UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS BIOLÓGICAS


# TESIS DOCTORAL

Estudio genómico del potencial adaptativo frente al cambio climático en coníferas sensibles a la sequía

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Belén Méndez Cea

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"Las ideas no duran mucho. Hay que hacer algo con ellas."

Ramón y Cajal.

“No dejes que le dé sed al árbol del que eres sol”.

Frida Kahlo.

A mi familia.

A mi tío José.

# Agradecimientos

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# Índice

# Resumen

Las coníferas constituyen uno de los grupos más antiguos de las plantas con semillas siendo el más ampliamente distribuido dentro de las gimnospermas. La familia Pinaceae es considerada la más abundante, ocupando un extenso rango de condiciones ambientales. Esta diversidad hace que este grupo sea idóneo como sistema experimental para desarrollar estudios acerca de la respuesta de los bosques a las variaciones ambientales. En esta tesis se han empleado cuatro especies de esta familia: *Cedrus atlantica*, *Abies pinsapo*, *Abies marocana* y *Pinus uncinata*.

*C. atlantica, A. pinsapo* y *A. marocana* son especie endémicas y relictas, catalogadas en peligro de extinción por la IUCN. Estas tres especies junto con *Pinus uncinata* están asentadas en la cuenca mediterránea, donde se prevé que los efectos del cambio climático sean más acusados. Tanto es así que varias de las poblaciones de estas especies podrían llegar en poco tiempo al límite de su capacidad de adaptación y, actualmente, ya se están observando rápidas respuestas a esas perturbaciones como eventos de decaimiento a escalas amplias, descensos de crecimiento y aumento de la mortalidad.

Entender la medida en la que estas especies forestales presentan capacidad adaptativa frente al cambio climático constituye una necesidad para futuros planes de conservación. Más aún, el enfoque desde un punto de vista genético permitirá tener un conocimiento a nivel molecular de los procesos fundamentales de adaptación y selección en un escenario climático cambiante. Nuestro conocimiento genético de las coníferas es limitado como consecuencia de las características propias de sus genomas, cuyo tamaño suele alcanzar del orden de 18–20Gbp, y con abundantes secuencias repetidas. Esto hace que cualquier estudio que consiga describir estas especies a nivel genómico, constituya un gran reto, pero genere información de considerable interés.

En la presente tesis se han empleado dos técnicas de genotipado basadas en NGS (*Next Generation Sequencing*) que son, *Genotyping By Sequencing* (GBS) y *double digest restriction-site associated DNA* (ddRAD-seq) para la obtención de matrices genéticas basadas en marcadores moleculares, concretamente polimorfismos de un único nucleótido (SNP), con las que desarrollar una amplia variedad de estudios.

Una de las aproximaciones metodológicas fundamentales para comprender los procesos de decaimiento es la comparación entre individuos sanos y decaídos. Este enfoque presenta la dificultad de obtener información genética de árboles muertos, cuyo único vestigio es su madera. Por esta razón, en esta tesis se ha desarrollado un método de extracción de ADN a partir de madera que permita llevar a cabo el genotipado de dichos individuos.

Si bien es cierto que se muestra un elevado flujo entre los núcleos dentro de cada especie, esta diversidad genética encontrada nos permite diferenciar poblaciones. Además, el uso de marcadores moleculares para el estudio de la estructura genética de las poblaciones de cedro del Atlas nos ha permitido identificar el posible origen de la repoblación española de Fiñana (Almería) gracias a su parecido a las muestras de la población del Alto Atlas en Marruecos. Por otro lado, se ha observado una variabilidad genética de las especies relictas mayor de la esperada.

Los estudios de huellas de selección aquí desarrollados mostraron una posible respuesta adaptativa frente a los cambios ambientales en todas las especies. En este sentido, se ha puesto de manifiesto la existencia de diferencias entre árboles viejos (en torno a 200 años) y jóvenes (menos de 50 años) al identificar diferencias significativas en las secuencias sometidas a selección. Estos resultados sugieren efectos genéticos como consecuencia del cambio climático. Así, se ha observado la existencia de una presión selectiva sobre ciertas poblaciones que estaría propiciando un cambio genético en los individuos jóvenes como respuesta a un ambiente más seco y cálido en el abeto marroquí.

La combinación de los dendrofenotipos, obtenidos a partir de las características de los anillos de crecimiento de los árboles, y de otros rasgos funcionales, con los datos genéticos en los estudios de asociación genotipo-fenotipo (GPA), ha permitido identificar asociaciones entre regiones genómicas y datos dendrocronológicos, resaltando la importancia del crecimiento secundario como variable sensible al estrés climático. Además, se han detectado respuestas significativas en otros rasgos

funcionales, tales como el área y la masa específica de las acículas, como respuesta adaptativa al ambiente del pino negro.

Los análisis de asociación de los caracteres genómicos con las variables ambientales (GEA) permiten identificar cuáles de estas pueden tener mayor efecto en la evolución de las especies forestales. Se ha puesto de manifiesto que la temperatura es la variable ambiental con un papel protagonista en cuanto a la supervivencia de todas las especies aquí estudiadas. Esto se ha visto reafirmado con los estudios predictivos basados en estimar la tasa de cambio de las frecuencias génicas necesaria para poder adaptarse a las variables climáticas futuras (RONA), donde se ha identificado la máxima vulnerabilidad con los cambios asociados con la temperatura para todas las especies estudiadas. Por tanto, es este factor el que va a suponer un alto riesgo para la supervivencia de las cuatro especies, siendo el cedro del Atlas y el pino negro los que han manifestado la menor capacidad de adaptación frente a escenarios futuros de calentamiento global.

Con todo ello, los estudios desarrollados en esta tesis suponen un avance en el conocimiento genético de estas especies de coníferas sensibles a la sequía y ponen de manifiesto la gran importancia que tienen las investigaciones multidisciplinares que integran datos genómicos y dendroecológicos, ofreciendo una visión global del potencial adaptativo de los árboles frente al cambio climático. Además, esta tesis abre la puerta al desarrollo de mejores protocolos de conservación de estas y otras especies vegetales relictas y de gran interés ecológico en un escenario tan inestable como el del cambio global.

**Palabras clave**: *Cedrus atlantica*, *Abies pinsapo*, *Abies marocana*, *Pinus uncinata*, cambio climático, polimorfismos de un único nucleótido, estudios de asociación genoma-ambiente, dendrofenotipos, riesgo de no adaptación, madera

# Abstract

Conifers constitute one of the oldest lineages of spermatophytes, being the most widely distributed among the gymnosperms. The Pinaceae family is considered the most abundant, inhabiting a wide range of environmental conditions. This diversity makes this group suitable as an experimental system to carry out studies on the response of forests to environmental variations. In this PhD thesis, four species of this family have been used: *Abies marocana*, *Abies pinsapo*, *Cedrus atlantica* and *Pinus uncinata*.

*A. marocana*, *A. pinsapo* and *C. atlantica* are endemic and relict tree species, listed as endangered by the IUCN. These three species, and marginally *P. uncinata*, are located in the Mediterranean basin, where the effects of climate change are expected to be worse than in other regions. Some of the populations of these species are likely near to their limit of tolerance. Indeed, rapid responses to changing climate are already being observed, such as large-scale growth decline, dieback, and mortality events.

Drought stress has become a crucial constraint of tree growth and vigor worldwide. Understanding the extent to which these forest species have adaptive capacity to cope ongoing climate change provides valuable information for future conservation planning. Furthermore, our genetic approach allows to understand the molecular basis and fundamental processes of adaptation and selection of trees under a changing climate. Nonetheless, our genetic knowledge about the conifers is still limited as a consequence of the characteristics of their genomes, whose size usually reaches 18–20 Gbp, and present abundant repeated sequences. As a result, the advance on the genomic knowledge of these species is challenging but generates useful information.

In this PhD thesis, two genotyping techniques based on NGS (Next Generation Sequencing) have been used, which are Genotyping By Sequencing (GBS) and double digest restriction-site associated DNA (ddRAD-seq) to obtain single nucleotide polymorphisms (SNPs), as genetic markers suitable to perform further ecological studies.

One of the fundamental methodological approaches to investigate dieback processes in forest ecosystems is the comparison of healthy versus declining trees. This approach presents the difficulty of obtaining genetic information from dead trees, which only suitable biological remnant for genetics is their wood. For this reason, in this PhD thesis we developed an extraction procedure to obtain suitable DNA from wood.

Despite our results showed overall weak differentiation and high degree of gene flow among the studied populations of all the species, some differential genetic characteristics were also found, for instance. Additionally, the study of the genetic structure of Atlas cedar provides insights to elucidate the origin of one of the Spanish plantations in Fiñana (Almería), showing the same gene pool of the High Atlas of Morocco. On the other hand, our results point out that relict tree species harbor higher genetic diversity than might be expected.

Selection studies showed a likely adaptive response to environmental changes in all the species. Thus, the existence of differences between old trees (around 200 years old) and young (less than 50 years old) were observed by identifying different sequences subjected to selection in the Moroccan fir. These results support genetic effects because of climate change, which might be acting as a selective pressure on certain populations and may promote genetic shifts in the young cohorts in response to a drier and warmer environment.

The combination of dendro-phenotypes, obtained from tree-rings characteristics, and other morphological traits, with genetic data (SNP) by genotype-phenotype association (GPA) studies allowed to identify genomic regions involved in growth patterns. These genomic and dendrochronological data associations highlighted the importance of secondary growth as a variable sensitive to climatic stress. In addition, significant responses have been observed in other functional traits, such as the leaf mass area, as an adaptive response to the environment in the black pine.

On the other hand, association studies of genomic traits with environmental variables (GEA) allow to identify which of these may have the greatest effect on the

evolution of forest species. In this context, temperature was the main environmental variable limiting the growth and dynamics of the studies tree species. Moreover, this is also supported by the predictions of the risk of non-adaptedness (RONA), which estimate the genetic offset or genomic vulnerability of species via allele frequency change under multiple scenarios of climate change. These models identified the key role of temperature for all the studied species. Specifically, rising temperature depicts a high risk for the survival of Atlas cedar and black pine, both being limited to adapt to future global warming scenarios.

Therefore, the studies carried out in this PhD thesis represent an advance in the genetic knowledge of these drought-sensitive coniferous species and highlight the importance of multidisciplinary investigations, integrating genomic and dendroecological data, to acquire a wider framework of the adaptive potential of trees to climate change. Furthermore, this PhD thesis provides valuable tools to improve conservation measures for endangered tree species, most of them relict and with great ecological value.

**Keywords**: *Cedrus atlantica*, *Abies pinsapo*, *Abies marocana*, *Pinus uncinata*, climate change, single nucleotide polymorphisms, genome-environment association studies, dendropenotypes, risk of non-adaptedness, wood

# Introducción general

## 1. Las coníferas como caso de estudio

La palabra conífera proviene del vocablo latino *conifer* compuesto por *conus,* que se traduce como piña o cono y por la terminación *fer* que proviene del verbo *ferre*, llevar. Así, el significado del término sería "el que lleva piñas". Actualmente el diccionario de la RAE incluye la siguiente definición para este término, "Dicho de un árbol o de un arbusto: del grupo de las gimnospermas de hojas persistentes aciculares o en forma de escamas, fruto en cono y ramas que presentan un contorno cónico."

Desde el punto de vista de la biología, los árboles y arbustos que forman parte del grupo de las coníferas son plantas leñosas y habitualmente muy longevas, cuyas hojas son simples y, mayoritariamente, perennes con una morfología acicular o similar (Rodríguez *et al*., 2022). Carecen de flores presentando en su lugar unas estructuras reproductivas denominadas conos o estróbilos que pueden ser masculinos o femeninos. Atendiendo a si los diferentes conos están o no en el mismo pie (árbol), se clasifica a las especies en monoicas o dioicas (Gernandt y Pérez-de la Rosa, 2014).

Las coníferas son uno de los grupos más antiguos dentro de las plantas con semillas (Williams, 2009). Según indica el registro fósil, se originaron hace unos 300 millones de años (Ma). Actualmente, muchas de las familias que lo conformaron en un inicio se encuentran extintas y las que han llegado hasta nuestros días, se sabe que surgieron en el periodo Jurásico (200–145 Ma) (Farjon, 2018).

Por otro lado, este conjunto de las coníferas constituye el grupo dentro de las gimnospermas más ampliamente distribuido por todo el mundo (Rautiainen *et al*., 2018), siendo en el hemisferio norte donde se encuentran el 70 % de las especies de este grupo (Neale y Wheeler, 2019) (**Figura 1**). Otro dato también relevante es el de su extensión en cuanto a bosques ya que ocupan el 39 % de los existentes en todo el planeta (Armenise *et al.,* 2012). En vista de las evidencias comentadas, parece razonable

señalar la gran importancia que tienen todas las especies pertenecientes al grupo de las coníferas *per se*.


**Figura 1:** Distribución mundial de las coníferas. Todas las zonas coloreadas en verde presentan áreas con bosques de coníferas. El recuadro marca el hemisferio norte del planeta en el que se encuentra el 70 % de todas las especies de coníferas actuales. (Modificado de Farjon, 2008).

La clasificación taxonómica de este grupo ha sido y es un tema muy controvertido que ha suscitado numerosos debates durante décadas. Actualmente, gracias a que los estudios genéticos han ido complementando a los morfológicos, se reconocen seis familias: Pinaceae, Podocarpaceae, Araucariaceae, Sciadopityaceae, Taxaceae y Cupressaceae (Gernandt *et al*., 2011). En lo referente al número total de especies que presentan las coníferas los datos oscilan entre 546 y 670, lo que le hace ser uno de los grupos más numerosos (Farjon, 2008; Gernandt *et al*., 2011; Eckenwalder, 2009; Christenhusz *et al*., 2011). Esta gran amplitud en el rango se explica debido a la dificultad que existe a la hora de diferenciar entre los niveles de especie y de subespecie dentro de los *taxa* (Farjon, 2018).

En cuanto a las características comunes a todas las especies pertenecientes a las coníferas, una de las más reseñables es la gran capacidad de adaptación que tienen para hacer frente a ese amplio abanico de ambientes diferentes en los que viven (Farjon y Filer, 2013). Esto les permite sobrellevar eventos que suponen un reto para su supervivencia como, por ejemplo, las variaciones climáticas que han ido sucediendo

desde su aparición. Esta capacidad ha propiciado que las coníferas sean siempre las primeras en asentarse en zonas que son inhóspitas para la mayoría de las especies de plantas, lo que les ha valido la consideración de especies pioneras. Gracias a esas adaptaciones han podido diversificar y sobrevivir durante todo este tiempo convirtiéndose en el grupo más ampliamente distribuido a lo largo del planeta (Neale y Wheeler, 2019).

Sin embargo, esta gran capacidad de adaptación no les excluye de ser uno de los grupos más amenazados del planeta. Muestra de ello es que la gran mayoría de las especies de coníferas (610) están incluidas en la Lista Roja de Especies (IUCN) y de estas, un 33,6 % englobadas en las categorías de amenazadas **(Tabla 1)** a fecha del año 2022. Esto está poniendo de manifiesto que, pese a las adaptaciones que presentan muchas de estas especies, existen peligros que las están afectando en gran medida haciendo que sus poblaciones se vean mermadas. Algunos de estos peligros están relacionados con la deforestación de los bosques, los incendios forestales y el cambio climático, amenaza de la que hablaremos más adelante.

**Tabla 1:** Resumen de las categorías existentes en la lista roja (IUCN) con el número de especies de coníferas que se encuentran recogidas en cada una de ellas en 2022. En la última columna se puede observar un porcentaje de las coníferas presentes en cada categoría contemplando como un único porcentaje las tres primeras dado que son las que están amenazadas.

| Categoría Lista Roja | Nº de coníferas | Porcentajes |
|---|---|---|
| **En peligro crítico de extinción** | 29 | 33,6 % |
| **En peligro de extinción** | 96 | |
| **Vulnerable** | 80 | |
| **Cercana al peligro** | 99 | 16,2 % |
| **Preocupación menor** | 298 | 48,8 % |
| **Falta de datos** | 8 | 1,3 % |

En cuanto a la importancia económica, el grupo de las coníferas siempre ha gozado de una gran relevancia en este ámbito a nivel global debido a su uso tanto en la industria maderera como en la de producción de papel, resinas y otros principios activos (Díaz-Sala *et al*., 2013). Así como tampoco se puede olvidar que este tipo de árboles se

han empleado en múltiples ocasiones como ornamentación (Rodríguez Valerón *et al*., 2021). Cabe destacar que, dentro de las familias de las coníferas, la Pinaceae es la más notable y la que goza de una mayor importancia económica (Farjon, 2018). Es de esta última familia de la que vamos a seguir hablando en la presente tesis.

## 1.1. La familia Pinaceae

Pinaceae es la familia más abundante de todas las que pertenecen al grupo de las coníferas (Farjon, 1990). Se encuentra asentada en su gran mayoría en el hemisferio norte (Wang y Ran, 2014) y está integrada por 11 géneros con un total aproximado de 230 especies descritas (Neale y Wheeler, 2019). Además, cuenta con tres de los cinco géneros de coníferas más numerosos, los cuales son: *Abies, Picea* y *Pinus* (Simpson, 2019). Si se atiende a sus rasgos morfológicos y reproductivos, esta familia se caracteriza por tener acículas perennes y por ser monoica, es decir, que un mismo pie tiene conos masculinos y femeninos, aunque también pueden ser sub-dioicas (Farjon, 2010).

Debido al amplio abanico de hábitats y, por tanto, de condiciones en las que se asientan las especies pertenecientes a esta familia a lo largo de todo el mundo, constituyen una magnífica oportunidad para tratar de conocer más acerca de la respuesta desde el punto de vista genético de las especies forestales frente a las variaciones ambientales.

Los tres géneros de la familia Pinaceae que se van a emplear en la presente tesis son: *Cedrus, Abies* y *Pinus*.

*Cedrus* es un género que cuenta únicamente con cuatro especies (Xiao *et al*., 2022). Es característico de las montañas del Himalaya (*Cedrus deodara* (Roxb.) G. Don.) en Asia y de las montañas mediterráneas del norte de África y Argelia (*Cedrus atlantica* (Endl.) Manetti ex Carrière), y del este de la cuenca mediterránea, en Chipre (*Cedrus brevifolia* Henry), Turquía, Siria y Líbano (*Cedrus libani* A. Rich.) (Linares *et al.,* 2013). *Abies*, presenta unas 50 especies bien definidas y distribuidas por todo el mundo (Semerikova y Semerikov, 2014). Está ampliamente diversificado en Asia, la cuenca

mediterránea y América del norte. El género *Pinus* cuenta con unas 113 especies descritas lo que le convierte en el más numeroso de los géneros de la familia Pinaceae y además muchas de estas especies son autóctonas del hemisferio norte (Neale y Wheeler, 2019). Se caracteriza por su amplio rango de distribución lo que le ha valido su categorización como el género con mayor diversidad taxonómica y biológica (Farjon, 2010).

Para conocer un poco más acerca de las especies que son objeto de estudio en este trabajo a continuación, se muestra una descripción más detallada de cada una.

### 1.1.1. *Cedrus atlantica*

*Cedrus atlantica* (Endl.) Manetti ex Carrière, comúnmente denominado cedro del Atlas, es una especie forestal con un porte más o menos cónico hacia su copa que puede llegar a alcanzar hasta 50 m de altura. Su tronco es recto con un diámetro máximo de 1,5 m y su corteza es lisa de un color ceniza que con la edad va adquiriendo una textura rugosa y un tono pardo-negruzco (Brunetti *et al*., 2001). Las hojas son aciculares, perennes, de un color verde azulado ligeramente blanquecino, con una longitud que va desde los 0,8 cm hasta los 4 cm y se agrupan radialmente sobre ramas cortas (Gilman y Watson, 1993).

El cedro del Atlas se caracteriza por ser una especie endémica y relicta de las montañas del norte de África, concretamente en Marruecos y en el norte de Argelia (Cheddadi *et al.,* 2009). La mayoría de los cedrales se asientan en el Medio y Alto Atlas donde tienen una extensión de unas 116.000 ha, lo cual representa el 80 % de la población mundial de cedros (Benabid, 1994; Linares *et al*., 2011b). En las montañas del Rif se extienden a lo largo de unas 12.000 ha (Cheddadi *et al*., 2017). En el caso de la zona de Argelia, el área de expansión de esta especie es de unas 50.000 ha (El Bakkali *et al.,* 2018) **(Figura 2)**. Además, el cedro del Atlas fue utilizado para realizar repoblaciones en España y otros países, sobre todo durante la segunda mitad del siglo XX, lo que hace que se puedan encontrar en la actualidad numerosas poblaciones fuera de su área de distribución natural (Camarero *et al*., 2021a).

**Figura 2:** Mapa de distribución y aspecto morfológico de *C. atlantica.* Los puntos verdes indican los núcleos de poblaciones en Marruecos y Argelia. (Foto J. C. Linares).

El rango de altitudes en el que se asienta esta especie está comprendido entre los 1.300 m y 2.600 m s.n.m (sobre el nivel del mar) (M'Hirit, 1994). En cuanto a los requerimientos de precipitaciones, cabe destacar que es uno de los factores más limitantes para la supervivencia del cedro del Atlas teniendo un rango que oscila entre los 500 y los 2.000 mm anuales (Cheddadi *et al.,* 2009). Dichas precipitaciones abundantes son fruto de la influencia del océano Atlántico en la cordillera del Atlas (CEPF, 2017), que hace que este lugar reúna las condiciones adecuadas para que esta especie pueda asentarse en la zona. Finalmente, los requerimientos edáficos que tiene el cedro del Atlas no son muy estrictos pudiendo encontrarse sobre una amplia variedad de tipos de sustratos (M'Hirit, 1999).

Pese a que las precipitaciones, como se ha comentado anteriormente, actúan como un factor limitante para el desarrollo de esta especie, es cierto que el cedro del Atlas es considerado como una especie más tolerante a un amplio rango de condiciones climáticas que otras coníferas (Linares *et al*., 2011b). Esto le permite sobrellevar veranos cálidos y secos, así como soportar temperaturas bajo cero en invierno e incluso, nevadas (Aussenac y Finkelstein, 1983; Aussenac, 1984; Benabid, 1994). Pese a esta tolerancia, desde el año 2013 se encuentra catalogado como especie en peligro de extinción en la

lista roja de especies amenazadas de la *International Union for Conservation of Nature* (IUCN) (Thomas, 2013). Actualmente, una de las principales amenazas que hace que el cedro del Atlas esté incluido en esta lista es el cambio climático (Camarero *et al.,* 2021b). El principal efecto del cambio climático es el incremento de la temperatura lo que lleva a una variación interanual de las precipitaciones. En el caso de la región mediterránea se produce un descenso de estas por lo que habrá una menor disponibilidad de agua para los árboles (Linder *et al*., 2008), lo que provocará un aumento de la evapotranspiración haciendo que estén sometidos a un gran estrés hídrico (Cheddadi *et al*., 2009). Dado que las sequías son cada vez más extremas y duraderas, la vulnerabilidad del cedro del Atlas frente a este estrés va en aumento y, además, se le suma que es una especie relicta, por lo que cualquier perturbación en el ambiente constituye un gran peligro para ella (Camarero *et al*., 2021b). De ahí que todos los estudios que puedan aportar un mayor conocimiento acerca de cómo responde esta especie frente al cambio climático sean de gran relevancia ya que, va a permitir desarrollar mejores actuaciones en pos de su supervivencia.

Sin embargo, no es la única amenaza a la que debe hacer frente. Desde los inicios, el ser humano ha sometido a esta especie a un intenso manejo (Lamb *et al*., 1991). Esto se debe en gran parte al comercio maderero que se ha desarrollado con el cedro del Atlas. Además, los cedrales se han visto afectados a lo largo de los años por un sobrepastoreo y una explotación intensiva lo que ha puesto en peligro la supervivencia de esta especie de gran valor ecológico (Linares *et al*., 2012b). Por esta razón, se ha tratado de buscar la protección de la especie lo que ha llevado a que en el año 2016 la cordillera del Atlas fuera declarada Reserva de la Biosfera por la UNESCO, convirtiéndose en una de las cuatro Reservas que tiene Marruecos actualmente (UNESCO; en.unesco.org/biosphere/arab-states/atlas- cedar).

### 1.1.2. *Abies pinsapo* y *Abies marocana*

*Abies pinsapo* Boiss., conocido popularmente como pinsapo, es un abeto de porte piramidal con tronco recto de hasta 1 m de diámetro que llega a alcanzar los 30

m de alto y con ramas horizontales. Sus hojas son aciculares, persistentes, de color verde oscuro, con una línea azulada en el envés y con disposición helicoidal sentadas sobre las ramas con una longitud de entre 6 y 16 mm (Linares y Carreira, 2006).

El pinsapo se caracteriza por ser un abeto relicto y endémico del que solamente se pueden encontrar tres núcleos en todo el mundo (**Figura 3**). Estos pinsapares están en el sur de España enmarcados dentro de espacios protegidos como son: el Parque Natural de Sierra de las Nieves en Ronda con el pinsapar de mayor extensión con 5.800 ha, el del Paraje Natural Los Reales de Sierra Bermeja en Estepona que cuenta con 70 ha, ambos situados en la provincia de Málaga y el último, el del Parque Natural de Sierra de Grazalema con 2.000 ha, localizado en la provincia de Cádiz (Junta de Andalucía). En España, la distribución total de los pinsapares ocupa una extensión de unas 8.146 ha que resulta de la suma de los tres núcleos descritos junto con otros pequeños bosques. Sin embargo, más de la mitad de los pinsapares que contribuyen a esa extensión se encuentran en muy baja densidad, es decir, no forman masas tupidas de bosque, sino que están aislados (Junta de Andalucía, 2011).

*Abies marocana* Trab., denominado comúnmente como abeto marroquí, es una especie que siempre ha estado muy relacionada con el pinsapo. Actualmente, forma dos núcleos en el norte de Marruecos (**Figura 3**) que se encuentran dentro del Parque Nacional de Talassemtane con una extensión de 2.000 ha (montaña del Rif, Jebel Lakraa) y se extiende hasta el límite del rango de la montaña situado en Jebel Tazaot donde cuenta con unas 1.000 ha (Ben-Said, 2022; Ben-Said *et al*., 2022).


**Figura 3**: Distribución y aspecto morfológico de las especies *A. pinsapo* y *A. marocana*. (Fotos B. Méndez-Cea y J. C. Linares).

Los requerimientos de humedad que tienen tanto el pinsapo como el abeto marroquí para poder vivir hacen imprescindible que habiten zonas donde las precipitaciones sean abundantes, normalmente superiores a los 1.000 mm anuales. Sabiendo esto, parece razonable que resulte extraño el lugar donde se asientan estas especies, ya que el clima mediterráneo del sur de España y del norte de Marruecos está caracterizado por tener una marcada estacionalidad con un periodo estival bastante seco y cálido que se corresponde con el verano (Lionello *et al*., 2006). Sin embargo, para asombro de muchos, la Sierra de Grazalema es el punto de España donde se registran las mayores precipitaciones, las cuales oscilan entre los 2.000–3.000 mm anuales (Fernández-Cancio *et al.,* 2007). Con el objeto de evitar la pérdida de humedad en los meses secos, debido a que el pinsapo es altamente sensible a la sequía (Linares *et al.,* 2011a), los pinsapares suelen asentarse en las laderas más umbrías de componente norte a una altitud comprendida entre los 1.000 y 1.800 m s. n. m (Fernández-Cancio *et al*., 2007). El abeto marroquí se encuentra asentado en un rango de alturas que va desde los 1.500 a los 2.000 m s. n. m (Aafi, 2000). Esta situación le permite mantener unas condiciones óptimas de humedad durante todo el año. En cuanto a los requerimientos

edáficos, el pinsapo no es nada específico en este aspecto por lo que se puede encontrar sobre una amplia variedad de sustratos (Linares y Carreira, 2006).

En el pasado, la distribución del pinsapo sufrió una abrupta disminución debido a la sobreexplotación del terreno y a los incendios forestales (Navarro-Cerrillo *et al*., 2021) lo cual es alarmante al tratarse de una especie relicta con un alto valor ecológico. De ahí que todas las medidas que se puedan tomar para su protección y conservación sean de vital importancia. Actualmente, el pinsapo se encuentra incluido en diferentes medidas legislativas tanto a nivel nacional como internacional. En el ámbito nacional, concretamente a nivel autonómico el pinsapo está catalogado como especie en peligro de extinción en el Catálogo Andaluz de Especies de la Flora Silvestre Amenazada fruto del Decreto 104/1994, de 10 de mayo (BOJA nº 107). Por otro lado, los pinsapares se encuentran protegidos por la Ley 2/1989, de 18 de julio (BOE-A-1989-20636), por la que se aprueba el Inventario de Espacios Naturales Protegidos de Andalucía y se crean las figuras de los Parques Naturales. A nivel estatal, el pinsapo y su hábitat están protegidos por la Ley 42/2007 de 13 de diciembre que es referente al Patrimonio Natural y de la Biodiversidad (BOE-A-2007-21490).

En el ámbito internacional, los pinsapares están incluidos en la Directiva 92/43/CEE (DOUE-L-1992-81200) que es relativa a la conservación de los hábitats naturales y de la fauna y flora silvestres (Junta de Andalucía, 2011). Además, los pinsapares de Sierra de las Nieves y de la Sierra de Grazalema fueron declarados Reservas de la Biosfera por la UNESCO en 1995 y en 1977, respectivamente.

Finalmente, la UNESCO en el año 2006 declaró Reserva de la Biosfera Intercontinental del Mediterráneo Andalucía (España)-Marruecos a los Parques Naturales en los que se encontraban los pinsapares (Sierra de Grazalema, Sierra de las Nieves y al Paraje Natural de Los Reales de Sierra Bermeja), y al Parque Natural de Talassemtane en Marruecos, lugar en el que se encuentra el abeto marroquí.

Hoy en día, la preocupación mayor en cuanto a la supervivencia del pinsapo y del abeto marroquí radica en los efectos del cambio climático ya que se ha convertido en la

principal amenaza a la que deben enfrentarse (López-Quintanilla *et al*., 2013; Moukrim *et al*., 2022). Por ello, tanto el pinsapo como el abeto marroquí están catalogadas como especies en peligro de extinción por la lista roja de especies amenazadas de la IUCN (Alaoui, 2011; Arista, 2011).

### 1.1.3. *Pinus uncinata*

*Pinus uncinata* Ram. ex de Candolle comúnmente denominado pino negro, es un pino de forma cónica con un tronco recto de entre 0,5 hasta 1 m de diámetro, de corteza grisácea o marrón oscura y que llega a alcanzar los 25 m de alto (Zaborowska *et al*., 2021). Sus acículas se disponen dos a dos sobre las ramas y se caracterizan por ser persistentes, de un color verde oscuro, rígidas y gruesas con una longitud comprendida entre 23 y 75 mm. Son árboles monoicos y el nombre de "uncinata" hace referencia a la forma de gancho que tienen las brácteas de los conos (The Gymnosperme Database).

El pino negro aparece de forma natural en las montañas del Este de Europa, concretamente en Andorra, Austria, Alemania, España, Francia, Italia y Suiza (**Figura 4**). En el caso de España, la mayor extensión de esta especie la encontramos en el Pirineo donde se agrupa el 99 % de la totalidad de individuos de pino negro que habitan en este país. El límite sudoccidental mundial de la distribución de esta especie se encuentra en las poblaciones relictas situadas en el Sistema Ibérico: Castillo de Vinuesa en Soria y Peñarroya en Teruel (Camarero, 2009). Por otro lado, dado que fue utilizado para realizar repoblaciones, también se pueden encontrar ciertas poblaciones en el norte de Europa y en la zona del Mediterráneo (Christensen, 1987; EUFORGEN).

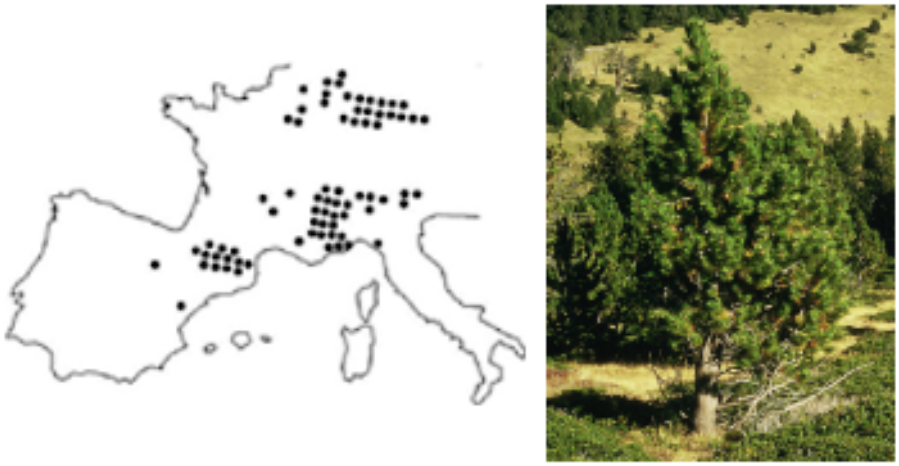

**Figura 4:** Distribución natural y aspecto de *Pinus uncinata*. (Mapa tomado de Camarero y Gutiérrez, 2008 y foto J. J. Camarero).

*Pinus uncinata* forma bosques de montaña en altitudes que van desde los 1.000 m hasta los 2.500 m s. n. m (Carreras *et al*., 1996). Las características de las zonas de alta montaña en las que habita esta especie hacen que sea tolerante al frío, a las nevadas y a los vientos fuertes. Cabe destacar que la temperatura es uno de los factores más limitantes para el crecimiento del pino negro, necesitando una temperatura media anual de 5 °C. En cuanto a los requerimientos de precipitaciones, suele estar asentado en lugares donde la precipitación anual se encuentra entre los 1.000 y 2.000 mm (Camarero, 2009). Si se atiende a las características del suelo que son necesarias para la supervivencia de esta especie, se puede observar que el pino negro no es específico de ningún tipo de sustrato, dándose en cualquier suelo (Camarero, 2009).

Esta especie ha sufrido alteraciones en su hábitat fruto del manejo que ha hecho el ser humano convirtiendo muchos de estos pinares en zonas de pastos de montaña. En cuanto al valor económico del pino negro se considera una especie poco productiva dado que su madera no es de muy buena calidad (Camarero, 2009). Pese a ello, el pino negro sí que goza de un importante valor ya que sus bosques al estar situados en lugares de elevada pendiente, permiten proteger frente a la erosión y controlar los aludes o avalanchas en estas zonas.

Al contrario que las otras tres especies estudiadas en este trabajo, actualmente, *Pinus uncinata* se encuentra en consideración menor según la Lista Roja de Especies Amenazadas de la IUCN (Farjon, 2017). Sin embargo, comparte con las demás especies la zona en la que se sitúan de forma natural, ya que en todos los casos se asientan en la zona del Mediterráneo bien la totalidad de las poblaciones o bien ciertos núcleos relictos.

## 2. La amenaza del cambio climático

La región mediterránea es considerada uno de los tres puntos calientes, *hotspots*, con mayor riqueza vegetal y una de las áreas con un mayor número de plantas endémicas (Myers *et al.,* 2000; Blondel y Aronson, 2010) (**Figura 5**). Su extensión es de 2.085.292 $km^2$ (CEPF, 2017). Muchos de los endemismos que encontramos principalmente en las penínsulas del Mediterráneo son el resultado de que dichas zonas actuaron como refugios glaciares amortiguando el clima extremo que, durante la mitad del Terciario, alteró las condiciones ambientales del planeta. Estas regiones se convirtieron en zonas de protección frente a dicho clima para muchas especies vegetales que encontraron en la cuenca mediterránea su hogar pese a no ser propias de la zona, como fue el caso del pinsapo y del cedro del Atlas (CEPF, 2017).

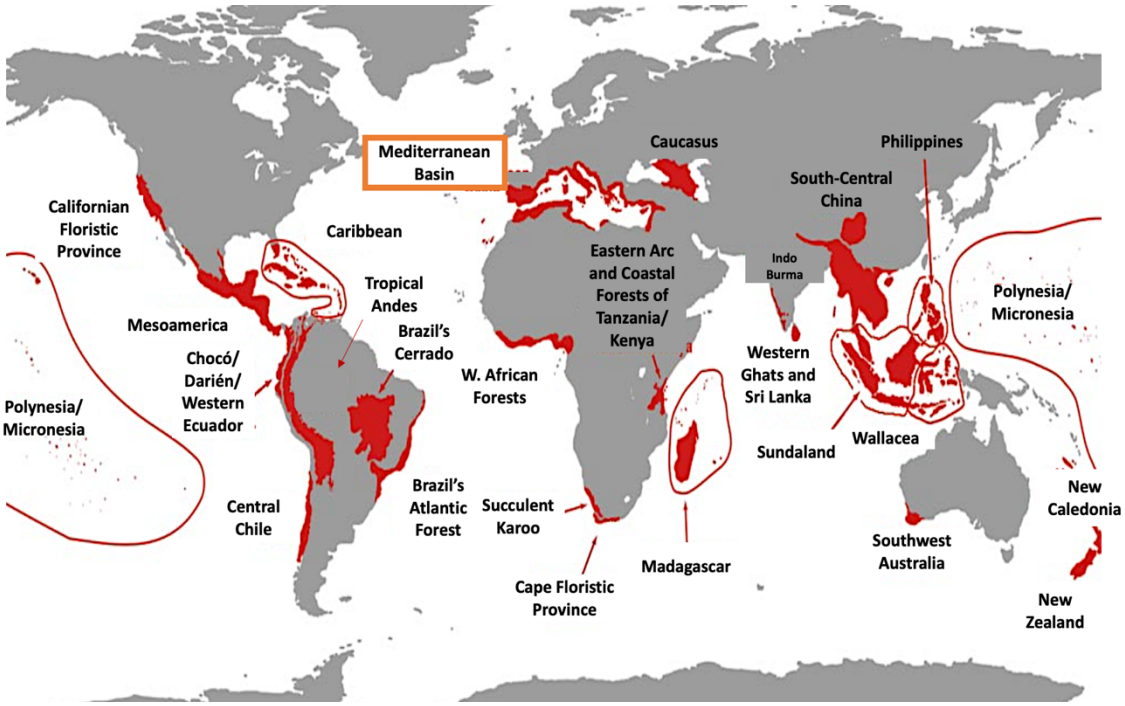


**Figura 5:** Distribución de los 25 *hotspots* de biodiversidad. El recuadro naranja destaca el punto caliente de la región mediterránea que es de interés para el presente estudio. (Modificado de Myers *et al*., 2000).

Hoy en día, toda esta biodiversidad de la cuenca mediterránea no está a salvo ya que tiene que hacer frente a múltiples amenazas (Peñuelas *et al*., 2017). La mayoría de ellas son consecuencia directa de ser una de las regiones del planeta con mayor densidad de población humana que, además, se ve incrementada por un turismo masivo e incontrolado (CEPF, 2017). Esto produce efectos en la vegetación y fauna debido a la alteración del sistema natural, a la agricultura intensiva, a la introducción de especies invasoras y, como no podría ser de otra forma, al cambio climático.

El clima de la cuenca mediterránea se caracteriza por tener inviernos fríos y húmedos, seguidos de veranos secos y calurosos. Las precipitaciones son irregulares y muy variables entre años (Lionello *et al*., 2006). Todo ello ha favorecido que esta región siempre se haya visto sometida a dos amenazas concretas: los fuegos y las intensas sequías, ambas directamente relacionadas con la fuerte estacionalidad de su clima (Blondel y Aronson, 2010). Dichos peligros han propiciado que los ecosistemas mediterráneos muestren una gran resistencia y resiliencia, lo que les ha brindado la posibilidad de hacer frente a las perturbaciones (Peñuelas *et al.,* 2017). Tanto es así, que incluso el fuego se ha llegado a considerar como una fuerza de cambio evolutiva para

muchas especies de esta cuenca (CEPF, 2017). Sin embargo, como consecuencia del cambio climático, actualmente la recurrencia de los incendios forestales supera la resiliencia de la vegetación para recuperar los patrones de productividad entre un evento y el siguiente (Trabaud y Prodon, 2002; Williams, 2013), produciendo un bloqueo en las etapas normales de regeneración de la vegetación tras un fuego.

Por todo ello, el cambio climático es un problema que está adquiriendo un estatus altamente preocupante a nivel mundial y también local, llegando a ser considerado en ciertos casos una amenaza real para los seres vivos como ocurre en la cuenca mediterránea. Se pueden encontrar muchas definiciones de cambio climático como por ejemplo la del Ministerio para la Transición Ecológica y el Reto Demográfico que dice: "se llama cambio climático a la variación global del clima en la Tierra" (MITECO). Pero ¿qué efectos tiene realmente el cambio climático que estamos sufriendo en la actualidad?

El principal efecto del cambio climático es un incremento en 1,5 °C de la temperatura media anual del planeta (IPCC, 2018). Como consecuencia de este incremento de temperatura se produce un aumento de la variación interanual de las precipitaciones lo que, en ciertos casos, disminuye la disponibilidad del agua que hay en el suelo y produce un aumento de la evapotranspiración de las plantas (García-Ruiz *et al.,* 2011) intensificando el estrés hídrico al que se ven sometidas éstas, lo que desemboca en un decaimiento poblacional al producirse la muerte de individuos. Además, se prevé que los eventos climáticos sean cada vez más severos y recurrentes lo que incluye precipitaciones extremas que desembocarán en inundaciones que producirán una mayor erosión del suelo y un lavado de nutrientes de este, perjudicando a las plantas que lo habitan (Allen *et al*., 2010; Anderegg *et al*., 2015; McDowell *et al*., 2020). Por todo esto, el cambio climático tiene un alto impacto en los hábitats y en las especies que viven en ellos (Peñuelas *et al*., 2013) y se ha convertido en una amenaza real para todo el planeta siendo más acusada en algunos puntos como, por ejemplo, en la cuenca mediterránea (Peñuelas *et al*., 2017). Es en esta zona donde se ha visto que los efectos del cambio climático son mucho más pronunciados que en el resto del

continente europeo debido a sus características climáticas previamente comentadas (CEPF, 2017).

Actualmente en la cuenca mediterránea ya se está observando ese incremento de la temperatura acompañado de la disminución de las precipitaciones, lo que está acrecentando la incidencia de los incendios forestales y produciendo una mayor desertificación (IPCC, 2018). Todo ello irá provocando, paulatinamente, la pérdida de la rica biodiversidad de la zona (Peñuelas *et al*., 2017; CEPF, 2017) (**Figura 6**).


**Figura 6:** En este mapa se muestra la deforestación que se ha producido en la primera década de los años 2000 en la cuenca del Mediterráneo. Las zonas más rojas son los lugares en los que se ha producido una mayor pérdida de bosques. Sacado de *Resilience Atlas* (https://www.resilienceatlas.org).

Para hacer frente a esta amenaza, los organismos se valen de diferentes estrategias como la migración hacia lugares donde las condiciones sean más adecuadas o, la adaptación frente a este novedoso y complejo escenario cambiante. Como las especies forestales al igual que el resto de las especies vegetales son organismos sésiles, suelen valerse del empleo de adaptaciones morfológicas y/o fisiológicas que les permitan reducir el impacto negativo que las condiciones extremas les provocan (Hampe y Jump, 2011). Además, se sabe que la variación de los rangos geográficos que ocupan las especies forestales no se produce lo suficientemente rápido como para permitir la subsistencia de estas especies (IPCC, 2018). Principalmente, esto es debido a que la tasa de cambio producida por el cambio climático puede ser más rápida que la

propia de las especies forestales. Los principales efectos del cambio climático han propiciado que, actualmente, ya se puedan observar los primeros decaimientos y muertes de individuos de diferentes especies forestales a nivel mundial (Allen *et al*., 2010; Reichstein *et al.,* 2013*;* Williams, 2013; Anderegg *et al*., 2015; McDowell *et al*., 2020).

Esto es solo el inicio de lo que está por llegar ya que se prevé que el número de muertes se incremente a lo largo del tiempo. Si a todo esto se le suma que la cuenca del Mediterráneo es una de las más susceptibles al cambio climático, vemos que es necesario hacer esfuerzos para poder incrementar el conocimiento de las especies forestales de dicha zona, más aún si esas especies son relictas y endémicas de la zona. De ahí que el ser capaces de entender cómo responden estas especies frente al cambio climático desde un abordaje poco empleado en este tipo de especies forestales como es el de la genética sea de gran interés.

## 3. Genética de las coníferas

Hoy en día se sabe que el tamaño del genoma nuclear de las coníferas está comprendido entre las 6,5 y 37 Gpb con una media de 15 Gpb (Ahuja y Neale, 2005), siendo el de las Pinaceae de unas 20 Gpb, aproximadamente. Teniendo como contexto que el genoma de cualquier angiosperma oscila entre 500 Mb y 1.000 Mb (Neale y Wheeler, 2019), se puede entender de forma clara la gran dimensión del genoma de las coníferas.

Este gran tamaño del genoma de las coníferas y la presencia de muchas secuencias repetidas en él hacen que cualquier trabajo con un enfoque de genética molecular cuyo objeto de estudio sean dichas especies forestales, se convierta en un proceso arduo en todos los sentidos y constituya un verdadero reto (Ojeda *et al.,* 2019). Es por esto por lo que no se tienen demasiados conocimientos genéticos de las especies de este grupo. Sin embargo, gracias a la aparición a partir del año 2010, de nuevas técnicas de secuenciación basadas en *next generation sequencing* (NGS), se ha abierto

un nuevo abanico de posibilidades hacia el conocimiento genético de este grupo. Estas nuevas metodologías permiten obtener resultados de interés con organismos no modelo así como, brindan la posibilidad de trabajar con genomas de tamaños tan grandes como los que tienen las coníferas. Además, hay que añadir que estas técnicas han mejorado en cuanto a la asequibilidad de sus precios se refiere, facilitando el inicio de una nueva era en el estudio de la genética de especies forestales.

La técnica *Whole Genome Sequencing* (WGS) se ha usado para estudios de secuenciación del genoma completo. Una de las primeras especies de coníferas que se secuenció fue *Picea abies* (L.) Karst (Nystedt *et al.,* 2013) con un tamaño de genoma de 20 Gbp. Posteriormente, se han ido añadiendo más coníferas a la lista de especies secuenciadas como son: *Picea glauca* (Moench) Voss. (Birol *et al.,* 2013), *Pinus taeda* L. (Zimin *et al.,* 2014), *Pinus lambertiana* Douglas (Stevens *et al.,* 2016), *Pseudotsuga menziesii* (Mirb.) Franco (Neale *et al.,* 2017) y *Abies alba* Mill. (Mosca *et al.,* 2019). Pese a ello, como puede observarse, esta lista sigue sin ser muy numerosa. Además, la mayoría de estos genomas obtenidos no son de buena calidad debido a la poca profundidad de lectura empleada para su realización y el elevado número de *scaffolds* de pequeño tamaño resultantes, lo que dificulta en mayor medida el trabajo con ellos (García-García *et al*., 2022).

La ausencia de un genoma completo secuenciado (genoma de referencia) y de una correcta anotación obstaculizan habitualmente el progreso de los estudios genéticos a nivel molecular e incluso, en algunos casos los impiden por completo. Sin embargo, esto no es del todo cierto. Actualmente existen varias técnicas basadas en NGS que permiten desarrollar estudios genéticos sin necesidad de contar con un genoma de referencia. Algunas de las más relevantes y empleadas en plantas son *Genotyping By Sequencing* (GBS) (Elshire *et al*., 2011) y *double digest restriction-site associated* DNA (ddRAD-seq) (Peterson *et al*., 2012), ambas herramientas se basan en la fragmentación del genoma mediante la utilización de enzimas de restricción (ER) y en la posterior secuenciación parcial de los genomas de los individuos estudiados.

El uso de las ER es barato, rápido, específico y altamente reproducible. Además, permite en muchas ocasiones acceder a zonas del genoma que son difíciles de detectar cuando se usan otros tipos de aproximaciones (Elshire *et al.,* 2011). Pero lo más relevante del empleo de las ER es que permite reducir la complejidad del genoma de estudio lo que facilita el trabajo con genomas muy grandes. Además, como no se necesita tener un conocimiento previo del genoma que se va a estudiar, abre la puerta a poder trabajar con especies no modelo las cuales pueden carecer de genoma de referencia. Esto hace que el GBS y el ddRAD-seq sean unas técnicas muy utilizadas cuando se manejan genomas como los de las coníferas cuya principal característica es su enorme tamaño.

Por otro lado, este tipo de técnicas permiten la obtención de una elevada cantidad de marcadores moleculares, tales como polimorfismos de un único nucleótido (SNP) o microsatélites (SSR). Estos marcadores constituyen la base de una amplia variedad de estudios como son los relacionados con la genética estructural, filogenética, detección de huellas de selección, estudios de asociación genoma ambiente (GEA) o estudios de asociación genotipo fenotipo (GPA). Además, dichas técnicas dan la oportunidad de estudiar una gran cantidad de individuos de una sola vez.

### 3.1. *Genotyping by sequencing* (GBS)

Dado que el GBS se basa en la fragmentación del genoma mediante el uso de ER, uno de los pasos más importantes a realizar cuando se trabaja con esta técnica, es la selección de la ER más adecuada tanto para el tipo de genoma de interés así como para el objetivo del estudio (Elshire *et al*., 2011). Por otra parte, se puede realizar una digestión con una única ER, así como llevar a cabo una doble digestión. Existe una amplia variedad de ER que pueden ser empleadas para el GBS. En este caso, se van a destacar dos de las ER más comunes utilizadas con genomas de plantas, las cuales son *Ape*KI y *Pst*I.

*Ape*KI es una ER de tipo II cuyo sitio de corte es 5’ G CWGC 3’ y es sensible a la metilación lo que hace que evite cortar en las zonas con una gran cantidad de

repeticiones que suelen estar altamente metiladas (Davey *et al*., 2011). Esta capacidad es muy útil al trabajar con coníferas, que, como ya se ha comentado anteriormente, presentan gran densidad de secuencias repetidas en su genoma. De ahí que al usar esta enzima se obtenga un mayor número de fragmentos pero que, al mismo tiempo, éstos sean de pequeño tamaño. En un estudio previo realizado con coníferas como *Picea glauca* o *Picea engelmanii* han empleado esta enzima (Gamal *et al*., 2015). En cuanto a *Pst*I también es una ER de tipo II cuyo sitio de corte es 5' CTGCA G 3' y no es sensible a la metilación. Esto hace que cuando se usa dicha ER se obtengan fragmentos más grandes pero una menor cantidad de los mismos.

El flujo de trabajo seguido en el GBS comienza con la fragmentación del ADN extraído previamente con la ER seleccionada seguida de la secuenciación y finalmente, el análisis bioinformático de los resultados obtenidos (**Figura 7**).

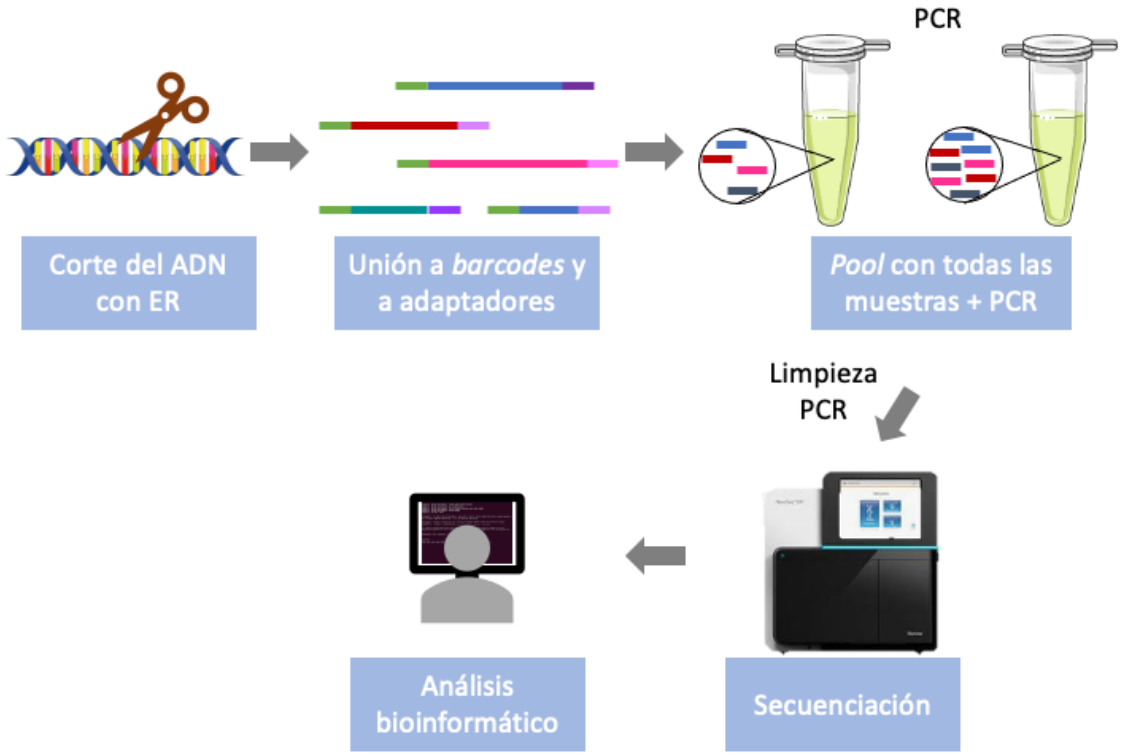


**Figura 7:** Esquema del flujo de trabajo que se ha de seguir para la realización de la técnica GBS. Comienza con la fragmentación del ADN mediante el uso de ER, posteriormente se unen los *barcodes* identificativos y los adaptadores. Seguidamente se juntan todas las muestras y se hace una PCR. Finalmente se secuencia y se analizan los datos obtenidos mediante herramientas bioinformáticas.

