other early-branching Iberian and south-western France endemisms like *Castellodrilus* (Figure 2). *Allolobophora*. s.l. *tiginosa* was recovered as a sister taxon to all the representatives of *Cataladrilus* with low boostrap but high posterior probability support values.

The earliest branching taxa in the genus were Prosellodrilus festae (from the island of Sardinia) followed by Prosellodrilus amplisetosus (with a putative native range in Aquitaine, France).

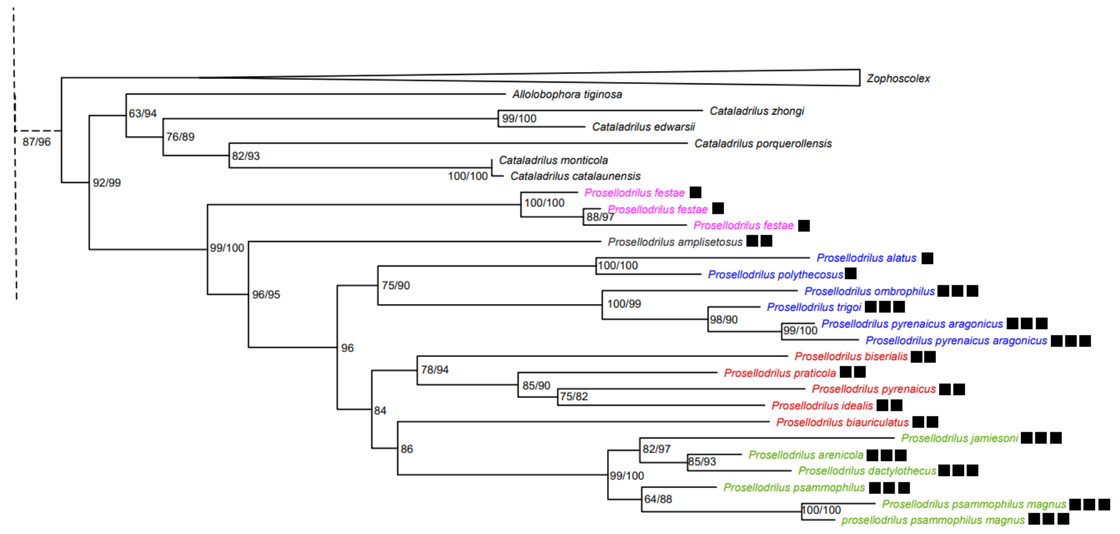


Figure 2: *Details of the maximum likelihood tree obtained using the molecular markers COI-16S-28S. Bootstrap values/posterior probability values are shown. One black square = weight under 0.5 g; two black squares = weight between 0.5 g and 1 g; three black squares = weight over 1 g. Blue names: Species of Prosellodrilus distributed in areas that were submerged before the formation of the Pyrenees. Red names: Species of Prosellodrilus that remained on the north face of the Pyrenees during and after uplift. Green names: Species of Prosellodrilus that remained on the south face of the Pyrenees during and after their uplift. Pink names: Species collected in Sardinia. Complete tree can be found in Supplementary material 1.*

Prosellodrilus amplisetosus was recovered as the sister taxon to a clade comprising all French and Spanish endemic species; these, however, were not recovered in reciprocally monophyletic French and Spanish clades. Instead, Spanish species formed two different clades nested within two French clades. One of those Spanish subclades comprised *P. trigoi, P. ombrophilus* and *P. pyrenaicus aragonicus*; surprisingly, the latter did not cluster with the representative of the parent taxon *P. pyrenaicus* (from France), but with the morphologically similar *P. trigoi*.

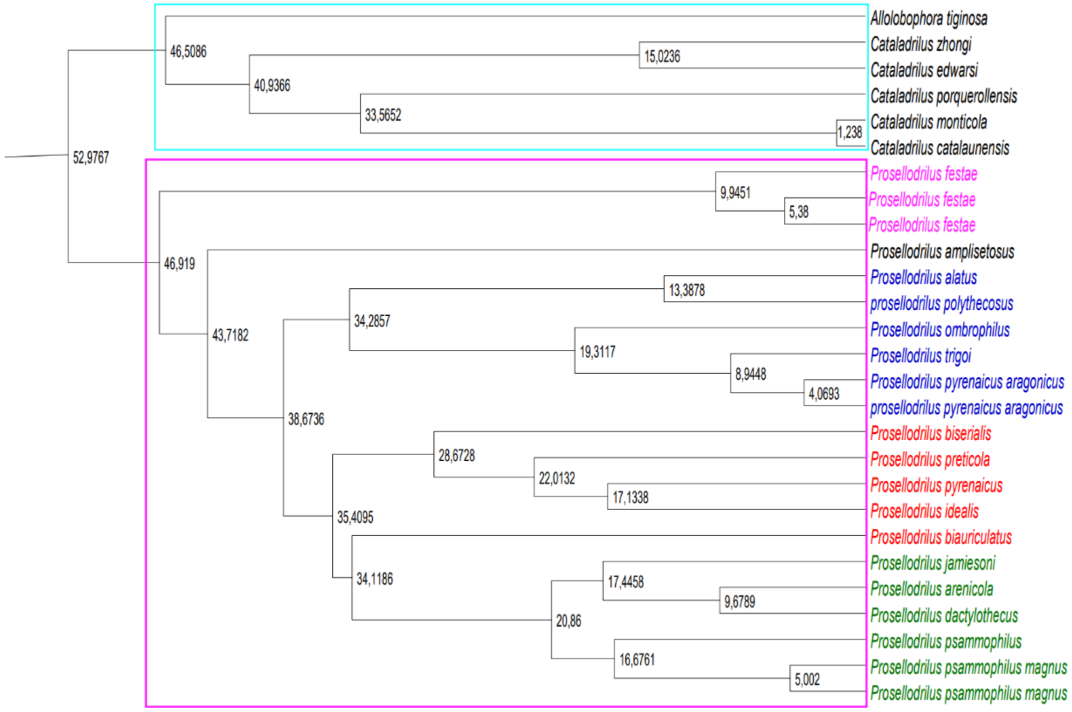


Figure 3: *Ultrametric tree obtained though the Maximum Likelihood tree with the molecular markers COI-16S-28S. Blue square = Cataladrilus; Pink square = Prosellodrilus. Blue names: Species of Prosellodrilus distributed in areas that were submerged before the formation of the Pyrenees. Red names: Species of Prosellodrilus that remained on the north face of the Pyrenees during and after their uplift. Green names: Species of Prosellodrilus that remained on the south face of the Pyrenees during and after their uplift. Pink names: Species collected in Sardinia. Paleogeographic reconstructions corresponding to the most relevant geological events modified from Calvet et al. (2021); numbers correspond to the grey stripes overlaid on the phylogenetic tree.*

The estimated age for *Prosellodrilus* (46 Ma) was older than the start of the break-off of Sardinia and the French-Iberian terrane (30 Ma, Calvet *et al.*, 2021). The estimated age for *Cataladrilus* was significantly younger (40 Ma) (Figure 3). It can be observed that the ancestral area for the genus *Prosellodrilus* is between the Eastern Pyrenees and Sardinia

