UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE CIENCIAS BIOLÓGICAS

TESIS DOCTORAL

Estudio de los efectos del cambio climático en el abeto común (Abies alba Mill.) mediante datos ómicos

Omics-based exploration of climate change effects on silver fir (Abies alba Mill.)

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Isabel García García

DIRIGIDA POR

Francisco Javier Gallego Rodríguez
Juan Carlos Linares Calderón
José Luis Hórreo Escandón

Madrid

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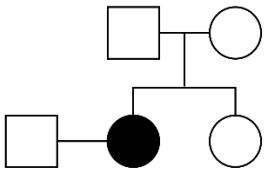
A mi familia



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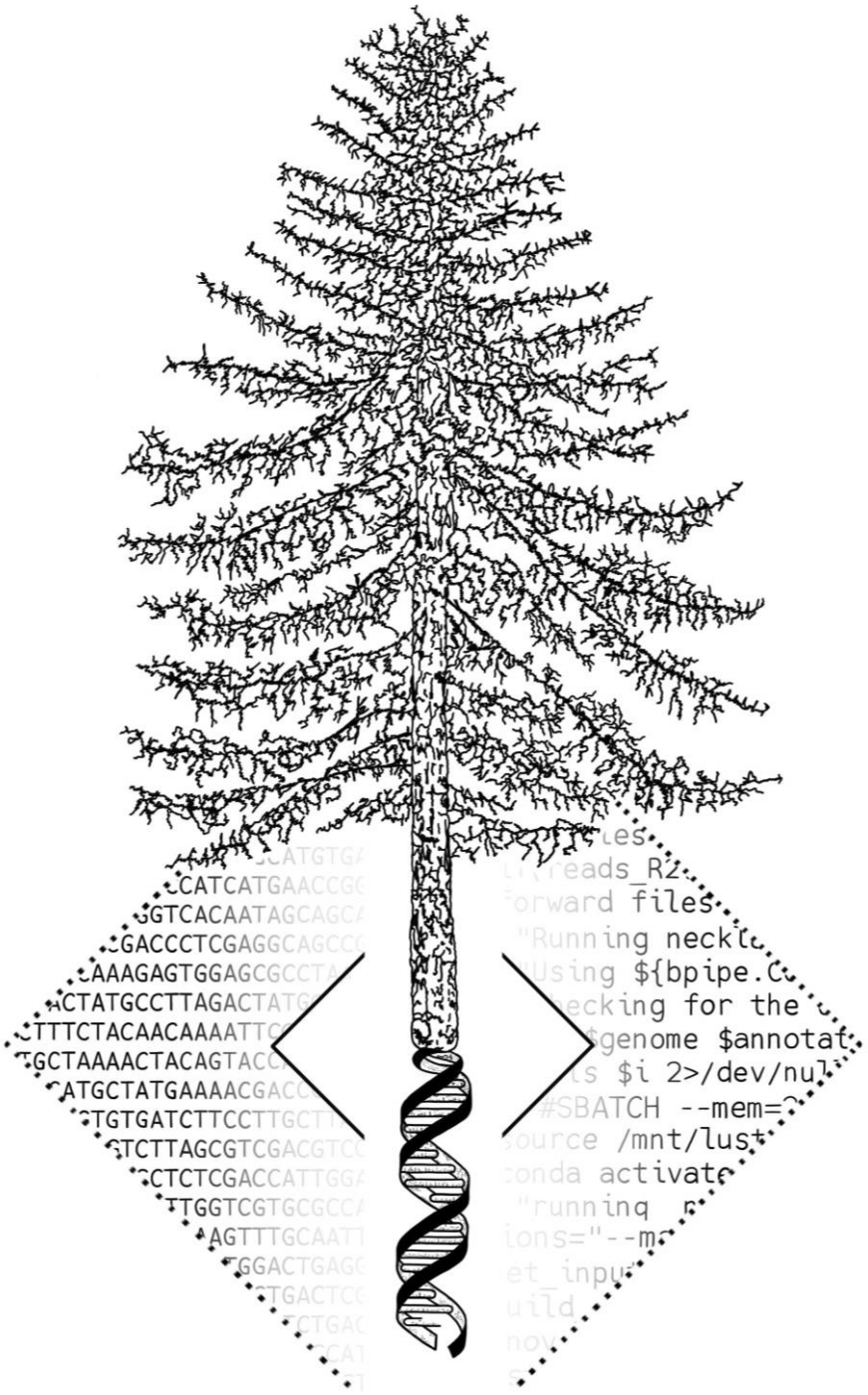

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RESUMEN

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ABSTRACT

RESUMEN

Los ecosistemas se están enfrentando a un estrés ambiental cada vez mayor debido al cambio climático. Diversos eventos climáticos extremos, como las sequías prolongadas, se están relacionando con episodios de decaimiento forestal en todo el mundo. Debido a su longevidad y alto tiempo generacional, algunas especies de árboles pueden tener dificultades para adaptarse lo suficientemente rápido a estos cambios ambientales. El abeto común (*Abies alba* Mill.) es uno de los árboles más afectados en los bosques europeos, con las poblaciones situadas más al sur, en la cuenca mediterránea, especialmente amenazadas. En este contexto, la presente tesis doctoral se centra en los Pirineos, el extremo suroccidental de la distribución de *A. alba*, donde se llevan documentando eventos de decaimiento desde la década de 1980. Dado que se espera que las condiciones ambientales empeoren en esta zona cada vez más propensa a la sequía, comprender cómo responde el abeto común a las alteraciones climáticas es de vital importancia. Para ello, se han analizado datos genómicos, epigenómicos y transcriptómicos, combinándolos con otros campos, como la dendrocronología y la ecología.

La sección de genómica trata datos de muestras de abeto común recolectadas de puntos calientes (zonas afectadas por decaimiento y mortalidad) y puntos fríos (zonas sin síntomas de decaimiento) en los Pirineos. Se identificaron polimorfismos de un único nucleótido (SNPs) tras una secuenciación de ADN con doble digestión asociada a sitios de restricción (ddRADseq) y se utilizaron para estudiar la capacidad de adaptación genética de las poblaciones en el extremo suroeste de la distribución de *A. alba* y el potencial impacto del cambio climático en su supervivencia, utilizando escenarios climáticos predichos en base a distintos niveles de emisiones de gases de efecto invernadero. Las diferencias encontradas en diversidad genética adaptativa y niveles de metilación del ADN entre árboles decaídos y no decaídos y la existencia de numerosos SNPs asociados a variables ambientales y con huellas de selección sugieren que el decaimiento forestal tiene una base molecular. Aunque los resultados obtenidos indican que los puntos fríos pueden servir de refugios temporales para la diversidad genética de la especie gracias a sus condiciones ambientales menos estresantes, los modelos genómicos y dendrocronológicos identifican al cambio climático como una amenaza significativa para los bosques de abeto común en los Pirineos, respaldados por reducciones demográficas pasadas vinculadas a eventos de calentamiento.

ABSTRACT

La sección de epigenómica se centra en definir un protocolo para realizar estudios epigenómicos en *A. alba* que permitiera identificar diferencias entre abetos decaídos y no decaídos. Sin embargo, debido a la complejidad del genoma del abeto común (gran tamaño y alto porcentaje de regiones repetitivas) y la falta de un genoma de referencia y anotación de alta calidad, ninguno de los dos métodos probados, es decir, ni la secuenciación con bisulfito del genoma completo (WGBS) ni de representación reducida (RRBS), que ofrecen información a nivel de secuencia, fueron efectivos. Por ello, se propuso un enfoque más general que no requiere conocimiento previo de las secuencias genómicas de interés: un ensayo de inmunoadsorción ligado a enzima (ELISA) colorimétrico para medir niveles globales de 5-metilcitosina.

Finalmente, en la sección de transcriptómica, se ensambló un transcriptoma *de novo* de *A. alba*, combinando secuencias de lectura larga y corta, logrando un ~95% de completitud. Este transcriptoma se utilizó para analizar datos de secuenciación de ARN de un experimento en invernadero donde se sometieron muestras de abeto común a sequía experimental para evaluar su resistencia. Posteriormente, las muestras volvieron a someterse a condiciones control para evaluar su recuperación y resiliencia. Las raíces mostraron una respuesta transcriptómica más fuerte a la sequía que la hoja, lo que las convierte en un órgano clave para evaluar el estrés por sequía en *A. alba*. Los niveles de metilación del ADN, la expresión génica y las variables fisiológicas comenzaron a volver a su estado original en condiciones control una vez retiradas del estrés, mostrando cierta resiliencia a la sequía. Sin embargo, la especie parece ser sensible a la sequía y propensa a morir por consumir sus reservas de carbono debido a su incapacidad para mejorar su eficiencia en el uso del agua. Por lo tanto, la severidad y duración de los episodios de sequía serán críticas para la supervivencia de los bosques de abeto común, especialmente en las etapas juveniles, caracterizadas por sistemas radiculares limitados y reservas de carbono escasas.

En resumen, esta tesis doctoral proporciona información valiosa para mejorar la comprensión de la adaptación de los árboles a las condiciones ambientales y sus respuestas a variaciones ambientales. Al combinar datos genómicos, epigenómicos y transcriptómicos con información de estudios ambientales y dendrocronológicos, esta investigación ofrece una perspectiva multidisciplinar que puede brindar conocimientos útiles para desarrollar estrategias de manejo de los bosques de abeto común en el contexto del cambio climático, lo que podría ser de particular importancia dado que estos bosques parecen encontrarse en riesgo de extinción local en los Pirineos. Además, se proponen diferentes variables genéticas, epigenéticas y transcriptómicas como indicadores para evaluar la vulnerabilidad de los bosques al cambio climático.

ABSTRACT

Ecosystems are facing growing environmental stress from climate change. Extreme climatic events, such as prolonged droughts, have been linked to forest dieback events worldwide. Due to their long lifespan and slow generational turnover, forest trees may struggle to adapt quickly enough to these rapid environmental changes. Silver fir (*Abies alba* Mill.) is among the most affected species within European forests, with populations at the southern limit of its distribution, in the Mediterranean basin, specially threatened. In this context, the present doctoral thesis focuses on the Pyrenees, *A. alba*'s southwestern rear edge, where decline events have been reported since the 1980s. With environmental conditions expected to worsen in an area increasingly prone to drought, understanding how silver fir responds to climate fluctuations is crucial. To that end, genomic, epigenomic and transcriptomic data have been analyzed and combined with data from other fields, such as dendrochronology and ecology.

The genomics section deals with data from silver fir samples collected from hotspots (stands affected by decline and mortality) and coldspots (stands without decline symptoms) of forest dieback in the Pyrenees. Single nucleotide polymorphisms (SNPs) were identified after double digest restriction-site associated DNA sequencing (ddRADseq) and used to study the genetic adaptive capacity of rear-edge *A. alba* populations and the impact of projected climate scenarios on their survival. Differences in adaptive genomic diversity and DNA methylation levels between declining and non-declining trees suggest a molecular basis for forest decline, which is also supported by the existence of numerous SNPs associated with environmental variables and showing signs of selection. While the obtained results suggest that coldspots may provide temporary refuges for the genetic diversity of the species thanks to their less challenging environmental conditions, genomic and dendrochronological models highlight climate change as a significant threat to silver fir forests in the Pyrenees, supported by evidence of past demographic reductions linked to warming.

The epigenomics section aimed to define a protocol for epigenomic studies in *A. alba* to identify epigenomic differences between declining and non-declining silver firs. However, due to the complexity of the silver fir genome (large size and high percentage of repetitive regions) and lack of a high-quality reference and annotation, neither of the two tested single-base resolution methods, namely whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS), was effective. Consequently, a more general approach, which does not require previous knowledge

about genomic sequences, was proposed and successfully tested: a colorimetric enzyme-linked immunosorbent assay (ELISA) to measure global 5-methylcytosine levels.

Finally, in the transcriptomics section a *de novo* transcriptome for *A. alba* was assembled, combining long- and short-read sequences and achieving ~95% of completeness. It was then used to analyze RNA sequencing data from a greenhouse experiment where silver fir samples were subjected to experimental drought to evaluate their resistance. Thereafter, samples were returned to control conditions to evaluate recovery and resilience. Roots exhibited a stronger transcriptomic response to drought compared to leaves, making them a key organ for drought stress assessment in *A. alba*. DNA methylation levels, gene expression, and physiological variables tended to return to their original state when the stress ended, suggesting some resilience to drought. However, the species seems to be drought-sensitive and prone to carbon starvation due to its inability to enhance water use efficiency. Thus, the severity and duration of drought events will be critical for the survival of silver fir forests, especially at juvenile stages, characterized by limited root systems and carbon reserves.

Overall, this doctoral thesis provided valuable information to enhance the understanding of tree adaptation to environmental conditions and responses to climate variability. By integrating epigenomic, genomic, and transcriptomic data with insights from environmental and dendrochronological studies, this research offers a multidisciplinary perspective that can provide valuable knowledge for developing strategies to manage silver fir forests under changing climatic conditions, which could be of particular importance as these forests seem to be at risk of local extinction in the Pyrenees. Further, different genetic, epigenetic, and transcriptomic traits are proposed as indicators to evaluate forest vulnerability to climate change.

INTRODUCTION

1. The climate threat: constant change vs sessile existence

Ecosystems are expected to experience increasing stress from climate change, both directly and through interactions with other stress factors [1]. Predictions point to a global temperature rise, as greenhouse gases increase their atmospheric concentration [2], along with more frequent extreme weather events, such as floods, droughts, and heatwaves [3]. This constantly changing environment is leading to shifts in species distribution [4], altering ecosystem composition and structure and triggering biodiversity loss when adaptation cannot be achieved [5]. Despite these challenging circumstances, species can follow several strategies to adapt to environmental alterations. Phenotypic plasticity, genetic selection, and migration are three key mechanisms that allow organisms to cope with novel conditions.

Phenotypic plasticity is the ability of one genotype to produce different phenotypes when exposed to varying environmental conditions [6]. It acts at individual level, and it is considered a rapid-response mechanism. It is highly related to epigenetic mechanisms that influence gene expression [7]. While significant efforts have been made to unravel the molecular processes underlying phenotypic plasticity in different species [e.g. 8–10], the interplay between plasticity and genetic variation as well as its impact on a population's extinction risk when facing abiotic stress remain poorly understood.

On the other hand, genetic selection represents a slower path towards long-term adaptive evolution. It happens at population level, as it leads to an increase of the frequency of some alleles, which are advantageous in a certain environment, over the generations through differential reproductive success of the individuals carrying them [11]. New mutations can potentially create beneficial alleles, but they hold less influence on adaptation than the already existing standing genetic variation in a population [12]. Therefore, the study of genetic variation is key to understanding the chances of adaptation and survival of a species under the current climate threat.

Finally, migration stands as another option for organisms when they are not able to adapt to the new conditions. However, looking for a better environment elsewhere is particularly difficult for sessile organisms, such as trees, which are forced to rely on future generations through pollen and seed dispersal rather than moving themselves [13], which could be slower than the speed of environmental change.

Predicting the fate of trees and forests has become a pressing concern in the light of rapid climate change. Around 30% of Earth's surface is covered by forests [14], which provide a wide range of ecological, economic, and social services, such as sheltering biodiversity, supplying food and medicinal resources, and offering recreational uses.

Among tree species, conifers represent a widely distributed phylogenetic group (Class Pinopsida / Coniferae), which can be found in all continents except for Antarctica [15]. Their origin dates from over 300 million years ago, when they were the predominant land plants until the rise of flowering plants (angiosperms) about 130 million years ago [16]. Despite being the predominant life forms in a wide range of ecosystems, proving their ability to adapt to many different climatic and soil conditions, about one in every three conifer species are currently designated as threatened or endangered [17].

The increase in frequency and duration of drought and heat stress episodes, brought by climate change, has been identified as one of the factors triggering the growing number of forest dieback events [18] which are being reported worldwide [e.g. 19–21] (Figure 1). This trend is expected to worsen in the next decades, with predictions pointing to increased mortality risk for several conifer species of different genus as temperatures rise and precipitations decrease [22]. Given their long-lived nature and long generation times [16], the continuously accelerating pace of environmental change is likely to surpass the adaptive capacity of forests [5].

All in all, the circumstances described above highlight the urgent need for mitigation and conservation strategies to address the challenges posed by climate change to forests specifically, and to ecosystems and biodiversity in general.

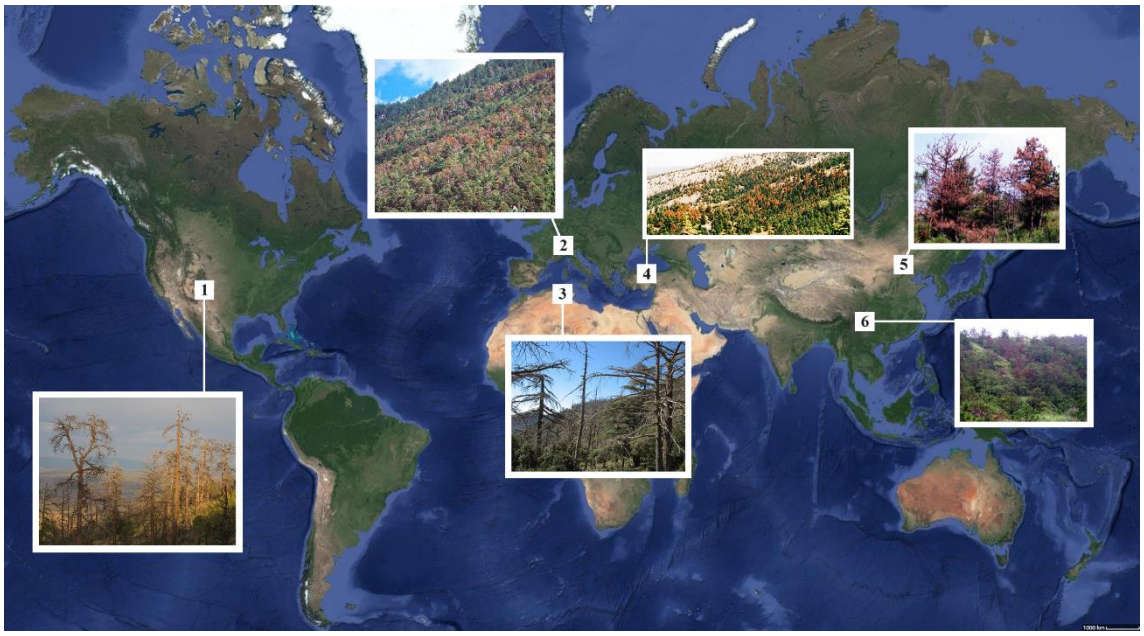

Figure 1: Forest dieback and mortality events of different conifer species across the world. 1. *Pinus ponderosa*, USA. 2. *Pinus sylvestris*, Switzerland. 3. *Cedrus atlantica*, Algeria. 4. *Abies cilicicia*, Turkey. 5. *Pinus tabulaeformis*, China. 6. *Pinus yunnanensis*, China. Modified from [18].

2. Omic studies in conifers

Omic studies involve the exploration and analysis of extensive amounts of data depicting the structure and function of a specific biological system at a particular level [23]. The development and improvement of the next generation sequencing (NGS) techniques enabled the emergence of diverse omics, such as genomics, epigenomics, transcriptomics, and proteomics among others.

Studying omics in conifers poses a significant challenge. The main difficulty lies in the inherent nature of their genomes, which hinders the assembly of high-quality references. Conifer genomes are remarkably large, with an average size of 15 Gb, and around 75% of highly repetitive DNA [24]. In addition, they have large gene families whose genes are not necessarily concentrated in specific regions but distributed across the genome [25]. These characteristics, along with other disadvantageous properties compared to model organisms (Figure 2), make working with these trees a challenge.

Nevertheless, reference genomes for 10 conifer species are available: *Abies alba* [26], *Larix kaempferi* [27], *Larix sibirica* [28], *Picea abies* [29], *Picea glauca* [30], *Pinus lambertiana* [31], *Pinus tabuliformis* [32], *Pinus taeda* [33], *Pseudotsuga menziesii* [34], and *Thuja plicata* [35].

Only that of *P. tabuliformis* achieved a chromosome-level assembly, while the rest are highly fragmented, with over 37 million scaffolds in the least favorable case (*A. alba*). Even though further improvement is still needed, they provide annotation and sequence information for between 45,000 and 100,000 genes.

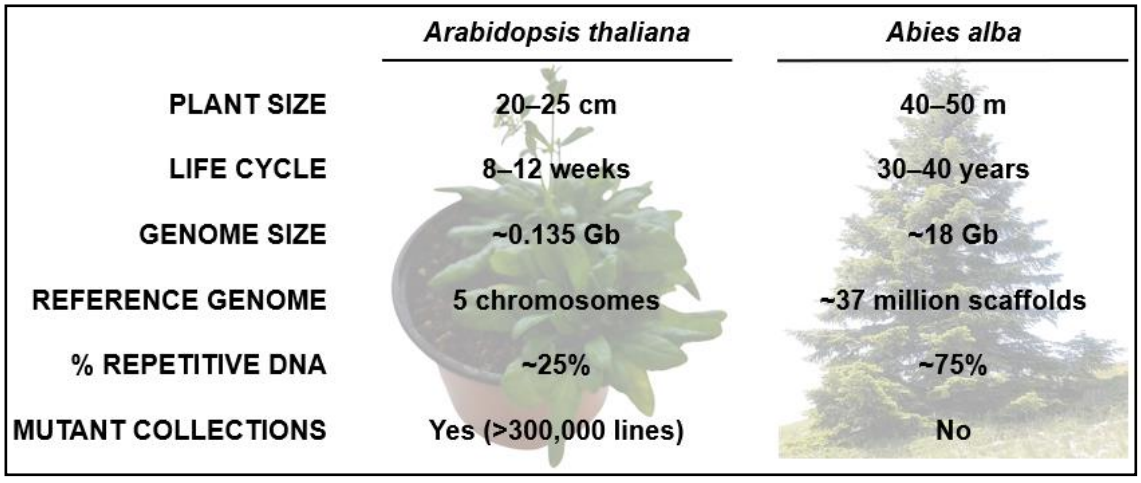

Figure 2: Comparison of some key characteristics between a model organism (*Arabidopsis thaliana*) and a conifer (*Abies alba*). Plant size, life cycle (time to reach maturity), genome size, level of assembly of their reference genomes, percentage of repetitive DNA, and existence of mutant collections are shown.

This section will delve into the essential aspects of the three major omics, namely genomics, epigenomics, and transcriptomics, paying special attention to their application to conifer studies. Regarding other omics, proteomics and metabolomics are typically among the most studied too. Both constitute understudied fields in conifers, as isolating and measuring proteins and metabolites is technically more challenging than working with DNA (genomics and epigenomics) or RNA (transcriptomics) [16]. Thus, they have not been explored in further detail in this thesis.

2.1. Genomics

Genomic studies aim to gain an exhaustive understanding of the structure and the function of a genome (an organism's complete set of genetic information) [36]. In the case of plants, it includes nuclear, mitochondrial and chloroplast DNA.

Genomic research on forest trees is motivated by the necessity to develop tools for conservation, restoration, and effective management of natural populations [37]. Given the complexity of conifer giga-genomes, projects aiming to sequence whole genomes are infrequent. However, other genomic approaches can be followed to characterize these trees, such as the study and discovery of molecular markers. Molecular markers are genetic *loci* that may be associated with some gene or trait of interest and can be easily tracked and quantified in a population [38]. They allow researchers to reconstruct phylogenies and demographic histories, identify genome-environment and genotype-phenotype associations, study genetic diversity, and predict their ability to adapt to future climatic conditions among other applications [e.g. 39–44].

Before the development of NGS techniques, microsatellites were the most used type of molecular marker [e.g. 45–47]. However, single nucleotide polymorphisms (SNPs) are increasingly becoming a common choice, as these molecular markers are more abundant and uniformly distributed across the genome, with relatively constant mutation rates and low levels of homoplasy [48]. Double-digest restriction-site-associated sequencing (ddRADseq) is one of the preferred protocols used for SNP discovery and genotyping in species with complex genomes or no available references [e.g. 49–51]. It enables a partial sequencing of the genome by digesting DNA with two restriction enzymes simultaneously. Then, genomic fragments are selected by size, creating sequencing libraries consisting of only the subset of fragments with a specific length produced by cuts with both enzymes [52]. This procedure reduces duplicate region sampling, as only a minor proportion of the fragments will fall within the desired size-selection range, minimizing the likelihood of sequencing the same region through

fragments with different length, and achieving a high sequencing depth (Figure 5). In addition, it increases the probability of sequencing the same genomic regions in different samples, as it favors some fragments. These two advantages compared to the classic RADseq, which uses just one enzyme, make ddRADseq suitable for reducing the complexity of studying conifer genomes while generating useful data. Furthermore, using methylation-sensitive enzymes (such as *Ape*KI) can contribute to a further simplification by enriching the representation of non-repetitive genomic regions, since repetitive sequences are usually methylated [53].

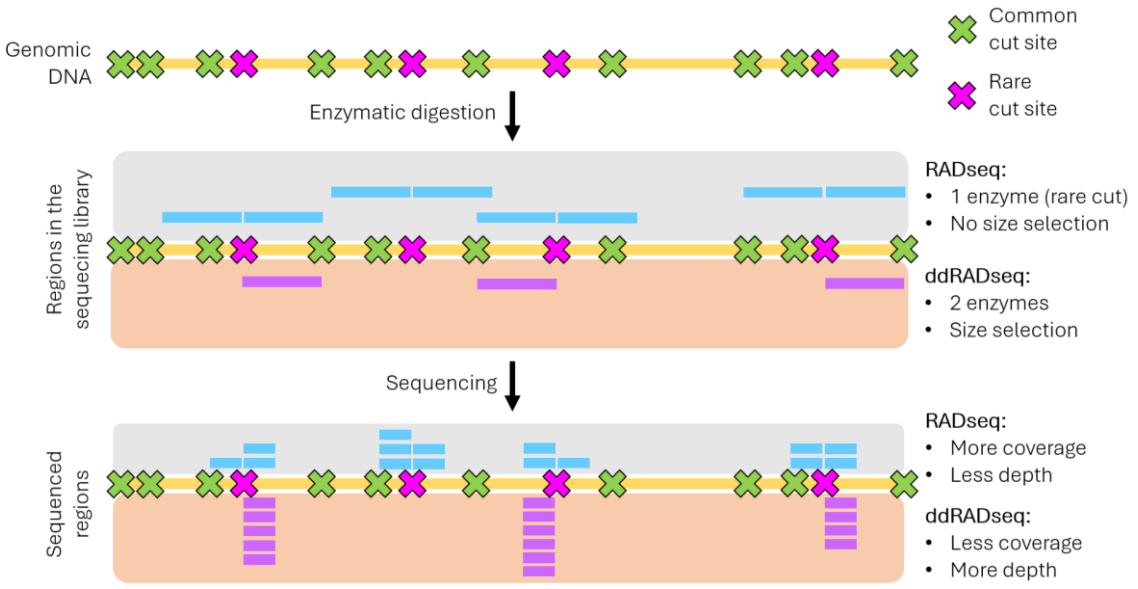

Figure 5: Comparison of two partial sequencing techniques based on enzymatic digestion: classical restriction-site-associated sequencing (RADseq) and its double-digest version (ddRADseq).

Association studies are of particular interest when working with SNP data in conifers. They use a regression approach to identify loci in which genetic variation is associated with variation in home environment, such as precipitation, temperature and soil variables, or trait values, such as leaf characteristics (Figure 6) [54]. Thus, genome-environment association studies (GEA) aim to identify loci that exhibit variations along environmental gradients, while genotype-phenotype association studies (GPA) look for loci that are correlated with specific phenotypes. Detecting these relationships allows the identification of signs of local adaptation and advantageous genotypes for certain conditions, such as alleles that could improve recovery after drought events. For example, phenotype–genotype–environment associations have successfully identified genes associated with adaptation to drought in different conifer species, such as *P. glauca* [55], *Sequoiadendron giganteum* and *Sequoia sempervirens* [56].

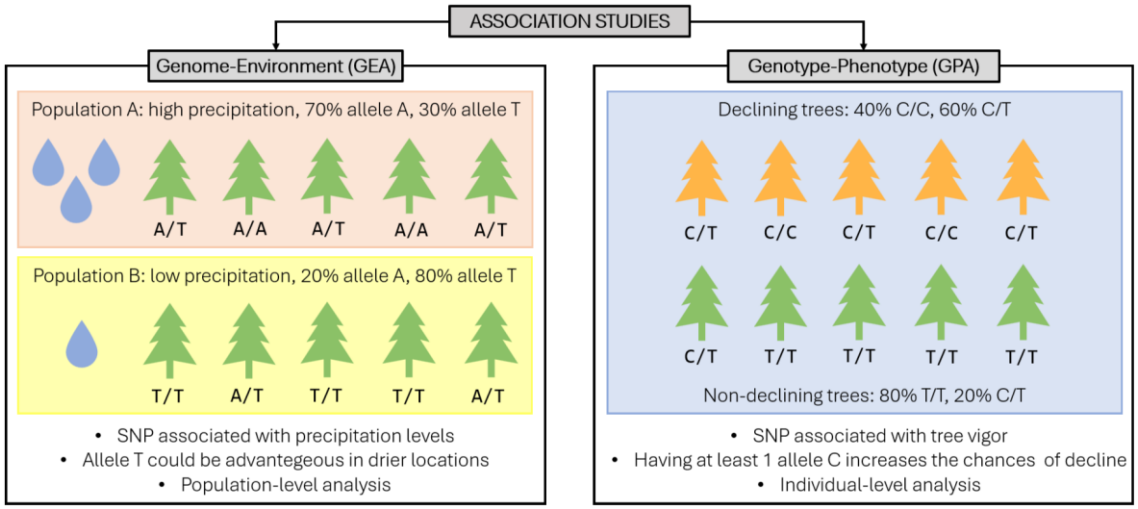

Figure 6: Types of association studies in conifers. Examples showing a genome-environment association (GEA) and a genotype-phenotype association (GPA) to illustrate their main characteristics.

2.2. Epigenomics

Epigenomic studies aim to identify the specific genomic sequences where epigenetic modifications occur and characterize their nature and effects [57]. These modifications include reversible DNA and histone alterations. In addition, this field deals with the study of epigenetic regulation of expression through RNA signaling [58].

Plants respond to stress through rapid, coordinated changes in transcriptional and post-transcriptional processes [59], which involve alterations of gene expression. Thus, epigenomic changes can facilitate adaptation to diverse stress conditions by modifying gene expression [60] and, therefore, play a role in phenotypic plasticity since they foster the emergence of different phenotypes [61]. Consequently, epigenetic modifications are especially important for sessile organisms, as they allow them to respond relatively rapidly to environmental variations, such as those caused by climate change [62].

Three classes of epigenetic regulation mechanisms are known (Figure 3): DNA methylation, histone modifications, and the action of non-coding RNAs (ncRNAs). Of these, DNA methylation, understood as the addition of a methyl group to a cytosine residue, is the most studied one, with many available techniques for its detection. In plants, it can occur in three sequence contexts (CpG, CHG, and CHH, where H is any nucleotide except G) [60]. It is particularly relevant in conifers as it is involved in silencing transposable elements (TE) [63], which are a substantial part of their giga-genomes. DNA methylation has been linked to changes in gene expression in response to environmental stress in plants [e.g. 64–66], as well as chromatin remodeling caused by histone

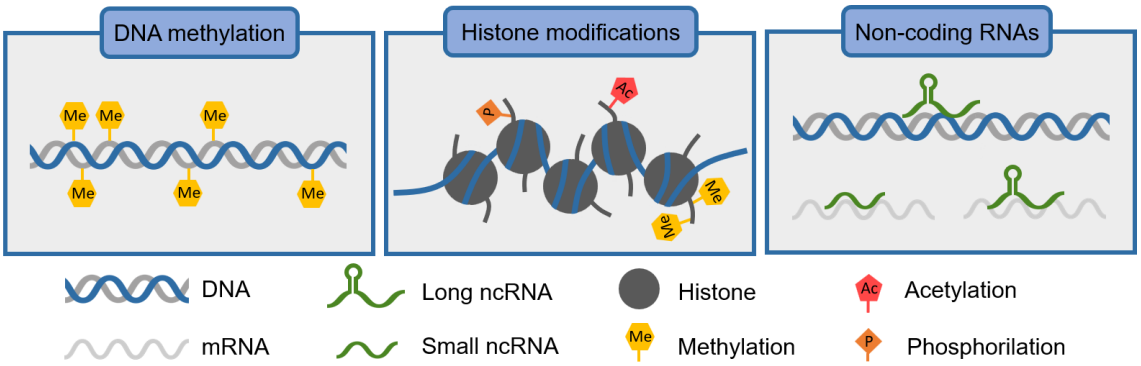

Figure 3: Main epigenetic mechanisms. Schematic representation of DNA methylation, histone modifications and the action of non-coding RNAs (ncRNAs).

modifications [e.g. 67–69], such as methylations and acetylations, which alter the positive charge on histones or the overall structure of nucleosomes, having an impact, therefore, on chromatin accessibility [70]. Transcriptional gene silencing / activation and translational repression provoked by ncRNAs, which target DNA regions or messenger RNAs (mRNAs) sharing sequence identity with them [71, 72], have also been reported in plants [e.g. 73–75].

Epigenomic studies in conifers are scarce, mainly due to the lack of reference genomes, which are often necessary for the data analysis step. This can lead to the study of epigenomic regulation of specific genes of interest rather than attempting an omic approach. Assembling a single-base resolution DNA methylome has only been possible so far in *P. tabuliformis* [32], thanks to the availability of a high-quality reference genome of this species. Whole genome bisulfite sequencing (WGBS) enabled this achievement (Figure 4). This technique is based on the conversion of unmethylated cytosines to uracils through bisulfite treatment, which does not affect methylated cytosines. Then, these changes can be easily detected via sequencing to identify methylation status of the cytosines in a genome.

There is a modification of this technique called reduced representation bisulfite sequencing (RRBS). While WGBS attempts to characterize the complete methylome of a sample, RRBS uses an enzymatic digestion to reduce the targeted portion of the genome, which is enriched in areas with a high CpG content, increasing the sequencing depth of the sequenced regions [76] and, thus, facilitating the identification of differentially methylation regions (DMRs) among samples (Figure 4). Typically, the methylation-insensitive restriction enzyme *Msp*I is used to generate short fragments that contain CpG dinucleotides at the ends, which are then selected based on their size (40–220 bp) before the bisulfite conversion step [77]. This technique can be used without a reference

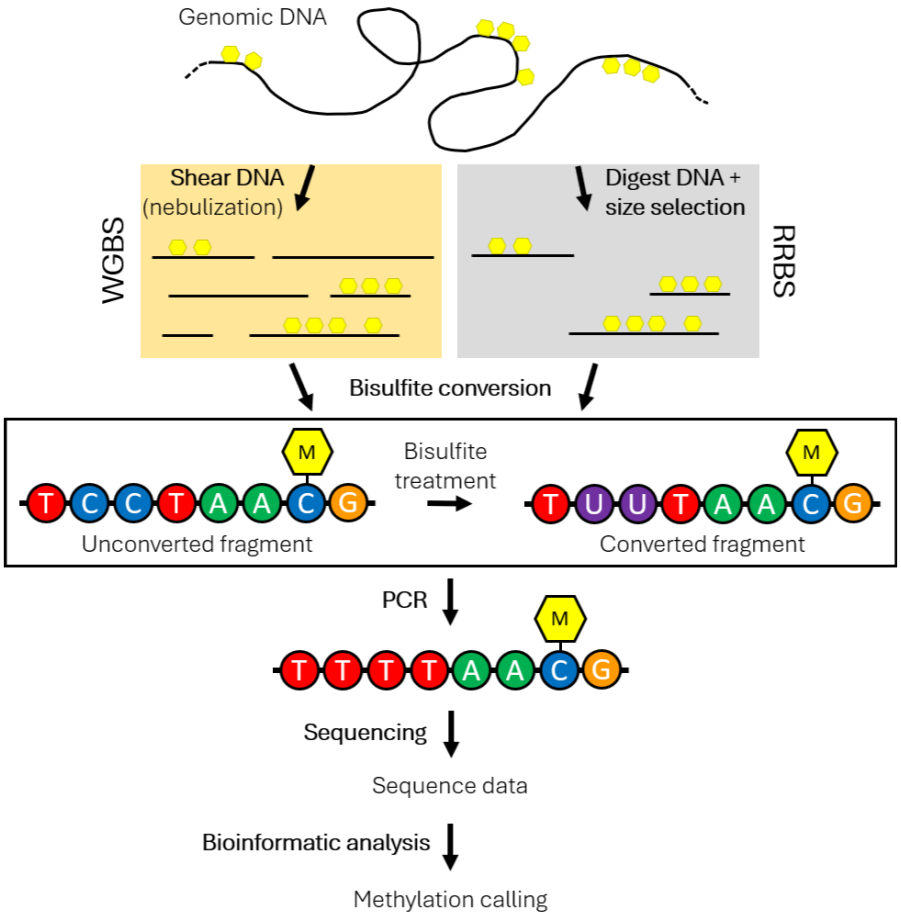

Figure 4: Bisulfite sequencing workflow. Steps in the whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS) techniques to detect methylation (M).

genome, as the original status of the sequenced portion of the genome can be directly inferred from the RRBS reads [78], which cover just a fraction of the total genomic DNA of the species, reducing computational costs. There are no available studies exploring DNA methylation patterns in conifers using this technique yet, but it has been tested on the oak tree *Quercus lobata*, concluding that methylation seems to play a role in locally adaptive evolution and plasticity in its natural populations [79].