El GBS ya se ha probado en el estudio de algunas coníferas como, por ejemplo: *Pinus contorta* Dougl. ex Loud. (Parchman *et al.,* 2012), *Picea glauca* (Moench) Voss (Parchman *et al.,* 2012; Chen *et al.,* 2013), *Pinus tabuliformis* Carr., *Pinus densata* Mast. y *Pinus yunnanensis* Franch. (Pan *et al*., 2015) y *Picea abies* (L.) H. Karst (Vallebueno-Estrada *et al*., 2022). En algunos de estos trabajos se ha visto que la eficiencia de la técnica se reduce cuanto mayor es el tamaño del genoma.

### *3.2. Double digest restriction-site associated DNA* (ddRAD-seq)

Esta técnica surgió a raíz de una modificación realizada al RAD-seq (Baird *et al*., 2008) con el objetivo de mejorar la eficiencia de la secuenciación. En cuanto al fundamento del ddRAD-seq cabe decir que es muy similar al del GBS descrito anteriormente. Sin embargo, existen ciertas diferencias entre ambos (**Figura 8**). La primera de ellas radica en que el ddRAD-seq utiliza siempre dos ER simultáneas para la digestión, generalmente una enzima de corte frecuente y otra que lo haga en menor medida. Al usar las dos, los fragmentos que se generan tienen que haber sido cortados por ambas enzimas. La segunda diferencia hace referencia a que, tras la unión de los adaptadores a los fragmentos digeridos, se realiza una selección por tamaños de forma precisa y reproducible. Este nuevo paso permite tener un mayor control sobre la longitud que van a tener los fragmentos que conformarán la librería (Peterson *et al*., 2012).

Existe una amplia variedad de combinaciones de ER que dan diferentes resultados y cuya eficacia dependerá de la especie con la que se esté trabajando. Por lo general, se suelen emplear las mismas enzimas que en el GBS.

En cuanto a los estudios realizados con esta técnica, se puede ver que también se ha empleado para trabajar con ciertas especies de la familia Pinaceae como por ejemplo *Keteleeria davidiana* var. *formosana* Hayata (Shih *et al*., 2018) y *Abies sachalinensis* (F. Schmidt) Mast (Goto *et al*., 2021) y también en especies de la familia Cupressaceae como *Platycladus orientalis* (L.) Franco (Jin *et al*., 2019) y *Cryptomeria japonica* var. *sinensis* (Thunb. ex L.f.) D. Don (Cai *et al*., 2020).

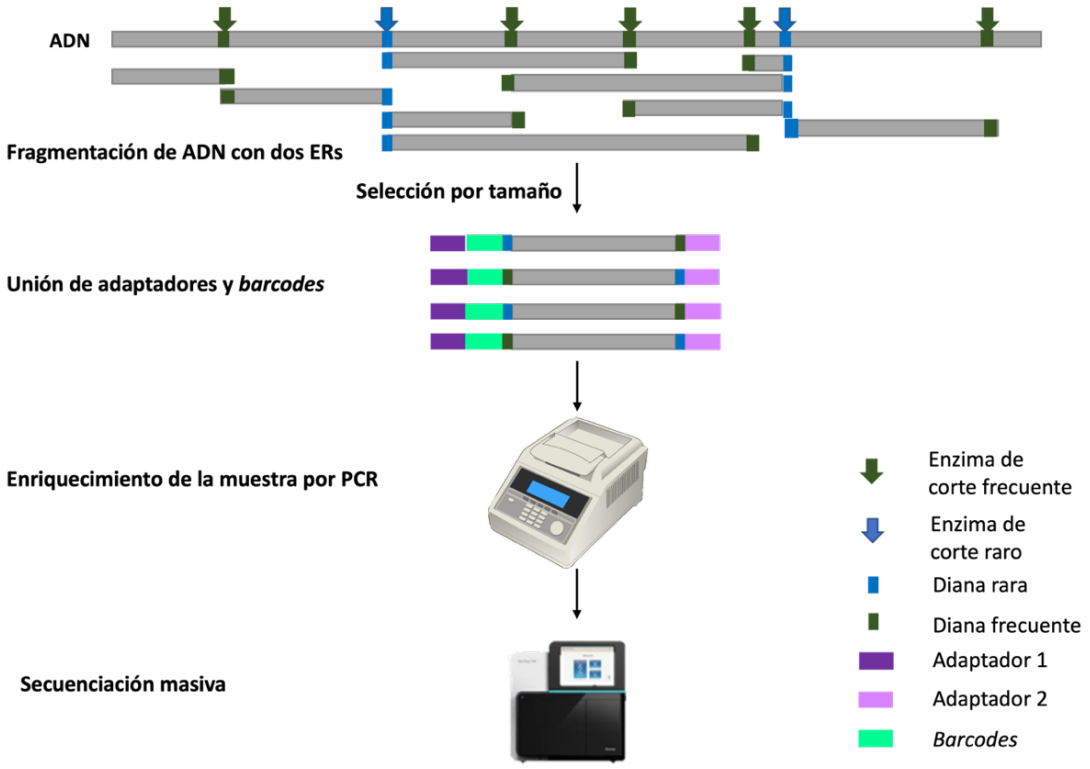


**Figura 8:** Se representa de forma resumida el flujo de trabajo que se sigue con la técnica ddRAD-seq. El primer paso es la fragmentación del ADN mediante el empleo de dos ER. Seguidamente se da una selección por tamaño y se añaden los adaptadores y los códigos que permiten llevar la trazabilidad de cada una de las muestras. Finalmente, se realiza una PCR y se procede a la secuenciación.

## 4. Estudios genéticos con el cedro del Atlas, el pinsapo, el abeto marroquí y el pino negro

Todo lo que se ha ido comentando acerca de los escasos conocimientos genéticos que se tienen de las coníferas puede dar una idea acertada sobre el bajo número de estudios realizados en dicho ámbito con las especies objeto de estudio de esta tesis. Además, hay una peculiaridad más con las especies con las que se ha trabajado y es que varias de ellas tienen la condición de relictas (cedro del Atlas, pinsapo y abeto marroquí), por lo que su rango de distribución es muy reducido lo que hace que sean poco atractivas para su estudio. Sin embargo, los estudios genéticos son muy importantes para este tipo de especies porque permiten conocer de forma más fiable la estructura de sus poblaciones, la variabilidad genética intra e inter poblacional, así como

nos dan la oportunidad de tratar de determinar la interacción genoma-ambiente (GEA) lo cual nos permite conocer la variación de los genotipos en función de las características ambientales a las que están expuestos. Actualmente debido a todo lo que está aconteciendo como consecuencia del cambio climático, ser capaces de conocer desde el punto de vista genético cómo responden las especies forestales frente a él se ha convertido en un reto de gran relevancia dado que este conocimiento nos permitirá desarrollar mejores estrategias de conservación de las especies.

Por otra parte, ser capaz de encontrar nuevos materiales biológicos de partida de los que se pueda extraer ADN funcional que permitiera realizar estudios posteriores, es de gran interés. Cuando un árbol muere, el vestigio que se encuentra disponible durante más tiempo es su madera. Por tanto, tener la capacidad de obtener ADN a partir de ese recurso abriría muchas opciones de estudios al permitir tener información genética de individuos muertos. Este proceso no está exento de complejidad al tratar con un ADN altamente degradado y fragmentado. Por esta razón, la extracción de ADN a partir de la madera no es un tema que esté ampliamente estudiado en la bibliografía científica. Pese a ello, sí que encontramos varios trabajos relacionados con la genética en los que las maderas han sido objeto de estudio con distintos fines como, por ejemplo: conocer más a fondo el ADN antiguo (Liepelt *et al.,* 2006; Parducci *et al.,* 2018), identificar la especie a la que pertenecen varias muestras (Jiao *et al.,* 2015) o bien, controlar el comercio de la industria maderera (Asif *et al.,* 2005; Fatima *et al.,* 2018) a fin de evitar fraudes y el comercio ilícito de especies protegidas.

En este apartado se muestra un breve resumen acerca de los diferentes estudios genéticos que se han realizado a lo largo de los años con las especies que son el objeto del presente trabajo (**Tabla 2**). Como se verá a continuación, no hay prácticamente estudios que hayan abordado el tema del cambio climático desde el ámbito de la genética, por lo que el desarrollo de esta tesis permitirá incrementar el conocimiento en este campo.

### 4.1. El cedro del Atlas

Los estudios que se han realizado con el cedro del Atlas en el ámbito de la genética son escasos. Los más tempranos se centraron en la citogenética de la especie donde la determinación del número cromosómico o la morfología de los cromosomas era lo empleado para ese objetivo. Así, se determinó que el número cromosómico es 2n=24 (ej.; Khoshoo, 1961). También se tiene constancia de algún trabajo citogenético en el que se lleva a cabo un estudio comparativo de los cariotipos de las distintas especies del género *Cedrus* (Dagher-Kharrat *et al.,* 2001).

Otro tipo de estudios genéticos son los centrados en el ámbito molecular. Para esto casos, se suelen usar marcadores moleculares como *Amplified Fragment Length Polymorphism* (AFLP) o *Simple Sequence Repeats* (SSR) y son varios los objetivos perseguidos como: realizar estudios de filogeografía (Terrab *et al.,* 2008); determinar la diversidad genética de los grupos estudiados (Terrab *et al*., 2006); construir filogenias (Terrab *et al.,* 2006; Karam *et al.,* 2019) o desarrollar estrategias de conservación (Bobo-Pinilla *et al.,* 2022). La mayoría de estos trabajos han mostrado que el cedro del Atlas mantiene una alta diversidad genética y que las mayores diferencias se suelen dar entre las poblaciones, así como que los núcleos de Marruecos y Argelia se pueden diferenciar a nivel genético.

Por otro lado, se ha estudiado el genoma mitocondrial del cedro del Atlas llegando a establecer comparaciones entre este y el de otras especies del género (Forgione *et al.,* 2019). También se han llevado a cabo trabajos relacionados con el ámbito de la transcriptómica lo que ha permitido la obtención de transcriptomas de *Cedrus atlantica*. El primero de ellos se realizó a partir de acículas y raíces (Karam *et al*., 2015). Posteriormente, Cobo-Simón (2020a) obtuvo un nuevo transcriptoma a partir de un RNA-seq realizado con muestras de acículas procedentes de un experimento de estrés hídrico.

En cambio, no hay apenas estudios con el cedro del Atlas en los que se empleen técnicas de NGS y actualmente, se carece de un genoma de referencia. Sin embargo, en

el trabajo de Karam *et al*. (2015) se utilizó la técnica de genotipado basada en NGS denominada RAD-seq, con la enzima de restricción *Pst*I, para describir nuevos marcadores moleculares que fueron validados en ciertos individuos.

### 4.2. El pinsapo y el abeto marroquí

Los marcadores moleculares más utilizados en los trabajos con estas especies han sido los microsatélites (SSR), tanto los nucleares (nSSR) como los pertenecientes a las mitocondrias (mtSSR) o cloroplastos (cpSSR). Estos trabajos suelen estar enfocados a estudiar su diversidad (ej.: Vendramin y Ziegenhagen, 1997; Terrab *et al.,* 2007), a desarrollar nuevos marcadores moleculares con el objeto de emplearlos en futuros trabajos de conservación (Sánchez-Robles *et al.,* 2012) o bien a describir la genética de poblaciones (ej.: Terrab *et al*., 2007; Jaramillo-Correa *et al*., 2010; Sánchez-Robles *et al*., 2014; Litkowiec *et al*., 2021). Estos trabajos han puesto de manifiesto una gran diversidad genética en las poblaciones estudiadas, así como han determinado que *A. pinsapo* y *A. marocana* son dos especies diferentes.

Se han realizado otros estudios basados en el uso de SSR e intermicrosatélites (ISSR) para determinar la variabilidad genética en gradientes altitudinales (Cobo-Simón *et al.,* 2020b). Además, se ha llevado a cabo un abordaje que ha permitido incrementar el conocimiento genético del pinsapo gracias al ensamblaje *de novo* de un transcriptoma que permitió, también, describir nuevos marcadores moleculares, SSR y SNP (Pérez-González *et al.,* 2018). Este transcriptoma no es el único que existe dado que, muy recientemente, se ha obtenido otro con el objeto de encontrar la base de las variaciones intraespecíficas que se observan en el fenotipo de las acículas de la población de pinsapo situada en la Sierra de las Nieves (Ortigosa *et al*., 2022). Este conocimiento permitirá poder desarrollar mejores métodos de conservación al conocer las bases moleculares de esas diferencias y sus implicaciones fisiológicas.

Partiendo de los genes que se identificaron en el transcriptoma descrito a partir del experimento de sequía se pudo llevar a cabo la búsqueda SNP en genes relacionados

con la respuesta a la sequía para poder valorar la supervivencia de la especie a dicho estrés (Cobo-Simón *et al*., 2021).

### 4.3. El pino negro

Son escasos los estudios genéticos que existen con esta especie como protagonista. Pese a ello, existen ciertos trabajos basados en SNP y SSR en los que se han realizado estudios de poblaciones con el objeto de determinar la diversidad genética de esta especie o de determinar la estructura genética de ciertas poblaciones (Dzialuk *et al*., 2009; Heuertz *et al*., 2010; Zaborowska *et al*., 2021). Por lo general, estos trabajos han mostrado que las poblaciones de *Pinus uncinata* de los Pirineos tienen un alto nivel de diversidad genética y que las poblaciones marginales muestran una fuerte diferenciación genética del resto. La comparación de las poblaciones de *P. uncinata* con las de otras especies del mismo género y de una región diferente han mostrado mayores diferencias entre las regiones geográficas que entre especies. Sin embargo, sí que se ha obtenido una estructura en cuanto a su filogeografía. Por otra parte, el trabajo desarrollado por Zaborowska *et al*. (2021) no solo se centró en el estudio de estructura de poblaciones sino que lo combinó con la identificación de regiones genómicas que pudieran estar involucradas en la respuesta adaptativa de esta especie a las grandes alturas mediante el uso de un array de SNP.

A la vista de los pocos estudios que existen, parece que es importante tratar de conocer más a esta especie desde el ámbito de la genética. Por lo tanto, cualquier dato nuevo en este campo será altamente relevante.

**Tabla 2:** Se muestra un resumen de algunos de los estudios con base genética que se han realizado en las especies con las que se ha trabajado en esta tesis. Se puede observar el nombre de la especie, la técnica molecular empleada, el objetivo del trabajo y finalmente, la referencia del artículo.

| Especie | Técnica molecular | Objetivo del trabajo | Referencia |
|---|---|---|---|
| ***Cedrus atlantica*** | Marcadores moleculares (SSR) | Estudios de filogeografía | Terrab *et al*., 2008 |
| | Marcadores moleculares (SSR) | Diversidad genética | Terrab *et al*., 2006 |
| | Marcadores moleculares (SSR) | Construir filogenias | Karam *et al*., 2019 |
| | Marcadores moleculares (AFLP) | Desarrollar estrategias de conservación | Bobo-Pinilla *et al*., 2022 |
| | RNA-seq | Transcriptoma *de novo* | Cobo-Simón, 2020a |
| ***Abies pinsapo*** | Marcadores moleculares (SSR) | Diferencias entre las especies del género *Abies* | Vendramin y Ziegenhagen, 1997 |
| | NGS | Descripción de marcadores moleculares (SSR) para *A. pinsapo* | Sánchez-Robles *et al*., 2012 |
| | RNA-seq | Ensamblaje *de novo* de un transcriptoma y descripción de nuevos marcadores moleculares | Pérez-González *et al*., 2018 |
| | RNA-seq | Buscar variaciones intraespecíficas | Ortigosa *et al*., 2022 |
| | Marcadores moleculares (nSSR, cpSSR e ISSR) | Determinar la variabilidad genética en gradientes altitudinales | Cobo-Simón *et al*., 2020b |
| | Marcadores moleculares (SNP) | Supervivencia frente a la sequía | Cobo-Simón *et al.,* 2021 |

| Especie | Técnica molecular | Objetivo del trabajo | Referencia |
|---|---|---|---|
| ***Abies pinsapo* y *Abies marocana*** | Marcadores moleculares (cpSSR) | Diversidad genética y estructura de poblaciones | Terrab *et al*., 2007 |
| | Marcadores moleculares (mtSSR) | Estudios de filogeografía | Jaramillo-Correa *et al*., 2010 |
| | Marcadores moleculares (AFLP y cpSSR) | Filogeografía del suroeste del Mediterráneo | Sánchez-Robles *et al*., 2014 |
| | Marcadores moleculares (nSSR) | Biogeografía y diversidad de poblaciones del género *Abies* | Litkowiec *et al*., 2021 |
| ***Pinus uncinata*** | Marcadores moleculares (cpSSR) | Diversidad genética de especies del género *Pinus* | Heuertz *et al*., 2010 |
| | Marcadores moleculares (SNP) | Estructura genética de las poblaciones y diversidad de varias especies | Zaborowska *et al*., 2021 |
| | Marcadores moleculares (cpSSR) | Determinar la estructura genética de poblaciones del Pirineo de pino negro | Dzialuk *et al*., 2009 |

## 5. El estudio interdisciplinar: un punto de encuentro entre la genética y la ecología

Como se ha indicado en el anterior apartado, los estudios relacionados con la genética no son muy abundantes en las especies de coníferas y menos aún, en las cuatro que nos ocupan en esta tesis ya que la condición de relictas de tres de ellas las hace menos accesibles. Cualquier tipo de estudio en este ámbito es de gran interés y permitirá determinar tanto la estructura genética como la variabilidad existente en las poblaciones (Peterson *et al*., 2014) facilitando la preservación de las diferentes especies. Además, estos datos permiten realizar búsquedas de SNP sometidos a selección (Peláez *et al*., 2020) permitiendo identificar una posible actuación de la presión selectiva en la respuesta adaptativa de estas especies frente a las variaciones ambientales ocasionadas por el cambio climático.

La búsqueda del enfoque multidisciplinar aporta un abordaje novedoso permitiendo obtener una forma más completa de comprender esta respuesta. De ahí, que en la presente tesis se hayan combinado los datos genómicos, concretamente SNP, con los ecológicos (Heer *et al.*, 2018), como son los datos ambientales poblacionales o los dendrofenotipos, mediante la realización de los estudios de asociación genoma ambiente (GEA) o los estudios de asociación genotipo fenotipo (GPA). Por otra parte, dado que lo interesante es determinar cómo se comportan estas especies en su propio medio natural (Estravis-Barcala *et al*., 2020), en esta tesis todos y cada uno de los estudios realizados se han llevado a cabo partiendo de datos obtenidos para los individuos en su medio natural.

El estrés por sequía se ha convertido en una limitación crucial para el crecimiento y el vigor de los árboles en todo el mundo (Anderegg *et al*., 2015; McDowell *et al*., 2020). Las condiciones más cálidas y secas exponen a los árboles a mayores tasas de respiración, elevada demanda de evaporación (déficit de presión de vapor en la atmósfera) y baja disponibilidad de agua en el suelo, lo que provoca una disminución en el crecimiento (Sánchez-Salguero *et al*., 2017). Además, el aumento de la variabilidad

climática puede hacer que eventos extremos como sequías y olas de calor sean más frecuentes y severos (Allen *et al*., 2010). De ahí, que tener la capacidad de predecir la vulnerabilidad de las especies frente a las perturbaciones ambientales sea un recurso de gran valor. Por eso, en la presente tesis se ha llevado a cabo una aproximación para describir dicha vulnerabilidad en las poblaciones estudiadas frente a las variaciones futuras que se producirán, por ejemplo, en la temperatura y las precipitaciones, mediante el uso de los estudios de riesgo de no adaptación (RONA, *risk of non-adaptedness*). Estas predicciones serán de gran utilidad para el desarrollo de métodos de conservación de las especies, pero aún estamos lejos de ser capaces de pronosticar al detalle la dinámica futura de los bosques en un escenario de cambio climático (**Figura 9**).

Por tanto, el desarrollo de los diferentes estudios indicados en este apartado va a permitir conocer más ampliamente cómo responden frente al cambio climático las cuatro especies de coníferas que son objeto de la presente tesis y abrirá la puerta a un mayor conocimiento genético de las especies forestales.

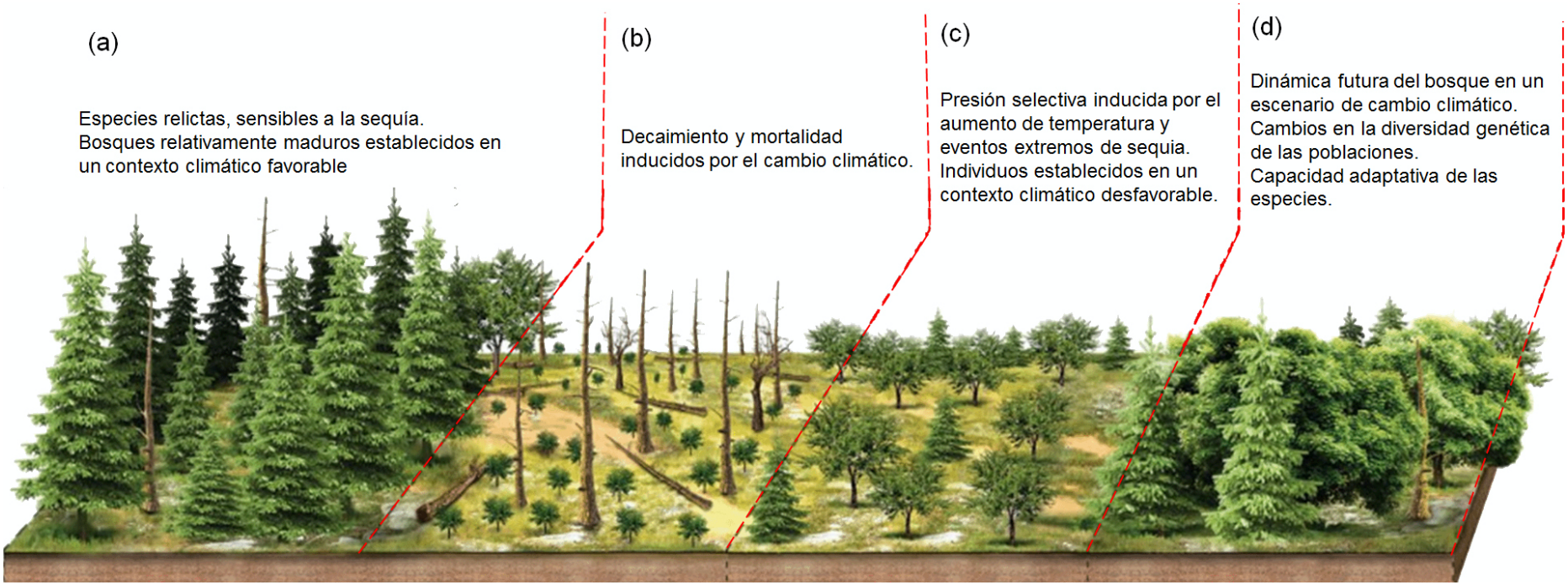


**Figura 9:** Diagrama conceptual de la dinámica reciente de bosques relictos sensibles a la sequía que han mostrado procesos de mortalidad y decaimiento. En el panel de la izquierda (a) se representa un bosque relativamente maduro, establecido en un contexto climático previo al calentamiento global. De estas poblaciones tenemos evidencias de sus patrones de crecimiento mediante datos dendrocronológicos y de su diversidad genética. En el siguiente panel (b) se representa una población con síntomas de decaimiento y mortalidad inducida principalmente por estrés climático, aunque pueden contribuir factores como plagas y patógenos, suelos poco desarrollados, etc. En poblaciones con estas características se han optimizado métodos de extracción de ADN a partir de la madera para poder analizar los genotipos de los individuos decaídos o muertos. Estos eventos de mortalidad suponen una presión selectiva para el establecimiento posterior de nuevas cohortes (c). Los individuos jóvenes se establecen en un contexto climático desfavorable, comparado con el de los adultos (a), que puede poner de manifiesto procesos de selección. En el último panel (d) se representa un bosque con nuevas especies que han reemplazado a parte de la especie sensible a la sequía, la cual mostrará capacidad adaptativa plasticidad fenotípica, cambios genéticos o epigenéticos y cuya vulnerabilidad puede cuantificarse mediante modelos de riesgo de no adaptación (RONA). Modificado a partir de McDowell *et al*., 2020.

# Objetivos

## OBJETIVO GENERAL

Para poder entender cómo responden ciertas especies de coníferas a las variaciones ambientales que se están produciendo como consecuencia del cambio climático es necesario un abordaje multidisciplinar en el que se incluya la genética. También se requiere el desarrollo de herramientas adecuadas que permitan realizar el análisis genómico de los datos generados. De esta forma, se podrán establecer mejores estrategias que permitan una adecuada conservación de las especies en cuestión.

La presente Tesis Doctoral tiene como objetivo principal incrementar el conocimiento genético de cuatro especies forestales, sensibles a las variaciones ambientales, que pertenecen a la familia Pinaceae: el cedro del Atlas, el pinsapo, el abeto marroquí y el pino negro. Para poder llevarlo a cabo se emplearon técnicas basadas en NGS para obtener nuevos marcadores moleculares, concretamente SNP, los cuales serán de gran relevancia tanto para este estudio como para los futuros. El interés de genotipar individuos muertos, aquellos que no han sido capaces de adaptarse frente a esas variaciones, hace que se haya puesto a punto un protocolo de extracción de ADN a partir de madera ya que esto permitiría el genotipado incluso cuando no se dispone de otro material de muestreo.

## OBJETIVOS ESPECÍFICOS

- Desarrollar un protocolo de extracción de ADN a partir de muestras de madera obtenidas de árboles muertos en dos especies endémicas y relictas (cedro del Atlas y pinsapo) y su potencial uso como herramienta para el estudio genético de individuos muertos en bosques con decaimiento.

- Desarrollar un *pipeline* adecuado para el análisis de los datos obtenidos con las técnicas GBS y ddRAD-seq para las cuatro especies estudiadas.

- Caracterizar la estructura genética, basada en SNP, de las poblaciones de cedro del Atlas, del pinsapo, del abeto marroquí y del pino negro.

- Identificar huellas de selección en el genoma de las especies estudiadas.

- Determinar la existencia de asociaciones entre variables genéticas y variables ambientales a nivel poblacional. Así como, describir las relaciones existentes entre los dendrofenotipos y las variables genéticas de dos de las especies estudiadas: abeto marroquí y pino negro.

- Pronosticar la vulnerabilidad de las especies estudiadas frente al cambio climático mediante el cálculo del riesgo de no adaptación (RONA).

# Bloque I. Extracción de ADN a partir de madera de árboles muertos

## Artículo: DNA extraction and amplification from Pinaceae dry wood

**Abstract:** Wood constitutes the unique source of DNA in dead trees, but extraction of adequate quality DNA from dry wood is usually challenging. However, many different molecular studies require the use of such DNA. We have standardized and validated a modified CTAB protocol to isolate DNA from dry wood from *Abies pinsapo* and *Cedrus atlantica* species. Due to the degradation and very little DNA that is normally present in the wood from dead trees we have developed a PCR based test to certify the quality of the extracted samples. In the present study, we have proved too the effectiveness of this methodology to isolate DNA from conifer dry wood samples of sufficient quality to perform further molecular genetic experiments.

# DNA extraction and amplification from Pinaceae dry wood

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## Abstract

Wood constitutes the unique source of DNA in dead trees, but extraction of adequate quality DNA from dry wood is usually challenging. However, many different molecular studies require the use of such DNA. We have standardized and validated a modified CTAB protocol to isolate DNA from dry wood from *Abies pinsapo* and *Cedrus atlantica* species. Due to the degradation and very little DNA that is normally present in the wood from dead trees we have developed a PCR based test to certify the quality of the extracted samples. In the present study, we have proved too the effectiveness of this methodology to isolate DNA from conifer dry wood samples of sufficient quality to perform further molecular genetic experiments.

Keywords: : *dry wood, DNA isolation, CTAB, 18S, Abies pinsapo, Cedrus atlantica.*

## Introduction

DNA isolation from wood of dead trees has not been studied extensively so far. Several studies about wood DNA isolation have been published with different purposes: increasing the knowledge of ancient DNA, development of forensics applications, identification of samples, control of timber, etc. As consequence, DNA has been isolated from different sources: fresh wood (White et al., 2000; Jiao et al., 2012; Fatima et al., 2018), fossil plants (Liepelt et al., 2006; Sønstebø et al., 2010; Parducci et al., 2018) or dry and processed wood (Asif & Cannon, 2005; Jiao et al., 2012, 2015; Almeida de Souza et al., 2017).

In this work, two species belonging to the Family Pinaceae have been used as models to test the effectiveness of a modified CTAB method (based on Kistler 2012) for dry wood DNA extraction in conifers: *Abies pinsapo* Boiss (Spanish fir) and *Cedrus atlantica* (Endl.) Manetti ex Carriere (Atlas cedar). Fresh wood from *Pinus sylvestris* L. (Scots pine) was used too as a positive control of the DNA extraction protocol.

*A. pinsapo* is a relict Mediterranean fir, endemic from southern Spain and northern Morocco, very drought sensitive (Linares et al., 2011a). Nowadays, it is classified as an endangered species (IUCN, 2018). On the other hand, the Atlas cedar is also an endemic conifer tree, located in northern Morocco and Algeria, over almost 130,000 Ha (Cheddadi et al., 2009). Several studies have also stated that Atlas cedar is highly vulnerable to drought (Cheddadi et al., 2009; Linares et al., 2011b). In these species, we are interested in comparing the genomes of surviving trees with those of dead trees regarding climate change.

In this work, 71 wood samples were used to perform DNA extraction. A total of 26 dry wood samples of *A. pinsapo*, which were obtained from Sierra de las Nieves Natural Park (Málaga, Spain), and 29 dry wood samples of *C. atlantica* from the High Atlas and Ifrane National Parks (both located in North Morocco). Besides, 19 samples of fresh wood of *P. sylvestris* from Picos de Urbión Natural Park (Soria, Spain) were used to compare the

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differences between the isolation of DNA in dry and fresh woods.

Firstly, we tried several plant DNA isolation kits with unsuccessful results in our dry wood samples. Then, we tested several manual methods, and finally selected a CTAB method to extract DNA from seeds (Kistler, 2012) to be proved in our material. After different adaptation experiments, we ascertained that a pre-treatment of wood samples was needed. First, it was necessary to remove the bark and sand from the surface with sandpaper. Then, wood shavings were obtained by means of the use of a scalpel, which was sterilized with ethanol 70 %. After that, wood was macerated by using a sterile pestle and liquid nitrogen until turning the samples into powder, which was placed into 2 mL tubes. Next, four to six tungsten balls were added and a fine powder was obtained by the use of Tissue Lyser II of Qiagen® for 4-6 minutes. This complete pre-treatment protocol is required to achieve a successful extraction from dry wood samples.

Subsequently, 100 mg of wood powder were used to carry out the DNA extraction with the method described by Kistler (2012). Regardless of the species, in the wood of dead trees there are only traces of degraded DNA. In our hands, such vestigial DNA could not be observed in 1 % agarose gels. In addition, as expected, analysis of dry wood DNA with Nanodrop™ showed spectrophotometric curves very far from the observed in the case of good-quality DNA (Figure 1). Then, any valuation of DNA via Nanodrop™ would give us invalid results. Consequently, we developed a method to determine the quality of the isolated DNA, which was based on a PCR amplification of an 18S ribosomal gene fragment. Briefly, we assumed that a positive PCR amplification of this 18S DNA fragment means that such sample is useful for any further genetic studies.

of DNA extracted from wood were added. Finally, the PCR programme used has the following steps: 3 minutes at 94°C; 35 cycles of: 1 minute at 94°C, 1 minute at 58°C and 01:20 minutes at 72°C; and 8 minutes at 72°C.

At that point, we tested this PCR protocol in our samples (Figure 2). As expected, a 100 % success rate in fresh wood from Scots pine was obtained (19/19). In the case of the dry wood samples, the results were variable. On one hand, for Atlas cedar 21 samples out of 29 were positively amplified (72.4 %). On the other hand, 14 out of 26 *Abies pinsapo* samples were positives too (46.2 %). Moreover, some of the samples that showed negative results in this first experiment were extracted again with positive results in the following amplification of the 18S fragment.

In addition, as proof of the usefulness of these isolated DNAs, a mtDNA SSR marker (*nad5-4*, Liepelt et al., 2002; Cinget et al., 2015) was positively genotyped in the isolated samples that showed a previous positive amplification of the 18S fragment. This PCR of the SSR was carried out in the same conditions that we described above, except the annealing temperature that was 55°C instead of 58°C.

Due to low quantity and huge degradation of DNA templates, the difficulties in amplifying DNA from wood is expected to be high and especially, when wood from dead trees is used. For this reason, designing short size amplicons are the best way to get successful amplifications in this kind of samples from wood. In addition, ome of the samples that were not isolated in the first experiment could be successfully extracted in subsequent experiments. This fact shows that in most cases is possible to obtain useful DNA traces from wood samples (fresh or dry).

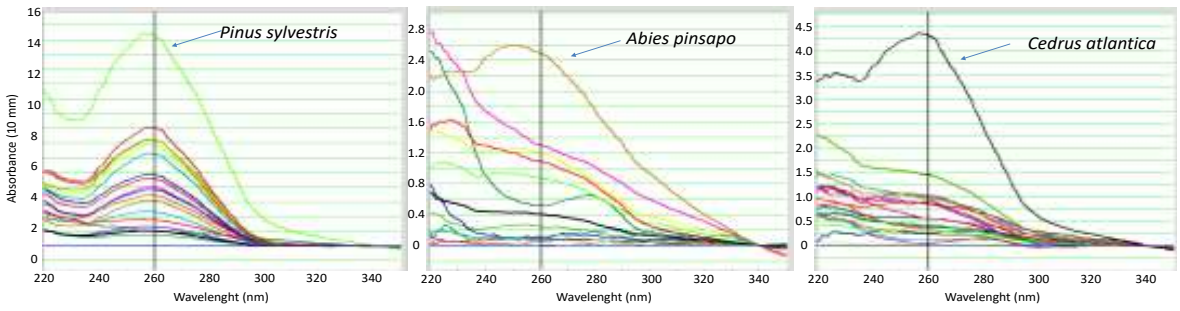


Figure 1
Spectrophotometric curves obtained via Nanodrop™ of the DNA isolated from the different samples of the three species. Arrows indicate the spectrophotometric curve of a DNA extracted from leaves of each species. Clear differences can be appreciated between control DNA (leaves) and the wood samples. Also, differences between dry wood (*A. pinsapo* and *C. atlantica*) and fresh wood (*P. sylvestris*) are evident

PCR amplification of this 18S 75-bp fragment was carried out in a final volume of 15 µl of the reaction mix, which was composed of: 7.5 µl of Master Mix of *DNAmplitools* (Biotools), 7.5 pmoles of each 18S universal primer (5´-CGCGAGAAGTCCACTAAACC-3' and 5'-CCTACGGAAACCTTGTTACGA-3´; Osakabe et al., 2013), 3 µl of sterile deionised water and 1.5 µl

*In summary, DNA from three forest species of Pinaceae Family has been isolated using a modified CTAB protocol. Moreover, the obtained DNA was functional, as it has been shown by PCR amplifications (18S fragment and mtDNA SSR). Thus, this extraction procedure of conifer wood samples isolates DNA traces to perform further molecular genetic research, even when trees are dead. For*

*this reason, our results would contribute, among others, to open unexplored paths in the research of the molecular basis of conifers response to changes in environmental conditions, such as recent climate change.*

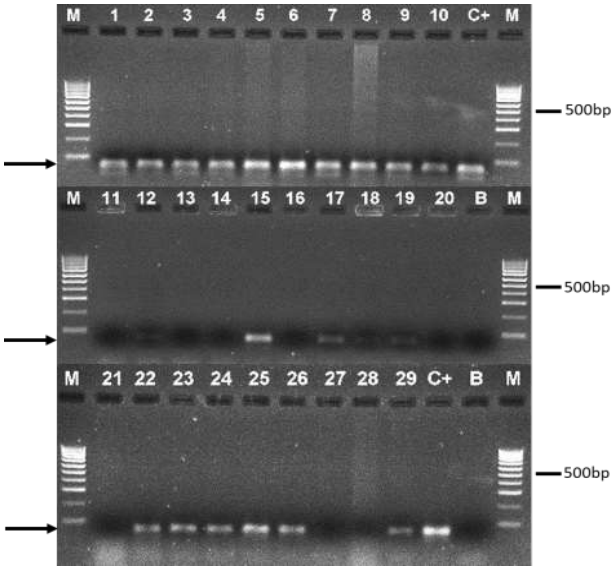


Figure 2
PCR of an 18S ribosomal gene fragment of 75 bp. Amplification products were analysed by electrophoresis in 2.5 % agarose gels. Lanes 1-10: DNA from fresh wood of *P. sylvestris*; Lanes 11-20: DNA from dry wood of *A. pinsapo*; Lanes 21-29: DNA from dry wood of *C. atlantica*; C+: DNA from leaves of each species; B: Blank; M: 100 bp ladder (Biotools). Arrows indicate the 18S PCR fragment.

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# Bloque II. Estudio genómico de poblaciones de cedro del Atlas y evaluación de su vulnerabilidad frente al cambio climático

## Artículo: Rising temperature, instead of precipitation, amends the expected vulnerability of Atlas cedar by enhanced risk of genetic non-adaptedness. (En preparación)

**Abstract:** The Atlas cedar *Cedrus atlantica* is a relict and endemic conifer from Morocco and Algeria, while plantations may be found in several locations aside its natural range. Recurrent droughts have been widely related to Atlas cedar dieback, growth decline, and mortality, while the genetic basis of potential adaptive capacity is still unknown. We used the double digest RAD-seq technique (ddRAD-seq) to investigate the genetic structure and variability of Atlas cedar in Morocco. Further, we investigate the genetic origin of some Spanish plantations. The obtained single nucleotide polymorphisms (SNPs) were used to perform genotype-environment associations (GEA) to define SNPs related to temperature and precipitations. Furthermore, the vulnerability of this species to environmental variations was estimated by the risk of non-adaptedness (RONA). The *de novo* assembly provides 2,336 SNP from 199 Atlas cedar trees. PCA results showed a divergence between Moroccan natural stands and Spanish plantations. High and Middle Atlas are related, while Western Rif depicts genetic differentiation. Moreover, one of the Spanish populations, Fiñana (Almería), formed a group with the Moroccan population of the High Atlas, suggesting a common genetic pool. Despite we found a total of 5 *loci* under selection, 1 *locus* in old trees, and 4 *loci* in saplings, respectively, no homolog proteins were detected in the databases. Annual mean temperature showed the highest number of associated *loci*, (5). Further, temperature seasonality, minimum temperature of the coldest month, and mean temperature of the driest quarter showed associations with 3 *loci*. RONA was higher in the High Atlas, where rising temperature was the main driver of expected genetic offset by allele frequency changes under the worse emissions scenario. By opposite, niche models reported that the most important bioclimatic variables driving the Atlas cedar distribution range are related to precipitation. This disagreement shows that the interpretation of the vulnerability of the species is not straightforward. Warming could amend our expectations based on niche suitability and future range distribution of endangered species.

**Keywords:** rear edge, *Cedrus atlantica*, drought sensitivity, tree age, selection signatures, genotype-environment associations, High Atlas, Middle Atlas, Rif, Talassemtane

## Introduction

*Cedrus atlantica* (Endl.) Carrière is a relict and endemic conifer species which is located in Atlas and Rif Mountain Ranges (Morocco and Algeria) (Cheddadi *et al*., 2009) showing its largest extension in Morocco (El Bakkali *et al*., 2018) (**Figure 1**). Rif Mountain Range is one of the Moroccan sites which hosts more endemic species and for this reason, it is considered a biodiversity hotspot (Myers & Cowling, 1999). Although, the biological relevance of Rif Mountains, this area is one of the most threatened by the human activity (Camarero *et al.,* 2021a). On the other hand, this species was used for plantation in Spain (Camarero *et al.,* 2021b) (**Figure 1**). Nowadays, this species is classified as endangered by the IUCN Red List of Threatened Species (Thomas, 2013) and it must cope with a new hazard which is the climate change.

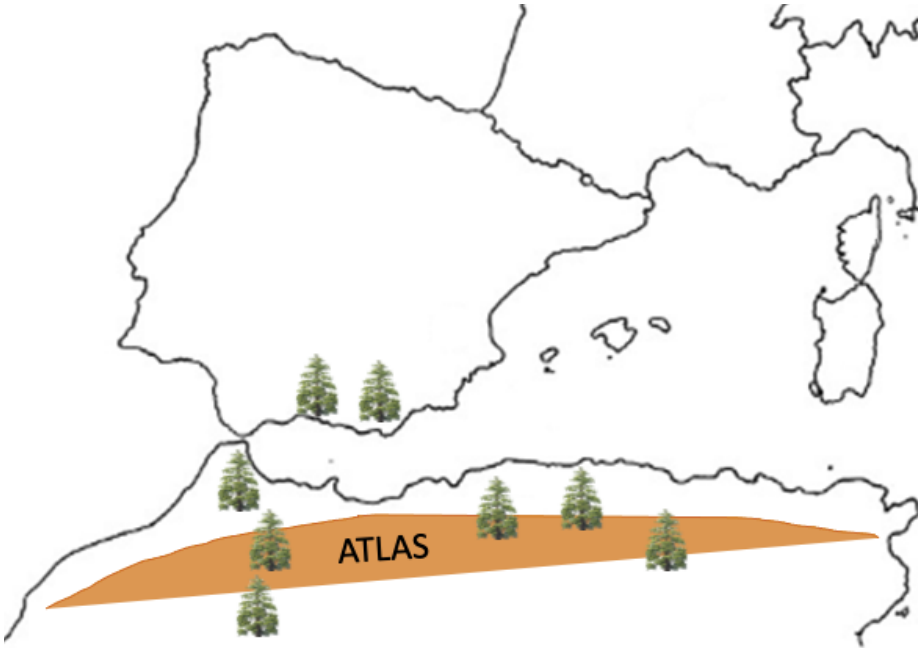


**Figure 1:** Distribution map of *Cedrus atlantica*. It is represented the natural nuclei of Morocco and Argelia (Atlas Mountains) and the two Spanish plantations.

In recent times, the heat waves, and recurrent drought events are more frequent in many Mediterranean areas, mainly in North Africa (Lelieveld *et al*., 2016). Increasing mortality rates of several tree species are alarming in the context of the pervasive environmental changes and habitats loss. The broad geographic distribution of forest dieback suggests a worldwide driver, such global warming, which is consistent with physiological theory and model results (McDowell *et al*., 2022). However, the genetic mechanisms underlying contrasting vulnerability among individual trees are poorly understood (Alberto *et al*., 2013). Several studies have revealed a link between these events of dryness, climate warming, decreased growth, and increased mortality in Atlas cedar (Linares *et al.,* 2011; Linares *et al.,* 2013; Navarro-Cerrillo *et al*., 2019; Camarero *et al.,* 2021a). One of the most affected areas by the environmental alterations is High Atlas, which is the driest place of Atlas cedar distribution. In any case, dieback episodes related to dryness are observed, as it was mentioned above, in other areas too such as Middle Atlas (Linares *et al.,* 2011). *C. atlantica* is an anisohydric species which means that it maintains high transpiration rates despite being under drought stress. For this reason, it shows higher vulnerability to dryness than other Mediterranean species which are isohydric trees.

Due to the characteristics of conifer genomes, which have large sizes, abundant repetitive sequences, and the absence of reference genome (García-García *et al.,* 2022), studies of *C. atlantica* related to genetics are not very common in the bibliography. The vast majority of the studies used molecular markers, microsatellites (SSRs), to describe genetic structure of populations, to estimate the genetic variability of several populations or to develop phylogeographic studies (Terrab *et al*., 2006; Karam *et al*., 2019). Recently, in our laboratory a drought stress experiment in this species was performed. Then, an RNA-seq experiment was carried out to obtain the first *de novo* transcriptome of Atlas cedar (Cobo-Simón, 2020). Despite this, studies based on NGS technologies in Atlas cedar are scarce. The development of new technologies based on NGS, such as the reduced representation sequencing, allows developing genetic studies without prior genetic knowledge. Moreover, the combination of genetics and environmental data give us the opportunity to have a better understanding of the response of forest species to rapid climate change and of future range dynamics of

them. This is so interesting because trees as sessile long-lived organisms need to cope rapidly with environmental perturbations. It is necessary to assess if their genetic change rate is fast enough to cope with climate change. Besides, this knowledge would allow in the future the development of efficient conservation strategies.

The present work has used the genotyping by sequencing technique (GBS) (Elshire *et al*., 2011) to study the genetic structure and variability of natural populations of Atlas cedar. As well as, the obtained SNPs have been used to investigate the origin of the plantations in Spain. On the other hand, the genetic matrix has been used to carry out genotype-environment associations study (GEA) to describe SNPs related to temperature and precipitations variables at population level. Lastly, the vulnerability of this species to environmental variations has been estimated using the risk of non-adaptedness (RONA) methodology (Pina-Martins *et al.,* 2019).

## Material and methods

### Sample collection

A total of six populations were studied. Three of them are natural populations located in the North of Morocco (Rif, Middle Atlas, and High Atlas). The other three are from southern Spain (Almería, Granada, and Málaga) where Atlas cedar was used to plantation in the second half of the last century (Camarero *et al*., 2021b). To our knowledge there are no records about the origin of the trees employed for these plantations.

### DNA extraction and GBS

246 fresh leaf samples were collected from Moroccan and Spanish populations of *C. atlantica*. 100 mg of this tissue were lyophilized and then, DNeasy Plant Mini Kit (Qiagen®, Germany) was used to extract the total DNA following the manufacturer's instructions with some modifications. To assess the concentration and the integrity of

the extracted DNA a spectrophotometer method, NanoDrop™, and an electrophoresis in 1 % agarose gel were employed, respectively. Afterwards, 213 samples were found to have the necessary quality to proceed with GBS (Elshire *et al*., 2011). This technique was performed at CRG-CNAG (Spain). GBS is a genotyping technique based on NGS which allows to work with non-model species because no prior genetic knowledge is needed. Restriction enzymes (RE) are used by this technique to reduce the complexity of the genomes allowing to work with large genomes, such as the conifer species. Two RE are the most common employed in GBS, which are *Pst*I (5'CTGCA/G'3) and *Ape*KI (5'G/CWGC3'). A pilot phase was carried out to determine the best RE for *Atlas cedar* species selecting *Pst*I which is a rare cutter enzyme.

Paired-end reads obtained were used to perform the assemblies and the SNP calling. The software ipyrad v.0.9.65 (Eaton & Overcast, 2020) was used to carry out both analyses. In this case, two assembly approaches were performed, *de novo* and reference. The Atlas cedar genome has not been sequenced at this time. Therefore, a published transcriptome has been used as a reference (Cobo-Simón, 2020). Subsequently, different filtering steps were performed using VCFtools v0.1.16 program package (Danecek *et al*., 2011) to create a SNP genetic matrix. The SNP retained in the matrix must met the following requirements: minimum allele frequency (MAF) of 5 %, maximum missingness of 50 %, be biallelic, only 1 SNP per *locus* was retained to avoid linkage disequilibrium, and finally, individuals with more than 50 % of missing data were removed.

Genetic structure of populations

Two approaches to describe the genetic structure of the Atlas cedar were carried out. First, a principal component analysis (PCA) was performed using the plink2 2.00a2.3 software (Chang *et al*., 2015) with the --pca option. Then, a graphic representation was obtained using the R v4.1.2 (R Core Team 2022) package, called ggplot2 v3.3.5 (Wickham, 2016). On the other hand, a sparse non-negative matrix factorization analysis (sNMF) was carried out. A cross-entropy study was performed using the snmf function of the LEA package v3.4.0 (Frichot & François, 2015) in R software v4.1.2 (R Core Team

2022) to obtain the most probable number of ancestry populations (named K) that best explains the structure of the populations, and the coefficients obtained were used for the graphical representation of the admixture with pong software package (Behr *et al.*, 2016). The cross-entropy parameters were set to 10 repetitions for each K, and a maximum value of the K range was established depending on the genetic matrix used for the study.

Genetic parameters such as fixation indexes ($F_{ST}$), to determine drift effect, migration rate (Nm) to describe the gene flow among populations, Nei's genetic distance, heterozygosity, private alleles, and polymorphic *loci* were calculated. Moreover, a molecular variance analysis (AMOVA) with a total of 9,999 permutations was performed to identify the proportion of genetic variation attributable to differences among and within populations. GenAlEx v6.5 software (Peakall & Smouse, 2006; 2012) was used to estimate them.

Selection signatures

BayeScan 2.1 software (Foll & Gaggiotti, 2008), with default parameters, was used to identify selection signatures. This software calculates the $F_{ST}$ coefficients for each *locus* and compare them among and within the populations of study to determine outliers. This version of BayeScan calculates q-values using the FDR correction (false discovery rate). The threshold was set at 5 % so all SNP with a q-value < 0.05 were significant to be under selection.

Those sequences containing significative SNPs were queried against the nucleotide database, and against the transcriptome shotgun assembly (TSA) database in the case of the results obtained from the reference assembly, using in both cases BLASTn (NCBI; Altschul *et al.,* 1990). When a match was obtained, a BLASTx (NCBI; Gish & States, 1993) against the non-redundant protein database was performed to identify protein homology.

Genotype-environment associations (GEA)

A total of 19 bioclimatic variables were obtained from WorldClim database (Fick & Hijmans, 2017) with a resolution of 30 s. The values of each variable for the localization of our study populations were extracted using the freeware QGIS 3.18 (Quantum Geographic Information System) (QGIS Development Team, 2022).

The Hmisc package v4.7-0 (Frank & Harrell, 2022) in R software v4.1.2 (R Core Team 2022) was used to perform a Pearson correlation to identify the intercorrelated variables. The threshold of the Pearson coefficient (r) was set at 0.9. Subsequently, an imputation step to fill the missing data of the genetic matrixes was conducted by using LEA R package v3.4.0 (Frichot & François, 2015).

To perform GEA studies the lfmm (latent factor mixed models) function of the LEA R package v3.4.0 (Frichot & François, 2015) was used. The parameters were 20 repetitions for each run with 100,000 iterations and a burn-in of 50,000. This function requires a K value which must be the same calculated in the cross-entropy analysis. In this case, p-values for each iteration between SNP and variable was obtained. FDR correction was performed to turn p-values into q-values. The threshold was established at 5 %. Finally, the sequence containing the significant SNP was used to carry out BLASTn and BLASTx as it was described in selection signature section.

Risk of non-adaptedness

To assess the change rate value required for a population to cope with the environmental alterations a risk of non-adaptedness (RONA) study was carried out (Rellstab *et al*., 2016; Pina-Martins *et al.,* 2019). The value of RONA shows an absolute average of the changes in allele frequency at *loci* associated to an environmental variable which is needed to the survival of the population to alterations in that variable. Previous studies have assessed that allele frequencies lower than 0.1 per decade indicate that the species could cope with the environmental perturbations, while the

values is higher than the range from 0.1 to 0.2 per decade it indicates that this species might not keep pace with climate change (Jump, 2006; Jump *et al.,* 2017).

pyRona v0.3.6 (Pina-Martins *et al*., 2019) was used to perform RONA study. Two emissions scenarios were analyzed: low emissions (RCP 2.6), whose temperature annual range increase is limited in 2 °C and high emissions (RCP 8.5), limited in a maximum of 4.9 °C. These two scenarios have been used to perform RONA studies with populations of *Pinus uncinata* (Méndez-Cea *et al*., 2023)

**Results**

Genetic structure of populations

After filtering the dataset obtained in the *de novo* assembly, a total of 2,336 SNP and 199 individuals were retained. PCA results showed a separation between Moroccan and Spanish population with a 51 % of explanation in PC1 axis (**Figure 2**). High and Middle Atlas are closer to each other than Western Rif. Hence, the second axis separated Western Rif to the other populations (19.82 % PC2) (**Figure 2**). Moreover, one of the Spanish populations, Fiñana (Almería), formed a group with the Moroccan population of the High Atlas. So, both groups have a common genetic pool. The cross-entropy analysis determined that K = 4 is the best value to explain the structure obtained in the populations of study. It corresponds with the nuclei observed in the PCA studies. These groups were formed by: Western Rif, Middle Atlas, High Atlas with Fiñana, Dornajo, and Yunquera.