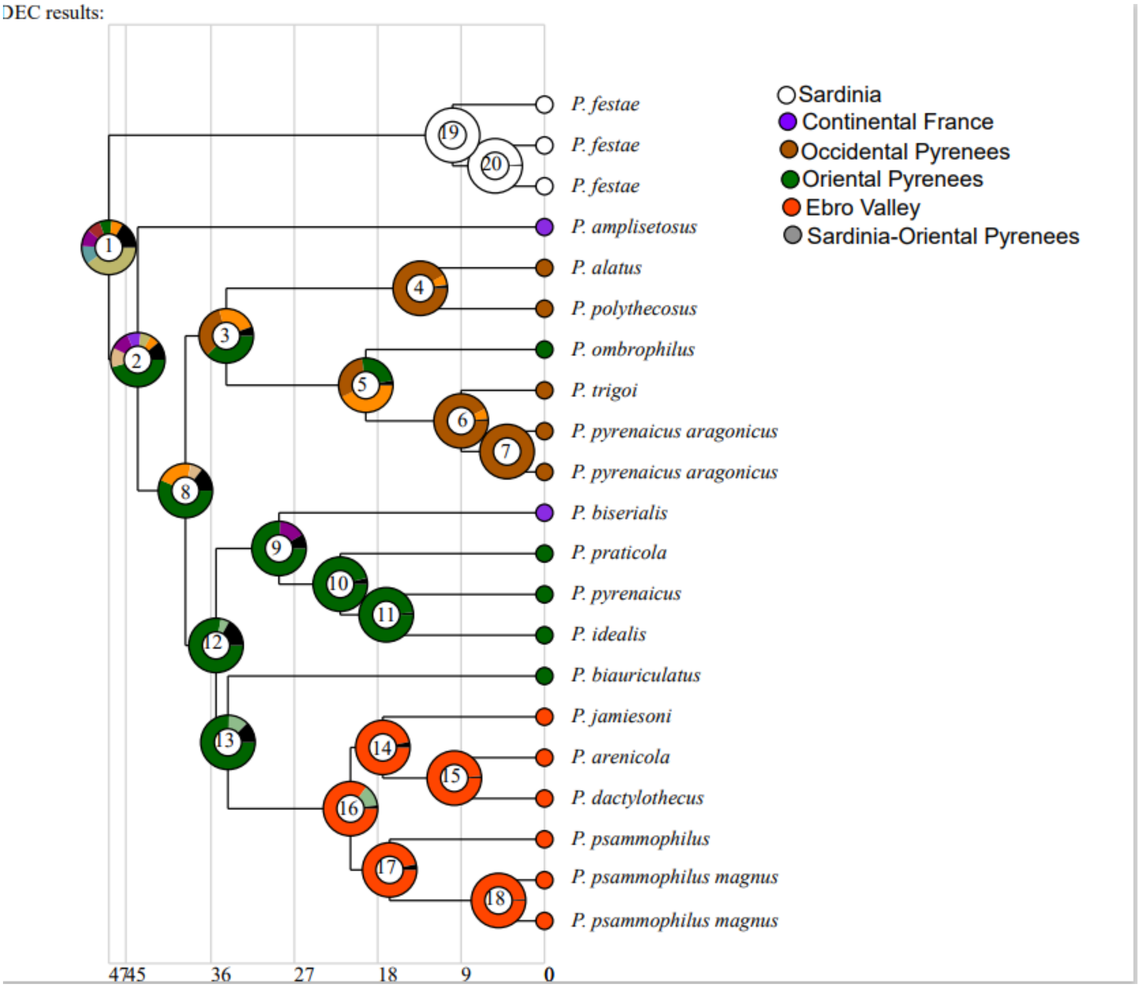
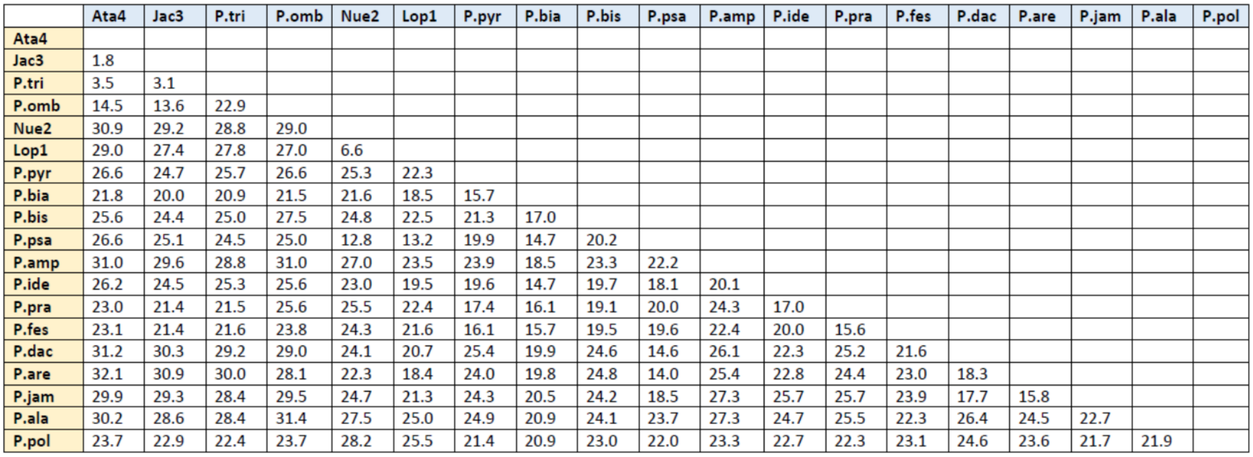


Figure 4. *Ancestral area reconstruction for each Prosellodrilus species obtained with BioGeoBEARS (RASP). Time (Ma) is represented on the bottom axis. Only the most likely state is shown for each node.*



	Ata4	**Jac3**	**P.tri**	**P.omb**	**Nue2**	**Lop1**	**P.pyr**	**P.bia**	**P.bis**	**P.psa**	**P.amp**	**P.ide**	**P.pra**	**P.fes**	**P.dac**	**P.are**	**P.jam**	**P.ala**	**P.pol**
Ata4																			
Jac3	1.8																		
P.tri	3.5	3.1																	
P.omb	14.5	13.6	22.9																
Nue2	30.9	29.2	28.8	29.0															
Lop1	29.0	27.4	27.8	27.0	6.6														
P.pyr	26.6	24.7	25.7	26.6	25.3	22.3													
P.bia	21.8	20.0	20.9	21.5	21.6	18.5	15.7												
P.bis	25.6	24.4	25.0	27.5	24.8	22.5	21.3	17.0											
P.psa	26.6	25.1	24.5	25.0	12.8	13.2	19.9	14.7	20.2										
P.amp	31.0	29.6	28.8	31.0	27.0	23.5	23.9	18.5	23.3	22.2									
P.ide	26.2	24.5	25.3	25.6	23.0	19.5	19.6	14.7	19.7	18.1	20.1								
P.pra	23.0	21.4	21.5	25.6	25.5	22.4	17.4	16.1	19.1	20.0	24.3	17.0							
P.fes	23.1	21.4	21.6	23.8	24.3	21.6	16.1	15.7	19.5	19.6	22.4	20.0	15.6						
P.dac	31.2	30.3	29.2	29.0	24.1	20.7	25.4	19.9	24.6	14.6	26.1	22.3	25.2	21.6					
P.are	32.1	30.9	30.0	28.1	22.3	18.4	24.0	19.8	24.8	14.0	25.4	22.8	24.4	23.0	18.3				
P.jam	29.9	29.3	28.4	29.5	24.7	21.3	24.3	20.5	24.2	18.5	27.3	25.7	25.7	23.9	17.7	15.8			
P.ala	30.2	28.6	28.4	31.4	27.5	25.0	24.9	20.9	24.1	23.7	27.3	24.7	25.5	22.3	26.4	24.5	22.7		
P.pol	23.7	22.9	22.4	23.7	28.2	25.5	21.4	20.9	23.0	22.0	23.3	22.7	22.3	23.1	24.6	23.6	21.7	21.9	

Table 2. *Uncorrected genetic distance for molecular marker COI (%) between Prosellodrilus species. P. pyr. Arag A and B = P. pyrenaicus aragonicus (Atarés and Jaca); P.tri = P. trigoi; P.omb. = P. ombrophylus; P. psa. Mag. A and B Lop1 = P.psammophilus magnus (Nueno and Loporzano); P.pyr = P. pyrenaicus; P.bia. = P. biauriculatus; P.bis. = P. biserialis; P.psa. = P. psammophilus; P.amp. = P. amplisetosus; P.ide. = P. idealis; P.pra. = P. praticola; P.fes. = P. festae; P.dac. = P. dactylothecus; P.are. = P. Arenicola; P.jam. = P. jamiesoni; P.ala. = P. alatus; P.pol. = P. polythecosus.*

When comparing the average body size (represented by average weight) of *Prosellodrilus* species (Figure 2), the largest species are separated in two Spanish clades. Most Spanish species have weights over 1.0 g, with the smallest species (*Pr. psammophilus*) still being larger than French species (weighing under 0.55 g). Ancestral character state reconstruction (Supplementary Material 3) recovered a weight between 0.5 and 1 g (state 1) as the ancestral state of the genus, and a weight over 1 g (state 2) as the ancestral state for the two main Spanish clades.

Regarding uncorrected average pairwise genetic distance for the COI molecular marker between species of the genus *Prosellodrilus*, most species showed very large genetic distances between them, most of them greater than 20% and reaching 30% in some cases, showing an average genetic distance much higher than that found among species of other Lumbricid genera. Some lower, noteworthy divergence values between taxa were the genetic distance between *P. psammophilus* and *P.*

psammophilus magnus (around 13%) or genetic distance between *P. trigoi* and *P. pyrenaicus aragonicus* (3.5%).