On the other hand, changes in the overall percentage of DNA methylation have been linked to diverse processes in conifers, which include aging [32], embryo development [80], and local adaptation [81]. Therefore, when the identification of specific regions or genes affected by epigenetic processes is not possible, measuring global methylation levels could provide some insights into the role of methylation in stress response. The main advantage of these techniques is that they do not require a reference genome, as they are based on colorimetry [e.g. 82] or mass spectrometry [e.g. 83] of the extracted DNA and do not include a sequencing step.

Regarding histone modifications, their role in adaptation in conifers remains unexplored, as its study is complex due to their highly heterogeneous nature [84]. However, ncRNAs have been explored in greater depth thanks to the development and improvement of RNAseq techniques and tools for computational prediction [e.g. 85–87]. Exploring their functions holds notable relevance, given that some ncRNAs have been identified as “genome guardians”, providing protection against invasive TE, as well as suppressors of the activity of the large amount of TE present in conifers [88].

2.3. Transcriptomics

Transcriptomic studies aim to characterize transcriptomes, which encompass the complete set of RNA molecules (transcripts) expressed in a certain sample at a certain time [89]. They include the analysis of both coding (messenger RNAs, mRNAs) and non-coding transcripts (ncRNAs), seeking to describe their sequence, structure, functions, locations, trafficking, degradation, expression levels… [90].

Initial studies focused on one or a small number of genes, calculating their approximate level of expression via Northern blot [91]. Then, microarrays enabled the simultaneous measurement of numerous genes. Later on, RNA sequencing (RNAseq) emerged and became the primary method for gene expression studies to the present date. More recently, long-read technologies have led to improvements in *de novo* transcriptome assembly and in-depth analyses, as they can be combined with full-length cDNA synthesis to achieve full-length mRNA sequencing [92].

RNAseq stands out as one of the most feasible methods for investigating genes in conifers, considering that whole genome sequencing remains challenging as it was previously mentioned. Consequently, gene expression in conifers is a highly explored field [16]. The relationship between a tree’s expression profile and abiotic factors constitutes an area of great interest. Experiments involving exposure to different stresses, such as drought [93], heat [94], and varying light intensities [95], have shown that conifers alter their gene expression patterns in response to them, enabling the identification of candidate genes involved in reacting to environmental changes.

For instance, signaling and transcription factor genes, such as AP2/ERF, bZIP, and MYB, have been linked to water stress in several conifer species, like *L. kaempferi* [96] and *Pinus massoniana* [97], through RNA sequencing after greenhouse experiments. These genes were found to be typically expressed early during drought stress and, therefore, can serve as indicators of efficient plant responses.

2.4. Interdisciplinary studies: dendrogenomics and beyond

Addressing today's complex environmental issues, such as climate change and how species respond to its effects, demands multidisciplinary approaches [98]. Therefore, genetic information must be combined with data from other fields of study in order to get a comprehensive view of forests' adaptive potential, which is crucial for predicting the consequences of climate change and designing conservation plans to alleviate its negative effects.

Consequently, a new integrative discipline emerged: dendrogenomics [99]. This interdisciplinary field combines data from dendrochronology and genomics. Dendrochronology studies the annual radial growth pattern of trees [100]. Trees of the same species growing at the same time under similar climatic conditions show similar patterns, which makes it possible to relate them to climatic events. Thus, tree rings formed annually in wood contain extensive information about how each tree responds to environmental factors that affect growth, such as drought events or phytopathogen outbreaks (Figure 7A) [101]. This offers a unique opportunity to synchronize fluctuations in annual wood growth with past stress events, creating dendrophenotypes [102], which can be then correlated with individual genotypes.

For example, some interesting dendrophenotypic traits are those related to tree-level responses to documented past stressful episodes: resistance (Rt), recovery (Rc), and resilience (Rs) [103]. They are calculated based on the ratios between different basal area increment (BAI) values, derived from the annual tree ring width (TRW) increase, which reflect pre-stress, under stress, and post-stress growth. In other words, they show how growth was affected by the stress period, such as prolonged drought events, at different time points (Figure 7B). Other noteworthy dendrophenotypes inform about climate sensitivity, which is inferred from the correlation between BAI values and different temperature and precipitation variables, such as winter precipitation or summer temperature. Then, the variation found in dendrophenotypes can be linked to the genetic variation found in molecular markers, allowing the identification of genotypes and genomic regions with a potential role in response to climatic events.

Despite being a recently established field (the term dendrogenomics was first used in 2018 [104], although studies combining dendrochronology and molecular markers started to appear in 2016 [105]), it has been explored in several conifer species [e.g. 102, 106, 107], proving its potential as a tool to identify adaptation processes and predict the impacts of climate change on forests to enable the design of strategies to mitigate its negative impacts.

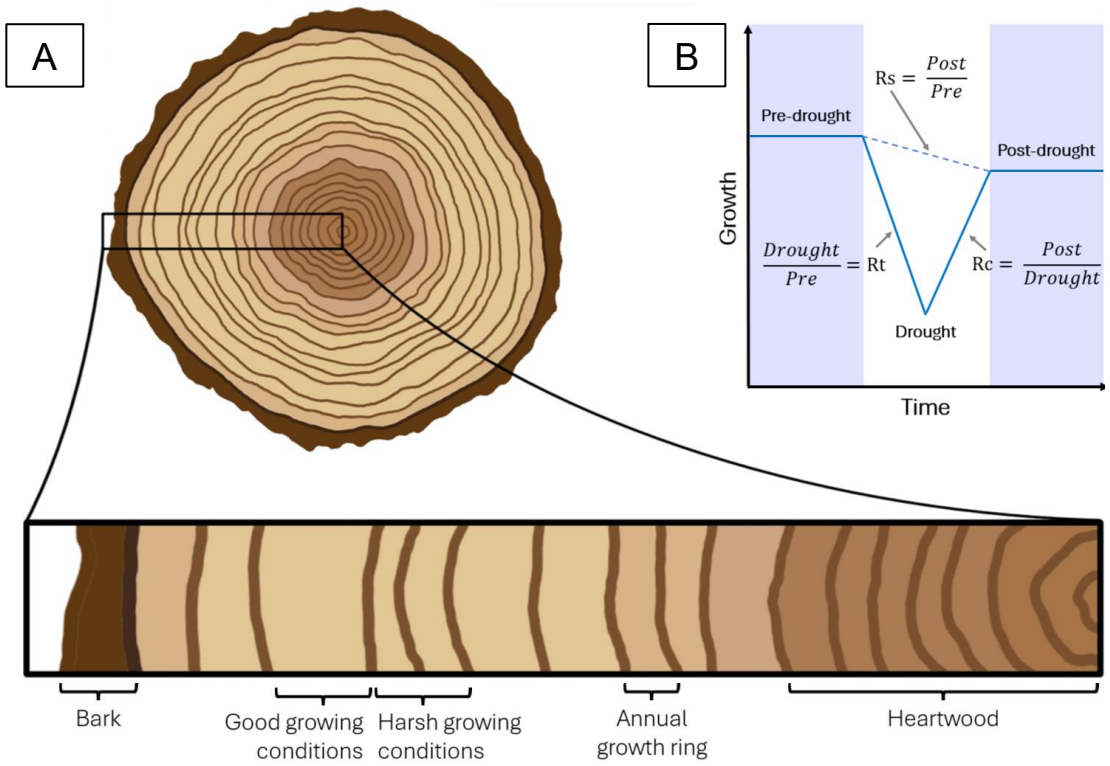

Figure 7: Dendrochronology basics. A. Interpretation of tree rings. B. Examples of dendrophenotypic traits in response to an ecological stress (drought period): resistance (Rt), recovery (Rc) and resilience (Rs).

Furthermore, dendrochronology is not the only field that can be combined with genetics in order to conduct meaningful analyses (Figure 8). For example, data typically produced in remote-sensing studies and field-based inventory data, which allow the characterization of spatio-temporal patterns in tree demography and forest structure [e.g. 108–110], could also potentially be included in genetic studies to gain a broader understanding of forest responses to climate change.

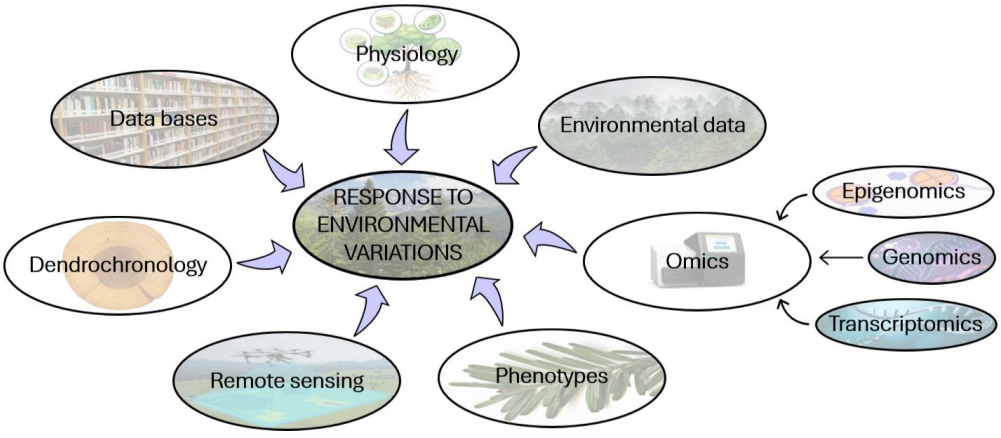

Figure 8: Multidisciplinary approach to study how trees respond to environmental variations.

3. Case study: silver fir

As previously mentioned, many conifer species are showing signs of dieback and high mortality rates, which seem to be worsened by the rapid emergence of challenging environmental conditions. Silver fir, *Abies alba*, is among the affected trees. This section will delve into its principal characteristics, its ecological importance, the challenges it is facing, and how to approach research on this conifer through omics data.

3.1. Characteristics and conservation status

Silver fir is a large evergreen conifer (Figure 9A), which can reach heights up to 60 meters and live for 500–600 years [111], reaching maturity at 30–40 years [112]. Its trunk is straight and exhibits a distinctive silver-grey color [113]. Young trees show a conical crown that gradually transforms into a rounded dome as the tree grows older [114]. Its needles are dark green, with a glossy upper side and two silver-green waxy bands of stomata on the lower side [112]. It is a monoecious species, with male and female flowers occurring separately on the same tree, at different heights in the crown [115].

It is distributed in montane areas in Europe, from the Pyrenees north to Normandy, east to the Alps and the Carpathians, and south to Italy, Bulgaria, and Greece (Figure 9B) [116]. This species usually appears in elevated areas (500–2000 meters above sea level) with constant high moisture conditions, mean annual precipitation of 700–1800 mm, and cool temperatures, favoring summers between 14 and 19ºC [112]. It prefers deep and moist soils, with neutral or acid pH, although it tolerates a wide variety of conditions [116].

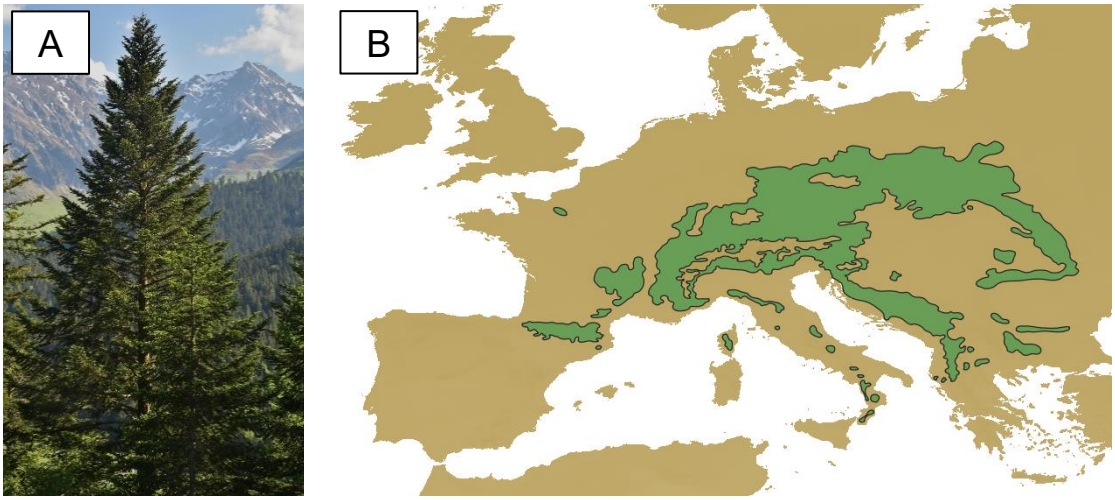

Figure 9: Appearance and distribution of silver fir, *Abies alba*. A. Silver fir specimen in the French Pyrenees, taken by Jeff Bisbee. B. Distribution of silver fir as defined by EUFORGEN, shown in green.

Silver firs are highly tolerant to shade, being able to survive long periods in the understory and respond promptly when light conditions become favorable, contributing to maintaining biodiversity in forests [117]. In addition, it is a key species for old-growth forests dynamics (Figure 10A) thanks to its resistance to wind, snow, and ice storms, thereby reducing the vulnerability of forest stands to natural disturbances [112]. It also holds economic importance, as its wood is used to produce paper and furniture, and its resin is used to produce turpentine [116]. On another note, it was the first species used as a Christmas tree in the 19th century [112].

Silver fir dieback has been observed across its natural range for over 150 years, with increased intensity from the end of the 20th century [118]. Initially, it was attributed to air pollution, specifically to SO_2 emissions, which reached their maximum in the beginning of the 1980s [116]. Then, global warming was identified as another factor affecting forest dieback, especially in the drought-prone areas of its distribution range (Figure 10B) [119]. In addition, diseases and plant pests are likely to proliferate and affect

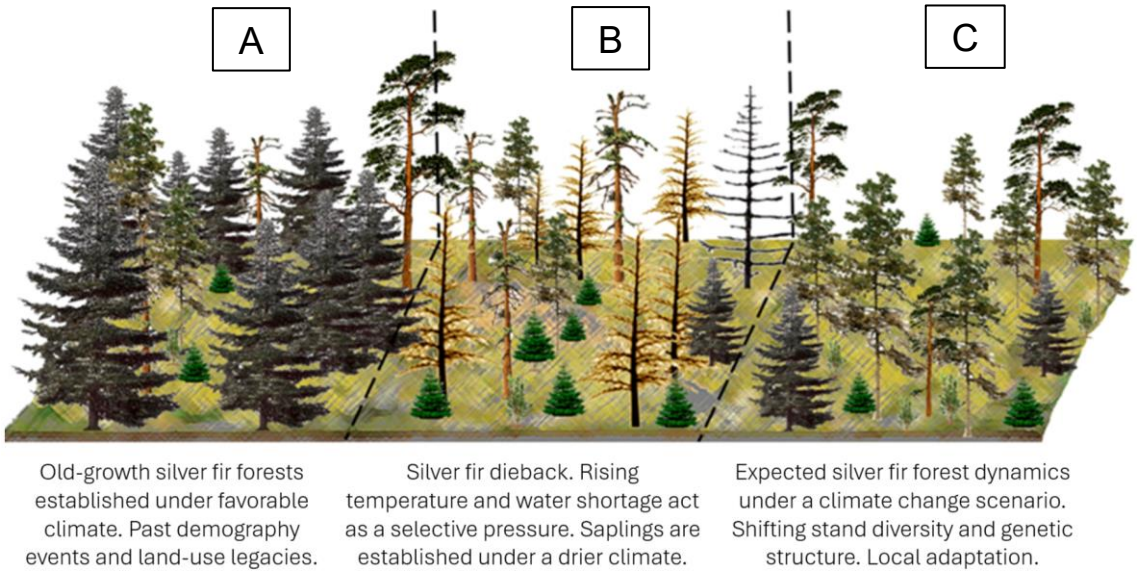

Figure 10: Conceptual framework of recent silver fir forests dynamics in drought-prone sites. The left panel (A.) represents a relatively mature silver fir forest, established in a climatic context prior to global warming. We have evidence of past demography and recent growth patterns for these populations through genetics and dendrochronological data. The next panel (B.) represents a silver fir population with symptoms of decline and mortality induced mainly by climatic stress, although factors such as logging, pests and pathogens and poorly developed soils may contribute. These mortality events represent a selective pressure for the subsequent establishment of new cohorts. Saplings are therefore established under an unfavorable climatic context, compared to that of adults. In the last panel (C.), silver fir forests might be shifting to mixed stands, where more drought-tolerant species, such Scots pine (*Pinus sylvestris*) may have replaced part of the drought-sensitive silver fir trees. Redrawn based on [5].

silver fir, as populations will suffer from increased susceptibility to them due to their constant exposure to stressful environmental conditions [112]. All in all, a shift in the current stand structure and diversity of silver fir forests is likely to occur [5], as well as a migration process towards higher elevations and northwards (Figure 10C) [120].

While this species is not considered to be endangered, there are some areas of particular concern. Special attention must be paid to populations situated at the lower latitudinal limit of the species' distribution area, as they are particularly vulnerable to fluctuations in climate [121]. Silver fir reaches its southern limit in the Mediterranean region, which is a climate change hotspot where aridity is predicted to increase [2, 122]. In fact, while radial growth has increased notably in populations in central Europe [123], declining trends have been reported in Spain and Italy, which seem to be linked to the rise in summer water deficit [124, 125].

The present study focuses on the Pyrenees, the southwestern distribution limit (a rear edge) of this species, where decline in silver fir forests has been reported since the 1980s, after a succession of dry years [126]. Reduced growth, crown defoliation, nutritional imbalances, and disrupted patterns of seed dispersal and regeneration have been linked to water stress in Pyrenean silver fir populations [127–131]. With predictions pointing to an increase in frequency and intensity of droughts in the Mediterranean area [132], the study of this species' response to climate fluctuations and adaptive capacity becomes a matter of upmost importance [5].

3.2. Research gap and significance of the study

Genetic characteristics of *A. alba* have been widely explored through diverse approaches. Microsatellites (nuclear, mitochondrial, and chloroplast) are the most used molecular marker to date. They have provided information about genetic diversity and differentiation of populations across its current distribution range [e.g. 133–135] and about the species geographic distribution in the past [e.g. 136–138], granting valuable tools for conservation biology [139]. More recently, studies employing SNPs have started to increase, thanks to the development of high-throughput genotyping methods via NGS. These molecular markers have allowed the appearance of association studies, which link genetics and environmental factors (typically temperature and precipitation variables) or phenotypes (typically dendrophenotypic traits), allowing to study adaptation and response to climatic stress [e.g. 102, 140, 141].

Regarding gene expression, the transcriptomic profile of cotyledons under drought stress has been characterized [142]. Unfortunately, the majority of the differentially expressed genes encode uncharacterized proteins, highlighting the need for further improvement of the silver fir reference genome and assembly of high-quality transcriptomes. Likewise, this lack of high-quality, annotated sequences has hindered the study of the epigenomics of this tree, which remains completely unexplored.

Thus, the present thesis aims to expand the knowledge about three of the main omics (genomics, transcriptomics, and epigenomics) in silver fir. To that end, the focus is placed on rear-edge populations, as they are especially vulnerable to global warming [121] and remained poorly studied from a genetic point of view. In addition, an initial approach to the study of epigenetics is proposed. Finally, expression profiles of different organs under drought conditions are set to be analyzed, along with other abiotic stresses, to better understand the transcriptomic response to climate change in this species and possibly find novel transcripts to improve the existing annotations.

3.3. Experimental design and bioinformatic analysis

The present study is divided into three different parts, each concerning a different omic. The genomics section analyzes data generated from samples which were chosen following an experimental design based on hotspots and coldspots of forest decline and mortality (Figure 11). Areas where trees are showing signs of decline (mixed with individuals which do not) are considered hotspots, while areas with similar environmental characteristics but where forest dieback is not noticeable are considered coldspots. Thus, declining and non-declining silver firs were sampled in hotspots along with non-declining trees from nearby coldspots in the Pyrenees. This approach provides information about the genetic characteristics of the entire study area and enables the exploration of differences between hotspots and coldspots, which had only been studied through dendrochronology analyses to date [130]. Moreover, both adults and saplings were sampled at each site, making it possible to check for intergenerational differences that may indicate emerging evolutionary processes induced by climate stress. In addition, the demographic history of *A. alba* in the Pyrenees was inferred, and predictions regarding its survival in the area were made based on its genetic adaptive capacity and forecasted climatic scenarios under global warming.

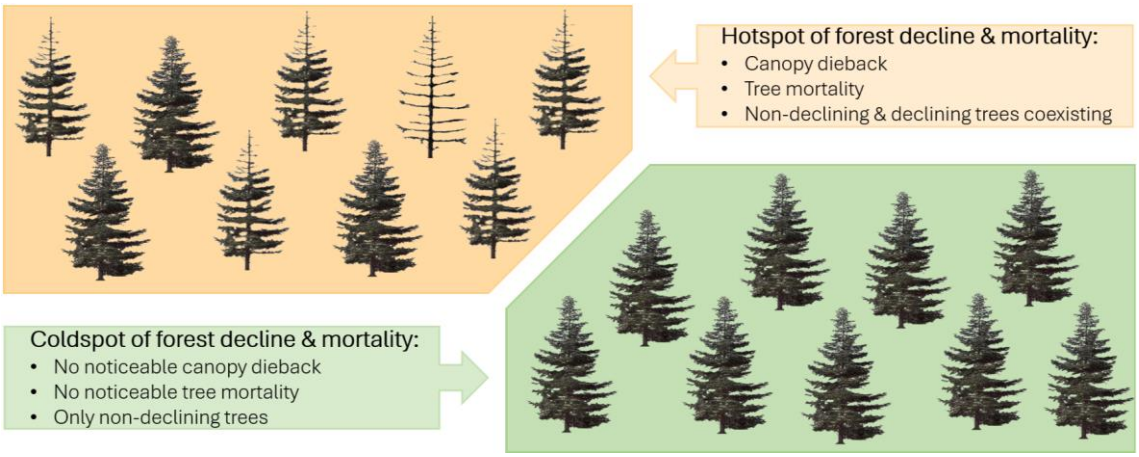

Figure 11: Characteristics of hotspots and coldspots of forest decline and mortality.

The epigenomics section deals with declining and non-declining samples, looking for differences in their single base-resolution DNA methylomes and global methylation levels. It explores which protocols might be best for conducting epigenomic studies in species with giga-genomes, such as *A. alba*.

Finally, the transcriptomics section takes into consideration data from a greenhouse experiment (Figure 12A). *A. alba* seedlings were grown in two different conditions: the control group was placed in optimal conditions for the species while the drought stress group was placed in an environment with reduced relative humidity (RH) and slightly higher temperature. Leaf and root transcriptomes were then studied employing short-read RNA sequencing data, aiming to identify differences in gene expression caused by drought in each organ and to characterize how silver fir responds to this type of stress.

In addition, data obtained through long- and short-read sequencing techniques were combined in order to assemble a high-quality transcriptome. Leaves, stems and roots were sampled from plants subjected to different types of stress (e.g. cold, heat, darkness) (Figure 12B) with the aim of increasing the number of expressed genes through their activation by different stimuli.

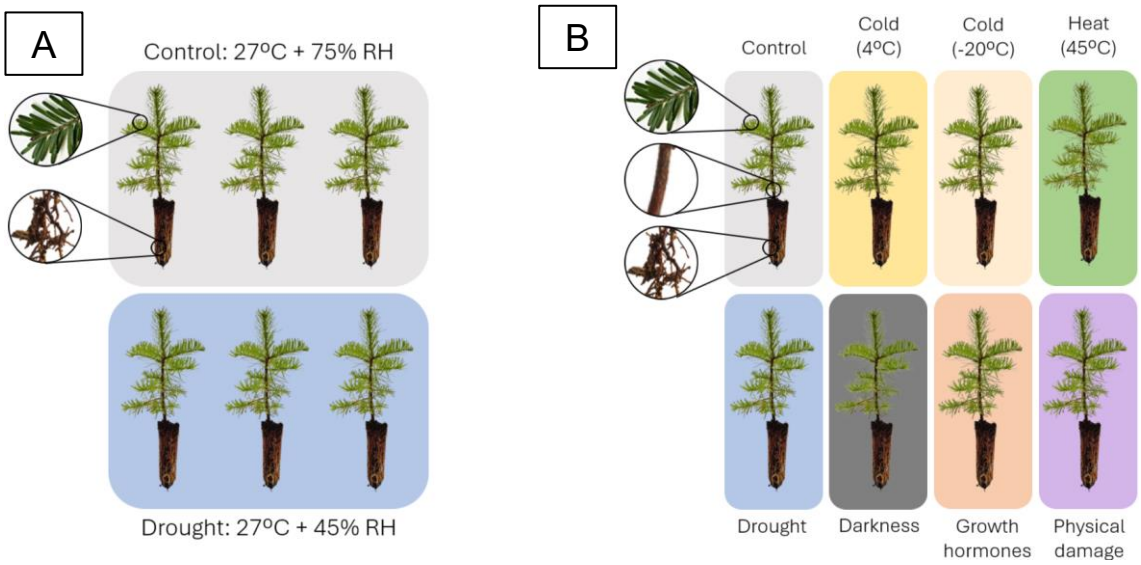

Figure 12: Transcriptomic section experimental design. A. Drought experiment. B. Transcriptome experiment.

Regarding the bioinformatic analyses, the genomics section (Figure 13A) is the most extensive one, in which population structure, genetic diversity, demographic history, kinship relationships, selection signatures, genome-environment and genotype-phenotype associations, and adaptive capacity to climate change are studied using SNPs obtained through ddRADseq.

The epigenomics section (Figure 13B) focuses on identifying differentially methylated regions between non-declining and declining trees, exploring data produced through two different methods based on bisulfite sequencing (WGBS and RRBS). As these techniques were not successful, primarily due to the lack of a high-quality reference, an alternative approach was proposed, namely measuring global methylation levels through colorimetric ELISA (enzyme-linked immunosorbent assay), which does not require previous knowledge about the target sequences. Following this method, differences in global methylation levels between declining and non-declining trees and between samples subjected to different controlled conditions were studied.

Finally, the transcriptomics section (Figure 13C) aims to create and annotate a highly complete reference transcriptome for *A. alba*, as previously mentioned, and to identify differentially expressed genes (DEGs) under drought conditions.

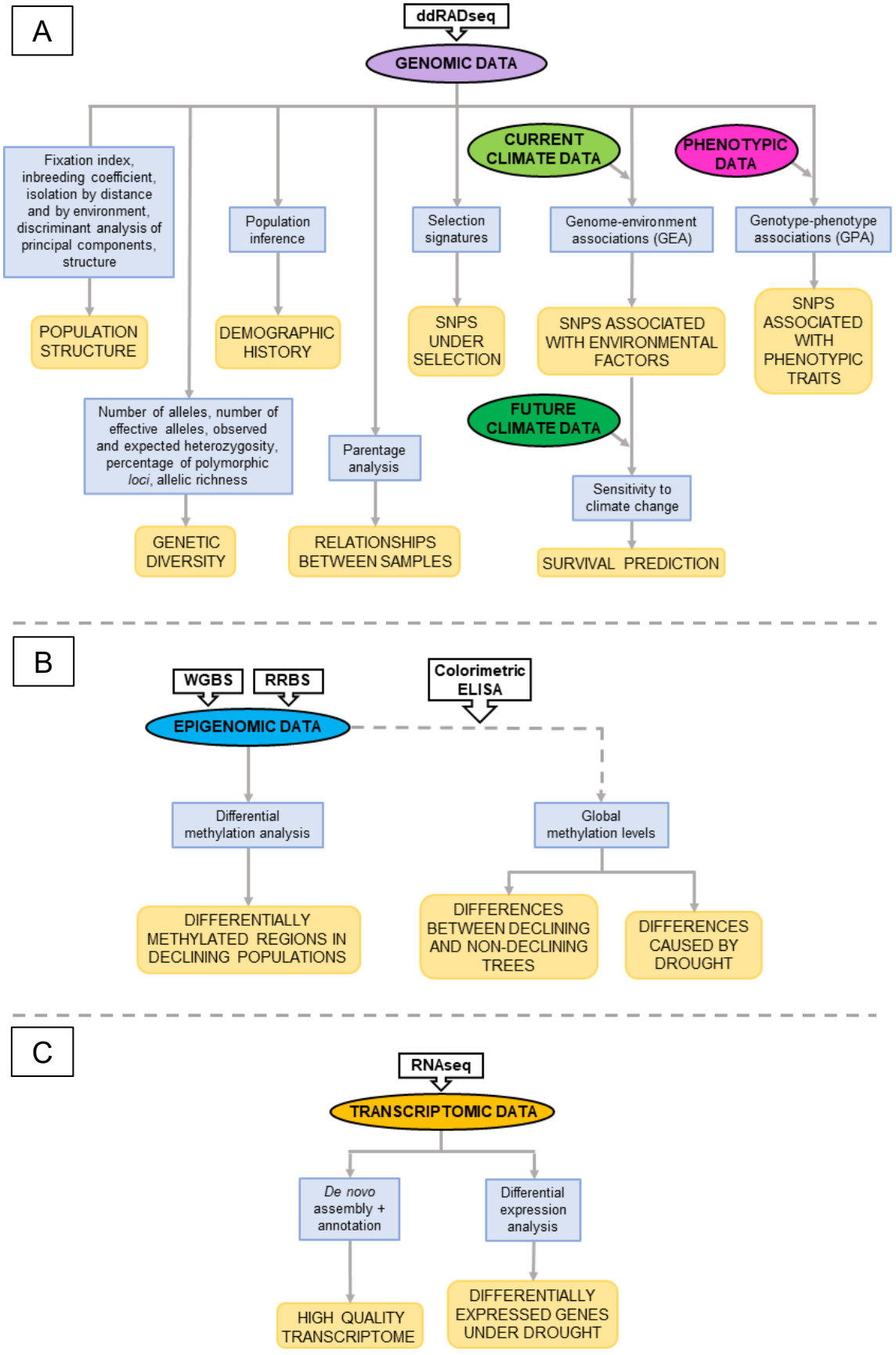

Figure 13: Bioinformatic analysis. A. Genomics. B. Epigenomics. C. Transcriptomics.

OBJECTIVES

OBJECTIVES

The main objective of this doctoral thesis is to gain a better understanding of **how trees adapt to environmental conditions and respond to climate variability**, a topic of particular relevance in the context of ongoing climate change, offering a comprehensive view through the integration of different types of genetic data (namely genomic, epigenomic, and transcriptomic) with data from other disciplines (e.g. climatic, dendrochronological, soil physicochemical properties, microbial communities).

To this end, the silver fir (*Abies alba* Mill.) was chosen as case study, leading to the definition of the following specific objectives:

- Identification of adaptive changes occurring in *A. alba* forests in the Pyrenees by analyzing the genetic structure and diversity of hotspots and coldspots of decline using genomic data.
- Identification of molecular markers, namely SNPs, in the *A. alba* genome involved in adaptation to the environment (under selection, associated with soil and climate environmental variables, or associated to dendrophenotypes).
- Prediction of the adaptive capacity of *A. alba* to climate change by calculating its genetic offset under future climate scenarios in the Pyrenees.
- Selection of an optimal protocol for epigenomic research in *A. alba* that allows the identification of epigenomic differences between declining and non-declining trees.
- Assembly and annotation of a high-quality transcriptome that can be used for expression studies in *A. alba*.
- Characterization of the transcriptomic response of *A. alba* to drought and identification of candidate genes involved in its response to vapor pressure deficit.

All in all, these objectives will advance the understanding of *A. alba*'s adaptive response to climate change from a multidisciplinary point of view, providing valuable insights into this conifer's declining process, resilience and evolutionary dynamics. Ultimately, this study seeks to contribute to the development of effective conservation and management strategies for *A. alba* in the Pyrenees, a rear-edge of the species.

OBJECTIVES

SECTION 1: GENOMICS

ARTICLE 1

Genomic insights into climate change-induced forest dieback in *Abies alba* hotspots of decline

Análisis genómico del decaimiento forestal causado por el cambio climático en puntos calientes de decaimiento de *Abies alba*

Comprender las respuestas genéticas adaptativas al cambio climático es una cuestión de máxima relevancia para mejorar las políticas de conservación y manejo. Este estudio profundiza en ello, centrándose en los bosques de abeto común (*Abies alba*) en el límite de su distribución, donde se ha reportado decaimiento forestal vinculado a factores climáticos, como el calentamiento y sequías recurrentes. Se definieron puntos calientes y puntos fríos de decaimiento y mortalidad forestal. Se utilizaron diferentes conjuntos de polimorfismos de un único nucleótido (SNPs), tanto a nivel del genoma completo como solo adaptativos, para estudiar sus características genéticas, con el objetivo de identificar diferencias en la diversidad genética entre cohortes de vigor (árboles decaídos y no decaídos) y de edad (adultos y jóvenes). Se evaluaron los niveles de metilación de ADN para investigar el posible papel de los procesos epigenéticos en la adaptación a estreses ambientales. Se realizaron análisis de parentesco para identificar la procedencia genética de los árboles en cada punto. Los resultados obtenidos indican que, aunque los SNPs adaptativos parecen proporcionar información más definida que los distribuidos por el genoma completo, la combinación de ambos es la estrategia óptima para este tipo de estudios evolutivos. Se observaron cambios en la diversidad genómica adaptativa y en la metilación del ADN entre las clases de vigor, revelando la existencia de una base molecular detrás de los eventos de decaimiento ocurriendo en los bosques de abeto común. Además, los parientes de los árboles muestran una mayor mezcla de orígenes en los puntos fríos, que podrían proporcionar un refugio temporal para la diversidad genética y el potencial adaptativo de la especie. En resumen, tanto las características genéticas como las epigenéticas deben investigarse para entender mejor cómo las especies forestales responden al estrés ambiental y se adaptan al cambio climático.

ARTICLE 1

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ARTICLE 1

RESEARCH

Genomic insights into climate change-induced forest dieback in *Abies alba* hotspots of decline

Isabel García-García[1] · Belén Méndez-Cea[1,2] · Francisco Javier Gallego[1] · Juan Carlos Linares[2] · Jose Luis Horreo[1]

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Abstract

Understanding adaptive genetic responses to climate change is an issue of utmost importance to improve conservation policies and adaptive management. This study deeps on it, focusing on rear-edge silver fir (*Abies alba*) forests, where decline has been reported and linked to climatic stressors, such as warming and recurrent drought events. Hotspots and coldspots of forest decline and mortality were defined. Different sets of single nucleotide polymorphisms (SNPs), namely genome-wide and adaptive, were used to study their genetic characteristics, aiming to identify differences in genetic diversity between vigor classes (declining and non-declining trees) and age cohorts (adult trees and saplings). Global DNA methylation levels were assessed to investigate a possible role of epigenetic processes in adaptation to stressful environments. Parentage and relatedness analysis were conducted to track the genetic lineage of trees from each site. Our findings indicate that, even though adaptive SNPs seem to provide more insightful information than the genome-wide set, the optimal approach for evolutionary studies is a combination of both. Changes in adaptive genomic diversity and DNA methylation were observed between vigor classes, revealing the existence of a molecular basis behind the ongoing decline events in silver forests. Besides, trees' relatives display a higher mixture of origins in coldspots, which could provide a temporary refuge for the species' genetic diversity and adaptive potential. All in all, both genetic and epigenetic characteristics should be considered in order to comprehend how forest trees respond to climate stress to achieve adaptation to climate change.

Keywords Adaptation · *Abies alba* · Forest decline · DNA methylation · Genetic diversity · Relatedness

Introduction

How species face climate change is a question of great importance in conservation and evolutionary biology (e.g. Hansen et al. 2001; Komatsu et al. 2019; Zhu et al. 2022). It is known that species evolve in response to global changes, as evidenced by the detection of adaptation processes such as changes in their population dynamics and genetic diversity (Horreo et al. 2016; Pauls et al. 2013). Particularly, global environmental alterations have been proven to be causing critical transitions and irreversible transformations of forests (Albrich et al. 2020; Batllori et al. 2020), with decline and mortality events being consistently reported since the 1970 decade across the world (Hammond et al. 2022) and linked to climate change effects (Allen et al. 2010; Hidalgo-Triana et al. 2023). However, there is still little information regarding the adaptation process of long-lived tree species, despite the potential usefulness of such knowledge to predict the fate of forests facing global change and act accordingly to prevent their disappearance, among other applications.