AMOVA showed that the higher proportion of genetic variation was attributed to differences among individuals (53 %) and the lower among populations (11 %). Fixation indexes were ranging between 0.023 and 0.241. As expected, $F_{ST}$ value obtained between High Atlas and Fiñana showed the lower value with the Spanish populations. The migration rate (Nm) values are lower than 1 between Rif and Dornajo and Yunquera and Almería. There are only two migration rates higher than 4 which are the value

between High and Middle Atlas, and Middle Atlas and Rif. The rest of populations showed values within 1.069 and 2.842. The percentage of polymorphic *loci* average was 80.19 ± 3.83%. Fiñana and High Atlas showed a very similar percentage (87.63 and 87.67, respectively).

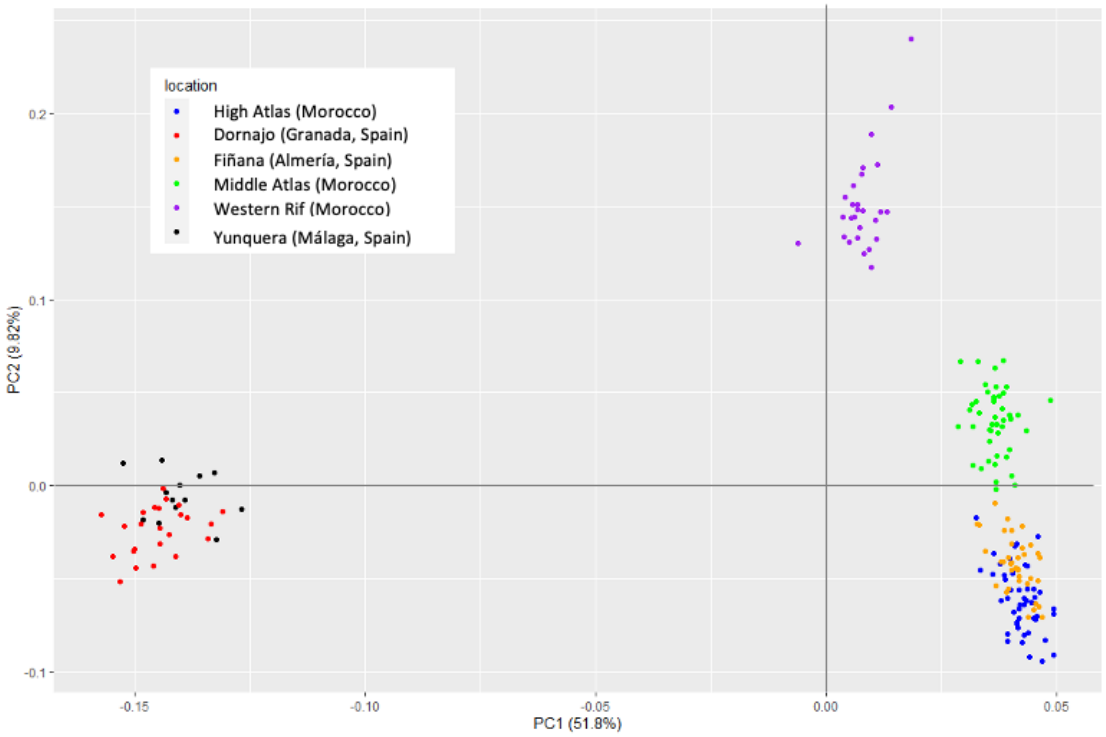


**Figure 2**: PCA results of *de novo* assembly with the six populations study in this work. Each of them is represented with a color depending on their location.

Nei's genetic distance showed values ranging from 0.012 to 0.108. The majority of the lowest values were obtained between Moroccan and Spanish populations except for the found among High Atlas and Fiñana which showed the minimum value of the range (0.012) (**Table S1**).

On the other hand, a dataset which only contained the genetic information of the Moroccan populations (natural populations) was created. In this case, a total of 1,885 SNP and 128 individuals were maintained. The explanation percentage of PC1 and PC2 reached 36.7 %. The first axis separated Western Rif from the other two Moroccan population (**Figure 3**). The second one allowed us to separate Middle Atlas from Rif and High Atlas. The cross-entropy analysis corroborated the result obtained with PCA, as it

showed a K value of 3, meaning that each of the Moroccan populations has its own gene pool.

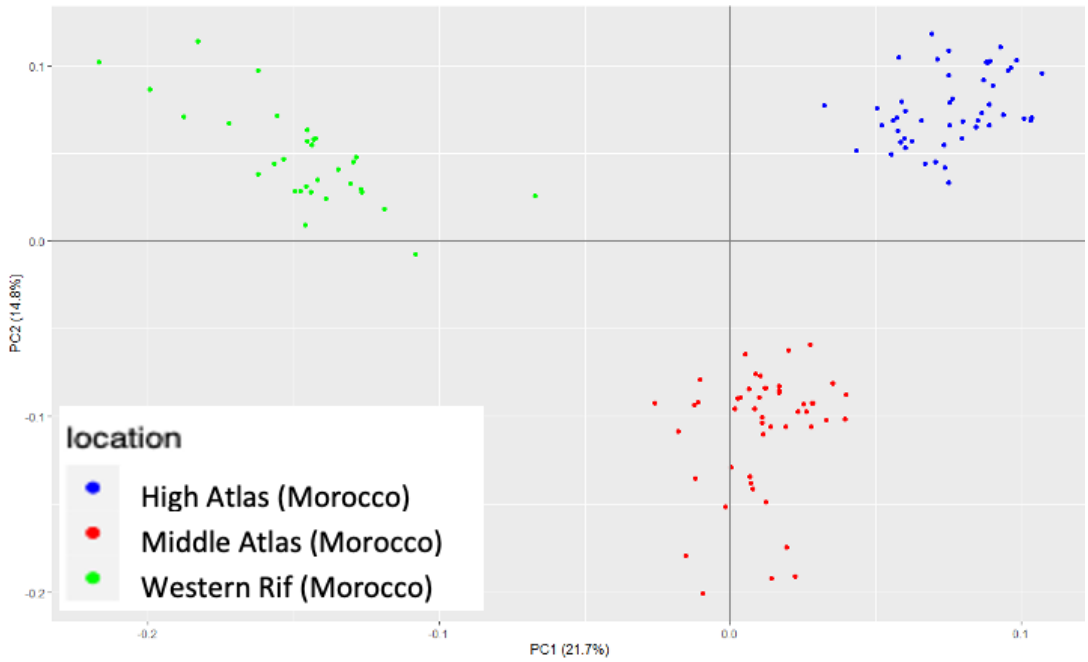


**Figure 3:** PCA results of *de novo* assembly with the three populations from Morocco used in this work.

To assess the presence of genetic differences among adults and saplings from these Moroccan populations, a PCA was carried out separating the individuals by ages and populations. In this case, no pattern was found, but those from the same population grouped together (**Figure S1**).

The variation among individuals showed the highest value (55 %) in this AMOVA study. In this case, the fixation indexes values were lower than the obtained with all populations with a range from 0.027 to 0.075. Western Rif showed the highest values. The migration rates presented values higher than 4 except for Western Rif and High Atlas (3.088). A high percentage of polymorphic *loci* was found with a mean of 93.78 ± 3.63 %. The maximum value of Nei´s genetic distance was 0.037 which was obtained between High Atlas and Western Rif.

The second assembly approach was performed using a reference and only with the dataset which contained the Moroccan populations. A total of 1,336 SNP and 126

individuals were retained. The results of PCA and admixture studies showed the same genetic structure obtained with *de novo* assembly using the populations of Morocco (**Figure 4**). However, the total explanation percentage achieved with the two first components (31.5 %) was slightly lower than the estimated with *de novo* assembly. Concerning the statistics analyses, the $F_{ST}$ values ranged from 0.018 to 0.049 which were lower than those obtained with *de novo* assembly. On the other hand, all the migration rates showed values higher than 4. The other indexes were identical to those estimated with *de novo* assembly data.

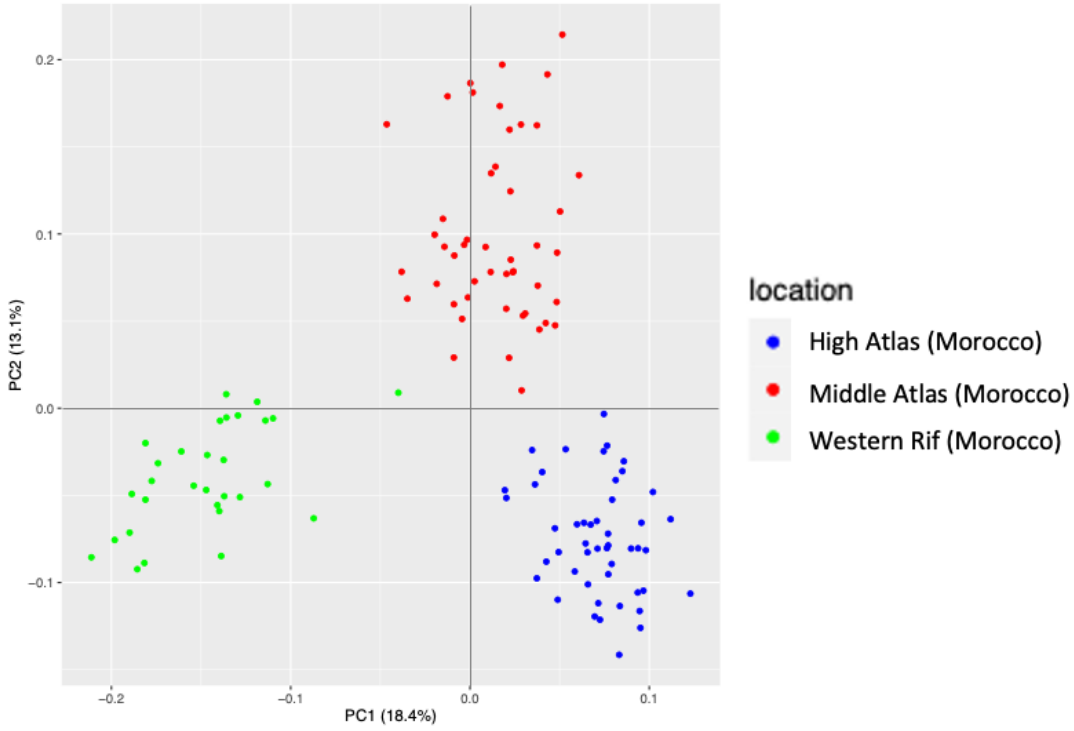


**Figure 4:** PCA results obtained with reference assembly carried out using Moroccan populations.

Selection signatures

This study was carried out using the Moroccan populations dataset and the *de novo assembly* genetic matrix. The results showed a total of 5 *loci* under selection with a q-value significance of 5 %. Three matches were obtained from the protein database (**Table S2**).

In order to describe differences between adults and saplings individuals of these populations, other two studies were performed. A total of 1 *locus* and 4 *loci* were identified in adults and youths, respectively. No homolog proteins were detected in the databases.

The reference assembly results showed 4 *loci* under selection and 3 of them were matched against the protein database. Moreover, one of these putative homologs were WAT1, that is a protein involved in the auxin pathway (**Table S3**).

Genotype-environment associations (GEA)

The Pearson correlation showed 8 variables highly correlated, so they were removed maintaining 11 bioclimatic variables to carry out the GEA studies: BIO1, BIO2, BIO3, BIO4, BIO5, BIO6, BIO8, BIO9, BIO12, BIO14, and BIO15.

A total of 23 associations were identified using the genetic matrix obtained with the *de novo* assembly. Annual mean temperature (BIO1) showed the highest number of associated *loci*, with 5. Seasonality temperature (BIO4), minimum temperature of the coldest month (BIO6), and mean temperature of the driest quarter (BIO9) showed associations with the same 3 *loci*. Mean diurnal range (BIO2) and annual precipitation (BIO12) shared the 2 *loci* associated. Maximum temperature of the warmest month (BIO5), precipitation of the driest month (BIO14), and precipitation seasonality (BIO15) only showed 1 *locus* associated which was the same for the three variables. Isothermality (BIO3) showed associations with 1 *locus* which is not shared with any variable. Finally, mean temperature of the wettest quarter (BIO8) showed associations with 1 *locus*.

In terms of the homologies found with the genome regions, only 1 sequence showed matches against the protein database. It is oxidase cytochrome c subunit 6b which is a protein involved in the electron exchange in mitochondrial respiration.

The GEA study performed using the genetic matrix of the reference assembly gave a total of 19 associations. Five of the variables showed the same 3 *loci* associated which were: annual mean temperature (BIO1), seasonality temperature (BIO4), maximum temperature of the warmest month (BIO5), minimum temperature of the coldest month (BIO6), and mean temperature of the driest quarter (BIO9). Precipitation seasonality (BIO15) showed 2 *loci* associated which were shared with the last variables. Two *loci* showed exclusively associations with isothermality (BIO3). In this case, the other 4 variables did not show any associations. Lastly, three matches were obtained against the protein database with several functions such as, carbohydrates metabolism or disease resistance (**Table S4**).

Risk of non-adaptedness

This study was performed using only the three populations from Morocco (**Figure 5**). The lowest values of change rate were obtained in the low emissions scenarios, as it was expected. The maximum change rate value is ranged between 2 and 4.7. Those values are reached by High Atlas population for both scenarios. Temperature annual range is the variable that most influences in the three populations and in both scenarios. It is remarkable that Middle Atlas is more vulnerable than Western Rif in the high emissions scenarios while in the low emissions Western Rif showed more vulnerability than Middle Atlas.

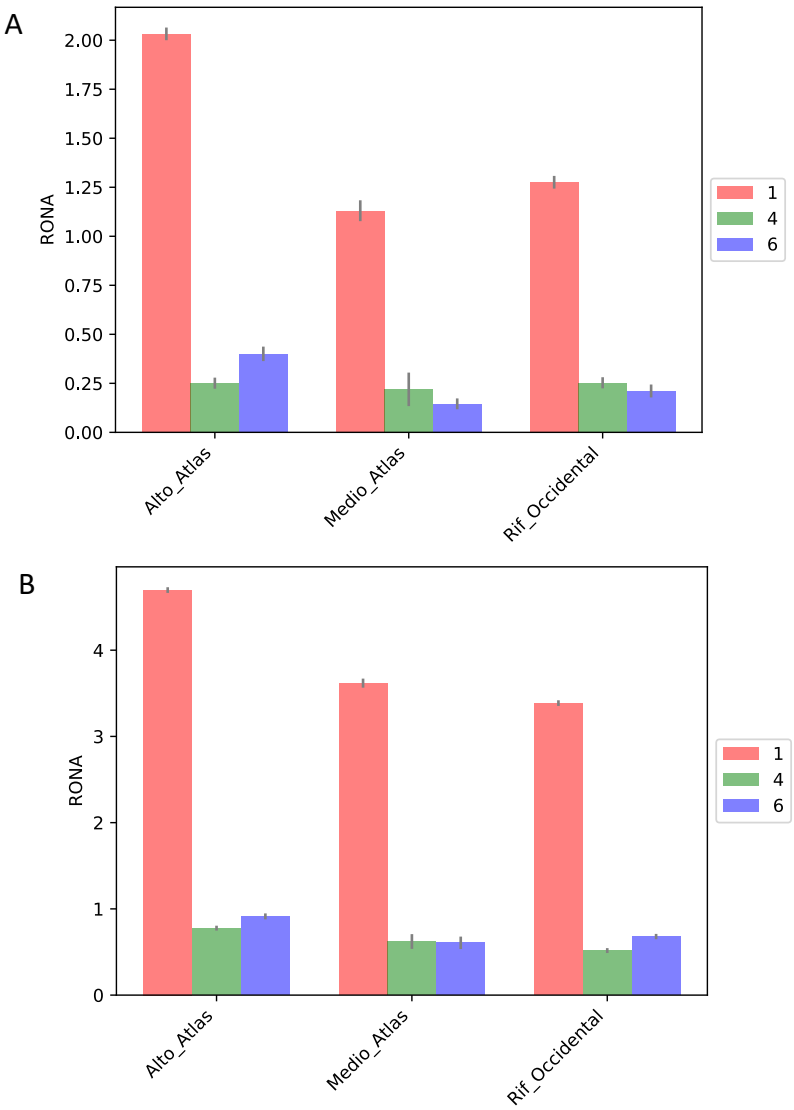


**Figure 5**: Graphical representation of the RONA values of Moroccan populations which are necessary to cope with the predicted variations of low emissions scenario (RCP2.6) (A) and high (RCP8.5) (B). There are showed the three bioclimatic variables with the lowest q-values which are: mean annual temperature (1), temperature seasonality (4), and minimum temperature of the coldest month (6).

## Discussion

To our knowledge, this study is the first to use GBS technique in C*edrus atlantica*. The development of new technologies based on NGS has allowed to work with non-model organisms such as conifers species without a reference genome (Elshire *et al.*, 2011). The reduced representation sequencing, such as GBS technology, minimizes the genome complexity of species with large genomes by fragmentation with restriction enzymes. The number of molecular markers maintained in this study after filtering

(1,885 SNPs for the Moroccan populations and 2,336 SNP for all populations) is lower than the obtained in other conifers species such as *Pinus contorta* which showed 17,765 SNP and *Picea glauca*, 17,845 SNP (Chen *et al.,* 2013). However, the number of SNP retained in *Quercus suber* (2,547 SNP) (Pina-Martins *et al.,* 2019) was much closer to the retained here for the dataset of all populations of Atlas cedar.

The amount of SNPs maintained after filtering steps using the reference assembly dataset was lower than those retained with the *de novo* approach (1,336 SNP *vs* 1,885 SNP). Despite this, the used of a transcriptome as a reference allows us to determine that all the SNPs identified are placed in genes regions. This is interesting due to these SNPs may be directly related to the functions of those genes in which they are present.

All the Moroccan populations study here showed their own genetic pool as it was showed in PCA and cross-entropy studies. In addition, Western Rif was the most differenced population. This could be explained by the environmental characteristics of this area, which is the northern limit of Atlas cedar distribution, with the maximum value of annual precipitation (2,000 mm) (Linares *et al*., 2012). Although each population has its own genetic pool there is a gene flow among them. As well, High Atlas and Western Rif showed the maximum fixation index value indicating a lower gene flow among them. The presence of the Rif and Atlas Mountain ranges could act as orographic barriers which avoid the pollen move through them hindering gene flow (Terrab *et al.,* 2006).

The Moroccan populations did not show a low genetic variability as it could be expected because it is a relict species. However, our results are consistent with other obtained with relict species such as *Abies pinsapo* (Cobo-Simón *et al*., 2020) or relict populations of *Pinus uncinata* (Méndez-Cea *et al.,* 2023) in which the decrease in the diversity was not observed either.

A noticeable result obtained in this study is the description of the origin of the plantation of one of the Spanish populations (Camarero *et al*., 2021b). The individuals present in Fiñana (Almería) come from High Atlas as shown in the PCA and cross-entropy

studies. Moreover, fixation indexes and Nei's genetic distance corroborated the results, indicating that the Spanish population has not diverged with respect of its Moroccan origin. The environmental conditions of these two populations are very similar in terms of dryness and aridity which is quite interesting. These conditions could explain the survival of the Fiñana population. In terms of the other Spanish populations, we do not know where they come from, but we can say that they are not from any of these three Moroccan populations which were studied in the present study. We hypothesize that they could come from other Algerian populations, but another study is required to assess this option.

The number of *loci* subjected under selection was not very high. Nevertheless, the presence of some significative SNP may indicate that selective pressure is acting in this species. In addition, some differences between adults and youths were observed due to a few *loci* were identified under selection using the dataset separated by ages. So, it is possible that the new generations of Atlas cedar are experiencing some genetic variations as response to recent environmental variations. However, the PCA study did not show any genetic differences between adults and youths. The selection may be occurring in a few very specific regions of the genome (which were evidenced by the significative SNPs obtained) and therefor in PCA is not detected because it is compared at the genome level. On the other hand, trees need long time generations, so it is possible that we are not able to describe this change in the next generation (Dauphin *et al.,* 2021).

The absence of a reference genome hindered the obtention of gene information from the genome regions containing the *loci* under selection. Despite this, some homologies could be found for these regions and one of the most interesting was a putative WAT1 protein. This protein is involved in the auxins signaling pathway and it controls the plants homeostasis (Ranocha *et al.,* 2013), that obviously could be related with an adaptation to drier climates.

It is expected that extreme climate events such as droughts will increase in both severity and intensity as a consequence of forecasted climate change. The severity of

drought stress and the temperature increase could modify the structure of forests in many regions mainly because one of the responses of trees is to migrate upwards looking for suitable conditions. Nowadays, it has been observed this kind of migration in Atlas cedar populations (Cheddadi *et al*., 2017). Moreover, in the last years, there are associations between extreme drought events in North Africa and an increase of Atlas cedar mortality (El Abidine, 2003). For instance, in the year range from 1960 to 2010 a 75 % decrease of Atlas cedar cense in the Rif Mountains has been observed (Cheddadi *et al.,* 2017). Therefore, it is of paramount importance to develop studies in these threatened species to understand how they could cope with climate change.

The combined study of environmental variables and genetic data allows studying the respond of tree species to climate perturbations (Méndez-Cea *et al*., 2023). Most of the significant *loci* identified in GEA analysis were associated with annual mean temperature indicating that this variable could limit the survival of Atlas cedar as it has been shown in previous ecological studies (Linares *et al*., 2013). In addition, variables related to precipitations showed some associations, and evidenced the effect of drought stress observed in several studies (Linares *et al*., 2011; Linares *et al*., 2013; Taoufik *et al.,* 2021).

Finally, the RONA values estimated for Atlas cedar shows a negative lag between the allele frequency change rate and the climate alterations being more pronounced in the High Atlas population. Variables related to temperature, mainly the annual mean temperature (BIO1), showed the greatest influence on the survival of Atlas cedar in both scenarios (Williams *et al*., 2013). The highest values obtained for High Atlas could be related to the intrinsic characteristics of this population which is the driest and the hottest area where Atlas cedar is placed (Camarero *et al.,* 2021a). For this reason, a reduction of this species is expected in this southernmost region (High Atlas) and our prediction indicates that it is possible to occur because of the highest RONA value obtained for this population in the high emissions scenario (near to 5). By opposite, a recent study based on niche models reported that the most important bioclimatic variables driving the modern distribution of *Cedrus atlantica* are related to precipitation, instead of temperature: winter precipitation (BIO19), precipitation of the driest month

(BIO14), and annual precipitation (BIO12) (Bouahmed *et al*., 2019; Xiao *et al*., 2022). This disagreement shows that the interpretation of the vulnerability of the species is not straightforward. Temperature (that is, global warming) could change our expectations based on niche suitability and future range distribution of endangered species, as there is a divergence between the variables that determine statistically current distribution and the risk of non-adaptedness.

Meanwhile, the values showed by Western Rif and Middle Atlas for low emission scenario indicated that both areas show lower risk than High Atlas. These results could be explained by the fact that Rif and Middle Atlas are the most suitable places for the development of Atlas cedar in Morocco (Benabid, 2000). However, in a low emission scenario, Rif showed a slightly higher value than Middle Atlas for annual mean temperature. Moreover, Western Rif has the highest precipitations and lowest temperature range of the Moroccan distribution of Atlas cedar. So, the individuals from this population might be less able to cope with temperature increases (Camarero *et al.,* 2021).

The change rates estimated for Atlas cedar, ranging from 0.15 to 4.7, are higher than the obtained in other relict species located in northern Morocco, which is the case of *Abies marocana*. This species showed a maximum change rate value of 0.9. In the light of the outcomes, Atlas cedar shows a high vulnerability to environmental alterations specifically to temperature perturbations. In summary, our studies indicate that Atlas cedar is prone to local extinction in their altitudinal range corroborating previous studies (Cheddadi *et al.*, 2009). In addition, its relict and endemic conditions increase the vulnerability of this species because of its population size.

To sum up, it is necessary to describe the genetic structure of populations to understand the relations among them. The selection fingerprints obtained seem to show that an adaptive response to climate change is taking place in these Moroccan populations. GEA studies indicated that environmental variables related to temperature are the most relevant for Atlas cedar populations. In this work, several candidate genes have been obtained which must be genotyped in other Atlas cedar populations in the

future and it is interesting to develop a functional characterization to describe their real function. Lastly, RONA studies are very useful to develop better strategies of conservation because they allow us to describe the vulnerability of individuals or populations.

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## Apéndice 1: Material suplementario

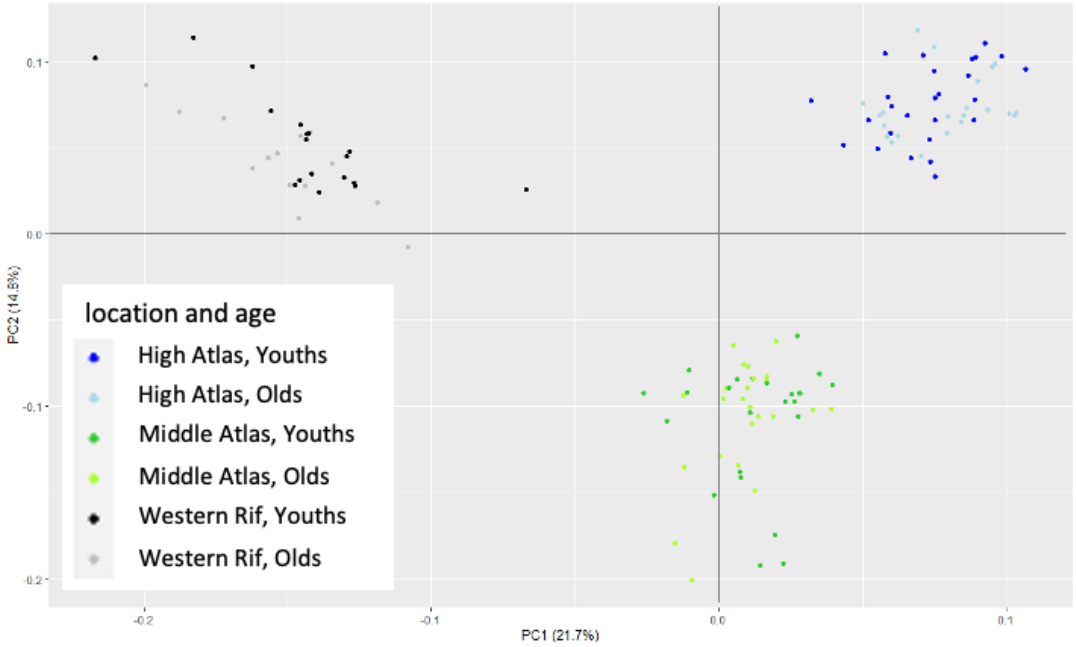


**Figure S1**: PCA results of *de novo* assembly with the three populations separated from Morocco by age.

**Table S1**: Nei genetic distance obtained with all populations *de novo* assembly dataset.

**Pairwise Population Matrix of Nei Genetic Distance**

| High Atlas | Middle Atlas | Western Rif | Yunquera | Almería | Granada | |
|---|---|---|---|---|---|---|
| **0.000** | | | | | | **High Atlas** |
| 0.019 | **0.000** | | | | | **Middle Atlas** |
| 0.035 | 0.030 | **0.000** | | | | **Western Rif** |
| 0.108 | 0.103 | 0.088 | **0.000** | | | **Yunquera** |
| 0.012 | 0.017 | 0.035 | 0.107 | **0.000** | | **Almería** |
| 0.106 | 0.102 | 0.087 | 0.019 | 0.105 | **0.000** | **Granada** |

**Table S2**: Results of the matches obtained with the significative *loci* identified in BayeScan study with the *de novo* assembly of Moroccan populations of Atlas cedar dataset. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identity | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **14** | Unknown mRNA *Picea sitchensis* | (Predicted) Accumulation and replication of chloroplast | Chloroplast organization and division | 0.0 |
| **950** | mRNA *Picea glauca* | (Probable) Galacturonosyltransferase | Mucilage biosynthesis<br>Mucilage extrusion from seed coat. | 0.0 |
| **1869** | Unknown mRNA *Picea sitchensis* | Phenylananine ammonia lyase | Plant metabolism enzyme<br>Cinnamic acid biosynthesis<br>L-Phenylalanine catabolism | 0.0 |

**Tabla S3**: Results of the matches found in the alignments against protein database with those *loci* which were significative in BayeScan analysis using reference assembly dataset. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identity | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **71** | mRNA *Picea sitchensis*<br>TSA *Pinus sylvestris* | (Putative) TIR-NBS-LRR | Disease plant response | 1e-92 |
| **668** | mRNA *Picea glauca*<br>TSA *Picea mariana* | WAT1 | Auxines pathway<br>Cell wall biogenesis<br>Defense response | 2e-143 |
| **1002** | mRNA *Picea glauca*<br>TSA *Larix laricina* | WAT1 | Auxines pathway<br>Cell wall biogenesis<br>Defense response | 2e-143 |

**Tabla S4**: Results obtained in GEA analysis using the reference assembly of Moroccan populations dataset. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identity | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **363** | mRNA *Picea glauca*<br>TSA *Pseudotsuga menziesii* | (Predicted) Mannan endo-1,4-beta-mannosidase 7 | Carbohydrates metabolism | 0.0 |
| **1035** | TSA *Picea glauca* | (Putative) Pentatricopeptide repeat-containing protein | Chloroplast RNA processing<br>Polycistronic mRNA processing<br>RNA modification | 0.0 |
| **1194** | mRNA *Picea glauca*<br>TSA *Pinus sylvestris* | NBS/LRR | Disease resistance | 7e-47 |

# Bloque III. Estudio genómico del potencial adaptativo frente al cambio climático en dos especies del género *Abies*: *Abies pinsapo* y *Abies marocana*

## Artículo 1: Warming appears as the main risk of non-adaptedness for western Mediterranean relict fir-forests under expected climate change scenarios. (En preparación)

**Abstract:** Circum-Mediterranean firs are considered one of the most drought-sensitive species to climate change. Understanding the genetic basis of trees adaptive capacity and intra-specific variability to drought avoidance is mandatory to define conservation measures. We focus here on *Abies marocana* and *Abies pinsapo*, both relict tree species, endemics from north Morocco and south Spain, respectively. We performed genotyping by sequencing, using double digestion RAD-seq (ddRAD-seq), to obtain a genetic matrix based on single nucleotide polymorphisms (SNPs). This matrix was utilized to study the genetic structure of *A. pinsapo* and *A. marocana* populations and to determine the presence of *loci* under selection in pinsapo. In addition, to understand how pinsapo and Moroccan fir cope with climate change, genotype-environment associations studies (GEA) were performed. Further, the vulnerability of these species to climate variations was estimated by the risk of non-adaptedness. The filtering of *de novo* assembly provided 3,982 SNPs, from 504 trees. PCA separated Grazalema from the rest of the Spanish populations. However, AMOVA results showed greater differences within-trees (50 %), compared to among-populations (3 %). The $F_{ST}$ value used to estimate the AMOVA, is 0.028 which indicated the absence of differences among the Spanish populations ($p = 0.0001$), but it supported significant differences between *A. pinsapo* and *A. marocana*. The percentages of polymorphic *loci* were high in all populations (about 97 %). We found 51 *loci* under selection. Homologies sequences were found for some proteins related to abiotic stress response such as dehydration-responsive element binding transcription factor, regulation of abscisic acid signaling and methylation pathway. Genotype-environment associations retained 5 bioclimatic variables: isothermality, maximum temperature of warmest month, temperature annual range, precipitation of wettest quarter, and precipitation of driest quarter. A total of 15 associations with 11 different *loci* were observed being the most numerous the maximum temperature of warmest month with 5 *loci*. This temperature sensitivity

was also supported by the risk of non-adaptedness, which yielded the higher risk for both *A. pinsapo* and *A. marocana* under the worse emissions scenario (RCP 8.5).

**Keywords**: circum-Mediterranean firs, selection signature, genotype-environment associations, risk of non-adaptedness, Sierra de las Nieves, Grazalema, Talassemtane.

## Introduction

The frequency and intensity of drought events are increasing worldwide, triggering extensive growth decline, dieback, and mortality in many forest ecosystems (DeSoto *et al*., 2020; McDowell *et al*., 2020). At a regional scale, the Mediterranean basin is highly vulnerable to the ongoing warming, a trend that is predicted to worsen (Ozturk *et al*., 2015). Consequently, drought-sensitive forests may be increasingly stressed, as water shortage impairs the functioning of trees by reducing their photosynthesis and growth rates (Choat *et al*., 2018). The circum-Mediterranean firs can be considered one of the most sensitive tree species to climate change (Aussenac, 2002; Linares, 2011; Caudullo & Tinner, 2016; Sánchez-Salguero *et al*., 2017). Furthermore, they are mainly relict tree species, which arises several uncertainties concerning their future prospects (Hampe & Jump, 2011). Ecological and evolutionary factors determine the population dynamics of these endangered tree species, while several underlying genetic responses remain elusive to our knowledge (Alberto *et al*. 2013). Thus, a proper understanding of key physiological and genetic mechanisms involved in the populations' persistence becomes imperative for maintaining forest biodiversity and ecosystem services (Anderegg *et al*., 2013).

Here, we focus on *Abies marocana* Trab. and *Abies pinsapo* Boiss., the westernmost circum-Mediterranean fir species, endemic from north Morocco and south Spain, respectively (Linares, 2011; Ben-Said, 2022). Both species are included in the IUCN Red List of Threatened Species as endangered species, being climate change one of the main threats to their conservation (Arista *et al*., 2011; Alaoui *et al*., 2011). Events of

forest dieback and mortality have been mainly reported for *A. pinsapo* (Linares *et al*., 2009; Navarro-Cerrillo *et al*., 2020a; Navarro-Cerrillo *et al*., 2022; Cortés-Molino *et al.*, 2023), while the sensitivity to climate change of *A. marocana* has been also pointed out (Esteban *et al.*, 2010; Navarro-Cerrillo *et al*., 2020b; Alaoui *et al*., 2021). Despite the increasing concern regarding the apparent increasing mortality rates of these relict tree species in the context of the pervasive environmental changes and habitats loss, the genetic mechanisms underlying contrasting vulnerability among individual are still poorly understood (Cobo-Simón *et al*., 2021; 2022).

Previous genetic studies performed in *A. pinsapo* and *A. marocana* focused mainly on molecular markers of nucleus and organelles, as chloroplast and mitochondria, but not on the entire genome. The main reasons are related to the intrinsic characteristics of the conifers' genomes, characterized by large size, high number of repetitive sequences and the absence of reference genomes for the most species (García-García *et al*., 2022). Hence, there are several studies determining genetic diversity and population structure (i.e., cpSSR) (Terrab *et al*., 2007); developing new molecular markers (i.e., nSSR) (Sánchez-Robles *et al*., 2012); describing genetic differentiation between *A. pinsapo* and *A. alba* using SSRs (Dering *et al*., 2014; Jaramillo-Correa *et al*., 2010); determining the genetic diversity of several pinsapo populations using SSR, intermicrosatellites (ISSR), and single nuclear polymorphisms (SNP) (Cobo-Simón *et al*., 2020); characterizing drought response genes of pinsapo applying SNPs (Cobo-Simón *et al*., 2021; 2022) or performing biogeographic studies with several species of *Abies* taxa (Litkowiec *et al*., 2021). Nonetheless, current advances in next generation sequencing (NGS) and in bioinformatics open a wide range of possibilities to perform studies with the conifer genomes. Furthermore, transcriptomes can be used as an alternative reference. Hence, in the case of *A. pinsapo*, Pérez-González *et al.* (2018) obtained a *de novo* transcriptome and recently, Ortigosa *et al*. (2022) published other transcriptome with the aim to determine intraspecific variations.

In this work, a genotyping by sequencing (GBS) technique named double digestion RAD-seq (ddRAD-seq) was used to obtain a genetic matrix based on single

nucleotide polymorphisms (SNPs). Our specific aims were: (i) to study the genetic structure of *A. marocana* and *A. pinsapo* populations and to determine the presence of *loci* under selection based on the obtained SNPs; (ii) to quantify genotype-environment associations (GEA) based on local climate variables and population genetics; (iii) to assess the vulnerability of *A. marocana* and *A. pinsapo* to future climate change scenarios based on the risk of non-adaptedness (RONA).

**Material and methods**

Species of study and field sampling

*Abies pinsapo* is a sub-dioicous fir with a pyramidal shape which can grow up to 30 m. Pinsapo is located on north-facing slopes between 1,000 and 1,800 m a.s.l. (Linares *et al*., 2009). This location allows pinsapo to maintain optimum conditions of humidity which is the most relevant issue to this species survival. Pinsapo is a relict species whose limited distribution is in the South of Spain. There are three nuclei which are placed in: Sierra de las Nieves with an area of about 5,800 ha, Sierra Bermeja with 1,212 ha, and Sierra de Grazalema, accounting for about 2,000 ha.

A total of 571 samples from six populations of *A. pinsapo* was collected. The populations studied located in the National Park of Sierra de las Nieves were Saucillo (SA) where 126 samples were sampled; Caucón (CA) with 259 samples, Ánimas (AN) with 51 samples; Pilones (PI) with 29 samples; and Pilar de Tólox (PT) with 16. Finally, 90 samples were sampled from the Biosphere Reserve Sierra de Grazalema (GR). To assess the genetic differences among saplings and old trees, individuals of these two cohorts were collected from SA, CA, and AN (**Table 1**).

*Abies marocana* is a monoecious species which appears in the high peaks between 1,500 and 2,000 m a.s.l. (Ben-Said, 2022). As it was described for pinsapo, this species is usually present in the north slope because it needs humid environments to

survive due to it is drought sensitivity (Aussenac, 2002). Moroccan fir is a relict species whose range distribution is in Northern Morocco.

In terms of *A. marocana*, two populations located in Talassemtane National Park were studied. Talassemtane (TA) accounts for about 2,000 ha and Jebel Tazaot (TZ), which is the mountain range of Talassemtane, is around 1,000 ha (Ben-Said, 2022; Ben-Said *et al*., 2022). The number of samples collected was 101: 53 from TA and 45 from TZ. As it was described for pinsapo populations, saplings and old trees were collected from these two populations (**Table 1**).

**Table 1**: Characteristics of each population of study. It Is indicated the localization of the populations and their elevation, mean annual temperature and precipitation. The last three columns are related to the total number of samples genotyped with ddRAD-seq and the number of saplings and old trees genotyped of each population.

| Population | Abbreviation | Latitude (°N) | Longitude (°W) | Elevation (m a.s.l.) | Mean Annual Temperature (°C) | Mean Annual Precipitation (mm) | Population (n) | Saplings (n) | Old trees (n) |
|---|---|---|---|---|---|---|---|---|---|
| Caucón | CA | 36.71 | -4.96 | 1114 | 12.0 | 975.0 | 226 | 24 | 25 |
| Saucillo | SA | 36.72 | -4.98 | 1352 | 11.2 | 1261.6 | 110 | 12 | 12 |
| Grazalema | GR | 36.78 | -5.40 | 1085 | 12.0 | 1277.5 | 79 | - | - |
| Pilar de Tólox | PT | 36.68 | -5.00 | 1669 | 10.2 | 1647.6 | 16 | - | - |
| Pilones | PI | 36.69 | -5.02 | 1700 | 10.3 | 1659.3 | 28 | - | - |
| Ánimas | AN | 36.70 | -5.01 | 1637 | 10.3 | 1616.6 | 50 | 18 | 17 |
| Talassemtane | TA | 35.17 | -5.18 | 1694 | 10.1 | 1821.0 | 53 | 23 | 30 |
| Tazaot | TZ | 35.23 | -5.10 | 1677 | 10.5 | 1644.3 | 45 | 26 | 19 |

DNA extraction and ddRAD-seq

The extraction starts with the lyophilization of 100 mg of each leaf fresh sample. Then, total DNA extraction was carried out by using DNeasy Plant Mini Kit (Qiagen®, Germany) following the manufacturer's instructions with some modifications. DNA concentration was measured on a NanoDrop™ spectrophotometer and the integrity of the samples extracted was determined by an electrophoresis in 1 % agarose gel. Only those samples which reached the necessary requirements for the ddRAD-seq technique (Peterson *et al*., 2012), were selected to their genotyping. In this case, a total of 509 samples of pinsapo and 98 of Moroccan fir were optimum for the technique. Subsequently, ddRAD-seq (Peterson *et al*., 2012) libraries were constructed using *Ape*KI/*Pst*I double digestion and sequenced by LGC Genomics (Germany). To know more about the methodology, please see Méndez-Cea *et al.*, 2023a.

Since the lack of pinsapo and Moroccan fir reference genomes, a *de novo* assembly of the paired-end sequences obtained was performed with them. However, there are available transcriptomes of *Abies pinsapo* which allow us to carry out a reference assembly using the sequences of this species. The transcriptome used for this study was previously obtained by our group (for more details, please see Pérez-González *et al*., 2018). Both assemblies and the SNP callings were performed using ipyrad v.0.9.65 (Eaton & Overcast, 2020). Several filtering steps were carried out using VCFtools v0.1.16 program packages (Danecek *et al*., 2011). With the aim to retain only quality biallelic SNPs the following parameters were used: a minimum allele frequency (MAF) of 5 %, a maximum missingness of 50 % and, to avoid linkage disequilibrium, only 1 SNP per *locus* was maintained. Moreover, a final step which consists of removing those individuals with less than 50 % of the filtered SNPs was performed. Several genetic matrixes were obtained depending on the data set used in the filtration steps.

Genetic structure of populations

For the obtained genetic matrix of pinsapo was used two approaches to study the genetic structure of populations: principal component analysis (PCA) and sparse non-negative matrix factorization analysis (sNMF). PCA was carried out using the plink2 2.00a2.3 software (Chang *et al*., 2015) with the --pca option and then, a graphic representation was obtained using the R v4.1.2 (R Core Team 2022) package called ggplot2 v3.3.5 (Wickham, 2016). For the second approach, a cross-entropy study was carried out using the snmf function of the LEA package v3.4.0 (Frichot & François, 2015) in R software v4.1.2 (R Core Team 2022) to determine the most probable number of ancestry populations (named K) that best explains the structure of the populations. In addition, the admixture coefficients were calculated too. For this analysis, a total of 10 repetitions for each K was performed and the maximum value of the K range was established at 8. Then, the .Q files obtained were employed to obtain a graphic representation of the admixture results using pong software package (Behr *et al.*, 2016).

Several statistical analyses of each genetic matrixes were performed using the GenAlEx v6.5 software (Peakall & Smouse, 2006; 2012). Parameters such as fixation indexes ($F_{ST}$), to determine population differentiation, and migration rate (Nm) to describe presence or not of gene flow among populations, were estimated. The Nei's genetic distance among populations was also estimated. Heterozygosity, private alleles, and polymorphic *loci* were calculated too. Finally, Shannon index was calculated to infer genetic diversity.

In addition, $F_{ST}$ coefficient was used to perform a molecular variance analysis (AMOVA) with the aim to determine the proportion of genetic variation attributable to differences among and within populations. A total of 9,999 permutations were used to carry out the AMOVA.

Selection signatures

BayeScan 2.1 software (Foll & Gaggiotti, 2008) were used to identify selection signatures. Default parameters were applied. For more details, please see Méndez-Cea *et al.,* 2023a. The threshold was established in 5 %.

The complete sequence which contains the SNPs of interest identified with BayeScan were queried against the nucleotide database, and in the case of the matrixes obtained from reference assembly, against the transcriptome shotgun assembly (TSA) database using in both cases BLASTn (NCBI; Altschul *et al.,* 1990). When a match was obtained, a BLASTx (NCBI; Gish & States, 1993) against the non-redundant protein database was performed too.

Genotype-environment associations (GEA)

The 19 bioclimatic variables from WorldClim database (Fick & Hijmans, 2017) with a resolution of 30 s, were used to perform this kind of studies. After obtaining the variables data from WorldClim, only the values of each population position of interest were retained using the freeware QGIS 3.18 (Quantum Geographic Information System) (QGIS Development Team, 2022). These points were taken out using point sampling tool which is included in QGIS.

Before carrying out the GEA study, a Pearson correlation with the bioclimatic variables and an imputation step were performed. The correlation was calculated using the Hmisc package v4.7-0 (Frank & Harrell, 2022) in R software v4.1.2 (R Core Team 2022). Since this correlation needs a minimum of 4 populations to be estimated, the coefficient could be obtained depending on the genetic matrix used. In all cases, the threshold of the Pearson coefficient, r, were established at 0.9. On the other hand, the imputation step was conducted by using LEA R package v3.4.0 (Frichot & François, 2015) to fill all the missing data of the genetic matrix before performing GEA.

The lfmm (latent factor mixed models) function of the LEA R package v3.4.0 (Frichot & François, 2015) were used to perform GEA studies. Each run was repeated 20 times with 100,000 iterations and a burn-in of 50,000. The K value used were the same which was obtained with cross-entropy analysis. The p-values results were corrected using FDR and q-values were obtained. The threshold was set at 5 % as in the case of BayeScan results. The sequences of those SNPs which showed some significant associations were queried against nuclear and protein NCBI database as it was described above.

Risk of non-adaptedness

Risk of non-adaptedness (RONA) value indicates the theoretical percentage of change in allele frequency at *loci* associated with environmental variables which is required for a given population to cope with future changes in that variable allowing it to survive (Rellstab *et al*., 2016; Pina-Martins *et al*., 2019). The estimation of this value is based on calculating the average absolute difference of the changes in allele frequencies between the current and future climate conditions of those *loci* which showed associations with environment variables. Low values of RONA predict a greater predisposition to adapt to new environmental conditions.

pyRona v0.3.6 (Pina-Martins *et al*., 2019) was used to calculate RONA value at population level. Two different climate scenarios which are predicted to take place by the end of this century (2081–2100), were studied: low emissions (RCP2.6), which limits the increase of global mean temperature to 2 °C, and high emissions (RCP8.5), whose limitation is 4.9 °C. A total of 19 bioclimatic variables of each scenario were tested. These variables were downloaded from WorldClim database as it was described in GEA section. The input file used was the lfmm results obtained in the GEA studies.

**Results**

Genetic structure of pinsapo populations

The filtering of *de novo* assembly of pinsapo populations dataset maintained 504 individuals and 3,982 SNPs. The first axis of PCA separated GR from the rest of the Spanish populations with an explanation percentage of 27.2 % (**Figure 1**). AN, PI, and PT are very similar while CA showed a gradient ranging from SA to the rest of the populations. The cross-entropy analysis, which was carried out with a K ranging from 1 to 8, did not provide much information due to the lower value obtained was 8. However, the admixture results showed that K = 3 is the best explanation for our results. In this case, GR showed its own gene pool again, SA composed the second group, and the rest of Spanish populations were grouped together.

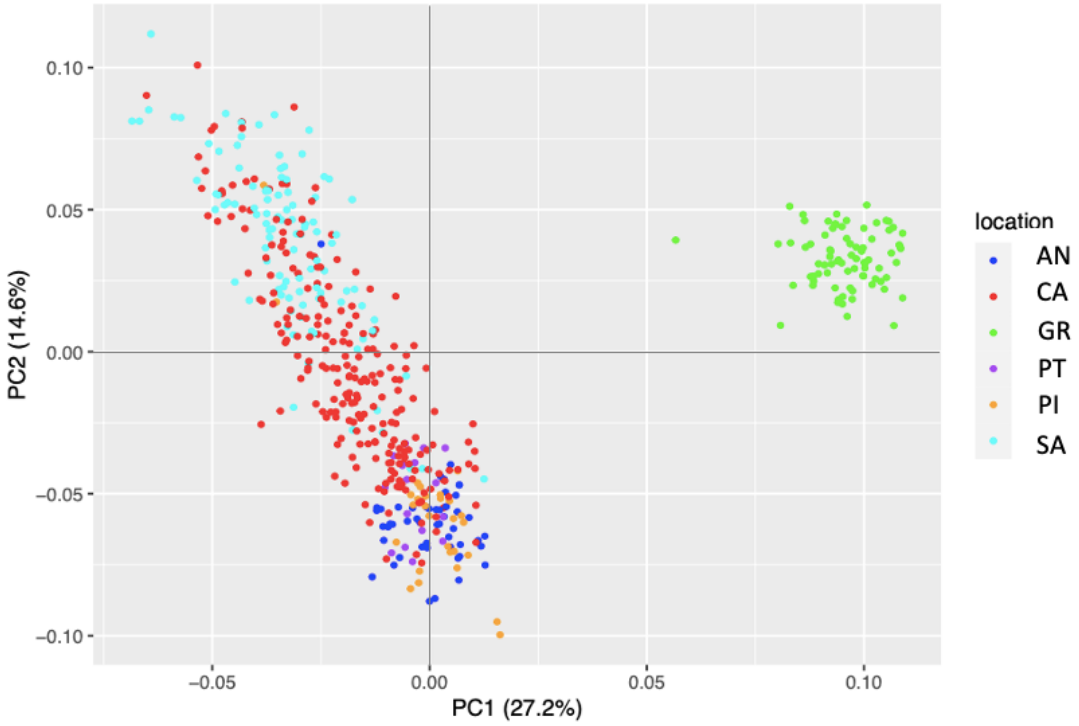


**Figure 1:** PCA representation with *de novo* assembly dataset of *Abies pinsapo* populations. Each of them has a different color which is indicated in the legend.

The AMOVA results of the pinsapo populations showed the greatest differences within individuals (50 %) and the lowest among populations (3 %). The $F_{ST}$ value used to

estimate the AMOVA, is 0.028 which indicated the absence of differences between the populations of study with a high significance (p-value = 0.0001).

Pairwise population $F_{ST}$ values were between 0.006 and 0.043 indicating that all the pinsapo populations behave as in complete panmixia (**Table 2**). Nevertheless, the minimum differences found in these values allow us to separate GR population from the others.

The percentages of polymorphic *loci* were high in all populations, reaching 100% in CA. The average was 97.49 $\pm$ 1.07 %. Shannon index (I) of each population was ranged from 0.467 to 0.501. The minimum was appeared in PT and the maximum in CA. In this case, no private alleles were found. Finally, all Nm values are very high which indicate that the populations of study have a strong gene flow among them. The extremely high value was found between CA and SA (35.923) and the lowest, among SA and PT (5.287).

**Table 2**: Pairwise population $F_{ST}$ obtained from pinsapo *de novo* assembly dataset. The higher index has obtained between GR and PT.

**Pairwise Population Fst Values**

| CA | SA | GR | PT | AN | PI | |
|---|---|---|---|---|---|---|
| **0.000** | | | | | | CA |
| 0.006 | **0.000** | | | | | SA |
| 0.029 | 0.034 | **0.000** | | | | GR |
| 0.021 | 0.029 | 0.043 | **0.000** | | | PT |
| 0.014 | 0.019 | 0.033 | 0.027 | **0.000** | | AN |
| 0.016 | 0.022 | 0.036 | 0.030 | 0.017 | **0.000** | PI |

Nei's genetic distance showed values near to 0 (from 0.006 to 0.045) indicating that all pinsapo populations are very similar. However, it is remarkable that GR showed the highest values which states this population is the most genetically different.

The pinsapo dataset from the reference assembly filtering retained 494 individuals and 1,642 SNPs. PCA results showed that GR separated from the rest of populations with an explanation percentage of 24.7 % (**Figure 2**). The cross-entropy study, which was carried out with a range from 1 to 8, did not provide much information as in the case of *de novo* assembly. The admixture results were interpreted by the PCA results and K = 3 was the best value to explain the genetic structure of our populations. In this case, GR had an own gene pool, SA and CA conformed other group, and the rest of the populations were grouped together.