DISCUSSION

Systematic implications

Results have shown *Cataladrilus* and *Prosellodrilus* genera as two monophyletic and well-supported genera, coinciding with previous works based only on morphology (Qiu & Bouché, 1998b). They are also evolutionarily close to other early-branching genera of the Lumbricidae family such as *Zophoscolex* or *Castellodrilus.* Previous phylogenetic studies had already suggested this phylogenetic position, but they were carried out using very few species from these two genera and did not use the type species of *Prosellodrilus* (Domínguez *et al.*, 2015, 2018).

Regarding the genus *Prosellodrilus*, the earliest branching taxon is *P. festae.* Previously not considered to belong to *Prosellodrilus* by Qiu & Bouché, 1998b, our results confirm its assignment to this genus and the presence of *Prosellodrilus* in Sardinia, with interesting biogeographical implications (see below).

Another interesting finding concerns the position of *Prosellodrilus amplisetosus* within *Prosellodrilus.* This widely distributed species has been controversial since it was described (Bouché, 1972) from France. It was later found in Galicia (Spain) (Souto *et al.,* 1991), but some authors rejected that those individuals belonged to the same species and described them as *Prosellodrilus occidentalis* (Qiu & Bouché, 1998a) or even as a new genus and species (*Kenleenus armadas*, Blackemore, 2012). These studies, however, were based on morphological characters only, which seem to not be enough to solve *Prosellodrilus* systematics. Even if re-examination of the Galician specimens suggested that they are indeed *P. amplisetosus,* further individuals suitable for DNA sequencing should be sampled to confirm it. This is further supported by the fact that *P. amplisetosus* has also been found in Ireland (Melody & Schmidt, 2012) and these Irish specimens are genetically very similar to the French ones (pers. comm.).

The result of this study shows that *P. pyrenaicus* and *P. pyrenaicus aragonicus* do not form a monophyletic clade, hence *P. pyrenaicus aragonicus* cannot be a subspecies of *P. pyrenaicus,* but instead the species *Prosellodrilus aragonicus stat.nov.* This is supported by the fact that *Prosellodrilus pyrenaicus* and *Prosellodrilus aragonicus* stat. nov. have important morphological differences; *P. aragonicus* stat. nov. is larger and heavier than *P. pyrenaicus* and the genetic distance for COI between these two species is around 25%, which is usually indicative of different species (Chang & James, 2011).

Prosellodrilus aragonicus stat. nov. formed a clade with *P. trigoi*, both being species that are very close geographically and restricted to Spain. In addition, upon closer inspection no morphological difference between representatives of these two species were found, and their COI genetic distance (3.1-3.5%) was well below the usual interspecific thresholds: this suggests that *P. aragonicus* stat. nov. and *P. trigoi* are the same species, with *P. trigoi* as the junior synonym.

Another rather problematic species that appears in the French-Iberian clade is *Prosellodrilus psammophilus*. This species is quite similar to other exclusively Spanish species such as *P. jamiesoni* and *P. arenicola*, and, in fact, these three species appear to form a monophyletic clade with *P. dactylothecus* and *P. psammophilus magnus*, as was described by Qiu & Bouché, 1998b based exclusively in morphology.

Some previous morphological studies have gone into great depth about the great resemblance of the species *P. psammophilus*, *P. psammophilus magnus*, *P. jamiesoni* and *P. arenicola*, even going so far as to vaguely suggest that these species were in fact a single species (Blakemore, 2012). However, the data obtained in this study, such as the genetic distance for COI of around 15–18% between these species, do not allow us to accept this hypothesis and, at least for the moment, *P. psammophilus*, *P. arenicola* and *P. jamiesoni* will continue to be treated as valid species. Further morphological studies including a larger number of mature specimens and a better knowledge of their distribution area would be necessary to delve into this topic.

Regarding the *Cataladrilus* genus, the addition of more representatives resulted in the recovery of *Allolobophora s.l. tiginosa* within the same clade: this contrasts with its phylogenetic position in Martínez-Navarro *et al.* (2022), where it formed a polytomy with *Prosellodrilus* and *Cataladrilus*. This species was found in Puéchabon (France), relatively close to the species *Cataladrilus porquerolensis*. Morphologically, *Allolobophora s.l. tiginosa* only differs from *Cataladrilus* species by its spermathecae position (10/11, 11/12, 12/13); this variation is no different from what can be found in other genera such as *Gatesona* Qiu & Bouché, 1998b or *Allolobophora* Eisen, 1873, and the genus already contains a species with a different number and position of spermathecae (*Cataladrilus multithecus* Qiu & Bouché, 1998b). The phylogenetic position, geographic range and morphology of *tiginosa* support its inclusion within *Cataladrilus*.

Evolution and biogeography

High mountain ranges appear to have acted as barriers during the evolution of earthworm taxa, giving rise to distinct clades isolated on opposite sides. Such a phenomenon was observed in the genus *Zophoscolex* as defined by Qiu & Bouché (1998), which was divided into *Zophoscolex* (French species) and *Castellodrilus* (Iberian species) when phylogenetic analyses were carried out (Jiménez-Pinadero *et al.,* 2021). This integrative work confirmed that *Prosellodrilus* and *Cataladrilus* contain species located on both sides of the Pyrenees, and hence this mountain range does not seem to have acted as a physical agent separating these two genera. Such a distribution on both sides of the Pyrenees has also been found for the French-Iberian endemism *Orodrilus* Bouché, 1972, as redefined by Jiménez-Pinadero *et al.* (2023).

The ancestral area of the genus *Prosellodrilus* would be an area between the Eastern Pyrenees and Sardinia, dating back 48 million years, when Sardinia had not yet separated from France (Figure 4, node1), which explains the presence of *Prosellodrilus* *festae* in Sardinia, and why it is the oldest and most divergent species of all, since it could have been isolated from the rest as Sardinia started separating from France 30 million years ago (Figure 4, nodes 19 and 20). In fact, isolation has been associated with greater divergence in some invertebrates (Novo *et al.*, 2010, Hutchinson & Templeton, 1999) which is consistent with the pattern of isolation by distance (Slatkin, 1993). Similar patterns were observed for hormogastrid earthworms by Novo *et al.* (2014) for which vicariance events due to plate separation could have caused speciation.

In the French-Iberian clade, there does not seem to be a separation between exclusively French species and exclusively Spanish species since species from both sides of the Pyrenees appear together in two subclades. This suggests, as supported by the time-calibrated tree, that those subclades diverged while the Pyrenees were still developing and could be easily crossed by those expanding lineages.

The beginning of the formation of the Pyrenees (38 Ma) caused land to emerge in the western area that had previously been submerged, which could have allowed the species of this genus to colonize those areas from the east through the moderately elevated hills, giving rise to the westernmost species ancestors that evolved independently. This led to the formation of two independent clades; one clade with the Ibero-French species further east and another clade with the Ibero-French species further west (Figure 4, node 8).

About 36 million years ago, the westward advance and strong uplift of the Pyrenees caused the western clade to be divided into two: a French clade (North of the Pyrenees) and a Spanish clade (South of the Pyrenees) (Figure 4, node 3), which also happened with the eastern clade as the mountains that divided Spain from France were being uplifted (Figure 4, node 12).

The presence of a large brackish lake near Huesca could have prevented the Spanish species from the east and west from expanding in opposite directions and coexisting until 20 million years ago when this lake dried up and some species such as *P.*

ombrophilus and *P. psammophilus* began to expand, eventually overlapping their ranges.

Both ancestral area and ancestral character state reconstruction suggest that the primitive form of this genus was small, as these are the species from Sardinia and eastern France where the genus originated. Large body size, exclusive to some Spanish species, only occurs in species whose distribution area is in Huesca and Navarra, which suggests that the large form arose twice in evolution independently in the species of the area, which could indicate that the environmental (soil characteristics, climate) or biotic interactions (competence or empty niche filling) in this colonized area drove species of this genus to evolve increased body size (homoplasy). Furthermore, these larger Spanish species create a large volume of surface casts of an easily recognizable type not observed in the smaller French species. This hints at those two clades having convergently evolved not just similar functional traits but also bioturbating and feeding behaviours; this transition to a different functional group could be tested by integrating them into the experimental framework of Capowiez *et al.* (2023), where only three French species of *Prosellodrilus* were studied.