Silver fir, *Abies alba* Mill., an ecologically and economically valuable species (Tinner et al. 2013) which covers extensive areas in Europe, is among the species showing signs of forest decline and dieback (Bledý et al. 2024). It finds its rear edge in the South of the continent, in the Mediterranean basin, where decline trends in its growth have been found and linked to climate change effects (Büntgen et

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✉ Juan Carlos Linares
jclincal@upo.es

[1] Dpto de Genética, Fisiología y Microbiología, UD Genética, Facultad de CC Biológicas, Universidad Complutense de Madrid, Madrid 28040, Spain

[2] Dpto de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, Sevilla 41013, Spain

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al. 2014; Gazol et al. 2015). Intense droughts are predicted to increase in frequency in the Mediterranean area (Ozturk et al. 2015), concurrent to warmer summers (Lionello et al. 2014), posing a challenge for silver fir forests, which will need to cope with the novel and demanding environment. Moreover, previous research has shown that the current distribution range of *A. alba* is missing a substantial part of its potential distribution towards the warmer edge in the south of Europe (Ruosch et al. 2016) and that the Pyrenean populations respond differently to environmental stress (Martínez-Sancho et al. 2021). Furthermore, genetic diversity that could be essential to achieve adaptation to a changing climate is likely held in rear-edge populations (Hampe and Petit 2005; Pearman et al. 2024), highlighting the need to study this type of forest.

High standing genetic diversity has been identified as a key characteristic for increasing the adaptive capacity to shifting environmental conditions (Barret and Schluter 2008), positioning genomics as a priority research field to identify potential tools to reduce forests vulnerability to the effects of climate change (Neale and Kremer 2011). In addition, epigenetics has recently emerged as an equally important field of study, especially for sessile organisms. As trees cannot migrate instantly, having to rely on new generations to "move" through pollen and seed dispersal to colonize new areas with more suitable conditions, epigenetic responses could provide a rapid source of phenotypic variation (Nicotra et al. 2015; Balao et al. 2018), thanks to their implication in gene regulation (Zhang et al. 2018), and, therefore, improve the ability of populations to deal with abiotic stress (Pascual et al. 2014).

The aim of this work was to identify adaptive changes occurring in silver fir populations in the central-western Spanish Pyrenees, where forest dieback episodes have been reported since the 1980 decade onward (Camarero et al. 2015; Valeriano et al. 2023). Thus, in order to understand the key mechanisms and processes involved in survival or decline processes (thus fitness and evolution) of forests, genetic and epigenetic differences between declining and non-declining trees, as well as between silver fir stands showing signs of dieback and increased mortality rates (hotspots) and stands without decline symptoms (coldspots), were assessed. These analyses were carried out using two different datasets in order to compare them and provide information on which kind of data is more suitable for this kind of studies: (1) genome-wide, complete set of identified SNPs, and (2) subset of only adaptive SNPs. In addition, parentage and relatedness analyses were conducted, as kinship levels are essential for identifying genetic signals of adaptation and reducing inbreeding for conservation and artificial selection in forestry breeding (Hall et al. 2020).

More specifically, we sought to: (i) compare the genetic diversity and identify genetic differentiation among western-Pyrenees silver fir populations, including hotspots and coldspots of forest decline, as well as declining and non-declining trees; (ii) test the hypothesis that declining and non-declining trees differ in their global DNA methylation levels, as an evidence of different short-term adaptation capacities through phenotypic plasticity; (iii) test the hypothesis that hotspots and coldspots of forest decline display contrasting patterns of parentage and relatedness between adult trees and the offspring saplings, as an evidence of climate-mediated selective pressures affecting fitness.

Materials and methods

Study sites and sampling design

Fresh leaf samples were collected from *A. alba* individuals from five stands in the central-western Spanish Pyrenees (Fig. 1; Table 1) (García-García et al. 2023), during the same morning in November 2019 under the same weather conditions (snowing, covered skies). A homogeneous environment is particularly important for epigenomic studies given the sensitivity of epigenomic processes to environmental conditions (Meyer 2015). Samples were conserved in liquid nitrogen until DNA extraction.

Three of the sampled sites (Cotatuero, CO; Paco Ezpela, PE; Paco Mayor, PM) are placed in the southern part of the studied area and show signs of forest dieback (e.g. high levels of crown defoliation), while the other two (Gamueta, GA; Selva de Oza, SO), in the north, do not. Thus, CO, PE, and PM constitute hotspots of tree mortality, while GA and SO constitute coldspots. Particularly, the PE forest shows more severe and widespread signs of decline. This population is situated at the lowest altitude and the westernmost location among those included in this study (Table 1), where stands suffer from higher water shortage than the rest of the Pyrenean populations (Camarero et al. 2011; Sancho-Knapik et al. 2014; Valeriano et al. 2023).

Tree vigor classes were established based on defoliation levels by visual assessment of crown transparency (Dobbertin 2005), considering neighboring individuals showing high (> 60%, declining trees) or low (< 40%, non-declining trees) canopy defoliation (Camarero et al. 2011, 2015; Hevia et al. 2019; González de Andrés et al. 2022). Thus, two types of tree vigor classes were sampled where possible: declining and non-declining adult individuals were sampled in CO, PE, and PM, while only non-declining trees were sampled in GA and SO as they are coldspots where declining trees cannot be found.

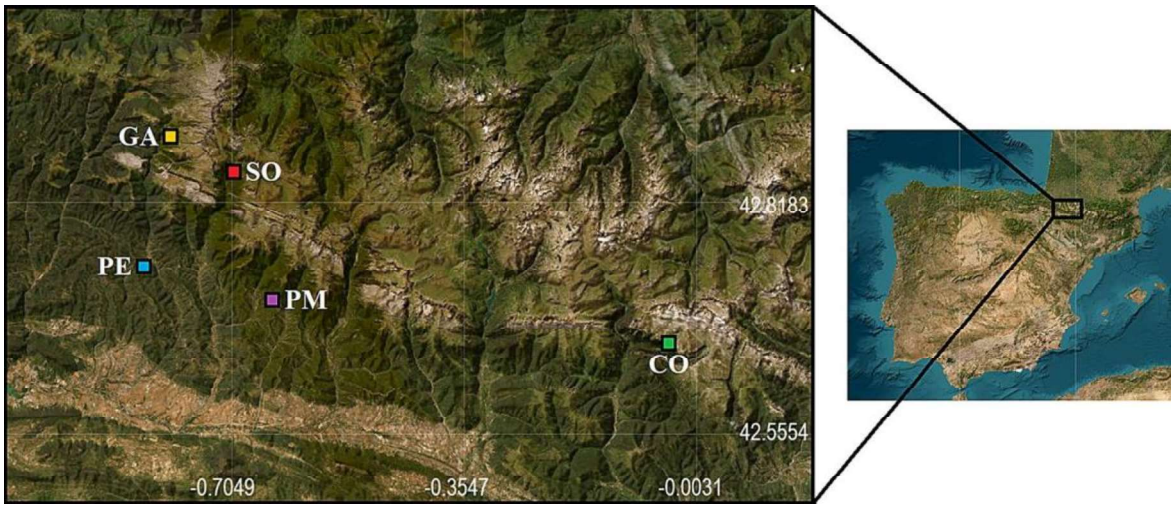

Fig. 1 Location of the study sites in the Central-Western Pyrenees, Spain: Cotatuero (CO); Gamueta (GA); Paco Ezpela (PE); Paco Mayor (PM); Selva de Oza (SO)

Table 1 Location of the study sites (CO, GA, PE, PM, SO) and number of sampled individuals. ND: non-declining trees; D: declining trees

	CO	GA	PE	PM	SO
Latitude	42.65	42.88	42.74	42.71	42.84
Longitude	-0.04	-0.79	-0.83	-0.64	-0.70
Altitude (m)	1456	1450	1116	1322	1220
ND adults	15	14	20	19	18
D adults	13	-	18	20	-
Saplings	19	4	36	38	20

In addition, two types of trees were sampled regarding their age: adults (over 40 years old, based on dendrochronological assessment through core analysis, see González de Andrés et al. 2022) and saplings (less than 1 m in height, younger than 10 years old, no symptoms of decline). The age of saplings was estimated by counting the number of scars in the main stem. These scars are left as marks after the fall of apical bud scales, once primary growth (stem and branches elongation) is resumed at the beginning of each year's growing season. This is possible since *A. alba* presents a strict preformed-growth pattern. Hence, each continuous and unique annual growth portion in the main stem and lateral branches of trees is separated and may be identifiable.

Declining and non-declining adult trees were randomly dispersed in the study sites. Hence, sampled trees were randomly selected within each typology, separated about 20 m among them. Thereafter, saplings were randomly selected among the nearest individuals found around a sampled adult tree. All in all, this sampling design allows therefore to compare the genetic characteristics of different types of populations and different cohorts (vigor classes and age).

SNP genotyping

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen®, Germany) and used to create PE libraries for ddRADseq (Peterson et al. 2012) which were sequenced by LGC Genomics (Germany) with 1 M read depth. Raw reads were trimmed using fastp v0.12.4 (Chen et al. 2018) and then mapped to a reduced version of the *A. alba* reference genome (Abal.1_1) (Mosca et al. 2019), to reduce computational costs due to its lengthy and highly fragmented nature, using ipyrad v0.9.65 (Eaton and Overcast 2020). Single nucleotide polymorphisms (SNPs) were then identified by the same software and filtered with VCFtools v0.1.16 (Danecek et al. 2011), keeping those that were biallelic, had a minor allele frequency higher than 5% and less than 50% of missingness (resulting in a dataset with ~ 20% missingness), and were situated at least 10,000 bp apart to remove strong linkage disequilibrium. Samples with more than 50% of missing data were removed. Consequently, the final data set retained 3,958 SNPs and 254 individuals (out of 55,803 SNPs and 266 individuals).

In addition, a subset of these SNPs was created, including exclusively the 102 SNPs that has been previously identified as adaptive in these populations (García-García et al. 2023). These genetic variants showed associations with environmental variables and with phenotypes related to response to drought. All analyses related to genetic diversity as well as differentiation among populations, tree age (adults *versus* saplings) and health (declining and non-declining) were done with both datasets in order to compare results.

Genetic characteristics

Genetic diversity was explored through several genetic parameters. Mean number of alleles per *locus* (Na), mean

number of effective alleles per *locus* (Neff), observed and expected heterozygosity (Ho, He), and percentage of polymorphic *loci* (PPL) with their respective standard error were calculated with GenAlEx v6.5 (Peakall and Smouse 2006, 2012). Allelic richness (AR) was obtained using the dartR v2.9.7 package (Gruber et al. 2018; Mijangos et al. 2022), as in q = 0 (Sherwin et al. 2017). Differences in genetic diversity between groups were tested through Wilcoxon matched-pairs signed rank tests, taking into consideration the obtained values for each *loci* in each group, after determining the non-parametric nature of the data with a Shapiro-Wilk test in GraphPad Prism 8 for Windows (Boston, Massachusetts, USA, www.graphpad.com). In addition, inbreeding coefficients (F_{IS}) and fixation indexes (F_{ST}) with their associated p-values were calculated with Genodive v3.06 (Meirmans 2020) with 9,999 permutations. Isolation by distance (IBD) and isolation by environment (IBE) were tested with genepop v1.2.2 (Rousset 2008). The 19 bioclimatic variables used as input for the IBE test were obtained from the WorldClim database (Fick and Hijmans 2017) with a cell grid resolution of 30 s using QGIS 3.18 (QGIS Development Team 2022).

Parentage and relatedness analysis

Parent-progeny relationships were studied in order to determine genetic identity between potential offspring (saplings) and parents (adults) with the 'apparent' R package (Melo and Hale 2019), which uses the Gower Dissimilarity metric (GD; Gower 1971) and does not require a priori knowledge of family structure. The general set of SNPs rather than just the adaptive was used in order to maximize the number of tested markers to improve the accuracy of the results. Self-crossing was enabled as *A. alba* is a monoecious species (Wolf 2003). Dyad analysis was enabled to identify at least one parent, which are significative for the mean GD (GDM) and the coefficient of variation (GDCV) tests, and to detect close relatives, which are significative only for the GDM test, in case just one or none of the parents had been sampled, which is highly probable working with natural forests made up of large numbers of individuals. Significant matches were those with a *p*-value lower than 0.05. Significant differences between proportions of native-foreign and declining/non-declining parents/relatives in hotspots compared to coldspots were tested through Fisher's exact tests for 2 × 2 contingency tables with GraphPad Prism 8 for Windows.

Table 2 Fixation indexes (F_{ST}) between the studied *A. Alba* populations in the pyrenees using the genome-wide set of SNPs below diagonal, and using the adaptive subset of SNPs above diagonal. * indicates p-value < 0.001

	CO	GA	PE	PM	SO
CO	-	0.117*	0.152*	0.126*	0.116*
GA	0.038*	-	0.066*	0.075*	0.066*
PE	0.034*	0.028*	-	0.041*	0.025*
PM	0.027*	0.035*	0.027*	-	0.016*
SO	0.027*	0.034*	0.019*	0.015*	-

DNA methylation levels

Global DNA methylation was quantified by measuring levels of 5-methylcytosine (5-mC) with the *MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit* (Epigentek Group Inc.). Declining and non-declining adult trees from hotspots were analyzed, using 100 ng of genomic DNA per sample as input. The absorbance at 450 nm of two biological replicates per sample was measured and their mean value was used for DNA methylation percentage calculation following the manufacturer instructions. Differences between vigor conditions within each site were tested through t-tests after checking for normality with a Shapiro-Wilk test in GraphPad Prism 8 for Windows.

Results

Genetic characteristics

Sampling sites

Fixation indexes (F_{ST}) showed significant genetic differentiation among all sampled sites using both the genome-wide set of SNPs as well as the adaptive subset; the latter resulting in higher F_{ST} values (Table 2). These results are in line with a previous principal component analysis (PCA) (Fig. 1 in García García et al. 2023) and AMOVA results (Table S2, García-García et al. 2023), which also indicated significant genetic differences among populations.

Regarding the adaptive subset, percentage of polymorphic *loci* ranged between 94.12 and 100, mean number of alleles per *locus* ranged between 1.941 and 2, mean number of effective alleles per *locus* 1.523–1.609, observed and expected heterozygosities ranged between 0.285 and 0.306 and 0.312–0.354, respectively, and allelic richness between 0.941 and 1 (Table 3). The GA population is the only one that shows significant reductions in adaptive diversity in relation to the other populations (Tables S1-S4).

Similar results were obtained using the genome-wide set of SNPs (Table S5). Again, GA displays significantly lower diversity values, along with PE (Tables S6-S9). Thus, neither type of genetic diversity allows to tell hotspots from coldspots. Inbreeding coefficient was significant in all populations (Table S5). Neither IBD (Appendix A) nor IBE (Appendix B) were detected.

Table 3 Description of the adaptive genetic diversity present in the five sampled stands of *A. Alba* in the pyrenees (considering adults and saplings), including mean number of samples (N), mean number of alleles per *locus* (na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (he), allelic richness (AR), and percentage of polymorphic *loci* (PPL), and their respective standard errors

	CO	GA	PE	PM	SO
N	38.382 ±0.796	14.863 ±0.377	63.804 ±1.070	64.059 ±1.323	32.382 ±0.611
Na	1.990 ±0.010	1.941 ±0.023	2.000 ±0.000	2.000 ±0.000	1.990 ±0.010
Neff	1.603 ±0.033	1.523 ±0.032	1.572 ±0.028	1.583 ±0.031	1.609 ±0.030
Ho	0.302 ±0.016	0.285 ±0.018	0.298 ±0.015	0.304 ±0.016	0.306 ±0.016
He	0.346 ±0.015	0.312 ±0.015	0.340 ±0.013	0.341 ±0.014	0.354 ±0.014
AR	0.990 ±0.099	0.941 ±0.236	1.000 ±0.000	1.000 ±0.000	0.990 ±0.099
PPL	99.02	94.12	100.00	100.00	99.02

Age

With respect to the adaptive subset, no significant differences between adults and saplings were found in F_{ST} values in any population. Accordingly, no differences between adults and samplings were found in genetic diversity, except for GA saplings, which showed significant reductions in genetic variability with respect adults (Table 4, Table S10). However, GA results should be viewed with caution as this study only counted with 4 saplings from this site, which by chance might not be representative of their category.

When using the genome-wide dataset, significant differences in fixation indexes were found between adults and saplings in CO (F_{ST} = 0.007, *p*-value = 0.003) and PM (F_{ST} = 0.002, *p*-value = 0.047), and saplings showed significant reductions in genetic variability with respect to adults in the CO, GA and SO populations (Tables S11-S12). Inbreeding coefficients were significant in all cases (Table S11).

Tree vigor

Interestingly, the adaptive subset identified significant differences in F_{ST} values between declining and non-declining individuals in PE (F_{ST} = 0.022, *p*-value = 0.003), while the other two hotspots remain undifferentiated. This population was the only that, in addition, showed an increase in Neff, He, and AR in non-declining compared to the declining individuals. On the other hand, declining trees showed significant reductions in Na and lower AR values compared to non-declining individuals in CO and PM (Table 5, Table S13).

The genome-wide analysis revealed no genetic differences between declining and non-declining trees (García-García et al. 2023). However, the case of PE is worth of notice, as it resulted in marginally significant differences (*p*-value = 0.055). Significant reductions in different parameters of genetic variability were found in declining trees in the three hotspots: in Na (CO, PE, PM), Neff (CO), Ho (CO), and He (CO, PM) (Tables S14-S15), and, conversely, declining trees in PE showed significantly higher Ho than non-declining trees. Inbreeding coefficients were significant in all stands and states of health (Table S14).

Parentage and relatedness analysis

Following the manual recommendations, individual 228 (declining tree from CO) was removed due to abnormally low values in its mean GDij|k and mean number of usable *loci* and the analysis was rerun. Again, GA results should be viewed with caution as this study only counted with 4 saplings from this site which might not be representative of its category.

Table 4 Description of the adaptive genetic diversity present in the age subgroups (A: adults; S: saplings) in each population, including mean number of samples (N), mean number of alleles per *locus* (na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (he), allelic richness (AR), and percentage of polymorphic *loci* (PPL), and their respective standard errors

	CO		GA		PE		PM		SO	
	A	S	A	S	A	S	A	S	A	S
N	22.529 ±0.472	15.853 ±0.390	11.784 ±0.297	3.078 ±0.112	32.951 ±0.566	30.853 ±0.554	31.951 ±0.762	32.108 ±0.608	15.735 ±0.277	16.647 ±0.357
Na	1.961 ±0.019	1.951 ±0.026	1.931 ±0.025	1.618 ±0.052	1.990 ±0.010	1.990 ±0.010	1.990 ±0.010	2.000 ±0.000	1.961 ±0.019	1.980 ±0.014
Neff	1.596 ±0.033	1.559 ±0.036	1.526 ±0.032	1.390 ±0.042	1.548 ±0.030	1.594 ±0.029	1.567 ±0.032	1.592 ±0.032	1.589 ±0.032	1.599 ±0.029
Ho	0.311 ±0.018	0.285 ±0.018	0.291 ±0.019	0.250 ±0.028	0.290 ±0.016	0.306 ±0.015	0.290 ±0.016	0.318 ±0.017	0.303 ±0.019	0.311 ±0.016
He	0.341 ±0.016	0.330 ±0.016	0.313 ±0.015	0.238 ±0.020	0.328 ±0.014	0.348 ±0.013	0.333 ±0.014	0.343 ±0.014	0.342 ±0.015	0.351 ±0.013
AR	0.961 ±0.195	0.970 ±0.171	0.931 ±0.254	0.650 ±0.479	0.990 ±0.099	0.990 ±0.099	0.990 ±0.099	1.000 ±0.000	0.961 ±0.195	0.980 ±0.139
PPL	96.08	96.08	93.14	63.73	99.02	99.02	99.02	100.00	96.08	98.04

Table 5 Description of the adaptive genetic diversity present in the tree vigor subgroups (ND: non-declining trees; D: declining trees) in each population, including mean number of samples (N), mean number of alleles per *locus* (na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (he), allelic richness (AR), and percentage of polymorphic *loci* (PPL), and their respective standard errors. Only CO, PE and PM are shown as GA and SO stands are composed of non-declining trees exclusively

	CO		PE		PM	
	ND	D	ND	D	ND	D
N	12.784 ±0.240	9.745 ±0.286	17.853 ±0.314	15.098 ±0.302	16.167 ±0.337	15.784 ±0.462
Na	1.941 ±0.023	1.853 ±0.035	1.941 ±0.023	1.961 ±0.019	1.971 ±0.017	1.882 ±0.035
Neff	1.576 ±0.034	1.578 ±0.035	1.517 ±0.031	1.562 ±0.032	1.556 ±0.032	1.543 ±0.036
Ho	0.313 ±0.020	0.311 ±0.022	0.277 ±0.017	0.307 ±0.019	0.281 ±0.018	0.297 ±0.018
He	0.331 ±0.016	0.328 ±0.017	0.310 ±0.015	0.330 ±0.015	0.328 ±0.014	0.322 ±0.016
AR	0.941 ±0.236	0.853 ±0.356	0.941 ±0.236	0.961 ±0.195	0.971 ±0.170	0.901 ±0.300
PPL	94.12	85.29	94.12	96.08	97.06	89.22

At least one parent was found for 100% of the saplings from GA, 40% of the saplings from SO, 36.11% of the saplings from PE, 50% of the saplings from PM, and 57.89% of the saplings from CO. Relatives were found for 75% of the saplings from GA, 75% of the saplings from SO, 77.78% of the saplings from PE, 73.68% of the saplings from PM, and 84.21% of the saplings from CO.

As expected, the majority of the relatives (Fig. 2a) and parents (Fig. S1a) of each sapling in each sampling site were from the area where they were sampled, except for SO, which presents a higher mixture of origins. In fact, significant differences were found in native-foreign proportion of relatives between hotspots and coldspots considered all together (p-value < 0.0001), showing that trees in coldspots have a higher proportion of foreign relatives than those in hotspots, where native relatives are dominant. On the other hand, when the tree vigor is considered, there appears to be no preference for declining or non-declining individuals as parents/relatives (Fig. 2b, Fig. S1b). In other words, non-declining individuals are not more likely to be parents/relatives than declining individuals and vice versa. Interestingly, PE was the only population without declining relatives or parents from other populations.

DNA methylation levels

Percentages of global DNA methylation were calculated after generating the standard curve with the positive control dilutions included in the kit, which gave an R^2 of 0.965, thus being a good fit for the observed data. Each hotspot was tested separately for differences between non-declining and declining individuals due to their differences in environmental conditions, which affect epigenetic patterns in each site. Significant differences were found in PM and CO, where declining trees show higher methylation levels than non-declining trees (Fig. 3).

Discussion

Information regarding the adaptation process of long-lived tree species is still scarce. Here, we aimed to identify the adaptive changes occurring in silver fir (*Abies alba*) populations affected by current global change, using a Pyrenean area as case study given its especial vulnerability as a rear edge. Although *A. alba* forests in the Pyrenees are relatively well characterized from a genetic point of view (e.g. Scotti-Saintagne et al. 2021; Major et al. 2021; Balao et al. 2020), they have been mostly studied as representative sampling sites of this area in the species' distribution range. This work presents, for the first time, a genomic study of silver fir populations following a hotspot-coldspot approach which also considers age and health state, showing an adaptive response to climate change, with DNA methylation being potentially involved in this evolutionary process, and finding that trees with genetic heritage from hotspots can grow in coldspots but not the contrary. With drought-induced forest decline and mortality events becoming a major worldwide concern (Hartmann et al. 2022), experimental designs like the here-used could help gain more in-depth understanding of the genetic responses of silver fir facing climate change. Comparison between the use of genome-wide SNP and an adaptive subset was additionally done in order to test which of them is more suitable for this kind of studies, which is a highly relevant topic about which there is still much debate (e.g. Zimmerman et al. 2020; Chen et al. 2021; Fernandez-Fournier et al. 2021; Kardos et al. 2021; Lind et al. 2024).

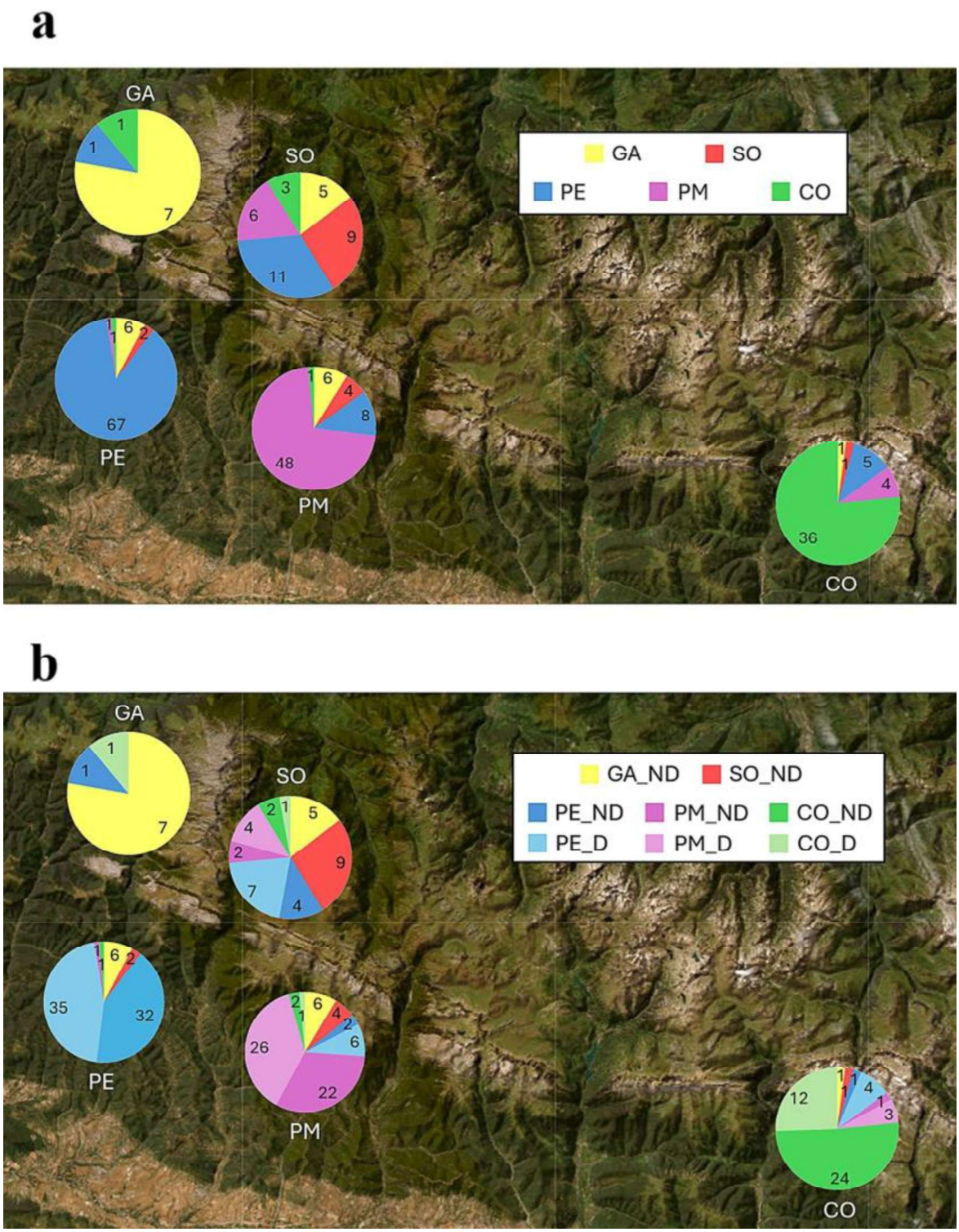

Fig. 2 Number of relatives (including parents) found for the sampled saplings in each population (GA, SO, PE, PM, CO). Colors indicate the population of origin of the adults (**a**) and their vigor class (**b**), where "_ND" stands for non-declining and "_D" stands for declining. Number of studied saplings in GA = 4; SO = 20; PE = 36; PM = 38; CO = 19

In relation to the latter, the genome-wide set, which includes almost 4,000 SNPs, showed similar but less clear results than the adaptive subset, with 102 SNPs (see material and methods section). Both inferred significant genetic differences (F_{ST}) among populations, but the adaptive subset showed higher values (Table 2), indicating a higher differentiation in the adaptive fraction of the genome than in the genome-wide variants. Similarly, no genetic differences were found between declining and non-declining trees in any population except for PE, with the genome-wide set giving a *p*-value of 0.055 and the adaptive subset giving a *p*-value of 0.03. This points to clearer information given

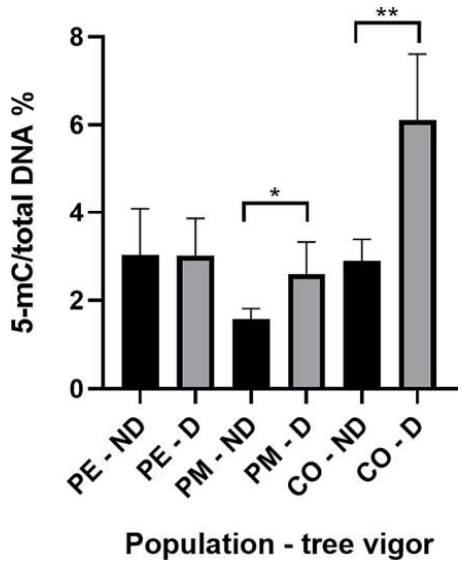

Fig. 3 Percentage of methylated cytosines (5-mC) per total DNA (A+G+C+T) in non-declining (ND) and declining (D) individuals in each declining population (PE, PM, CO). * indicates p-value < 0.5; ** indicates p-value < 0.01. Total sample size: 24 (4 per group)

by the adaptive subset in comparison with the genome-wide, which is in line with the higher differentiation values mentioned above. Furthermore, when testing for changes in genetic variation between declining and non-declining trees, both datasets showed lower genetic variability in declining trees in the CO and PM populations and higher in PE (Tables S13, S15), but the adaptive subset was capable to detect it in more estimators (Neff, He, and AR, while the genome-wide set only detected it in Ho). Lastly, both datasets showed different results when testing for genetic differences between adults and saplings: the adaptive subset did not find differences between them in F_{ST} values in any population, contrary to the genome-wide, which found differences in CO and PM; and the adaptive subset found reduced genetic diversity in saplings in GA, while such differences were identified in GA, CO and SO using the genome-wide set (Tables S10, S12). The genome-wide set provides therefore less conclusive results, indicating genetic differences between adults and saplings in PM, but no changes in their genetic diversity. All this points to the subset of adaptive SNPs as the most informative in this work, and, thus, it will be the dataset employed in the discussion hereafter. This is a crucial decision because the use of neutral or adaptive SNPs can affect evolutionary and conservation conclusions of this type of studies (e.g. Silva et al. 2020; Xuereb et al. 2021).

Fixation indexes (F_{ST}) showed, as commented above, and according to a previous study (Sancho-Knapik et al. 2014), each sampled site is significantly different from the rest. They have been consequently treated as independent populations for further analysis. Coldspots could not be distinguished from hotspots solely looking at their genetic diversity, as no differences were detected between them (except for GA, which has a low number of samples that could be affecting its results). No genetic differences were found between saplings and adults, nor were there changes in genetic diversity between said age groups in any population (Tables S10) (GA should not be considered for this comparison due to its low number of saplings). In line with this, declining trees did not show genetic differences compared to non-declining trees in any population but in PE. Precisely in this population (PE), silver fir decline has been described as more severe and widespread because of its environmental characteristics, including higher water shortages (Camarero et al. 2011; Sancho-Knapik et al. 2014; Crespo-Antia et al. 2024). This suggests that PE has been suffering from higher levels of environmental stress than the rest of the studied sites, presumably for a longer time as conditions were already harsh in this population, and, thus, genetic changes towards adaptation have been occurring through a higher number of generations than in the rest of the hotspots. This hypothesis totally agrees with the here-detected genetic differences, as well as with the absence of non-declining relatives or parents from other populations (Fig. S2b, S1b): they cannot survive in such conditions. Furthermore, in this population (PE), and contrary to what happens in the other populations, non-declining trees show lower genetic diversity than declining trees, agreeing with the occurrence of an evolutionary reduction of genetic variability caused by directional selection (Hoffmann and Sgrò 2011). This is supported by the adaptive SNP subset finding such changes in genetic diversity in more parameters than the genome-wide set (Tables S13, S15). As genetic variability is extremely important for the future of the species (Waldvogel et al. 2020; Barret and Schluter 2008) and the effects of climate change-induced selection seem to be visible in PE, where selection has likely been acting on non-declining trees (the ones which are likely to survive in the near future), leading to reduced genetic variability, these populations could be defined as at risk.

Nevertheless, foreign relatives being dominant within coldspots while native relatives are the most common in hotspots (Fig. 2a) supports that trees with genetic heritage from all origins might be equally capable of growing in areas with mild climate (coldspots), but not the contrary. The lack of IBD and IBE indicates that this "migration" from hotspots (south) to coldspots (north) is possible. Thus, coldspots, in the north, where the weather is less challenging for silver fir, as it does not cope will with aridity (Macias et al. 2006; Gazol et al. 2015; Valeriano et al. 2023) and conditions are more humid than in the south (Vigo and Ninot 1987), could serve a refuge for the species' genetic diversity.

Another aspect to consider regarding adaptation is epigenomic factors (McCaw et al. 2020). There is increasing evidence about how methylation processes regulate gene

expression providing a rapid response to stress through transient phenotypic plasticity, prior to any adaptive genetic changes over generations (Rajpal et al. 2022). In fact, drought-tolerant plants have been found to have more stable methylomes under drought conditions and heat-resistant genotypes show lower levels of methylation than heat-sensitive individuals (Liu and He 2020; Sun et al. 2022). In agreement with this, lower DNA methylation levels were found in non-declining trees in PM and CO, (Fig. 3), suggesting that declining trees from both populations would be more sensitive to the hard climatic present conditions.

In contrast to it, DNA methylation levels show no differences between declining and non-declining trees in PE (Fig. 3), which, in combination with the above-commented possible existence of a selective pressure in this population for a long time, could lead to the formulation of the following hypothesis: adaptive changes might occur in rear-edge silver fir forests following a step-by-step pattern. Speculatively speaking, epigenetic processes may play a key role in *A. alba*'s first response to stressing environmental conditions through phenotypic plasticity, while a genetic change arriving later would be necessary for long-term adaptation to strong climate stresses. Therefore, it is possible that a response through gene expression modulation, achieved via epigenetic modification, has been occurring for generations. This could have led to the death of non-responsive trees, which may explain why no epigenetic differences between declining and non-declining trees are currently detectable. Then, genetic changes through directional selection might have begun to happen across generations, potentially leading to the present difference between declining and non-declining individuals, making some individuals survive and others start a decline process. Thus, even though declining trees in PE seem to exhibit a "normal" epigenetic response, being able to modify their DNA methylation levels similarly to non-declining trees, it is plausible that their genetic background may not be sufficient to ensure their survival under harsh environmental conditions, possibly contributing to their decline. On the other hand, the other hotspots (CO and PM) would still be in the first step, where there are methylation differences but not genetic yet.

However, our experiments cannot prove a relationship between our genetic and epigenetic analyses. Nonetheless, the here-obtained results match the above-outlined hypothesis and offer an explanation for this situation on their own. Further studies are required in order to test this theory, focusing on finding the link between DNA methylation levels and genetic changes across generations, given its potential high relevance on evolution and conservation. Regardless of whether our step-by-step hypothesis is true, what does seem clear is the involvement of both genomic and epigenomic factors in silver fir's response to climate stress.

On summary, we report evidence of inbreeding as well as reductions in genetic variability in declining silver fir forests from the Pyrenees, climate change inducing selective pressures in the rear edge of the species and hotspots (southern populations) being at risk. In response to it, individuals from these hotspots are being pushed to northern coldspots. However, global warming could potentially act faster than the pace at which trees can alter their allele frequencies to achieve adaptation to the new conditions or move to new areas (García-García et al. 2022). Hence, it is of upmost importance to enhance habitat conservation and adaptive forest management, and to expand the existing knowledge about how evolutionary responses occur in order to ensure the preservation of forests (Leites and Benito Garzón 2023).