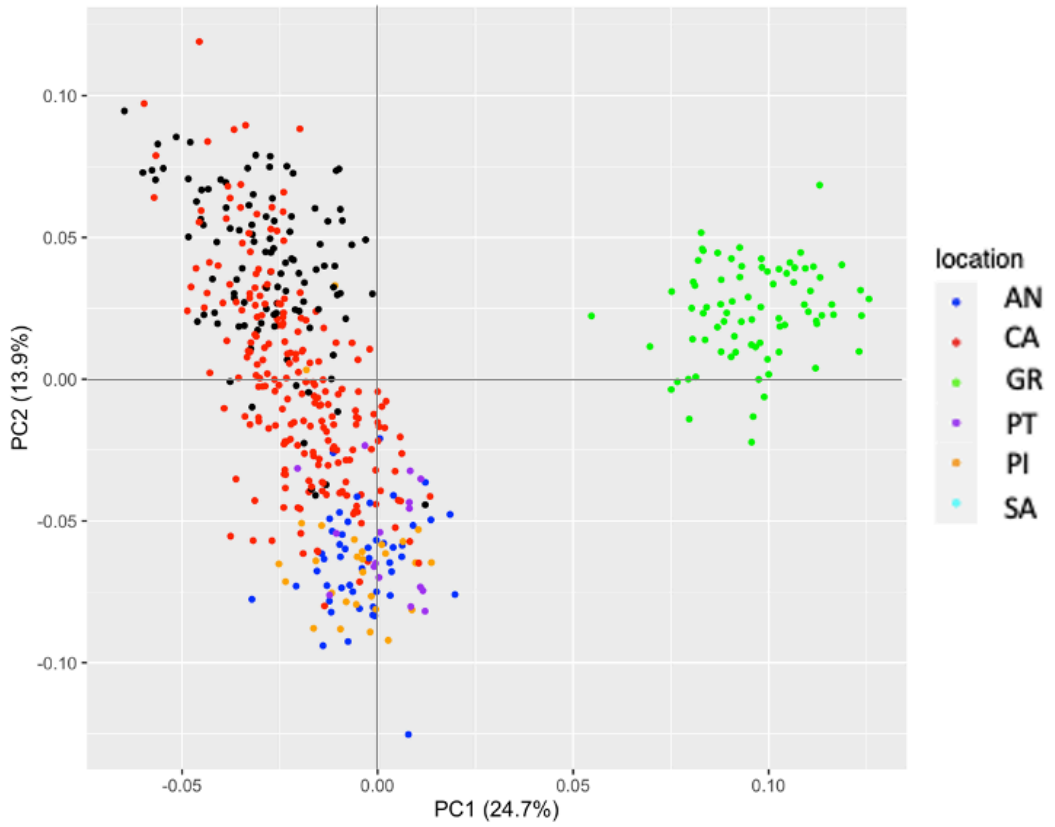


**Figure 2**: PCA representation of genetic matrix obtained from reference assembly with *A. pinsapo* populations. The meaning of the color is indicated in the legend.

Predictably, the statistical analyses showed the same results which were obtained with *de novo* assembly dataset. However, it is remarkable the Nm value between CA and SA which was higher (42.561) than the estimated with *de novo* assembly. As well as Nei's genetic distance was ranging from 0.005 to 0.037, being the higher value of the range lower than the estimated with the *de novo* assembly dataset.

Selection signatures

BayeScan analysis with the *de novo* assembly pinsapo populations showed 51 *loci* under selection (q-value 5 %). The complete sequence of the 10 SNPs which had the lowest q-values and those which showed associations with bioclimatic variables in GEA studies, were queried against the databases. Homolog sequences were found for 9 of these sequences and 6 of them hit with the protein database (**Table S1**). It is remarkable that two of the scaffolds used gave the same result for both the nucleotide sequence and the proteins. A protein whose function are involved in the dark reaction photosynthesis was obtained. However, the most interesting proteins found are those which are related with abiotic stress response such as dehydration-responsive element binding transcription factor, and the late embryogenesis abundant protein (LEA3-1) which is produced during the seed embryogenesis.

The reference dataset of the pinsapo populations showed in BayeScan 24 *loci* under selection. The 10 complete sequences analyzed gave match with sequences in NCBI Database. However, only 4 of them obtained hit with proteins in the Database (**Table S2**). Two proteins are enzymes involved in photosynthesis and in Calvin cycle, respectively. Other homology is found with serine/arginine-rich splicing factor SR45 which is related to the regulation of abscisic acid (ABA) signaling and methylation pathway.

Genotype-environment associations

The genetic matrix of pinsapo populations assembly with *de novo* approach was used to perform GEA study. First, the Pearson correlation was calculated which allowed us to retain 5 bioclimatic variables which were: isothermality (BIO3), maximum temperature of warmest month (BIO5), temperature annual range (BIO7), precipitation of wettest quarter (BIO16), and precipitation of driest quarter (BIO17). A total of 15 associations with 11 different *loci* were observed being the most numerous the

maximum temperature of warmest month (BIO5) with 5 *loci*. Some associations were shared between two or more bioclimatic variables.

Once the alignments were performed, nine sequences matched with nucleotide sequences and six of them gave us information about homologies with other proteins. Some of these proteins are related with transmembrane transport, transcription regulation or abiotic stress response (**Table S3**). Six *loci* were under selection including the *locus* involved in the abiotic stress response. No matches were found for one of them in the protein database (**Table S2**; **Table S3**). The allele frequencies of these *loci* were calculated for three of the six populations here studied, which were: CA, SA, and AN. The results indicated that CA and SA populations are very similar each other, whilst they are so different from AN. The allele frequencies estimated separating the individuals of these populations into old trees and saplings was slightly different within CA and SA populations for SNP 448, within SA population for SNP 2400, and SNP 2528; and within AN population for SNP 3808 (**Figure 3**).

The GEA study with the reference assembly carried out with pinsapo populations genetic matrix showed 12 associations. The bioclimatic variable called isothermality (BIO3) did not show associations with any *locus*. This study was performed with the same 5 bioclimatic variables which was retained in *de novo* assembly. The largest number of associations were observed with BIO7 (7 *loci*). The complete sequences of all these *loci* hit against nucleotide database but only 5 of them matched with protein database. Some of the protein function which showed homologies was involved in ubiquitination, transmembrane transport, or stomatal closure (**Table S4**).

The GEA study with Moroccan fir populations was carried out with the 19 bioclimatic variables since the Pearson correlation could not be performed due to this genetic matrix had only 2 populations. The same *locus* (SNP 5594) was associated with all the variables and the alignment results showed homology with a protein whose function is related with protein phosphorylation (LRK1). Méndez-Cea *et al*., (2023b) identified this *locus* under selection too so the allele frequencies of each nucleus were

calculated. TA had an allele frequency of the reference of 0.97 while TZ showed 0.41, for the same allele. When the two sites were separated by age, the saplings from TZ showed higher allele frequency of the alternative one (0.67) than the adults (0.5) while TA did not show differences for this *locus* between age groups (**Figure 3**).


**Figure 3**: Frequencies of reference allele (1) and alternative allele (2) in SNPs associated with bioclimatic variables in the GEA study of *A. pinsapo* (CA, SA, and AN populations), and *A. marocana* (TA and TZ populations). Allele frequencies are showed for the whole population, old trees and saplings.

Risk of non-adaptedness (RONA)

The change rate value obtained in the low emissions scenario for pinsapo populations was ranging from 0.01 to 0.47, while in the high emissions the ranging was 0.02–0.97. In the case of Moroccan fir, the change rate value was between near 0 to 0.39 for the low emissions expected and ranging from lower to 0.02 to 0.90. Previous studies in trees showed than the change values were less than 0.1 per decade, the species might cope with the climate alterations, while if these changes were higher than the range from 0.1 to 0.2 per decade, the species showed a lag between allele frequencies and environment perturbations (Jump *et al*., 2006). Considering these results, the high emission scenario showed higher risk for the pinsapo and Moroccan fir survival than the other scenario, as it was expected.

The isothermality (BIO3) had shown the maximum change rates for each of pinsapo populations in the low emissions scenario except for GR population which displayed high values in temperature annual range (BIO1) (**Figure 4A**). The Moroccan fir populations reached the higher rates with temperature annual range (BIO1) like Grazalema. On the other hand, the annual precipitation (BIO12) was the bioclimatic variable related to precipitations which showed the maximum change rate value in both species and scenarios (**Figure 4B**).

In terms of the high emissions scenario, *A. pinsapo* and *A. marocana* showed the maximum rates with temperature annual range (BIO1). Depending on the pinsapo population, the second bioclimatic variable which needs higher change rate value were related to temperature (SA, CA, and PT) or precipitation (PI). Since, the second maximum value obtained with Moroccan fir populations (TA and TZ) was always related with annual range precipitation (BIO12).

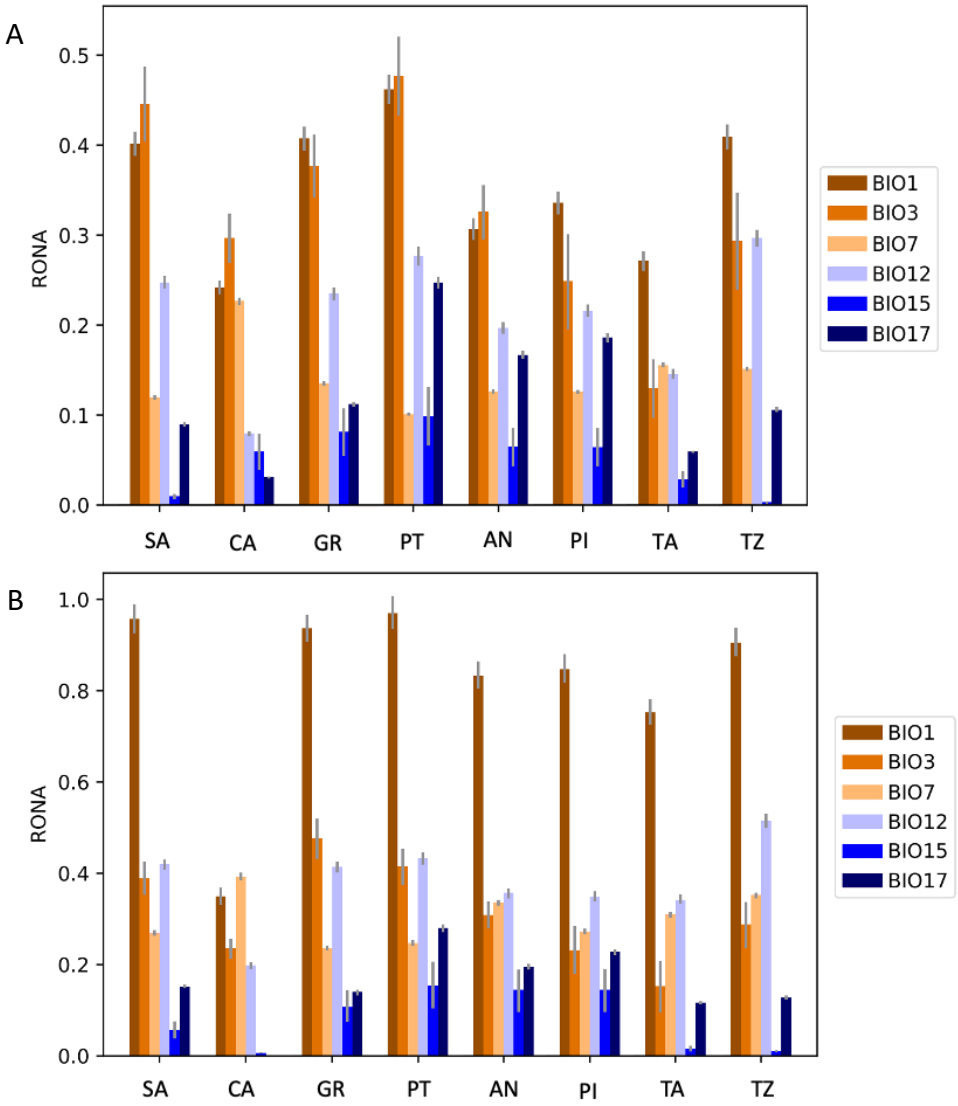


**Figure 4**: RONA results. Change rate values of each pinsapo and Moroccan fir populations studied for the low emissions scenario (A) and the high emissions scenario (B). The bioclimatic variables related to temperature are colored in orange and those related to precipitation appear in blue.

## Discussion

The development of new genotyping techniques based on NGS technology, such as the used in this work, ddRAD-seq, allows to rise the genomic knowledge about these species. The amount of SNPs detected by ddRAD-seq varied depending on the molecular marker density in the genome of study (Shirasawa *et al.*, 2016). Since the genome was the same, the number of SNPs maintained after filtering steps was always higher with

*de novo* assembly than with reference. This could be explained by the representation of those SNPs in the reads obtained. Due to the large size genome of this species (García-García *et al*., 2022), the likelihood of obtaining the same fragment in most of the individuals sequenced is low. Hence, the SNP was lower represented, and it could not be retained.

The lack of a reference genome but the availability of pinsapo transcriptomes gave us the chance to perform two different assembly approaches. Using a transcriptome as a reference allowed us to obtain functional information since all the SNPs described are placed in genes (Card *et al*., 2014). However, the absence of a correct annotation prevents us to know in some cases the biological function of the regions of interest.

Mainly, the $F_{ST}$ values over 0.15 are interpreted as a significant indication of divergence (Frankham *et al*., 2002). Considering the $F_{ST}$ values obtained, which are under 0.15, the six pinsapo populations behave as in complete panmixia, moreover the Nm values indicate that these populations have a high gene flow. In addition, AMOVA results showed that the higher differences are within populations and the lower among populations. However, there are some genetic differences, which separated them into three genetically different groups, being GR the most dissimilar one. Studies carried out with cpSSR showed differences within GR and Sierra de las Nieves (Terrab *et al*., 2007; Cobo-Simón *et al*., 2020; Sánchez-Robles *et al*., 2022), which is the place where the rest of the Spanish populations studied in the present work are located.

The geographic distribution of Sierra de Grazalema (Cádiz, Spain) and Sierra de las Nieves (Málaga, Spain) could be involved in the differences found. Obviously, the differences in location are associated with different climatic conditions. In fact, GR is the point of Spain with the highest annual precipitation (Castillo, 2000). Hence, the individuals from this population may have its own genetic pool because they do not cope with harsh drought stress conditions. In addition, the low dispersion of the pinsapo pollen (lower than 3 km) could act as a limit factor to genetic exchange between populations (Arista & Talavera, 1994).

The great dispersion observed in the PCA for the CA population could be explained by the fact that it is a rear edge of pinsapo distribution. Since the individuals of this population are under limiting climatic conditions, they should have genetic tools to cope with them (Cobo-Simón *et al*., 2020; 2021). This is confirmed by the 100 % polymorphic *loci* found in CA population and by the Shannon index which showed the highest value.

Regarding the *loci* under selection obtained with pinsapo populations, one of them also showed associations in GEA studies, increasing the robustness of the results and highlights the possible relevance of this *locus*. The putative protein homolog identified for this *locus* which was under selection and associated with bioclimatic variables is named dehydration-responsive element binding transcription factor (DREB).

Generally, the expression of DREBs is induced by abiotic stresses such as low and high temperatures, drought, and salt salinity. Due to the involvement in abiotic stresses response, the DREB genes of several economically important organisms have been modified with the aim to increase their stresses resistance, such as freezing tolerance in potato (i.e., Behnam *et al*., 2007), and dehydration tolerance in soybean (i.e., de Paiva Rolla *et al*., 2014) or rice (i.e., Zhao *et al*., 2010). In terms of studies with angiosperms, it has been described the role development by DREB in cold, drought, and salt stress response of *Populus euphratica* Olivier (Chen *et al*., 2009). Regarding conifers, it has been reported the involvement of DREB in the drought stress response of *Picea abies* (L.) Karst (Haas *et al.,* 2020). So, this *locus* could be interesting to future studies.

Since temperature increase and extreme drought events have been reported in the range of *A. pinsapo* and *A. marocana* (Linares *et al*., 2009; Méndez-Cea *et al*., 2023b), the GEA results provide insights on the genetic response of these relict species to the ongoing climate change. *A. pinsapo* populations showed associations with temperature and precipitation variables, indicating that both are important to its development (Alba-Sánchez *et al*., 2010; López-Tirado & Hidalgo, 2014), while recent studies performed on *A. marocana* pointed out to a main role of temperature (Alaoui *et al*., 2021; Méndez-Cea *et al*., 2023b).

Drought events are causing widespread tree mortality across many forest biomes with profound effects on the ecosystem dynamics and carbon balance (Anderegg *et al*., 2013; McDowell *et al.*, 2020). Our results suggest that the effect of future droughts will almost certainly be worsened by increases in air temperature associated with global warming (**Figure 4**). Thus, climate change is expected to intensify regional-scale droughts and related forests die-off (Choat *et al*., 2012; DeSoto *et al*., 2020). Accordingly, focusing attention on the physiological basis of drought-induced tree mortality is nowadays required (McDowell *et al.*, 2008; Anderegg *et al.*, 2012; Choat *et al*., 2018). Failure of the plant hydraulic system has been identified as a relevant mechanism involved in tree die-off and mortality during drought, where old trees might exhibit greater sensitivity (Bennett *et al.*, 2015). Here, we obtained contrasting allele frequencies between old trees and saplings in some SNPs identified by GEA (**Figure 3**).

The results obtained by comparing saplings and old trees gave a higher number of *loci* under selection in these two age groups than within the whole population. This contrasting selection signatures may be reflecting the fact that the studied young trees could have been subjected to a selective pressure, as they have been stablished under a warmer and dryer climate scenario, compared to old trees, mainly stablished around the XIX century. Old trees play keystone roles in forests and can be disproportionately important to ecosystem carbon storage (McDowell *et al*., 2020). On the other hand, the saplings' cohorts depict the forest of the future, including the coming allele frequencies structure (**Figure 3**). Droughts may have a more detrimental impact on the growth and mortality of old, usually the larger, trees (DeSoto *et al*., 2020). This pronounced drought sensitivity of larger trees might be related to greater inherent vulnerability to hydraulic failure and the higher evaporative demand experienced by their towering crowns (Bennett *et al.*, 2015). Assuming that future extreme drought events, whose frequency is expected to worsen, will have a more detrimental impact on the growth and mortality of old trees, it should be expected exacerbating feedbacks to climate change, affecting forest biodiversity and ecosystem services (Anderegg *et al*., 2013; McDowell *et al*., 2020).

Reducing uncertainty about tree vulnerability and mortality projections should be founded on robust physiological processes (Anderegg *et al*., 2012). However, the proposed mechanisms of drought-induced mortality, including hydraulic failure and carbon starvation, are still unresolved (Choat *et al.*, 2018). To that extent, some protein homologies obtained here may provide useful insights on the physiological processes underling drought-induced tree mortality. One of the homology proteins identified with *de novo* pinsapo dataset in the GEA studies was a transcription factor of MYB family. This is one of the most relevant and abundant family responsible of plant abiotic stress response/tolerance and they are often implied in the ABA-response. So much so that there are studies, which have reported gene expression changes of these transcription factors family during drought stress response in conifer species (Lorenz *et al*., 2011; Du *et al*., 2018; de María *et al*., 2020).

The protein related with stomatal closure which showed association between the reference pinsapo dataset and temperature annual range (BIO7) is called aluminum-activated malate transporter 12-like. In *Arabidopsis thaliana* (L.) Heynh., it is expressed in the guard cells and as a response to a drought stress, this protein induced the stomatal closure as a response to ABA (Meyer *et al*., 2010). This function is very interesting due to most of the gymnosperms such as *Abies pinsapo* which is an isohydric species, close their stomata avoiding water loss in a dryness conditions (Brodribb *et al*., 2014).

In the case of Moroccan fir, the identification of only one *locus* associated with all the bioclimatic variables which is also under selection gave us an insight of the relevance that this region could have. The allele frequencies differences found among populations and between ages indicated that the selection pressure is acting. In addition, a protein homology was identified with a Leucine-rich repeat serine/threonine-protein kinase (LRK1). This protein kinase family can be involved in the abiotic stress response (Shiu & Bleecker, 2003). For this reason, this region could be an interesting target to future genotyping in Moroccan fir populations.

Finally, the RONA studies allowed us to describe the genetic vulnerability of the species under future scenarios. Simulating the local adaptation at population level and

their adaptative potential to future conditions is very useful to determine the risk of the species. The use of this kind of studies, such as RONA, allows us to integrate the current and future environment data with genetic information to obtain a robust prediction of species adaptation (Feng & Du, 2022). Conifers, as pinsapo and Moroccan fir, are long-lived species with long generation times. These characteristics cause them to show a lag in their adaptation to current and future environmental perturbations (Browne *et al*., 2019). So, the faster climate change is, the greater adaptation lag occurs (Jump & Peñuelas, 2005). Previous studies suggested that conifers species may have difficult to cope with the rapid climate change (Dauphin *et al*., 2021).

Despite recent observational, experimental, and modeling studies suggesting increased vulnerability of relict trees to hotter drought, to our knowledge, this is the first estimation of climate change-induced vulnerability based on genetics and future climate scenarios in *A. pinsapo* and *A. marocana*. RONA modelling supports that warming might be the main limiting factor for the survival of *A. pinsapo* and *A. marocana*, according to several studies performed on drought-sensitive species (Williams *et al*., 2013; Allen *et al.,* 2015). Our results support that *A. pinsapo* and *A. marocana* should overcome high risk of non-adaptedness under any warming climate scenario (**Figure 4**). Similar results were obtained by dendrochronological studies and climate-growth models for the whole circum-mediterranean firs group (Sánchez-Salguero *et al.,* 2017; Méndez-Cea *et al*., 2023b). Regarding *A. marocana*, previous studies aimed on the potential distribution of Moroccan fir, based on species distribution models (SDMs), showed that the main variables conditioning the presence of *A. marocana* were the average temperature of the warmest quarter and the maximum temperature of the warmest month (Alaoui *et al*., 2021). Further, similar methodological approaches performed for *A. pinsapo* also obtained a significant effect of temperatures (Alba-Sánchez *et al*., 2010; López-Tirado & Hidalgo, 2014). Notwithstanding, it is noteworthy that these SDMs reported as the most important bioclimatic variable driving the modern distribution of *A. pinsapo* winter rainfall, instead of temperature. This disagreement shows that the interpretation of the vulnerability of the species is not straightforward. Temperature (that is, global warming) could change our expectations based on niche suitability and future range distribution of endangered

species, such the circum-Mediterranean firs (Caudullo & Tinner, 2016; Sánchez-Salguero *et al*., 2017). Both species have a limited distribution because their relict condition, so any alteration in the environmental conditions of their distribution range is a real threat for them making more vulnerable to this kind of alterations (Esteban *et al*., 2010; Cortés-Molino *et al*., 2023). Interestingly, the Moroccan fir population from TZ, with a drier climate, showed the highest vulnerability to variations in annual precipitation at both emission scenarios.

We present here likely genetic vulnerability drivers, which were, to date, scarcely known. Overall, we obtained estimations of change in allele frequencies higher than 0.1, so both species are at risk because they might not keep pace with climate change due to their change rates are slower than those of the expected environmental shift. The RONA values obtained for *Quercus suber* at high emissions scenario (RCP8.5) were ranged from 0.07 to 0.38 (Pina-Martins *et al*., 2019). The estimated values for both pinsapo and Moroccan fir in our study at the RCP8.5 scenario (0.97) are higher than the ones in *Q. suber*. Then, these species are apparently more vulnerable to climate change.

There are other kinds of approaches to assess the vulnerability of the populations which are becoming increasingly important. The development of tools based on machine-learning is becoming a promising way to assess the vulnerability of populations in forest species (Ellis *et al*., 2012). This technology allows to make predictions at different spatial-temporal scales and can estimate the individual response of SNPs to different environmental variations (Fitzpatrick & Keller, 2015).

To sum up, the development of new genotyping technologies based on NGS that reduce the genome complexity, such as ddRAD-seq, permits to perform genetic studies in conifer species. On the other hand, the use of a transcriptome as reference may give the opportunity to identify SNPs located in genes. In terms of the genetic structure, the pinsapo populations have showed that all the nuclei behave as in complete panmixia, existing gene flow among them (see also Cobo-Simón *et al*., 2020; Sánchez-Robles *et al*., 2022). Despite this, GR has an own genetic pool that differentiate it from the rest of the

populations. GEA results indicated that temperatures and precipitations have effects in both species.

It is widely assumed that warming will led to hotter droughts and nonlinear increasing atmospheric moisture demand. As a result, tree mortality can occur faster in hotter drought, consistent with fundamental physiology (Linares *et al*., 2012; Sánchez-Salguero *et al*., 2015; Choat *et al.*, 2018) supporting greater vulnerability perspectives. Thus, we highlight urgent challenges for research, management, and policy-making communities. The combined analysis of genome-wide single nucleotide polymorphisms and local environmental data provided genetically-based evidences of tree-level sensitivity to climate, estimations of the risk of non-adaptedness, as well as preliminary evidences of shifting allele frequencies in young cohorts. Nonetheless, the molecular mechanisms of adaptation remain largely unknown for long-lived tree species. Finally, pinsapo and Moroccan fir shows high vulnerability to, mainly, temperature variations. The RONA value estimation indicated that both populations are at risk because they might not keep pace with climate change. This kind of studies provide a complete understanding of forest species respond to climate change. All these information could be used for future conservation strategies.

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## Apéndice 2: Material suplementario

**Table S1:** Protein homologies obtained using the BayeScan results of the *de novo* assembly of pinsapo populations dataset. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | NCBI database | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|---|
| **448** | Nucleotide | mRNA *Picea glauca* | Apyrase 2-like | ATP binding<br>Hydrolase function | 6e-109 |
| **922** | Nucleotide | mRNA *Picea glauca* | Thermospermine synthase | Auxin polar transport<br>Phloem and xylem histogenesis<br>Xylem vessel member cell differentiation | 2e-38 |
| **1563** | TSA | Transcribed RNA sequence of *Pseudotsuga menziesii* | Heavy metal-associated isoprenylated plant protein 45 | Heavy metal detoxification (Cd)<br>Transcriptional response to cold and drought<br>Plant-pathogen interactions | 3e-26 |
| **2322** | Nucleotide | mRNA *Picea sitchensis* | Light-independent protochlorophyllide reductase subunit N | Chlorophyllide synthesis<br>Dark reaction of photosynthesis | 5e-65 |
| **2400** | Nucleotide | mRNA *Picea sitchensis* | D-xylose-proton symporter-like3 (chloroplast) | Transmembrane transporter | 0.0 |

| | | | | | |
|---|---|---|---|---|---|
| **2517** | Nucleotide | mRNA *Picea glauca* | Thermospermine synthase | Auxin polar transport<br>Phloem and xylem histogenesis<br>Xylem vessel member cell differentiation | 2e-38 |
| **2528** | Nucleotide | mRNA *Nelumbo nucifera* | (Predicted) Transcription factor MYB39-like | Suberin biosynthesis<br>Regulation of transcription | 6e-169 |
| **2700** | Nucleotide | mRNA *Picea sitchensis* | Late embryogenesis abundant protein (LEA3-1) | Stress tolerance responses (drought) | 2e-50 |
| **3808** | Nucleotide | mRNA *Picea sitchensis* | Dehydration-responsive element binding transcription factor | Abiotic stress response | 4e-58 |

**Table S2**: Protein homologies obtained using the BayeScan results of reference assembly pinsapo populations dataset. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | NCBI database | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|---|
| **325** | TSA | Transcribed RNA sequence *Abies pinsapo* | Phosphoenolpyruvate carboxylase | Convert phosphoenolpyruvate and bicarbonate into oxaloacetate and inorganic phosphorus<br>Photosynthesis | 1e-31 |
| **370** | Nucleotide | mRNA *Cucumis melo* | WRKY transcription factor 9 | Defense response | 0.0 |
| **974** | Nucleotide | Chloroplast genome of *Abies alba* | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RUBISCO) | Calvin cycle: dioxide carbon fixation | 4e-111 |
| **1313** | Nucleotide | mRNA *Picea sitchensis* | Serine/arginine-rich splicing factor SR45 | RNA-directed DNA methylation pathway<br>Splicing site selection of introns<br>Negatively regulation of ABA signaling | 6e-52 |

**Table S3**: Results of GEA study with the genetic matrix of the pinsapo populations obtained in the *de novo* assembly. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | NCBI database | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|---|
| **448** | Nucleotide | mRNA *Picea glauca* | Apyrase 2-like | ATP binding<br>Hydrolase function | 6e-109 |
| **2400** | Nucleotide | mRNA *Picea sitchensis* | D-xylose-proton symporter-like3 (chloroplast) | Transmembrane transporter | 0.0 |
| **2517** | Nucleotide | mRNA *Picea glauca* | Thermospermine synthase | Auxin polar transport<br>Phloem and xylem histogenesis<br>Xylem vessel member cell differentiation | 2e-38 |
| **2528** | Nucleotide | mRNA *Nelumbo nucifera* | (Predicted) Transcription factor MYB39-like | Suberin biosynthesis<br>Regulation of transcription | 6e-169 |
| **3808** | Nucleotide | mRNA *Picea sitchensis* | Dehydration-responsive element binding transcription factor. | Abiotic stress response | 4e-58 |
| **3961** | Nucleotide | mRNA *Larix sibirica* | DDE-type integrase/transposase/recombinase | DNA integration | 1e-08 |

**Table S4:** GEA results obtained with pinsapo population dataset from the reference assembly. It is shown the sequence type, the name and functions of the proteins, and the E-value for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | NCBI database | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|---|
| **249** | Nucleotide | mRNA *Picea glauca* | E3 ubiquitin-protein ligase | Promotion of protein ubiquitination and degradation | 6e-139 |
| **691** | Nucleotide | mRNA *Picea sitchensis* | Class I chitinase | Hydrolyzation of N-acetylglucosamine polymer chitin | 1e-133 |
| **697** | Nucleotide | mRNA *Picea glauca* | NEDD8-activating enzyme E1 regulatory subunit | Ubiquitination | 5e-125 |
| **872** | Nucleotide | mRNA *Picea sitchensis* | D-xylose-proton symporter-like 3 (chloroplast) | Transmembrane transport | 0.0 |
| **1609** | TSA | Transcribed RNA of *Pinus patula* | Aluminum-activated malate transporter 12-like | Stomatal closure induced by dark, ABA, water deficient | 5e-120 |

## Artículo 2: Tree-level Growth Patterns and Genetic Associations Depict Drought Legacies in the Relict Forests of *Abies marocana* (En revisión en la revista *Plants*)

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**Abstract:** The frequency and intensity of drought events are increasing worldwide. Trees are sessile organisms with long generation time, which makes them vulnerable to climate change and hindered to fast adaptations. Here, we evaluate tree-growth patterns and climate sensitivity to precipitation, temperature, and drought in the relict Moroccan fir *Abies marocana*. We selected two study sites, formerly stated as harboring contrasting *A. marocana* taxa (*A. marocana* and *A. tazaotana*, respectively). For each tree, dendrochronological methods were applied to quantify growth patterns and climate-growth sensitivity. Further, ddRAD-seq was performed in the same trees and close saplings to obtain single nucleotide polymorphisms (SNPs) and related genotype-phenotype associations. First, genetic differentiation between the two studied remnant populations of *A. marocana* was weak. We found significant differences in the SNPs subjected to selection in the saplings, compared to the old trees, suggesting that relict tree populations might be subjected to genetic differentiation and local adaptation to climate dryness. SNPs provide evidences of possible selection in the new cohorts, already established and grown in a warmer environment. Our results illustrate the potential of tree-rings and genome-wide analysis to improve our understanding of the adaptive capacity of drought-sensitive forests to cope with ongoing climate change.

**Keywords:** *Abies marocana*; drought sensitivity; tree age; selection signature; genotype-phenotype associations; dendrochronology.

## 1. Introduction

Trees are sessile organisms with long generation time, which makes them vulnerable to climate change and hindered to fast adaptations [1-3]. The frequency and intensity of drought events are increasing worldwide, triggering extensive dieback and mortality in many forest ecosystems [4-6]. At a regional scale, the Mediterranean basin is highly vulnerable to the ongoing warming, a trend that is predicted to worsen [7]. As a consequence, drought-sensitive forests may be increasingly stressed, as water shortage impairs the functioning of trees by reducing their photosynthesis and growth rates [8,9]. The circum-Mediterranean firs can be considered one of the most sensitive tree species to climate change [10,11]. This taxonomic group is subdivided in two sections: *Abies* and *Piceaster* [12]. It is remarkable that the *Piceaster* group is formed by the most ancient lineages, currently relict tree species, namely: *Abies pinsapo* Boiss., *Abies marocana* Trab., *Abies tazaotana* Villar, and *Abies numidica* Carr. [12]. These species are drought-sensitive, although they have several physiological mechanisms which allow them to cope with water stress, such as an early stomatal closure [13]. Furthermore, relict tree species rely on mechanisms promoting population persistence, which arises several uncertainties concerning their future prospects [14]. Relict populations persist geographically isolated in enclaves of benign environmental conditions within a regional climate significantly hotter and dryer than those tolerated by the species [11]. Hence, complex ecological and evolutionary factors determine population dynamics, and points out the need for a proper understanding of the key mechanisms implied in population persistence [14].

In this work two remnant forests of the Moroccan fir (*A. marocana*) were investigated. Recent studies reported increasing mortality induced by drought stress in the relative *A. pinsapo* in south Spain [15]. Our knowledge about drought resilience in *A. marocana* forests is still limited, while useful studies about tree growth and stand dynamics have been recently performed [16]. On the other hand, previous genetic

studies focused on *A. marocana* used molecular markers, such as microsatellites based on single sequence repeats (SSRs), mainly mitochondrial or chloroplast SSR, and single nucleotide polymorphisms (SNPs), aimed to determine genetic diversity and populations' genetic structure [17-20].

The genetic knowledge about Moroccan fir species is still scarce. One of the main reasons is the large size of the conifer genomes and the high number of repetitive sequences [3]. In addition, the absence of reference genomes for most tree species makes challenging these genetic studies [3]. Nevertheless, in recent times, the advances in next generation sequencing (NGS) offer a wide range of possibilities to perform studies with giga-genomes, even without a reference. One of these techniques is the genotyping by sequencing (GBS) using a double digest restriction-site associated DNA (ddRAD-seq) [21]. This technique provides new molecular markers and increases the genetic knowledge of non-model species. In addition, the molecular markers obtained with this technique allow to carry out association studies between single nucleotide polymorphisms (SNPs) and local adaptations to the environment [22].

Despite the above mentioned advances in genotyping techniques, our knowledge on the potential for evolutionary responses to climate change is very limited for most tree species [3,23]. Indeed, there is still no agreement on the relationship between individual genetic diversity and tree growth, with positive relationships [24-26] or no associations [27,28]. Recently, the combination of genetic and dendroecological data has been proposed as a reliable framework to obtain a new class of phenotypes (dendrophenotypes) for genetic associations [29-33]. Through the investigation of tree-rings, dendroecology allows the quantification of climatic constraints that are exerted on trees [11]. Hence, the analysis of the relationships between growth and climate variables enables to assess tree-level sensitivity to climatic constraints, reflecting individual growth-limiting factors [31]. Here, such tree-ring characteristics reflecting climate sensitivity have been used as phenotypic traits in the context of quantitative genetics. Thus, we performed genotype-(dendro)phenotype associations (GPA) to identify genetic regions involved in tree growth patterns and their responses to climate stress [31].

Our specific aims are: (i) to quantify local climate, genetic diversity and secondary growth trends of the relict tree *A. marocana*, considering two nuclei potentially harboring genetic differentiation; (ii) to quantify tree-level climate sensitivity as a phenotypic trait; (iii) to quantify the genetic structure of such contrasting sites and between old trees and saplings, in order to account for spatial (between sites) and temporal (between cohorts) shifts in genetic diversity; (iv) to perform genotype-(dendro)phenotype associations to identify genetic regions involved in long-term *A. marocana* growth patterns and its response to climate stress at individual tree-level.

## 2. Results

### 2.1. Climate and dendrochronology studies

Total annual precipitation was not reduced in the study area over the 20$^{th}$ Century, indeed, the trend obtained for the period 1921-2020 was significantly positive (**Figure S1a; Table 1**), mainly due to a positive trend in winter precipitation. The analysis of the period 1961-2020 depicts contrasting trends in the intra-annual patterns of precipitations, with summer precipitation showing a significant decline, while the rest of seasons showed not significant changes (**Table 1**). By opposite, trends in mean temperature were consistently positive, supporting a warming of the study area (**Figure S1a; Table 1**), mainly during winter and spring, supporting an estimation of about 1.7 °C mean annual temperature rise between 1961 and 2020.

Extreme drought events (**Figure S1b**) increased in frequency toward the last thirty of the 20$^{th}$ Century and the onset of the 21$^{st}$. Hence, except that of 1945, all the extreme drought events registered in the dataset take place after 1983, and six drought events have occurred in a period of roughly two decades, between 1995 and 2016. The growth pattern (BAI time series) was very similar between TA and TZ sites ($r = 0.66$ for the period 1900–2018, $p < 0.01$; $r = 0.78$ for the period 1961–2018, $p < 0.01$; **Figure 1 and Figure S2**). *A. marocana* trees were sampled at similar elevation (around 1700 m, **Table 2**) and old trees showed similar age in both study sites (about 220 years old; **Table 2, Figure 1a**), although mean tree diameter (DBH) was slightly narrower in TZ site (**Figure**

**1b**). No significant differences were observed between mean BAI comparing the periods 1900–2018 (**Figure 1c; Table S1**) and 1961–2018 (**Figure 1f; Table S1**), while BAI trend was positive in TZ and significantly different to TA (almost steady) during the periods 1900–2018 (**Figure 1d; Table S1**). Recent BAI trends (1961–2018) were slightly negative and not different between sites (**Figure 1g; Table S1**), however, growth variability, estimated as the CV, was significantly higher in TZ site all over the studied period (**Figure 1e, h; Table S1**).

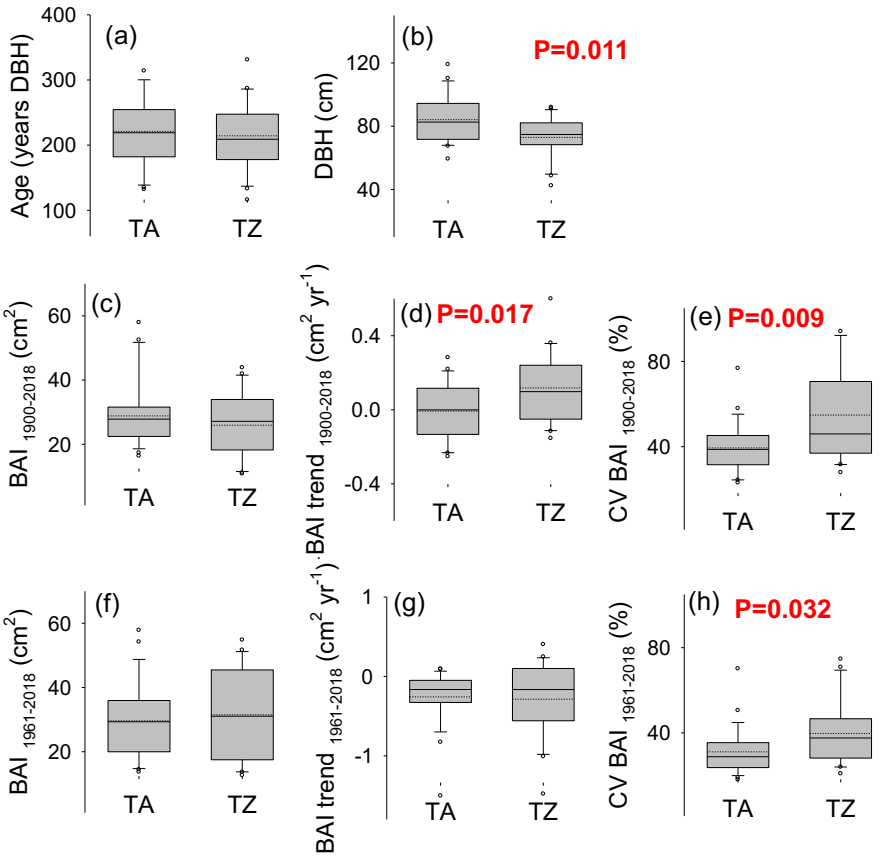


**Figure 1.** Comparison of tree-scale growth patterns estimated as the basal area increments (BAI) of tree-rings obtained in *Abies marocana* for Talassemtane (TA) and Tazaot (TZ). Tree age (a) was estimated at coring height, according to tree-rings dating. DBH (b) indicates the tree diameter at 1.3 m from the ground. Mean growth was computed comparing the periods 1900–2018 (c) and 1961–2018 (f). BAI trend (d, g) was estimated as the slope of BAI against the time. CV (e, h) indicates the coefficient of variation, estimated as (100*) the ratio of the BAI standard deviation divided by the mean. Significant p-values for ANOVA are indicated.

**Table 1**. Temporal trends observed in seasonal and annual total precipitation and mean temperature according to Mann-Kendall statistic for the period 1921–2021 and 1961–2021. Seasons are noted as winter (Wi), spring (Sp), summer (Su) and autumn (Au), including December–February, March–May, June–August and September–November, respectively. Annual estimates consider the hydrological year (prior September to current August). Mean change in each variable over the considered time span was computed, only if the slope was statistically significant, by multiplying the slope estimate (mm or °C year $^{-1}$) per the number of years.

| Time span | | 1921–2021 | | | | 1961–2021 | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | Season | mean (mm, °C) | Mean change (slope*time span; mm, °C) | MK-Stat | p-value | mean (mm, °C) | Mean change (slope*time span; mm, °C) | MK-Stat | p-value |
| Total precipitation | Wi | 468 | 165 | 2.06 | 0.0394 | 521 | ns | -1.23 | 0.2179 |
| | Sp | 291 | ns | 0.76 | 0.4490 | 299 | ns | 0.10 | 0.9207 |
| | Su | 23 | -30 | -2.67 | 0.0076 | 20 | -26 | -2.94 | 0.0032 |
| | Au | 322 | ns | 1.59 | 0.1118 | 337 | ns | 0.58 | 0.5586 |
| | Yr | 1105 | 219 | 2.05 | 0.0406 | 1178 | ns | -0.83 | 0.4044 |
| Mean temperature | Wi | 7.7 | 2.4 | 7.28 | <0.0001 | 8.1 | 1.8 | 4.47 | <0.0001 |
| | Sp | 11.2 | 0.8 | 3.09 | 0.0020 | 11.1 | 2.0 | 5.86 | <0.0001 |
| | Su | 18.5 | ns | -1.24 | 0.2145 | 18.2 | 1.7 | 4.34 | <0.0001 |
| | Au | 14.4 | ns | 1.48 | 0.1392 | 14.4 | 1.5 | 3.81 | 0.0001 |
| | Yr | 12.9 | 0.9 | 3.50 | 0.0005 | 13.0 | 1.7 | 5.71 | <0.0001 |

**Table 2.** Location and growth pattern estimated as the basal area increments (BAI) of tree-rings obtained in *Abies marocana* for Talassemtane and Tazaot. Tree age was estimated at coring height, according to tree-rings dating. DBH indicates the tree diameter at 1.3 m from the ground. CV indicates the coefficient of variation, estimated as (100*) the ratio of the BAI standard deviation divided by the mean. BAI trend was estimated as the slope of BAI against the time. Within-tree BAI autocorrelation was estimated as the first order correlation of the $BAI_{(i)}$ and the $BAI_{(i+1)}$. Among trees BAI inter-correlation was estimates as the correlation of the individual BAI time series with the mean BAI time series. ***** indicates significant differences (ANOVA, $p < 0.05$).

| | Talassemtane | Tazaot | |
|---|---|---|---|
| Latitude (°N) | 35.14 ± 0.006 | 35.26 ± 0.001 | |
| Longitude (°W) | -5.14 ± 0.001 | -5.10 ± 0.001 | |
| Elevation (m a.s.l.) | 1652.80 ± 23.890 | 1722.33 ± 10.423 | |
| Tree age (years) | 221.72 ± 12.274 | 214.95 ± 11.459 | |
| DBH (cm) | 84.44 ± 3.066 | 73.18 ± 2.821 | * |
| BAI mean 1900-2018 ($cm^2$) | 28.97 ± 2.109 | 26.07 ± 2.177 | |
| CV 1900-2018 (%) | 39.60 ± 2.379 | 55.03 ± 5.568 | * |
| BAI trend 1900-2018 ($cm^2 year^{-1}$) | -0.01 ± 0.031 | 0.12 ± 0.042 | * |
| Within-tree BAI autocorrelation 1900-2018 | 0.71 ± 0.028 | 0.76 ± 0.035 | |
| Among-trees BAI intercorrelation 1900-2018 | 0.50 ± 0.033 | 0.49 ± 0.074 | |
| BAI mean 1961-2018 ($cm^2$) | 29.75 ± 2.355 | 31.62 ± 3.124 | |
| CV 1961-2018 | 31.29 ± 2.232 | 39.92 ± 3.343 | * |
| BAI trend 1961-2018 ($cm^2 year^{-1}$) | -0.25 ± 0.069 | -0.28 ± 0.102 | |
| Within-tree BAI autocorrelation 1961-2018 | 0.58 ± 0.038 | 0.68 ± 0.030 | |
| Among-trees BAI intercorrelation 1961-2018 | 0.60 ± 0.039 | 0.53 ± 0.065 | |

Climate sensitivity of the mean growth (mean basal area increment) was similar in both study sites, supporting a significant sensitivity to summer climate conditions (**Figure 2**). Mean BAI showed positive correlations with water availability (SPEI) and negative correlations with temperature of prior and current summer (Sup and Su, respectively; **Figure 3**). Spring (Sp) and year (Yr) were also significant (negative correlations) in both study sites, while SPEI during the prior autumn (Aup) and summer precipitation were significant (positive correlations) only in TA; temperature during the prior autumn was significant (negative correlation) only in TZ.

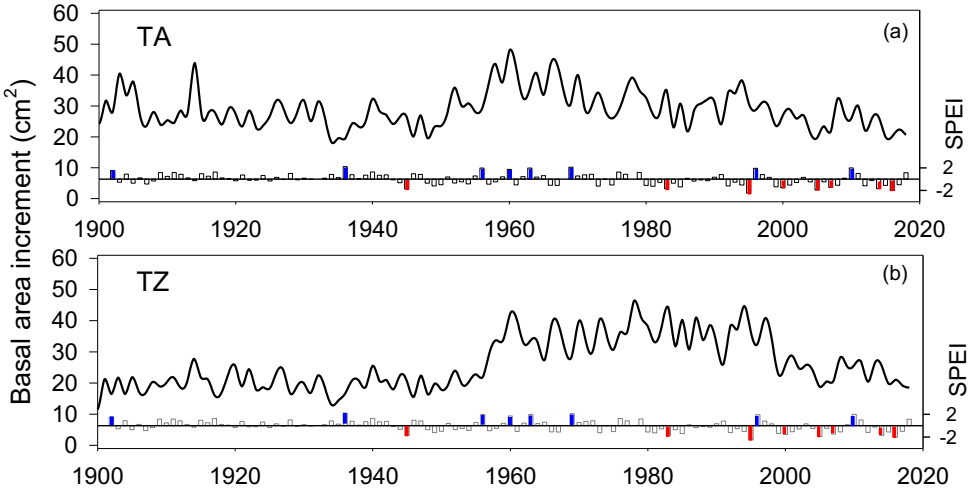


**Figure 2.** Growth pattern estimated as the basal area increments of tree-rings obtained in *Abies marocana* for Talassemtane, TA (a) and Tazaot, TZ (b) over the period 1900–2020. Lines represent the mean. Bottom bars indicate the annual SPEI, where extreme moist (blue) and drought (red) events are highlighted.

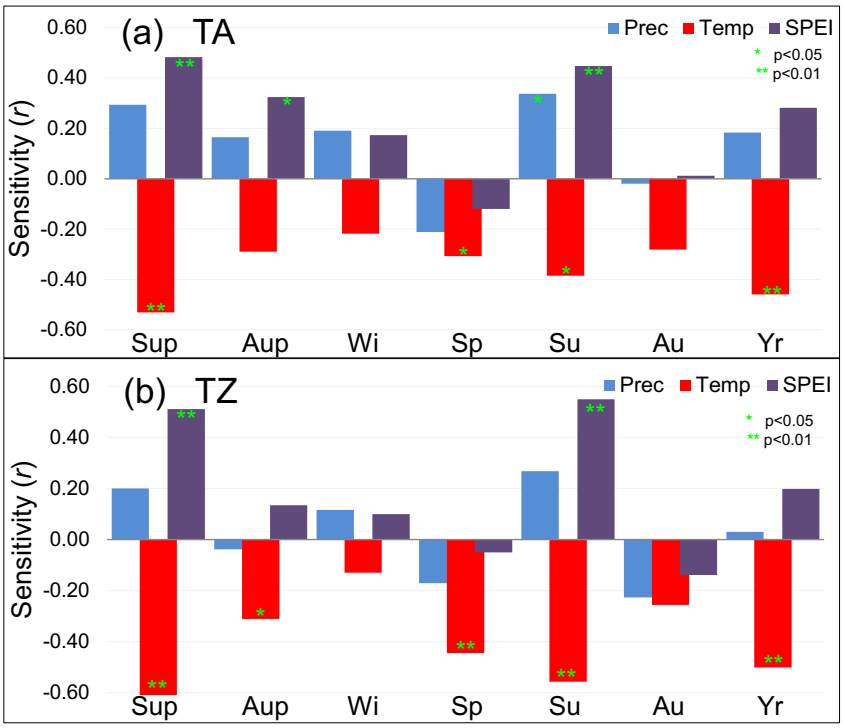


**Figure 3.** Climate sensitivity (correlation) of mean growth (mean basal area increment) for the period 1961-2018 of *Abies marocana* trees from Talassemtane, TA (a) and Tazaot, TZ (b) ranges. Sensitivity was tested at seasonal and annual scales for mean temperature (red), total precipitation (blue) and the SPEI drought index (purple). Seasonal scale was defined by mean (sum) of three-monthly scale temperature (precipitation), while seasonal-scale SPEI data were directly obtained from the database using the available three months estimates (noted as SPEI_3); similarly, annual estimates consider the hydrological year (prior September to current August), and the August estimate of SPEI_12 (Yr). Seasons are noted as winter (Wi), spring (Sp), summer (Su) and autumn (Au), including December–February, March–May, June–August and September–November, respectively. Further

we consider seasonal correlations for summer and autumn of the previous year of tree-ring formation (Sup and Aup, respectively). * and ** indicate significant correlations after Bonferroni correction at a threshold of $p < 0.01$ and $p < 0.05$, respectively.

## 2.2. Genetic structure of populations

The vcf file obtained in the assembly was filtered and a total of 98 individuals and 6,131 SNPs were maintained. PCA showed 2 groups: one formed by Talassemtane and the other one by Tazaot. (**Figure 4**). The percentage of explanation of this PCA was a 19.9 % in the first axis. It is noteworthy that the individuals from Talassemtane displayed high dispersion while those of Tazaot were more similar to each other. The cross-entropy analysis indicated that K = 2 is the best explanation for the genetic structure of these populations and admixture study corroborated this result.

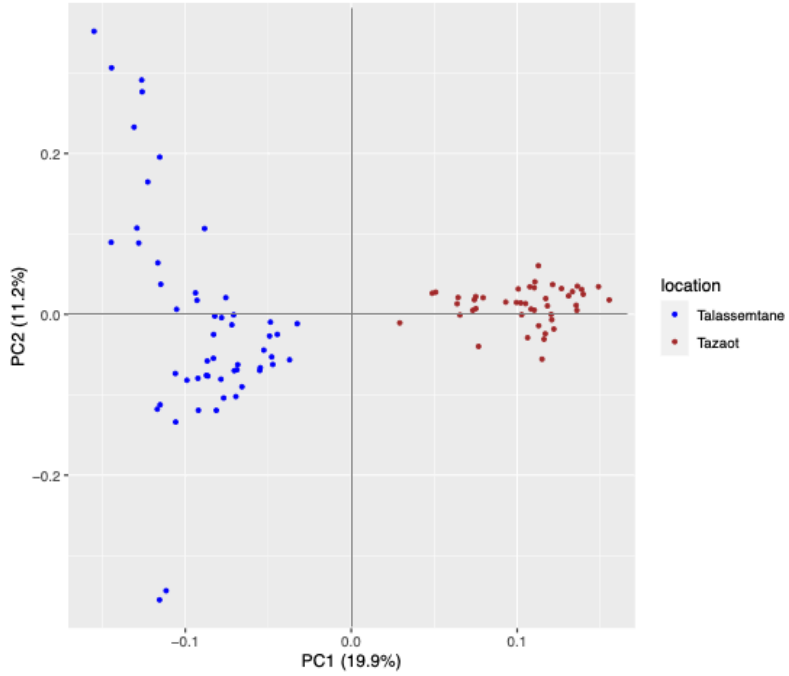


**Figure 4**: PCA results with Moroccan populations. Talassemtane site is colored in blue and Tazaot in brown. It is shown the axis of the two principal components (PC) and the percentage of each axis.

However, the AMOVA (**Table S2**) indicated low differences among populations, with a 2 % of the total, while the highest differences were observed within individuals, 57 %. These results are based on the $F_{ST}$ value (0.018), which showed that there are no differences between populations with a high significance ($p = 0.0001$). The number of

private alleles was higher in Tazaot than Talassemtane. Shannon indexes values were low but slightly higher in Tazaot. For Talassemtane and Tazaot the grand mean values of number of effective alleles are 1.524 and 1.528, respectively. Nei's genetic distance (0.021) indicates that both populations are very close. Finally, Nm value (13.393) shows the presence of a high gene flow among populations which reduces the population differentiation. Therefore, both populations might behave as in panmixia.