CONCLUSIONS

1. *Cataladrilus* and *Prosellodrilus* are two well-supported monophyletic genera that include their type species respectively, so they are valid genera.

2. *Cataladrilus* and *Prosellodrilus* are very closely related, early branching lumbricid genera and are closely related to other early branching genera such as *Zophoscolex* and *Castellodrilus*.

3. The species *Prosellodrilus pyrenaicus aragonicus* does not form a monophyletic clade with the species *Prosellodrilus pyrenaicus*, appearing separately on the phylogenetic tree instead. Therefore, they cannot be the same species. *Prosellodrilus*

pyrenaicus aragonicus and *P. trigoi* seem to be the same species instead, for which the synonymization proposal is *P. aragonicus.*

4. *Prosellodrilus psammophilus magnus* appears to be a subspecies of *Prosellodrilus psammophilus*, as previously described based solely on morphology.

5. *Prosellodrilus psammophilus* (and *P. psammophilus magnus*), *P. jamiesoni*, *P. arenicola* and *P. dactylothecus* are closely related and morphologically very similar, however, results obtained in this study indicate that they can be maintained as valid species for the moment and more comprehensive studies are needed to make further taxonomic decisions.

6. Diversification of the genus *Prosellodrilus* appear to have been strongly influenced by the westward emergence and uplift of the Pyrenean range.

7. Large body size appears to have originated within *Prosellodrilus* in Spain, in a small area between Huesca and Navarra, but since all Spanish species with large body sizes are not in the same clade and small body size was inferred to be the ancestral character state, it seems to indicate that large size has evolved twice within this genus (homoplasy).

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SUPORTING INFORMATION

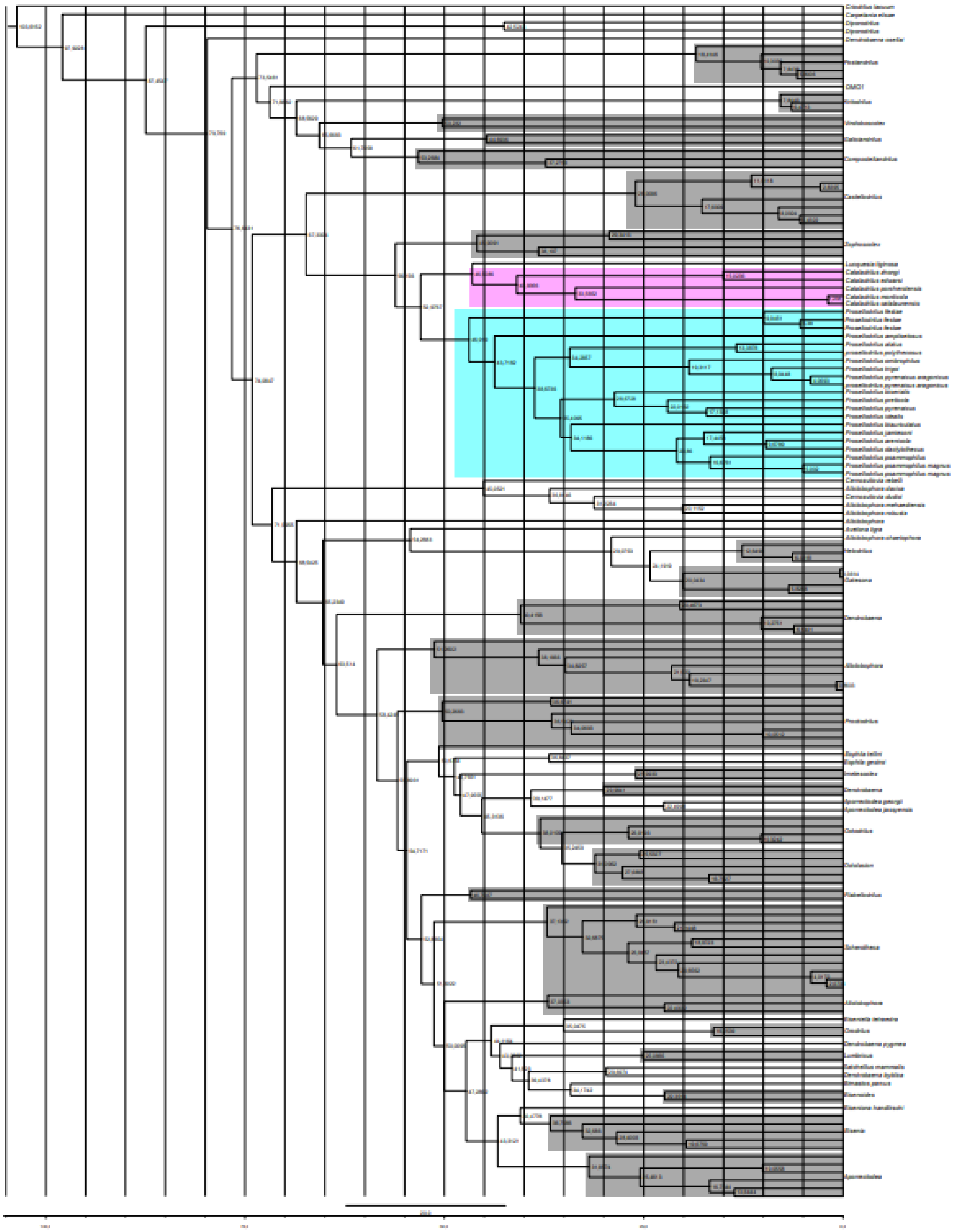

Figure S1: Maximum likelihood tree obtained using the molecular markers COI-16S-28S.

CAPÍTULO 4: UN NUEVO GÉNERO ENDÉMICO DE LA PENÍNSULA IBÉRICA EN LA BASE DE LOS LUMBRÍCIDOS

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TRABAJO EN PREPARACIÓN

Nota. Se ha realizado ya la mayor parte del estudio morfológico y molecular de dos de las especies implicadas en este nuevo género, *Dendrobaena pseudorrosea* y *Dendrobaena osellai*. Falta para completar este trabajo capturar ejemplares de *Dendrobaena ruffoi* para su estudio morfológico y molecular, aunque esta especie tiene una distribución restringida en la zona Este peninsular y puede resultar complicado capturarla. Si no se consiguiese en las épocas favorables del otoño y primavera próximos, se procedería a publicar este nuevo género con los datos disponibles en el momento.

INTRODUCCIÓN

En el árbol filogenético de la Figura 2 de Jiménez Pinadero *et al.*, 2023, capítulo 2 de esta memoria, se observa que en una posición basal de la Familia Lumbricidae aparece un pequeño clado formado por dos especies *Dendrobaena pseudorrosea* Moreno, Jesús y Díaz Cosín, 1981 (= *Iberoscolex pseudorroseus* Qiu y Bouché, 1998) y *Dendrobaena osellai* Zicsi, 1970 que presentan quetas separadas, color claro o pardo (ni púrpura ni rojo intenso) y glándulas de Morren intramurales sólo en dos o tres segmentos. Este clado es el grupo hermano del resto de los lumbrícidos, por lo que su estudio puede tener gran interés para profundizar en la filogenia del grupo más abundante de lombrices de tierra en la Península Ibérica y en el continente europeo.

Los soportes del árbol son relativamente bajos con ML y muy altos en bayesiano, pero este grupo aparece de forma constante y bien diferenciada en varios árboles realizados en diferentes ocasiones.