Genomic approaches have provided valuable tools to design conservation plans over the last decades (McMahon et al. 2014), but an epigenetic perspective should be taken into consideration as well (Rey et al. 2019), as differences can be found between declining states. Our results support that marginal populations deserve priority conservation efforts, as they might be especially important for the long-term conservation of genetic diversity, phylogenetic history and evolutionary potential of silver fir (Hampe and Petit 2005). These range-limit populations are currently in climate change hotspots, where drier and warmer summer conditions will represent the major climatic constraints to the persistence of silver fir. So, what conservation strategies might be useful to preserve silver fir forests in the Pyrenees? Maintaining gene flow between forests, especially towards coldspots where trees coming from every region seem to be able to grow, is crucial to prevent habitat fragmentation and the loss of potentially advantageous genetic variants in isolated populations. Thus, the construction of wide roads, hiking trails, and ski slopes should be minimized or avoided in order to avoid the interruption of pollen flow between forests. Further, the specific regions undergoing DNA methylation modifications should be identified, as these could act as molecular markers of stress and be used to evaluate the state of populations.

Conclusions

Our findings show that changes in genomic diversity are occurring, in response to climate change, in declining silver fir forests from the Pyrenees, which are of special importance as they constitute the southwestern edge of this species' natural distribution. Changes in global DNA methylation levels between declining and non-declining trees have also been detected, pointing to epigenomic processes being involved in adaptation to climate stress. Coldspots, where a mixture of origins can be found and current environmental

stress is not imminently harmful, may be offering a temporary refuge for silver fir genetic diversity and adaptive potential. All in all, research efforts must be increased in order to understand in more detail how forests respond to global change and its derived environmental consequences. In addition, we present a comparison between the use of neutral or adaptive genetic markers to study adaptation to climate change in natural populations, concluding that the adaptive subset seems to provide less diluted information than the genome-wide set, but also revealing that a combination of both would be the best option for these kinds of evolutionary studies.

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Author contributions I.G-G and B.M-C carried out the formal analysis. I.G-G wrote the manuscript and prepared the figures and tables. B.M-C, FJG, JCL and JLH reviewed the manuscript. FJG, JCL and JLH designed the study.

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Data availability Raw ddRADseq sequences are available at NCBI's Sequence Read Archive (SRA), under the BioProject with accession number PRJNA947483.

Declarations

Competing interests The authors declare no competing interests.

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SUPPLEMENTARY MATERIAL

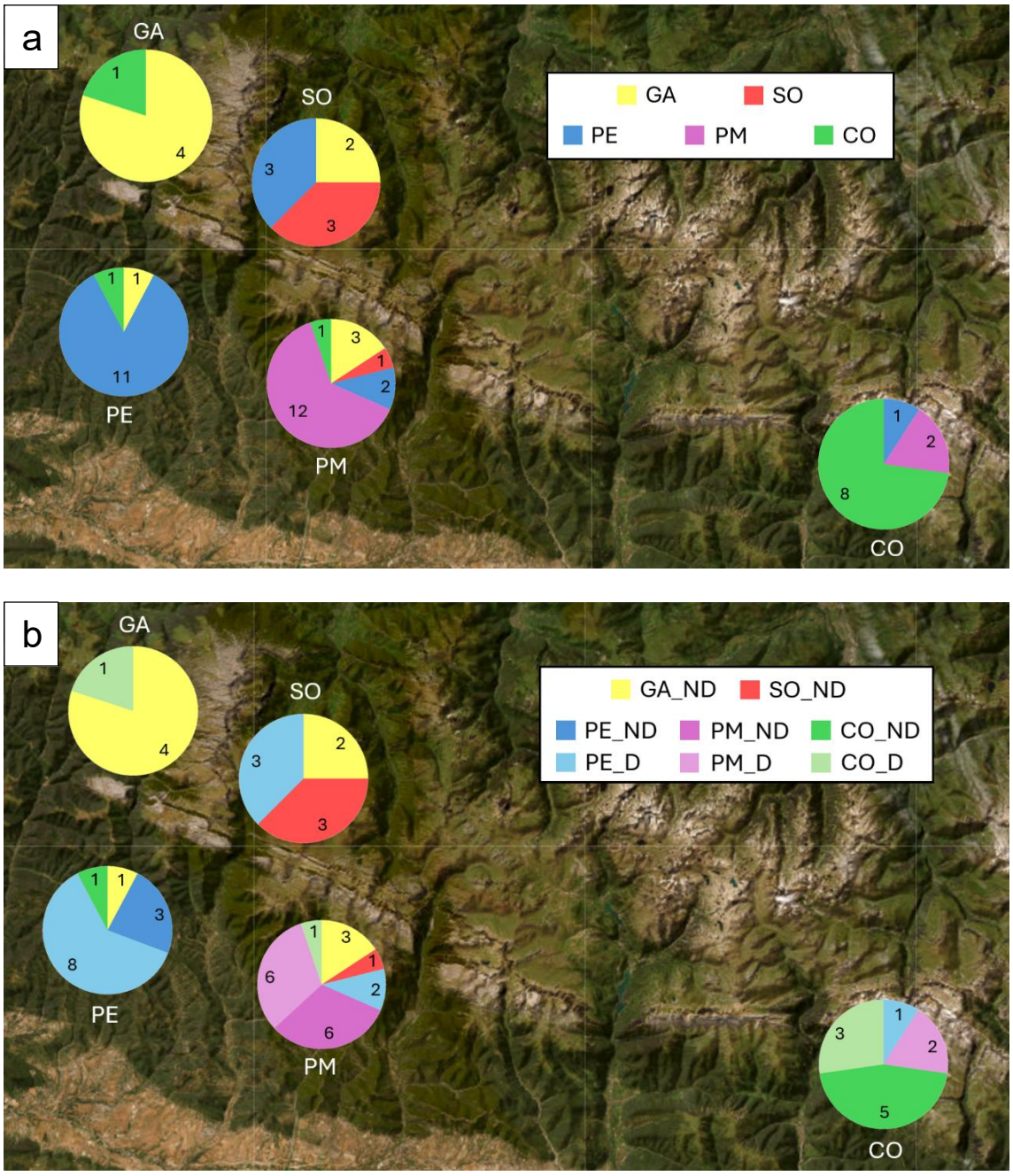

Fig. S1: Number of parents found for the sampled saplings in each population (GA, SO, PE, PM, CO). Colors indicate the population of origin of the adults (a) and their vigor class (b), where "_ND" stands for non-declining and "_D" stands for declining. Number of studied saplings in GA = 4; SO = 20; PE = 36; PM = 38; CO = 19.

Table S1: Wilcoxon matched-pairs signed rank test results for mean number of alleles per *locus* (Na) between sampling sites (CO, GA, PE, PM, SO) for the adaptive subset of SNPs. Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-20	-1	-1	0
GA	0.1250	-	-21	-21	-20
PE	> 0.9999	0.0313	-	=	-1
PM	> 0.9999	0.0313	=	-	1
SO	> 0.9999	0.1250	> 0.9999	> 0.9999	-

Table S2: Wilcoxon matched-pairs signed rank test results for mean number of effective alleles per *locus* (Neff) between sampling sites (CO, GA, PE, PM, SO) for the adaptive subset of SNPs. Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	102	604	255	-38
GA	0.0471	-	-806	-1,035	-1,440
PE	0.3152	0.1796	-	638	906
PM	0.6723	0.0797	0.2886	-	-958
SO	0.9503	0.0129	0.1255	0.1102	-

Table S3: Wilcoxon matched-pairs signed rank test results for observed heterozygosity (Ho) between sampling sites (CO, GA, PE, PM, SO) for the adaptive subset of SNPs. Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-722	382	-9	-63
GA	0.2227	-	-682	-829	-771
PE	0.5135	0.2565	-	216	462
PM	0.9887	0.1612	0.7202	-	-475
SO	0.9161	0.1861	0.4427	0.4232	-

Table S4: Wilcoxon matched-pairs signed rank test results for expected heterozygosity (He) between sampling sites (CO, GA, PE, PM, SO) for the adaptive subset of SNPs. Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-1,098	434	207	-185
GA	0.0628	-	-1,007	-1,039	-1,460
PE	0.4710	0.0930	-	341	656
PM	0.7314	0.0741	0.5713	-	-972
SO	0.7591	0.0117	0.2610	0.0901	-

Table S5: Description of the genetic diversity present in the five sampled stands of *A. alba* in the Pyrenees (considering adults and saplings), including mean number of samples (N), mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (He), allelic richness (AR), percentage of polymorphic *loci* (PPL), and inbreeding coefficient (F_{IS}; * indicates p-value < 0.001), and their respective standard errors.

	CO	GA	PE	PM	SO
N	35.170 ± 0.148	13.489 ± 0.076	61.051 ± 0.181	61.156 ± 0.229	31.247 ± 0.103
Na	1.994 ± 0.001	1.874 ± 0.006	1.996 ± 0.001	1.997 ± 0.001	1.988 ± 0.002
Neff	1.591 ± 0.005	1.505 ± 0.006	1.564 ± 0.005	1.584 ± 0.005	1.577 ± 0.005
Ho	0.315 ± 0.002	0.288 ± 0.003	0.303 ± 0.002	0.312 ± 0.002	0.313 ± 0.003
He	0.347 ± 0.002	0.303 ± 0.003	0.333 ± 0.002	0.344 ± 0.002	0.340 ± 0.002
AR	0.994 ± 0.074	0.895 ± 0.307	0.996 ± 0.061	0.997 ± 0.053	0.988 ± 0.109
PPL	99.44	88.53	99.62	99.72	98.79
F_{IS}	0.106*	0.103*	0.098*	0.102*	0.097*

Table S6: Wilcoxon matched-pairs signed rank test results for mean number of alleles per *locus* (Na) between sampling sites (CO, GA, PE, PM, SO). Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-97,286	119	187	3,958
GA	<0.0001	-	100,918	103,206	93,397
PE	0.2962	<0.0001	-	54	-891
PM	0.0801	<0.0001	0.5572	-	-1,073
SO	0.0013	<0.0001	<0.0001	<0.0001	-

Table S7: Wilcoxon matched-pairs signed rank test results for mean number of effective alleles per *locus* (Neff) between sampling sites (CO, GA, PE, PM, SO). Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-2,211,383	-1,140,282	-277,869	-564,332
GA	<0.0001	-	1,504,166	2,019,729	1,761,238
PE	<0.0001	<0.0001	-	1,020,548	661,578
PM	0.0514	<0.0001	<0.0001	-	-151,106
SO	<0.001	<0.0001	<0.0001	0.2886	-

Table S8: Wilcoxon matched-pairs signed rank test results for observed heterozygosity (Ho) between sampling sites (CO, GA, PE, PM, SO). Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-1,417,741	-821,552	-320,794	-202,209
GA	<0.0001	-	925,727	1,240,421	1,170,286
PE	<0.0001	<0.0001	-	626,122	719,385
PM	0.0239	<0.0001	<0.0001	-	160,008
SO	0.1519	<0.0001	<0.0001	0.2591	-

Table S9: Wilcoxon matched-pairs signed rank test results for expected heterozygosity (He) between sampling sites (CO, GA, PE, PM, SO). Sums of signed ranks (W) are shown above diagonal; *p*-values are shown below diagonal.

	CO	GA	PE	PM	SO
CO	-	-2,475,099	-1,216,428	-251,578	-577,792
GA	<0.0001	-	1,760,159	2,273,589	2,005,143
PE	<0.0001	<0.0001	-	1,082,057	689,599
PM	0.0757	<0.0001	<0.0001	-	-140,839
SO	<0.0001	<0.0001	<0.0001	0.3161	-

Table S10: Wilcoxon matched-pairs signed rank test results (sums of signed ranks, W, and *p*-values) for mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), and expected heterozygosity (He) **between adults and saplings in the studied populations (CO, GA, PE, PM, SO) for the adaptive subset of SNPs.**

		CO	GA	PE	PM	SO
Na	W	-3	-528	0	1	5
	p-value	> 0.9999	< 0.0001	> 0.9999	> 0.9999	0.6250
Neff	W	-878	-1,916	1,051	568	163
	p-value	0.1377	0.0004	0.0708	0.3450	0.7744
Ho	W	-1,005	-1,063	802	1,103	212
	p-value	0.0842	0.0350	0.1689	0.0577	0.7004
He	W	-885	-2,140	978	102	372
	p-value	0.1345	< 0.0001	0.0929	0.3462	0.5120

Table S11: Description of the genetic diversity present in the age subgroups (A: adults; S: saplings) in each population, including mean number of samples (N), mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (He), allelic richness (AR), percentage of polymorphic *loci* (PPL), and inbreeding coefficient (F_{IS}; * indicates *p*-value < 0.001), and their respective standard errors.

	CO		GA		PE		PM		SO	
	A	S	A	S	A	S	A	S	A	S
N	21.067 ± 0.081	14.103 ± 0.083	10.699 ± 0.059	2.790 ± 0.020	31.917 ± 0.092	29.234 ± 0.098	30.363 ± 0.133	30.793 ± 0.105	15.468 ± 0.046	15.779 ± 0.063
Na	1.984 ± 0.002	1.900 ± 0.005	1.847 ± 0.006	1.517 ± 0.010	1.983 ± 0.002	1.984 ± 0.002	1.988 ± 0.002	1.992 ± 0.001	1.965 ± 0.003	1.958 ± 0.003
Neff	1.595 ± 0.005	1.532 ± 0.006	1.496 ± 0.006	1.326 ± 0.008	1.559 ± 0.005	1.557 ± 0.005	1.578 ± 0.005	1.578 ± 0.005	1.569 ± 0.005	1.561 ± 0.005
Ho	0.325 ± 0.003	0.296 ± 0.003	0.290 ± 0.003	0.263 ± 0.005	0.304 ± 0.003	0.301 ± 0.003	0.311 ± 0.003	0.312 ± 0.003	0.315 ± 0.003	0.309 ± 0.003
He	0.347 ± 0.002	0.318 ± 0.003	0.319 ± 0.003	0.285 ± 0.004	0.330 ± 0.002	0.329 ± 0.002	0.340 ± 0.002	0.340 ± 0.002	0.334 ± 0.002	0.331 ± 0.002
AR	0.984 ± 0.125	0.923 ± 0.267	0.875 ± 0.331	0.633 ± 0.482	0.983 ± 0.129	0.984 ± 0124	0.988 ± 0.108	0.992 ± 0.088	0.965 ± 0.183	0.958 ± 0.200
PPL	98.41	91.16	86.21	58.82	98.31	98.43	98.81	99.22	96.51	95.81
F_{IS}	0.092*	0.124*	0.099*	0.110*	0.094*	0.102*	0.104*	0.099*	0.092*	0.101*

Table S12: Wilcoxon matched-pairs signed rank test results (sums of signed ranks, W, and *p*-values) for mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), and expected heterozygosity (He) **between adults and saplings in the studied populations (CO, GA, PE, PM, SO).**

		CO	GA	PE	PM	SO
Na	W	-55,310	-792,156	250	456	-2,926
	p-value	<0.0001	<0.0001	0.6879	0.0440	0.0523
Neff	W	-1,451,353	-2,357,910	-159,181	83,302	-286,291
	p-value	<0.0001	<0.0001	0.2539	0.5523	0.0342
Ho	W	-1,328,006	-725,069	-239,864	199,003	-243,226
	p-value	<0.0001	<0.0001	0.0842	0.1506	0.0667
He	W	-1,565,234	-2,401,148	-165,038	56,146	-265,725
	p-value	<0.0001	<0.0001	0.2332	0.6854	0.0483

Table S13: Wilcoxon matched-pairs signed rank test results (sums of signed ranks, W, and *p*-values) for mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed (Ho) and expected heterozygosity (He) **between non-declining and declining trees in the studied hotspots of dieback (CO, PE, PM) for the adaptive subset of SNPs.**

		CO	PE	PM
Na	W	-63	9	-54
	p-value	0.0225	0.7266	0.0332
Neff	W	-45	1,269	-157
	p-value	0.9355	0.0288	0.7888
Ho	W	-230	1,069	586
	p-value	0.6715	0.0620	0.2786
He	W	-165	1,162	-235
	p-value	0.7648	0.0455	0.6836

Table S14: Description of the genetic diversity present in the tree vigor subgroups (ND: non-declining trees; D: declining trees) in each population, including mean number of samples (N), mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), expected heterozygosity (He), allelic richness (AR), percentage of polymorphic *loci* (PPL), and inbreeding coefficient (F_{IS}; * indicates *p*-value < 0.001) and their respective standard errors. Only CO, PE and PM are shown as GA and SO stands are composed of non-declining trees exclusively.

	CO		PE		PM	
	ND	D	ND	D	ND	D
N	12.336 ± 0.042	8.731 ± 0.050	17.413 ± 0.048	14.404 ± 0.052	15.525 ± 0.056	14.838 ± 0.083
Na	1.964 ± 0.003	1.910 ± 0.005	1.955 ± 0.003	1.946 ± 0.004	1.968 ± 0.003	1.937 ± 0.004
Neff	1.585 ± 0.005	1.562 ± 0.005	1.547 ± 0.005	1.550 ± 0.005	1.569 ± 0.005	1.557 ± 0.005
Ho	0.328 ± 0.003	0.320 ± 0.003	0.301 ± 0.003	0.308 ± 0.003	0.311 ± 0.003	0.310 ± 0.003
He	0.341 ± 0.002	0.328 ± 0.002	0.322 ± 0.002	0.323 ± 0.002	0.334 ± 0.002	0.327 ± 0.002
AR	0.964 ± 0.185	0.911 ± 0.284	0.956 ± 0.206	0.946 ± 0.227	0.968 ± 0.176	0.938 ± 0.239
PPL	96.44	91.06	95.53	94.57	96.79	93.78
F_{IS}	0.084*	0.096*	0.098*	0.086*	0.106*	0.101*

Table S15: Wilcoxon matched-pairs signed rank test results (sums of signed ranks, W, and *p*-values) for mean number of alleles per *locus* (Na), mean number of effective alleles per *locus* (Neff), observed heterozygosity (Ho), and expected heterozygosity (He) **between non-declining and declining trees in the studied hotspots of dieback (CO, PE, PM).**

		CO	PE	PM
Na	W	-39,639	-4773	-17,060
	p-value	<0.0001	0.0220	<0.0001
Neff	W	-578,999	84,119	-196,198
	p-value	<0.0001	0.5409	0.1469
Ho	W	-348,230	354,554	-34,267
	p-value	0.0088	0.0085	0.7959
He	W	-679,317	24,894	-278,937
	p-value	<0.0001	0.8560	0.0381

APPENDICES

Appendix A: Isolation by distance (IBD) results as outputted by Genepop v1.2.2.

5 populations (10 pairs)

genetic estimates (F/(1-F)):

0.019091

0.025962 0.014011

0.034649 0.026582 0.026973

0.026671 0.032738 0.03363 0.038863

Ln(distance):

-1.80781

-1.64842 -1.94367

-0.229275 -0.375706 -0.50585

-1.92688 -2.31781 -1.48406 -0.242741

Geographical distance range of pairs allowed for regression: (0.0001 -- 1e+09);

Fitting F/(1-F) to a + b ln(distance) → a = 0.0332902, b = 0.00430434

ABC bootstrap results for SLOPE:

Point estimate and 95% confidence interval: 0.00430434 [0.0031948 , 0.00545761]

Unidirectional P-value for [slope=0] : 6.71314e-16

Mantel test, 1000 permutations

Test of isolation by distance (One tailed Pvalue):

Pr(correlation > observed correlation) =0.258 under null hypothesis

ABC bootstrap results for INTERCEPT:

Point estimate and 95% confidence interval: 0.0332902 [0.0314739 , 0.0352388]

Appendix B: Isolation by environment (IBE) results as outputted by Genepop v1.2.2.

5 populations (10 pairs)

genetic estimates (F/(1-F)):

0.019091

0.025962 0.014011

0.034649 0.026582 0.026973

0.026671 0.032738 0.03363 0.038863

Ln(distance):

0.819635

0.212982 0.111328

1.51964 0.843456 1.20994

1.25532 0.217259 0.854194 0.174573

Geographical distance range of pairs allowed for regression: (0.0001 -- 1e+09);

Fitting F/(1-F) to a + b ln(distance) → a = 0.0263938, b = 0.00211075

ABC bootstrap results for SLOPE:

Point estimate and 95% confidence interval: 0.00211075 [0.000837088 , 0.00341319]

Unidirectional P-value for [slope=0] : 0.000545752

Mantel test, 1000 permutations

Test of isolation by distance (One tailed Pvalue):

Pr(correlation > observed correlation) =0.319 under null hypothesis

ABC bootstrap results for INTERCEPT:

Point estimate and 95% confidence interval: 0.0263938 [0.024821 , 0.0280597]

ARTICLE 1

ARTICLE 2

Climate and soil microsite conditions determine local adaptation in declining silver fir forests

Las condiciones climáticas y del suelo determinan la adaptación local en los bosques en decaimiento de abeto común

El cambio climático está amenazando la supervivencia de especies de árboles sensibles a la sequía, como el abeto común (*Abies alba*). Aunque el decaimiento por sequía ya se había estudiado en esta conífera, no se había explorado el papel de la diversidad genética a nivel de árbol ni su relación con los patrones de crecimiento y las condiciones del suelo. Se utilizó la técnica de secuenciación de ADN con doble digestión asociada a sitios de restricción (ddRADseq) para caracterizar genéticamente cinco bosques de abeto común en el Pirineo español, incluyendo árboles decaídos y no decaídos. Se utilizaron polimorfismos de un solo nucleótido (SNPs) para investigar las relaciones entre genética, decaimiento, variación intraespecífica de rasgos (funcionales dendrofenotípicos y foliares), condiciones bioclimáticas locales y propiedades de la rizosfera. Pese a que no se observaron diferencias genéticas entre los árboles decaídos y no decaídos, se encontraron abundantes asociaciones entre el genoma y el ambiente con huellas de selección, lo que sugiere una fuerte influencia del clima y las propiedades fisicoquímicas y la diversidad microbiana del suelo en la adaptación local. Estos resultados ofrecen nuevas perspectivas sobre cómo se interrelacionan la genética y diferentes factores ambientales, y remarcan la necesidad de incorporar datos genéticos en los estudios sobre el decaimiento de los bosques de abeto común para obtener una mejor comprensión de su adaptación local.

ARTICLE 2

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Climate and Soil Microsite Conditions Determine Local Adaptation in Declining Silver Fir Forests

Isabel García-García [1], Belén Méndez-Cea [1], Ester González de Andrés [2], Antonio Gazol [2], Raúl Sánchez-Salguero [3], David Manso-Martínez [1], Jose Luis Horreo [1], J. Julio Camarero [2], Juan Carlos Linares [3] and Francisco Javier Gallego [1,*]

[1] Departamento de Genética, Fisiología y Microbiología, Unidad de Genética, Facultad de CC Biológicas, Universidad Complutense de Madrid, 28040 Madrid, Spain
[2] Instituto Pirenaico de Ecología (IPE-CSIC), 50059 Zaragoza, Spain
[3] Departamento de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, 41013 Sevilla, Spain
* Correspondence: fjgalleg@ucm.es

Abstract: Ongoing climatic change is threatening the survival of drought-sensitive tree species, such as silver fir (*Abies alba*). Drought-induced dieback had been previously explored in this conifer, although the role played by tree-level genetic diversity and its relationship with growth patterns and soil microsite conditions remained elusive. We used double digest restriction-site-associated DNA sequencing (ddRADseq) to describe different genetic characteristics of five silver fir forests in the Spanish Pyrenees, including declining and non-declining trees. Single nucleotide polymorphisms (SNPs) were used to investigate the relationships between genetics, dieback, intraspecific trait variation (functional dendrophenotypic traits and leaf traits), local bioclimatic conditions, and rhizosphere soil properties. While there were no noticeable genetic differences between declining and non-declining trees, genome–environment associations with selection signatures were abundant, suggesting a strong influence of climate, soil physicochemical properties, and soil microbial diversity on local adaptation. These results provide novel insights into how genetics and diverse environmental factors are interrelated and highlight the need to incorporate genetic data into silver fir forest dieback studies to gain a better understanding of local adaptation.

Keywords: *Abies alba*; climate warming; forest die-off; ddRADseq; SNPs; dendroecology; soil nutrients; soil microbiome; PLFAs

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1. Introduction

Climate-change-induced disturbances are increasing in frequency and severity, threatening the persistence of numerous tree species, as well as the diversity and dynamics of forest ecosystems worldwide [1]. Together with variations in the average value of climatic variables, forests will also have to deal with the risks posed by the increase in adverse weather events, broadly represented by extreme droughts and heat waves [2,3].

Tree populations can follow two main strategies to cope with major changes in climatic conditions: migration and adaptation [4]. Migration rates will likely be surpassed by the current pace of climate change [5] and could be hindered by natural barriers and human-induced habitat transformations [6]. On the other hand, the genetic diversity of populations could be key to mitigating the effects of climate change [7] and achieving adaptation through the selection of potentially beneficial alleles present in the populations [8]. Therefore, understanding the relationship between genomes and adaptive phenotypic characteristics and the extent to which they are influenced by the environment may be essential for predicting the fate of tree species coping with climate change [9,10].

Regarding genomic information, single nucleotide polymorphism (SNP) genotyping allows one to make extensive use of genetic markers, as they are found at a high frequency across genomes [11]. Genotyping by sequencing (GBS) methods for SNP discovery are

particularly useful to study the genetic structures of natural populations [12,13], identify genome–environment associations [14,15], and detect selection signatures [16,17]. In addition, they are particularly appropriate for conifer genomes, allowing for a reduction in their intrinsic complexity derived from their large sizes and high percentage of repetitions [18] by reducing the sequenced portion. More specifically, double digest restriction-site-associated DNA sequencing (ddRADseq) including methylation-sensitive enzymes in the digestion step seems to be especially beneficial [14], as demonstrated by the increasing number of studies following this approach, e.g., [15,19,20]. Repetitive DNA sequences and transposable elements are highly methylated in plants [21], so the use of at least one methylation-sensible enzyme is conducive to a reduction in their representation and to an increase in the coverage of coding regions [22].

The combination of genetic data with environmental factors and dendrochronology can help provide a comprehensive vision of how genomes are shaped by their interactions with the environment, and how much of an impact they make on functional traits and growth patterns [23].

Genome–environment associations look for statistical associations between allele frequencies and ecological variations with the aim of identifying genes involved in local adaptation [24]. With climate change notably altering the abiotic environmental factors that are known to affect plants [25], the need to understand the molecular basis of such evolutionary processes is particularly important in order to be able to design conservation plans if necessary to protect plant species.

While genome–environment associations can help identify putatively adaptive *loci* [26], genotype–phenotype associations give cause for other approaches in the path to understanding adaptation. Particularly, dendrophenotypes offer an exceptional opportunity to determine how each tree responded to documented stress periods in the past by analyzing past growth rates, and have become a potent resource for genetic association studies [27]. Leaf functional traits can also add relevant information regarding responses to drought events. For instance, high leaf mass area seems to lead to better tolerance to dehydration [28], just like leaf dry matter content is positively correlated with endurance to dry conditions [29,30].

In this context, this study focuses on silver fir (*Abies alba* Mill.), which is among the tree species affected by extensive dieback and increased mortality rates [31,32]. Forest decline in populations of silver fir has been reported since the 1980s in the Pyrenees (Europe), coinciding with a succession of dry years [33]. Although it was generally agreed that this conifer is quite resistant to drought [34,35], water stress has been linked to growth decline [36], crown defoliation [37], nutritional impairments in carbon–water balance [38], and uncoupled patterns of seed dispersal and regeneration [39] in Pyrenean silver fir forests in recent decades. Predictions point to more frequent and intense droughts in the Mediterranean area [40], concurrent to global warming [41], which will further challenge the species' ability to cope with the new environmental conditions.

Consequently, the main objective of this study was to identify molecular markers (SNPs) in the *A. alba* genome that could be used to characterize silver fir populations in the Pyrenees and contain enlightening information regarding their potential adaptive capacity to climate change. Hence, we combined genotype information with environmental data, soil physicochemical properties and soil microbial diversity, and functional and dendrophenotypic traits with the following aims: (i) to describe and compare the genetic characteristics of declining and non-declining stands, as well as declining and non-declining trees, (ii) to detect genomic regions under selection in such trees, and (iii) to identify genetic markers (SNPs) associated with environmental and phenotypic variables potentially involved in responses to climate stress. This information could help gain a better insight into this conifer's declining process.

2. Results

2.1. SNP Genotyping

After trimming, an average of 96.87% of the reads were retained (93.54% minimum, 98.37% maximum). An average of 68.74% of those reads were successfully mapped to the reference genome (37.42% minimum, 85.19% maximum). A total of 55,803 SNPs were identified in the 266 sequenced samples, of which 3958 SNPs and 254 individuals were retained after the filtering step (Table S1).

2.2. Genetic Characteristics of Populations

The SNP dataset showed a percentage of polymorphic *loci* of 99.44, 88.53, 99.62, 99.72, and 98.79 in the five studied stands (CO, GA, PE, PM, and SO, respectively; see Section 4.1 for details). Significant genetic differences, measured by fixation index (F_{ST}), were detected between all population pairs (Table 1). The analysis of molecular variance (AMOVA) also showed significant genetic differences among populations (Table S2). When accounting for F_{ST} differences between declining and non-declining individuals, no statistical differences were found within any population (Table 2).

Table 1. Fixation indexes (F_{ST}) above diagonal between the studied *A. alba* populations in the Pyrenees and their associated *p*-values below diagonal.

Populations	CO	GA	PE	PM	SO
CO	-	0.038	0.034	0.027	0.027
GA	0.000	-	0.028	0.035	0.034
PE	0.000	0.000	-	0.027	0.019
PM	0.000	0.000	0.000	-	0.015
SO	0.000	0.000	0.000	0.000	-
Number of samples	47	18	74	77	38

Table 2. Fixation indexes (F_{ST}) and their associated *p*-values between non-declining (ND) and declining (D) *A. alba* individuals within each declining population (declining individuals were present only in CO, PE, and PM populations; see Section 4.1 for details).

	CO	PE	PM
F_{ST}	0.004	0.004	0.002
p-value	0.142	0.055	0.192
Number of ND samples	15	20	19
Number of D samples	13	18	20

The principal component analysis (PCA) showed, in agreement with the F_{ST} and AMOVA results, that each population can be genetically distinguished, with their respective individuals grouping together (Figure 1). CO was the most isolated one, in consonance with a larger geographic distance between itself and the other study sites (see Section 4.1). The two healthy populations (GA and SO) appeared close to each other, in the center of the plot. GA overlapped with PE, while SO partially overlapped with PM. This pairing was in line with the existence of geographical barriers between the two populations more to the west and the two more to the east (GA and SO are separated by a mountain, and PE and PM are on different sides of a valley).

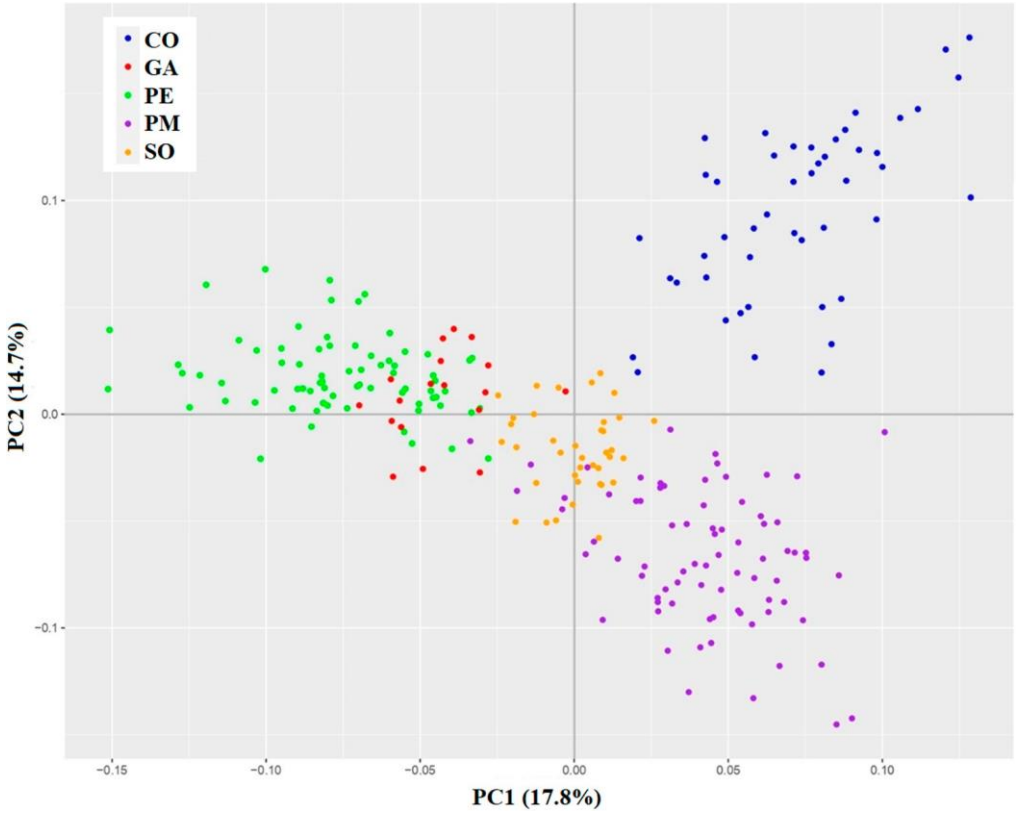

Figure 1. Principal component analysis (PCA) of the 3958 *A. alba* SNPs showing the first and second principal components. Each color represents a different population (CO, GA, PE, PM, SO).

2.3. Selection Signatures

Selection signatures were found in 41 SNPs taking into account the five genetically different populations (Figure S1). The rest of the comparisons (considering health state and age) did not uncover any genomic region under selection. Eleven of those SNPs were located within an annotated gene or close to one (Table 3). Among the proteins produced by these genes, there was an abscisic acid (ABA) receptor, which is part of a family involved in responses to adaptive stresses in plants, coordinating complex networks that allow responses to drought, salinity, and temperature fluctuations [42]. A heavy metal-associated protein (HIPP) also showed signs of selection. While they are mainly known for their detoxification purposes, it has been seen that they also play a role in response to ABA, and, therefore, in response to different abiotic stress conditions [43,44]. In addition, a chaperone could also be found among the obtained proteins, which is also linked to the ABA signaling pathway. These proteins can sense the epigenetic environment created by some chromatin-remodeling factors that interact with ABA, playing a role in inducing stress memory by ensuring the dynamic epigenetic mechanisms that control gene expression [45]. Their allele frequencies in each population can be seen in Figure 2.

2.4. Genome–Environment Associations (GEA)

Genome–environment associations were found between 47 different SNPs and the five bioclimatic variables, giving a total of 71 associations (Table S3). Of these, 4 also showed selection signatures, and 15 were located within an annotated gene or close to one (Table 4). BIO15 (precipitation seasonality) showed the highest number of associations, while BIO3 (isothermality) only had one. Among the proteins associated with precipitation variables, there was an aquaporin, which is part of a family of water channels crucial for maintaining water balance in plants [46]. There was an SNP in one of the introns which are part of the gene that encodes this protein, with the frequency of its allele 2 (T) increasing as BIO15 increased (Figure 3a). Temperature variables also showed associations with proteins related

to response to abiotic stress, such as RAD7, which is involved in DNA repair after UV light damage [47]. In this case, the frequency of allele 1 (T) of its associated SNP increased as BIO2 (mean diurnal range) increased (Figure 3b).

Table 3. Molecular markers (SNPs) under selection found within or close to an annotated gene ("Position") in *A. alba*, specifying their exact location in the genome, as well as the protein encoded by such genes.