### 2.3. Selection signatures

The dataset showed 20 *loci* under selection. The 10 most significative SNPs were used to find homologies. 8 matches were obtained for nucleotide sequences and 5 of them were similar to some protein (**Table S3**). Among them, a peroxidase 5-like protein is the most interesting one, which is involved in environmental stress and oxidative stress responses. To assess the differences between old trees and saplings in the Moroccan dataset, another BayeScan study was carried out. In this case, 72 *loci* under selection were obtained of which 20 are the same as those obtained in the BayeScan analysis with populations of Morocco without age separation. The 16 sequences most significative, of which 9 were used to find matches with Moroccan populations previously, were queried against nucleotide database and 14 matches were found. Finally, 7 of them could be defined at protein level (**Table S4**).

### 2.4. Genotype-phenotype associations (GPA)

The 17 dendrophenotype variables related to growth which were used to perform this study are: age, DBH, BAI mean 1900–2018, BAI trend 1900–2018, BAI autocorrelation 1900–2018, CV 1900–2018, BAI intercorrelation 1900–2018, Growth mean 1970–2018, Growth trend 1970–2018, BAI autocorrelation 1970–2018, CV 1970–2018, BAI intercorrelation 1970–2018, BAI mean 1984–2016, Growth mean before 1980–1983. GPA study showed 26 associations with 6 of these 17 dendrophenotype variables related to growth trend, which were: CV 1900–2018, CV 1970–2018, DBH, BAI

mean 1984–1986, BAI mean 2004–2005, and BAI trend 1970–2018. BAI mean 1984–1986 was associated with 9 *loci*, CV 1900–2018 with 8 *loci*, BAI mean 2004–2005 with 4 *loci*, DBH and BAI trend 1970–2018 with 2 *loci* and CV 1970–2018 with 1 *locus*. It is remarkable the presence of several *loci* which are associated to more than one variable. This is the case of CV 1900–2018 and BAI mean 1984–1986 which shared 3 *loci*, and CV 1900–2018 and CV 1970–2018 shared 1 *locus*. A total of 7 associations were identified with 1 resilience variable which was stress drought recuperation in 1999.

In terms of homology results, 16 matches were found against the nucleotide database and 5 of them obtained hits for protein homology. Some of the proteins identified are related to ethylene biosynthesis (SNP 3192), or RNA binding (**Table S5**). Several allele frequencies of these SNPs associated showed differences between sites (**Figure 5**). Climatic sensitivity dendrophenotypes did not show associations with any SNPs of the genetic matrix.


**Figure 5**: SNPs which showed associations with dendrophenotypes variables, specifically with DBH (SNP 1732), and CV BAI 1900-2018 (SNP 2712, SNP 3192, SNP 3996, SNP 5332). It is shown the percentage of each allele: blue for the reference allele and grey for the alternative. The fixation index ($F_{ST}$) and migration rate (Nm) between the two sites are indicated above and below the arrow.

## 3. Discussion

Lacks of genetic differences between sites are supported by intra-specific synchrony in growth patterns, although growth variability (CV) was significantly related to SNPs subjected to selection. Synchrony in growth response to climate (that is, climate sensitivity) supports similar climate-induced selective pressure in old trees over the study area [31]. Our results also suggest an increasing growth synchrony among trees during the last century (here estimated by the among-trees BAI inter-correlation), according to increasing warming, as has been recently reported over wide spatial and temporal scales (see [34] and references there in). Intra-specific synchrony in annual tree growth, both between- and within–sites, reflects a species-specific dependence on environmental variability in *A. marocana*. Here, TA and TZ mean growth shows a correlation of 0.78 for the period 1961-2018, while mean correlations among neighboring trees were 0.60 and 0.53 in TA and TZ, respectively, for the period 1961-2018. This covariance of the year-to-year variability in growth of individual neighboring trees should be expected to intensify due to climate warming and drying conditions, supporting that within-population growth synchrony may be used as an integrative ecological measure of environmental stress, as well as to forecast effects of global climate change on tree growth [34, 35].

It is widely acknowledged that tree-ring analysis provides past forest growth conditions at annual resolution, as well as several important insights on trees sensitivity and adaptive capacity [9-11]. However, studies with natural populations which combine genomic and dendrophenotype data at individual level are still scarce [i.e., 29-33, 36]. Nevertheless, some results indicate that susceptibility to drought could be determined at the genomic level. For instance, the analysis of dendroecological and genetic data (SNPs in candidate genes) from *Abies alba* Mill., revealed that dendrophenotypes can be a powerful resource for genetic association studies [29]. Hence, fifteen genes were associated with the dendrophenotypes, including genes linked to photosynthesis and drought stress [29]. Also, by combining SNPs and dendrochronology, a study performed in *Nothofagus dombeyi* (Mirb.) Blume forests from northern Patagonia provided genetically-based individual tree vulnerability to drought [30]. Indeed, this study found

a set of 33 adaptive SNPs by comparing healthy and drought-induced declining trees, 8 of which were related to water stress. Further, association analysis between genomic variants and dendrophenotypic traits yielded 6 SNPs that were associated with growth patterns [30]. Another study, combining dendrochronology and population genetic analyses performed in *Nothofagus macrocarpa* [(DC.) Vásquez et Rodr.], found no relationship between growth patterns and genetic diversity [33]. In this case, tree genetic variability was estimated by nuclear microsatellite markers, which might be reflecting the limitations of methods such simple sequence repeats (SSR) markers to account for genotype-phenotype associations [27,28]. Nonetheless, the use of individual tree-level dendrophenotypes in genetic association studies would greatly benefit our understanding on the response of trees to environmental stressors over time [31].

As regards the relict forests of *A. marocana,* dendrochronology has revealed past forest growth conditions, providing significant clues on forest dynamics [16]. By opposite, genomic studies on Moroccan fir were mainly focused on diversification, genetic diversity and inter-specific relationships [i.e., 20, 37, 38]. Notwithstanding, the ability to identify genes or genomic regions related to adaptation to climate requires the evaluation of traits, such climate-growth sensitivity, that precisely reflect the extent to that climate exerts a selective pressure [31, 36]. Hence, the development of new techniques based on NGS technology, such as ddRAD-seq, allows to develop new molecular markers and describe the genetics of this species. To our knowledge, this work is the first combining ddRAD-seq data and dendrophenotypes on this tree species. It is highlighted that our study was carried out using a high number of new molecular markers, developed in the same study. These findings contrast to previous studies, which used molecular markers described formerly in other related species [17-20,38].

The taxonomy of the two studied sites has been under debate, sometime stating the existence of two species, namely *A. marocana* and *A. tazaotana* in TA and TZ, respectively [12]. Our results support that the Moroccan fir populations act as one population, meaning that these nuclei are in panmixia. Notwithstanding, the PCA and cross-entropy analyses have distinguished two groups, suggesting some genetic

differences among them. Previous studies using SSRs concluded that there is no evidence to distinguish *Abies tazaotana* at the species level [19, 20]. Recently, a genomic study based on RAD-seq technology, including several species of the *Abies* genus, also concluded that the Moroccan fir represents a single species [37].

TA showed a high scattering in the PCA which could be due to the existence of a gradient among sampled trees of about 4 km, where elevation ranges from around 1600 m to near to 1800 m, while the study site of TZ was always close to 1700 m in elevation and the distance among sampled trees was not much more than 1 km. Nevertheless, both sites showed similar climate sensitivity, supporting comparable environmental conditions and pointing out the relevance of the summer conditions as the main limiting season for drought-sensitive trees [9]. Hence, tree BAI showed positive correlations with water availability and negative correlations with temperature of prior and current summer, which is precisely the season where warming trends are expected to become worsen within the Mediterranean basin [7]. This agreement between observed climate sensitivity and expected climate trends enhances the threaten condition of relict *A. marocana* forests, as has been already stated for the whole circum-Mediterranean firs and *A. alba* across its southern distribution limits in Spain, Italy, and Romania [10,11].

Summer drought is the main factor impairing tree radial growth, reducing productivity, and triggering tree defoliation and mortality events in the Mediterranean forest ecosystems [4,8,15]. However, tree-level responses to drought vary according to specific physiological characteristics and local adaptations [1-3,13]. Indeed, several SNPs associated with growth variables showed contrasting allele frequencies in TA and TZ, respectively (Figure 5). These differences suggest that a given allele may be more advantageous for one population than the other, which might be related to local conditions. Nonetheless, both *A. marocana* study sites were sensitive to summer drought, as they reduce their radial growth according to summer water shortage (Figure 3).

Despite it is expected that warming and extreme climate events such as droughts will increase as a consequence of forecasted climate change [7], *A. marocana* harbor a

significant genetic diversity [17-20,38]. According to BayeScan results, it is noteworthy the large amount of *loci* under selection. Regarding the results obtained by comparing saplings and old trees, it is remarkable that this dataset gave a higher number of *loci* under selection than the global one. So, there are significant differences among old and juvenile trees. This contrasting selection signatures may be reflecting the fact that the studied saplings could have been subjected to a selective pressure, as they have been stablished under a warmer and dryer climate scenario (Table 1), compared to old trees, mainly stablished around the onset of the 19$^{th}$ century.

We found two SNPs under selection present in both study sites and both age groups that depict homology with a peroxidase 5-like protein and LRK1 protein. The peroxidase function is related to several responses to stress, such as drought, oxidative damage or pathogen defense. Several studies reported that the activity of peroxidases is affected by drought stress, increasing its activity in the conifers needles to reduce the damage of reactive oxygen species (ROS) [39, 40]. As peroxidases are often up regulated under drought conditions, the obtained SNP could be relevant for the survival of *A. marocana* in the upcoming drier climate scenario. On the other hand, LRK family is involved in development, pathogen defense, and abiotic stress responses [41]. Despite we evaluated several traits that precisely reflect drought-induced selective constraints, many of these *loci* under selection did not match with the protein database checked by us. This shortcoming illustrates the need to dedicate more research efforts to take access, for instance, to the sequenced genome of more conifer tree species and to identify genes or genomic regions related to adaptation to climate [23,31].

Contrary to our expectations, we do not obtained associations between the dendrophenotypes defined by climatic sensitivity and SNPs, according to the GPA. It should be expected to find associations among individuals' sensitivity to climate variables and the genome-wide single nucleotide polymorphisms. Indeed, several evidences reported here and by previous studies illustrate the extent to that temperature and precipitation drive tree growth and stand dynamics in *A. marocana* and relative species [15,16]. Then, the absence of associations with tree-level climate

sensitivity might be due to the significant growth synchrony and shared climate sensitivity found overall among the sampled trees (**Figures 2 and 3**).

On the other hand, variables related to growth trends did not show associations in the GPA, while tree size and growth variability did. Stem diameter (DBH) increases steadily over a tree's lifespan, whereas disentangling the effects of stem size and age on growth is challenging, as both increase together [42,43], nonetheless, some studies suggest that growth is most strongly influenced by tree size, specifically, supporting that large trees are more sensitive to drought [2]. Here we obtained 2 associations with DBH but none with age supporting a size-effect. Previous studies have shown also differences in growth responses to drought mainly explained by tree size [33]. Moreover, the variability (here estimated by the CV) in tree growth provides an additional piece of evidence about tree growth response to stress related to the SNPs analyzed. Identifying how growth variability influences drought sensitivity is not straightforward [44]. When growth variability is driven by inter-annual climate fluctuations, we can expect a larger vulnerability to drought with the increase in growth variability, as can be observed in gymnosperms from dry regions whose growth is severely constrained by drought [4]. This is also supported by the negative relationship found between BAI trend and its CV during the period 1970-2018 ($r=-0.73$, $p<0.001$), as well as previous studies reporting a negative relationship between mean BAI and growth variability [44]. The variability in growth may be also related to land-use legacies such logging and pollarding, which poses an intriguing question regarding the observed relationship between tree genotypes, that is, the differential SNPs obtained according to growth variability, and forests management. On summary the complex picture found in this study suggests that further studies considering how growth variability is related to vulnerability to drought and land-use legacies, at the individual level, are clearly required [4-6,14].

All in all, the two nuclei studied here depict a single population from a genetic point of view, although some differences can be stated (Figure 4). Regarding the selection signatures, our results support enhanced selective pressures within the saplings group. Besides, our results support a negative effect of rising temperature and drought events in the secondary growth of old *A. marocana* trees, as has been also

reported worldwide from several drought-sensitive forests [4-6]. Tree growth decline and dieback are predicted to increase even further, while understanding the genetic basis of tree adaptive capacity to changing environments is essential to the conservation of relict and endangered species [14,23]. Here, the combined analysis of genome-wide single nucleotide polymorphisms and dendrophenotypes provided genetically-based evidences of tree-level sensitivity to climate, as well as preliminary evidences of climate change-induced selection in sapling cohorts. Nonetheless, the molecular mechanisms of adaptation remain largely unknown for long-lived tree species [23]. Finally, we would point out future research lines, such the newly developed methods to quantify the risk of non-adaptedness, which can predict the genetic offset or genomic vulnerability of species via allele frequency change under multiple scenarios of climate change [36].

## 4. Materials and Methods

### Study sites

The study was carried out in the mountain range of Talassemtane and in Jebel Tazaot (both included in Talassemtane National Park). The range occupied by this fir in Talassemtane (TA) is around 3,760 ha, while the range of Tazaot (TZ) extends over approximately 300 ha, where fir grows mostly on northern slope [45-47].

*A. marocana* is a monoecious species which appears in the high peaks between 1,500 and 2,000 m a.s.l. [12] and it is present in humid and perhumid areas, being a drought-sensitive species [13,42]. In the lower elevation limits of dense *A. marocana* forests the vegetation is Mediterranean, dominated by *Quercus rotundifolia* Lam. (holm oak), *Quercus faginea* Lam. (gall oak) and *Pinus pinaster* Ait. (Maritime pine), *Pinus halepensis* Mill. (Alepo pine) forests. Upslope, *A marocana* is the dominant tree to roughly 1,700 m, where it grows with other relict trees, such as *Acer granatense* Boiss. (Spanish Maple), *Taxus baccata* L. (yew), *Cedrus atlantica* Manetti (Atlas cedar), and *Pinus nigra salzmannii* (Dunal) Franco (laricio pine).

The Moroccan fir is endemic and relict, limited to the Rif mountains of North Morocco; this fir is included in the IUCN Red List of Threatened Species as endangered species [48] and one of the most serious hazards for its survival is climate change. Soils are usually shallow, rocky, and developed on limestones.

The mean temperature range in the study area goes from 12 to 14 °C, with a maximum of 33 °C, and a minimum of 0 °C, reaching −3 °C at high elevation. The mean annual precipitation is above 500 mm and can exceed 2,000 mm at high mountain peaks [49]. As a whole, the rainfall patterns are distinctly Mediterranean, with approximately 90 % of all precipitation falling between October and April, followed by a long summer drought.

Climate data

To quantify climate-growth relationships, monthly mean temperature (T, units in ºC; 0.25° resolution) were downloaded from the EOBS database v23.1e for the period 1985–2020 [50] using the Climate Explorer webpage (https://climexp.knmi.nl/). To quantify drought severity, we used the Standardized Precipitation Evapotranspiration Index (SPEI) [51]. Climate variables were estimated based on monthly, seasonal, and annual estimate of mean temperature, total precipitation, and SPEI. In order to consider seasonal variations of BAI sensitivity to climate, the climatic variables were aggregated at the three-monthly scale, thus defining four seasons, winter, spring, summer and fall, as December-February, March-May, June-August and September-November, respectively. Further we consider these seasonal correlations for summer and autumn of the previous year of tree-ring formation to test the effect of prior climate conditions on current growth. Finally, the effect of yearly average was also tested. Note that winter was not discarded from the analysis as growth and photosynthesis can be substantially influenced by climatic conditions during this period and are often ongoing at hotter years. Temporal trend of seasonal/annual mean temperature and total precipitation were estimated using the Mann-Kendall test [52, 53].

Field sampling and dendrochronological methods

Field sampling was carried out at Talassemtane (TA, 35.14 N, -5.14 W, 1,653 m a.s.l.) and Tazaot (TZ, 35.26 N, -5.10 W, 1,722 m a.s.l.) ranges, which are home to natural unevenly aged *Abies marocana* stands that have not been intensively managed since the middle 20$^{th}$ Century [12]. (**Figure 6**).

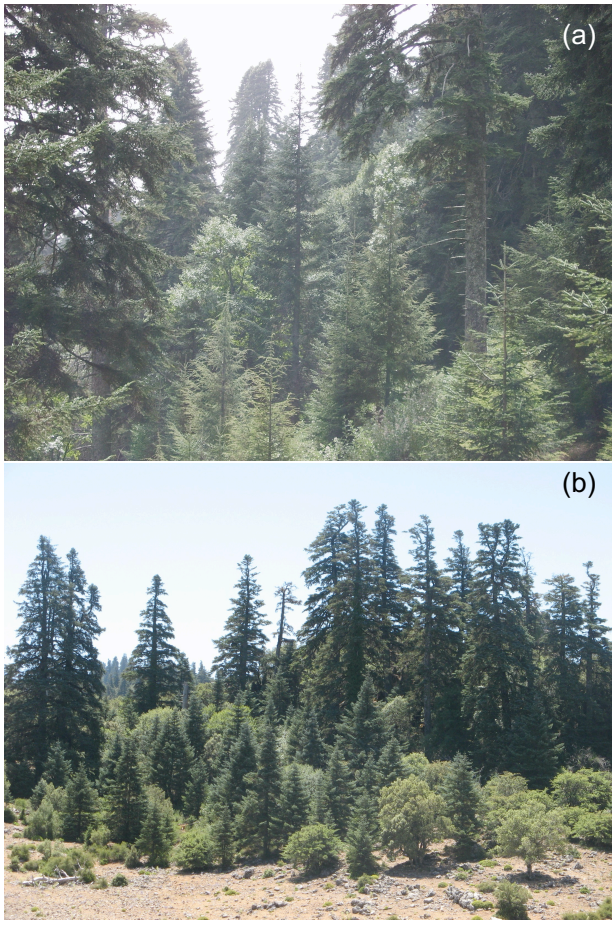


**Figure 6.** High tree species diversity in *Abies marocana* forest of Talassemtane (a) and Tazaot (b) ranges, north Morocco. Photo: Juan Carlos Linares.

In late autumn 2018 we sampled 25 mature, dominant, and healthy old trees (wood cores and needles), and 25 close saplings (needles) in TA site, and 20 old trees and 20 saplings in TZ site. Old trees were cored to the pith at breast height perpendicularly to the slope. Each tree was measured for trunk circumference and bark thickness. Bored cores were 5 mm in diameter; at least three cores per tree were extracted, and pith was reached in at least one of each set. Dendroecological analysis was conducted for all cores for age determination and radial growth measurement.

The cores were sanded until the tree rings were clearly visible under a binocular microscope. All samples were visually cross-dated. Tree ring widths were measured to 0.01 mm using a LINTAB measuring device (Rinntech, Germany), and the cross-dating quality was checked using COFECHA [54]. Growth patterns were estimated as the basal area increments (BAI) of tree-rings, for a more accurate reflection of annual radial growth around the circumference of the tree. Tree age was estimated at coring height (roughly 1.3 m from the ground), as well as the tree diameter (DBH). The coefficient of variation (CV) was estimated as the ratio of the BAI standard deviation divided by the mean and expressed in %. BAI trends were estimated as the slope of BAI against the time. Within-tree BAI autocorrelation was estimated as the first order correlation of the BAI(i) and the BAI(i+1). Among trees BAI inter-correlation was estimates as the correlation of the individual BAI time series with the mean BAI time series. Differences between sites for tree age, DBH, mean BAI, CV, BAI trend, within-tree BAI autocorrelation, and among-trees BAI intercorrelation were compared by ANOVA using a significance level of $p < 0.05$. Climate sensitivity was estimated by the Pearson correlation coefficient (*r*), with significance corrected using Bonferroni adjustment [55]. All the analyses were calculated using R software [56].

DNA extraction and ddRAD-seq

Fresh needles samples were collected in the same old trees and close saplings from the above mentioned dendrochronological sampling. Then, 100 mg of each sample were lyophilized, and total DNA extraction was carried out by using DNeasy Plant Mini Kit (Qiagen®, Germany) following the manufacturer's instructions with some modifications. DNA concentration was measured on a NanoDrop™ spectrophotometer and the integrity of the samples extracted was determined by an electrophoresis in 1 % agarose gel.

Subsequently, ddRAD-seq [21] libraries were constructed using *Ape*KI/*Pst*I double digestion and sequenced by LGC Genomics (Germany). ddRAD-seq is a NGS technique which can be used with large genomes and in absence of a reference genome. This technique allows us to obtain high number of molecular markers, especially single

nucleotide polymorphisms (SNP) and to carry out several kinds of studies such as genome-environment and genotype-phenotype association studies [21]. One of the main advantages of using restriction enzymes is the reduction of the genome complexity due to the fragmentation. *Ape*KI (5'G/CWGC3'), a methylation-sensitive enzyme, is blocked by overlapping CpG methylation, which implies that it avoids cutting the methylated regions present in the genomes. As the presence of repetitive sequences in conifers genomes is very common, and generally heavily methylated, the use of this enzyme reduces the presence of this kind of sequences in ddRAD-seq libraries. On the other hand, *Pst*I (5'CTGCA/G'3) is not sensitive to methylation but it is a rare cutter enzyme. Therefore, the combination of both enzymes increases representation of coding regions [57] and, subsequently, the possibility to sequence the same fragments in different samples.

The reads obtained were paired-ended with a depth of 1 M. FastQC v0.11.9 [58] was used to determine the quality of the raw reads. Subsequently, adapter sequences were trimmed and those sequences which were low-quality, were removed using fastp v0.12.4 [59]. A *de novo* assembly was carried out, and the SNP callings were performed using ipyrad v.0.9.65 [60]. Several filter steps were carried out until the obtention of the genetic matrix. All filtrations were performed using VCFtools v0.1.16 [61] with the aim to retain those SNPs which were biallelic, with a minimum allele frequency (MAF) of 5 %, maximum missingness of 50 %, and to avoid the linkage disequilibrium, we retain only 1 SNP per *locus*. Individuals with less than 50 % of the filtered SNPs were removed. Once the SNP matrix was created, several analyses were carried out using it.

Genetic structure of populations

Two approaches to study the genetic structure of the populations were performed: principal component analysis (PCA) and sparse non-negative matrix factorization analysis (sNMF). PCA was carried out using the plink2 2.00a2.3 software [62] with the --pca option and then, a graphic representation was obtained using the R v4.1.2 [56] package called ggplot2 v3.3.5 [63]. For the second approach, a cross-entropy study was carried out using the snmf function of the LEA package v3.4.0 [64] in R

software v4.1.2 [56] with the aim to obtain the most probable number of ancestry populations (named K) that best explains the structure of the populations. For this analysis, a total of 10 repetitions for each K ranging 1 to 4 was performed. In addition, the admixture coefficients were obtained and then employed to obtain a graphic representation of the admixture results using pong [65]. Several statistical analyses were performed using the GenAlEx v6.5 software [66, 67] Parameters such as fixation indexes ($F_{ST}$), to determine population differentiation, and migration rate (Nm) to describe presence or not of gene flow among populations, were estimated. The Nei's genetic distance between populations was also estimated. Shannon index was inferred to describe genetic diversity. In addition, $F_{ST}$ coefficient was used to perform a molecular variance analysis (AMOVA) with the aim to determine the proportion of genetic variation attributable to differences among and within populations. A total of 9,999 permutations were used to carry out the AMOVA.

Selection signatures

Selection signatures in the genetic matrix were detected using BayeScan 2.1 software [68]. Default parameters were applied. This software calculates the $F_{ST}$ coefficients for each *locus* and compare them among and within the populations of study with the aim to determine differences. This version of BayeScan directly calculates q-values using the false discovery rate (FDR) correction. In this study the threshold was established in 5%, so all SNPs with a q-value < 0.05 were significant to be under selection. To know more about the genetic variants under selection, the sequences which contain the SNPs were queried against the nucleotide database, and in the case of the matrixes obtained from reference assembly, against transcriptome shotgun assembly (TSA) database using in both cases BLASTn (NCBI) [69]. When a match was obtained, the sequence was used to perform a BLASTx (NCBI) [70] against the non-redundant protein database, with the aim to gain knowledge about the protein which could be derived from the SNP scaffold.

Genotype-phenotype associations (GPA)

To determine the existence of associations between dendrophenotypes and the SNPs obtained, a genome-phenotype associations study was carried out. Since the phenotype data was only available for old trees, a reduced genetic matrix consisting of 44 individuals and 6,029 SNPs was used for GPA. The variables studied were described in the Field sampling section and dendrochronological methods (**Figure 7**). The rrBLUP v4.6.1 R package was used to perform GPA [71, 72]. To obtain q-values, FDR correction was applied to p-values estimated in the GPA, and only those associations with a significance of 5 % were used to find homologies with nucleotide sequences and proteins in NCBI databases.

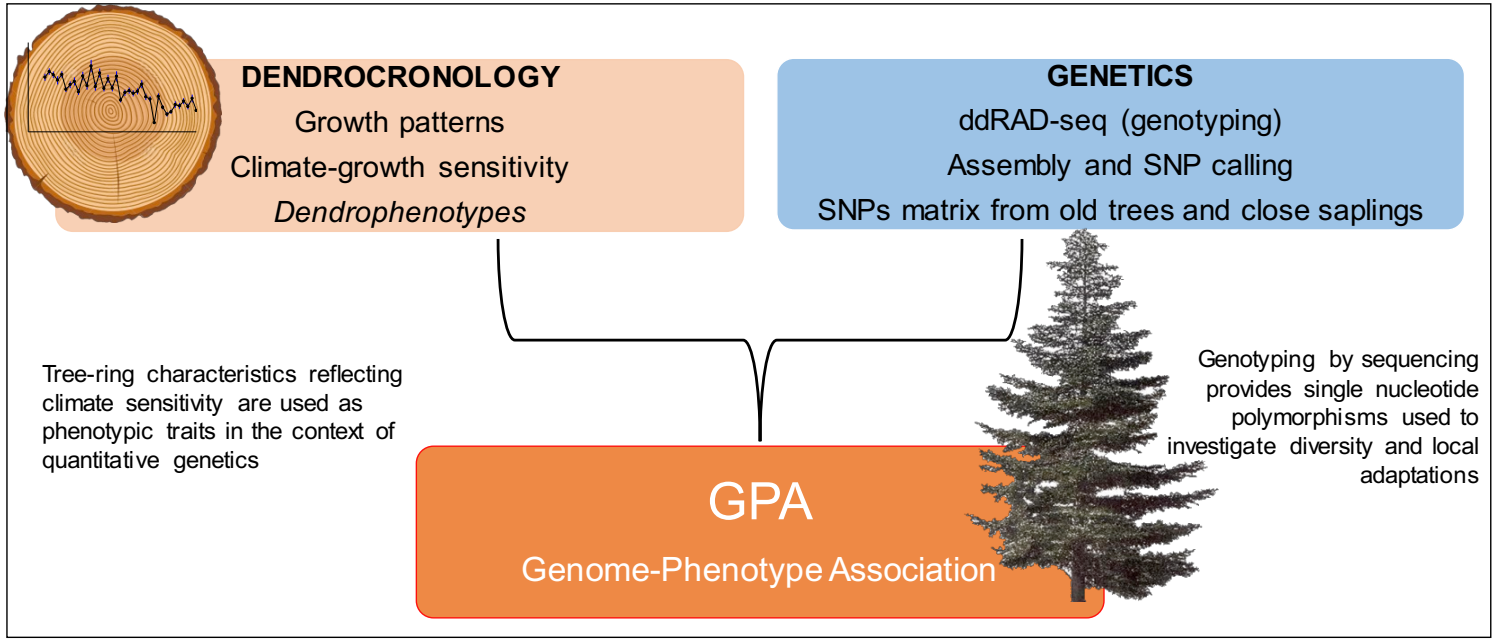


**Figure 7.** Framework of the study combining dendrochronological methods applied to quantify growth patterns and climate-growth sensitivity and the genotyping technique called ddRAD-seq, performed in the same trees and close saplings to obtain single nucleotide polymorphisms (SNPs) and related genotype-phenotype associations.

**Supplementary Materials**: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1

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## Apéndice 3: Material suplementario

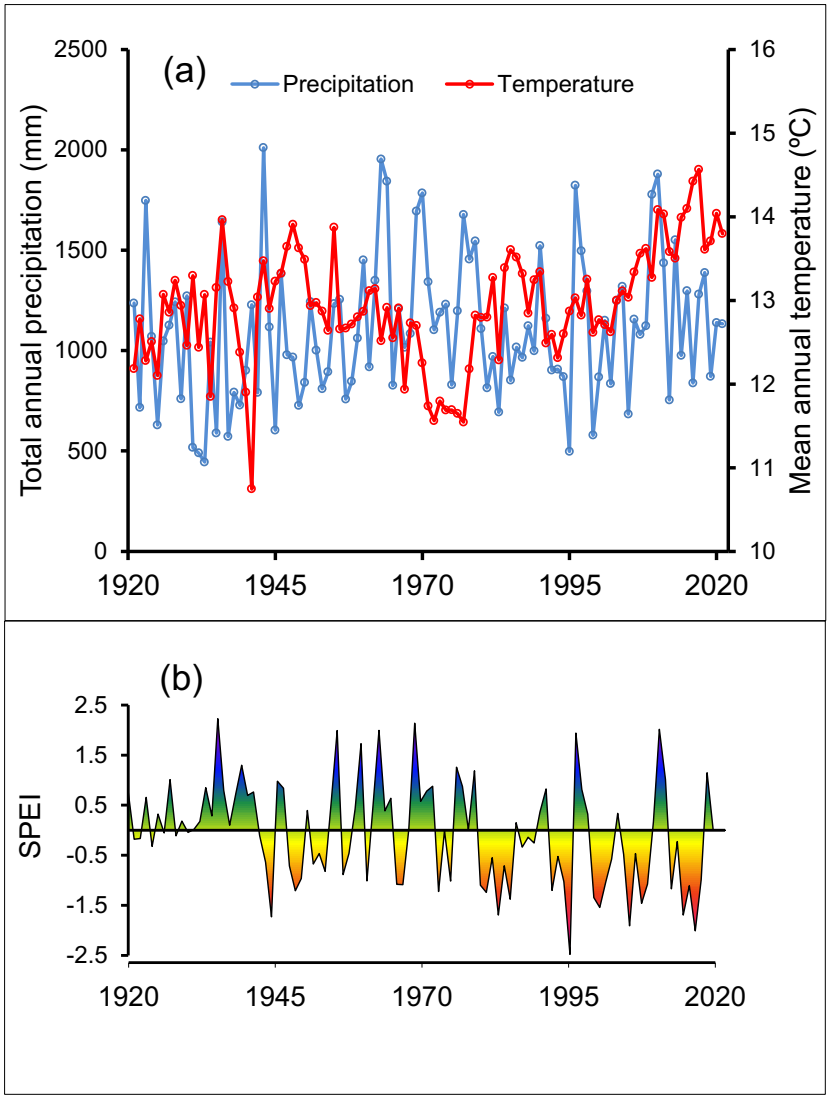


**Figure S1.** Mean temperature and total precipitation (a), and SPEI (b) data of the study area for the period 2021-2020.

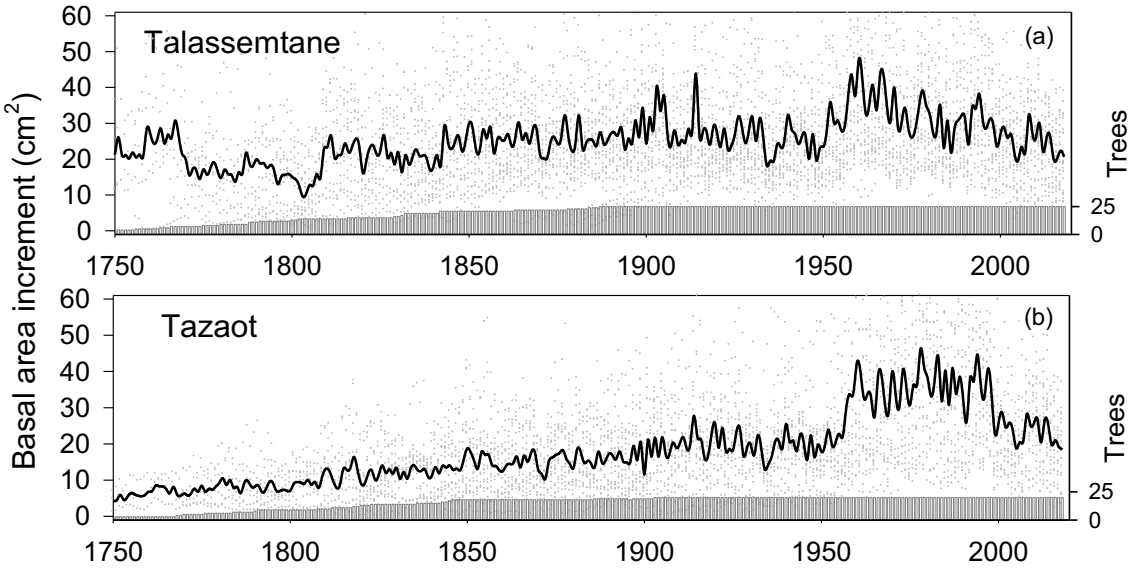


**Figure S2.** Growth pattern estimated as the basal area increments of tree-rings obtained in *Abies marocana* for Talassemtane (a) and Tazaot (b). Lines represent the mean while points indicate raw measurements. Bottom bars indicate the sample size (number of trees with growth data for this year).

**Table S1**. Results obtained by ANOVA comparing tree age, size, and growth patterns in Talassemtante and Tazaot populations.

| | | Intercept | Population | Error | Total |
|---|---|---|---|---|---|
| Degree of Freedom | | 1 | 1 | 43 | 44 |
| Age | SS | 2118674.00 | 509.00 | 140284.00 | 140793.00 |
| 1900-2018 | MS | 2118674.00 | 509.00 | 3262.00 | |
| | F | 649.418 | 0.156 | | |
| | p | 0.000 | 0.695 | | |
| DBH | SS | 276039.100 | 1410.600 | 8663.800 | 10074.400 |
| 1900-2018 | MS | 276039.100 | 1410.600 | 201.500 | |
| | F | 1370.039 | 7.001 | | |
| | p | 0.000 | **0.011** | | |
| BAI mean | SS | 33663.850 | 93.010 | 4469.080 | 4562.090 |
| 1900-2018 | MS | 33663.850 | 93.010 | 103.930 | |
| | F | 323.903 | 0.895 | | |
| | p | 0.000 | 0.349 | | |
| BAI trend | SS | 0.146 | 0.175 | 1.227 | 1.402 |
| 1900-2018 | MS | 0.146 | 0.175 | 0.029 | |
| | F | 5.114 | 6.145 | | |
| | p | 0.029 | **0.017** | | |
| BAI autocor | SS | 23.960 | 0.035 | 0.958 | 0.993 |

| | | | | | |
|---|---|---|---|---|---|
| 1900-2018 | MS | 23.960 | 0.035 | 0.022 | |
| | F | 1075.540 | 1.555 | | |
| | p | 0.000 | 0.219 | | |
| CV | SS | 99509.010 | 2643.860 | 15177.010 | 17820.880 |
| 1900-2018 | MS | 99509.010 | 2643.860 | 352.950 | |
| | F | 281.932 | 7.491 | | |
| | p | 0.000 | **0.009** | | |
| BAI intercor | SS | 10.858 | 0.003 | 2.732 | 2.735 |
| 1900-2018 | MS | 10.858 | 0.003 | 0.064 | |
| | F | 170.885 | 0.047 | | |
| | p | 0.000 | 0.830 | | |
| BAI mean | SS | 41839.350 | 38.800 | 7034.000 | 7072.800 |
| 1961-2018 | MS | 41839.350 | 38.800 | 163.580 | |
| | F | 255.771 | 0.237 | | |
| | p | 0.000 | 0.629 | | |
| BAI trend | SS | 3.162 | 0.010 | 6.847 | 6.856 |
| 1961-2018 | MS | 3.162 | 0.010 | 0.159 | |
| | F | 19.859 | 0.061 | | |
| | p | 0.000 | 0.806 | | |
| BAI autocor | SS | 17.663 | 0.093 | 1.197 | 1.290 |
| 1961-2018 | MS | 17.663 | 0.093 | 0.028 | |
| | F | 634.488 | 3.332 | | |
| | p | 0.000 | 0.075 | | |
| CV | SS | 56353.260 | 827.310 | 7235.590 | 8062.890 |
| 1961-2018 | MS | 56353.260 | 827.310 | 168.270 | |
| | F | 334.899 | 4.917 | | |
| | p | 0.000 | **0.032** | | |
| BAI intercor | SS | 14.295 | 0.061 | 2.538 | 2.599 |
| 1961-2018 | MS | 14.295 | 0.061 | 0.059 | |
| | F | 242.177 | 1.027 | | |
| | p | 0.000 | 0.317 | | |

**Table S2**: Results obtained by AMOVA studies with Talassemtante and Tazaot populations. The rows indicate the partitions genetic variability in two components: among and within populations. The columns show degrees of freedom (df), sum of squares (SS), mean squares (MS), estimate of variance (Est.var.) and the percentage of total variation (%). The percentage of variation is higher within individuals than among populations.

| Summary AMOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | **df** | **SS** | **MS** | **Est. Var.** | **%** |
| **Among Pops** | 1 | 4515.948 | 4515.948 | 26.029 | 2% |
| **Among Indiv** | 96 | 190280.027 | 1982.084 | 587.692 | 41% |
| **Within Indiv** | 98 | 79056.500 | 806.699 | 806.699 | 57% |
| **Total** | 195 | 273852.474 | | 1420.421 | 100% |

**Table S3**. Results of the matches found in the alignments against protein database with those *loci* which were significant in BayeScan analysis with Moroccan fir populations. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **1127** | Transcribed RNA sequence *Abies pinsapo* | Formyltetrahydrofolate deformylase 1 (mitochondrial) | Biosynthesis of purines<br>Metabolism of amino acids | 2e-104 |
| **1369** | mRNA *Picea glauca* | Disease resistance protein | Pathogen response (viruses, bacteria, or fungi) | 4e-58 |
| **2458** | mRNA *Picea glauca* | Peroxidase 5-like | Response to environmental stress, pathogen, and oxidative stress<br>Auxin catabolism<br>Suberization | 5e-65 |
| **5262** | mRNA *Pinus taeda* | Clavata 1-like protein | Cell differentation<br>Meristem structure regulation<br>Peptidyl-serin autophosphorylation | 8e-09 |
| **5594** | mRNA *Picea glauca* | LRK1 | Protein phosphorylation | 2e-151 |

**Table S4**. BayeScan results of *de novo* assembly Moroccan populations dataset separated adults and youths. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **1127** | Transcribed RNA sequence *Abies pinsapo* | Formyltetrahydrofolate deformylase 1 (mitochondrial) | Biosynthesis of purines<br>Metabolism of amino acids | 2e-104 |
| **1369** | mRNA *Picea glauca* | Disease resistance protein | Pathogen response (viruses, bacteria, or fungi) | 4e-58 |
| **2458** | mRNA *Picea glauca* | Peroxidase 5-like | Response to environmental stress, pathogen, and oxidative stress<br>Auxin catabolism<br>Suberization | 5e-65 |
| **2769** | mRNA *Picea glauca* | Alpha-D-phospohexomutase superfamily | Catalyze a phosphoryl transfer on sugar substrates | 0.0 |
| **3755** | Transcribed RNA sequence of *Picea glauca* | WD-40 repeat family protein | Signal transduction<br>Protein trafficking<br>Transcriptional mechanisms | 2e-05 |
| **5262** | mRNA *Pinus taeda* | Clavata 1-like protein | Cell differentation<br>Meristem structure regulation<br>Peptidyl-serin autophosphorylation | 8e-09 |
| **5594** | mRNA *Picea glauca* | LRK1 | Protein phosphorylation | 2e-151 |

**Table S5.** Results obtained at protein level with those SNPs which showed association with some variables in GPA studies. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| **196** | mRNA *Picea glauca* | Nudix hydrolase 3-like | Regulatory and signaling roles in metabolism | 3e-62 |
| **3145** | TSA Abies balsamea | TOM1-like protein 6 | Ubiquitin-dependent protein catabolic process | 5e-129 |
| **3192** | mRNA *Picea glauca* | S-adenosylmethionine synthase | Ethylene biosynthesis | 0.0 |
| **5531** | mRNA *Picea glauca (*clone) | APO protein 1 (chloroplastic) | It may participate in 4Fe-4S cofactor incorporation into psaA and/or psaB during translation | 3e-140 |
| **5944** | TSA *Abies pinsapo* | Ribonuclease H | DNA binding Transferase activity | 2e-67 |

# Bloque IV. Estudio genómico del potencial adaptativo frente al cambio climático de poblaciones españolas de *Pinus uncinata*

## Artículo: Weak genetic differentiation but strong climate-induced selective pressure toward the rear edge of mountain pine in north-eastern Spain

**Abstract:** Local differentiation at distribution limits may influence species' adaptive capacity to environmental changes. However, drivers, such gene flow and local selection, are still poorly understood. We focus on the role played by range limits in mountain forests to test the hypothesis that relict tree populations are subjected to genetic differentiation and local adaptation. Two alpine treelines of mountain pine (*Pinus uncinata* Ram. ex DC) were investigated in the Spanish Pyrenees. Further, an isolated relict population forming the species' southernmost distribution limit in north-eastern Spain was also investigated. Using genotyping by sequencing, a genetic matrix conformed by single nucleotide polymorphisms (SNPs) was obtained. This matrix was used to perform genotype-environment and genotype-phenotype associations, as well as to model risk of non-adaptedness. Increasing climate seasonality appears as an essential element in the interpretation of SNPs subjected to selective pressures. Genetic differentiations were overall weak. The differences in leaf mass area and radial growth rate, as well as the identification of several SNPs subjected to selective pressures, exceeded neutral predictions of differentiation among populations. Despite genetic drift might prevail in the isolated population, the $F_{ST}$ values (0.060 and 0.066) showed a moderate genetic drift and Nm values (3.939 and 3.555) indicate the presence of gene flow between the relict population and both treelines. Nonetheless, the SNPs subjected to selection pressures provide evidences of possible selection in treeline ecotones. Persistence in range boundaries seems to involve several selective pressures in species' traits, which were significantly related to enhanced drought seasonality at the limit of *P. uncinata* distribution range. We conclude that gene flow is unlikely to constrain adaptation in the *P. uncinata* rear edge, although this species shows vulnerability to future climate change scenarios involving warmer and drier conditions.

**Keywords**: rear edge, treeline, selection signature, genotype-environment associations, genotype-phenotype associations, risk of non-adaptedness

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# Weak genetic differentiation but strong climate-induced selective pressure toward the rear edge of mountain pine in north-eastern Spain

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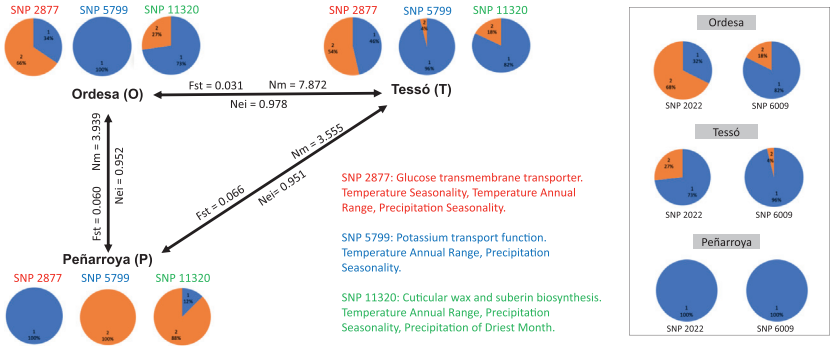
## HIGHLIGHTS

- Low heterozygosity and genetic differentiation were not observed in the relict population.
- Genetic data support gene flow among populations
- Genotype/environment analyses revealed effects of thermal seasonality and soil organic matter.
- Leaf mass area showed association with several single nucleotide polymorphisms.
- *P. uncinata* shows significant risk of non adaptedness based on expected changes in climate.

## GRAPHICAL ABSTRACT




## ARTICLE INFO

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Genotype-phenotype associations
Risk of non-adaptedness

## ABSTRACT

Local differentiation at distribution limits may influence species' adaptive capacity to environmental changes. However, drivers, such gene flow and local selection, are still poorly understood. We focus on the role played by range limits in mountain forests to test the hypothesis that relict tree populations are subjected to genetic differentiation and local adaptation. Two alpine treelines of mountain pine (*Pinus uncinata* Ram. ex DC) were investigated in the Spanish Pyrenees. Further, an isolated relict population forming the species' southernmost distribution limit in north-eastern Spain was also investigated. Using genotyping by sequencing, a genetic matrix conformed by single nucleotide polymorphisms (SNPs) was obtained. This matrix was used to perform genotype-environment and genotype-phenotype associations, as well as to model risk of non-adaptedness. Increasing climate seasonality appears as an essential element in the interpretation of SNPs subjected to selective pressures. Genetic differentiations were overall weak. The differences in leaf mass area and radial growth rate, as well as the identification of several SNPs subjected to selective pressures, exceeded neutral predictions of differentiation among populations. Despite genetic drift might prevail in the isolated population, the Fst values (0.060 and 0.066) showed a moderate genetic drift and Nm values (3.939 and 3.555) indicate the presence of gene flow between the relict population and both treelines. Nonetheless, the SNPs subjected to selection pressures provide evidences of possible selection in treeline ecotones. Persistence in range boundaries seems to involve several selective pressures in species' traits, which were significantly related to enhanced drought seasonality at the limit of *P. uncinata* distribution range. We conclude that gene flow is unlikely to constrain adaptation in the *P. uncinata* rear edge, although this species shows vulnerability to future climate change scenarios involving warmer and drier conditions.

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## 1. Introduction

Current climate change enhances the interest of the evolutionary significance of species' geographic range limits and peripherally isolated populations (Angert et al., 2020). Elucidating the processes determining the limits of a species range is particularly vital in predicting how species will respond to warming climate, as range-edge populations are assumed to be more sensitive to environmental changes (Hoffmann and Blows, 1994; Sexton et al., 2009; Holt, 2003; Angert et al., 2020). Hence, the fate of some species may depend upon the capacity of populations to cope with current climate trends, where the southernmost range margin often represents the point beyond drier climatic conditions exceed the tolerance limits of a given species (Bridle and Vines, 2007). Furthermore, range-edge populations are particularly important, in the face of adaptive management to rapid climate change, because dispersal, establishment, and extinction processes determine theoretically the pace of range shifts and whether a species' geographic range contracts or expands (Alexander et al., 2018).

Global warming provides a strong directional environmental change. This selective pressure may either enhance or reduce genetic differentiation and local adaptation at the limits of species' geographical ranges, depending on the magnitude and directionality of gene flow (Hoffmann and Sgrò, 2011; Shaw and Etterson, 2012; Alberto et al., 2013). Adaptive capacity to climate change in range-edge populations is influenced, among others, by the availability of genetic variation, the strength of natural selection and the extent of gene flow (Hoffmann and Sgrò, 2011).

In mountain forested landscapes, long-term isolation and genetic drift may limit or preclude to more extent the adaptation of relict sparser tree populations at the periphery, even if they locally experience intense directional selection (Hampe and Petit, 2005; Hampe and Jump, 2011; Kottler et al., 2021). By opposite, the altitudinal treelines provide a clinally shift, for instance in the temperature optimum for tree growth (Camarero and Gutiérrez, 2004). Differentiation might occur at these ecotonal tree populations (Bontrager et al., 2021). Meanwhile this divergence from the main forest population depends not only on gene flow and selection, but it might involve genetic drift or any other stochastic component, such founder event mechanisms (Angert et al., 2020).

Changes of treeline positions have occurred worldwide, matching with recent climate warming and leading to upward migrating in some treelines limited by low temperature (e.g., Harsch et al., 2009; Du et al., 2018; Sigdel et al., 2018). Besides, densification processes within the treeline ecotones have been also widely observed (Camarero et al., 2017; Feuillet et al., 2019; Davis et al., 2020). Hence, alpine treeline ecotones may originate a relatively fast component of genetic variation, ranging from dense forest to sparse individuals located at higher elevation, essentially similar to that ordinarily considered by genetic models of evolution at the edge of geographical ranges. Despite landscape features may influence patterns of gene flow and spatial genetic structuring, comparisons between the fine-scale genetic structures of contrasting range-limit populations are scarce. This knowledge is relevant, as fine-scale genetic structures may be reflecting the limitations for dispersal from the forests and lags in the establishment of recruits in the treeline ecotone (Alexander et al., 2018).

Hence, contrasting rear-edge populations may provide a demographical framework where the selective pressure of climate change might be detected (Bontrager et al., 2021). We focus on the role played by range limits in mountain forests comparing relict and treeline populations to investigate patterns of genetic differentiation and local adaptation using mountain pine (*Pinus uncinata* Ram. ex DC) as experimental system (Gazol et al., 2022). We compare a relict population forming the southernmost species' distribution limit in Europe and two treelines located in the Spanish Pyrenees.

Studies about how forest species respond to environmental variations are essential to understand how they cope with climate change. Several studies on the response of *P. uncinata* to climate are available (e.g., Camarero and Gutiérrez, 2007; Camarero et al., 2021; Sánchez-Salguero et al., 2017; Sanmiguel-Vallelado et al., 2019). However, they focus on morphological traits, with few exceptions which determine genetic variation between populations (Dzialuk et al., 2009) using chloroplast microsatellites (cpSSRs) or describe evolutionary history (Heuertz et al., 2010; Zaborowska et al., 2019) using mitochondrial DNA markers. Others have used a small amount of neutral nuclear RAPD (Random Amplified Polymorphic DNA) markers (Monteleone et al., 2007), and have established links between genetic diversity and fitness (González-Díaz et al., 2020) using cpSSRs.

Huge genome sizes, absence of reference genomes and annotations, and massive amounts of data to analyze are the main reasons limiting genome-wide studies within conifer species (García-García et al., 2022) such as *P. uncinata*. Nonetheless, current genotyping by sequencing (GBS) methods enable the implementation of single nucleotide polymorphisms (SNPs) analysis, which provides a genome-wide coverage, even for non-model organisms, allowing to check for adaptive *loci*, as well as neutral ones (Unamba et al., 2015). Specifically, as tree density decreases upwards, from the forest limit to the treeline, climate, and micro-site conditions (mainly temperature, wind, light, and soil properties) shift gradually (Batllori et al., 2009; Gazol et al., 2022). According to that, we expect a clinal pattern of selection that favors different values of quantitative traits, derived by local selection pressure and gene flow. Even with random establishment of individuals, we hypothesized a net flux of migrants from the forest to the treeline, whose genetic differentiation reflects the balance between selection/local adaptation and gene flow. In contrast, isolated relict tree populations on the rear edge might reflect genetic divergence likewise as a result of genetic drift, to more extent than by responses to local selection pressures. Despite this challenging framework, next-generation sequencing (NGS) GBS techniques, which are based on using restriction enzymes to fragment the genomes and reduce their complexity, provide an innovative tool. This work proves the usefulness of double digest restriction-site associated DNA sequencing (ddRAD-seq) technique to obtain single nucleotide polymorphisms (SNP) markers holding relevant information about the genetics of this species.

Hence, we obtained a genetic matrix based on SNP data, which allow us to determine diversity and differentiation among- and within-populations. Thereafter, SNPs subjected to selection were detected to determine the presence of associations between SNPs and bioclimatic variables, providing genotype-environment associations (GEA). On the other hand, the availability of functional traits data for the study individuals (Gazol et al., 2022), allows to investigate the genotype-phenotype associations. Finally, we modeled the risk of non-adaptedness (RONA) to estimate changes in allele frequency required within a given population to cope with the expected changes in climate under different emission scenarios (Pina-Martins et al., 2018).

We hypothesize lower genetic diversity and higher differentiation in the relict population, compared to the treeline ecotones, as a legacy of long term-isolation. We also hypothesize that neutral, non-adaptive, differentiation prevails in the relict population, according to genetic drift. By opposite, SNPs subjected to selection are hypothesized to be more frequent in the treeline ecotones, according to temperature rise. Finally, we hypothesize higher risk of non-adaptedness to the expected changes in climate under different emission scenarios in the relict population, according to drought increase.

## 2. Material and methods

### 2.1. Study sites description

We compared three *Pinus uncinata* Ram. ex DC sites corresponding to two structurally different treelines and a relict population (Fig. 1). The two treeline ecotones were located in the Central Spanish Pyrenees, in 'Ordesa y Monte Perdido' and 'Aigüestorters i Estany de Sant Maurici' National Parks, referred as Ordesa (O) and Tessó (T), thereafter. The O site (42.63° N, 0.08° W, 2100–2110 m a.s.l.) represents an abrupt treeline with strong differences in tree cover and height from the forest to the treeline. The aspect of this site is south, and the slope is 10–20°. Soils are rocky and mixed with calcareous and acid spots. The T site (42.58° N, 1.03° E, 2330–2360 m a.s.l.) represents a diffuse treeline with a gradual decrease in tree cover and height upwards (Camarero and Gutiérrez, 2004).