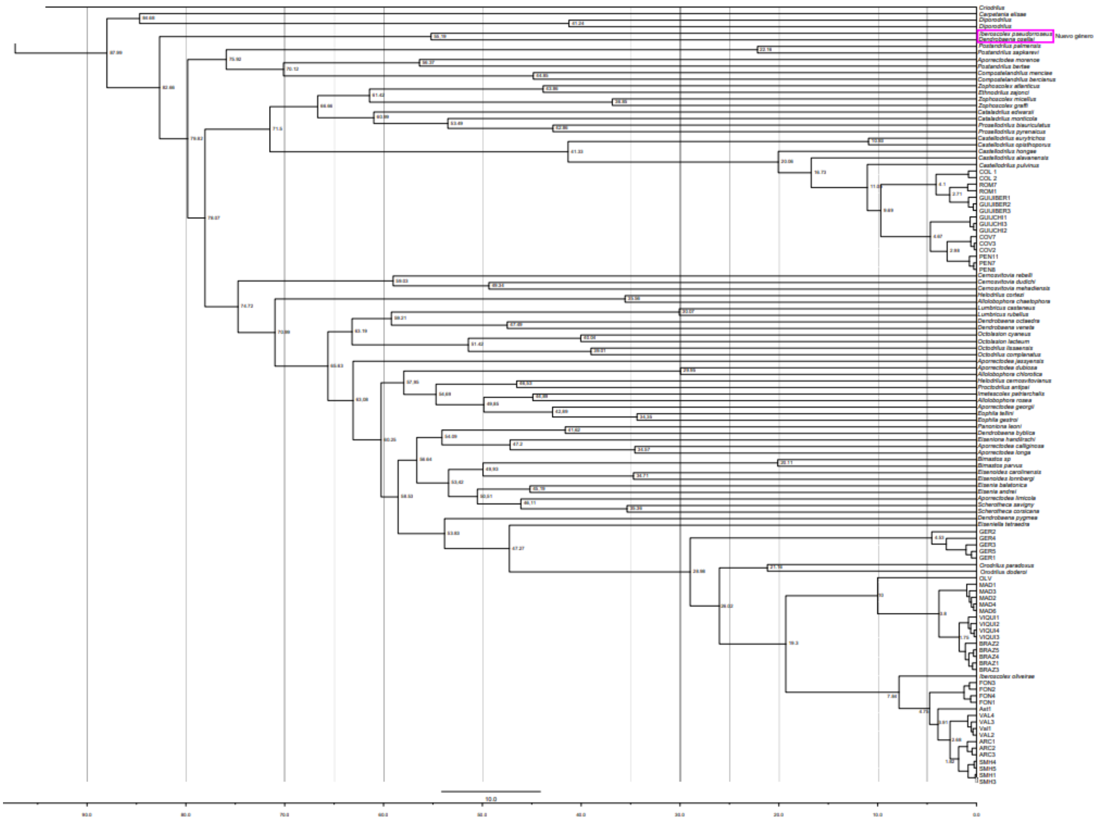

Figura 2 de Jiménez-Pinadero et al., 2023. Árbol filogenético ultramétrico de la familia Lumbricidae utilizando los marcadores moleculares COI-16S-28S-ND1-12S.

Filogenéticamente este pequeño grupo está muy bien separado del resto de los lumbrícidos, separación que se ha datado en 82 Ma, Jiménez Pinadero *et al* (2023), edad más que suficiente para considerarlo muy antiguo dentro de la filogenia del grupo. Las dos especies citadas se han separado entre sí hace un mínimo de 50 Ma lo que indica que ambas especies y su ancestro común ya se habían diferenciado dentro de un nuevo género en ese momento, y soporta su carácter genérico y su antigüedad.

Posteriormente hemos podido ver que hay una tercera especie que por su morfología podría estar relacionada con estas dos; se trata de *Dendrobaena ruffoi* Zicsi, 1970 cuya localidad típica es Frías de Albarracín, Teruel y que no se ha podido estudiar molecularmente porque no se han capturado ejemplares a pesar de haber muestreado por la zona. Pérez Onteniente y Rodríguez Babío, 2007, 2008, 2009 y Pérez Onteniente, 2012 la han encontrado posteriormente además en las provincias de Castellón y Valencia, aunque sus ejemplares se diferencian en algunos caracteres de los descritos por Zicsi, 1970 en la localidad típica (ver más abajo). Esta especie es similar morfológicamente a las dos especies que forman el pequeño clado basal de los lumbrícidos, por lo que es posible que esté emparentada con ellas, y hemos decidido incluirla al menos provisionalmente en ese grupo. Evidentemente esta decisión tendrá que ser confirmada mediante marcadores moleculares en cuanto seamos capaces de obtener nuevos ejemplares que nos permitan aplicar estas técnicas.

Dada la información existente en este momento sobre este pequeño grupo de especies creemos que se les puede asignar la categoría taxonómica de un nuevo género, pero en aras de la estabilidad sistemática y nomenclatorial hemos optado por no asignarle un nombre genérico hasta que reunamos más evidencia morfológica y molecular. En cualquier caso queremos dejar constancia aquí de la existencia de este pequeño grupo de especies que se han separado tempranamente del resto de los lumbrícidos y que muy posiblemente representen un nuevo género dentro de la Familia.

CARACTERES DEL NUEVO GÉNERO

Pigmentación ausente o poco patente pardo rojiza clara (ni púrpura ni rojo intenso); clitelo a veces ocre. Longitud media alrededor de 50 mm o un poco más pequeña. Número de segmentos de 121 a 182. Prostomio epilóbico abierto o cerrado. El clitelo es largo, abarca entre 10 y 14 segmentos, empieza entre 26 y 30 y termina entre 35 y 43. Tubérculos pubertarios generalmente cortos -tres o cuatro segmentos-, a veces hasta seis, empiezan entre 32 y 38 y acaban entre 34 y 41. Quetas separadas, *ab* puede ser igual o hasta dos veces mayor que *cd*. Habitualmente poros de las espermatecas en 9/10 y 10/11, pero en algunos ejemplares se han citado hasta 12/13. Poros masculinos en 15 con poróforos. Septos engrosados 5/6-8/9. Corazones 6, 7-11. Vesículas seminales 11 y 12, a veces un tercer par en 9. Ovisacos en 14. Glándulas de Morren en 11, 12,13 intramurales y sin desarrollo aparente en 10 ni 14. Buche 15-16. Molleja 17-19. El tiflosol cuando se conoce es simple. Vesículas nefridianas en forma de S en *D. pseudorrosea*, desconocidas en las otras especies.

Especies incluidas: *Dendrobaena osellai* Zicsi, 1970, *Dendrobaena pseudorrosea* Moreno, Jesús y Díaz Cosín, 1981, ¿*Dendrobaena ruffoi*? Zicsi, 1970.

Dendrobaena osellai Zicsi, 1970

Sinonimias. *Kritodrilus osellai* Pérez Onteniente, 2012: 65; no *Allolobophora osellai* Zicsi, 1981 (= *Italobalkaniona* o *Pietromodeona osellai*)

Localidad tipo. Sierra de los Monegros, Peñarroya, Teruel, España.

Descripción. Pigmentación clara, pardo rojizo claro en ejemplares fijados (Zicsi, 1970); ausente o rosácea en vida (Pérez Onteniente y Rodríguez Babío, 2008). Forma subcilíndrica con clitelo aplastado y ensanchado. Longitud media 5,15 cm (4,3-6 cm). Diámetro 2,5-3 mm. Número medio de segmentos 145 (123-166). Prostomio epilóbico abierto. Clitelo largo en silla de montar 26-39 (27, 28-39, 40 Pérez Onteniente y Rodríguez Babío, 2008). Tubérculos pubertarios 33-37 (33, 34-38 Pérez Onteniente y Rodríguez Babío, 2008), Figura 1,

Quetas separadas. Papilas *a* y *b* en 11, y *a* del 28 al 30 o 31 y del 34 al 40 (Pérez Onteniente y Rodríguez Babío, 2008). Primer poro dorsal 5/6 (5/6 a 7/8, Pérez Onteniente y Rodríguez Babío, 2008). Poros nefridianos en línea, área B. Poros masculinos en 1/2 15, área B, con atrios masculinos redondeados que no afectan a los segmentos contiguos. Poros de las espermatecas simples en 9/10, 10/11 (¿9/10-12/13 Pérez Onteniente y Rodríguez Babío, 2008?).

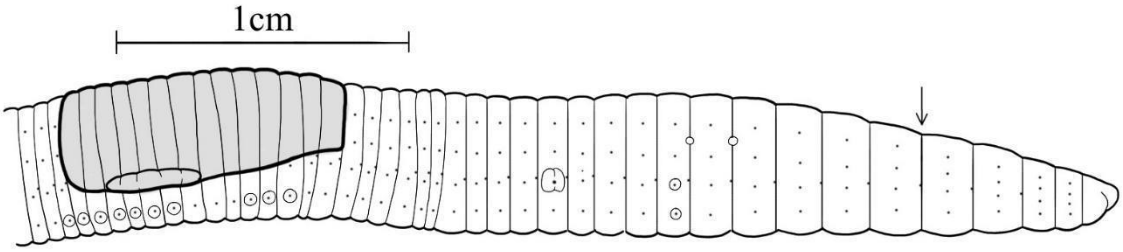

Figura 1. *Dendrobaena osellai.*

Septos engrosados 5/6-8/9. Corazones 6-11. Dos pares de vesículas seminales en 11 y 12, el par posterior es mayor. Testículos y embudos masculinos en 10 y 11. Epidídimos en 11 y 12. Ovarios y embudos femeninos en 13. Ovisacos en 14. Espermatecas en 9/10,10/11 (segmentos 10 y 11). Glándulas de Morren 12 y 13. Buche 15-16. Molleja 17-19.