SNP	Location	Position	Protein	Protein ID
333	aalba5_s00002910:109,731	Within the gene	Abscisic acid receptor (PYL3)	AALBA5B1118705T1
799	aalba5_s00008638:65,727	Within the gene	La-related protein 1B (LARP1B)	AALBA5B999098T2
1034	aalba5_s00012107:25,977	Within the gene	Heavy metal-associated protein (HIPP39)	AALBA5B430765T1
1241	aalba5_s00014817:64,736	Within the gene	Fucosidase (FUCO1)	AALBA5B698786T1
2059	aalba5_s00027378:40,879	Within the gene	Mediator of RNA polymerase II (MD26C)	AALBA5B1091291T1
2477	aalba5_s00034336:46,604	20,152 bp downstream	Kinase (CRK42)	AALBA5B567750T1
2591	aalba5_s00036159:10,277	5615 bp upstream	Transcription factor (PHL3)	AALBA5B939227T1
2665	aalba5_s00037486:53,893	939 bp downstream	TATA-box-binding-interacting homolog (RIN1)	AALBA5B827346T1
2697	aalba5_s00038192:32,220	25,073 bp downstream	Chaperone (BAG5)	AALBA5B1063708T1
3215	aalba5_s00047689:19,730	2651 bp upstream	Kinase (BSK2)	AALBA5B446846T1
3952	aalba5_s00062077:3573	35,864 bp upstream	Squalene epoxidase (ERG1)	AALBA5B441483T1

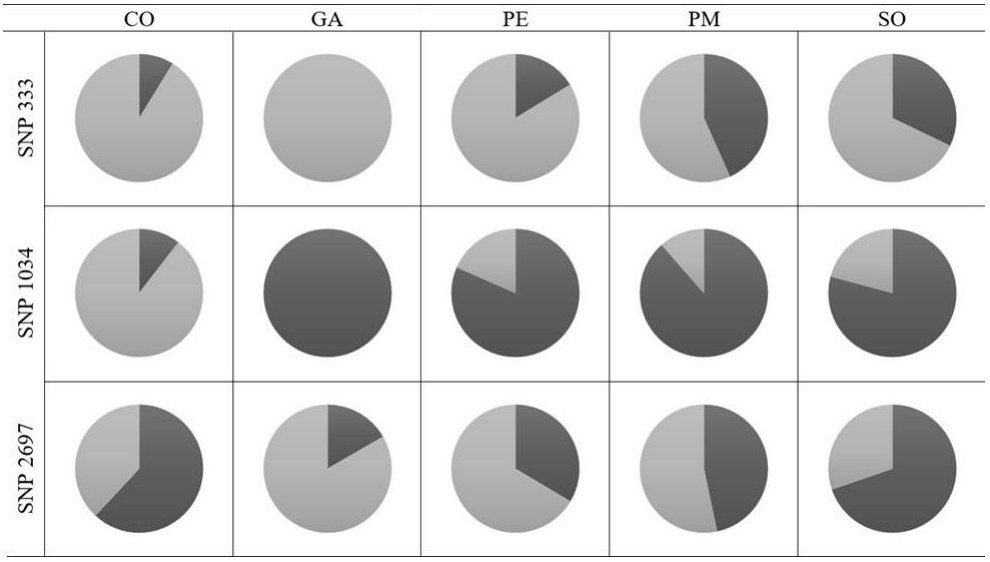

Figure 2. Allele frequencies of SNPs showing selection signs (SNP 333, SNP 1034, SNP 2697) in the studied *A. alba* populations (CO, GA, PE, PM, SO). Dark gray represents the frequency of allele 1; light gray represents the frequency of allele 2.

Genome–environment associations were found between 35 different SNPs and the eight abiotic soil variables, giving a total of 80 associations (Table S4). Of these, 5 also showed selection signatures, and 13 were located within an annotated gene or close to one (Table 5). The Saxton value showed the highest number of associations, while the percentage of silt only had two.

Table 4. Molecular markers (SNPs) associated with bioclimatic variables found within or close to an annotated gene ("Position") in *A. alba*, specifying their exact location in the genome, as well as the protein encoded by such genes. SNPs with selection signatures are marked in bold.

SNP	Location	Position	Protein	ID
159	aalba5_s00001283:86,646	21,883 bp upstream	UDP-glycosyltransferase (U86A2)	AALBA5B1024847T1
179	aalba5_s00001426:131,290	Within the gene	BEACH domain-containing protein (BCHC2)	AALBA5B098257T1
681	aalba5_s00007005:35,095	Within the gene	Aquaporin (SIP2-1)	AALBA5B253951T1
983	aalba5_s00011431:62,999	21,010 bp downstream	Tetratricopeptide repeat-like superfamily protein (PPR35)	AALBA5B348236T1
1808	aalba5_s00023261:31,104	Within the gene	Kinase (NEK4)	AALBA5B973686T1
2061	aalba5_s00027387:15,057	30,900 bp upstream	MOTHER of FT and TFL1 (MFT)	AALBA5B1139649T1
2119	aalba5_s00028425:12,399	328 bp upstream	Chaperone, chloroplastic (DJA7A)	AALBA5B512522T1
2459	aalba5_s00034077:43,568	772 bp upstream	Mitogen-activated kinase (M3K17)	AALBA5B244254T1
2591	aalba5_s00036159:10,277	5615 bp upstream	Transcription factor (PHL3)	AALBA5B939227T1
2645	aalba5_s00037229:12,665	111 bp upstream	Homeobox (PKNOX1)	AALBA5B627517T1
2794	aalba5_s00040228:24,711	20,262 bp upstream	DNA repair protein (RAD7)	AALBA5B261613T1
2940	aalba5_s00042872:51,622	18,194 bp downstream	Helicase (MAA3)	AALBA5B767119T1
3063	aalba5_s00045131:27,419	17,345 bp downstream	Phosphatase (P2C60)	AALBA5B289418T1
3313	aalba5_s00049423:3040	909 bp upstream	Translation initiation factor (SDA1)	AALBA5B666590T1
3400	aalba5_s00051396:12,444	Within the gene	Uncharacterized transmembrane protein (UGPI7)	AALBA5B360123T1

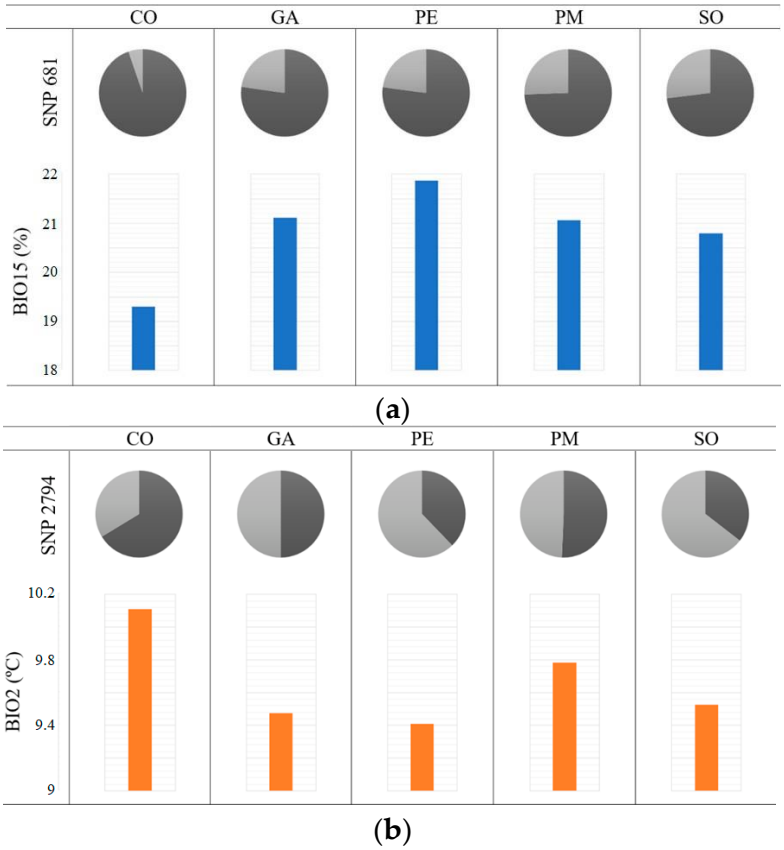

Figure 3. Allele frequencies of SNP 681, associated with a precipitation variable (BIO15, precipitation seasonality, represented in blue) (**a**), and SNP 2794, associated with a temperature variable (BIO2, mean diurnal range, represented in orange) (**b**). Dark gray represents the frequency of allele 1 in each population; light gray represents the frequency of allele 2 in each population.

Table 5. Molecular markers (SNPs) associated with abiotic soil variables found within or close to an annotated gene ("Position") in *A. alba*, specifying their exact location in the genome, as well as the protein encoded by such genes. SNPs with selection signatures are marked in bold.

SNP	Location	Position	Protein	ID
238	aalba5_s00002010:71,026	22 bp downstream	Endo-1,3(4)-beta-glucanase (ENG1)	AALBA5B495784T1
287	aalba5_s00002479:90,851	1649 bp downstream	Chaperone (DNJ10)	AALBA5B1036913T1
764	aalba5_s00008117:59,024	25,055 bp upstream	Coatomer subunit delta-2 (COPD2)	AALBA5B407046T1
1808	aalba5_s00023261:31,104	Within the gene	Kinase (NEK4)	AALBA5B973686T1
2587	aalba5_s00036112:6656	407 bp upstream	Translocase of chloroplast (TC120)	AALBA5B952204T1
2665	aalba5_s00037486:53,893	939 bp downstream	TATA-box-binding-interacting homolog (RIN1)	AALBA5B827346T1
2794	aalba5_s00040228:24,711	20,262 bp upstream	DNA repair protein (RAD7)	AALBA5B261613T1
3000	aalba5_s00044048:11,938	Within the gene	3-oxoacyl synthase, mitochondrial (KASM)	AALBA5B636711T1
3120	aalba5_s00046057:16,198	309 bp downstream	Sorbitol dehydrogenase (DHSO)	AALBA5B083042T1
3313	aalba5_s00049423:3040	909 bp upstream	Translation initiation factor (SDA1)	AALBA5B666590T1
3870	aalba5_s00060613:17,756	21,499 bp upstream	Ribonuclease HI (RNH)	AALBA5B308154T1
3874	aalba5_s00060695:20,797	21,154 bp upstream	UDP-glucose 4-epimerase (GALE2)	AALBA5B999424T1
3952	aalba5_s00062077:3573	35,864 bp upstream	Squalene epoxidase (ERG1)	AALBA5B441483T1

Regarding the eight biotic soil variables, 33 different SNPs were significantly associated with them, giving a total of 81 associations (Table S5). Of these, 4 also showed selection signatures, and 13 were located within an annotated gene or close to one (Table 6). The concentration of Gram-positive bacteria showed the highest number of associations, while fungi only had one.

Table 6. Molecular markers (SNPs) associated with biotic soil variables found within or close to an annotated gene ("Position") in *A. alba*, specifying their exact location in the genome, as well as the protein encoded by such genes. SNPs with selection signatures are marked in bold.

SNP	Location	Position	Protein	ID
159	aalba5_s00001283:86,646	21,883 bp upstream	UDP-glycosyltranferase (U86A2)	AALBA5B1024847T1
238	aalba5_s00002010:71,026	22 bp downstream	Endo-1,3(4)-beta-glucanase (ENG1)	AALBA5B495784T1
681	aalba5_s00007005:35,095	Within the gene	Aquaporin (SIP2-1)	AALBA5B253951T1
764	aalba5_s00008117:59,024	25,055 bp upstream	Coatomer subunit delta-2 (COPD2)	AALBA5B407046T1
983	aalba5_s00011431:62,999	21,010 bp downstream	Tetratricopeptide repeat-like superfamily protein (PPR35)	AALBA5B348236T1
1808	aalba5_s00023261:31,104	Within the gene	Kinase (NEK4)	AALBA5B973686T1
2587	aalba5_s00036112:6656	407 bp upstream	Translocase of chloroplast (TC120)	AALBA5B952204T1
2591	aalba5_s00036159:10,277	5615 bp upstream	Transcription factor (PHL3)	AALBA5B939227T1
2665	aalba5_s00037486:53,893	939 bp downstream	TATA-box-binding-interacting homolog (RIN1)	AALBA5B827346T1
3120	aalba5_s00046057:16,198	309 bp downstream	Sorbitol dehydrogenase (DHSO)	AALBA5B083042T1
3313	aalba5_s00049423:3040	909 bp upstream	Translation initiation factor (SDA1)	AALBA5B666590T1
3870	aalba5_s00060613:17,756	21,499 bp upstream	Ribonuclease HI (RNH)	AALBA5B308154T1
3874	aalba5_s00060695:20,797	21,154 bp upstream	UDP-glucose 4-epimerase (GALE2)	AALBA5B999424T1

A high proportion of SNPs were found to be associated with several types of environmental variables and/or to be under selective pressure (Figure 4), showing the influence of the environment on this species genome and the existence of a correlation between soil and climatic variables.

2.5. Genotype–Phenotype Associations (GPA)

Genotype–phenotype associations were found between 19 SNPs and 2 functional traits (out of 5) and 7 dendrophenotypic traits (out of 46) related to response to past drought events, with resilience to the 1998 drought (Rs98) as the variable with the highest number of

associations (Table S6). Of these, eight were located within an annotated gene or close to one, and none of them showed either signatures of selection or associations with environmental variables (Table 7).

Figure 4. Venn diagram showing the number of SNPs associated with different environmental variables (bioclimatic variables, soils—abiotic, soils—biotic) or showing selection signs, and the coincidences among them.

Table 7. Molecular markers (SNPs) associated with phenotypic traits found within or close to an annotated gene ("Position") in *A. alba*, specifying their exact location in the genome, as well as the protein encoded by such genes.

SNP	Location	Position	Protein	ID
1	aalba5_s00000025:18,4813	Within the gene	Probable kinase (Y1960)	AALBA5B157798T1
259	aalba5_s00002236:37,636	6142 bp upstream	Farnesyl pyrophosphate synthase (FPPS)	AALBA5B673769T1
887	aalba5_s00009946:20,716	22 bp upstream	Zinc finger 10 (ZFP10)	AALBA5B909389T1
1769	aalba5_s00022666:63,643	55,996 bp downstream	Expansin (EXPA8)	AALBA5B425104T1
2319	aalba5_s00031591:9355	Within the gene	RecQ-mediated genome instability protein (RMI-1)	AALBA5B1147052T1
3101	aalba5_s00045724:48,524	4919 bp downstream	Ribonuclease H (RNHX1)	AALBA5B088054T1
3238	aalba5_s00048104:19,924	284 bp upstream	SUN domain-containing protein (SUN1)	AALBA5B001978T1
3878	aalba5_s00060735:27,446	16,784 bp downstream	Aluminum-activated malate transporter (ALMTE)	AALBA5B787661T1

Regarding the 2005–2006 drought, there were two proteins with functions involved in drought response associated with resistance and resilience to this extreme climatic period. One of them was a zinc finger transcription factor which has been linked to drought and heat response multiple times, e.g., [48–50]. Homozygous individuals for the T allele of its associated SNP had a higher resistance to the 2005–2006 drought (Figure 5a). The other one was an expansin, which contributes to drought-resistance by maintaining cell turgor and taking part in ABA-mediated cell-wall extension [51]. Homozygous individuals for the C allele of its associated SNP showed a higher resilience to the 2005–2006 drought (Figure 5b).

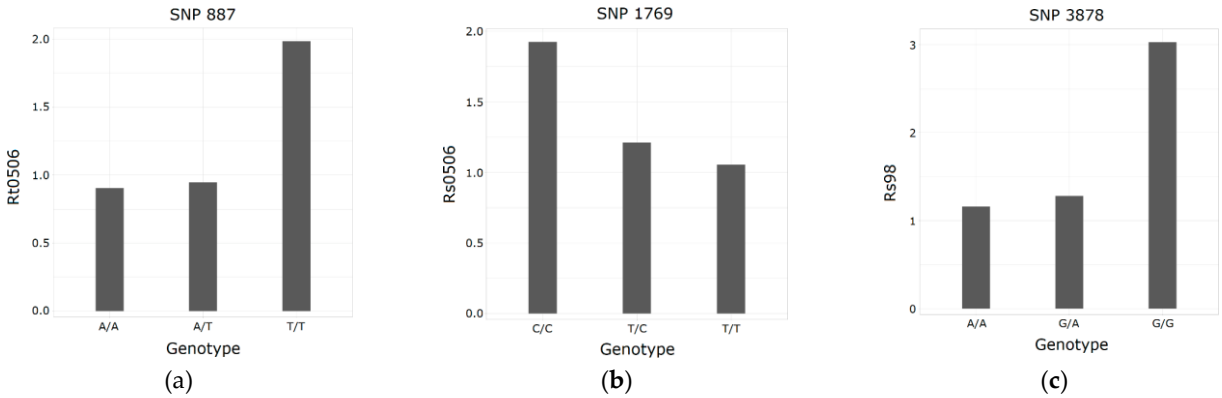

Figure 5. Mean values of resistance to the 2005–2006 drought (Rt0506), resilience to the 2005–2006 drought (Rs0506), and resilience to the 1998 drought (Rs98) with respect to genotypes (allele 1/allele 2)of SNP 887 (**a**), SNP 1769 (**b**), and SNP 3878 (**c**), respectively.

Also associated with resilience, but to the 1998 drought period, there was an aluminum-activated malate transporter, which influences the cellular gamma-aminobutyric acid (GABA) concentration [52]. This amino acid is rapidly accumulated in response to multiple stresses, including drought [53]. Homozygous individuals for the G allele of its associated SNP had a higher resilience to the 1998 drought (Figure 5c).

3. Discussion

This study aimed to gain a better understanding of how genetics could be involved in tree decline and mortality, determining local variability in healthy trees and neighboring individuals showing a declining process towards death. Furthermore, we provide information about the genetic characteristics of contrasting Pyrenean populations of silver fir and their relationship with the environment and phenotypic traits, finding climate and soil microsite conditions as the main factors affecting their local adaptation.

Previously, microsatellites were the most widely employed method to study genetic diversity, structure, and adaptation in silver fir populations, e.g., [54–56]. More recently, SNPs have gained popularity as molecular markers in this species, predominantly in the form of genotyping a predefined list of them in candidate genes, e.g., [27,57,58]. In spite of its robustness, it is a limited approach, not accounting for genetic variability in non-coding regions. Additionally, its potential for adaptation in forest trees could rely on quantitative trait *loci* (QTL), which tend to be scattered across the genome [4], rather than just specific genes of major effect. QTL mapping requires a large number of genetic markers to be successful [59], stressing the need for genome-wide identification of SNPs. RADseq had already proved to be a useful technique for genome-wide genotyping in *A. alba* [60], and our results show that ddRADseq can be used for that purpose too.

However, genome-wide identification of SNPs in conifers, although possible, remains a complex process hindered by the lack of reference genomes and their quality. For instance, 70% of the reads used in this study mapped to the silver fir reference genome, possibly due to the highly fragmented nature of this assembly. This means 30% of the generated information was lost. It is clear that efforts must be made to improve the existing references and sequence new species.

To our knowledge, this is the first study comparing the genetics of declining or non-declining silver fir individuals. Our results showed that there were no genetic differences between them within their respective populations. *A. alba* is known to have high phenotypic plasticity, mainly attributed to the effect of environmental factors rather than genetic variability [61]. Thus, epigenomics could be playing a more important role in the fate of each tree in populations subjected to increasingly unfavorable environmental conditions than their differences in genetic molecular markers, especially since climate change is surpassing their adaptive changes in allele frequency rates [5,18]. The lack of selection

signatures between adult trees and saplings could also be indicative of a slow adaptation process happening in the studied populations which is not detectable yet and, thus, clearly slower than the ongoing climatic changes. This means this species could probably be threatened in the Pyrenees if conditions continue as they are currently.

Regarding the comparison between populations and considering that they are placed in regions with different environmental conditions, it is not surprising that several SNPs showed signs of selection. Some of these could be used as molecular markers of nearby genes that are involved in stress responses, mainly related to ABA signaling pathways. Whole-genome scans in other coniferous species have demonstrated that genes under selection are often involved in responses to abiotic stress and are rapidly evolving to enable trees to cope with such demanding conditions [62,63]. Therefore, this finding suggests that the environment is impacting the silver fir's genome by causing different levels of abiotic stress to the populations. The fact that approximately 22% of these SNPs were associated with environmental variables supports this affirmation.

Temperature and precipitation had a similar number of associated SNPs whose allele frequencies changed between populations in line with the variable's variation. Precipitation seasonality (BIO15) had the highest number of associations, including an aquaporin, which has been directly linked to drought stress in *A. alba* [64]. Additionally, this variable seems to drive the growth timing of populations of silver fir [65], revealing itself as a key environmental factor for this species [66]. On the other hand, the most relevant temperature variable was mean diurnal range (BIO2), which has been identified as a potential modifier of the effect of drought on tree growth, alleviating the degree of stress caused by drought events when its range is narrow [67].

Soil variables also had a high number of associations, with a large percentage of SNPs associated with both biotic and abiotic parameters. Vegetation, microbial community, and physicochemical properties of soil are closely linked to each other, interacting in a process known as plant–soil feedback [68]. It has been described that plant genotype can significantly influence microbial community composition [69], which, in turn, has an impact on soil properties, such as pH, organic matter content, and soil structure [70,71], and vice versa, with the microbial community being the one affected when there are changes in soil conditions and vegetation [72,73]. Therefore, it is no wonder that almost 50% of the associated markers appear both for soil composition and soil microorganisms and are strongly related to the sampled trees' genotype.

In addition, it has been described that forest management affects soil microbial composition, increasing soil microbial stress [74] and affecting its resistance and resilience to drought [75,76]. For instance, CO, which has been protected by the "Ordesa and Monte Perdido" National Park since 1918 with strict conservation policies [38], presents higher biomasses of the different tested groups of microorganisms (except for fungi) than the rest of the studied stands (Table S9), which suffered from intense logging activity in the past [33]. Therefore, although climate change and drought are usually the most discussed reasons behind tree dieback, human perturbations, such as logging and selective thinning, should be considered too.

Finally, the genotype–phenotype associations were fewer and almost unique for each variable. While genome–environment associations identify general trends due to their population-level nature, phenotype data can produce much more finely tuned results, as they consider tree-level information. Thus, a strong genetic signal related to growth traits is less likely to be found [27,77,78].

In accordance with this, only 7 out of the 46 dendrophenotypic variables related to growth patterns were associated with genetic markers. More specifically, these traits define how each tree responded to past drought periods. Survival to one-time extreme events, such as extended drought periods, might not be driven as much by adaptation as by each tree's genetic variability, which could be key to increasing its chances of carrying a variant that could help it cope with the adverse climatic situation [8]. Thus, the small number of associations found for the variables related to response to past drought events could

be pointing to genome regions that were helpful in maintaining certain growth rates and vitality while dealing with a specific unfavorable event but were not particularly useful in the long run. Additionally, none of these regions showed signs of selection, proving that they are not directly involved in long-term adaptation to the environment.

Two traits related to leaf characteristics also showed a low number of associated SNPs, in line with a weak differentiation in leaf morphology between individuals [38]. Lastly, the lack of associations to growth pattern variables based on tree-level BAI might be due to the influence of the environment on growth rates rather than the effect of the genotype itself, as the succession of severe drought events in combination with increasingly higher temperatures during the last 40 years has led to growth decline in different silver fir populations across the Pyrenees [33,79].

In summary, integrative studies incorporating genetic, phenotypic, and environmental data represent a powerful tool for the uncovering adaptation mechanisms of natural tree populations, as demonstrated by the growing number of publications following thisapproach, e.g., [14,27,58].

In this regard, improved reference genomes for conifer species are of utmost importance in the pursuit of understanding their genetic vulnerability to climate change. In addition to allowing more efficient genome-wide identification of molecular markers and adaptive candidate *loci*, they would pave the way for the accurate identification of epigenomic marks [18]. Epigenetic mechanisms reflect to a large extent the effect of environmental factors on gene expression [80], which would complement the existing information about how these variables affect the genome. Given that our results did not identify any genomic region under selection between declining and non-declining trees, epigenomic studies should also be put in the spotlight following their potential explanatory power regarding trees' response to climate change.

4. Materials and Methods

4.1. Study Species and Sites

Silver fir (*Abies alba* Mill.) is a large conifer distributed in montane areas in Europe (Figure 6). It can live up to half a millennium, reaching its reproductive maturity at 30–40 years old [81]. It is commonly found in elevated areas (500–2000 m a.s.l) with 700–1800 mm of mean annual precipitation [82]. It is highly shade-tolerant, finding its best growth conditions in locations with at least 1500 mm of annual precipitation and mean temperature around 9 °C [83]. It can grow in soils with a pH from acid to neutral, preferring moist soils but not too wet [81]. The silver fir forests of the Pyrenees constitute the European southwestern limit of the distribution of this species, where it usually grows in mesic sites on north-facing slopes, creating pure or mixed forests with European beech (*Fagus sylvatica* L.) or Scots pine (*Pinus sylvestris* L.), with the understory vegetation dominated by several shrubs such as European box (*Buxus sempervirens* L.) [38].

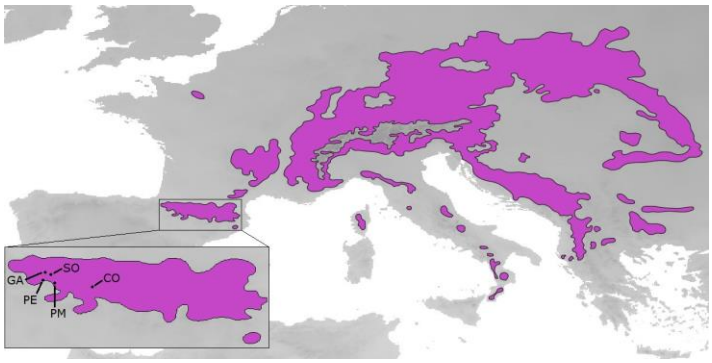

Figure 6. Silver fir, *Abies alba*, distribution in purple, as described by EUFORGEN, and study sites in the Pyrenees (GA, CO, PE, PM, SO), marked with black circles.

The study sites were the four *A. alba* stands included in a previous study [38] plus an extra one, all of them placed in north-western Aragón, in the central-western Spanish Pyrenees (Figure 6, Table 8). Three of these sites show signs of forest decline, with declining and non-declining trees coexisting (Cotatuero, CO; Paco Ezpela, PE; and Paco Mayor, PM), while the other two are populated only by healthy individuals (Selva de Oza, SO; and Gamueta, GA). Samples from different types of individuals were collected at all sites: adult trees (between 40 and 220 years old, with a mean age of 111) and saplings growing nearby (no taller than 1.5 m). Additionally, adult trees were sampled regarding two additional categories at sites where it was possible: declining individuals and non-declining (healthy) individuals. Their health state was determined by their crown defoliation levels [38], considering only trees that did not show signs of any disease. This sampling design allowed us to look for differences between age cohorts and health cohorts, as well as between stands. Tree age was determined by the preformed-growth pattern found in firs [15].

Table 8. Brief characterization of the study sites, including their geographical position and climate information. Altitude was measured in meters above sea level, mean temperature (T) was measured in ˚C, and annual precipitation (P) was measured in mm.

Populations	Cotatuero (CO)	Gamueta (GA)	Paco Ezpela (PE)	Paco Mayor (PM)	Selva de Oza (SO)
Latitude	42.65	42.88	42.74	42.71	42.84
Longitude	-0.04	-0.79	-0.83	-0.64	-0.70
Altitude	1456	1450	1116	1322	1220
Mean T	5.22	6.23	9.74	8.55	7.47
Annual P	1167	1120	867	944	1028

4.2. SNP Genotyping

Fresh leaf samples were collected from 266 *A. alba* individuals. In total, 100 mg of each sample was lyophilized and used for total genomic DNA extraction with DNeasy Plant Mini Kit (Qiagen®, Berlin, Germany). DNA yield and purity were determined using a NanoDrop™ spectrophotometer and visually inspected after electrophoresis on agarose gels. Paired-End (PE) libraries for ddRADseq [84] were constructed using *Ape*KI and *Pst*I restriction enzymes and sequenced by LGC Genomics (Berlin, Germany), with a sequencing depth of 1M. Quality of raw reads was assessed using FastQC v0.11.9 [85]. They were trimmed using fastp v0.12.4 [86] to remove low-quality bases/reads and adapter contamination. The processed reads were mapped to the *A. alba* reference genome (Abal.1_1) [87] using ipyrad v0.9.65 [88]. This reference genome had over 37 million scaffolds, with a mean scaffold length of 488.5 pb; therefore, in order to reduce the computational cost of this process while retaining its usefulness as much as possible, a shorter version of this genome was created and used as reference. It was made of the first 62,061 scaffolds, which were the longest and had annotated genetic features. The same software (ipyrad) was then used to perform an SNP calling. The obtained SNPs were filtered with VCFtools v0.1.16 [89] to retain high-quality, informative SNPs for subsequent analyses: biallelic, minor allele frequency (MAF) > 5%, maximum missingness < 50%, and only 1 SNP per 10,000 bp to remove strong linkage disequilibrium.

4.3. Genetic Characteristics of Populations

Different descriptive statistics were calculated. GenAlEx v6.5 [90,91] was used to calculate the percentage of polymorphic *loci* and allele frequencies of relevant SNPs in each population. Genodive v3.06 [92] was used to test population differentiation due to genetic structure (fixation indexes, F_{ST} [93]) and to perform an analysis of molecular variance (AMOVA [94,95]) with 9999 permutations. In addition, plink2 v2.00a2.3 [96] was used to carry out a principal component analysis (PCA).

4.4. Selection Signatures

Detection of selection signatures was performed using BayeScan 2.1 [97] with default parameters. SNPs with q-values < 0.05 were considered to be under selective pressure. Different configurations of groups were tested looking for significant differences in allele frequency measured by their F_{ST} coefficient: sampling points as groups, condition as groups (non-declining vs. declining), condition as groups within each population, age as groups (adult vs. young), and age as groups within each population.

4.5. Genome–Environment Associations (GEA)

Three types of environmental variables were considered for genome–environment associations (GEA): (1) bioclimatic variables, (2) abiotic soil variables, and (3) biotic soil variables (microbial communities). The associations were identified using BayEnv2 [98] with 100,000 iterations after estimation of the marker's covariance matrix as indicated by the manual. SNP–variable combinations scoring a Bayes Factor (BF) > 100 were considered to be associated [99].

For each sampling point's location, 19 bioclimatic variables were obtained from the WorldClim database [100], with a grid cell resolution of 30 s, using QGIS 3.18 (QGIS Development Team, 2022). Highly correlated variables (Pearson's coefficient > 0.9) were removed, keeping only 5 bioclimatic variables (Table 9), of which 3 were related to temperature (BIO1, BIO2, BIO3) and 2 were related to precipitation (BIO12, BIO15).

Table 9. Bioclimatic variables for each *A. alba* population's location. BIO1: annual mean temperature (°C). BIO2: mean diurnal range (°C). BIO3: isothermality (%). BIO12: annual precipitation (mm). BIO15: precipitation seasonality (%).

Variable	CO	GA	PE	PM	SO
BIO1	5.22	6.23	9.74	8.56	7.47
BIO2	10.1	9.48	9.41	9.78	9.53
BIO3	36.62	35.75	37.33	37.48	36.35
BIO12	1167	1120	867	944	1028
BIO15	19.30	21.11	21.87	21.06	20.79

The 8 abiotic soil variables were obtained at individual-level [38] and then their mean values were calculated for each population (Table 10). Briefly, soil samples were processed to determine percentage of N, percentage of C (organic and total, used to calculate the C/N ratio), assimilable P (ppm), and 4 soil texture variables (percentage of clay, silt, and sand, and Saxton value to integrate them in a single variable).

Table 10. Abiotic soil variables for each *A. alba* population (mean value ± standard error): percentage of nitrogen (% N), carbon-to-nitrogen ratio (C/N), percentage of organic carbon (% org C), assimilable phosphorus (P); percentage of clay (% clay), percentage of silt (% silt), percentage of sand (% sand), and Saxton value (Saxton).

Variable	CO	GA	PE	PM	SO
% N	0.87 ± 0.10	0.34 ± 0.02	0.37 ± 0.01	0.32 ± 0.02	0.32 ± 0.01
C/N	18.93 ± 0.36	17.46 ± 0.39	18.32 ± 0.38	15.93 ± 0.41	14.04 ± 0.44
% org C	16.44 ± 1.79	5.97 ± 0.45	6.87 ± 0.33	5.11 ± 0.30	4.53 ± 0.30
P (ppm)	66.98 ± 7.02	18.36 ± 1.62	13.50 ± 1.28	12.05 ± 1.83	8.16 ± 0.87
% clay	6.89 ± 0.45	15.69 ± 0.56	21.74 ± 0.61	25.36 ± 0.86	24.88 ± 0.55
% silt	29.91 ± 0.72	42.36 ± 0.84	40.70 ± 0.67	43.90 ± 0.85	50.65 ± 0.72
% sand	63.20 ± 1.07	41.95 ± 1.24	37.56 ± 1.21	30.74 ± 1.49	24.47 ± 1.08
Saxton	2.11 ± 0.05	1.63 ± 0.04	1.02 ± 0.04	0.85 ± 0.07	1.23 ± 0.05

Finally, the 8 biotic soil variables (Table 11) were based on Phospholipid Fatty Acid (PLFA) analysis [101] to assess the microbial community found in the soils where the

sampled trees lived. General fatty acid methyl esters (FAMEs) were obtained and then individually identified to calculate the biomasses of eukaryotes, Gram-negative bacteria, Gram-positive bacteria (not including actinobacteria), Actinomycetes, fungi, arbuscular mycorrhizal (AM) fungi, and anaerobic bacteria.

Table 11. Biotic soil variables for each *A. alba* population (mean values ± standard error in nmoles): general phospholipid fatty acid (FAME) content, eukaryote biomass, Gram-negative bacteria biomass, Gram-positive bacteria biomass, Actinomycetes biomass, fungi biomass, arbuscular mycorrhizal (AM) fungi biomass, and anaerobic bacteria biomass.

Variable	CO	GA	PE	PM	SO
General FAME	63.57 ± 4.12	41.91 ± 3.00	29.96 ± 1.16	24.86 ± 1.72	30.63 ± 1.96
Eukaryote	7.99 ± 0.91	4.45 ± 0.47	6.31 ± 0.32	5.13 ± 0.68	4.91 ± 0.89
Gram-negative	181.97 ± 10.71	116.56 ± 9.22	113.75 ± 3.43	90.33 ± 5.63	123.61 ± 8.62
Gram-positive	98.25 ± 5.30	70.02 ± 4.39	74.67 ± 2.04	64.81 ± 3.88	78.15 ± 4.75
Actinomycetes	36.34 ± 2.67	20.82 ± 1.36	24.93 ± 1.09	20.21 ± 1.27	23.85 ± 1.42
Fungi	15.51 ± 1.30	11.36 ± 1.47	16.10 ± 1.39	15.58 ± 1.82	11.87 ± 1.84
AM Fungi	13.95 ± 0.90	7.49 ± 0.68	9.80 ± 0.39	8.62 ± 0.59	10.63 ± 0.89
Anaerobe	3.22 ± 0.27	1.76 ± 0.11	1.81 ± 0.07	1.64 ± 0.11	1.88 ± 0.14

4.6. Genotype–Phenotype Associations (GPA)

Two types of tree-level phenotypic data were considered for genotype–phenotype associations (GPA): (1) functional traits related to leaf characteristics, and (2) dendrophenotypic traits related to growth patterns, climate sensitivity, and response to past drought events. Only adult trees were used for this analysis as saplings were not big enough to sample wood cores from their logs. The associations were determined using the R package rrBLUP v4.6.1 [102,103], including the 19 bioclimatic variables as fixed effects. SNP–trait combinations with q-values < 0.05 after FDR correction were considered to be associated.

The 4 functional traits (Table S7) included leaf area (LA, in mm^2), which was measured while needles were fresh, and leaf mass per area (LMA, in mg mm^2), which was calculated after they were dried [38]. Leaf dry weight (LDW, in g) was also measured, as well as leaf dry matter content (LDMC, in g g^{-1}) [101].

The 46 dendrophenotypic traits were obtained from dendrochronological studies carried out using data obtained from sampled cores [38]. For the 13 dendrophenotypic traits (Tables 12 and S8) related to tree-level growth patterns, several variables based on the basal area increment (BAI) were calculated [15], which reflect the growth of the whole tree rather than just the one-dimensional ring width. The periods 1979–1999 and 1999–2019 were selected to account for a time span prior to extensive decline symptoms in the studied *Abies alba* forests, and a near-to-present period when this fir dieback was widely reported [33].

For the 21 dendrophenotypic traits (Tables 13 and S9) related to climate sensitivity, BAI values were correlated with different temperature and precipitation variables [15]. The period used to investigate climate sensitivity was from 1961 to 2019 to ensure the reliability of the climate data and because it included growth data from all sampled trees, excluding juvenile effects [38].

For the 12 dendrophenotypic traits (Table S10) related to tree-level response to documented drought periods, resistance (Rt), recovery (Rc), and resilience (Rs) indexes were calculated based on the ratios between different BAI values (reflecting pre-drought, drought, and post-drought growth during documented past extreme drought events) for the 1986 drought, the 1998 drought, the 2005–2006 drought, and the 2012 drought [38].

Table 12. Description of functional dendrophenotypic traits related to tree-level growth patterns, which were based on the basal area increment (BAI). See [15,38] for details.

Trait	Description
BAI5, BAI10, BAI20	Mean BAI in the last 5, 10, and 20 years, respectively (cm^2)
BAI mean 1979–1999, BAI mean 1999–2019	Mean BAI for the indicated time span (cm^2)
BAI trend 1979–1999, BAI trend 1999–2019	Slope of the BAI for the indicated time span ($cm^2\ year^{-1}$)
Within tree BAI autocorr. 1979–1999, Within tree BAI autocorr. 1999–2019	Tree-level first-order Pearson's autocorrelation of the BAI over time for the indicated time span
Among trees BAI intercorr. 1979–1999, Among trees BAI intercorr. 1999–2019	Pearson's correlation between the tree-level BAI and population mean BAI for the indicated time span
CV 1979–1999, CV 1999–2019	Tree-level coefficient of variation of the BAI (standard deviation/mean 100) for the indicated time span (%)

Table 13. Description of functional dendrophenotypic traits related to tree-level climate sensitivity. They represent the Pearson's correlation coefficient of the basal area increment (BAI) and the described climate variable for the time span from 1961 to 2019. SPEI stands for Standardized Precipitation Evapotranspiration Index. See [15,38] for details.