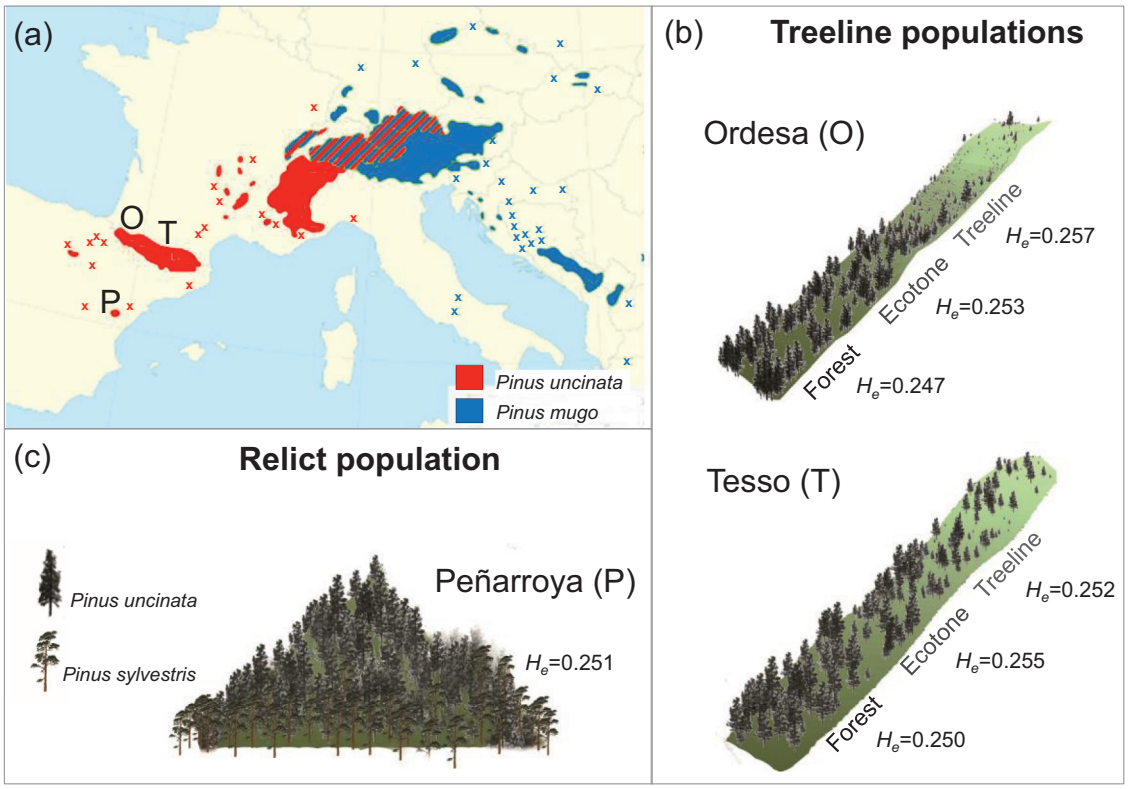


**Fig. 1.** (a) The map depicts the range of *Pinus uncinata* and the relative *Pinus mugo*, as well as the location of the three study sites in south-western Europe. Study sites: (b) sharp treeline (Ordesa, O), gradual treeline (Tessó, T); and (c) a relict population, restricted to the upper elevation of the mountain range (Peñarroya, P) where are populations of *Pinus sylvestris*; see more details about the study sites in Gazol et al. (2022). The values of expected heterozygosity (*He*) are noted.

The treeline was defined as the highest elevation of 2-m tall trees where cover was below 20 %, whilst the forest limit corresponded to the highest elevation of continuous forest patches with tree cover >40 %. The aspect of the site is north-eastern, the slope is 25–30° and soils are calcareous and developed on shales. The rear-edge, relict population represents the southernmost distribution limit of *P. uncinata*, located in Peñarroya (site P, thereafter; 40.39° N, 0.57° W, 2010 m a.s.l.). This relict population grows in a flat terrain, where soils are developed on limestones (Gazol et al., 2022).

The climate is continental in the three sites. In the treelines O and T sites the mean annual temperatures have a range between 3° and 5 °C and total annual precipitation is ca. 1200–1660 mm (Camarero and Gutiérrez, 2002, 2004). Meanwhile the mean annual temperature in site P is 9 °C and its total annual precipitation is ca. 680 mm (Camarero and Gutiérrez, 2002).

### 2.2. Tree growth, functional traits and soil characteristics

Tree radial growth patterns were obtained by using dendrochronological methods, which allowed calculating the mean growth rate (either as mean ring width or as mean basal area increment) and the tree age at 1.3 m (see Gazol et al., 2022) top. Two cores were extracted at 1.3 m and perpendicular to the slope using 5-mm increment borers (Haglof, Sweden). Cores were air dried, glued onto supports, sanded, scanned at 2400 dpi resolution and visually cross-dated. Then, tree rings were measured with a 0.001 mm resolution using the CDendro software (Larsson and Larsson, 2018). In the treelines, we selected ten trees near the forest, ten in the ecotone and ten in the treeline. In the relict population, 15 trees were sampled and similarly measured. In total, 75 trees were sampled and processed (see Gazol et al., 2022 for details).

Leaf area (LA), specific leaf area (SLA), leaf mass area (LMA), leaf dry matter content (LDMC), and wood specific gravity (WSG) were used as basic functional traits characterizing competition for light and structural costs (Niinemets, 2001; Wright et al., 2004; Nardini, 2022). These traits were measured in the same tree. Dendro-phenotypic traits such as resistance and recovery to extreme events, using estimate of secondary growth (basal area increments) resistance and recovery to 2012 drought event were estimated. Relationships with climate sensitivity were also investigated using climate variables significantly related to secondary growth (tree-ring width indexes) by dendrochronological analyses (April and May mean temperatures; Gazol et al., 2022).

Finally, soil samples were collected around the trunk (<50 cm in distance) and below the canopy projection of each sampled tree, to obtain soil texture, soil pH, soil carbon and nitrogen concentrations, and organic matter (see Gazol et al., 2022 for details).

### 2.3. SNP genotyping

Fresh leaf samples (needles) were collected from 75 *P. uncinata* individuals. 100 mg of each sample were lyophilized, and total genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen®, Germany) following the manufacturer's instructions with some modifications. DNA concentration was measured on a NanoDrop™ spectrophotometer (Thermo Scientific) and an electrophoresis in agarose gel was carried out to determine DNA quality. 72 samples had the required quality to proceed with NGS. ddRAD-seq (Peterson et al., 2012) libraries were constructed using *Ape*KI and *Pst*I restriction enzymes and sequenced by LGC Genomics (Germany). ddRAD-seq is a technique based on NGS technology which requires no prior genomic knowledge about study species. It is suitable for huge genome size species due to restriction enzymes cutting reduces the complexity of the genome. The ddRAD-seq allows us to describe a large amount of new molecular markers such as SNPs which could be used to carry out multiple types of studies as genotype-phenotype or genotype-environment associations studies (Peterson et al., 2012).

Paired-end reads were obtained with a read depth of 1 M. Quality of raw reads was checked using FastQC v0.11.9 (Andrews, 2010). Then, adapter sequences were trimmed, and low-quality reads were removed using fastp

v0.12.4 (Chen et al., 2018). A de novo assembly of the retained reads and a SNP calling were performed using ipyrad v.0.9.65 (Eaton and Overcast, 2020). The VCFtools v0.1.16 program package (Danecek et al., 2011) was used to filter for high-quality, informative SNPs for genetic structure, selection, and association analyses (biallelic, minimum allele frequency of 5 %, maximum missingness of 50 %, and 1 per *locus* to avoid linkage disequilibrium). Similarly, individuals with <50 % of the retained SNPs were removed. In addition, a more restrictive set of SNPs was created to perform the statistical genetic analysis, retaining SNPs that met the previous criteria but with a maximum missingness value of 25 %.

### 2.4. Genetic structure of tree populations

Two different methods were applied to study the genetic structure of populations with the aim to obtain robust results: (1) principal component analysis (PCA) and (2) sparse non-negative matrix factorization analysis (sNMF). For the first approach, the plink2 2.00a2.3 software (Chang et al., 2015) --pca option was used. For the second, admixture coefficients were obtained using the snmf function of the LEA package v3.4.0 (Frichot and François, 2015) in R software (version 4.1.2 R Core Team, 2020). In total, 10 repetitions were run for different K values, number of ancestry populations, ranging from 1 to 6 and represented using the software package called pong (Behr et al., 2016). The cross-entropy criterion was used to determine the value of K that best explains the obtained structure.

The GenAlEx v6.5 software (Peakall and Smouse, 2006, 2012) was used to perform the statistical analyses of the genetic matrix. Population differentiation and gene flow were estimated by fixation indexes (Fst) and migration rate (Nm) statistics, respectively. The Nei's genetic distance among populations was also estimated. Heterozygosity, private alleles, and polymorphic *loci* were calculated. Shannon index was calculated to infer genetic diversity. Lastly, allele frequencies of those SNPs which were relevant for our study were calculated.

A molecular variance analysis (AMOVA) based on Fst coefficient was performed to determine the proportion of genetic variation attributable to differences among and within populations. AMOVA was estimated based on 9999 permutations.

### 2.5. Detection of selection signatures

Detection of selection signatures was carried out with the BayeScan 2.1 software (Foll and Gaggiotti, 2008) using default parameters. Fixation indexes (Fst) with their respective q-values were obtained. This version of BayeScan directly calculates q-values using the false discovery rate (FDR) correction. Those SNPs with a q-value <0.05 were considered candidate genetic variants under selective pressure. To look for possible biological functions underlying their potential importance, the scaffolds with SNPs under selection (obtained from the ipyrad assembly) were queried against the BLAST (NCBI; Altschul et al., 1990) nucleotide database. When a match was obtained, the scaffolds were also queried against the non-redundant protein sequences database using BLASTx (NCBI; Gish and States, 1993). The *E*-value obtained in each BLAST describes the random noise of the background and give us information about the accuracy of the similarity of the protein functions found in BLAST.

### 2.6. Associations studies

Two types of environmental variables were tested for association with genotype. For the first one the 19 bioclimatic variables from the WorldClim database (Fick and Hijmans, 2017), with a grid cell resolution of 30 s were used at population level. And the second one employed individual-level measurements of 8 soil variables (sand, lime and clay soil percentage, soil pH, soil organic carbon, organic matter, soil nitrogen, and carbon-to-nitrogen ratio) obtained as described in the soil data section.

After downloading the variables from WorldClim, the freeware QGIS 3.18 (Quantum Geographic Information System) (QGIS Development Team, 2022) was used to extract the bioclimatic variables for each population's geographic position. Point sampling tool, included in QGIS, allowed us to take out information about those geographical points where are located the three study areas.

Genotype-environment associations (GEA) were assessed using the lfmm (latent factor mixed models) function of the LEA R package v3.4.0 (Frichot and François, 2015) in R software (version 4.1.2 R Core Team, 2020). After an imputation step to fill in the missing data, each run was repeated a total of 20 times with 100,000 iterations and a burn-in of 50,000. Again, p-values were transformed into q-values with FDR correction and the significance threshold was 5 %. Then, the significant scaffolds were analyzed looking for homologies with the nucleotide and protein NCBI databases as it was described previously in the detection of selection signatures section.

A genome-wide association study (GWAS) was carried out to determine the associations between the imputed SNPs matrix and functional and dendro-phenotypic traits. The measurement of each trait is described in tree growth, functional traits and soil characteristics section.

GWAS was performed with the rrBLUP v4.6.1 R package (Endelman, 2011; Endelman and Jannink, 2012). A FDR correction was applied with the significance threshold set to 5 % and the SNPs which showed associations were analyzed looking for homologies with the nucleotide and protein NCBI databases as it was described previously.

### 2.7. Risk of non-adaptedness

The value of RONA shows the theoretical percentage of change in allele frequency at *loci* associated with environmental variables required for a given population to survive changes in that variable (Pina-Martins et al., 2018). Consequently, the lower the RONA is, the more likely a population is to be able to adapt to the given new environmental conditions.

We used pyRona v0.3.6 (Pina-Martins et al., 2018) to calculate the RONA of each tree population to two different climate scenarios, predicted to happen by the end of this century (2081–2100): low emissions (RCP2.6), which limit the increase of global mean temperature to 2 °C, and high emissions (RCP8.5), whose limitation is 4.9 °C. The information was obtained using WorldClim database and QGIS (for more detail, see genotype-environment association section). We used the lfmm results from the GEA analysis as input, with their q-values.

## 3. Results

### 3.1. Genetic structure of tree populations

A total of 11,904 SNPs and 71 individuals were retained after the filtering steps. These molecular markers were used to create the genetic matrix of *P. uncinata*. PCA showed, with a 23.5 % of variance attributed to the first principal component and 16.7 % to second principal component, 3 different groups: O and T (core treeline populations) and P (rear-edge population). One of the PCA axes separated O and T treelines from the P rear-edge site (Fig. 2). In addition, it is remarkable that 8 individuals of site T are separated of their own population: 5 of them belonging to forest, 2 to treeline and 1 to the treeline ecotone separating the forest from the treeline.

In order to explore those isolated points of site T, a PCA with only this population was carried out. This analysis revealed a cluster consisting mainly of ecotone and treeline samples and, again, the group with 8 individuals that was seen in the previous PCA, consisting mainly of trees located in the forest limit.

The cross-entropy analysis determined that $K = 2$ is the value that best explained the obtained genetic structure (Fig. S1). In contrast to the PCA, this analysis showed a marked genetic differentiation between a group formed by O and T treelines, and the remaining P relict population. Hence, the following analyses were carried out taking into consideration this genetic structure.

On the other hand, the statistical analyses were carried out with the most restrictive set of SNPs (75 %) which is composed of 72 individuals and 5374 SNPs.

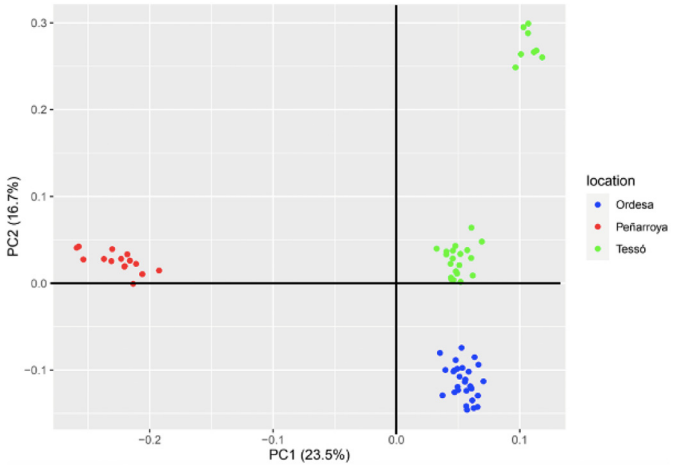


**Fig. 2.** First two principal components of PCA based on genetic data (SNP matrix) obtained from ddRAD-seq. It is shown three different groups one for each population of study. Colors are related with the location of each point: blue for Ordesa, red for Peñarroya and green for Tessó.

The AMOVA indicated large differences within populations with 95 % of the total (Table S4). This is related with the Fst value obtained (0.047) which showed, with highly significance ($p < 0.0001$), that there are no significant differences (p-value >0.05) between populations. Pairwise population Fst values are between 0.031 and 0.066 (Fig. 4; Table S1). The highest values are between P and the other two populations. However, those values are near 0, pointing that all the populations are in complete panmixia.

The gene flow which is measure with migration rate, Nm, showed a range value from 3.555 to 7.872. The higher value is found between O and T which indicates a panmictic behavior. On the other hand, P shows the lower values which indicates the presence of gene flow with the other two populations (Fig. 4; Table S2).

The average percentage of polymorphic alleles is 90.47 ± 5.21 % being lower in site P (80.05 %). The number of private alleles is very similar in the O and T treelines (0.012 ± 0.001 and 0.011 ± 0.001, respectively), but lower in site P (0.007 ± 0.001). In terms of Shannon index values, genetic diversity is higher in O and T than in P. Observed heterozygosity ($H_o$) has a range from 0.240 to 0.245 and expected heterozygosity ($H_e$) ranges from 0.251 to 0.267. The lowest values for both parameters derive from the P relict populations. The average values of $H_o$ and $H_e$ are 0.243 ± 0.001 and 0.261 ± 0.001, respectively.

Several differences among populations are showed in allele frequencies (Fig. 4). It is remarkable that site P has the highest percentage of fixated alleles followed by site T.

Finally, Nei's genetic distance allows us to explain the differences which are found between our populations. All of them are close to 1 which indicates that our 3 populations are very similar among them (Fig. 4; Table S3).

### 3.2. Detection of selection signatures

The BayeScan analyses showed 39 *loci* under selection (Table S5), The results indicate that these *loci* showed significant differences (q-value 5 %) in their Fst values. Homology with previously described sequences was found for 17 of these sequences, and only 10 of them could be defined at protein level. It is important to emphasize that these matches with protein sequences are a first approach to the probable function of our study sequences. Table S6 shows sequence type, the name and functions of the proteins, and the *E*-value obtained for each match.

The most interesting proteins obtained were NADP-specific glutamate dehydrogenase, which is involved in the ammonium and nitrogen assimilation, aquaglyceroporin, which mediates water, glycerol, and other molecules flow through membranes, GABA transporter whose functions are related to stress response, and a protein related to the response to light stimuli.

### 3.3. Associations studies

The LEA package found 129 associations with 5 of the 8 soil variables (Table S7). Sand soil percentage was associated with 17 *loci*, soil pH with 15 and lime soil percentage with 3. Soil nitrogen and carbon-to-nitrogen ratio showed the highest number of associations with 40 and 54 *loci*, respectively. Additionally, one of these *loci* which are associated with soil nitrogen, is under selection too.

It is remarkable the presence of several *loci* associated with more than one variable. Such is the case of the soil nitrogen and carbon-to-nitrogen ratio variables, which shared 5 *loci*. Lime, sand, and pH soil shared 3 *loci*. Sand and lime percentage shared 2 associations. Finally, lime percentage and soil pH shared 1 *locus*. The alignments against the nucleotide database gave 42 matches, and 20 of them also got hits for protein homology (Table S8). Some of the identified proteins were related to stress response, such as glutathione peroxidase, β-1,3-glucanasa, E3 ubiquitin-protein ligase listerin and GABA transporter. It is highlighted that the *locus*, which is similar to GABA transporter, is under selection.

On the other hand, 395 associations were observed with the 19 bioclimatic variables (Table S9). The largest number of associations were found with variables related with temperature seasonality BIO4 (34 *loci*), minimum temperature of coldest month BIO6 (37 *loci*), temperature annual range BIO7 (44 *loci*), and precipitation seasonality BIO15 (43 *loci*). The majority of *loci* were shared with two or more variables (for more details, see Table S9). Considering these results, the relation found between genetic data and temperature gives us evidence of the important role which is performed by temperature in the survival of this species.

Moreover, it is important to highlight that 25 of the *loci* associated with bioclimatic variables were under selection. In terms of the function of some of these *loci*, it should be emphasized those related with nitrogen and ammonium assimilation, potassium transport, and light response.

Once the alignments were performed, 44 hits with nucleotide database were obtained, and 23 showed protein homologies too (Table S10). It is remarkable that some of these proteins are related with: electrons exchange in mitochondrial, response to light stimulus, and ion transports.

The results obtained with rrBLUP for GWAS study showed 22 associations with 7 of the 15 functional traits (Table S11) which were: mean tree-ring width (TRW), basal area increment (BAI), LDMC, LMA, SLA, WSG, and tree age. LDMC was associated with 8 *loci*, WSG with 5, BAI with 3, LMA with 3, TRW with 2, and tree age with 1 *locus*. As it is shown, LDMC had the higher number of associations. In this case only WGS shared 1 *locus* with LDMC and LMA shared other with LDMC. All of these associations had a q-value significance of 5 % (Table S11).

In total, 12 matches have been found against the nucleotide database and 7 of them obtained hits for protein homology (Table S12). The majority of the protein functions identified were related with metabolism or nucleic acid issues.

A total of 8 dendro-phenotypic variables were used to carry out another GWAS study with the same SNP matrix. In this case, the results showed no associations.

### 3.4. Risk of non-adaptedness

The low emission scenario (Fig. 3a) showed lower risk of non-adaptedness that the high emission one (Fig. 3b), as it could be expected, as the latter's variation in its BIOs values is higher. However, at least 4 environmental variables required changes in allele frequency higher than 50 % in both scenarios. Moreover, BIO4 (temperature seasonality) stands out, reaching values of around 200 % for the low emission scenario and higher than 500 % for the high emission one, becoming the most threatening environmental variable in both predictions. All populations showed similar RONA values, but the relict P populations was less capable to adapt to changes in variables related to temperature (BIO1-BIO11), while changes in precipitation-related variables (BIO12-BIO19) were not as challenging (Fig. 3).

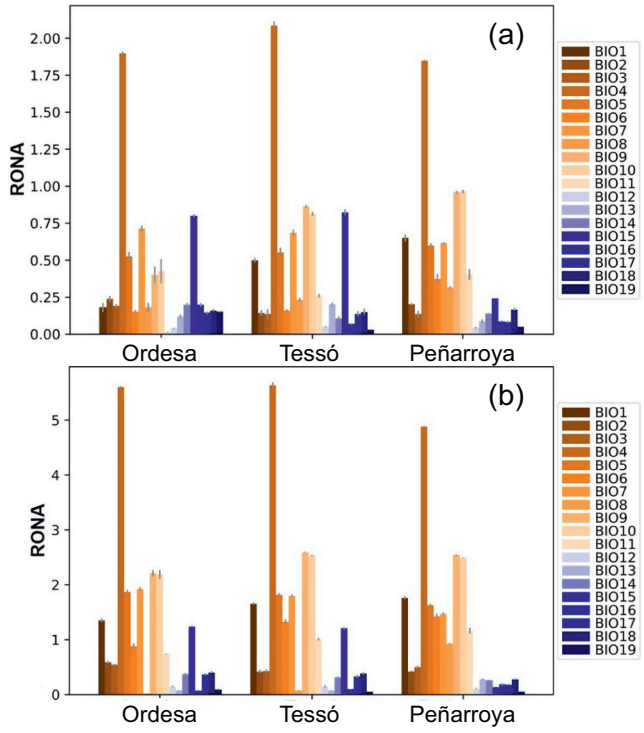


**Fig. 3.** Risk of non-adaptedness (RONA) of each population to two different climatic scenarios. Environmental variables related to temperature are represented in orange colors (BIO1-BIO11), whilst environmental variables related to precipitation are represented in blue colors (BIO12-BIO19). (a) Low emission scenario RCP2.6. (b) High emission scenario RCP8.5.

## 4. Discussion

Identifying constraints to adaptation at range edges remains a key challenge (Angert et al., 2020). In this work, we provide new insights regarding how evolutionary forces interact to shape adaptation at contrasting range margins of mountain pine. For this purpose, we focused on peripheral populations, which encompass the full altitudinal transition from closed forest to the treeline. This provides an analogy of species range expansion gradient while controlling for factors such as regional climate and colonization history. Furthermore, our sampling approach allows for fine-scale spatial analysis of genetic structuring at the rear distribution edge formed by the relict population.

Range limits are correlated with a number of abiotic and biotic factors, but underlying mechanisms are poorly understood (Sexton et al., 2009). It should be hypothesized a concomitant variation in the relative importance of genetic drift and gene flow due to limited size in the relict population (Peñarroya, P) and declining density upslope in the treelines (Ordesa, O and Tessó, T), respectively. Genetic drift leading to erosion of local genetic diversity and enhanced population differentiation should be expected to prevail at the relict P population and might occur near the upper margins of the treelines because of the smaller size and sparser distribution of tree populations, randomness associated with founding events, and limitations for seed and pollen flow (Hampe and Jump, 2011).

The results obtained in the cross-entropy study show two groups with a visible genetic differentiation: one consisting of O and T treeline sites, and another one consisting of the P relict, rear-edge populations on its own (e.g., Sjölund et al., 2019). On the contrary, PCA results suggested three separate populations, instead of two, indicating that some genetic differences do exist between the two treeline sites. This differentiation could be related with their constrain structures since O shows an abrupt treeline while T presents a gradual transition (Camarero and Gutiérrez, 2002, 2004), which implies a wider range of environmental conditions, so genetic variability could be expected along this gradient (Bontrager et al., 2021). It is important to highlight that Peñarroya site represents the southernmost distribution limit of the species. Dzialuk et al. (2009) found that the populations from the southernmost range were the most genetically different by using cpSSRs, which is consistent with the results obtained in our study. Moreover, some evidence about the existence of genetic differences between O and P populations could be found in a previous study of González-Díaz et al. (2020) by using SSR as molecular markers.

In contrast to our preliminary hypothesis of genetic isolation, results support unambiguously a situation closer to a drift-gene flow equilibrium, where, despite the isolation and relative low populations sizes, gene exchange events via pollen or seeds seems not to have been infrequent, at least to the extent to determine significant genetic isolation. This interpretation is strongly supported by low values of genetic differentiation (Fst) and high migration rates (Nm), while within-population genetic diversity (He) does not vary predictably along the forest-treeline gradient (O and T sites; Fig. 1), nor was significantly reduced in the relict population (P site; Fig. 1). A previous study showed low differentiations between populations of mountain pine (Dzialuk et al., 2009). However, the genetics studies found in the bibliography are performed using SSRs and we cannot make comparisons with the same kind of data obtained in the present study (SNPs). Predictions regarding trait divergence at relict populations were supported (see also Gazol et al., 2022). However, genetic variations across the leading edge defined by forest-to-treeline ecotones were not observed.

Nonetheless, we obtained significant evidence of possible selection in several SNPs. Some of these marks are in genes likely related with compounds involve in stress response such as transporters of ions, water, or GABA. When we obtained the alleles frequencies of the SNPs under selective pressures, some differences between populations are evidenced. For example, the SNP 2022, which is related with light gravitropic response (Fig. 4), showed the presence of both alleles in O and T sites, being more represented the reference one. It could be appreciated a decrease in the frequency of the alternative among these 2 populations, 18 % in O and 4 % in T. However, the P site presented only the reference allele (Fig. 4). This difference could be explained by the light variations which are found in P due to its drier Mediterranean climate with more radiation than northward treeline sites.

Other SNP under selective pressures is the SNP 6009 which is similar to an aquaglyceroporin (Table S3). Studies show modifications in the expression of this kind of proteins during drought stress periods (e.g., Mahdieh et al., 2008; Ding and Chaumont, 2020). However, the mechanisms which link drought stress response to aquaglyceroporin are poorly studied. In our study, the allele frequencies for this SNP showed differences among populations. Meanwhile O and T had a representation of both alleles, P showed only the reference allele (Fig. 4). These variations could indicate some advantages of having a higher proportion of the reference allele for the population with less amount of precipitation. Another example of these kind of SNPs is SNP 8051 which is a GABA transporter. Moreover, this SNP showed association with N variable in GEA study. Its allele frequencies show the same fixed allele in T and P sites, meanwhile the O site had more representation of the second allele. Due to GABA is involved in plant response to abiotic stresses, it increases the drought resistance in plants (Mekonnen et al., 2016), and this metabolite are related with C:N balance (e.g., Fait et al., 2011; Batushansky et al., 2014). This difference showed in allele frequencies may be caused by environmental variations, mainly in the soil composition, that are found in the distribution range of the T treeline and in the relict P population.

Therefore, the presence of possible selection pressure marks in this kind of genes, evidences the effect of climate variations on this species' genome and points out the relevance of keeping an eye on its genetics to be able to anticipate their response to climate change in the coming years.

The genetic differences observed among tree populations (Fig. 4) suggest that random genetic drift was not the prevailing force shaping the genetic structure of *P. uncinata* at its rear edge. It should be expected that random genetic drift increases the among-population component of diversity. This expectation is weakly supported by the genetic differentiation showed in PCA of the relict population, while this hypothesis appears not

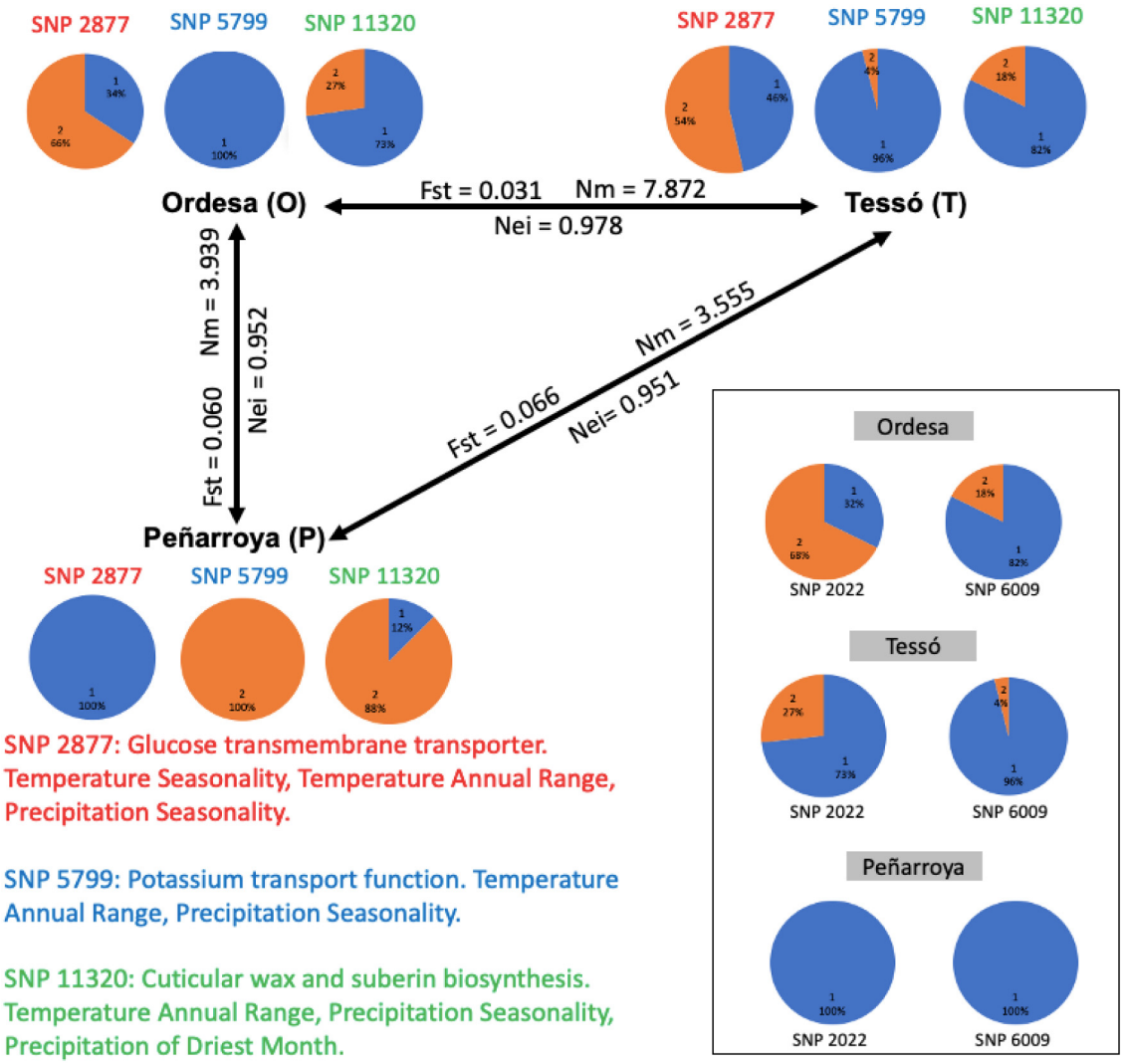


**Fig. 4.** Examples of SNPs showing associations in GEA studies with our three *P. uncinata* populations. It is shown Fst, Nm and Nei's genetic distance (Nei) values among populations. The allele percentages of these SNPs, whose functions are inferred for homology, are represented with circles: color blue for the reference allele and orange for the alternative allele. A brief description of the protein function which present homology for each SNP and of the bioclimatic variables which showed associations with these SNPs are indicated. The inset describes the allele percentages of two SNPs under selection.

reinforced at the reduced spatial scale of the forest-ecotone-treeline transitions. Furthermore, despite random genetic drift is also expected to reduce gene diversity within small populations (Hampe and Jump, 2011), our results support the maintenance of relatively high genetic diversity, which was in agreement with the conclusions reached by González-Díaz et al. (2020).

At a spatial scale shorter than those of the among-populations differences, as the population range spreads from the forest to the treeline, allele frequencies in each treeline transition might drift independently without any relation to the fine-scale spatial distances. Thus, random sampling of gametes may create a wide degree of scatter between points (Fig. S2). It must be noted, however, some relationship between genetic and treeline position was also observed in T. The presence of 8 individuals from T separated from their main nucleus in the PCA is remarkable. Since most part of forest samples can be found in this isolated group, a loss of genetic traits which were shared in the past with the ecotone and treeline areas could have happened.

Relict populations are assumed to have limited performance due to genetic drift and inbreeding depression (Hampe and Jump, 2011; Bontrager et al., 2021). Nonetheless, evidence for increased vulnerability to climate change in the relict population is lacking, based on risk of non-adaptedness estimations (Fig. 3). Furthermore, the comparison of this relict population with the O and T treelines did not support the hypothesis that relict populations tend to have increased genetic isolation and loss of heterozygosity (Fig. 1). By opposite, adequacy between phenotypes and local environment (that is, local adaptation), might be hypothesized for the relict P population according to obtained SNPs frequencies and its relationships with climate seasonality and dryness (Fig. 4). However, the small population size may be limited to cope with changing environmental conditions if they are subjected to strong demographic stochasticity and competitive exclusion by neighboring species such *P. sylvestris* (Fig. 1). Gazol et al. (2022) found that Peñarroya did not show definite adaptation to cope with drier conditions in leaf traits or climate-growth sensitivity, despite the general trend of rear-edge populations being more vulnerable to drought, compared to populations located in the core distribution range (Camarero et al., 2021; Gazol et al., 2022).

Contrasting to the relict populations, the treelines show a stepping-stone pattern of density decrease from the forest to the upper ecotone (Camarero et al., 1998; Camarero and Gutiérrez, 2002, 2004), depicting a small-scale species range limit. Hence, an increased frequency of locally adapted genes might be expected, as climate warming acts as a selective pressure over tree recruitment and growth near or beyond

the tree line (Bontrager et al., 2021). Specifically, it should be expected that some genotypes boost population fitness at the treeline (Hargreaves and Eckert, 2019). However, we found no relationship between genetics and treeline position, suggesting that rapid climate change was not limiting gene flow from the forest to the expanding treeline. Notwithstanding, the role of epigenetics changes and phenotypic plasticity should not be discarded (Alberto et al., 2013; García-García et al., 2022).

Lack of evidence regarding climate-driven selection may be partially reflecting that land-use changes have played a more important role than climate in driving forest dynamics at a landscape scale over the last half century (e.g., Améztegui et al., 2010). The expansion of *P. uncinata* has been observed mainly by increasing canopy cover of pre-existing forests, either through enhanced growth of pre-existing individuals or the recruitment of new ones (Améztegui et al., 2010).

The associations found with GEA and GWAS studies allow us to identify the relationship between genetics and environmental and phenotypical variables, respectively. The presence of a high number of associations, in GEA studies, with nitrogen and C:N ratio reveals the importance of soil nutrients for tree growth. These associations could be related with less fertile soils in the treeline as compared with the forest, as was previously described by Gazol et al. (2022). Regarding the comparisons between sites, these authors reported that C:N ratio has the maximum in the O forest trees and the minimum in the P site.

Differences in allele frequencies allow us to have an idea about the relevance of the alleles and the homology protein (Fig. 4). For example, a potassium transporter (SNP 5799) showed huge differences in allele frequencies among populations. Mainly one of the alleles is fixed in site O and the other one in site P. Moreover, it is highlighted that this SNP is under selection too. It is relevant due to studies describing the important role of potassium in plant stress response to water shortage (Wang et al., 2013).

The ability to alter traits in response to a changing environment is particularly important for trees (Des Marais et al., 2013). Traits related to the carbon and water balance may define tree performance in a given environment, and therefore plasticity in these traits should provide adaptive capacity when conditions change (Wright et al., 2004). Leaf traits depict variations in resource investment widely associated with different evolutionary strategies across plant species (Reich et al., 2003; Wright et al., 2004). Low values of leaf mass area (LMA) reveal low investment in tissue density and nutrients, which has been related to high rates of photosynthesis and resource acquisition, but at the cost of longevity. On the other hand, high values of LMA reveal high invest in long-lived leaf material (Reich, 2014). The associations found with LMA and LDMC agree with the among-populations differences found in other leaf traits and soil properties (Gazol et al., 2022). In addition, in some cases, a relationship between genotype and LMA values was found (Fig. S3).

Several anatomical traits leading to high LMA are mechanistically correlated to physiological traits conferring tolerance to dehydration (Nardini, 2022). Given that the relict P population is subjected to drier conditions, a selective pressure for needles stiffness should be expected, as needles with high mass per unit area occur more frequently in water-limited habitats (Niinemets, 2001). However, the results regarding LMA were contrary to our expectations. In addition, Gazol et al. (2022) performed dendroecological analyses which revealed a stronger dependency of growth on water availability in this relict, rear-edge population than in the two alpine treeline sites. By opposite, as some of the anatomical modifications associated with high LMA and leaf mechanical stiffness provide leaf mechanical resistance, in addition to drought tolerance, the higher values of LMA obtained in the treelines (Fig. S2) might confer adaptive advantages to trees growing in this harsh environment, which is also cold- and nutrient-limited (Nardini, 2022).

Lastly, RONA values allowed us to predict the adaptation capacity of our populations to different climatic scenarios predicted for the end of the 21st century. The results show that they are at risk of non-adaptedness to both the low and high emission scenarios. As pines generation times are long, taking about 20–25 years to reach sexual maturity (Camarero et al., 2017), the magnitude of change, which was obtained in this study, is extremely difficult to achieve in 60–80 years. Environmental variables related to temperature seem to pose a higher threat, which is consistent with the results obtained from GEA and selection analyses. The SNPs subjected to selection processes and associated with environmental variables were related to 7 out of 11 temperature BIOs and only 1 out of 8 precipitation BIOs. RONA studies have been performed with other species such as *Eucalyptus microcarpa* (Jordan et al., 2017), or *Quercus suber* (Pina-Martins et al., 2018).

Temperature seasonality (BIO4) is the most hazardous environmental variable for every population in both predictions. An increase of extreme meteorological events, including high and low temperature episodes, is expected in the future (Hoegh-Guldberg et al., 2018), leading to considerable changes in this BIO, such that any of our three populations' allele frequencies are likely to be able to match.

It is of particular concern that, while only 4–5 environmental variables seem to be threatening at the low emission scenario, this number rises to 8–10 when looking at the high emission expectation.

## 5. Conclusions

Forests dynamics in range boundaries seem to involve several selective pressures in species' traits, which were significantly related to the characteristics of harsh and marginal climates. Contrasting patterns of local adaptation among relict populations and treeline ecotones may be influenced by microsite conditions, while the dispersal and upward establishment in the treeline need to overcome several limitations to effectively result in further range shifts to higher elevation.

Increasing dryness and climate seasonality toward the species border appears as an essential element in the interpretation of regional genetic structuring of *P. uncinata* at its rear edge, and a factor which may influence the evolutionary potential of peripheral populations by local adaptation. Nevertheless, genetic differentiations were overall weak. Our results showed reduced among-populations genetic differences, while heterozygosity values were similar, contrasting to expectations of increasing differentiation and limited genetic diversity in relict populations. Further, among-population gen flow seems to prevail over any process of genetic drift or inbreeding.

By opposite, the differences in several phenotypic traits, such leaf mass area and growth rates, as well as the identification of several SNPs subjected to selection, exceeded neutral predictions of differentiation among populations. Despite limited differences among populations, the SNPs subjected to selection provide evidences of strong selection in marginal climates.

Relict populations are assumed to have limited performance due to genetic drift and inbreeding depression. Nonetheless, evidence for increased vulnerability to climate change in the relict population was not higher than in the treelines. Finally, it should be noted that, despite we obtained several evidences of selective pressures, likely driven by climate, land-use changes have also played a determinant role driving recent *P. uncinata* forest dynamics at a landscape scale.

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## CRediT authorship contribution statement

**Belén Méndez-Cea:** Investigation, Formal analysis, Writing – original draft, Writing – review & editing. **Isabel García-García:** Investigation, Writing – review & editing. **Antonio Gazol:** Resources. **J. Julio Camarero:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Ester González de Andrés:** Resources, Writing – review & editing. **Michele Colangelo:** Resources. **Cristina Valeriano:** Resources. **Francisco Javier Gallego:** Conceptualization, Funding acquisition, Writing – review & editing. **Juan Carlos Linares:** Conceptualization, Funding acquisition, Writing – review & editing.

## Data availability

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159778.

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## Apéndice 4: Material suplementario

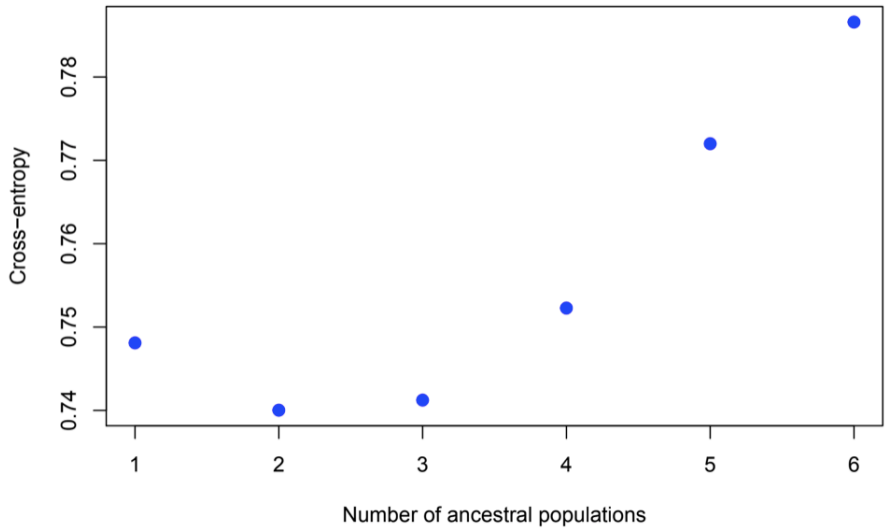


**Figure S1**: Cross-entropy result. The lower value of cross-entropy is K = 2 so this is the best explanation for the genetic structure derived from the study populations.

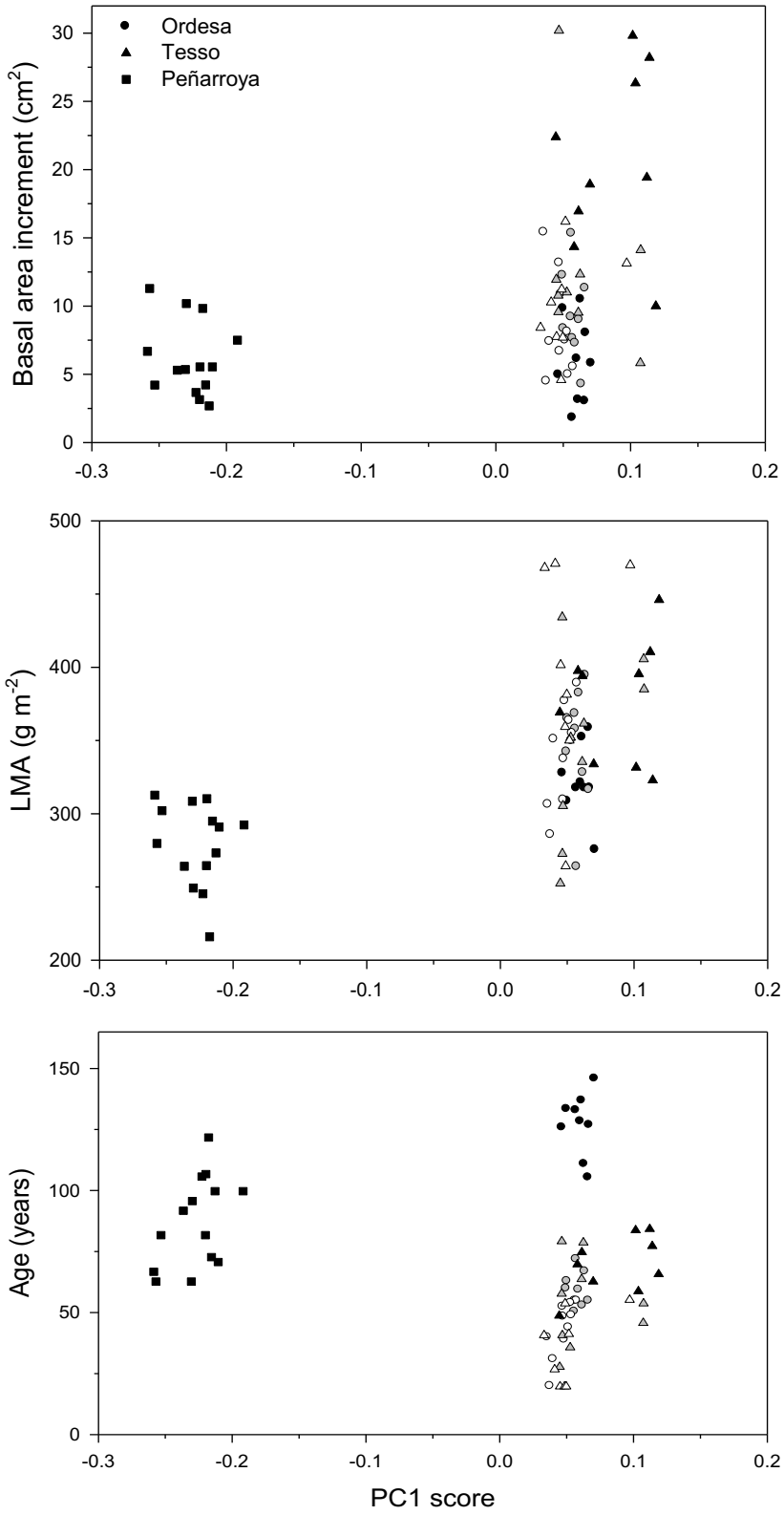


**Figure S2**: Principal Component Analysis (PC) scores obtained for (a) mean tree basal area increment (cm2), (b) leaf mass area (LMA, g m2), and (c) age (years at 1.3 m). In the two treeline populations (Ordesa, Tessó), trees located in the forest, ecotone and treeline are indicated by black, grey, and white symbols, respectively.

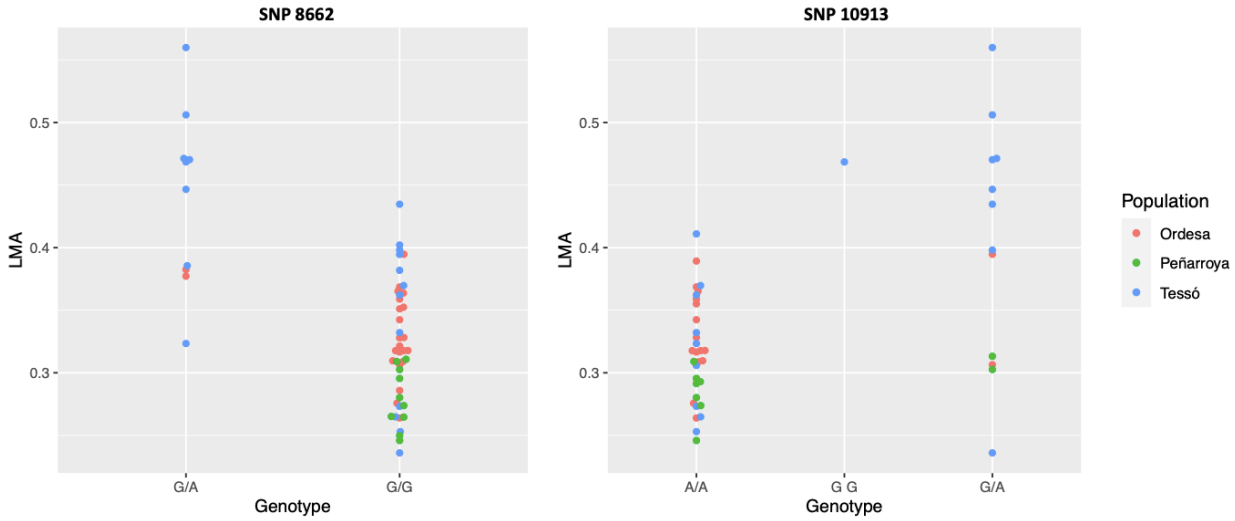


**Figure S3**: Scatter plots of two SNPs, 8662 and 10913, which showed associations in GWAS study with the LMA. The colours of the points are indicated the population of origin. It could be appreciated the relationship between LMA and the genotype.