Comentarios. Pérez Onteniente et al., 1998, señalan diferencias de sus ejemplares con la especie de Zicsi en caracteres internos y externos “que serán tratados en un trabajo posterior” que nunca fue abordado. Pérez Onteniente y Rodríguez Babío, 2007, 2008 y Pérez Onteniente, 2012 señalan algunas diferencias en caracteres como clitelo, tubérculos y poros de las espermatecas, pero no aportan datos sobre los órganos internos de sus ejemplares, lo que no permite confirmar el número real de espermatecas. La descripción incompleta de estos autores no permite descartar que sus ejemplares representen en realidad una especie diferente, lo que habrá que rechazar o confirmar en el futuro.

Categoría ecológica. Por las características morfológicas *Dendrobaena osellai* podría asignarse a la categoría de las epiendogeas.

Hábitat. Ambientes preferidos: Bosques de pinos y encinas y vaguadas con matorral de aliagas y romero en zonas de altitud media o alta. Segrega una capa de mucus que aglutina las partículas de suelo a su alrededor para camuflarse.

Distribución mundial. Endemismo iberobalear.

Distribución iberobalear: Citada por Zicsi, 1970 en Teruel, por Pérez Onteniente y Rodríguez Babío, 2007, 2008, 2009 y Pérez Onteniente, 2012 en algunas zonas del Este Peninsular (Castellón, Valencia, Albacete) y por Jesús *et al.*, 2001, 2002 en Madrid.

Fig. 2 Distribución de Dendrobaena osellai.

Dendrobaena pseudorrosea Moreno, Jesús y Díaz Cosín, 1981

Sinonimias. *Kritodrilus pseudorroseus* Pérez Onteniente y Rodríguez Babío, 2008; *Iberoscolex pseudorrosea* Qiu y Bouché, 1998.

Localidad tipo. El Escorial (Sierra de Guadarrama), Madrid, España.

Descripción. En vivo coloración rosácea con gradiente anteroposterior y dorsoventral, parte posterior casi transparente, zona ventral color marfil; clitelo ocre; fijados se vuelven blanco grisáceos; en el momento de la fijación expulsan líquido celomático amarillento. Longitud 36-45 mm. Diámetro 3-4 mm. Número de segmentos 121-128. Peso de los individuos fijados 130-200 mg. Prostomio epilóbico abierto 1/2. Algunos segmentos trianillados y postclitelares bianillados. Clitelo en silla de montar aplanado ventralmente 26, 27-35, 36 siendo visibles las quetas y las líneas intersegmentarias. Tubérculos pubertarios 32–34, en línea engrosada en los laterales del clitelo. En dos individuos se encontraron espermatóforos en la zona clitelar.

Quetas separadas distancia entre quetas en el segmento 40, *aa*: 17; *ab*: 11,6; *bc:* 12,5; *cd:* 9; *dd:* 20,8. Papilas como pequeñas areolas sobre quetas *a* en 14, 29 y 30. Primer poro dorsal 4/5, 5/6 poco visible, más patente 6/7. Poros de los nefridios a nivel del área B. Poros masculinos en 15, atrios glandulares bien desarrollados. Poros femeninos en 14 cerca de *b*, con pequeños labios glandulares. Poros de las espermatecas en 9/10 y 10/11 a nivel de *c.*

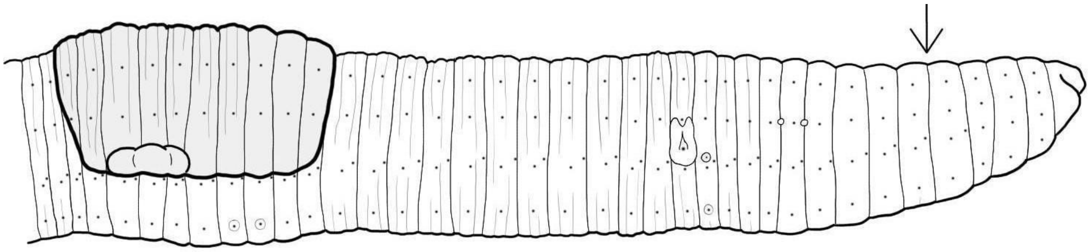

Fig. 3 Dendrobaena pseudorrosea

Musculatura pennada. Septos engrosados 5/6-8/9. Corazones laterales 7-11. Tres pares de vesículas seminales en 9, 11 y 12, pequeñas y aparentemente vacías. Testículos y embudos masculinos en 10 y 11, los embudos son pequeños e iridiscentes; sin epidídimos. Los conductos deferentes se unen aparentemente en 11/12 y terminan en unas evidentes glándulas atriales internas redondeadas y blanquecinas en la posición de los poros masculinos. Ovarios y embudos femeninos en 13. Ovisacos en 14. Dos pares de espermatecas en 10 y 11, pequeñas y esféricas en las que no se aprecian espermatozoides (¿transferencia y almacenaje de esperma en espermatóforos?). Glándulas de Morren con laminillas en 11-13, sin dilataciones extramurales. Segmento 14 valvular. Buche 15-16. Molleja 17-19. Tiflosol simple, comienzo gradual en 20, 21, termina en 94-104 con 21-24 segmentos atiflosados. Vesículas nefridianas en forma de S.

Hábitat. Los individuos de la serie tipo se encontraron en un robledal, en una depresión situada entre rocas y llena de hojarasca poco degradada, con las siguientes características del suelo: C: 5,61%, N: 0,176%, relación C/N: 31,34, Porosidad: 43,92%, Humedad: 19,95%, Aireación: 23,97%, pH: 6,0 y F>2 mm: 14,59%. Se trata de un suelo húmedo, rico en materia orgánica, poroso y ligeramente ácido.

Comentarios. Morfológicamente es parecida a *Kritodrilus calarensis* con la que coincide en clitelo y tubérculos, pero puede diferenciarse por las distancias entre quetas, el número de espermatecas y otros caracteres. Moreno, Jesús y Díaz Cosin, 1981 señalan que los ejemplares asignados por Zicsi, 1970 a *Dendrobaena calarensis* en los Montes Universales (Teruel) y Moncayo (Zaragoza) realmente pertenecen a *D. pseudorrosea* (según Zicsi com. per.).

Distribución mundial. Endemismo ibérico.

Distribución iberobalear. Un poco por toda la Península. Posiblemente presente una distribución más amplia que la reflejada en el mapa.

Fig. 4 Distribución de Dendrobaena pseudorrosea

Dendrobaena ruffoi Zicsi 1970

Sinonimias. *Kritodrilus ruffoi* Pérez Onteniente y Rodríguez Babío, 2008; Pérez Onteniente, 2012; non *Octodrilus ruffoi* Zicsi, 1981

Localidad tipo. Frías de Albarracín, Teruel, España.

Descripción. Sin pigmentación (Pérez Onteniente y Rodríguez Babío, 2008), pigmentación pardo rojizo claro en ejemplares fijados (Zicsi, 1970). Forma cilíndrica con aplastamiento y ensanchamiento clitelar. Longitud media 50,5 mm (39-62 mm). Diámetro 2,5-3 mm. Número medio de segmentos 165 (147-182). Prostomio epilóbico cerrado. Clitelo en forma de silla 30-42 (29, 30-42, 43 Pérez Onteniente y Rodríguez Babío, 2008). Tubérculos pubertarios 38-41, (1/2 37, 38-41 Pérez Onteniente y Rodríguez Babío, 2008). Quetas separadas, *aa* dos veces y media más grande que *ab*; *ab* un poco más grande que *bc*; *bc* más grande que *cd*; *dd* algo más grande que *aa* Zicsi, 1970. *aa*= 2*ab*; *ab*=*bc*=2*cd*; *dd* > *aa*,(Pérez Onteniente y Rodríguez Babío, 2008).