Trait	Description
PsuP, PauP	Previous summer and autumn precipitation, respectively
Pwi, Psp, Psu, Pau, Pye	Winter, spring, summer, autumn, and year precipitation, respectively
TsuP, TauP	Previous summer and autumn temperature, respectively
Twi, Tsp, Tsu, Tau, Tye	Winter, spring, summer, autumn, and year temperature, respectively
spei_sup, spei_aup	Previous summer SPEI, previous autumn SPEI
spei_wi, spei_sp, spei_su, spei_au, spei_ye	Winter, spring, summer, autumn, and year SPEI, respectively

5. Conclusions

The sampled stands of silver fir in the central-western Spanish Pyrenees showed genetic differences among them. However, no significant genetic differentiation was obtained between declining and non-declining trees within populations. Associations between genome and environment were abundant, linking tree genetics, climate, and soil composition and microbial communities. A high percentage of them also showed signs of selection, supporting a strong impact of environmental factors on this species' genome. Genotype–phenotype associations were scarce, indicating that growth patterns might beshaped by mechanisms beyond the tree's genotype itself, such as phenotypic plasticity in response to extreme climatic events. All in all, we provide an integrative vision of how thesilver fir genome, the environment, and its phenotype are interrelated, which could be a valuable source of information in the context of climate change.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants12142607/s1, Figure S1: Detection of selection signatures considering all populations. SNPs to the right of the vertical line show significant signatures of selection (q-value < 0.05).; Table S1: Original number of samples from each sampling point and final number of retained samples after filtering; Table S2: Analysis of molecular variance (AMOVA); Table S3: Genome–environment associations (GEA) with bioclimatic variables. SNPs with selection signatures are marked in bold; Table S4: Genome–environment associations (GEA) with abiotic soil variables. SNPs with selection signatures are marked in bold; Table S5: Genome–environment associations (GEA) with biotic soil variables. SNPs with selection signatures are marked in bold; Table S6: Genotype–phenotype associations; Table S7: Tree-level values of functional traits related to leaf characteristics; Table S8: Tree-level values of dendrophenotypic traits related to growth patterns; Table S9: Tree-level values of dendrophenotypic traits related to climate sensitivity; Table S10: Tree-level values of dendrophenotypic traits related to responses to documented drought periods.

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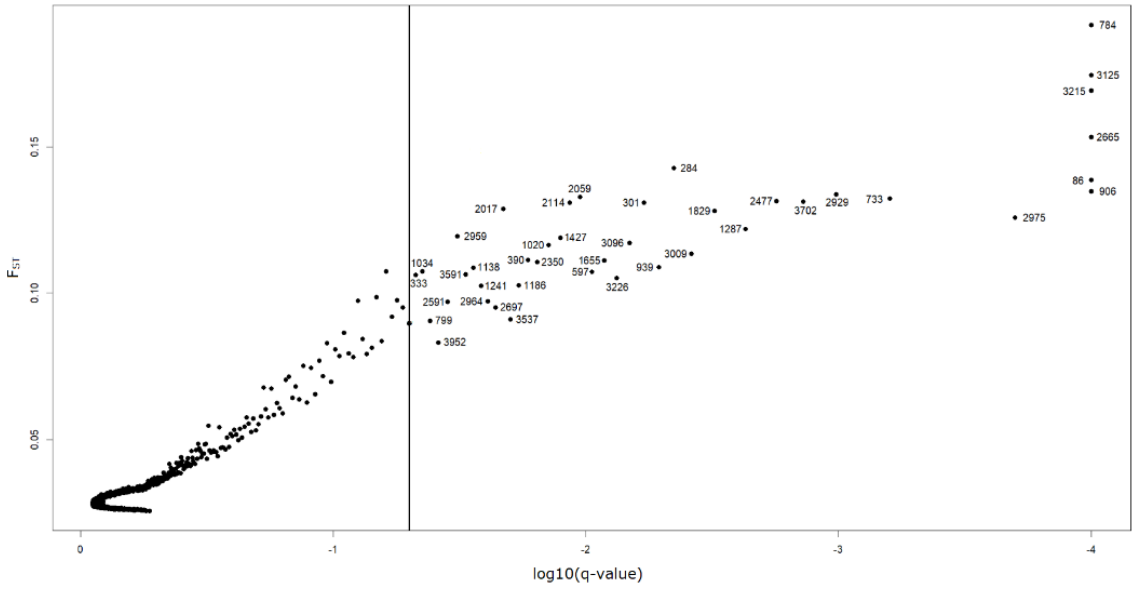
Supplementary materials

1. Figures

Figure S1. Detection of selection signatures considering all populations. SNPs to the right of the vertical line show significant signatures of selection (q-value < 0.05).



2. Tables

Table S1. Original number of samples from each sampling point and final number of retained samples after filtering.

Population		Sequenced samples	Retained samples
CO	Non-declining	15	15
	Declining	15	13
	Saplings	21	19
GA	Non-declining	15	14
	Young	5	4
PE	Non-declining	20	20
	Declining	18	18
	Saplings	39	36
PM	Non-declining	19	19
	Declining	20	20
	Saplings	39	38
SO	Non-declining	20	18
	Saplings	20	20

Table S2. Analysis of molecular variance (AMOVA).

Source of variance	F-stat	F-value	% of total variance	p-value
Within individuals	F_{IT}	0.123	87.7	-
Among individuals	F_{IS}	0.099	9.7	0.000
Among populations	F_{ST}	0.026	2.6	0.000

Table S3. Genome-environment associations (GEA) with bioclimatic variables. SNPs with selection signatures are marked in bold.

SNP	BIO1	BIO2	BIO3	BIO12	BIO15
159	X	X		X	X
179					X
278					X
350	X			X	
448		X			
608		X			
681					X
752		X			X
784		X			
811					X
858		X			
939		X			
948		X			X
983	X	X			X
1172					X
1188	X		X	X	
1344					X
1517		X			X
1544	X			X	
1808		X			
1934					X
2061					X
2119		X			
2128		X			
2131					X
2249					X
2438					X
2452		X			
2459	X				
2484	X				
2591	X			X	X
2645		X			
2794		X			
2940					X
2968		X			
2981					X
3063	X			X	
3111	X			X	X
3306	X			X	
3313					X
3337					X
3380					X
3400	X			X	
3450	X				
3491	X			X	
3537	X	X		X	X
3779	X			X	
Total	16	18	1	12	24

Table S4. Genome-environment associations (GEA) with abiotic soil variables. SNPs with selection signatures are marked in bold.

SNP	%N	C/N	% organic C	P (ppm)	% clay	% silt	% sand	Saxton
197					X			X
238	X		X	X				X
287					X			
368							X	
764					X			X
900		X						
948	X		X	X				
971		X			X		X	X
983				X	X		X	X
1074					X		X	
1287								X
1592	X							
1673	X		X	X				
1808	X							
1829		X			X		X	
2131					X			X
2392								X
2587				X	X		X	X
2665					X		X	X
2775								X
2794				X				
2814				X				
2835	X		X	X	X		X	
2928						X		
3000		X						
3120	X							
3226	X			X				X
3286		X						
3313	X		X	X	X		X	X
3380								X
3537	X		X	X	X	X	X	X
3742	X		X					
3870					X			X
3874	X		X	X				
3952								X
Total	12	5	8	12	14	2	10	17

Table S5. Genome-environment associations (GEA) with biotic soil variables. SNPs with selection signatures are marked in bold.

SNP	General FAME	Eukaryote	Gram negative	Gram positive	Actinomycetes	Fungi	AM Fungi	Anaerobe
159			X	X				X
197	X		X	X				X
238	X		X	X	X			X
288			X	X	X		X	
434				X				
681			X					
764	X							
948		X	X	X	X			X
971	X							
983	X							
1074	X							
1425			X					
1555				X	X		X	
1673								X
1712			X	X				
1756						X		
1808				X	X			X
2131	X							
2587	X							
2591								X
2665	X			X	X			
2835	X							
2997				X				
3049							X	
3104				X				
3120		X			X			X
3226	X		X	X	X		X	X
3313	X		X	X	X			X
3380			X	X	X			X
3537	X		X	X	X		X	X
3742		X						X
3870	X							
3874		X	X	X	X		X	X
Total	14	4	13	17	12	1	6	14

Table S6. Genotype-phenotype associations.

SNP	LMA	LDMC	Rc86	Rt98	Rs98	Rt0506	Rc0506	Rs0506	Rs12
1				X	X				
259	X								
797		X							
887						X			
1086							X		
1104					X				
1277					X				
1669	X								
1769								X	
2270			X						
2319					X				
2431	X								
2797					X				
2882		X							
3101									X
3205		X							
3238				X	X				
3718									X
3878					X				
Total	3	3	1	2	7	1	1	1	2

Table S7. Tree-level values of functional traits related to leaf characteristics.

Due to its extensive size, Table S7 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/Plants_supplementary_excel/blob/main/Supplementary%20tables%20S7-S10.xlsx

Table S8. Tree-level values of dendrophenotypic traits related to growth-patterns.

Due to its extensive size, Table S8 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/Plants_supplementary_excel/blob/main/Supplementary%20tables%20S7-S10.xlsx

Table S9. Tree-level values of dendrophenotypic traits related to climate sensitivity.

Due to its extensive size, Table S9 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/Plants_supplementary_excel/blob/main/Supplementary%20tables%20S7-S10.xlsx

Table S10. Tree-level values of dendrophenotypic traits related to response to documented drought periods.

Due to its extensive size, Table S10 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/Plants_supplementary_excel/blob/main/Supplementary%20tables%20S7-S10.xlsx

ARTICLE 2

ARTICLE 3

Fate of the fallen: modelling past and expected demography of silver fir forests affected by dieback through the genomic and dendroclimatic timeline

El destino de los caídos: modelado de la demografía pasada y esperada de los bosques de abeto común en decaimiento a través de la línea temporal genómica y dendroclimática

Identificar los factores que conducen a la maladaptación es crucial para tomar decisiones sobre conservación y manejo, especialmente en bosques donde el cambio climático está provocando una reducción en el crecimiento de los árboles. Se integraron métodos genómicos y dendroecológicos para inferir y predecir la demografía pasada y futura en cinco bosques de abeto común (*Abies alba* Mill.) en el límite suroeste de su distribución (el Pirineo español) para estudiar su riesgo de maladaptación. Se utilizaron polimorfismos de un único nucleótido (SNPs) para estimar la historia demográfica, las asociaciones genoma-ambiente y la compensación genética, como indicador de vulnerabilidad climática, bajo los escenarios de emisiones RCP 2.6 y 8.5. El incremento del área basal (BAI), estimado a partir de anillos de árboles, se utilizó como un indicador de los patrones de crecimiento a largo plazo para evaluar la sensibilidad del crecimiento al clima, modelándose predicciones para los escenarios de emisiones previamente mencionados. Se obtuvo un gradiente ascendente de riesgo de maladaptación desde los Pirineos orientales hacia los occidentales, coincidiendo con un aumento de la aridez. Los modelos genómicos y dendrocronológicos y la demografía inferida coinciden e indican que el cambio climático es actualmente una amenaza para la supervivencia de los bosques de abeto común en los Pirineos, vinculando los descensos demográficos y de crecimiento al calentamiento. Además, estos resultados respaldan la teoría de la dinámica poblacional a largo plazo, ilustrando que los cambios globales a gran escala tienen efectos generalizados y dominantes, mientras que los cambios localizados desencadenan respuestas locales específicas: los cambios a escala local generan condiciones de crecimiento diferentes en los bosques estudiados y promueven adaptaciones locales, mientras que los cambios a gran escala, como el aumento de las temperaturas y la reducción de las precipitaciones provocados por el cambio climático, afectan de manera generalizada a todas las poblaciones de los Pirineos, mostrando patrones similares en las predicciones de demografía, compensación genética y BAI.

ARTICLE 3

Fate of the fallen: modelling past and expected demography of silver fir forests affected by dieback through the genomic and dendroclimatic timeline

Abstract: Determining the factors that lead to maladaptation is crucial to make informed conservation and management decisions, particularly in forests where growth decline due to climate change is widely supported. We combined genomic and dendroecological methods to infer and forecast past and future demography in five silver fir (*Abies alba* Mill.) forests at its southwestern range margin (the Spanish Pyrenees) to study their risk of maladaptation. Single nucleotide polymorphisms (SNPs) were used to estimate demographic history, genome-environment associations and genetic offset, as an indicator of climate vulnerability, under the RCP 2.6 and 8.5 emission scenarios. Basal area increment (BAI) was estimated from tree-rings as an indicator of long-term tree growth patterns and to evaluate tree growth sensitivity to climate. BAI predictions were modelled for the same emission scenarios. We obtained increasing risk of maladaptation from the eastern to the western Pyrenees, in line with increasing aridity. Genomic and dendrochronological modelling and past demography agree and indicate that climate change is currently a threat to the survival of silver fir forests in the Pyrenees, linking demographic and tree-growth declines to warming. Further, these results support the long-term population dynamics theory, illustrating that large-scale global changes have widespread, dominant effects, while localized changes trigger site-specific responses: local-scale changes create different growth conditions in the studied forests and drive site-specific adaptations, while large-scale changes, like rising temperatures and reduced precipitation brought by climate change, broadly affect all populations across the Pyrenees, which show consistent patterns in demography, genetic offset, and BAI predictions.

Key words: Climate change; Genetic offset; Emission scenarios; Secondary growth; Tree rings

1. Introduction

With climate change constraining the persistence of numerous tree species (Allen *et al*., 2015; McDowell *et al*., 2020), the biogeography and genetic diversity of populations could be key to achieve local adaptations (Aitken *et al*., 2008). As regards demography, the response of species to changing environments is likely to be determined by population responses at range margins, where rear edge populations may be a long-term store of genetic diversity (Hampe & Petit, 2005). Further, it has been recently demonstrated that ecological changes affect not only short-term populations dynamics of a species (years; e.g. Chamaillé-Jammes *et al*., 2006), but also, both directly and indirectly, their long-term (thousands of years; Horreo *et al*., 2016). Therefore, ecological changes play an important role in shaping population dynamics and genetic characteristics, including differentiation processes, and make it possible to trace the effects of these changes far into the past. In relation to this, two major relevant findings should be noted: i) there exists a link between changes in life history traits (e.g. generation time) and long-term population growth/evolutionary rates under climatic constraints (Turrero *et al*., 2012), and ii) major global climatic changes have a greater influence on the long-term demography of widely distributed species than local changes (Horreo & Fitze, 2021). However, there remains a gap between long-term population dynamics theory and long-term data replicated at regional scale to test its reliability (Connor *et al*., 2018), which could lead to improving our understanding of the eco-evolutionary processes associated with climate change, as well as our ability to predict the adaptive capacity of species to cope with future environmental changes.

Species can respond to shifting environmental conditions either by changing their range, migrating to places that could serve as a more suitable habitat, or by adapting to their local habitat (Aitken *et al*., 2008), which requires phenotypic plasticity or genetic adaptation, that relies on population genetic diversity and size (Waldvogel *et al*., 2020). However, migration may pose challenges for many species (e.g. sessile organisms), leaving adaptation as the only option to persist in the face of climate change (Sang *et al*., 2022), which can also be severely limited in organisms with long generation times (Dauphin *et al*., 2021).

Forest trees, as long-lived, sessile organisms, could be affected by climate change with unforeseen intensity in the future (Dauphin *et al*., 2021; Hartmann *et al*., 2022). Their long generation times hinder their capacity to keep pace with the rapid effects of climate change through genetic changes across generations and slow their expansion to other areas, which can only be achieved through pollen and seed dispersion (Petit & Hampe,

2006). In relation to this, declining processes have been reported repeatedly in forests across the world over the last decades (e.g. Kurz *et al*., 2008; Linares *et al*., 2011; Liu *et al*., 2013) and linked to climate change effects, such as drought stress caused by warming (Barber *et al*., 2000; Camarero *et al*., 2011). Concerningly, climate predictions indicate that temperatures will continue to rise, and drought events will be more frequent and prolonged (IPCC, 2022), which will challenge tree species' ability to cope with water shortage. Hence, there is great interest in forecasting the impacts of climate change on forests in order to be able to protect their biodiversity (Petchey *et al*., 2015; Gazol *et al*., 2020). A multidisciplinary approach could be key to identifying the ecological factors that affect populations and led to certain responses to past climate fluctuations and predicting their responses to future environmental changes (Fordham *et al*., 2014).

On one hand, tree-ring records, directly related to individual fitness (Philipson *et al*., 2014), store information about how documented stress periods in the past, such as drought events or heat waves, affected tree growth (Heer *et al*., 2018), being a reliable source of information about how trees responded to past climatic variability (Wilmking *et al*., 2020). This makes dendrochronology studies a valuable tool to forecast climate-growth sensitivity (Matías *et al*., 2017) and predict events of forest decline. Indeed, dendroecological studies evidenced contrasting growth variability and climate sensitivity over the distribution range that are in line with post-glacial phylogeny and genetic diversity (Bosela *et al*., 2016). On the other hand, genomic data also holds potential value for identifying ecological factors that affect populations. Hence, the molecular imprint of local adaptation in natural populations can be tracked back thousands of years and linked to known climate fluctuations through the Quaternary (de Lafontaine *et al*., 2018). Genetic variants associated with current environmental factors can also lead to discovery of adaptive patterns (Rellstab *et al*., 2015). Knowing how allele frequencies of certain *loci* vary according to temperature and precipitation gradients allows the measurement of the amount of adaptive genetic change between current and potential future climates necessary to maintain species present adaptation to the environment (Fitzpatrick & Keller, 2015). This distance is known as genetic offset, and it can serve as an indicator for the risk of non-adaptedness of populations to climate change (Pina-Martins *et al*., 2019).

In this study, by combining ecologic, genomic and dendrochronological data, we infer long-term silver fir (*Abies alba* Mill.) demography both in the past and in the future replicating analyses at regional scale in five populations of the Pyrenees, located on its rear-edge, which are being particularly threatened by climate change-induced environmental fluctuations (Habibzadeh *et al*., 2021). This was done by looking at

changes in effective sizes and population trends and relating them to global ecological changes and fitness (secondary growth rates and climate-growth sensitivity). The major aim was to confront long-term population dynamics theory and long-term data by assessing their demography, genetic vulnerability, adaptive capacity, and the effect of global environmental changes on fitness. Results will be of great importance for evolutionary and conservation studies by confirming long-term population dynamics theory reliability thanks to the here-proposed integrative view of how a drought-sensitive tree species respond to global changes.

2. Materials and methods

2.1. Study sites and sampling design

The study sites were montane silver fir (*Abies alba* Mill.) stands located in north-western Aragón, in the central-western Spanish Pyrenees (Figure 1, Table 1, see detailed information about the sampling sites at González de Andrés *et al*., 2022), which represent the southwestern limit of the distribution of the species in Europe. In this transition zone, between the Mediterranean and temperate biomes, population performance responds negatively to drought, with expectations of further impacts. Moreover, it is expected that genes involved in genetic adaptation will vary along the geographical range of species, where rear edge populations might be disproportionately important (Hampe & Petit, 2005). Five stands were sampled: three of them characterized by abundant dominant trees with high levels of crown defoliation (Cotatuero, CO; Paco Ezpela, PE; and Paco Mayor, PM), and the other two sites currently without decline symptoms (Gamueta, GA, and Selva de Oza, SO), see García-

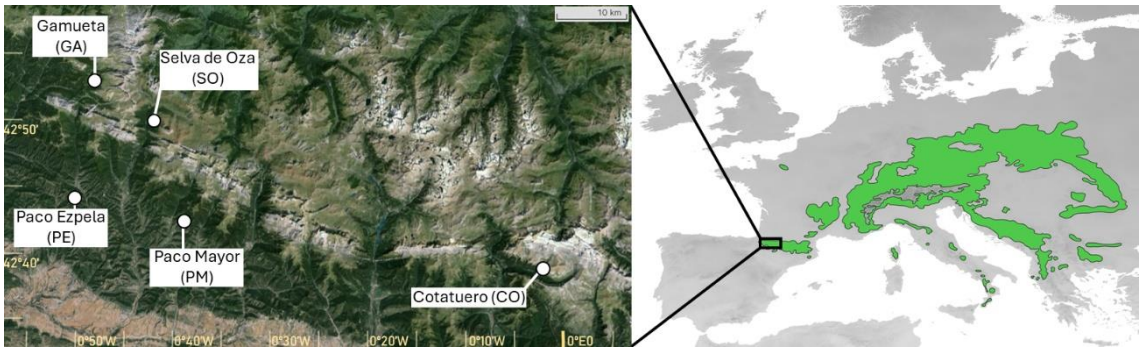


Figure 1: *Abies alba* distribution in green as described by EUFORGEN (right) and study sites in the western-central Pyrenees (left): Gamueta (GA), Selva de Oza (SO), Paco Ezpela (PE), Paco Mayor (PM) and Cotatuero (CO).

Table 1: Location of the study sites and type of population regarding forest decline.

	Gamueta (GA)	Selva de Oza (SO)	Paco Ezpela (PE)	Paco Mayor (PM)	Cotatuero (CO)
Type	Coldspot	Coldspot	Hotspot	Hotspot	Hotspot
Latitude	42.88	42.84	42.74	42.71	42.65
Longitude	-0.79	-0.70	-0.83	-0.64	-0.04
Altitude	1450 m	1220 m	1116 m	1322 m	1456 m

García (2023, 2024) for further details. Historical management also differed among study sites: PE, PM, GA and SO show signs of intense logging activity in the past (Cabrera, 2001; Camarero *et al*., 2011; Sangüesa-Barreda *et al*., 2015), while CO has not been logged for at least 50 years since it is located in a National Park, with strict conservation policies since 1918.

2.2. SNP genotyping

Genomic DNA was extracted from leaf samples using the DNeasy Plant Mini Kit (Qiagen®, Germany) and sequenced following the double digest restriction-site associated DNA (ddRADseq) methodology (Peterson *et al*., 2012) in LGC Genomics (Germany) with 1M read depth. Raw reads were trimmed using fastp v0.12.4 (Chen *et al*., 2018) and mapped to a reduced version of the *A. alba* reference genome (Abal.1_1) (Mosca *et al*., 2019) using ipyrad v0.9.65 (Eaton & Overcast, 2020). This shorter reference retained scaffolds with annotated genetic features and the longest length, allowing the analysis to keep its usefulness while diminishing the high computational costs derived from the fragmented nature of the assembly. Single nucleotide polymorphism (SNP) calling was then performed with ipyrad v0.9.65 and the results were filtered with VCFtools v0.1.16 (Danecek *et al*., 2011), retaining biallelic SNPs with minor allele frequency higher than 5% and less than 20% of missing data. In addition, only 1 SNP per 10,000 bp was kept in order to remove strong linkage disequilibrium, and samples with more than 50% of missingness were removed.

2.3. Population structure

Two clustering approaches were used to assess population structure in the study area: (1) structure and (2) discriminant analysis of principal components (DAPC). STRUCTURE v2.3.4 (Pritchard *et al*., 2000) was used for the first clustering method, running 10 independent repetitions with a burn-in period of 50,000 replicates and a sampling period of 500,000 replicates for clusters (*K*) from 1 to 5. The optimal number of genetic clusters was determined with StructureSelector (Li & Liu, 2018) based on two different approaches: i) the Puechmaille method (Puechmaille, 2016) with the 4 available estimators (MedMedK, MedMeaK, MaxMedK, MaxMeaK; threshold of 0.7), and ii) the Evanno method (Evanno *et al*., 2005), which uses deltaK values. Results were plotted with pong v1.5 (Behr *et al*., 2016). The latter approach was the multivariate method implemented in adegenet v2.1.5 (Jombart, 2008) for DAPC. Optimal number of principal components (PCs) was chosen based on the percentage of cumulative variance retained (100 PCs, 60% variance), and optimal *K* was decided based on the Bayesian Information Criterion (BIC), choosing the *K* that marked the elbow in the curve of BIC values.

2.4. Demographic history

Past demographic history of silver fir populations in the Pyrenes was inferred from site frequency spectrum (SFS) information using stairwayplot v2.1.2 (Liu & Fu, 2015; 2020). The easySFS python script (Gutenkunst *et al*., 2009) was used to calculate the folded SFS, as ancestral alleles are not known in these populations. Then, blueprint files were created following the Stairway plot manual to obtain the demographic history of all the study sites together, individually and regarding the inferred genetic clusters, which were plotted in R v4.3.1 (R Core Team, 2024), along with their respective growth rates (changes in effective size/years). Mutation rate was set at 4.94e-10 (described for the genus *Abies* in De La Torre *et al*., 2017) and generation time was set at 35 years (Mauri *et al*., 2016). A non-parametric Spearman correlation was carried out in GraphPad Prism 8 for Windows (Boston, Massachusetts, USA, www.graphpad.com) in order to compare the obtained results for every inference.

2.5. Genome-environment associations

Local adaptation to climate factors was studied through the identification of genome-environment associations (GEA). The 19 bioclimatic variables (BIOs) offered by the WorldClim database (Fick & Hijmans, 2017) were obtained for each population's location (Table S1) with a grid cell resolution of 30 s, using QGIS 3.18 (QGIS Development Team, 2022). The associations between the climatic gradients of these BIOs and SNP allelic frequencies variations were identified using BayEnv2 (Günther and Coop, 2013) with 100,000 iterations, after estimation of the marker's covariance matrix as indicated by the manual. SNP-BIO combinations scoring a Bayes Factor (BF) higher than 100 were considered to be strongly associated (Jeffrey, 1961).

2.6. Genetic sensitivity to climate change

Genetic sensitivity of each population was calculated as its genetic offset (GO), which reflects the genetic distance between the current and required genomic composition in a set of adaptive loci under a changed environment (Rellstab *et al*, 2021). GO under two predicted climatic scenarios for 2081–2100 was calculated. The considered situations were low concentrations of greenhouse gases in the atmosphere (MIROC6 ssp126 update of RCP2.6), and high concentrations of greenhouse gases in the atmosphere (MIROC6 ssp585 update of RCP8.5). Bioclimatic variables for each population and scenario (Table S2, Table S3) were obtained as described in the genome-environment associations section. These 19 BIOs were used as predictors, in combination with a set of adaptive SNPs (the same significant climate-associated SNPs used to calculate the genetic variability), to build a model following the machine-learning regression tree approach implemented in gradientForest v0.1-32 in R, using 300 trees. R^2 weighted importance of the variables and their cumulative importance were examined to identify the predictors with the strongest impact on the model. GO was then calculated as the Euclidean distances between current and future genetic composition.

In addition, current and future climate conditions were obtained for 25,000 points, randomly scattered through the *A. alba* distribution in the Pyrenees (EUFORGEN), so that their GO attributed to climate-associated variants could be calculated using the previously described model. The results were plotted using ggmap v3.0.1 (Kahle & Wickham, 2013) to visualize areas where GO is predicted to be higher and areas that are less likely to suffer great changes.

2.7. Dendrochronological methods

Radial growth of sampled trees (Table 1) was evaluated using standard dendrochronology procedures (see details at González de Andrés *et al*., 2022). Two cores at 1.3 m height were extracted from each tree, perpendicular to the maximum slope, using 5 mm Pressler increment borers (Haglöf, Sweden). The wood samples were air-dried, glued onto wooden mounts, and polished until the xylem cellular structure was visible (Fritts, 1976). All samples were visually cross-dated, and tree-ring width was measured to a precision of 0.01 mm using a LINTAB measuring device (Frank Rinn, Heidelberg, Germany). Cross-dating was further validated using the COFECHA software, which calculates moving correlations among individual tree series (Holmes, 1983). Tree-ring width series were transformed to basal area increment (BAI) series because it is a two-dimensional measure of stem increment in area that is known to better reflect growth of the whole tree than the one-dimensional ring width (Biondi & Qeadan, 2008). BAI series were calculated using the following equation, assuming concentric rings:

$$BAI = \pi (R^2_t - R^2_{t-1}),$$

where R^2_t and R^2_{t-1} are the radii corresponding to the years t and $t - 1$, respectively. When cores did not reach the pith, we estimated the missing core length by subtracting the core length from the tree radius measured in the field in order to calculate the BAI. We opted for basal area increment as a measure of tree growth since it minimizes the radial growth variations associated with increasing tree size and retains both the long and the short-term changes in tree growth (Matías *et al*., 2017). Detection of outliers was performed on raw BAI data and, accordingly, values above the threshold were removed for subsequent analysis.

2.8. Tree growth modelling and forecast

Tree growth modelling was performed using BAI data (see details in Matías *et al*., 2017). For each site (GA, SO, PE, PM and CO), we first fitted the tree age using the most accurate function (linear, polynomial or sigmoidal) and kept the residuals as an estimate of BAI without ontogenetic effects. After that, mixed-effects models were used to identify the effects of 10 seasonal climatic variables. Thus, we considered seasonal precipitation (P) and temperature (T) during the autumn prior to tree-ring formation: prior September, prior October and prior November (Paup, Taup, respectively); winter: prior December, current January, current February (Pwi, Twi, respectively); spring: current

March, April and May (Psp, Tsp, respectively); summer: current June, July and August (Psu, Tsu, respectively); and autumn: current September, October and November (Pau, Tau, respectively). These climate sensitivity modelling was performed on the residuals of the previous function, using climatic variables as fixed factors and tree as a random factor (Zuur *et al*., 2009). Model selection was performed using backward stepwise regression to minimise the Akaike Information Criterion corrected for sample size (AIC_c). The final model was selected for each site as the one with the lowest number of variables among those with the lowest AIC_c (Burnham & Anderson 2002). Finally, first order autocorrelations (BAI of the previous year, *BAIp*) were used to explain the variations of the residuals of the climate-growth model. The selected models were run to forecast BAI of each site for the period 1885-2100, using KNMI23 selection of CMIP6 scenario runs under moderate (Representative Concentration Pathway 2.6, RCP 2.6) and severe (Representative Concentration Pathway 8.5, RCP 8.5) climate change scenarios, respectively. Climate models were downloaded from KNMI Climate Explorer (https://climexp.knmi.nl/). We simulated annual BAI of a population of 1000 individuals with initial ages between 5 and 100 years. Accordingly, the individual trees would be a maximum 298 years old at the end of the simulated period. All analyses were performed using the packages “nlme” and “mgcv” in R (R Development Core Team 2024). Data are shown as mean ± SE throughout the text. Mean values were compared by ANOVA followed by Tukey HSD test. The level of significance was $p \leq 0.05$ (Zuur *et al*., 2009).

2.9. Data availability

Genomic raw sequences can be accessed at NCBI’s Sequence Read Archive (SRA), under the BioProject with accession number PRJNA947483.

3. Results

3.1. Population structure

After filtering, 3,958 SNPs and 254 individuals were retained. This genetic data set was used to study population structure following two different approaches. The inferred number of genetic clusters (*K*) in the total dataset obtained from the STRUCTURE analysis was $K = 3$ (Figure 2A), determined by both the Puechmaille and the Evanno criteria. These clusters grouped populations as follows: i) GA and PE (western area), ii) PM and SO (center), and iii) CO (east). DAPC results totally agree with this grouping (Figure 2B), as well as a previous principal component analysis carried out with these samples (García-García *et al*., 2023).

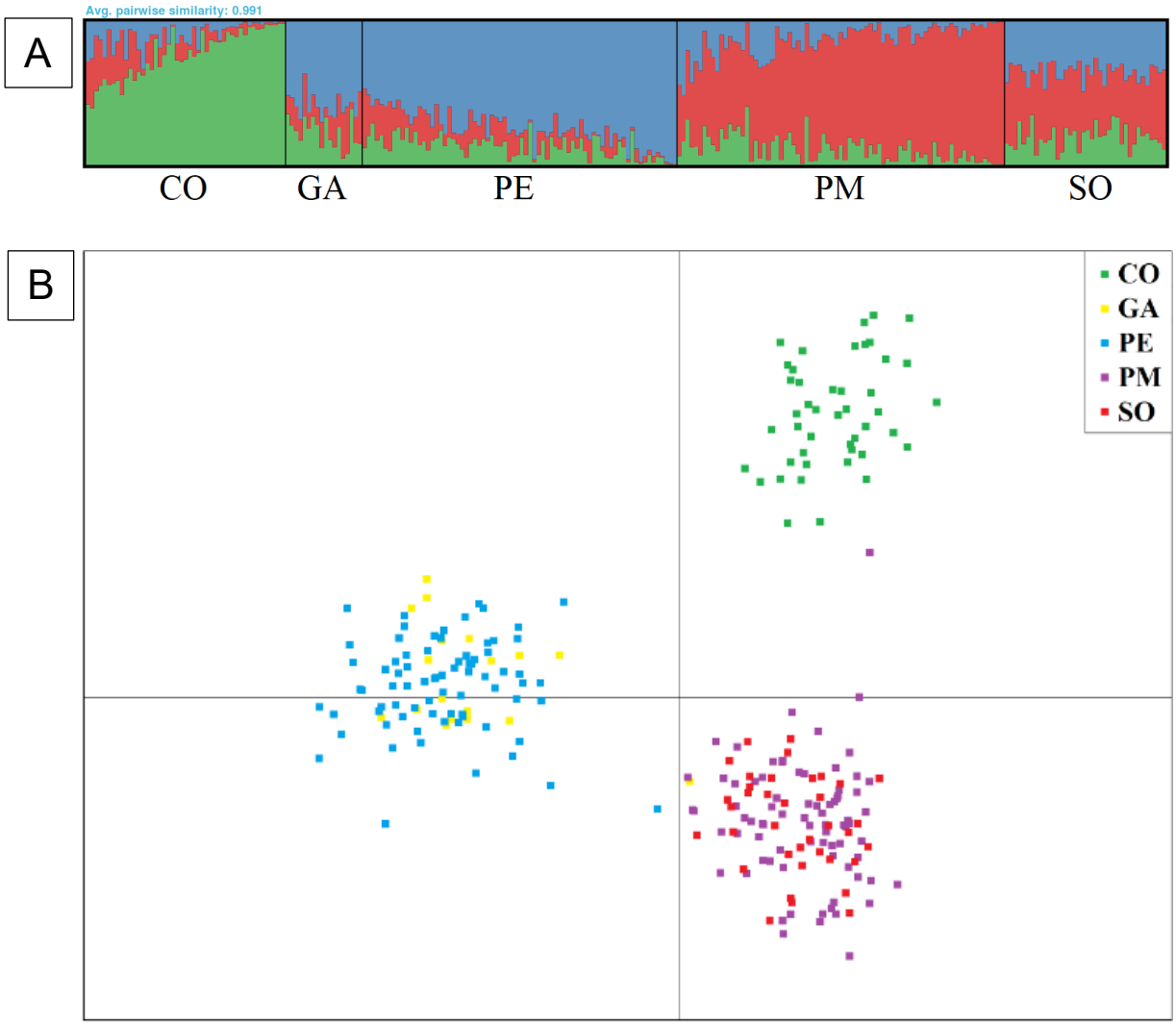


Figure 2: Genetic structure of the studied *A. alba* sampling points in the Pyrenees (CO, GA, PE, PM, SO). A. STRUCTURE results for the optimal number of genetic clusters (*K* = 3). Colors represent the different inferred *K*s. B. Discriminant analysis of principal components (DAPC) results for *K* = 3 with 100 retained principal components (PCs). Colors represent different sampling points.

3.2. Demographic history

Silver fir forests in the Pyrenees have experienced a marked decrease in effective size over the last million years. This reduction was more pronounced during 4 time periods: (1) 0.96–0.90 mya, (2) 0.74–0.61 mya, (3) 0.53–0.42 mya, and (4) 0.24–0.16 mya (Figure 3). When analyzing each of the five populations separately, as well grouping them by the inferred genetic units (GA/PE, PM/SO, and CO; see above), the same downward tendency and pattern were observed (Figures S1, Figure S2), with significant correlations among each pair of results (Table S4).