**Table S1**: Pairwise population Fst values. Values near 0 indicate low genetic differentiation between tree populations.

| **Pairwise Population Fst Values** | | | |
|---|---|---|---|
| **Ordesa** | 0.000 | | |
| **Tessó** | 0.031 | 0.000 | |
| **Peñarroya** | 0.060 | 0.066 | 0.000 |
| | **Ordesa** | **Tessó** | **Peñarroya** |

**Table S2**: Results of effective migration rate (Nm) for pairwise population. All values are higher than 1 which indicates the presence of genetic flow through the three populations. Nm between Ordesa and Tessó treelines indicates that both populations are in panmixia.

| **Pairwise Population Nm Values Based on Fst Values** | | | |
|---|---|---|---|
| **Ordesa** | 0.000 | | |
| **Tessó** | 7.872 | 0.000 | |
| **Peñarroya** | 3.939 | 3.555 | 0.000 |
| | **Ordesa** | **Tessó** | **Peñarroya** |

**Table S3**: Nei genetic identity. Values are near to 1 indicating a high similarity among the three populations of study.

| Pairwise Population Matrix of Nei Genetic Identity | | | |
|---|---|---|---|
| **Ordesa** | 1.000 | | |
| **Tessó** | 0.978 | 1.000 | |
| **Peñarroya** | 0.952 | 0.951 | 1.000 |
| | **Ordesa** | **Tessó** | **Peñarroya** |

**Table S4**: Summary of the AMOVA results. The rows indicate the partitions genetic variability in two components: among and within populations. The columns show degrees of freedom (df), sum of squares (SS), mean squares (MS), estimate of variance (Est.var.) and the percentage of total variation (%). The percentage of variation is higher within populations than among populations.

| Summary AMOVA Table | | | | | |
|---|---|---|---|---|---|
| **Source** | **df** | **SS** | **MS** | **Est. Var.** | **%** |
| **Among Pops** | 2 | 7580.742 | 3790.371 | 57.061 | 5% |
| **Within Pops** | 141 | 161885.834 | 1148.126 | 1148.126 | 95% |
| **Total** | 143 | 169466.576 | | 1205.188 | 100% |

**Table S5**: SNP identification of those 39 *loci* which were obtained under selection in BayeScan analysis.

| SNPs under selection |
|---|
| 86, 113, 205, 257, 326, 980, 1295, 1458, 2022, 2140, 3715, 4008, 4250, 4453, 4546, 4842, 5105, 5147, 5799, 5978, 6009, 6096, 6408, 6649, 6899, 7549, 7980, 8051, 8253, 8303, 8443, 9871, 10041, 10158, 10415, 10442, 10568, 11247, 11866 |

**Table S6:** Results of the matches found in the alignments against protein database with those *loci* which are under selection. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| 113 | *Picea glauca* mRNA | NADP-specific glutamate dehydrogenase | Ammonium assimilation<br>Nitrogen assimilation<br>Glutamate catabolysim | 1e-08 |
| 205 | *Pinus taeda* sequence complete clon | DDE-type integrase/transposase/recombinase | Nucleic acid union<br>DNA integration | 0.0 |
| 1295 | *Colletotrichum karsti* mRNA (fungi) | Transmembrane amino acid transporter | Transmembrane amino acid transporter | 0.0 |
| 1458 | *Picea sitchensis* unknown mRNA | Disease resistance protein | Disease resistance | 1e-82 |
| 2022 | *Picea glauca* mRNA | Protein gravitropic in the light | Light gravitropic response | 2e-91 |
| 5147 | *Picea glauca* mRNA | Cytokinin riboside 5'-monophosphate phosphoribohydrolase LOG3 (fosforibohidrolasa) | Hydrolase activity, hydrolyzing N-glycosyl compounds<br>Cytokinin biosynthetic process | 4e-126 |
| 5799 | Anonymus *locus Pinus contorta* var. bolanderi | Potassium transporter | Potassium ion transport | 2e-42 |
| 6009 | *Pinus taeda* mRNA | Early embryogenesis aquaglyceroporin | Water, glicerol and other solutes transporter | 0.0 |
| 8051 | Predichas de *Camellia sinensis* | GABA transporter 1-like isoform X3 | GABA transporter<br>Abiotic and biotic stress response<br>Regulation of plant growth | 0.0 |
| 11247 | *Araucaria heterophylla* cds mitochondrial | NADH dehydrogenase or succinate dehydrogenase | Electron transport chain | 3e-10 |

**Table S7:** Results obtained in GEA study with the soil variables. First column shows the name of the variable, the second is the total number of associations for each variable and the last one, indicated the SNP identification of the *loci* with associations.

| Soil variables | Number of associations | SNP identification |
|---|---|---|
| **Lime** | 17 | 139, 166, 872, 1405, 3010, 3396, 4123, 4124, 4190, 6143, 8010, 8280, 9935, 11054, 11311, 11394, 11664 |
| **Sand** | 3 | 4190, 11394, 11664 |
| **pH** | 15 | 851, 1214, 2692, 2909, 2923, 3396, 4060, 4190, 4349, 5614, 7196, 8059, 8570, 9663, 11327 |
| **N** | 40 | 152, 206, 250, 781, 1097, 1226, 1367, 1533, 2314, 2411, 2836, 2849, 3347, 3731, 3760, 4014, 4265, 4563, 5000, 5236, 6025, 6128, 6316, 6329, 6871, 7222, 7485, 7970, 8051, 8095, 8119, 8215, 8444, 8904, 9237, 10442, 10548, 10701, 10957, 10972 |
| **C:N ratio** | 54 | 137, 407, 568, 600, 686, 767, 828, 898, 1038, 1409, 1488, 1793, 2314, 2411, 2882, 2939, 2998, 3070, 3347, 3611, 3646, 3801, 3844, 4429, 4518, 4637, 4780, 5082, 5123, 5240, 5422, 7086, 7300, 7915, 7996, 8087, 8215, 8395, 8444, 8708, 8855, 8908, 8992, 9476, 9804, 10190, 10606, 10972, 11003, 11047, 11056, 11126, 11310, 11457 |

**Table S8**: Results obtained in GEA study with soil variables. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the similarity of the protein function is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| 568 | *Picea abies* mRNA | Wuschel WOX8/9 (homeobox protein) | DNA union<br>Transcription factor<br>Process regulator | 0.0 |
| 600 | (Predicted) *Abrus precatorius* | Trafficking protein particle complex II-specific subunit 120 homolog | Transport of proteins in post-Golgi trafficking pathways | 0.0 |
| 781 | *Pinus tabuliformis* mRNA | Glutatión peroxidasa 1 | Oxidative stress response | 2e-121 |
| 1226 | *Picea glauca* mRNA | Serina/treonin protein-quinasa | Serin/treonin fosforilation | 0.0 |
| 2692 | *Picea glauca* mRNA | Heparan-alpha-glucosaminide N-acetyltransferase-like isoform X1 | Acetyltransferase activity<br>Lysosomal transport | 0.0 |
| 2882 | *Pinus pinaster* mRNA | Transcription elongation factor SPT4 homolog 1 | Regulation of transcription elongation from RNA polymerase II promoter.<br>mRNA processing.<br>Positive regulation of DNA-templated transcription. | 1e-53 |
| 4060 | *Picea glauca* mRNA | Charged multivesicular body protein 5-like | Vesicular transport | 5e-78 |
| 4518 | *Camellia sinensis* mRNA | Apoptosis inhibitor 5-like protein AP15 | Regulation of apoptotic process | 0.0 |

| | | | | |
|---|---|---|---|---|
| 5123 | *Picea glauca* mRNA | (Probable) Xyloglucan endotransglucosylase/hydrolase protein 7 | Cell wall biogenesis and organization<br>Xyloglucan metabolic process | 3e-88 |
| 6128 | *Amborella trichopoda* mRNA | Pentatricopeptide repeat-containing protein | Chloroplast mRNA processing<br>Polycistronic mRNA processing<br>RNA splicing | 0.0 |
| 6316 | *Picea sitchensis* mRNA | Beta-1,3-glucanase | Carbohydrate metabolic process<br>Pathogen defense response<br>Resistance to abiotic stress | 2e-140 |
| 6329 | *Picea glauca* mRNA | Transcriptor factor TCP24-like | Leaf development<br>Negative regulation of cell population proliferation<br>Regulation of secondary shoot formation<br>Regulation of transcription | 2e-20 |
| 8051 | (Predicted) *Camellia sinensis* mRNA | GABA transporter 1-like isoform X3 | GABA transporter<br>Abiotic and biotic stress response<br>Regulation of plant growth | 0.0 |
| 8059 | *Picea glauca* mRNA | Histona H2A | DNA packaging | 1e-41 |
| 8087 | *Picea glauca* mRNA | E3 ubiquitin-protein ligase listerin | Vegetative growth control<br>Abiotic and biotic stress tolerance<br>DNA reparation | 9e-158 |
| 8395 | Complete sequence of *Pinus taeda* | DDE-type integrase/transposase/recombinase | Nucleic acid union<br>DNA integration | 3e-106 |

| | | | | |
|---|---|---|---|---|
| 8908 | *Picea glauca* mRNA | Cellulose synthase interactive 1-like isoform X | Cellulose biosynthetic process<br>Response to water deprivation<br>Pollen wall assembly | 0.002 |
| 10701 | *Picea glauca* mRNA | Persulfide dioxygenase ETHE1 | Endosperm development<br>Seed development<br>Glutathione and hydrogen sulfide metabolic processes | 1e-143 |
| 11310 | *Picea glauca* mRNA | Ras-related protein RABB1c-like | Vesicular traffic<br>Protein transport | 4e-128 |
| 11457 | Ribosomal proteins of *Cedrus deodora* | Retrovirus-related Pol polyprotein from transposon TNT 1-94 | Endonuclease activity<br>DNA integration<br>Reverse transcriptase | 9e-63 |

**Table S9:** Associations found in GEA study using WorldClim variables. It is shown the name of variable, the meaning of each variable, the total number of associations and the SNP identification.

| Variable | Compute | Number of associations | SNP identification |
|---|---|---|---|
| **BIO1** | Annual Mean Temperature [°C] | 12 | 1275, 1458, 4480, 6096, 8360, 8443, 8839, 9755, 10041, 10606, 11247, 11527 |
| **BIO2** | Mean Diurnal Range [Mean of monthly (max temp–min temp)] [°C] | 19 | 791, 1275, 1295, 1441, 3990, 4453, 4546, 5370, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 10158, 11242, 11619 |
| **BIO3** | Isothermality (BIO2/BIO7) (* 100) [°C] | 4 | 1275, 6096, 8839, 9755 |
| **BIO4** | Temperature Seasonality (standard deviation *100) [°C] | 34 | 205, 257, 326, 767, 791, 1295, 1536, 2140, 2430, 2877, 3715, 4250, 4453, 4546, 4788, 5114, 5370, 6853, 6899, 7025, 7831, 8253, 8292, 8708, 8815, 9249, 9826, 9871, 9896, 10158, 10442, 10568, 11242, 11619 |
| **BIO5** | Max Temperature of Warmest Month [°C] | 17 | 791, 1275, 1295, 1441, 1458, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896 |
| **BIO6** | Min Temperature of Coldest Month [°C] | 37 | 767, 1769, 1831, 2013, 2748, 2752, 2849, 3045, 3208, 3274, 3587, 3777, 4111, 4480, 4642, 4898, 5003, 6096, 6723, 7549, 7823, 7980, 7996, 8350, 8360, 8443, 8893, 9496, 9900, 9979, 10041, 10606, 10982, 11167, 11182, 11247, 11527 |
| **BIO7** | Temperature Annual Range (BIO5–BIO6) [°C] | 44 | 81, 205, 791, 1130, 1275, 1290, 1295, 1441, 1566, 1627, 2140, 2164, 2533, 2707, 2877, 3243, 3593, 3715, 3899, 3990, 4001, 4453, 4455, 4546, 4788, 5370, 5799, 6649, 7831, 8253, 8292, 8658, 8839, 9599, 9755, 9775, 9871, 9896, 10158, 11242, 11268, 11320, 11583, 11619 |
| **BIO8** | Mean Temperature of Wettest Quarter [°C] | 17 | 791, 1275, 1295, 1441, 1458, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896 |

| | | | |
|---|---|---|---|
| **BIO9** | Mean Temperature of Driest Quarter [°C] | 11 | 1275, 1458, 4480, 6096, 8360, 8443, 8839, 9755, 10606, 11247, 11527 |
| **BIO10** | Mean Temperature of Warmest Quarter [°C] | 6 | 1275, 6096, 8360, 8443, 8839, 9755 |
| **BIO11** | Mean Temperature of Coldest Quarter [°C] | 10 | 1458, 4480, 6096, 8360, 8443, 8839, 10041, 10606, 11247, 11527 |
| **BIO12** | Annual Precipitation [mm] | 18 | 791, 1275, 1295, 1441, 3990, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 11242 |
| **BIO13** | Precipitation of Wettest Month [mm] | 17 | 791, 1275, 1295, 1441, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 11242 |
| **BIO14** | Precipitation of Driest Month [mm] | 27 | 791, 1275, 1295, 1441, 1566, 1627, 2533, 3243, 3990, 4453, 4546, 5370, 6649, 8253, 8292, 8658, 8839, 9755, 9775, 9871, 9896, 10158, 10415, 11242, 11268, 11320, 11619 |
| **BIO15** | Precipitation Seasonality (Coefficient of Variation: mean/SD*100) [%] | 43 | 205, 257, 791, 1295, 1536, 1627, 2140, 2164, 2430, 2877, 3593, 3715, 3990, 4001, 4133, 4250, 4453, 4455, 4501, 4546, 4788, 5370, 5799, 6649, 6853, 6899, 7025, 7831, 8253, 8292, 9114, 9249, 9826, 9871, 9896, 10158, 10442, 10568, 10705, 11242, 11268, 11320, 11619 |
| **BIO16** | Precipitation of Wettest Quarter [mm] | 16 | 86, 791, 1275, 1295, 1441, 4453, 4546, 5370, 6096, 6649, 8253, 8443, 8839, 9755, 9871, 9896 |
| **BIO17** | Precipitation of Driest Quarter [mm] | 21 | 791, 1275, 1295, 1441, 2533, 3990, 4453, 4546, 5370, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 10158, 10415, 11242, 11619 |
| **BIO18** | Precipitation of Warmest Quarter [mm] | 21 | 791, 1275, 1295, 1441, 2533, 3990, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 10415, 11242, 11619 |
| **BIO19** | Precipitation of Coldest Quarter [mm] | 21 | 791, 1275, 1295, 1441, 2533, 3990, 4453, 4546, 5370, 6096, 6649, 8253, 8658, 8839, 9755, 9775, 9871, 9896, 10415, 11242, 11619 |

**Table S10**: Results obtained at protein level with those SNPs which showed association with some WorldClim variables in GEA study. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| 205 | *Pinus taeda* sequence complete clone | DDE-type integrase/transposase/recombinase | Nucleic acid union<br>DNA integration | 0.0 |
| 1290 | *Pinus pinaster* clone sequence | Actin-depolymerizing factor | Actin-depolymerizing factor | 5e-72 |
| 1295 | *Colletotrichum karstii* mRNA (fungi) | Transmembrane amino acid transporter | Transmembrane amino acid transporter | 0.0 |
| 1458 | *Picea sitchensis* mRNA | Disease resistance protein | Virus resistance<br>Blue light response<br>Response to absence of light | 1e-82 |
| 1627 | *Pinus taeda* clone | Pol protein | Viral genome integration into host DNA | 2e-63 |
| 2013 | *Picea sitchensis* mRNA | Transcription initiation factor IIE subunit alpha isoform X1 | Transcription initiation from RNA polymerase II promoter<br>Metal ion binding<br>RNA polymerase II complex binding | 0.0 |
| 2533 | *Picea sitchensis* mRNA | E3 ubiquitin-protein ligase UPL3 | Ubiquitination<br>Proteasomal degradation of target proteins | 0.0 |
| 2877 | WGS *Vitis vinifera* | Bidirectional sugar transporter SWEET1 | Glucose transmembrane transporter activity<br>Mannose, fructose, and galactose transporter | 4e-44 |

| | | | | |
|---|---|---|---|---|
| 3045 | *Pinus taeda* clone | Endonuclease-reverse transcriptase | Endonuclease activity<br>RNA-directed DNA polymerase activity | 2e-112 |
| 3578 | (Predicted) *Capsella rubella* mRNA | Exportin-7 isoform X1 | Protein export from nucleus | 0.0 |
| 3899 | *Picea glauca* mRNA | Transmembrane emp24 domain-containing protein p24beta3 | Vesicular protein trafficking | 2e-101 |
| 4111 | *Carica papaya* mRNA | Pentatricopeptide repeat-containing protein | Chloroplast mRNA processing Polycistronic mRNA processing<br>RNA splicing | 0.0 |
| 4480 | (Predicted) *Zingiber officinale* mRNA | Auxin-induced protein 6B-like | Auxin-activated signaling pathway (stress response) | 6e-61 |
| 4788 | *Picea sitchensis* mRNA | Inner membrane protein (PPF-1) | Insertion of integral membrane proteins into thylakoid membrane<br>Senescence inhibitor | 0.0 |
| 4898 | *Picea glauca* mRNA | Galacturonosyltransferase 11 | Pectin biosynthesis in seeds<br>Mucilage extrusion from seed coat | 0.0 |
| 5799 | Anonymus *locus Pinus contorta* var. *bolanderi* | Potassium transporter 7 | Potassium transport | 2e-42 |
| 6723 | *Picea glauca* mRNA | Acyl-protein thioesterase 2-like isoform X3 | Acylglycerol catabolic process | 4e-143 |

| | | | | |
|---|---|---|---|---|
| 9114 | *Picea glauca* mRNA | Charged multivesicular body protein 5-like | Vesicles traffic | 5e-78 |
| 9900 | *Picea glauca* mRNA | Alpha-1,4 glucan phosphorylase L isozyme | Carbohydrate metabolic process | 0.0 |
| 10041 | *Picea glauca* clone mRNA | TVP38/TMEM64 family membrane protein | Little-known | 5e-82 |
| 10982 | *Picea sitchensis* mRNA | Bifunctional nuclease 1-like | Defense response to fungus<br>Protein ubiquitination<br>Nuclease activity | 3e-145 |
| 11247 | *Araucaria heterophylla* cds mitochondrial | NADH dehydrogenase or succinate dehydrogenase | Electron transport chain | 3e-10 |
| 11320 | *Cynara cardunculus* var. scolymus mRNA | 3-ketoacyl-CoA synthase 10-like | Fatty acid biosynthetic process<br>Response to cold<br>Response to light stimulus | 0.0 |

**Table S11:** Results obtained in GWAS study with functional traits. The first column called variables indicates the name of the functional traits which showed associations. The other column is the SNP identification of each sequence associated with the variable.

| Variable (code) | SNP identification |
|---|---|
| **Basal area increment (BAI)** | 7662, 8181, 11349 |
| **WGS** | 2400, 5252, 6832, 7790, 11128 |
| **Specific Leaf Area (SLA)** | 1572 |
| **Leaf Dry Matter Content (LDMC)** | 182, 490, 1710, 2214, 3701, 5403, 8662, 11128 |
| **Leaf Mass Area (LMA)** | 8662, 10913 |
| **Mean tree-ring width (TRW)** | 6698, 11409 |
| **Tree age at 1.3 m** | 3228 |

**Table S12**: Results obtained at protein level with those SNPs which showed association with some functional traits in GWAS study. It is shown the sequence type, the name and functions of the proteins, and the E-value obtained for each hit (the closer the values are to 0, the greater the probability that the protein function similarity is correct). Protein functions were obtained from UniProt.

| SNP identification | Sequence type | Protein name | Protein function | E-value |
|---|---|---|---|---|
| 1572 | mRNA de *Prosopis alba* | Dihydroorotase (mitochondrial) | Catalyze the reversible hydrolytic conversion of dihydroorotate and carbamoyl aspartate in the pyrimidine biosynthesis | 0.0 |
| 2400 | *Picea sitchensis* unknown mRNA | Nuclear transport factor 2-like | Gene silencing by RNA (methylation) | 1e-73 |
| 3701 | *Picea glauca* mRNA | Zinc finger | DNA recognition, RNA packaging, transcriptional activation, regulation of apoptosis, protein folding and assembly, and lipid binding | 1e-04 |
| 7662 | *Picea glauca* mRNA | DNA replication licensing factor MCM3 homolog 2 | DNA strand elongation involved in DNA replication<br>Mitotic DNA replication initiation<br>Double-strand break repair via break-induced replication | 0.0 |
| 7790 | *Picea glauca* clone mRNA | Protein KTI12 homolog | Leaf development<br>Root elongation | 1e-130 |



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|---|---|---|---|---|
| 8181 | *Picea glauca* clone mRNA | Nucleoline (predicted) | Regulation of:<br>DNA and RNA metabolism, chromatin structure, rDNA transcription, rRNA maturation, cytokinesis, nucleogenesis, cell proliferation and growth, the folding, maturation and ribosome assembly and nucleocytoplasmic transport of newly synthesized pre-RNAs | 0.048 |
| 11409 | *Picea glauca* mRNA | Insulin-degrading enzyme-like 1,<br>peroxisomal | Pathogen response<br>Response to injuries | 9e-157 |

# Discusión integradora

Esta tesis es uno de los primeros trabajos en los que confluyen datos genéticos y variables relacionadas con la ecología de diferentes coníferas para comprender en qué medida pueden responder dichas especies a las perturbaciones ambientales que se están dando como consecuencia directa del cambio climático (Alberto *et al*., 2013). Este trabajo presenta un notable avance en el conocimiento y un alto grado de originalidad, pero a la vez tiene cierta complejidad, debido a la escasez de estudios genéticos previos en todas las especies empleadas. Sus genomas no están secuenciados y, por ello, los resultados derivados de esta tesis no sólo profundizan en los procesos de adaptación a alteraciones ambientales, sino también, incrementan el conocimiento genético fundamental de las especies estudiadas. Ambas aportaciones son clave para la definición de medidas efectivas de protección de la diversidad genética y la conservación de estas especies. Además, los datos moleculares obtenidos han permitido realizar predicciones de vulnerabilidad bajo diferentes escenarios futuros de cambio climático, permitiendo predecir su probabilidad de supervivencia y estimar el grado de vulnerabilidad de las distintas poblaciones.

## 1. Herramientas de análisis genómico

El abordaje desde un punto de vista genético con cualquier especie de conífera es complejo debido a las características intrínsecas que tiene su genoma, con tamaños que rondan las 20 Gbp y un altísimo porcentaje de secuencias repetidas (García-García *et al.,* 2022). Por eso, la elección de la herramienta de genotipado más adecuada para la obtención de marcadores moleculares ha sido un paso crucial ya que, de esta decisión dependerá en gran medida la calidad de los datos obtenidos.

En este trabajo se han empleado dos técnicas de genotipado basadas en NGS: GBS y ddRAD-seq, utilizando la misma profundidad de lectura (1 M). En ambos casos, la secuenciación parcial obtenida del genoma se ha ensamblado *de novo* y, en los casos en los que hubiera algún transcriptoma disponible, también frente a una referencia. Seguidamente, se identificaron los SNP y se llevó a cabo el filtrado de los mismos. Este último paso es crucial ya que elimina ciertos problemas derivados del genotipado

permitiendo obtener unos marcadores fiables sobre los que asentar estudios posteriores.

Ambas técnicas han dado buenos resultados en cuanto al número de SNP descritos. Sin embargo, tras los diferentes filtrados realizados se ha mantenido un mayor número de marcadores con el ddRAD-seq (*A. pinsapo*, *A. marocana* y *P. uncinata*) con un rango entre 3.982 y 11.904 SNP en comparación con el GBS de *C. atlantica* cuyo rango es inferior, 1.885–2.336 SNP. Esta diferencia mostrada podría deberse a que en el caso de la técnica ddRAD-seq únicamente se seleccionan aquellos fragmentos del genoma que hayan sido cortados simultáneamente por las dos ER empleadas (Peterson *et al*., 2012). Esto incrementaría las posibilidades de que el SNP incluido dentro de esa secuencia estuviera representado en la mayoría de los individuos estudiados y, por tanto, que superara los límites fijados para los diferentes parámetros de filtrado. Dichos parámetros de análisis empleados han sido idénticos en todos los casos y son: una frecuencia del alelo menor (*minor allele frequency*, MAF) superior al 5 %, una ausencia de datos del SNP (*missingness*) como máximo del 50 %, que sean marcadores bialélicos y que estén representados en al menos el 50 % de los individuos secuenciados. Este último es un filtrado de individuos el cual no suele ser muy común en este tipo de análisis pero que, debido a su gran utilidad se decidió añadir. Dicho parámetro permite mejorar el conjunto de las variables moleculares obtenidas haciendo que descienda el valor de *missingness* (Cerca *et al*., 2021) y, además permitirá que se mantengan tanto los SNP como los individuos de mayor calidad (O'Leary *et al*., 2018) pudiendo, en algunos casos, eliminar ciertas muestras que serán *outliers* en los estudios posteriores.

Es cierto que puede parecer que estos parámetros son poco restrictivos si los comparamos con los empleados con otras especies como *Olea europaea* L. (D'Agostino *et al*., 2018), *Picea abies* (L.) Karst (Korecký *et al*., 2021), *Pinus contorta* Douglas ex Loudon o *Picea glauca* (Moench) Voss. (Chen *et al*., 2013) en los que se eliminan aquellos SNP que tengan más del 90 % de *missingness*. Sin embargo, en la presente tesis es necesario mantener una menor restricción debido al gran tamaño de genoma que tienen las especies estudiadas (18–20 Gbp) y a la ausencia de referencias. Lógicamente, a mayor tamaño del genoma menos probable es que se secuencien los mismos

fragmentos en las diferentes muestras estudiadas. Además, el límite de ausencia de datos debe establecerse en función de los valores obtenidos en los propios trabajos (Cerca *et al*., 2021), de ahí que el valor de *missingness* aceptado se haya establecido en el 50 %.

La principal diferencia que existe entre las dos técnicas de genotipado empleadas es que en el ddRAD-seq se han utilizado dos ER con distintas frecuencias de corte y en el GBS una sola. En un estudio realizado por Guillardín-Calvo y colaboradores (2019) se compararon los resultados obtenidos con ambas técnicas de genotipado (GBS y ddRAD-seq) para dos especies del género *Quercus*. Encontraron un mayor número de SNP en bruto con el GBS (una media entre réplicas de 128.082 SNP) que con el ddRAD-seq (una media entre réplicas de 15.448 SNP). Sin embargo, la tasa de error por *locus* fue menor con el ddRAD-seq por lo que finalmente se empleó esta técnica obteniendo 5.635 SNP en el filtrado final tras eliminar los errores técnicos. En el trabajo de Pina-Martins y colaboradores (2019) se utilizó la técnica GBS para el estudio de *Quercus suber* y se obtuvieron un total de 2.547 SNP tras el filtrado final. Balao y colaboradores (2020) emplearon la técnica RAD-seq y tras el filtrado se mantuvo un total de 6.090 SNP. Por tanto, los valores que se han obtenido en esta tesis tras los filtrados no están muy alejados de los de otros trabajos.

En la introducción, se han mostrado ejemplos de estudios realizados en el ámbito de la genética con las coníferas que nos ocupan en este trabajo. En la mayor parte de ellos se emplearon SSR como marcadores moleculares para realizar estudios de filogeografía, estructura genética de las poblaciones o diversidad genética (ej.: Terrab *et al*., 2008; Jaramillo-Correa *et al*., 2010; Heuertz *et al*., 2010; Zaborowska *et al*., 2021). Si bien es cierto que estos tipos de estudio han permitido caracterizar las poblaciones de las especies que se han empleado en esta tesis, también ponen de manifiesto el escaso uso de técnicas de genotipado basadas en NGS con dichas especies. Recordemos de nuevo la ausencia de genomas de referencia para estas coníferas estudiadas. En cambio, sí que hay transcriptomas descritos de pinsapo y de cedro del Atlas que se obtuvieron mediante RNA-seq en trabajos previos llevados a cabo en nuestro grupo de investigación (Pérez-González *et al*., 2018; Cobo-Simón, 2020). Estos transcriptomas se pudieron

emplear como referencia permitiendo realizar el abordaje del ensamblaje con referencia y se vio que, en ambos casos, el número de SNP obtenido tras los filtrados fue considerablemente inferior al de la aproximación con el ensamblaje *de novo*. Para el caso del pinsapo, el mínimo de SNP que se mantuvo fue de 1.642 frente a los 3.982 del *de novo* y para el cedro del Atlas, se retuvieron 1.336 SNP frente a los 1.885 SNP del *de novo*. Sin embargo, la gran ventaja que tiene este ensamblaje con referencia es que pese a conseguir un menor número de SNP, se sabe que van a estar localizados en genes y, por tanto, pueden estar relacionados directamente con la función de los genes donde se localizan.

Si bien el número de muestras es sensiblemente inferior al utilizado en otras especies, por ejemplo, en estudios que utilizan organismos modelo, animales o especies cultivadas, debe de tenerse en consideración la mayor complejidad de estos estudios en los que se complementan los datos puramente genéticos/genómicos de cada individuo con variables ambientales y rasgos funcionales, entre los que destacaríamos los dendrocronológicos (Housset *et al*., 2018).

Una vez ajustadas las tecnologías para el desarrollo de los marcadores moleculares (SNP) hay que hacer frente al análisis de los datos obtenidos, lo cual constituye un verdadero reto bioinformático. Aquí, se deben seleccionar aquellas herramientas bioinformáticas que permitan el análisis en profundidad de los datos generados. Además, dado que se carece de genoma de referencia para cualquiera de las especies estudiadas en esta tesis, hay que emplear herramientas que nos permitan realizar un ensamblaje *de novo*. Los genomas de las coníferas muestran una gran divergencia entre especies, por lo que no es recomendable el empleo de genomas de referencia de especies relacionadas ya que, es probable que el alineamiento no sea correcto y que se pierdan muchos SNP (Shu y Moran, 2020).

En el presente trabajo se ha diseñado un protocolo propio de análisis compatible con los datos obtenidos del GBS y del ddRAD-seq. Dicho protocolo se divide en dos partes: el pretratamiento de los datos en bruto, que son los que se reciben tras la

secuenciación masiva y la realización de los diferentes estudios de interés con las matrices genéticas obtenidas (**Figuras 10 y 11**).

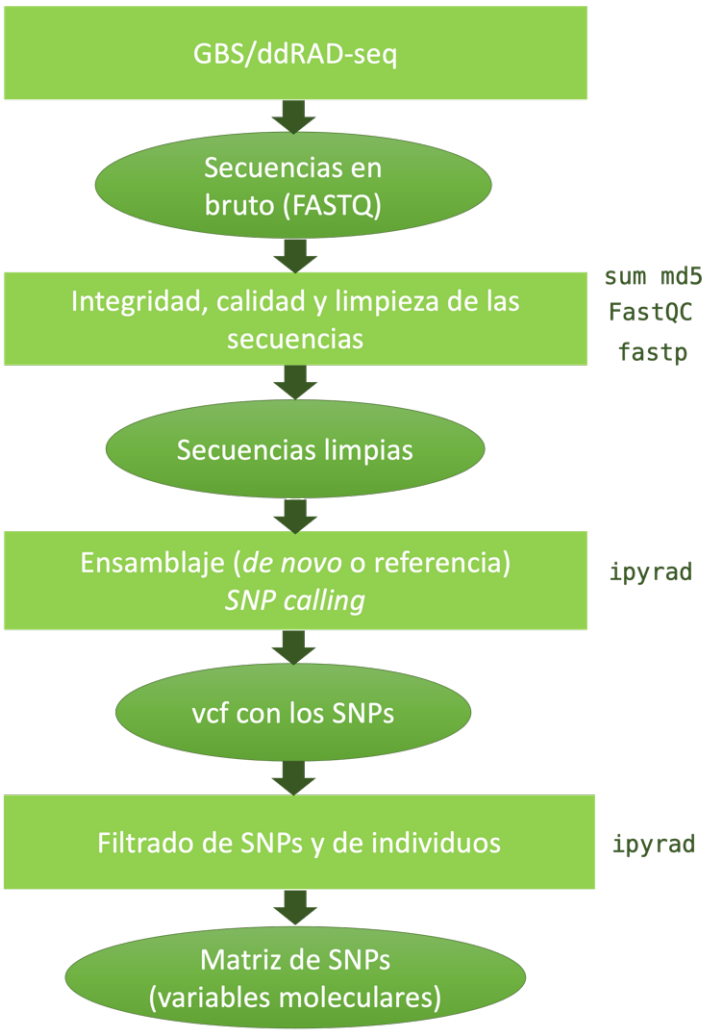


**Figura 10:** *Pipeline* desarrollada para la realización del pretratamiento de las secuencias en bruto obtenidas con las técnicas de genotipado basado en NGS. En los óvalos se muestran los datos que se van generando, en los cuadrados el tipo de análisis. A la derecha de los análisis se nombran los softwares empleados para cada uno de los trabajos.

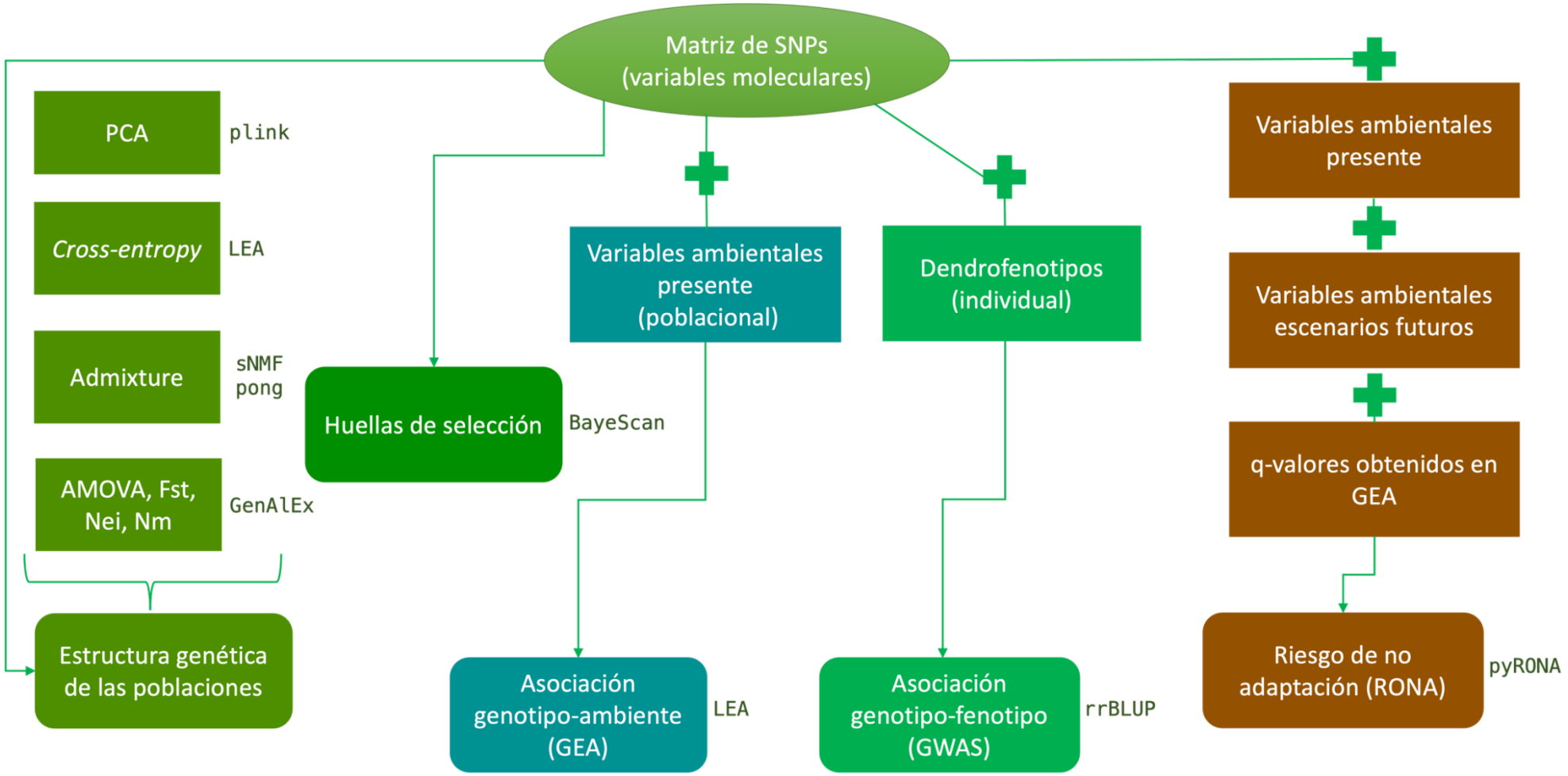


**Figura 11:** *Pipeline* desarrollada para la realización de los diferentes estudios que se han llevado a cabo en esta tesis. Para todos ellos, se ha utilizado la matriz genética obtenida tras el filtrado. En los óvalos se muestran los datos que se van generando, en los cuadrados el tipo de análisis. A la derecha de los análisis se nombran los *softwares* empleados para cada uno de los trabajos.

## 2. Estructura genética de las poblaciones

El empleo en esta tesis de individuos procedentes de poblaciones naturales ha supuesto un considerable esfuerzo de muestreo debido al amplio número de núcleos estudiados (**Figura 12**). Se trata de una ardua tarea pues es preciso tomar una amplia variedad de datos (variables ambientales, suelos, características de las hojas, sensibilidad climática del crecimiento, etc.) recogidos, en la gran mayoría de los casos, a nivel individual con la dificultad que ello conlleva. Además, se ha dispuesto de una amplia variedad de datos dendrocronológicos permitiendo llevar a cabo estudios de asociación entre ellos y los datos genéticos de cada individuo. Heer y colaboradores (2018) pusieron ya de manifiesto la importancia del uso de los datos dendroecológicos a nivel individual para poder establecer estas asociaciones con la información genética de *Abies alba*, una especie muy similar a las investigadas en esta tesis. De este modo, se puede obtener una visión global acerca de la respuesta de las especies forestales a los diferentes tipos de estrés ambiental (Housset *et al.*, 2018).

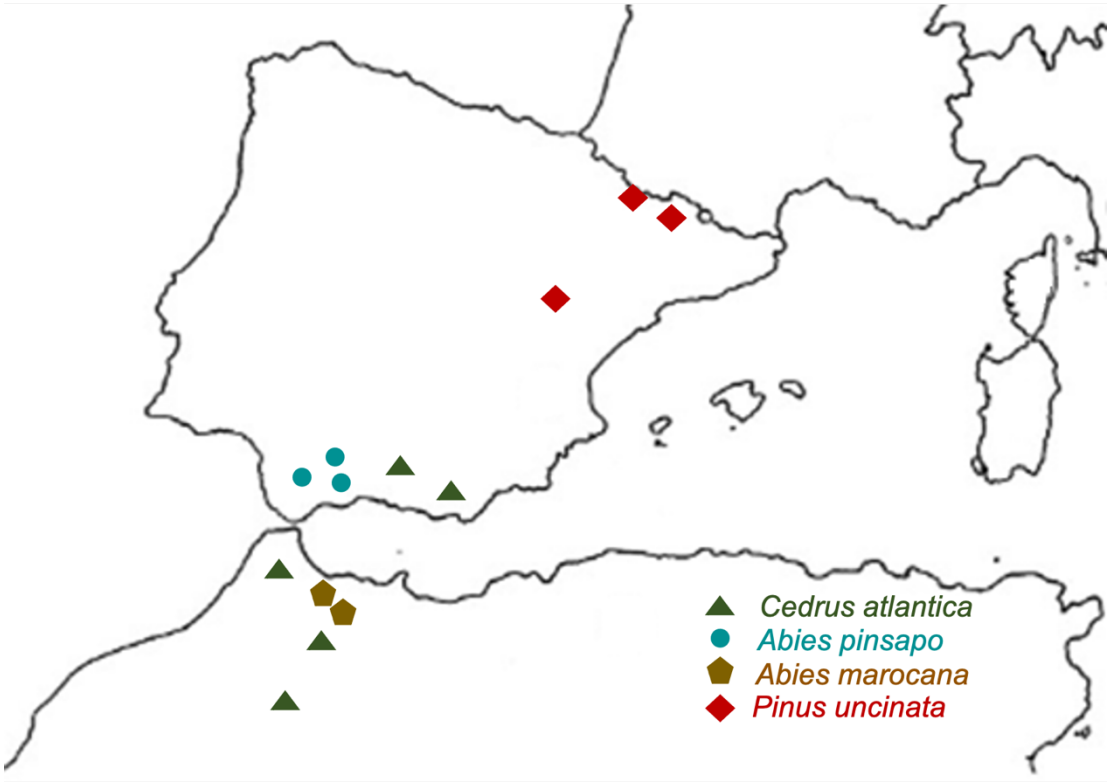


**Figura 12:** Localización de los diferentes núcleos muestreados para las cuatro especies de coníferas estudiadas en la presente tesis.

En las tres poblaciones de Marruecos del cedro del Atlas existe una correlación entre las poblaciones geográficas y las genéticas ya que se han encontrado tres acervos genéticos diferentes, es decir, uno por cada una de las poblaciones muestreadas. Los resultados obtenidos indican que, pese a que la diversidad genética entre las poblaciones no es muy alta, sí que muestran una diferenciación genética entre ellas que es lo que permite que sean considerados como grupos genéticos diferentes. En un trabajo previo con cpSSR se vio que existía una baja diversidad genética entre estas tres mismas poblaciones, pero consideraron que no podían ser tratadas como una única población homogénea (Terrab *et al*., 2006). Esta diferenciación genética poblacional guardaría relación con las variaciones ambientales de cada una de las áreas analizadas (Camarero *et al*., 2021b) que se habrían producido a lo largo del tiempo (Cheddadi *et al*., 2009, 2017; Bouahmed *et al*., 2019).

Por otro lado, el trabajo con el cedro del Atlas ha permitido obtener información acerca de la procedencia de una de las repoblaciones españolas estudiadas lo cual es interesante dado que se carecía de registro alguno de su origen. Dichas repoblaciones se produjeron en los años 50 y 60 del siglo XX en Fiñana (Almería), Dornajo (Granada) y Yunquera (Málaga). Gracias a la obtención de datos genéticos de diferentes poblaciones de cedro de la Península Ibérica y Marruecos, se planteó resolver el posible origen geográfico de las reintroducciones realizadas. En el caso de la de Fiñana, se ha podido determinar que el origen se encuentra en el Alto Atlas gracias a que en el PCA realizado con todas las poblaciones, los individuos de Fiñana agrupan con los de esta población de Marruecos.

En el caso de las poblaciones de Dornajo y Yunquera, dada la diferenciación genética encontrada entre dichas poblaciones y las ibéricas así como las marroquíes, no se pudo determinar su procedencia. Si bien, teniendo en cuenta la existencia de núcleos poblacionales de cedro en diferentes regiones de Argelia, podría plantearse esta zona como posible origen de la reintroducción en estas dos áreas españolas.

Por otro lado, se ha confirmado la existencia de dos especies diferenciadas como son el pinsapo y el abeto marroquí. Así, se habla de *A. pinsapo* para referirse a los

núcleos que se encuentran asentados en España y de *A. marocana* para los situados en el norte de Marruecos. Estos resultados confirman lo obtenido en estudios moleculares previos (ej., Terrab *et al.,* 2007; Dering *et al*., 2014; Sánchez-Robles *et al*., 2014; Balao *et al*., 2020; Litkowiec *et al*., 2021). La marcada diferenciación genética encontrada entre los individuos de pinsapo y los abetos marroquíes se ve apoyada por unos valores muy reducidos de flujo genético entre las mismas. La presencia del Estrecho de Gibraltar podría suponer una barrera geográfica (Terrab *et al*., 2007) que haya facilitado la especiación de los individuos y la reducción del flujo genético entre las diferentes áreas.

Los núcleos de pinsapo conforman un gradiente climático que va desde el punto más al oeste, situado en Grazalema, hasta el más al este, en Saucillo (Linares, 2011). Se puede interpretar que la separación encontrada tanto en el PCA como en el *admixture* entre la población de Grazalema y el resto de las localizaciones, englobadas todas dentro de la Sierra de las Nieves responde a dicho gradiente. Estudios moleculares previos corroboran los resultados obtenidos en esta tesis (ej., Terrab *et al*., 2007; Cobo-Simón *et al*., 2020). Existen dos escenarios de evolución planteados por Cobo-Simón y colaboradores (2020) que podrían explicar nuestros resultados. El primero basado en resultados de nSSR, indica que la población de Saucillo habría divergido de una población ancestral y que posteriormente, el resto de las poblaciones se habrían separado al mismo tiempo produciéndose después un cuello de botella. Esto podría encajar con lo que se observa en el grupo de las poblaciones de Sierra de las Nieves, donde se ve que Saucillo es la que menos se parece al resto. La segunda hipótesis basada en los datos obtenidos con cpSSR, muestra que Grazalema habría divergido directamente de la población ancestral. Dicha hipótesis explicaría esa diferenciación en el acervo genético que tiene Grazalema y que hace que se separe del resto de poblaciones. Pese a que ambas hipótesis parecen explicar los resultados obtenidos en esta tesis, la gran separación encontrada con Grazalema en el PCA parece indicar que existe una mayor posibilidad de que sea la segunda hipótesis la que se haya producido.

Por otro lado, la media del porcentaje de *loci* polimórficos de todas las poblaciones estudiadas de pinsapo es de 97,49 $\pm$ 1,07 %. En un estudio realizado con SNP en dos genes nucleares de pinsapo, *PIP1;3* y *GORK*, se obtuvo un 100 % de *loci*

polimórficos en estas mismas poblaciones, salvo en Pilar de Tólox donde el valor descendió hasta un 50 % (Pérez-González *et al*., 2018). En la presente tesis, Caucón mostró la mayor variabilidad con un 100 % de *loci* polimórficos y el menor valor se dio en Pilar de Tólox. Dado que Caucón es la población del límite sur del pinsapo se va a encontrar sometida a condiciones climáticas que pueden limitar en mayor medida su supervivencia. Por ello, deberá tener una mayor variabilidad genética que permita a sus individuos responder frente a esas condiciones limitantes.

Normalmente, en las especies forestales situadas en el hemisferio norte se suele dar una pérdida de diversidad genética en un gradiente latitudinal que va desde el norte hacia el sur de la distribución (Schierenbeck, 2017), siendo mayor la diversidad en el centro de la distribución ya que las zonas límites tendrán núcleos más pequeños y aislados (Brussard, 1984; Lawton, 1993; Vucetich y Waite, 2003). Sin embargo, en el caso del pinsapo no se produce esta pérdida de diversidad. También es cierto que el eje norte-sur de esta especie es muy reducido ya que los núcleos de pinsapo se encuentran todos muy cercanos entre sí en la zona sur de España. Por lo general, mantener una alta diversidad genética permite tener una mayor posibilidad de sobrevivir frente a las variaciones ambientales (Rajora y Zinck, 2021). Esto se cumple para aquellas especies que tienen rangos de distribución amplios, pero no para las de rangos limitados. Éstas últimas, a las que pertenecen las especies relictas, tienen una menor posibilidad de sobrevivir dado que la pérdida de individuos producida como consecuencia de esas perturbaciones en el ambiente va a contraer en mayor medida su distribución haciéndolas más susceptibles a desaparecer (Jump y Peñuelas, 2005).

En cuanto a los núcleos del abeto marroquí no se pueden confirmar diferencias genéticas significativas entre la zona de Talassemtane y la de Tazaot, al menos con los estudios de *cross-entropy*. Este resultado se ve reafirmado por trabajos previos (ej., Dering *et al*., 2014; Sánchez-Robles *et al*., 2014; Litkowiec *et al*., 2021) y puede deberse a la presencia de flujo genético entre ambos núcleos. El alto valor de Nm (13,393) obtenido indica que ambos sitios actúan prácticamente como una única población. Pese a ello, se ha puesto de manifiesto la existencia de ciertas diferencias que permiten

separar en pequeña medida ambos grupos en el PCA, pudiendo existir algún tipo de relación con las diferencias intrínsecas de los ambientes de ambas zonas.

Aunque la mayoría de los estudios realizados con *A. pinsapo* y *A. marocana*, como se ha comentado anteriormente, se han llevado a cabo con SSR (**Tabla 2**), recientemente Balao y colaboradores (2020) emplearon una técnica basada en NGS, denominada RAD-seq. Utilizaron diferentes especies del género *Abies,* entre las que se encuentran el pinsapo y el abeto marroquí, con el objetivo de describir la historia evolutiva de los abetos circum-Mediterráneos y obtener un árbol filogenético. Es una de las primeras aproximaciones en el empleo de este tipo de técnicas de genotipado.

Para el estudio de la estructura genética de *P. uncinata* se muestrearon tres poblaciones, dos de ellas situadas en Huesca y una en Teruel. Mostraron una estructura que permitió diferenciar a Ordesa y Tessó (Huesca) por un lado y a Peñarroya (Teruel), población relicta que se encuentra en el límite sur de la distribución de esta especie, por otro. Estudios previos basados en caracteres morfológicos mostraron ciertas diferencias entre esta población relicta y las otras que confirmarían los resultados aquí obtenidos desde el punto de vista genético (Gazol *et al*., 2022).

Hartl y Clark (1997) establecieron distintos rangos de valores de $F_{ST}$ que permitieran evaluar, en cierta medida, el grado de influencia de la deriva genética en las diferencias encontradas entre las poblaciones de una especie. En este contexto, se podría interpretar que el cedro ha experimentado desde un bajo grado de deriva (entre la población del Medio Atlas y las del Alto Atlas y el Rif Occidental) a uno medio que es el que se da con todas las repoblaciones en España, así como entre el Alto Atlas y el Rif ($F_{ST}$ = 0,075). Este valor más elevado de $F_{ST}$ está indicando que existe un flujo genético ligeramente menor que el que se da con las otras poblaciones. Una posible explicación puede venir dada por el hecho de que la población del Rif está bastante aislada y las cordilleras del Rif y del Atlas actuarían como barreras orográficas limitando el paso de polen de una a otra (Terrab *et al*., 2006).

Para el caso del pinsapo parece que la deriva genética no ha tenido un papel principal en la separación de las poblaciones ya que los valores de $F_{ST}$ obtenidos de la comparación de poblaciones dos a dos se encuentran en un rango comprendido entre 0,006 y 0,043, siendo más bajos que los del cedro ($F_{ST}$ = 0,075). En este caso, la población de Grazalema es la que muestra los valores más altos del rango. Una posible explicación a que esta población sea la que más se aleja genéticamente de las demás teniendo un acervo propio sería que es la población situada más al oeste del pinsapo y con unas características de precipitaciones muy diferentes a las demás, siendo este punto uno en los que más llueve de España (comunicación personal de J.C. Linares). En estudios previos con estas mismas poblaciones, el valor más alto de $F_{ST}$ se obtuvo para las comparaciones con Grazalema usando cpSSR y para Saucillo empleando nSSR (Cobo-Simón *et al*., 2020). En el estudio de Terrab y colaboradores (2007) realizado con cpSSR, se describió una baja diferenciación genética en *A. pinsapo* ($F_{ST}$ = 0,04). El bajo efecto de la deriva pone de manifiesto que el pinsapo ha mantenido un gran flujo genético entre las poblaciones. Esta idea se ve reforzada por las altas tasas de migración obtenidas en la presente tesis, siendo la encontrada entre Caucón y Saucillo (Nm = 35,923) la mayor de todas.

La especie *A. marocana* también ha mostrado una reducida influencia de la deriva en la diferenciación de los sitios estudiados ($F_{ST}$ = 0,018) comparada con la del cedro ($F_{ST}$ = 0,075). Mientras que, estudios previos realizados con cpSSR en pinsapo y abeto marroquí, mostraron una diferenciación similar en ambas especies (Terrab *et al*., 2007), con los SNP de esta tesis se ha obtenido una menor diferenciación genética en el abeto marroquí que en el pinsapo.

*Pinus uncinata* muestra un efecto reducido de la deriva en la comparación entre Ordesa y Tessó ($F_{ST}$ = 0,031) mientras que en el caso de Peñarroya frente a las otras dos poblaciones es algo superior ($F_{ST}$ = 0,060–0,066). Dicha deriva encontrada en Peñarroya puede estar asociada a su condición relicta que también la diferencia genéticamente del resto. En esta especie se ha obtenido la media de porcentajes de *loci* polimórficos más baja (90,47 ± 5,21 %), siendo Peñarroya la población con el menor porcentaje de polimorfismos con un 80,05 % y la mayor cantidad de alelos fijados. Esto se corresponde

con que esta población es relicta y su tamaño poblacional es menor lo que incrementa el efecto de la deriva favoreciendo la fijación de alelos.

Por lo general, en las especies/poblaciones relictas se suele esperar una baja diversidad genética como consecuencia de ese tamaño poblacional pequeño y de su aislamiento, pero a la vez, estos núcleos suelen caracterizarse por estar genéticamente diferenciados del resto (Hampe y Jump, 2011; Rehm *et al.,* 2015). Las especies relictas analizadas en esta tesis no mostraron ni una baja diversidad ni una alta diferenciación genética entre sus poblaciones lo que contrasta con otras especies relictas del Mediterráneo que sí que suelen ser más diferentes (Hampe *et al*., 2003; Petit *et al*., 2003). Además, se ha descrito la existencia de flujo génico entre todas las poblaciones estudiadas lo que hace que se alcance un estado que podría ser cercano al de panmixia en la mayoría de los casos. Nuestros resultados son similares a otros trabajos como el realizado en pinsapo de Cobo-Simón y colaboradores (2020) en el que se encontró una diversidad mayor de la esperada en las mismas poblaciones estudiadas en el presente trabajo.

## 3. Huellas de selección

Determinar la existencia de diferencias genéticas entre los individuos adultos y los jóvenes en una población podría indicar tendencias de cambio en respuesta a un estrés ambiental que estaría condicionando el desarrollo de los nuevos individuos. Sin embargo, esas variaciones ambientales se están produciendo con una rapidez superior a la tasa de generación de las especies forestales lo que dificulta que se puedan encontrar diferencias moleculares en una sola generación. Por eso, ninguna de las poblaciones de las distintas especies estudiadas ha mostrado una estructura genética que separe a los adultos de los jóvenes (Dauphin *et al*., 2021).

A lo largo de los años, se han llevado a cabo trabajos relacionados con la búsqueda de genes sometidos a selección en diferentes especies de coníferas, como *Pinus sylvestris* L. (Wachowiak *et al*., 2009; Kujala y Savolainen, 2012), *Pinus mugo* Turra.

y *Pinus uliginosa* G. E. Neumann (Wachowiak *et al*., 2011), *Pinus uncinata* (Zaborowska *et al*., 2021), *Pinus pinaster* Ait. (Eveno *et al*., 2008), *Pinus radiata* D. Don (Dillon *et al*., 2013) y *Pinus contorta* (Eckert *et al*., 2012). En la mayoría de los casos, se han estudiado genes candidatos de respuesta a varios tipos de estrés como, por ejemplo, a la sequía o al frío. Algunos de los genes nucleares empleados son los de las deshidrinas, del ácido abscísico (ABA), de la embriogénesis tardía (LEA) o de fitocromos (ej.: Wachowiak *et al*., 2009; 2011). En cuanto a las especies del género *Abies,* la cantidad de estudios de este tipo es menor. Se puede encontrar alguno como el realizado con varias especies de árboles de montaña entre las que se encuentra *Abies alba*, y en el que prácticamente no se identificaron huellas de selección significativas para los 70 genes estudiados con dicha especie (Mosca *et al*., 2012a). Sin embargo, en ninguno de estos trabajos previos se ha llevado a cabo un estudio completo del genoma, por lo que tanto la estrategia como los análisis desarrollados en la presente tesis son de gran interés.

Como se ha mostrado en la anterior sección, en términos generales existen pocas diferencias genéticas entre las poblaciones de las especies estudiadas. Sin embargo, este resultado no impidió encontrar ciertos SNP que parecen estar sometidos de forma significativa a algún proceso evolutivo como podría ser, por ejemplo, la selección.

Se han identificado SNP sometidos a algún proceso evolutivo con todas las especies estudiadas siendo el mayor número el descrito con pinsapo (51 SNP) y el menor, el de las poblaciones de cedro de Marruecos (4 SNP). Todo esto indica que esas regiones en las que están los SNP significativos son susceptibles de desempeñar un papel en los procesos de adaptación local de dichas especies (Dauphin *et al*., 2021).

Se llevó a cabo una segunda estrategia en aquellas poblaciones en las que en la toma de muestras se colectaron individuos adultos y jóvenes. Esto se pudo realizar con las poblaciones de Marruecos de cedro del Atlas y con los núcleos de abeto marroquí. La búsqueda de SNP significativos con el cedro separando las muestras por edades, permitió identificar únicamente 2 SNP. Mientras que, en el caso del abeto marroquí fueron 72 SNP los significativos siendo este supuesto en el que se consiguió describir el mayor número de SNP.