Primer poro dorsal 7/8, rudimentarios 5/6, 6/7 (Pérez Onteniente y Rodríguez Babío, 2008). Poros nefridianos en línea, en el área B. Poros masculinos 1/2 15, área B, con poróforos redondeados que no afectan a los segmentos contiguos. Poros de las espermatecas en 9/10, 10/11, simples.

Septos engrosados 5/6-8/9. Corazones 6-11. Dos pares de vesículas seminales en 11 y 12, el par posterior mayor. Testículos 10 y 11. Epidídimos 11 y 12. Embudos masculinos 10 y 11. Ovarios y embudos femeninos en 13. Ovisacos en 14. Espermatecas en 9/10, 10/11 (segmentos 10 y 11). Glándulas de Morren 12 y 13 sin divertículos. Buche 15-16. Molleja 17-19.

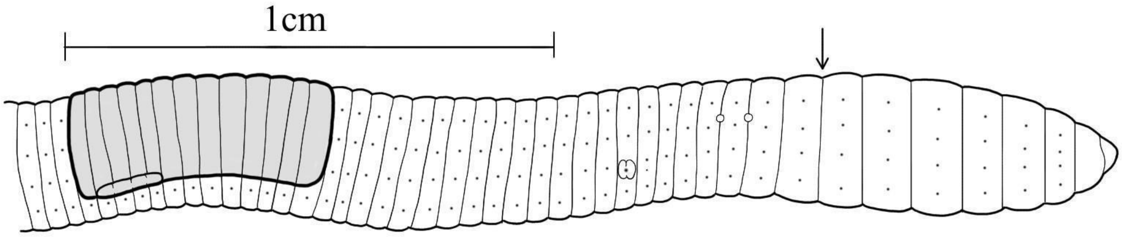

Fig. 5 Dendrobaena ruffoi.

Categoría ecológica. Por las características morfológicas podría asignarse a la categoría de las epiendogeas.

Hábitat. Ambientes preferidos: Bosques de pinos silvestres y prados en zonas de gran altitud.

Distribución mundial. Endemismo ibérico.

Distribución iberobalear. Teruel, Castellón, Valencia, Zicsi, 1970; Pérez Onteniente y Rodríguez Babío, 1998, 2008.

Fig. 6 Distribución de Dendrobaena ruffoi.

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DISCUSIÓN GENERAL

Importancia de incluir la especie tipo en los análisis filogenéticos

La importancia de llevar a cabo estudios moleculares y de incluir a la especie tipo de los géneros estudiados queda especialmente reflejada en el género *Zophoscolex*, donde se puede observar que, las especies incluidas en este estudio y que se suponían todas ellas pertenecientes a este género, pertenecen, al menos, a tres géneros distintos (*Zophoscolex*, *Castellodrilus* y *Cataladrilus*), lo que viene a reforzar la idea de que los caracteres somáticos, tanto externos como internos, no son a veces suficientes para discriminar las relaciones evolutivas en las lombrices de tierra y establecer una sistemática sólida. Esto es consecuencia directa del hecho de que el medio edáfico impone serias constricciones al modelo corporal de las lombrices de tierra, y lleva a la estasis morfológica por lo que son pocos los caracteres disponibles, que además pueden presentar variabilidad intraespecífica.

El género *Zophoscolex* como tal quedaría restringido a sólo tres especies: *Z. atlanticus* (especie tipo, y, por tanto, la que soporta el nombre del género), *Z. micellus* y *Z. graffi*. Estas tres especies se sitúan en la zona sur de Francia (Norte de la cordillera de los Pirineos), y, por tanto, este género quedaría restringido solo a Francia, no teniendo presencia en España.

Por otra parte, la especie *Zophoscolex zhongi*, que se encuentra en el Pirineo Catalán (España), parece formar un clado monofilético con otras especies del género *Cataladrilus* (Qiu & Bouché, 1998b), por lo que esta especie debería ser transferida a ese género y renombrada como *Cataladrilus zhongi*. La inclusión de *Z. zhongi* en el género *Cataladrilus* puede resultar sorprendente, ya que esta especie presenta notables diferencias morfológicas con las otras especies de este género, sin embargo, hay que tener en cuenta que en este estudio no se han podido estudiar otras especies del género *Zophoscolex* muy cercanas geográficamente a *Z. zhongi* y muy similares morfológicamente a esta, como *Zophoscolex byanensis* o *Zophoscolex diazi*, que podrían ayudar a aclarar esta situación.

Las otras siete especies del género *Zophoscolex* contempladas en este estudio (*Z. alavanensis, Z. chitae, Z. eurytrichos, Z. opisthoporus, Z. ibericus, Z. hongae* y *Z. pulvinus*), todas ellas distribuidas únicamente en la Península Ibérica, forman un clado monofilético, pero separado del clado donde se encuentra *Z. atlanticus*, por lo que estas especies no forman parte del género *Zophoscolex*, sino que representan un género en sí mismo. Las relaciones evolutivas entre las especies de este género sí que parecen mantenerse tal y como las describieron Qiu & Bouché en 1998a.

Este nuevo género es endémico de la Península Ibérica y se propone como nombre para él *Castellodrilus*. Este género, por tanto, sería endémico de la Península Ibérica, y no se encontraría en Francia.

En el caso del género *Iberoscolex*, la inclusión de la especie tipo también ha sido clave a la hora de poder aclarar la validez de este género; todas las especies ibéricas contempladas en este estudio (*I. oliveirae, I. albolineatus, I. gerardoi*, *I. carpetanus*), salvo *I. pseudorroseus,* sí que aparecen formando un clado monofilético junto con *I. microepigeus*, la especie tipo del género, y, además, el clado se encuentra separado de la especie *Eiseniona handlirschi*, especie tipo del género *Eiseniona,* por lo que queda demostrado que estas especies constituyen un género aparte y no pertenecen a *Eiseniona*.

Sin embargo, no se puede hablar del género *Iberoscolex* como género endémico de la Península Ibérica, ya que, sorprendentemente, los análisis moleculares muestran que junto a las especies de *Iberoscolex* aparecen dos especies pertenecientes al género *Orodrilus* (*Orodrilus doderoi*, especie tipo de este género, y *Orodrilus paradoxus*).

Este hecho hace que sea necesario sinonimizar los géneros *Orodrilus* e *Iberoscolex*, pues ambos aparecen como un clado monofilético que incluye a las especies tipos de ambos géneros, y dado que el género *Orodrilus* fue descrito antes que el género

Iberoscolex, se propone que las especies de *Iberoscolex* contempladas en este estudio pasen a formar parte del género *Orodrilus*.

Esto tiene algunas implicaciones importantes, ya que, por una parte desaparece el género *Iberoscolex* como género endémico de la Península Ibérica, y por otra parte, el género franco-ibérico *Orodrilus*, que se suponía como un género exclusivo de las montañas y de distribución limitada, tiene una distribución mucho más amplia de lo esperada, distribuyéndose por el sur de Francia y por gran parte de la Península Ibérica.

En cuanto a *I. pseudorroseus*, parece ser una especie mucho más basal en la filogenia de lo que serían las otras especies de este género, y parece estar evolutivamente cerca de otras especies basales como *Dendrobaena osellai*, con la que es muy posible que forme un nuevo género endémico de la Península Ibérica, pero se necesitan más estudios para poder resolver esta incógnita, tal y como se ha comentado en el capítulo 4 de esta memoria en el que se avanza la posible existencia de este endemismo ibérico.

La validez del género *Prosellodrilus* como un género monofilético endémico del sur de Francia y el noreste de la Península Ibérica se ve apoyada por los estudios filogenéticos llevados a cabo en este estudio, que incluyen por primera vez en un mismo árbol filogenético a la especie tipo del género, *P. idealis*, y a especies tanto ibéricas como francesas. Algo similar ocurre con el género *Cataladrilus*, que también queda validado como género monofilético.

Implicaciones biogeográficas e historia evolutiva

Las relaciones evolutivas interespecíficas dentro de los géneros, así como los patrones biogeográficos de distribución de las especies que los componen, parecen estar altamente influenciados por la historia geológica de la zona, y es que se ha demostrado en varias ocasiones, que la formación de cadenas montañosas puede influenciar las relaciones evolutivas entre especies, dando lugar a procesos de especiación por aislamiento geográfico (especiación alopátrida) o dando lugar a la aparición de especies relícticas (García-*Jacas et al.,* 2022; Domínguez *et al*., 2018).