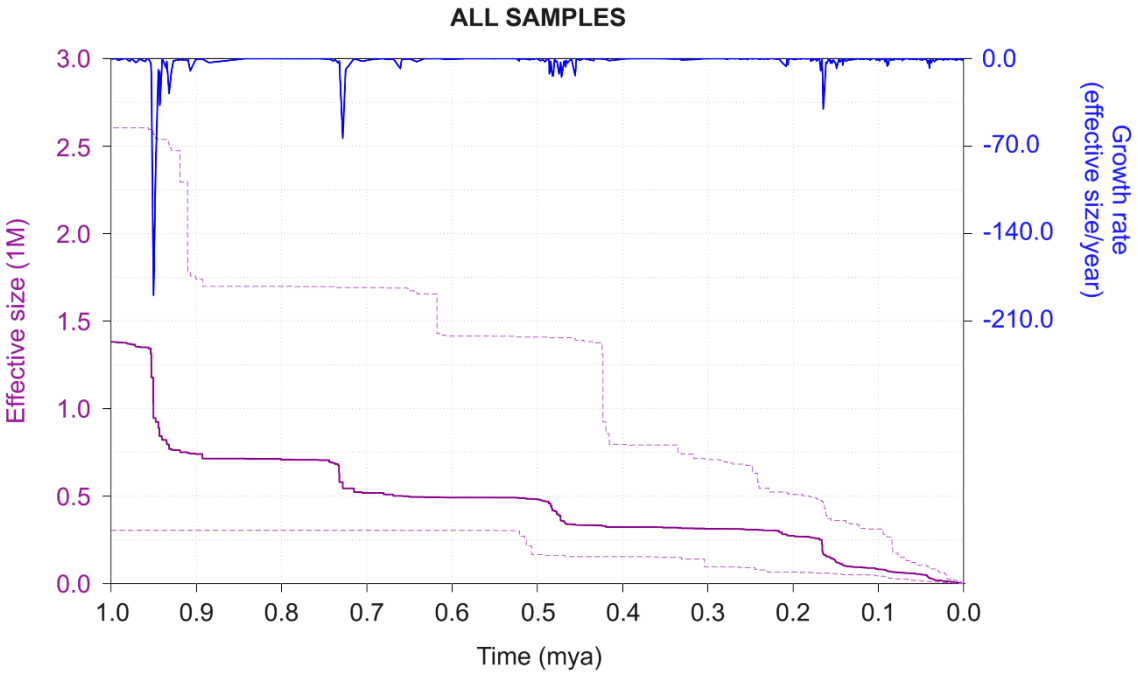


Figure 3: Inferred demographic history of the *Abies alba* forests in the Pyrenees. Purple lines indicate effective size in millions at every time point in millions of years ago (the solid line represents the median; the dashed lines represent the 95% confidence interval). The blue line indicates the speed of changes in effective size over the years.

3.3. Genome-environment associations

A total of 277 genome-environment associations were found between 18 BIOs and 57 different SNPs, with the highest number of associations found for BIO7 (temperature annual range) and none for BIO8 (mean temperature of the wettest quarter) (Table S5). Of these, 18 were located within an annotated gene or close to one (Table 2). Some of the proteins encoded by those genes include two cytochromes P450, which show association with a temperature variable (BIO7, temperature annual range), and an aquaporin, which is associated with BIO13 (precipitation of wettest month), BIO14 (precipitation of driest month), BIO15 (precipitation seasonality), BIO17 (precipitation of driest quarter), and BIO18 (precipitation of warmest quarter).

Table 2: List of SNPs associated with bioclimatic variables that could be used as molecular markers for annotated genes in *A. alba,* specifying their location in the genome, position regarding the gene they are closer to, and protein encoded by the gene.

SNP	Location	Position	Protein	ID
159	aalba5_s00001283:86,646	21,883 bp upstream	UDP-glysocyltranferase (U86A2)	AALBA5B1024847T1
179	aalba5_s00001426:131,290	Within the gene	BEACH domain-containing protein (BCHC2)	AALBA5B098257T1
629	aalba5_s00006464:75,131	2,738 bp downstream	Cytochrome P450 (CYP704C1)	AALBA5B1110432T1
681	aalba5_s00007005:35,095	Within the gene	Aquaporin (SIP2-1)	AALBA5B253951T1
983	aalba5_s00011431:62,999	21,010 bp downstream	Tetratricopeptide repeat-like superfamily protein (PPR35)	AALBA5B348236T1
1806	aalba5_s00023261:31,104	Within the gene	Kinase (NEK4)	AALBA5B973686T1
2061	aalba5_s00027387:15,057	30,900 bp upstream	MOTHER of FT and TFL1 (MFT)	AALBA5B1139649T1
2119	aalba5_s00028425:12,399	328 bp upstream	Chaperone, chloroplastic (DJA7A)	AALBA5B512522T1
2459	aalba5_s00034077:43,568	772 bp upstream	Mitogen-activated kinase (M3K17)	AALBA5B244254T1
2591	aalba5_s00036159:10,277	5,615 bp upstream	Transcription factor (PHL3)	AALBA5B939227T1
2645	aalba5_s00037229:12,665	111 bp upstream	Homeobox (PKNOX1)	AALBA5B627517T1
2794	aalba5_s00040228:24,711	20,262 bp upstream	DNA repair protein (RAD7)	AALBA5B261613T1
2904	aalba5_s00042872:51,622	18,194 bp downstream	Helicase (MAA3)	AALBA5B767119T1
3063	aalba5_s00045131:27,419	17,345 bp downstream	Phosphatase (P2C60)	AALBA5B289418T1
3313	aalba5_s00049423:3,040	909 bp upstream	Translation initiation factor (SDA1)	AALBA5B666590T1
3400	aalba5_s00051396:12,444	Within the gene	Uncharacterized transmembrane protein (UGPI7)	AALBA5B360123T1
3640	aalba5_s00055804:20,741	Within the gene	Cytochrome P450 (CYP90B01)	AALBA5B283308T1
3898	aalba5_s00061110:42,148	Within the gene	Probable carboxylesterase	AALBA5B153451T1

3.4. Sensitivity to climate change

BIO2 (mean temperature diurnal range) has the strongest impact on the model, followed by BIO15 (precipitation seasonality) (Figure 4). As a general trend, temperature and precipitation variables show similar levels of contribution to explaining the model. Hence, there is not a dominant type of environmental factor, but both exert a combined effect on the genetic composition of this species and, consequently, on its GO prediction.

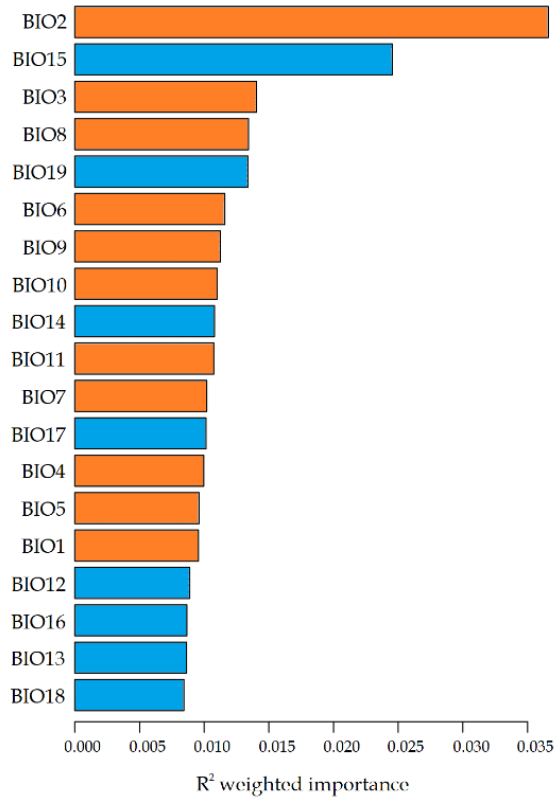


Figure 4: Mean R^2 weighted importance of the climatic variables included in the gradient forest model. Temperature variables appear in orange; precipitation variables appear in blue.

The projection of the GO across this species' distribution in the Pyrenees yields a mean value of 0.12 in the low-emission scenario and a mean value of 0.19 in the high-emission scenario (Figure 5), meaning that a greater genetic change would be necessary to adapt to the most severe scenario. It also shows that the eastern part of the mountain range is likely to suffer the effects of climate change to a lesser extent in both scenarios (Figure 5). Therefore, these predictions suggest increased vulnerability to climate change within the western and central Pyrenees silver fir populations, precisely where the studied populations are located, in line with our field observation.

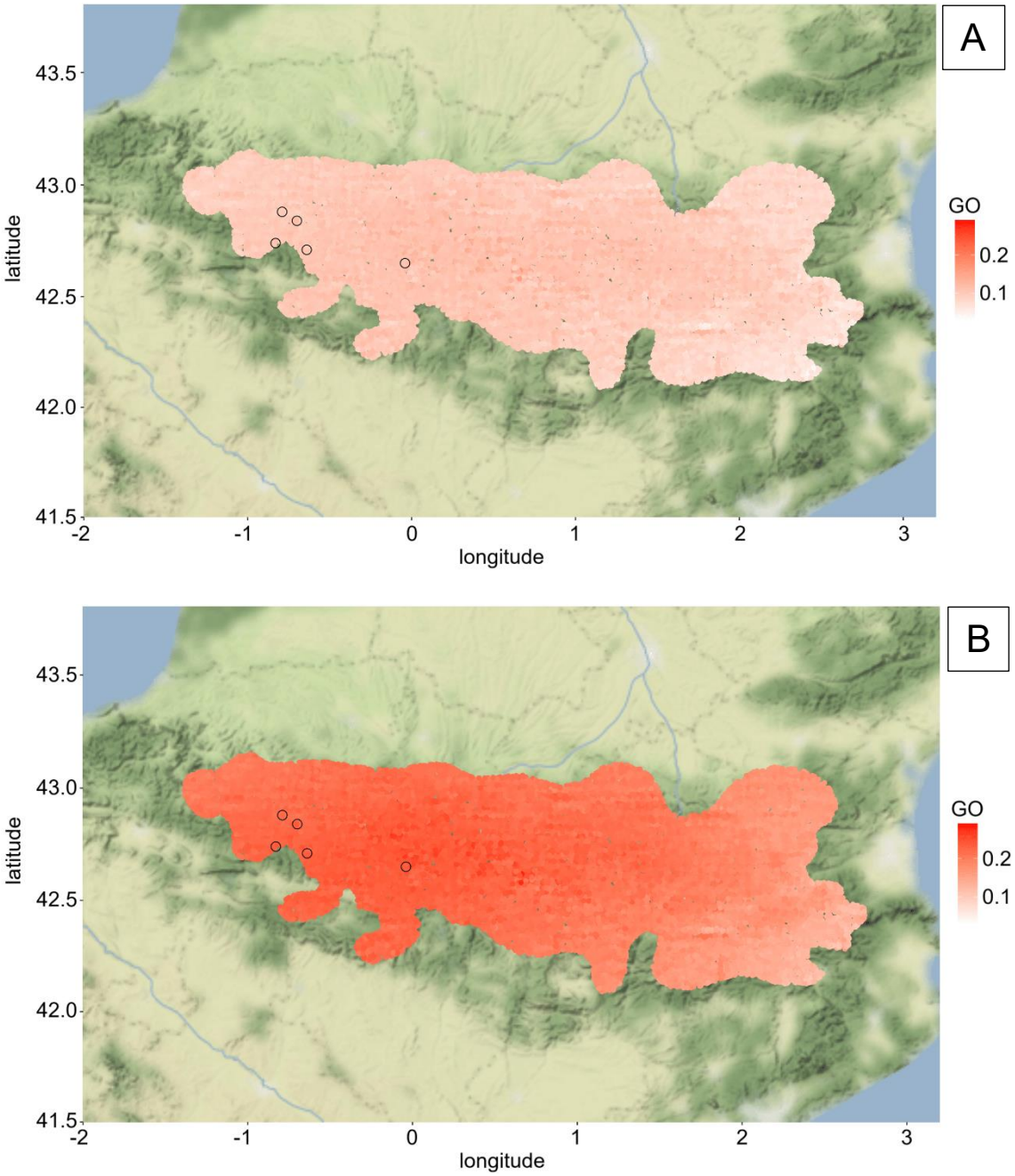


Figure 5: Genetic offset (GO) projection across the *A. alba* distribution in the Pyrenees. Circles indicate sampled sites. A. Low emission scenario RCP2.6 for the year 2100. B. High emission scenario RCP8.5 for the year 2100.

3.5. Tree growth modelling and forecast

Growth is mainly controlled by temperature in four of the studied sites, except for CO, where precipitation variables sum up over 50% of importance in the model (Table 3). Temperature variables show a negative correlation with growth, except for Tsp (spring temperature) on one hand, and Taup (previous autumn temperature) only in CO on the other. Regarding precipitation variables, Paup (previous autumn precipitation) influences growth in every site except for SO, where no correlation between growth and precipitation has been detected. See Figure S3 and Appendix A–E for more details.

The forecasted growth trends point to decreasing growth during the 2001–2050 period in both scenarios in every site except for CO, where we obtained steady growth trends (Figure 6). Then, differences between scenarios were more definite during the 2051–2100 period, turning to almost steady growth in the RCP2.6 while BAI visibly decreases in the RCP8.5 (except in CO, where it remains steady throughout the complete time period in both scenarios, in line with its growth trend observed during the previous ~100 years).

Table 3. Regression coefficients of climate models. The within-population relative weight of each coefficient (percentage) appears between parentheses. Climate variables abbreviations: P, precipitation; T, temperature; sp, spring; su, summer; au, autumn; wi, winter. Seasons abbreviation followed by "p" corresponds to the year prior to tree-ring formation.

Variable	GA	SO	PE	PM	CO
Taup	-1.0125 (33.6)	-1.2885 (21.7)		-0.8174 (14.0)	0.3943 (43.7)
Twi					
Tsp	0.5567 (12.3)	1.2609 (14.1)	1.2500 (22.4)	1.7341 (19.7)	
Tsu		1.2942 (41.4)	-0.7223 (36.9)	-0.8741 (28.4)	
Tau	-1.1747 (39.1)	-1.3482 (22.8)		-1.7280 (29.7)	
Paup	0.0088 (15.0)		0.0081 (11.2)	0.0093 (8.2)	0.0066 (30.7)
Pwi					
Psp			0.0133 (18.3)		0.0055 (25.6)
Psu					
Pau			0.0081 (11.2)		

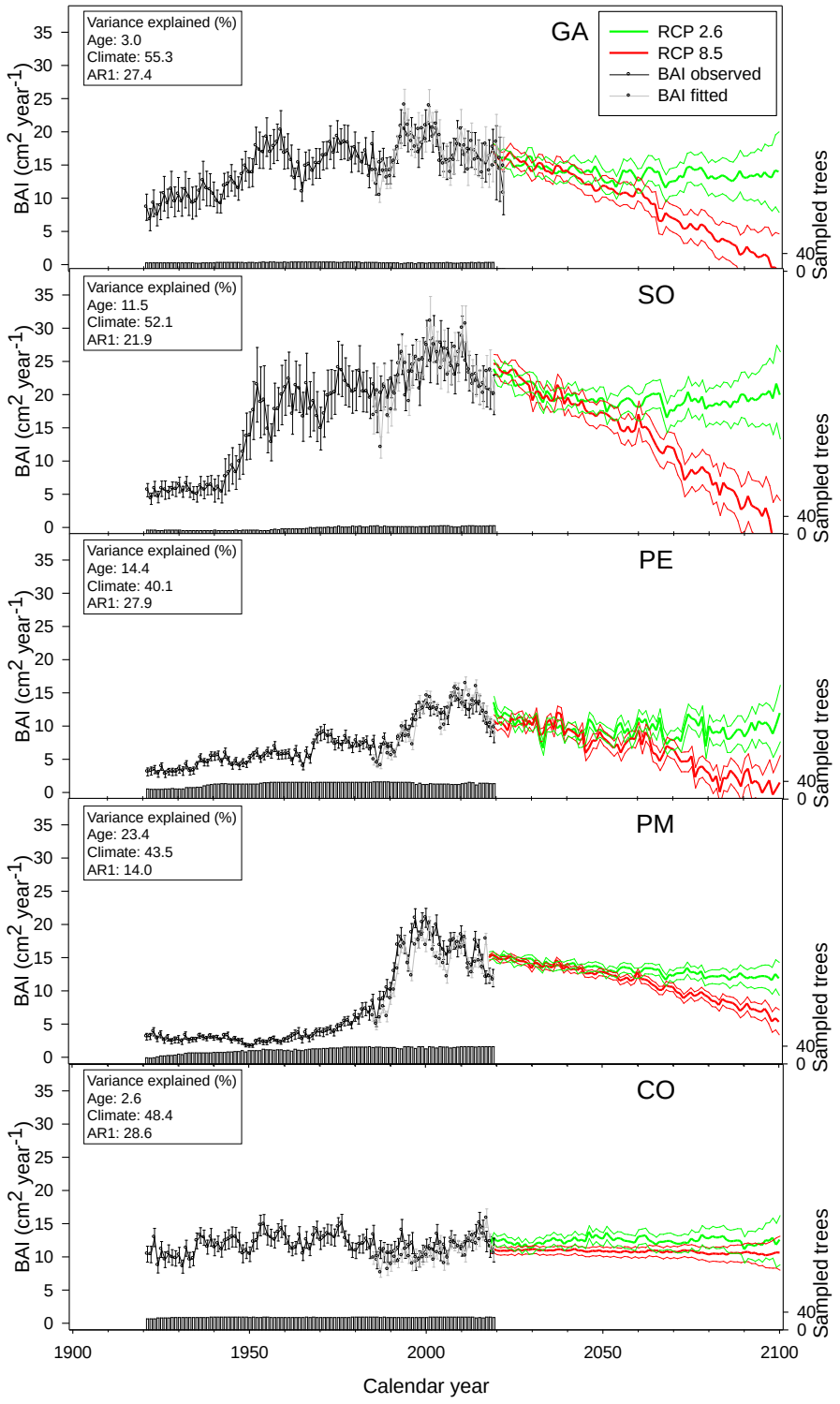


Figure 6. Growth patterns (basal area increment, BAI) observed, fitted and forecasted. The inset indicates the partial variance explained by tree age-effect, climate sensitivity and first-order autoregressive correlation (AR1), respectively. Black lines show observed BAI values (means ± SE) and grey lines are fitted values obtained through linear mixed-effect models (LMEM). Green and red lines show BAI forecasts under moderate (Representative Concentration Pathway 2.6, RCP2.6) and severe (Representative Concentration Pathway 8.5, RCP8.5) climate change scenarios, respectively. The number of sampled trees is indicated with grey bars (right Y axes).

Differences in forecasted mean growth are higher in the RCP2.6 scenario, where each site displays a unique response, except for PM and CO during the second half of the XXI century, which show no differences between them (Figure 7). The RCP8.5 scenario results in a more homogeneous response, with CO grouping with PE in the first half of the XXI Century and similar mean growth in the CO-PM and SO-PM pairs in the second half of the XXI century (Figure 7). See Figure S4 and Appendix F – J for more details.

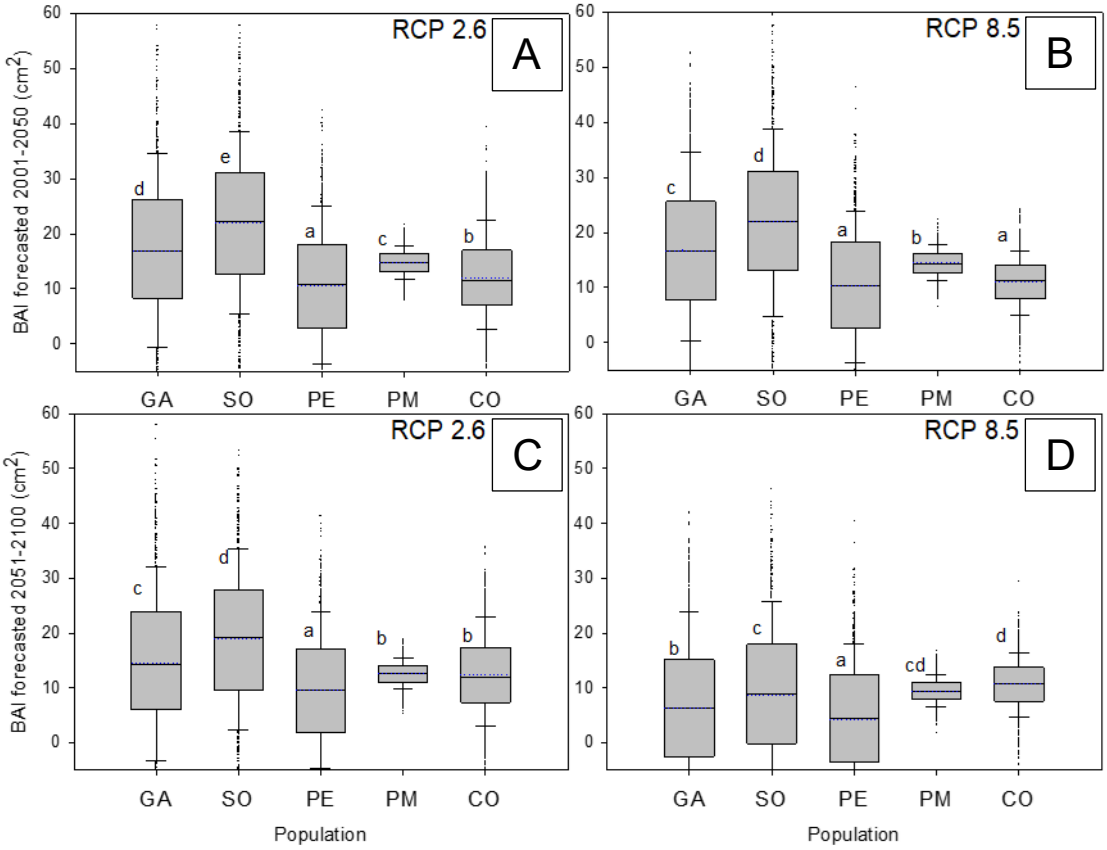


Figure 7. Forecasted mean growth expressed as the mean basal area increment (BAI) predicted under RCP2.6 and RCP8.5 scenarios for the period 2001-2050 (A, B) and 2051-2100 (C, D). In the box-plot figure, error bars represent the 5th/95th percentiles; boxes stand for the standard error (n = 1000 simulations per site); solid lines represent the median; dashed lines are the mean values, and points are outliers. Different letters indicate significant differences for ANOVA, *p*-value < 0.05.

4. Discussion

This study solidly supports the reliability of long-term population dynamics theory, which remained a major gap in evolutionary theory so far (Connor *et al*., 2018), by replicating at regional level past and future demographic inferences and combining genomic, ecology, dendrochronology and fitness data. To that end, knowledge of the genetic structure of the studied populations at regional scale is essential, which in this species seems to be affected by both geographical and environmental factors (Mosca *et al*., 2014). Three differentiated genetic units were identified (Figure 2) across the 5 studied populations. These units are in line with geographical barriers rather than phenotypic characteristics (namely tree vigor). The GA-PE and SO-PM pairs are relatively close, while they are ~50 km apart from CO (see Figure 1), with declining trees present only in southernmost populations (PE, PM, CO).

But the observed local genetic characteristics in these *A. alba* populations are not only caused by geography: local environmental factors also affect them, as supported by significant genetic differentiation of each population based on F_{ST} values (García-García *et al*., 2024) and the existence of numerous genome-environment associations (Table S5), some of them relating temperature and precipitation with genes involved in abiotic stress response (Table 2). For example, BIO7 (temperature annual range), which has been identified as a key bioclimatic factor in forest distribution models including species belonging to the genus *Abies* (Dakhil *et al*., 2019), had the highest number of associations, including SNPs related to proteins that are known to play a role in response to thermal stress. Therefore, both geography and local ecological conditions play relevant roles in the genomic characteristics of silver fir at regional scale, as previously proposed (Mosca *et al*., 2014), and, thus, can have relevant consequences on its demography, as genetic diversity in this species provides information about its demographic history (Major *et al*., 2021).

When looking backwards on silver fir demography in the area, we find different clearly marked peaks of population decreases (Figure 3). The first and most abrupt decrease in effective size identified in this study (0.96–0.90 mya) agrees with the divergence between the Alpine and the Pyrenean silver fir lineages (0.83–1.1 mya; Scotti-Saintagne *et al*., 2021), indicating the isolation of silver fir in the Pyrenees, separating from a big initial ancestral population from the Alps. Afterwards, three other marked decreasing peaks were identified. The first of them (0.74–0.61 mya) lies close to the Matuyame-Brunhes event (which is the last Earth's magnetic reversal, around 0.77 mya; Suganuma *et al*., 2015), which produced a change towards a warmer temperate

subtropical climate happened in Western Europe (Almogi-Labin, 2011), and led to the impoverishment of forests and increase of dry meadows and open land (Cuenca-Bescós *et al*., 2011). Then, another population decline occurred between 0.53 and 0.42 mya, coinciding with the Mid-Brunhes event (MBE, around 0.45 mya), which was a climatic transition to warmer interglacial period with lower levels of rainfall (Blain *et al*., 2012). Lastly, the reduction observed between 0.24 and 0.16 mya perfectly overlaps with the glacial buildup from Marine Isotope Stage 7 to 6 (0.24–0.17 mya), when occurred the fastest glaciation-deglaciation event in the last 800,000 years with a very quick decrease of ice volume due to increased temperatures (Choudhury *et al*., 2020). Thus, all these events represent a rapid change towards warmer weather, which led to a decrease in effective size of *A. alba* forests in the Pyrenees. Importantly, all this occurs at regional level (forests in the western Pyrenean area; Figure 3), as well as population level (Figure S1) and genetic unit level (Figure S2), suggesting that global changes could have shaped past population demography, overriding local scale conditions, even if signs of local adaptation can be detected at present.

The dynamics of populations that inhabit the southernmost margins of the silver fir distribution range might be critically important in determining the adaptive capacity of this tree to expected climate change (Hampe & Petit, 2005). Thus, selection for local adaptation can be expected in these marginal populations, as well as gradually reduced gene flow, and effective population size declines, resulting in potential distinct ecotypes (Alberto *et al*., 2013). Although the demographic events that shaped the current spatial structure of genetic diversity of silver fir go back to the Pleistocene, as described above, human pressure during the Holocene likely led to an abrupt range decline too (Linares, 2011), while recent drought events have caused widespread decreasing productivity and dieback of silver fir forest, mainly over its southwestern range limits (Gazol *et al*., 2015).

In addition to the negative effect of drought events on the recent demographic history of the species, the here-identified genome-environment associations support the occurrence of warm-induced past decreases in demography by relating environmental stress to adaptation processes (and, thus, evolutionary changes driven by a stressful environment). For example, two cytochromes P450 were associated with a temperature variable (BIO7, temperature annual range). These enzymes have been linked to response to several kinds of abiotic stress (Pandian *et al*., 2020), including heat and cold tolerance (Baron *et al*., 2012; Li *et al*., 2013; Tao *et al*., 2017). Precipitation variables also showed associations with genetic markers involved in stress response, including an aquaporin, associated with BIO13 (precipitation of wettest month), BIO14 (precipitation of driest month), BIO15 (precipitation seasonality), BIO17 (precipitation of driest quarter),

and BIO18 (precipitation of warmest quarter). This family of water channels plays a crucial role in maintaining water homeostasis in plants (Srivastava *et al*., 2014) by reducing their activity and expression to preserve water content under abiotic stress situations (Martinez-Ballesta & Carvajal, 2014). Interestingly, it is also associated with several temperature variables, namely BIO4 (temperature seasonality), BIO6 (min temperature of coldest month), and BIO7 (temperature annual range), in line with aquaporins being involved in plant response to different abiotic stresses, including temperature variations (Shang *et al*., 2022), as heat and cold also exert a general impact on plant hydraulics (Maurel *et al*., 2015). All in all, the GEA results are completely in line with the existence of genes which are directly affected by climatic changes, like the ones that produced drastic falls in demography.

This finding can also be seen in the tree growth analysis (Figure 6), which is indeed directly related to silver fir fitness (Linares & Camarero, 2012). They show that major changes (high increase of temperatures, RCP8.5) affect populations (all but CO, which is the non-logged population, which will be discussed later) in the same way, with drastic reductions in BAI, thus fitness, compromising their future. However, this does not occur in less severe conditions (moderate increase of temperatures, RCP2.6), where local conditions do produce differences: coldspots (GA and SO) are less affected, maintaining a higher BAI than hotspots. In sum, ecological changes affect silver fir demography through changes in their fitness (BAI) and genetic characteristics (specifically genes related to abiotic stress), affecting at regional scale when severe, but at local scale when moderate.

Further, the genetic sensitivity to climate change analysis identifies an east-to-west gradient, predicting higher GO in the western and central populations than in the eastern region, in line with field observations that report more severe and widespread decline in western silver fir forests (Camarero *et al*., 2011; Sangüesa-Barreda *et al*., 2015; Valeriano *et al*., 2023). This means that greater changes in genetic composition will be needed in the western-central region of the Pyrenees in order to survive. Site-dependent patterns in mean growth outline east-to-west gradients of increasing vulnerability at regional scale too. However, the long generation time of this species works against its survival in the area, as their local adaptation will be disrupted if the rate of change needed to adapt to the rapidly changing environmental factors is slower than the speed of climate change (Aitken & Bemmels, 2015). Thus, silver fir populations in the Pyrenees are prone to local extinction in their current locations. Furthermore, previous research showed that aging trends and growth levels were similar during the last millennia among the main regions where *A. alba* can be found (Büntgen *et al*., 2014), with a high degree of common

growth variability. This highly synchronized growth behavior may indicate that our growth projections might be also expected, regarding the overall trends, across the distribution range of silver fir.

Indeed, recent drought events have caused widespread dieback and mortality in almost the whole silver fir populations located in drought-prone sites (Gazol *et al*., 2015). Dendroecological studies evidenced contrasting responses of *A. alba* over the distribution range that are in line with genetic and biogeographical features (Bosela *et al*., 2016). Specifically, the growth of silver fir increased considerably during the last decades across central Europe (Buntgen *et al*., 2014), whereas there was a significant growth decline toward the Mediterranean ecotone in southern France (Cailleret *et al*., 2014), Italy (Carrer *et al*., 2010) and the Pyrenees (Macias *et al*., 2006; Linares & Camarero, 2012). Furthermore, declining growth patterns have also been reported in the south-eastern European mountains in Slovenia, Croatia, Slovakia and Bosnia-Herzegovina (Diaci *et al*., 2011). Thus, these populations in drought-prone sites, like the ones studied here, deserve priority conservation efforts, as they are currently in climate change hotspots, where drier and warmer summer conditions will represent the major climatic constraints to the persistence of silver fir (Gazol *et al*., 2015; Valeriano *et al*., 2023). In addition to summer drought, our results also confirm the well-established growth sensitivity of silver fir to water deficit during the autumn prior to the growing season, as most populations yielded negative relationships with the temperature of the previous autumn, except the westernmost (CO), which will be discussed later due to its particularities. Likewise, the machine learning model also identified BIO15 (precipitation seasonality) as the second most explanatory variable, supporting this affirmation. Low soil moisture and elevated evaporative demand during late summer and autumn may be the main climatic growth limitation for silver fir in the coming future. Further, the presence of local adaptations in marginal populations might be critical in determining the fate of this tree under a climate change scenario (Alberto *et al*., 2013), as currently observed among the studied populations (hotspots of forests decline as opposed to coldspots nearby). However, growth models obtained under the most unfavorable climate scenario (RCP8.5) predict that growth decline may affect populations that are not currently showing decline symptoms, suggesting that the expected pervasive warming might override local adaptation, leading to increased sensitivity to drought stress and widespread silver fir decline and mortality. In fact, events of forest decline and dieback have been widely reported in the Pyrenees (Büntgen *et al*., 2014; Camarero *et al*., 2015; Camarero *et al*., 2011; Gazol *et al*., 2020; González de Andrés *et al*., 2022), in line with the expected growth decline obtained from dendrochronology data in this study. The

occurrence of successive severe droughts and increasingly warmer temperatures during the 1980s–1990s may have impaired growth and resilience and led to very low growth rates that preceded dieback (Cailleret *et al*., 2017; Camarero *et al*., 2015; DeSoto *et al*., 2020). Indeed, the extreme 1985–1986 drought has been identified as a tipping point for the growth decline of several silver fir populations in the southwestern Spanish Pyrenees (Camarero *et al*., 2011), being aggravated by successive droughts in 2005 and 2012 (Valeriano *et al*., 2023).

All in all, these results support the long-term population dynamics theory, which hypothesizes a differential impact of environmental changes depending on their scale. The effects of local-scale changes can be observed in the fact that coldspot areas provide more favorable environmental conditions for the species' growth than hotspots, where forest decline is occurring, and in the identification of local adaptations through genome-environment associations. On the other hand, the effects of large-scale global changes, such as rising temperatures and decreasing precipitation, are apparent across the entire Pyrenees, impacting both hotspots and coldspots alike. This is reflected in the consistent patterns observed across populations in terms of demography, genetic offset, and BAI predictions. Thus, global changes affect all populations in a generalized manner, while local changes have specific effects on the areas where they occur, inducing population-specific responses. This results in a hierarchical relationship between both types of changes, in which large-scale environmental shifts have a dominant and widespread impact, prevailing over minor differences in local conditions, that can buffer the impact of environmental changes up to a certain limit. So, can silver fir forests in the Pyrenees rely on local conditions to avoid regional extinction? Our results suggest they exhibit high vulnerability to climate change, with local factors, such as microclimates caused by orography, as well as biological traits, like adaptive genetic diversity, unlikely to counterbalance the widespread and intense increase in regional aridity, particularly over a long-term timeframe.

As a final remark, and coming back to CO, which is the only population whose predicted BAI does not decrease under severe conditions, it should be noted that it is the only population included in this study that has not been managed in the last decades. Forest logging decreases competition for the available resources among the remaining trees, leading to growth releases (Camarero *et al*., 2011; Sangüesa-Barreda *et al*., 2015), while a lack of management creates more stable growth patterns, in line with the here-shown tree-ring data (Figure 6). Therefore, intense selective logging may also affect demography, especially under severe drier climate scenarios, producing population decreases in long-term managed populations (Sangüesa-Barreda *et al*., 2015).

5. Conclusions

The here-presented findings align with the long-term population dynamics theory, indicating that large-scale global changes exert widespread and dominant effects (risk of extinction in the Pyrenees under climate change scenarios), whereas localized differences drive site-specific responses (declining processes happening only in certain forests). Both local and global scale inferences agree, and independent, diverse data point in the same direction, confirming the reliability of the presented conclusions and the long-term population dynamics theory. Furthermore, they reveal that environmental changes shape silver fir demography by affecting their fitness (BAI) and genomic regions related to abiotic stress. In addition, genomic and dendrochronological modelling point to a climatic threat posed by climate change to the Pyrenean silver fir forests. Altogether, combining genetic and eco-physiological variables to predict vulnerability to climate change should become a field of high interest due to the existence of feedbacks between genomic and physiological functioning involved in coping with drought stress.

6. References

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SUPPLEMENTARY MATERIAL

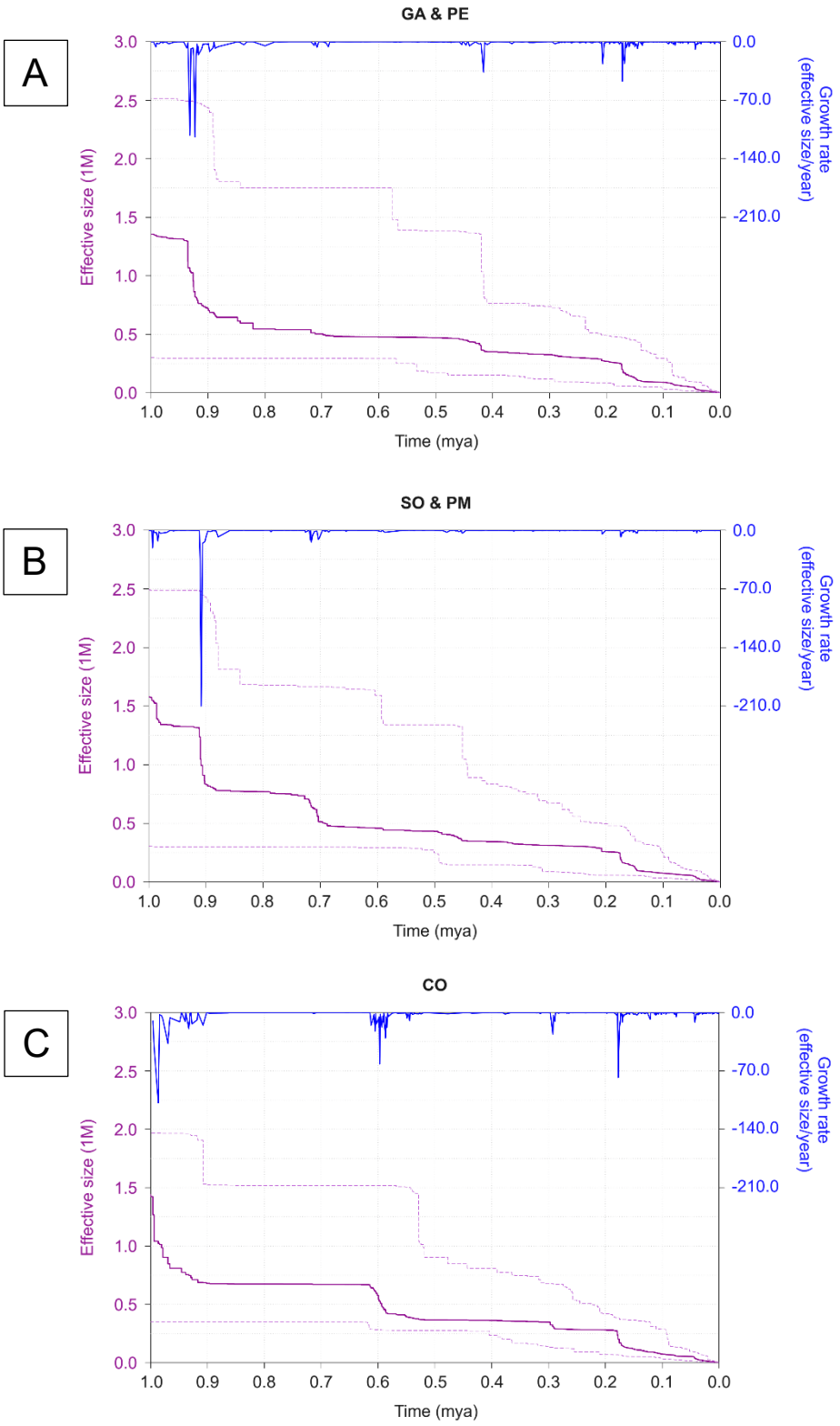


Figure S1: Inferred demographic history of each *Abies alba* inferred genetic cluster. Purple lines indicate effective size in millions at every time point in millions of years ago (the solid line represents the median; the dashed lines represent the 95% confidence interval). The blue line indicates the speed of changes in effective size over the years. A. PE & GA. B. PM & SO. C. CO.