A la vista de estos resultados se puede decir que en los dos núcleos de *A. marocana* está teniendo lugar algún cambio en las frecuencias alélicas propiciado por una posible presión selectiva. De esos 72 SNP significativos, 20 fueron también identificados en este mismo estudio realizado con las poblaciones de Talassemtane y Tazaot sin distinción por edades. Por tanto, esos SNP estarían relacionados con las diferencias intrínsecas que hay en las características climáticas de ambos núcleos. Mientras que, por otro lado, los 52 SNP identificados exclusivamente en el análisis por edades sí que podrían estar mostrando ciertas diferencias en las frecuencias alélicas entre adultos y jóvenes. De ello se puede deducir que, las variaciones ambientales que se están dando en esos dos núcleos sí que están repercutiendo en los individuos jóvenes y se está evidenciando en ciertos cambios genéticos.

En términos generales, los SNP obtenidos en los estudios de selección permiten identificar regiones del genoma sometidas a algún proceso evolutivo, pero no se puede determinar el tipo de fuerza evolutiva que está actuando. Una parte de los SNP identificados se encuentran en secuencias codificantes, pero debido, sobre todo, a la ausencia de genomas de referencia en muchos casos no ha sido posible determinar la función de los correspondientes genes. Pese a ello, sí que se han podido encontrar ciertas homologías con secuencias de proteínas descritas en otras especies. Las más interesantes son aquellas que ya se han descrito en otras especies como las relacionadas con la respuesta al estrés abiótico, dado que en la presente tesis se ha estudiado la respuesta desde el punto de vista genético al cambio climático. Algunas de las más interesantes se discuten a continuación.

En cedro, se han encontrado dos secuencias con homología con una proteína denominada WAT1. Esta proteína está involucrada en el crecimiento secundario de las fibras de la pared celular (Ranocha *et al*., 2010) y en la ruta de señalización de las auxinas. Ranocha y colaboradores (2013) determinaron que es un transportador de auxinas situado en el tonoplasto vacuolar que regula la homeostasis de las plantas. Las posibles funciones se podrían considerar básicas para el mantenimiento del organismo, de ahí que esté sometida a algún proceso evolutivo.

La identificación de posibles factores de transcripción es importante dado que podrían formar parte de un mecanismo de respuesta frente a variaciones en el ambiente. Este es el caso en pinsapo, donde hemos encontrado una homología con una secuencia de la familia de factores de transcripción DREB (*dehydration-responsive element binding transcription factor*). Concretamente, estos factores de transcripción suelen actuar en respuesta a distintos tipos de estrés abióticos como son la temperatura, la sequía o la salinidad. En *Picea abies*, Haas y colaboradores (2020) describieron la implicación de un factor de transcripción de esta familia en respuesta a la sequía. Por tanto, la caracterización de la secuencia que contiene este SNP podría ser relevante para futuros estudios de respuesta del pinsapo a dicho tipo de estrés.

Las poblaciones de abeto marroquí mostraron también diferentes secuencias con homologías de interés. Una de ellas está relacionada con la proteína LRK1. La familia LRK está involucrada en el desarrollo, la respuesta a patógenos y al estrés ambiental (Shiu y Bleecker, 2003). Por otro lado, Kang y colaboradores (2017) encontraron que LRK2 está relacionado con la tolerancia a la sequía en arroz. Estas posibles funciones hacen que dicha proteína se postule como una potencial candidata con vistas al futuro.

Con todo ello, este tipo de estudios de procesos evolutivos han permitido que se identifiquen posibles huellas de selección en los genomas de estas especies de coníferas. Los SNP que presentan diferencias significativas en sus frecuencias alélicas entre las poblaciones de estudio son la vía para identificar esas huellas. Se ha obtenido un número muy diferente de SNP en las especies analizadas. Es razonable pensar que aquellas especies en las que se han detectado más SNP pudieran tener un acervo genético con mayor capacidad de respuesta adaptativa al cambio climático. En este sentido, se puede hipotetizar a la luz de los resultados de esta tesis que, especies como *Abies pinsapo* o *Abies marocana* pueden tener una capacidad superior de adaptarse al escenario de calentamiento global en el que nos encontramos. Por otra parte, los casos en los que se han podido identificar genes concretos en esas regiones genómicas de interés permiten apuntar a la posible existencia de diferentes mecanismos adaptativos en cada especie.

## 4. Asociaciones genotipo-ambiente y genotipo-fenotipo

Los estudios de asociación genotipo-ambiente (GEA) y genotipo-fenotipo (GPA) han ido adquiriendo una mayor relevancia en el ámbito de los estudios forestales. El empleo de estos tipos de análisis permite conocer las relaciones existentes entre los genotipos de los individuos de una población y las variables ambientales de los sitios en los que habitan (GEA) o entre el genotipo y el fenotipo de los individuos (GPA). Como es bien sabido, los individuos de una misma población no tienen la misma carga genética y pequeñas variaciones genéticas pueden marcar la diferencia entre la muerte o la supervivencia de un individuo. De ahí que los estudios de asociación nos ayuden a comprender por qué ciertos individuos se adaptan mejor que otros a condiciones ambientales adversas.

Son varios los estudios de GEA que se pueden encontrar en la bibliografía relacionados con alguna especie perteneciente al grupo de las coníferas como, por ejemplo, *Picea glauca* (Depardieu *et al*., 2021), *Abies alba*, *Larix decidua*, *Pinus cembra* y *Pinus mugo* (Mosca *et al*., 2012b). En esta tesis se han analizado para las cuatro especies de coníferas estudiadas las posibles asociaciones de los SNP con las variables ambientales relacionadas con la temperatura y con las precipitaciones que están registradas en WorldClim.

En el caso del cedro del Atlas, se han identificado un total de 23 asociaciones entre SNP y variables ambientales. Cinco de las asociaciones se han observado con la temperatura media anual siendo este valor el máximo encontrado con una misma variable. Recientemente se ha visto que las variables bioclimáticas que más influyen en la composición genética de esta especie son las relacionadas con el incremento de la temperatura (Bobo-Pinilla *et al*., 2022).

Por otro lado, estudios de ecofisiología realizados previamente han mostrado que la sensibilidad a la sequía es el factor ambiental más relacionado con el decaimiento de las poblaciones de cedro del Atlas (Linares *et al*., 2011b; Navarro-Cerrillo *et al*., 2019; Camarero *et al*., 2021b). También, Linares y colaboradores (2013) encontraron que el

crecimiento de esta especie se veía afectado por el incremento de la temperatura. Nuestros resultados indican que la temperatura podría tener un mayor impacto que la sequía ya que se han identificado menos asociaciones con las variables relacionadas con precipitaciones. Sin embargo, ambas conclusiones no son contradictorias ya que, a mayor temperatura mayor será la tasa de evapotranspiración que provocará un incremento de la pérdida de agua. La principal estrategia fisiológica del cedro del Atlas, al ser una especie anisohídrica, es mantener sus estomas abiertos pese a estar sometido a sequía para prolongar la fotosíntesis el mayor tiempo posible, por lo que retarda al máximo la activación de los mecanismos de ajuste osmótico (Taoufik *et al*., 2021). Sin embargo, esta capacidad podría llegar a perjudicarle ya que sus tejidos quedan expuestos a sufrir una deshidratación máxima que puede provocar la muerte del individuo (Allen *et al*., 2010; Choat *et al*., 2012).

En el caso del pinsapo, se han identificado un total de 15 asociaciones entre SNP y variables ambientales siendo el menor número descrito para las especies estudiadas. También en esta ocasión, se ha determinado un total de 5 asociaciones con una misma variable que es la máxima temperatura del mes más cálido. Dado que el pinsapo es una especie que necesita grandes cantidades de agua para poder sobrevivir y que se encuentra asentada en sitios de clima Mediterráneo, cualquier variación en la temperatura afectará en mayor medida a su supervivencia. Más aún cuando esa alteración se da en el mes más cálido. Este ambiente le ha hecho tener ciertas adaptaciones como un gran tamaño de traqueidas que le permiten transportar una mayor cantidad de agua a las acículas cuando se producen pérdidas por evapotranspiración, haciendo que pueda sobrellevar mejor la alta demanda de evaporación que tiene durante los meses de verano (Peguero-Pina *et al*., 2011). También se ha visto que el pinsapo, al contrario que el cedro del Atlas, cierra sus estomas de forma temprana para evitar la pérdida de agua tanto cuando hay falta de humedad en el suelo como cuando la tasa de evapotranspiración es muy elevada a consecuencia de la temperatura ambiente (Sánchez-Salguero *et al*., 2015).

En esta especie, al igual que en el cedro, se ha encontrado una relación entre el crecimiento y el incremento de la sequía observándose que las altas temperaturas en el

inicio del verano producen una disminución del crecimiento (Navarro-Cerrillo *et al*., 2020). Cuando en invierno se dan temperaturas más altas, el pinsapo crece gastando la reserva de recursos hídricos y de carbono que se acumulan en esa época del año para el verano. Este proceso provocará un mayor riesgo de defoliación en verano derivando en una mayor mortalidad (Navarro-Cerrillo *et al*., 2020). Por tanto, estas variaciones en las temperaturas harán que el pinsapo no pueda disponer de las reservas necesarias para su supervivencia en los meses en los que su tasa fotosintética es menor como consecuencia del cierre de sus estomas que coincide con los periodos más secos y calurosos del año.

El caso del abeto marroquí es reseñable pues un mismo SNP ha mostrado asociación con las 19 variables analizadas. Además, este mismo SNP se ha identificado en los análisis de huellas de selección. Esto puede estar indicando que en esa región del genoma se encontrarían uno o varios genes con un papel fundamental en la adaptación de esta especie a las variaciones ambientales. Sus frecuencias génicas muestran que el alelo de referencia se encuentra en una frecuencia de 0,97 en la población de Talassemtane y de 0,41 en la de Tazaot. Dado que Tazaot es la población que tiene unas condiciones ambientales más secas, se podría pensar que el alelo alternativo es el que le aporta cierta ventaja para adaptarse a ese tipo de ambiente (Méndez-Cea *et al*., *In rev.*).

Se calcularon, también, las frecuencias dividiendo a las poblaciones por edades y se observó que en el caso de Talassemtane no había diferencias, mientras que en Tazaot sí mostraban diferencias significativas. Los individuos jóvenes de Tazaot tenían una mayor frecuencia del alelo alternativo (0,67) que los adultos (0,5). Las condiciones ambientales están siendo cada vez más acusadas en la zona de Tazaot, la cual ya mostraba inicialmente condiciones más áridas que en Talassemtane. Lo que parece observarse es una selección positiva del alelo alternativo en los individuos jóvenes, el cual les podría conferir cierta ventaja en el escenario cambiante. Todo ello se ve reforzado por el hecho de la homología encontrada con esta secuencia que es la proteína LRK1. Dicha familia se caracteriza, como se ha indicado en la sección de huellas de selección, por estar involucrada, entre otras cosas, en la respuesta al estrés ambiental

(Shiu y Bleecker, 2003). A la vista de estos resultados, en un futuro, se podría plantear el desarrollo de un método de genotipado de esta región para poder caracterizar la vulnerabilidad de los individuos y poblaciones frente a las perturbaciones ambientales.

Es importante destacar que en el caso de *P. uncinata* no solamente se emplearon las variables de WorldClim extraídas a nivel poblacional, sino que además se contó con variables de suelos obtenidas individualmente. El número total de las asociaciones identificadas con las variables ambientales fue considerable, 524. El rango anual de temperatura y la estacionalidad de las precipitaciones son las variables que mostraron un mayor número de asociaciones. Dado que esta especie se encuentra situada en la parte más alta de la ladera cualquier perturbación puede alterar su supervivencia ya que no puede, como ocurre en otras especies, ascender en busca de mejores condiciones para sobrevivir. Se ha visto con anterioridad que tanto la temperatura como el estrés hídrico afectan a la formación de las semillas del pino negro. Por tanto, ambas variables van a ser las más limitantes a la hora de que este pino pueda expandirse (Camarero y Gutiérrez, 2007).

El crecimiento de los árboles puede estar afectado directamente por la concentración de nutrientes presentes en el suelo (Hagedorn *et al*., 2019) lo que demuestra la gran relevancia que tienen las variables estudiadas de suelo. En un trabajo previo realizado por Gazol y colaboradores (2022) con las mismas poblaciones utilizadas en esta tesis, se vieron variaciones en el contenido de los suelos. Las concentraciones de carbono y de nitrógeno eran muy diferentes entre las poblaciones y la ratio carbono-nitrógeno (C:N) era menor en Peñarroya que en los otros dos lugares estudiados. El estudio conjunto de variables de campo con las variables genéticas nos permite tener una visión más completa sobre la importancia que tienen estas características ambientales para la supervivencia de esta especie. Así, con los estudios de asociación realizados con estas variables de suelo se ha determinado que las diferencias observadas en el campo tienen una base genética identificando que la ratio C:N y el contenido de nitrógeno son los factores más limitantes para el pino negro.

Por otra parte, como ya se ha dicho anteriormente, en algunas de las especies analizadas hemos realizados también estudios de asociación genotipo-fenotipo (GPA). En nuestro caso se han buscado asociaciones entre datos dendrocronológicos a nivel individual y los SNP generados en los experimentos ddRAD-seq. Este tipo de trabajos con datos individuales requieren un esfuerzo considerable, pero aportan resultados muy interesantes y valiosos en el ámbito de la ecología (Heer *et al*., 2018). En la presente tesis, el GPA se ha realizado en las poblaciones de abeto marroquí y en las de pino negro (Méndez-Cea *et al*., 2023). El empleo de datos individuales para tratar de describir cómo es su respuesta a las perturbaciones ambientales, es algo bastante novedoso y que no ha sido estudiado en dichas especies. Poder analizar genomas individuales es una herramienta muy potente que permite determinar la existencia de relaciones entre dendrofenotipos y ciertas regiones genómicas que nos darán una idea de la relevancia que puede tener el genotipo en la capacidad de adaptación de los individuos.

Diferentes conjuntos de variables dendrocronológicas se han medido para ambos casos que se enmarcan en varios grupos de datos. El primero es el de la tendencia, que muestra patrones de crecimiento a lo largo del tiempo. Otro con la sensibilidad climática del crecimiento que muestran tendencias significativas a lo largo del tiempo entre el crecimiento y las variables de temperatura, precipitación e índices de sequía (SPEI), tanto a nivel mensual como anual. Finalmente, el último conjunto de datos está relacionado con la respuesta de los individuos frente a determinados eventos extremos de sequía, cuantificándose indicadores de resistencia, recuperación y resiliencia en el crecimiento **(Figura 13)** (Lloret *et al*., 2011).

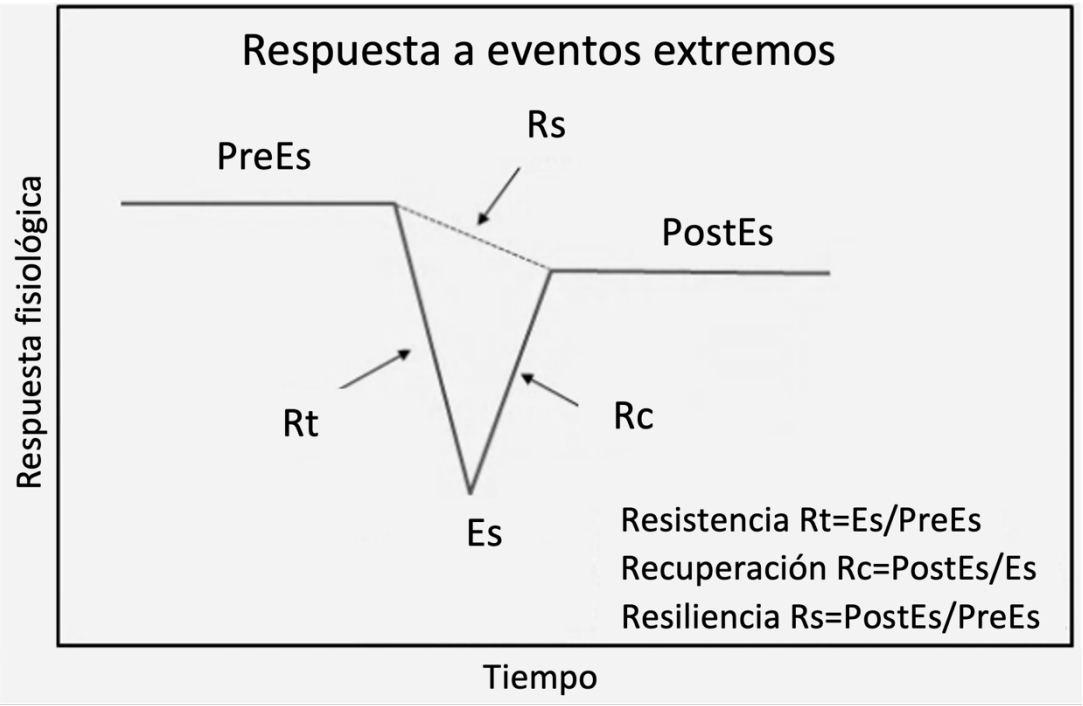


**Figura 13:** Estimación de indicadores de resistencia (Rt), recuperación (Rc) y resiliencia (Rs) en estudios ecológicos. PreEs caracteriza el estado previo al estrés, el cual suele ser, en nuestro caso, una sequía extrema, PostEs representa un periodo inmediatamente posterior al evento de estrés, y Es indica el momento en el que ocurre el estrés. Las fórmulas que permiten calcularlas se muestran abajo a la derecha. (Esquema de Juan Carlos Linares basado en Lloret *et al*., 2011).

En el caso del abeto marroquí, los dendrofenotipos de crecimiento y de resiliencia mostraron diferentes asociaciones con los SNP, 26 y 7, respectivamente. Mientras que, los relacionados con la sensibilidad climática no dieron ninguna asociación. Esto puede parecer contradictorio a lo que se podría esperar, sin embargo, como se ha comentado anteriormente, el crecimiento está altamente relacionado con las condiciones ambientales a las que está sometido el individuo. Los árboles necesitan reservas de carbono que puedan ser utilizadas en el crecimiento. Cuando se produce una sequía, especies como el abeto marroquí, que son sensibles a la sequía (Aussenac, 2002), van a disminuir la tasa fotosintética haciendo que disminuya la cantidad de carbono disponible y es por esto por lo que esos eventos de sequía suelen coincidir con un menor crecimiento en muchas especies forestales (DeSoto *et al*., 2020).

En el caso del GPA realizado en el pino negro se midieron de nuevo variables dendrofenotípicas y adicionalmente caracteres morfológicos de las acículas. No se

obtuvo ninguna asociación con los dendrofenotipos relacionados con la sensibilidad al clima, pero sí con los de crecimiento mostrando un total de 6 asociaciones. Estos resultados son similares a los obtenidos con el abeto marroquí (Méndez-Cea *et al*., *In rev*.). Por tanto, se pone de manifiesto que las variables relacionadas con el crecimiento pueden darnos una idea muy acertada de cómo responden genéticamente las especies a las variaciones ambientales (Méndez-Cea *et al*., 2023). Así como que, desde el punto de vista genético, parece que son más importantes los patrones de crecimiento del individuo a lo largo de los años que los que contemplan únicamente un año en concreto en el que se ha producido una sequía severa.

Se identificaron 16 asociaciones con los rasgos morfológicos. Dichas asociaciones se dieron con variables relacionadas con medidas de las acículas. Concretamente las variables de masa por el área foliar (*leaf mass area)* y el contenido de materia seca de la hoja (*leaf dry matter content*) cuentan con 2 y 8 asociaciones, respectivamente. Este resultado es un indicio de la importancia de las características de las hojas en la adaptación ambiental de estos árboles y que apoya resultados previos en este sentido (Gazol *et al.*, 2022). Por tanto, a la vista de lo obtenido en este GPA se puede decir que las diferencias fenotípicas observadas en las acículas de varios núcleos poblacionales tienen una fuerte base genética.

Diferentes trabajos han determinado que cuanto mayor sea la masa por el área foliar mayor longevidad y retención de nutrientes tendrán las hojas, así como estarán más protegidas frente a la sequía (de la Riva *et al*., 2016; Mooney y Dunn, 1970; Nardini, 2022). Según esto, las acículas de Peñarroya, al estar sometidas a unas condiciones ambientales más secas deberían tener una elevada masa por área, sin embargo, presentan la menor de todas las poblaciones estudiadas. Esta característica de hojas pequeñas incrementa la superficie de absorción por unidad de biomasa tisular haciéndolas más eficaces en la toma de recursos (Wright *et al*., 2004) y disminuye la pérdida de agua. Por tanto, en Peñarroya se podría postular la existencia de una presión selectiva para mantener el tamaño de hoja pequeño incrementando así la eficacia en la adquisición de nutrientes con mayores tasas fotosintéticas. Es importante comentar que los datos obtenidos por Gazol y colaboradores (2022) indicaron que existía una fuerte

dependencia entre el crecimiento de los individuos de la población de Peñarroya y la disponibilidad de agua por lo que, dichas características encontradas en sus acículas pueden ser determinantes para el crecimiento de los individuos en esta zona.

En los experimentos de genómica en coníferas se genera un menor número de marcadores de tipo SNP en comparación con otras especies de animales y de plantas con genomas más pequeños. Además, la ausencia de genomas de referencia es otro factor limitante a la hora de hacer este tipo de estudios de asociación, GEA y GPA. Pese a ello, como se ha demostrado en esta tesis, se pueden identificar regiones de interés en el genoma implicadas en la respuesta a las variaciones ambientales de esa especie. Al igual que se ha manifestado en el apartado de huellas de selección, esa ausencia de genomas de referencia limita en muchos casos la identificación de genes candidatos localizados en la proximidad de los SNP que muestran asociaciones con variables ambientales o fenotípicas de interés. Pese a ello, hay ciertos casos en esta tesis, que se van a mostrar a continuación, en los que se han podido identificar posibles genes candidatos responsables de la respuesta al estrés en estas especies de coníferas.

Por ejemplo, para el caso del cedro del Atlas se ha identificado un factor de transcripción de respuesta a etileno (ABR1) que puede ser muy interesante ya que podría regular la activación/inactivación de la expresión de los genes en situaciones de estrés. Experimentos realizados en el organismo modelo *Arabidopsis thaliana* han demostrado que se produce una gran acumulación de tránscritos de ABR1 cuando la planta está sometida a estrés hídrico y a bajas temperaturas (Pandey *et al*., 2005).

Con las regiones del pinsapo se han encontrado varias asociaciones con factores de transcripción de la familia MYB, la cual está altamente relacionada con la respuesta y la tolerancia a diferentes tipos de estrés abiótico. Esta familia está ampliamente distribuida entre las plantas y se caracteriza por ser una de las más numerosas dentro de los factores de transcripción (Li *et al*., 2019). En *A. thaliana* se ha visto que diferentes genes MYB están relacionados con la respuesta a la sequía (Cominelli *et al*., 2005) y hay algunos de ellos que regulan el cierre estomático a través de la cascada de señalización de la ruta de ABA (Seo *et al*., 2011). Además, se ha identificado una asociación con un

factor de transcripción DREB que también está sometido a selección y ya se ha hablado de su posible relevancia en el apartado de huellas de selección.

Dentro de las homologías encontradas con el abeto marroquí las más relevantes para nuestro estudio son aquellas que aparecen en varias ocasiones y están relacionadas con la biosíntesis de etileno. El etileno es una molécula endógena de las plantas cuyos niveles aumentan cuando debe responder a algún tipo de perturbación o en ciertos momentos del desarrollo (Kende, 1993). Además, está involucrada en la respuesta al estrés abiótico (Chen *et al*., 2022) función que hace que sea interesante para este tipo de trabajos.

Finalmente, en el pino negro se ha identificado un transportador de GABA, ácido γ-aminobutírico, que es bastante interesante dado que GABA incrementa la resistencia a la sequía en las plantas (Mekonnen *et al*., 2016) y, además, está relacionado con el balance carbono-nitrógeno (Fait *et al*., 2011; Batushansky *et al*., 2014). También se encontró una similitud con una proteína denominada *auxin-induced protein 6B-like,* relacionada con la activación de la ruta de las auxinas. Las auxinas son unas hormonas propiamente de plantas que están involucradas en la regulación del crecimiento y desarrollo de estos organismos. Sin embargo, se sabe que también responden frente a estímulos externos como son la sequía o el calor generando un gradiente de auxinas que estará más concentrado en el lugar inicial donde se ha producido el estrés (Blakeslee *et al*., 2019). En *A. thaliana* se ha visto que hay una relación directa entre el incremento de los niveles auxinas y la capacidad de tolerar la sequía (Shi *et al*., 2014).

Por tanto, los estudios de asociación llevados a cabo en esta tesis han permitido obtener una idea general de qué variables ambientales son las que más influyen en cada una de las especies estudiadas. Se ha visto en el GEA, que tanto para el cedro del Atlas como para el pinsapo las variables relacionadas con la temperatura son las más influyentes. El abeto marroquí ha mostrado un único SNP que sin embargo muestra una asociación consistente con todas las variables estudiadas, indicando la gran relevancia que puede tener esta región del genoma ya que está sometida a selección también. Mientras que, para el pino negro son relevantes prácticamente en la misma medida, la

temperatura anual y la estacionalidad de las precipitaciones. Además, para esta especie el nitrógeno y la ratio C:N del suelo son muy importantes para su desarrollo debido al número de asociaciones identificadas. Por otro lado, los análisis de asociación con variables fenotípicas individuales (GPA) realizados con el abeto marroquí han puesto de manifiesto asociaciones con dendrofenotipos de crecimiento y de resiliencia, pero se ha visto la ausencia de ellas con la sensibilidad del crecimiento al clima. Con el pino negro se han identificado asociaciones con los dendrofenotipos de crecimiento, pero no con los de sensibilidad, al igual que con el abeto marroquí. Además, con el pino negro se ha descrito la gran relevancia que tiene la morfología de las acículas obteniendo una explicación genética a las diferencias encontradas en el campo. Finalmente, a pesar de las limitaciones para encontrar homologías de las secuencias identificadas con genes descritos previamente se han localizado posibles genes de potencial interés. De este modo, destacan las familias de factores de transcripción ABR1 en el cedro, MYB en el pinsapo o la síntesis de etileno en el abeto marroquí. Y, por último, un transportador de GABA y la ruta de las auxinas con el pino negro.

## 5. Vulnerabilidad de las especies estudiadas frente a las variaciones ambientales

En los últimos años se ha venido observando cierto decaimiento en las poblaciones de las especies estudiadas en la presente tesis y se ha descrito que los pinos al igual que los abetos tienen una mayor vulnerabilidad frente a sequías intensas y duraderas (Gazol *et al*., 2018). Por otro parte, se ha puesto de manifiesto el incremento en el rango altitudinal de muchos individuos de las poblaciones de estas especies (Cheddadi *et al*., 2017; Linares y Carreira, 2006). Tener la capacidad de obtener ADN de aquellos individuos que han muerto constituye un recurso fundamental para poder genotiparlos y tratar de encontrar la base genética que permita discernir entre los individuos capaces de hacer frente a las variaciones ambientales y aquellos que no lo son. De ahí que, la puesta a punto del protocolo de extracción de ADN a partir de madera muerta realizado en la presente tesis vaya a ser de gran utilidad para el futuro.

Por otro lado, la predicción de la vulnerabilidad es muy importante para determinar cuáles son las variaciones ambientales que más afectan a las especies estudiadas y qué individuos y/o poblaciones pueden ser más resilientes en respuesta a estas perturbaciones. Además, estos trabajos adquieren una mayor relevancia cuando están centradas en especies endémicas como son todas las que se han usado en este trabajo, a excepción del pino negro. Estas especies pueden ser más sensibles a pequeñas variaciones ambientales al estar adaptadas a entornos muy concretos que son los que han permitido su supervivencia a lo largo de los años (Fois *et al*., 2016). De ahí que, determinar el potencial adaptativo y la vulnerabilidad a las perturbaciones a las que deben hacer frente, conformen una importante base para futuros trabajos de conservación de las especies (Xiao *et al*., 2022).

Si los estudios ecofisiológicos se combinan con la información genética de las especies, la comprensión de la respuesta a las variaciones ambientales será más completa. Además, dichos datos pueden utilizarse para determinar qué capacidad tiene una especie para sobreponerse a los cambios ambientales y conocer así su vulnerabilidad a distintos tipos de escenarios ambientales (Schierenbeck, 2017). Por ello, es importante caracterizar la variabilidad y la estructura genética de las poblaciones para así poder estimar su capacidad adaptativa. En términos generales, altos niveles de heterocigosidad están relacionados con una mayor capacidad para hacer frente a las perturbaciones ambientales (Bonin *et al.,* 2007). Aquellas poblaciones que tienen una distribución muy limitada y presentan perturbaciones frecuentes y severas, pueden sufrir un descenso drástico de la heterocigosidad (Davies *et al*., 2016). Por tanto, las poblaciones relictas o endémicas suelen ser también desde un punto de vista genético, más susceptibles a cualquier variación en el clima.

En este contexto, se han desarrollado herramientas para el estudio del riesgo de no adaptación (*risk of non-adaptedness*, RONA) permitiendo obtener un valor que muestre qué tasa de cambio es necesaria que se produzca en la población para poder hacer frente a las perturbaciones ambientales. Esta tasa está relacionada con los cambios que se han de dar en las frecuencias alélicas, que en el caso estudiado en esta tesis se calcula a partir de los valores de los SNP que muestran asociaciones en el GEA.

Esto permitirá estimar si la tasa de cambio genético en la población va a darse lo suficientemente rápido como para poder adaptarse a esa alteración. Generalmente, en las poblaciones naturales se pueden producir adaptaciones frente a las perturbaciones ambientales de una forma rápida (Shaw y Etterson, 2012), pero siempre hay que tener en cuenta los tiempos de generación propios de cada especie (Jump *et al*., 2006). Además, este tipo de estudios permiten definir cuál o cuáles son las variables ambientales que se presentan como más limitantes para la supervivencia de la especie estudiada en cuestión. Por tanto, el fin último del RONA es el de realizar predicciones de respuesta de vulnerabilidad.

En esta tesis se ha podido realizar dicha predicción para todas las especies estudiadas. Se han realizado en dos escenarios futuros (Trayectorias de Concentración Representativas, RCP) determinados por el Grupo Intergubernamental de Expertos sobre el Cambio Climático (IPCC). Un escenario, denominado de bajas emisiones (RCP 2.6), que es más optimista con un incremento máximo de la temperatura media en 2 °C y otro más restrictivo (RCP 8.5), con un máximo de 4,9 °C denominado de altas emisiones. Ambos escenarios son predicciones para el rango de años del 2080–2100. En todas las especies se ha observado que las variables relacionadas con la temperatura son las que necesitan una mayor tasa de cambio para su supervivencia. Mientras que las relacionadas con las precipitaciones parecen presentar menos vulnerabilidad.

Como se muestra en la **figura 14**, el incremento de la temperatura es más acusado en el futuro de altas emisiones que en el de bajas y, además, se producirá en todos y cada uno de los lugares en los que se asientan las poblaciones estudiadas en esta tesis.

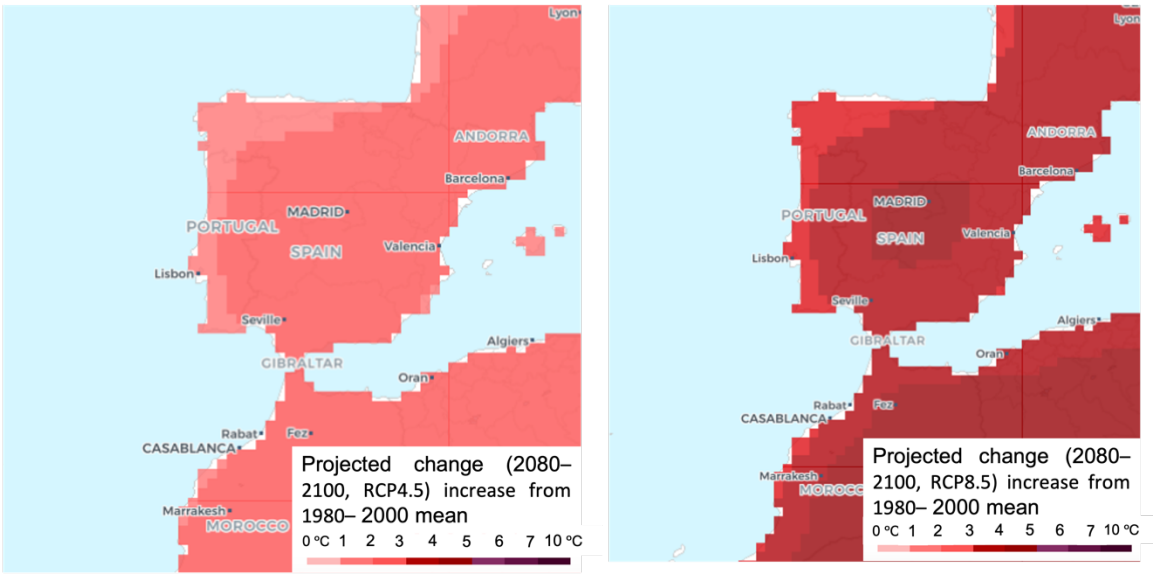


**Figura 14:** Representación del incremento de temperatura esperado para el escenario de bajas emisiones (izquierda) y el de altas emisiones (derecha). Se observan subidas de temperatura en todos los puntos de recolección de muestras de las especies aquí estudiadas (norte de Marruecos, Andalucía y Aragón). Cuanto más oscuro el color, mayor es el incremento. Sacado de *Resilience Atlas* (https://www.resilienceatlas.org).

Actualmente, la zona sur del Mediterráneo es una de las que más está sufriendo ese incremento de la temperatura anual, observándose ya un descenso de las precipitaciones en la cordillera del Atlas (Barkhordarian *et al*., 2013) y en la zona sur de España (Duque-Lazo et al., 2018).

Como ya se ha comentado con anterioridad, el cedro del Atlas es una especie anisohídrica lo que le permite mantener su actividad fisiológica activa durante más tiempo cuando se encuentra sometida a un estrés hídrico (Thomas, 2013). A lo largo de los años se han realizado muchos trabajos con datos de dendroecología y de ecofisiología que han tratado de explicar los episodios de muerte ocasionados en varias poblaciones de esta especie encontrando una asociación entre estas muertes y los severos eventos de sequía (ej.: Linares *et al.,* 2011b; Linares *et al*., 2013; Taoufik *et al*., 2021).

Pese a que se ha determinado que esta especie tiene una gran tolerancia al clima (Cheddadi *et al.,* 2017), los resultados de RONA aquí obtenidos indican que el cedro del Atlas se muestra muy vulnerable a las variaciones de la temperatura, lo cual coincide

con estudios basados en el nicho ecológico y modelos de distribución de esta especie (Bouahmed *et al*., 2019; Xiao *et al*., 2022). Dicho resultado puede deberse a la vulnerabilidad que presenta frente al incremento de las temperaturas durante el invierno, de ahí que variaciones en la temperatura anual sean las que puedan provocar un mayor problema para la supervivencia de esta especie. Recientemente, Cheddadi y colaboradores (2022) han indicado que en un escenario de incremento de la temperatura estacional en 2 °C junto con una reducción de las precipitaciones en un 20 %, provocaría un entorno inadecuado para el 70 % de los bosques actuales de cedro del Atlas.

Concretamente en el RONA, la población del Alto Atlas es la que muestra valores más altos tanto en el escenario de bajas emisiones como en el de altas emisiones con una tasa de cambio de 2 y superior a 4, respectivamente. Esta gran vulnerabilidad de esta población puede deberse a que es la zona que presenta las condiciones más extremas en la actualidad lo que le lleva a estar sometida a una aridez máxima en comparación con las otras dos estudiadas; aunque, por ejemplo, el Medio Atlas también ha sufrido fuertes sequías (Linares *et al*., 2011b). En cualquier caso, la situación es preocupante para todas las poblaciones aquí estudiadas ya que se prevé que desaparezcan a causa de ese incremento de las temperaturas que hará que los puntos donde se asientan se vuelvan más áridos y migrar en altitud no es una opción dado que ya ocupan zonas muy altas en la montaña (Thomas, 2013).

La ausencia de estudios similares de vulnerabilidad con RONA en cedro impiden que se establezcan comparaciones con el rango de tasa de cambio estimado en esta especie para los dos escenarios de cambio futuros (2–4,7). Pese a ello, Bobo-Pinilla y colaboradores (2022) realizaron un estudio de vulnerabilidad en el cedro del Atlas, empleando otro método de modelización conocido como *gradient forest*, y con las predicciones del escenario de altas emisiones para las mismas variables aquí estudiadas. Han encontrado que aquellas variables relacionadas con la temperatura, concretamente la estacionalidad de la temperatura, la isotermalidad y la temperatura media anual, son las que tienen una mayor influencia en la composición genética de las poblaciones

indicando que van a ser las que tengan un mayor riesgo para la adaptación de la especie. Estos resultados corroboran los obtenidos en la presente tesis para el cedro del Atlas.

En el caso del pinsapo, actualmente también se ha evidenciado que el incremento de las temperaturas que se está produciendo de forma paulatina está provocando un descenso en las precipitaciones anuales en toda la zona sur de España, lo cual parece que va a ir en aumento (Duque-Lazo *et al*., 2018). Los estudios de vulnerabilidad muestran unas tasas de cambio máximas que oscilan entre un 0,47 en el escenario de bajas emisiones y un 0,95 en el de altas emisiones. Estos valores son de los menores encontrados para las especies con las que se ha trabajado en la presente tesis. De nuevo, las variables relacionadas con la temperatura son las que parecen afectar más a la supervivencia del pinsapo lo que corrobora ciertos estudios previos en los que se han realizado modelos de predicción de hábitat con esta especie (Williams *et al*., 2013; Navarro-Cerrillo *et al*., 2021). La población que muestra una menor vulnerabilidad es la de Caucón y esto puede venir dado porque es una población con un alto grado de dispersión encontrándose sometida a una amplia variedad de condiciones ambientales lo que hace que tenga mucha variabilidad (Cobo-Simón *et al*., 2020). Se ha visto que en el escenario de bajas emisiones la mayor vulnerabilidad aparece mayoritariamente con la isotermalidad, salvo en el caso de Grazalema y Pilones, junto con las precipitaciones anuales. Este resultado indica que el rango de variación de la temperatura diurna respecto al rango anual tiene una gran influencia en el pinsapo. Sin embargo, en un futuro de altas emisiones la mayor vulnerabilidad se da siempre con la temperatura media anual.

La vulnerabilidad del abeto marroquí obtuvo un valor máximo de 0,42 en el futuro de bajas emisiones y de 0,95 en de altas emisiones, siendo un rango bastante similar al del pinsapo. En este caso también las variables relacionadas con la temperatura son las que tienen un mayor efecto. La variable relacionada con las precipitaciones anuales alcanza su máximo en ambos escenarios en la población de Tazaot. Esto puede deberse a que esa población puede estar menos adaptada al clima mediterráneo ya que es una zona supramediterránea en la que se dan unas temperaturas ligeramente inferiores y precipitaciones superiores a las encontradas en

Talassemtane (Ben-Said, 2022). Además, como se ha visto en nuestros estudios de estructura genética llevados a cabo con esta población, existe menos variabilidad que en Talassemtane. De ahí que cualquier perturbación que se produzca en el hábitat de esta población, vaya a suponer un mayor reto para su supervivencia y comprometa más directamente a su vulnerabilidad (Méndez-Cea *et al*., *In rev*).

En el pino negro, es interesante destacar que es la especie con la que se ha obtenido el mayor requerimiento de tasa de cambio que va desde el máximo del escenario de bajas emisiones situado en 2,5 hasta una tasa de 5,5 en el de altas emisiones. Estos resultados indican que el pino negro presenta la mayor vulnerabilidad de todas las especies estudiadas en esta tesis. Para una especie altamente relacionada con el *Pinus uncinata* la cual es *Pinus mugo*, se ha predicho de forma clara una fuerte reducción o incluso la extinción de las áreas de su distribución ya que las condiciones óptimas que conforman el hábitat de esta especie en la montaña se van a ir viendo reducidas (Palombo *et al*., 2013). De ahí, que se prevea que durante este siglo se dé un ascenso en la ladera de las áreas ocupadas por ambas especies. Por otro lado, se ha visto que el crecimiento de *P. mugo* está altamente influenciado por la temperatura tanto en el inicio como en el final de la época de crecimiento (Palombo *et al*., 2013) lo cual podría ser aplicable al *P. uncinata*.

Los datos obtenidos en el RONA con el pino negro hacen ver que, de nuevo, las variables relacionadas con la temperatura son las que más ponen en peligro su supervivencia. Aunque, en general, la vulnerabilidad mostrada en las tres poblaciones es similar, en la población de Peñarroya se observa una menor vulnerabilidad con las precipitaciones. Esto puede deberse a que Peñarroya es la población situada más al sur de la distribución por lo que podría tener un mayor potencial adaptativo frente a las sequías, como hemos visto que ocurre con el tamaño de sus acículas. El punto máximo de la tasa de cambio se encuentra en la población de Tessó en ambos escenarios y se da con la estacionalidad de la temperatura lo que indica que es este núcleo el que presenta la mayor vulnerabilidad. Sin embargo, es cierto que Peñarroya parece mostrar una vulnerabilidad ligeramente mayor en relación con la temperatura. Esta menor capacidad

de adaptación debe de estar relacionada con su condición de relicta (Méndez-Cea *et al*., 2023).

Para comprender todos estos valores de tasa de cambio necesarios para enfrentarse a las perturbaciones ambientales, se debe tener una noción sobre cuáles son las tasas de variación en las frecuencias alélicas que presentan las especies. A modo de contexto se puede emplear el valor de la frecuencia de cambio que tiene por ejemplo la especie *Fagus sylvatica* que se estimó en un rango de 0,1–0,2 por década (Jump *et al*., 2006). Sin embargo, sería más interesante, para poder hacerse una idea más acertada, conocer qué valores de tasas de cambio tienen las coníferas. En el trabajo de Dauphin y colaboradores (2021) se calculó una media de variación en las frecuencias alélicas de la conífera *Pinus cembra* de 1,23 x $10^{-2}$ por generación. A la vista de estos resultados, aquellas especies que tengan unos valores de tasa de cambio superiores a ese rango, como es el caso de todas las especies aquí estudiadas, se prevé que tengan un desfase negativo entre la variación en su frecuencia alélica y la adaptación a las perturbaciones.

Los estudios de RONA realizados en otras especies permiten contextualizar los datos aquí obtenidos. En un trabajo previo realizado con *Quercus suber* L. ninguno de los valores de tasa de cambio llegó a ser superior a 0,38 (Pina-Martins *et al*., 2019). Mientras que, la tasa obtenida en RONA para *Quercus pubescens* alcanzó un valor de 0,48 (Rellstab *et al*., 2016). Por otro lado, la máxima tasa mostrada por *Eucalyptus microcarpa* frente a las variaciones de temperaturas fue cercana a 0,3 (Jordan *et al*., 2017). Finalmente, el máximo de la tasa de cambio para *Pinus cembra* fue de 0,10 (Dauphin *et al*., 2021). A la vista de estos datos parece, por tanto, que los valores estimados para todas las especies que han sido objeto de esta tesis indican que sus vulnerabilidades son considerablemente altas.

Los análisis realizados en esta tesis han puesto de manifiesto que el impacto del incremento de la temperatura es superior al del descenso de las precipitaciones, ya que las variables que muestran mayor vulnerabilidad están relacionadas con la temperatura (Williams *et al.,* 2013). Esto también fue lo que se observó en el estudio realizado con *Pinus cembra* donde los valores de RONA obtenidos con las variables de temperatura

fueron más elevados que con las precipitaciones (Dauphin *et al*., 2021), con unas tasas máxima de 0,10 para las temperaturas y de 0,04 para las precipitaciones.

Las especies muy longevas y con grandes tiempos de generación, como son las coníferas, suelen mostrar cierto desfase en su adaptación frente a las condiciones ambientales que se están dando actualmente (Browne *et al*., 2019), así como a las que se puedan producir en escenarios futuros (Wilczek *et al*., 2014). En las poblaciones relictas este desfase puede provocar grandes pérdidas influyendo, por tanto, en la extinción de las poblaciones relictas. En el caso de los bosques, los desfases afectan directamente a la dispersión de los bosques y al reclutamiento de individuos para el establecimiento en el ecotono de los árboles. Dichos desfases se dan entre la presión selectiva que se esté produciendo en ese lugar y la capacidad que tienen esas poblaciones para desarrollar ciertos cambios en sus dinámicas lo que le permitan mantener las poblaciones pese a esas perturbaciones (Alexander *et al*., 2018). En el caso de las poblaciones relictas, hay diferentes factores que van a hacer que esos desfases que favorecen su persistencia se acorten o alarguen como son la longevidad, la deriva genética, la competencia, etc. Por su parte, en la expansión de la línea del bosque (*treeline*) existen también estos desfases, pero en este caso lo que se va a ver modificado es su dispersión y su rango de establecimiento, con factores como la distancia de dispersión, la competencia, el calentamiento, la plasticidad fenotípica, etc. (**Figura 15**).

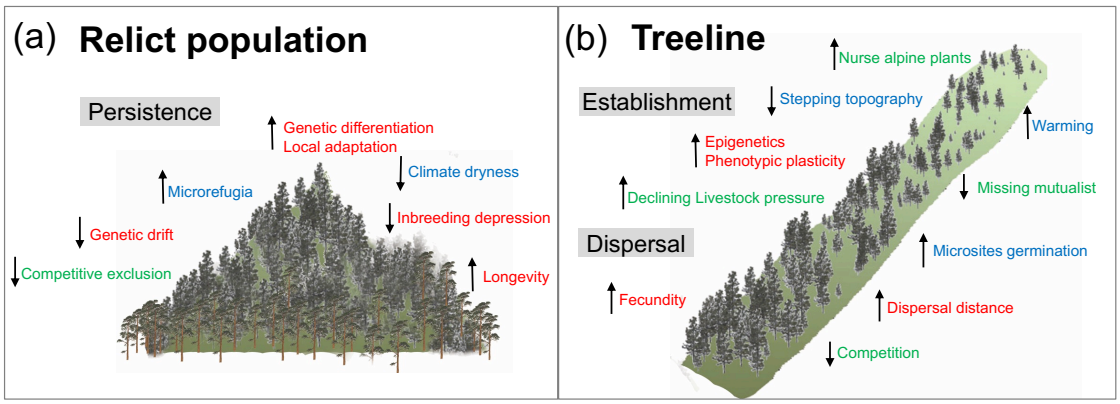


**Figura 15:** Representación de los desfases que se pueden encontrar en el mantenimiento de las poblaciones relictas (a) y en el establecimiento, así como en la dispersión de los *treelines* (b). Las flechas hacia abajo indican aquellos procesos que están asociados con el acortamiento del desfase y las que están hacia arriba muestran procesos que alargan esos desfases. (Figura: J.C. Linares basada en Alexander *et al.*, 2018)

Los análisis de vulnerabilidad desarrollados en esta tesis nos han permitido determinar cuáles son las variables más influyentes para las especies de coníferas aquí estudiadas. Así como, también hemos podido hacer predicciones acerca de la capacidad que tendrían estas mismas especies de sobreponerse a las perturbaciones ambientales que ya se están produciendo como consecuencia del cambio climático. Así se ha descrito que, para todas las especies estudiadas, la mayor vulnerabilidad la encontramos con las alteraciones en la temperatura más que con las de las precipitaciones. El cedro del Atlas y el pino negro han mostrado la máxima vulnerabilidad de todas las estudiadas. En cuanto al pinsapo y al abeto marroquí, los valores han sido más bajos y bastante similares entre ellas mostrando, por tanto, una vulnerabilidad más moderada (Méndez-Cea *et al*., 2023).

Los estudios realizados en la presente tesis han brindado la oportunidad de aportar nuevo conocimiento genético de 4 especies de coníferas, asentando una base sólida sobre la que se podrán llevar a cabo nuevos trabajos. Estos permitirán profundizar más acerca de cómo responden estas especies frente a las perturbaciones ambientales que cada vez serán más acusadas. Las perspectivas futuras que serían interesantes poder desarrollar son varias.

En primer lugar, se debería invertir recursos en la obtención de genomas de referencia con su correspondiente anotación de buena calidad, de todas las especies aquí estudiadas. Si bien es cierto que este proceso sería complejo debido a las características intrínsecas de estas especies, la obtención de genomas nos daría la oportunidad de determinar con mayor precisión la relevancia de los *loci* significativos que se han descrito en los diferentes análisis de huellas de selección o GEA/GPA. En segundo lugar, aquellos SNP asociados a las variables ambientales o a dendrofenotipos más interesantes para cada una de las especies, sobre todo, aquellos relacionados con la respuesta al estrés, se deberían emplear para llevar a cabo el genotipado en otras poblaciones de la especie en la que se han identificado como significativos para así, corroborar su relevancia. En el caso en el que ese SNP esté en un gen, se debería realizar una caracterización del mismo para conocer realmente cuál es su función. Finalmente, con el objeto de poder crear métodos de conservación más adecuados y específicos para

cada especie, se podrían realizar estudios con inteligencia artificial que permitieran hacer predicciones de vulnerabilidad más concretas y así ser capaces de adelantarse a la pérdida de núcleos de especies. Esto adquiere una mayor relevancia cuando, como en el caso de la presente tesis, se trabaja con especies o poblaciones relictas cuyos rangos de distribución son muy limitados. Tanto es así que en el caso de perder una de esas poblaciones la especie experimentaría un descenso en un porcentaje elevado de su área de distribución.

# Conclusiones

Los resultados obtenidos en esta Tesis Doctoral permiten extraer las siguientes conclusiones.

1. La extracción de ADN de la madera permitirá realizar estudios genéticos en árboles que han mostrado procesos de decaimiento inducidos por sequía, con el fin de identificar marcadores genéticos de predisposición.

2. La utilización de transcriptomas como referencia para realizar el ensamblaje en especies de las que se carece de un genoma secuenciado tiene gran utilidad. Si bien, el número de SNP obtenidos tras los filtrados con este método es inferior al descrito con el ensamblaje *de novo*, estos SNP son más informativos al encontrarse todos ellos en secuencias codificantes.

3. Todas las especies estudiadas mostraron cierto grado de diferenciación entre poblaciones, a pesar del alto nivel de flujo génico observado habitualmente. También, se ha evidenciado una variabilidad genética mayor de la esperada para especies y poblaciones relictas.

4. La identificación de huellas de selección en las cuatro especies estudiadas sugiere una respuesta adaptativa frente a las variaciones ambientales. Sin embargo, el número de *loci* significativos identificados ha sido muy variable, poniendo de manifiesto diferencias en la capacidad adaptativa de cada una de las especies.

5. Se han encontrado diferencias significativas entre adultos y jóvenes en los estudios de huellas de selección realizados en *Abies marocana*, lo que nos permite plantear una posible presión selectiva que estaría propiciando un cambio genético en las cohortes más jóvenes.

6. Los estudios GEA han mostrado un mayor número de asociaciones con variables relacionadas con la temperatura que con las de precipitación, confirmando el

papel clave que el calentamiento global puede ejercer en la supervivencia y la dinámica futura de las especies forestales.

7. Los estudios GPA, realizados en el abeto marroquí y el pino negro, han puesto de manifiesto la sensibilidad de varios caracteres dendrocronológicos en relación con la respuesta al estrés ambiental. En concreto, el crecimiento y la resiliencia a eventos de sequía parecen tener un papel primordial. Por otra parte, en el caso de *Pinus uncinata* también es muy importante la morfología de las acículas en la adaptación al ambiente.

8. Varios de los SNP identificados en los estudios de asociación se encuentran en secuencias codificantes con posible homología con genes previamente descritos en otras especies. Algunos de esos genes se han descrito en respuesta a la sequía. De entre ellos destacan varias familias de factores de transcripción (ABR, DREB y MYB); proteínas relacionadas con auxinas (WAT1); transportadores de GABA, así como proteínas de la familia LRK.

9. La proteína LRK, identificada en estudios de huellas de selección y asociada en GEA con todas las variables ambientales en el abeto marroquí, sugiere un importante papel de este gen en la respuesta adaptativa de *Abies marocana* a la sequía.

10. Todas las especies han mostrado una alta vulnerabilidad frente al incremento de las temperaturas predicho bajo diferentes escenarios, siendo este factor el más influyente para su supervivencia, pero en distintos grados. Los valores de RONA indican que el cedro de Atlas y el pino negro son las especies con una menor capacidad de adaptación y, por tanto, con un mayor riesgo para su supervivencia.

**Conclusión final**: Las especies de coníferas estudiadas parecen estar sufriendo un proceso adaptativo en respuesta al cambio climático. El diferente número de SNP identificados en los estudios de asociación indica que cada especie podría tener diferente capacidad de respuesta al estrés ambiental. Los genes candidatos

descritos pueden ofrecer las claves de diferentes mecanismos adaptativos, que deben ser objeto de futuras investigaciones en las, por ejemplo, se caractericen funcionalmente. Finalmente, los estudios de vulnerabilidad ponen de manifiesto la distinta vulnerabilidad de las especies y el papel crucial de la variabilidad genética frente a escenarios futuros de cambio climático.

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