De esta manera, se puede observar como la cordillera de los Pirineos parece actuar de barrera que impide la presencia de especies de los géneros *Zophoscolex* y *Castellodrilus* a ambos lados de los Pirineos, encontrándose las especies del primero en la parte norte de la cordillera y las del segundo en la parte sur.

Sin embargo, en el caso del género *Prosellodrilus*, la formación de los Pirineos no parece ser un elemento que haya funcionado como una barrera que impidiera que el género se distribuyera a ambos lados de la cordillera, sino que parece haber sido un elemento que permitió la conexión de distintas zonas geográficas aisladas y una distribución más amplia del género (Hoorn *et al.,* 2013)

El área ancestral de este género se encontraría en una zona entre el este de Francia y Cerdeña, y sería anterior a la separación de Cerdeña del resto de Francia, lo que explicaría la presencia de *Prosellodrilus festae* en la isla y su gran divergencia con el resto de especies estudiadas, ya que *P. festae* pudo quedar aislado en Cerdeña cuando se produjo la separación de la isla por el océano y haber evolucionado de forma independiente adaptándose a las características ecológicas y edáficas de la isla.

En cuanto al resto de especies, todas localizadas a ambos lados de los Pirineos, no parece haber una separación entre especies localizadas en el norte de la cordillera (Francia), y especies localizadas en el sur (España), ya que las especies de ambos lados de los Pirineos aparecen juntas en distintos subclados, sin embargo, si se estudia en detalle la historia geológica de estas montañas y se compara con la distribución de las especies en los clados, se puede observar una clara relación entre las diferentes etapas de formación de los Pirineos y el área de distribución y las relaciones evolutivas de las diferentes especies.

La peculiar orografía de la Península Ibérica y su compleja historia geológica hacen que sea una buena zona para estudiar patrones biogeográficos de distribución, pudiéndose comparar, en este caso, diversos parámetros de diversidad genética en géneros con especies que presentan un área de distribución parecida y bastante amplia en esta zona, concretamente analizando diferentes poblaciones de los géneros endémicos *Castellodrilus* (*C. ibericus* y *C. chitae*) e *Iberoscolex* (*I. microepigeus, I. carpetana* e *I. albolineatus*). Las especies analizadas de ambos géneros presentan una fuerte estructura y un fuerte aislamiento poblacional, así como una correlación entre la distancia genética en la secuencia de COI y la distancia geográfica de las poblaciones, siguiendo lo establecido en estudios anteriores realizados con diferentes organismos edáficos (Caesar *et al.,*2005; Costa *et al.,* 2011) pero contrastando fuertemente con estudios previos realizados con lombrices cosmopolitas (Fernández *et al.,* 2016).

Sin embargo, las poblaciones de *Castellodrilus* presentan una diversidad genética mucho menor que las poblaciones de *Iberoscolex*, con un menor número de haplotipos, lo cual podría estar relacionado con la categoría ecológica de cada género, ya que las especies de *Castellodrilus* son epiendogeas, y en las capas intermedias y profundas del suelo las condiciones son más homogéneas, mientras que las especies de *Iberoscolex* son epigeas, y en las capas más superficiales del suelo las condiciones son muy cambiantes y dependen mucho del ambiente. Se podría decir, por tanto, que las especies de *Castellodrilus* son más especialistas, estando muy adaptadas a unas condiciones concretas, mientras que las de *Iberoscolex* son más generalistas, con diferentes haplotipos para adaptarse a diferentes condiciones del medio.

Futuras líneas de investigación

Tras la realización de estos trabajos, se han abierto nuevas incógnitas y se han despejado algunas vías para seguir progresando en el conocimiento de varios de los géneros de Lumbrícidos endémicos de la Península Ibérica y el Sur de Francia:

En primer lugar sería de gran importancia poder introducir en la filogenia las especies de cada género que no se han podido incluir en estos trabajos, especies como

Zophoscolex albacetensis, para comprobar si realmente no pertenece al género *Zophoscolex* sino a *Castellodrilus* y se sigue manteniendo lo descrito en el trabajo anterior, o especies como *Zophoscolex andorranensis*, localizada justo en los Pirineos y que podría servir como nexo de unión entre las especies de ambos lados de la cordillera.

Otra línea de investigación que queda abierta pasaría por estudiar la filogeografía de las especies más extendidas del género *Prosellodrilus*, como *Prosellodrilus pyrenaicus* o *Prosellodrilus psammophilus* y poder compararla con la de otros géneros endémicos de la zona como *Castellodrilus* e *Iberoscolex*. Además, sería interesante estudiar la asignación de estas especies a su grupo funcional (Capowiez *et al.,* 2024 para analizar si existe una relación entre el grupo funcional y el mayor tamaño corporal (p. ej. peso) de las especies Ibéricas en comparación con las francesas, de manera que se pudiera saber más sobre su modo de vida e incluso sobre los servicios ecosistémicos que proporcionan.

Podría ser interesante estudiar la diversidad y filogeografía de la especie *Prosellodrilus amplisetosus*, ya que presenta un fuerte conflicto taxonómico, su distribución es muy amplia en comparación con la del resto de especies de este género, estando presente en España, Francia, las Islas Británicas e incluso Argentina, en un raro caso de especie invasiva (o peregrina, como se han denominado en la bibliografía de lombrices de tierra) entre las lombrices endogeas de géneros de distribución restringida.

Por último habría que intentar obtener ejemplares de *Dendrobaena ruffoi* para estudiarlos molecularmente y validar su identidad específica y ver si forma parte del mismo clado que las otras especies del nuevo género.

CONCLUSIONES GENERALES

1. El género *Zophoscolex* no es un género monofilético, y sus especies pertenecen, al menos, a tres géneros distintos.

2. El género *Zophoscolex* propiamente dicho quedaría restringido a la zona norte de los Pirineos, y a él pertenecerían la especie tipo (*Z. atlanticus*) y las especies francesas incluídas en este estudio.

3. Las especies ibéricas de *Zophoscolex* incluídas en este estudio formarían un género propio endémico de la Península Ibérica, el género *Castellodrilus*.

4. Las especies del género *Iberoscolex* aparecen en un clado separado de la especie tipo del género *Eiseniona*, por lo que no pertenecerían a este género.

5. Las especies de los géneros *Iberoscolex* y *Orodrilus* aparecen formando un clado monofilético que incluye a las especies tipo de ambos géneros, por lo que habría que sinonimizar el más moderno.

6. *Iberoscolex* y *Orodrilus* se sinonimizan como *Orodrilus* al haber sido descrito este género con anterioridad.

7. Desaparece el género *Iberoscolex* como género endémico de la Península Ibérica.

8. El género franco-ibérico *Orodrilus* presenta una distribución mucho más amplia de lo que se conocía y no está restringido a las montañas.

9. Las especies estudiadas de *Orodrilus* y *Castellodrilus* presentan fuerte estructura poblacional y aislamiento genético.

10. La categoría ecológica parece tener un papel determinante en la diversidad haplotípica de las especies. Así pues, las especies de *Castellodrilus* (epiendogeas), que viven en las capas intermedias del suelo donde las condiciones son más homogéneas, presentan un número de haplotipos por población mucho más bajo que las especies estudiadas de *Orodrilus* (epígeas), que viven en las capas edáficas más superficiales

donde las condiciones son mucho más fluctuantes y donde los cambios ambientales afectan mucho más.

11. Los géneros *Prosellodrilus* y *Cataladrilus* son géneros monofiléticos y, por tanto, géneros válidos que se distribuyen a ambos lados de los Pirineos.

12. *Prosellodrilus pyrenaicus aragonicus* no es una subespecie de *Prosellodrilus pyrenaicus*, y todo parece indicar que debería ser sinónimo de *Prosellodrilus trigoi*, pasándose a llamar *Prosellodrilus aragonicus*.

13. La diversificación del género *Prosellodrilus* parece estar fuertemente influenciada por el proceso de orogenia de la cordillera de los Pirineos.

14**.** Hay un pequeño grupo de especies que aparecen en un clado de posición muy basal como taxón hermano del resto de los lumbrícidos, y que por su antigüedad y caracteres podrían eventualmente representar un nuevo género.

15. En este clado se incluyen molecularmente *Dendrobaena pseudorrosea* y *Dendrobaena osellai*.

16. Por su semejanza morfológica es posible también que *Dendrobaena ruffoi* forme parte de este nuevo género, por lo que sería de la mayor relevancia poder obtener ejemplares de esta especie y estudiarlos molecularmente.

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