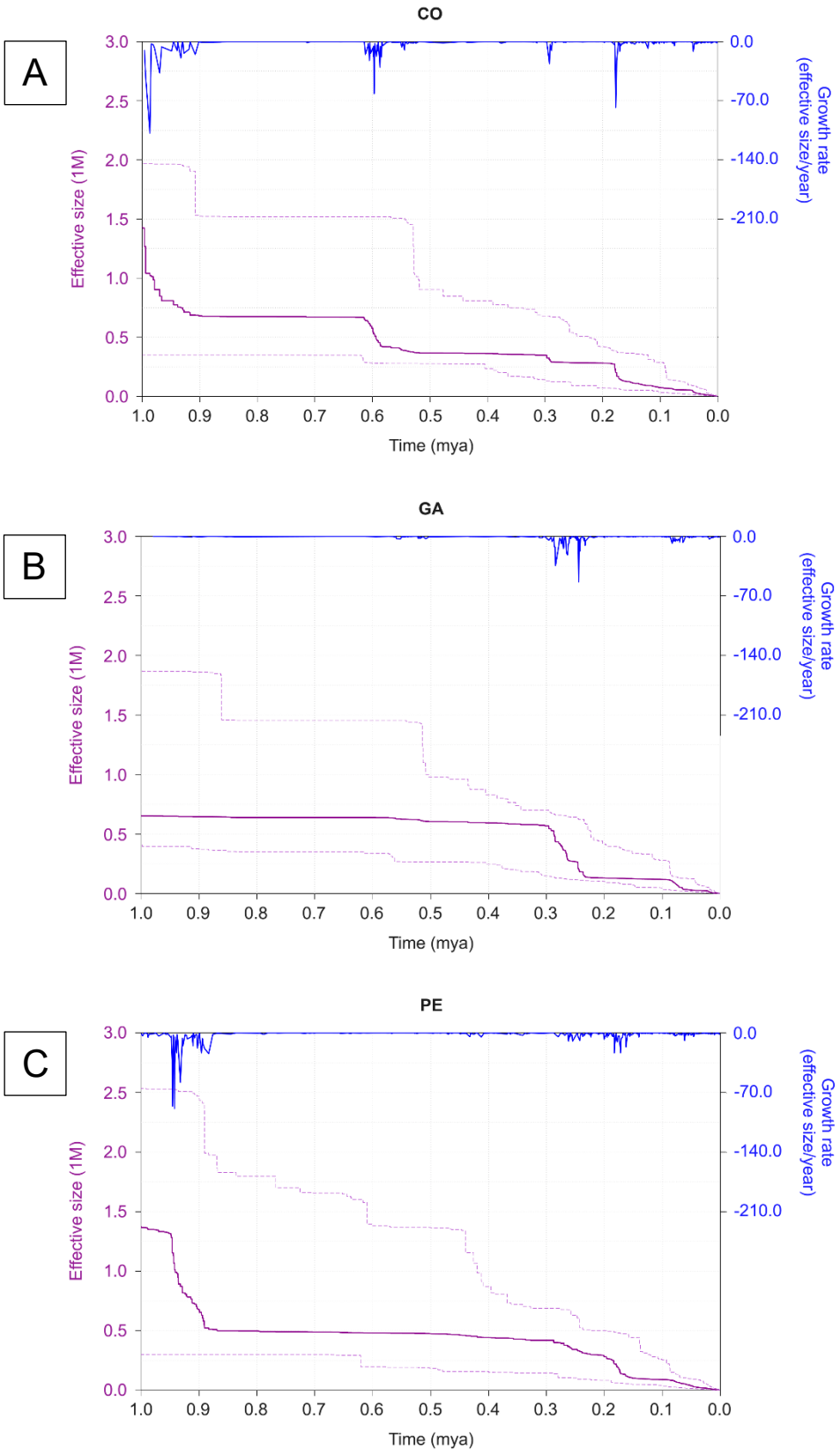


Figure S2: Inferred demographic history of each *Abies alba* sampling point. Purple lines indicate effective size in millions at every time point in millions of years ago (the solid line represents the median; the dashed lines represent the 95% confidence interval). The blue line indicates the speed of changes in effective size over the years. A. CO. B. GA. C. PE. D. PM. E. SO. [Part 1 of 2]

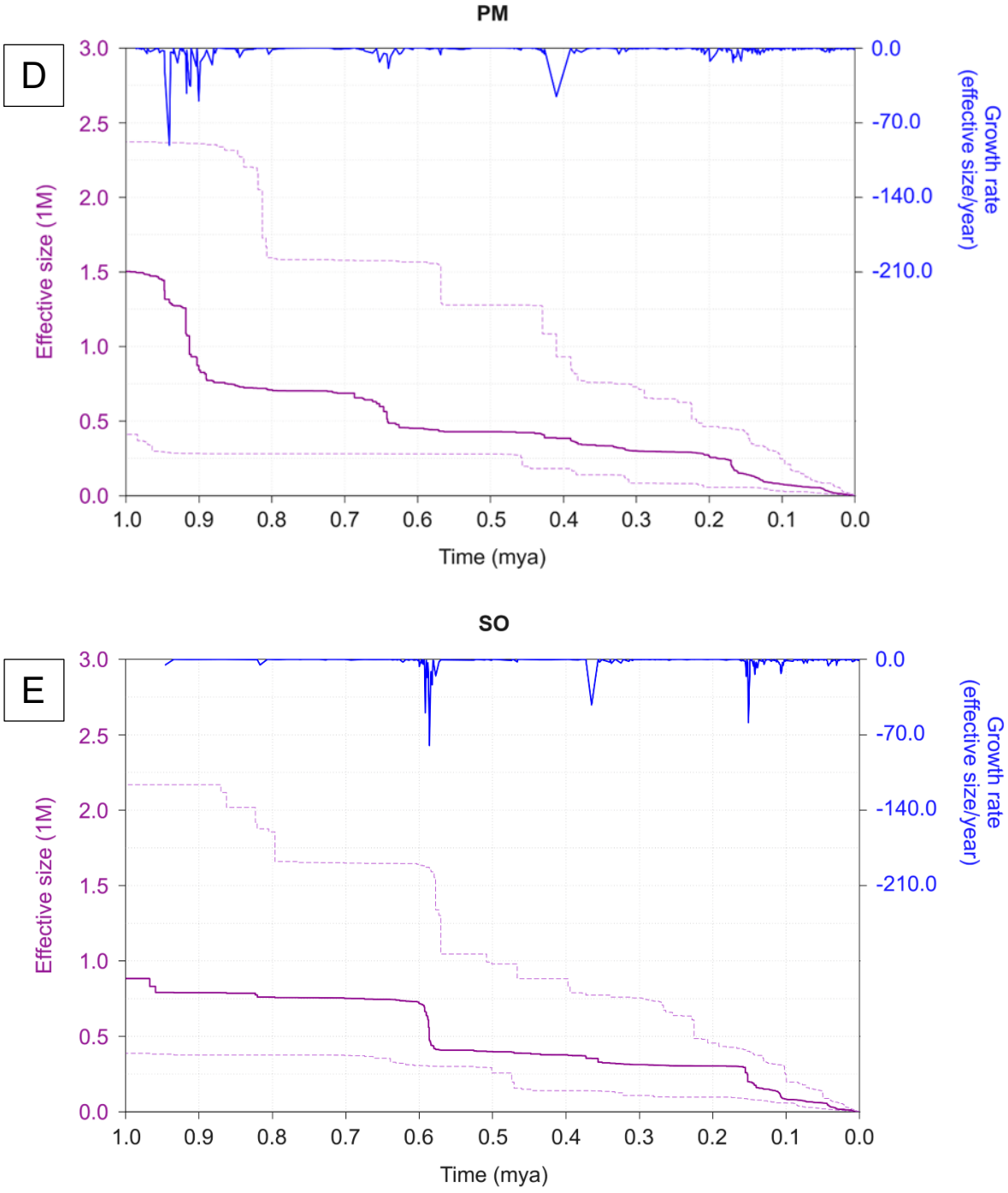


Figure S2: Inferred demographic history of each *Abies alba* sampling point. Purple lines indicate effective size in millions at every time point in millions of years ago (the solid line represents the median; the dashed lines represent the 95% confidence interval). The blue line indicates the speed of changes in effective size over the years. A. CO. B. GA. C. PE. D. PM. E. SO. [Part 2 of 2]

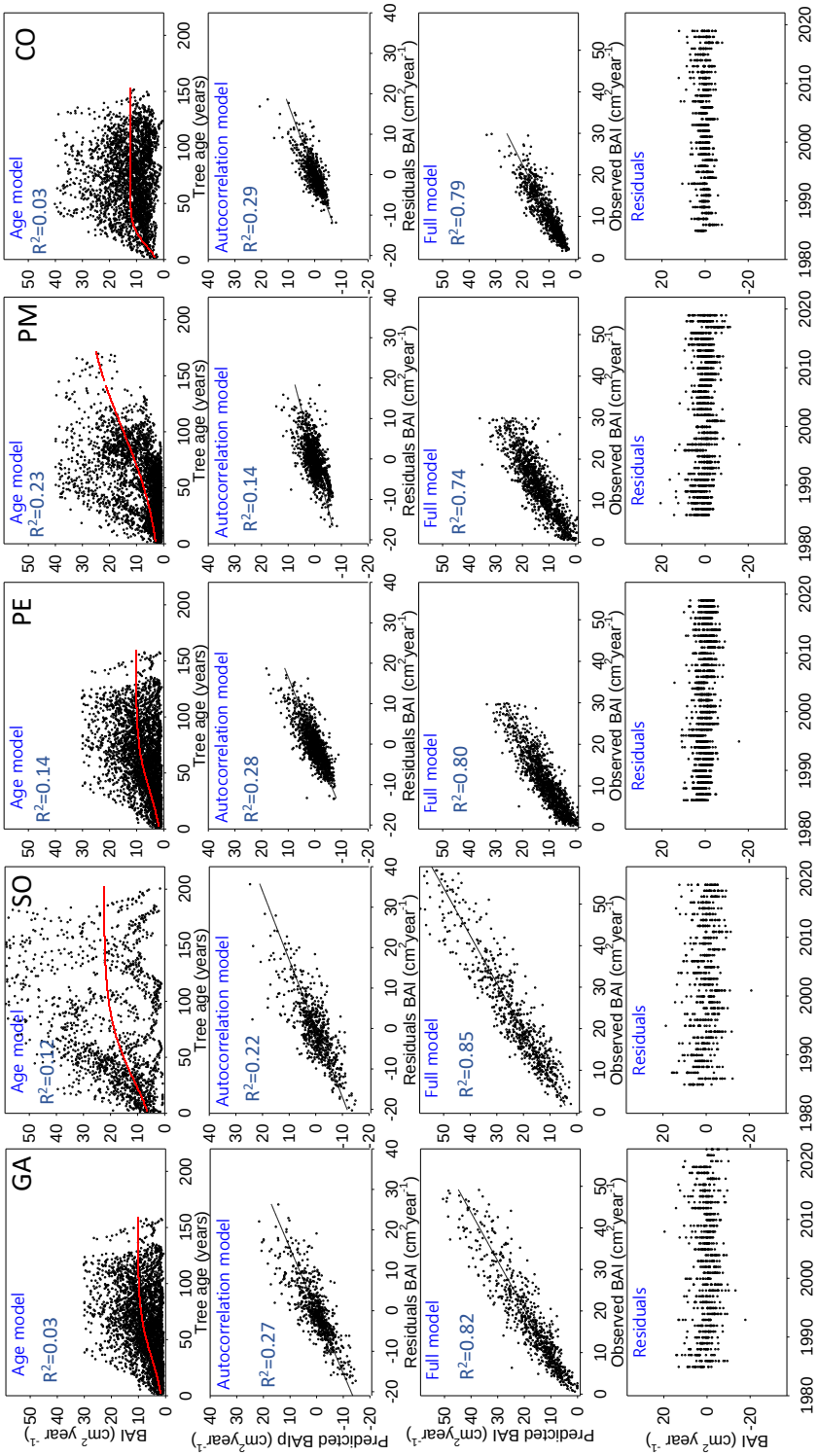


Figure S3: Components of the growth (BAI, basal area increment) modelling exercise considering tree age, BAI autocorrelation and the full model. Plots present the regressions between BAI residual or observed vs. predicted BAI. The lowermost plots show the BAI residuals variability through time. Insets indicate the coefficient of determination (R2) of tree age-effect and autocorrelation partial correlations, and the full model. Populations are abbreviated as: GA, Gamueta; SO, Selva de Oza; PE, Paco Ezpela; PM, Paco Mayor; and CO, Cotatuero.

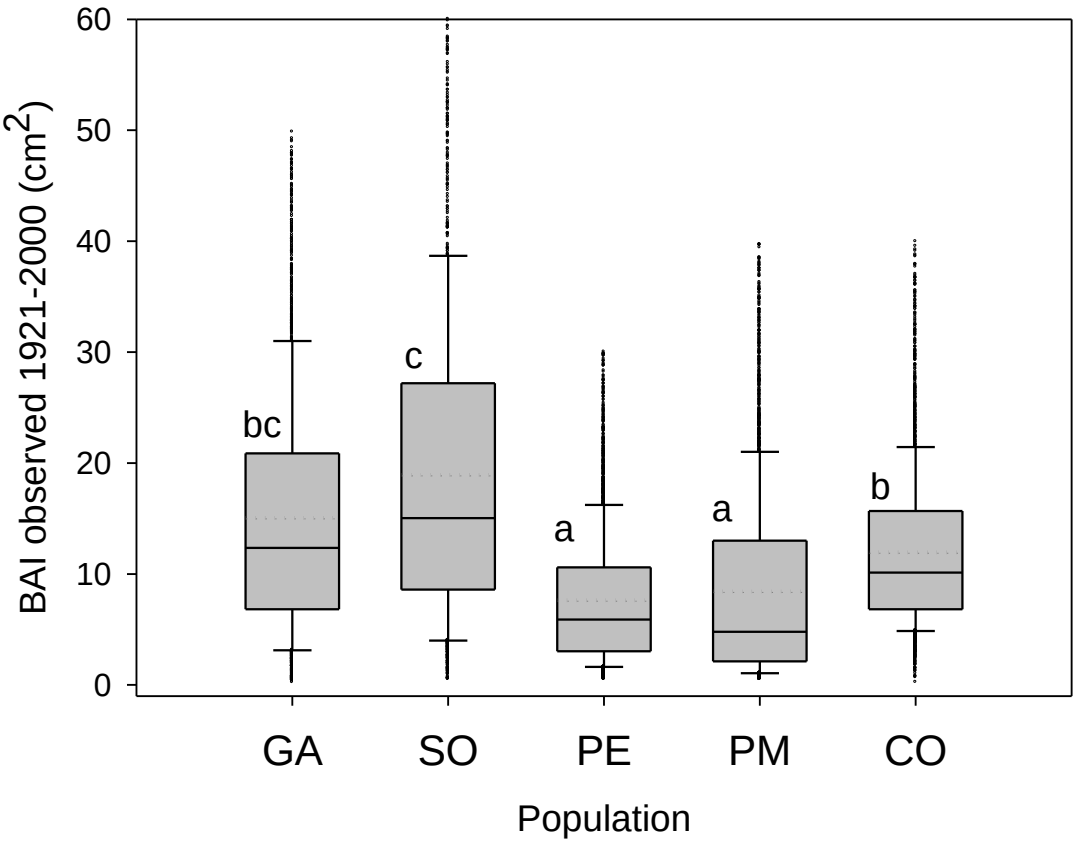


Figure S4: Observed mean growth (basal area increment, BAI) for the period 1921-2000 of trees sampled in Gamueta, GA (n=21); Selva de Oza, SO (n=20); Paco Ezpela, PE (n=40); Paco Mayor, PM (n=40); and Cotatuero, CO (n=24). In the box-plot figure, error bars represent the 5th/95th percentiles, boxes stand for the standard error, solid lines represent the median, dashed lines are the mean values, and points are outliers. Different letters indicate significant differences for ANOVA, *p*-value<0.05.

Table S1: Each population's bioclimatic variables. BIO1: annual mean temperature (ºC). BIO2: mean diurnal range (ºC). BIO3: isothermality (%). BIO4: temperature seasonality (%). BIO5: max temperature of warmest month (ºC). BIO6: min temperature of coldest month (ºC). BIO7: temperature annual range (ºC). BIO8: mean temperature of wettest quarter (ºC). BIO9: mean temperature of driest quarter (ºC). BIO10: mean temperature of warmest quarter (ºC). BIO11: mean temperature of coldest quarter (ºC). BIO12: annual precipitation (mm). BIO13: precipitation of wettest month (mm). BIO14: precipitation of driest month (mm). BIO15: precipitation seasonality (%). BIO16: precipitation of wettest quarter (mm). BIO17: precipitation of driest quarter (mm). BIO18: precipitation of warmest quarter (mm). BIO19: precipitation of coldest quarter (mm).

	CO	GA	PE	PM	SO
BIO1	5.22083	6.22917	9.73750	8.55833	7.47083
BIO2	10.10833	9.47500	9.40833	9.78333	9.52500
BIO3	36.62440	35.75472	37.33466	37.48404	36.35496
BIO4	625.16711	605.77319	568.88300	583.53870	594.36926
BIO5	21.5	22.0	24.7	24.0	22.9
BIO6	-6.1	-4.5	-0.5	-2.1	-3.3
BIO7	27.6	26.5	25.2	26.1	26.2
BIO8	2.35000	0.51667	6.90000	5.70000	4.66667
BIO9	13.43333	14.11667	17.26667	16.26667	15.23333
BIO10	13.43333	14.11667	17.26667	16.26667	15.23333
BIO11	-1.68333	-0.56667	3.46667	2.13333	0.78333
BIO12	1167	1120	867	944	1028
BIO13	127	116	90	100	107
BIO14	63	56	43	47	52
BIO15	19.30247	21.11128	21.86723	21.06077	20.79447
BIO16	342	334	263	282	306
BIO17	220	197	154	171	184
BIO18	220	197	154	171	184
BIO19	308	315	244	259	286

Table S2: Each population's bioclimatic variables for the MIROC6 ssp126 update of RCP2.6 scenario (low concentration of greenhouse gases in the atmosphere).

	CO	GA	PE	PM	SO
BIO1	5.57917	7.05417	12.65000	10.42500	8.66250
BIO2	11.32500	10.54167	10.61667	10.85000	10.87500
BIO3	35.28037	35.02215	36.99187	36.65540	36.00993
BIO4	732.37305	689.07532	644.88898	664.67865	684.89923
BIO5	25.1	25.2	29.8	28.2	26.8
BIO6	-7.0	-4.9	1.1	-1.4	-3.4
BIO7	32.1	30.1	28.7	29.6	30.2
BIO8	-1.20000	0.53333	6.33333	4.05000	2.08333
BIO9	15.23333	16.01667	21.06667	19.13333	17.53333
BIO10	15.23333	16.03333	21.11667	19.15000	17.58333
BIO11	-2.15000	-0.38333	5.71667	3.33333	1.20000
BIO12	1279	1169	790	925	1070
BIO13	146	131	90	103	119
BIO14	60	54	36	44	51
BIO15	24.71828	26.17723	26.65796	25.18192	25.23211
BIO16	406	371	255	292	337
BIO17	215	186	127	152	173
BIO18	215	188	128	155	175
BIO19	369	345	235	270	314

Table S3: Each population's bioclimatic variables for the MIROC6 ssp585 update of RCP8.5 scenario (high concentration of greenhouse gases in the atmosphere).

	CO	GA	PE	PM	SO
BIO1	10.09167	11.25833	16.99167	14.79167	12.91667
BIO2	12.88333	11.96667	12.15000	12.41667	12.33333
BIO3	32.69881	33.05709	34.51704	34.30019	33.78996
BIO4	933.05176	857.74768	823.35461	848.19550	859.57263
BIO5	34.9	33.8	38.8	37.3	35.6
BIO6	-4.5	-2.4	3.6	1.1	-0.9
BIO7	39.4	36.2	35.2	36.2	36.5
BIO8	1.63333	3.33333	9.16667	6.86667	4.88333
BIO9	22.45000	22.58333	27.88333	26.10000	24.21667
BIO10	22.63333	22.58333	27.93333	26.10000	24.26667
BIO11	0.33333	2.05000	8.21667	5.80000	3.65000
BIO12	1137	1043	702	823	951
BIO13	133	122	83	97	110
BIO14	54	46	30	37	42
BIO15	27.04473	29.67850	30.55195	28.59488	28.79618
BIO16	378	346	237	273	313
BIO17	185	156	105	128	144
BIO18	187	156	106	128	145
BIO19	353	330	224	259	299

Table S4: Non-parametric Pearson correlation between each pair of results: all populations together, each population separately (GA, PE, SO, PM, CO) and each of the 3 identified genetic clusters (CO, SO & PM, and GA & PE). * indicates p-value < 0.001.

	All pops	GA	PE	SO	PM	CO	SO & PM	GA & PE
All pops	-							
GA	1.000*	-						
PE	1.000*	0.894*	-					
SO	1.000*	0.883*	0.901*	-				
PM	0.999*	0.833*	0.983*	0.918*	-			
CO	0.999*	0.886*	0.967*	0.980*	0.951*	-		
SO & PM	1.000*	0.882*	0.976*	0.900*	0.988*	0.950*	-	
GA & PE	1.000*	0.866*	0.990*	0.948*	0.986*	0.966*	0.979*	-

Table S5: Genome-environment associations. BIO8 showed none.

SNP	BIO1	BIO2	BIO3	BIO4	BIO5	BIO6	BIO7	BIO8	BIO9	BIO10	BIO11	BIO12	BIO13	BIO14	BIO15	BIO16	BIO17	BIO18	BIO19
159	X	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X	
179															X				
278							X								X				
288							X												
350	X			X	X	X	X		X	X	X	X	X	X		X	X	X	
448		X																	
608		X																	
629							X												
681				X		X	X						X	X	X		X	X	
752		X													X				
784		X		X			X						X						
811															X				
858		X																	
939		X																	
948		X													X				
983	X	X		X	X	X	X		X	X	X		X	X	X		X	X	
1172							X								X				
1188	X		X	X	X	X	X		X	X	X	X	X	X		X	X	X	X
1344															X				
1378				X			X										X	X	
1517		X					X								X				
1544	X								X	X	X	X	X			X			
1808		X																	
1834							X												
1858				X		X	X										X	X	
1934															X				
2061				X		X	X						X		X		X	X	
2119		X																	
2128		X																	
2131															X				
2249															X				
2438															X				
2446							X												
2452		X																	
2459	X			X		X	X						X	X			X	X	
2484	X			X							X		X	X			X	X	
2591	X			X	X	X	X		X	X	X	X	X	X	X		X	X	
2645		X																	
2794		X																	
2940							X								X				
2968		X																	
2981															X				
3063	X			X		X	X				X	X	X	X			X	X	
3111	X			X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
3306	X			X	X	X	X		X	X	X	X	X	X		X	X	X	X
3313							X								X				
3337							X								X				
3380															X				
3400	X			X		X	X		X	X	X	X	X			X	X	X	
3450	X			X		X					X		X	X			X	X	
3491	X			X	X	X	X		X	X	X	X	X	X		X	X	X	
3537	X	X		X	X	X	X		X	X	X	X	X	X	X	X	X	X	X
3640							X												
3702							X												
3779	X			X		X	X		X	X	X	X	X	X		X	X	X	
3898				X			X										X	X	
3937																			X
Total	16	18	1	21	9	17	31	0	12	12	15	12	19	15	24	10	20	20	5

APPENDICES

Appendix A: Gamueta Population Report

Linear mixed models explaining the effect of climate on basal area increment (BAI) after accounting for ontogenetic effects (age model). Selected models are highlighted in bold. Null model considered the effect of BAI as a constant. The best models were selected based on the Akaike Information Criteria corrected by sample size (AICc). K represents the number of variables included in the model plus constant and error terms, Δi is the difference in AICc respect the best model, and Wi is the relative probability to be the best model for the observed data. Taup, Twi, Tsp, Tsu, Tau are standardised temperatures of previous autumn and winter, spring, summer and autumn of current year, respectively. Similar names for standardised precipitation values (Paup, Pwi, Psp, Psu, Pau).

Population	Gamueta			
Trees (n)	21			
Data (n)	721			
Random effect	Tree			
	Fixed effects	K	Δi	Wi
	Taup+Tsp+Tau+Paup	4	0.00	0.5877
	Taup+Tau+Paup	6	2.59	0.2653
	Taup+Tau	5	2.83	0.1426
	Taup	3	10.63	0.0029
	Taup+Tsp+Tau+Paup+Pwi	7	11.97	0.0015
	Taup+Tsp+Tau+Paup+Pwi+Pau	8	22.58	0.0000
	Taup+Tsp+Tsu+Tau+Paup+Pwi+Pau	9	24.59	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Pau	10	27.49	0.0000
	null model	2	27.50	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psu+Pau	11	38.19	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	12	49.63	0.0000

Age-effect model

Call: lm(formula = bai ~ poly(age, 2), data = ac)

Residuals:

Min	1Q	Median	3Q	Max
-14.419	-6.045	-1.835	4.711	29.691

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	11.6618	0.1425	81.84	< 2e-16 ***
poly(age, 2)1	177.7469	8.0405	22.11	< 2e-16 ***
poly(age, 2)2	-55.3183	8.0405	-6.88	7.18e-12 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 8.041 on 3181 degrees of freedom

Multiple R-squared: 0.1442, Adjusted R-squared: 0.1437

F-statistic: 268 on 2 and 3181 DF, p-value: < 2.2e-16

Climate-effect model

Linear mixed-effects model fit by REML

Data: NULL

AIC	BIC	logLik
5018.577	5050.593	-2502.289

Random effects: Formula: ~1 | tree

	(Intercept)	Residual
StdDev:	8.530037	7.335201

Fixed effects: b ~ Taup + Tsp + Tau + Paup

	Value	Std.Error	DF	t-value	p-value
(Intercept)	11.939007	3.740776	696	3.191586	0.0015
Taup	-1.012497	0.267667	696	-3.782674	0.0002
Tsp	0.556704	0.278250	696	2.000732	0.0458
Tau	-1.174680	0.274855	696	-4.273819	0.0000
Paup	0.008834	0.002888	696	3.058849	0.0023

Correlation:

	(Intr)	Taup	Tsp	Tau
Taup	-0.632			
Tsp	-0.221	0.039		
Tau	-0.438	0.056	-0.298	
Paup	-0.400	0.269	0.018	-0.050

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-3.03091077	-0.58811748	-0.07531665	0.51386436	3.59729035

Number of Observations: 721 Number of Groups: 21

Autocorrelation-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
4244.072	4262.383	-2118.036	StdDev:	5.880278	4.292428

Fixed effects: c ~ baip Correlation: (Intr)

	Value	Std.Error	DF	t-value	p-value	baip -0.268
(Intercept)	-13.044212	1.3426397	699	-9.71535	0	
baip	0.734224	0.0202595	699	36.24095	0	

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-4.2001980	-0.5847729	-0.1028285	0.4877086	4.5195380

Number of Observations: 721 Number of Groups: 21

Final model

Call: lm(formula = bai ~ results$fit.final)

Residuals:

Min	1Q	Median	3Q	Max
-15.6246	-2.3557	-0.4393	1.9255	13.8338

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.26344	0.31709	0.831	0.406
results$fit.final	0.98338	0.01768	55.633	<2e-16 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.885 on 683 degrees of freedom

Multiple R-squared: 0.8192, Adjusted R-squared: 0.819

F-statistic: 3095 on 1 and 683 DF, p-value: < 2.2e-16

Appendix B: Selva de Oza Population Report

Linear mixed models explaining the effect of climate on basal area increment (BAI) after accounting for ontogenetic effects (age model). Selected models are highlighted in bold. Null model considered the effect of BAI as a constant. The best models were selected based on the Akaike Information Criteria corrected by sample size (AICc). K represents the number of variables included in the model plus constant and error terms, Δi is the difference in AICc respect the best model, and Wi is the relative probability to be the best model for the observed data. Taup, Twi, Tsp, Tsu, Tau are standardised temperatures of previous autumn and winter, spring, summer and autumn of current year, respectively. Similar names for standardised precipitation values (Paup, Pwi, Psp, Psu, Pau).

Population	Selva de Oza
Trees (n)	20
Data (n)	653
Random effect	tree

Fixed effects	K	Δi	Wi
Taup+Tsp+Tsu+Tau	6	0.00	0.9786
Taup+Tsp+Tsu+Tau+Paup	7	8.27	0.0157
Taup+Tsp+Tsu+Tau+Paup+Pwi	8	11.74	0.0028
Taup+Tsp+Tau	5	11.76	0.0027
Taup+Tau	4	18.78	0.0001
Taup+Tsp+Tsu+Tau+Paup+Pwi+Psp	9	18.80	0.0001
Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp	10	20.66	0.0000
Taup	3	26.26	0.0000
Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Pau	11	32.11	0.0000
null model	2	41.22	0.0000
Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	12	42.76	0.0000

Age-effect model

Formula: bai ~ SSlogis(age, Asym, xmid, scal)

Parameters:	Estimate	Std. Error	t value	Pr(>|t|)
Asym	22.4735	0.7705	29.168	< 2e-16 ***
xmid	28.2683	3.0298	9.330	< 2e-16 ***
scal	29.2038	3.8183	7.648	3.22e-14 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 12.58 on 1886 degrees of freedom

Number of iterations to convergence: 5

Achieved convergence tolerance: 1.695e-06

Climate-effect model

Linear mixed-effects model fit by REML

Data: NULL

AIC	BIC	logLik
4647.178	4678.495	-2316.589

Random effects: Formula: ~1 | tree

	(Intercept)	Residual
StdDev:	10.32574	7.943961

Fixed effects: b ~ Taup + Tsp + Tsu + Tau

	Value	Std.Error	DF	t-value	p-value
(Intercept)	38.11059	6.027870	629	6.322397	0e+00
Taup	-1.28853	0.290962	629	-4.428521	0e+00
Tsp	1.26089	0.324268	629	3.888420	1e-04
Tsu	-1.29424	0.342989	629	-3.773423	2e-04
Tau	-1.34815	0.308132	629	-4.375240	0e+00

Correlation:

	(Intr)	Taup	Tsp	Tsu
Taup	-0.376			
Tsp	0.016	0.039		
Tsu	-0.751	-0.006	-0.223	
Tau	-0.394	0.065	-0.302	0.103

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.53614999	-0.60962828	-0.08358075	0.49884581	4.50687729

Number of Observations: 653 Number of Groups: 20

Autocorrelation-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
4057.723	4075.637	-2024.861	StdDev:	8.763923	5.009284

Fixed effects: c ~ baip

	Value	Std.Error	DF	t-value	p-value	Correlation:
(Intercept)	-17.711617	2.054532	632	-8.620755	0	(Intr)
baip	0.690232	0.022627	632	30.504884	0	baip -0.283

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-4.23322175	-0.69035504	-0.05899584	0.61801972	3.67061396

Number of Observations: 653 Number of Groups: 20

Final model

Call: lm(formula = bai ~ results$fit.final)

Residuals:

Min	1Q	Median	3Q	Max
-19.9962	-3.4126	-0.3779	2.9934	18.6052

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.54624	0.42014	1.30	0.194
results$fit.final	0.97669	0.01593	61.32	<2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 4.928 on 651 degrees of freedom

Multiple R-squared: 0.8524, Adjusted R-squared: 0.8522

F-statistic: 3760 on 1 and 651 DF, p-value: < 2.2e-16

Appendix C: Paco Ezpela Population Report

Linear mixed models explaining the effect of climate on basal area increment (BAI) after accounting for ontogenetic effects (age model). Selected models are highlighted in bold. Null model considered the effect of BAI as a constant. The best models were selected based on the Akaike Information Criteria corrected by sample size (AICc). K represents the number of variables included in the model plus constant and error terms, Δi is the difference in AICc respect the best model, and Wi is the relative probability to be the best model for the observed data. Taup, Twi, Tsp, Tsu, Tau are standardised temperatures of previous autumn and winter, spring, summer and autumn of current year, respectively. Similar names for standardised precipitation values (Paup, Pwi, Psp, Psu, Pau).

Population	Paco Ezpela			
Trees (n)	40			
Data (n)	1290			
Random effect	tree			
	Fixed effects	K	Δi	Wi
	Tsp+Tsu+Paup+Pwi+Psp+Pau	8	0.00	0.3864
	Tsp+Tsu+Tau+Paup+Pwi+Psp+Pau	9	0.03	0.3809
	Tsp+Tsu+Paup+Psp+Pau	7	**1.41**	0.1912
	Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Pau	10	4.47	0.0414
	Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	11	16.67	0.0001
	Tsp+Tsu+Psp+Pau	6	19.96	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	12	20.76	0.0000
	Tsp+Psp+Pau	5	32.79	0.0000
	Tsp+Psp	4	45.58	0.0000
	Psp	3	103.45	0.0000
	null model	2	139.52	0.0000

Age-effect model

Formula: bai ~ SSlogis(age, Asym, xmid, scal)

Parameters:	Estimate	Std. Error	t value	Pr(>|t|)
Asym	10.2510	0.2229	45.99	<2e-16 ***
xmid	32.7102	1.4739	22.19	<2e-16 ***
scal	20.4773	1.4779	13.86	<2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.166 on 4058 degrees of freedom

Number of iterations to convergence: 4

Achieved convergence tolerance: 4.747e-06

Climate-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
7983.345	8024.607	-3983.672	StdDev:	4.305287	4.992941

Fixed effects: b ~ Tsp + Tsu + Paup + Psp + Pau

	Value	Std.Error	DF	t-value	p-value
(Intercept)	-4.953404	2.9729819	1245	-1.666140	0.0959
Tsp	1.249991	0.1463795	1245	8.539384	0.0000
Tsu	-0.722341	0.1713325	1245	-4.216022	0.0000
Paup	0.008138	0.0014337	1245	5.676202	0.0000
Psp	0.013317	0.0018367	1245	7.250150	0.0000
Pau	0.008119	0.0014160	1245	5.733935	0.0000

Correlation:	(Intr)	Tsp	Tsu	Paup	Psp
Tsp	-0.276				
Tsu	-0.863	-0.040			
Paup	-0.149	-0.030	-0.016		
Psp	-0.642	0.307	0.434	-0.089	
Pau	-0.214	0.072	-0.026	0.075	0.137

Standardized Within-Group Residuals:	Min	Q1	Med	Q3	Max
	-2.68839273	-0.67478084	-0.08750474	0.55901846	3.77067908

Number of Observations: 1290 Number of Groups: 40

Autocorrelation-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
6714.29	6734.933	-3353.145	StdDev:	2.459349	3.100815

Fixed effects: c ~ baip

	Value	Std.Error	DF	t-value	p-value	Correlation:
(Intercept)	-7.211888	0.4316543	1249	-16.70756	0	(Intr)
baip	0.648687	0.0147268	1249	44.04791	0	baip -0.38

Standardized Within-Group Residuals:	Min	Q1	Med	Q3	Max
	-5.143442542	-0.672183456	-0.002908932	0.584399074	4.504613287

Number of Observations: 1290 Number of Groups: 40

Final model

Call: lm(formula = bai ~ results$fit.final)

Residuals:	Min	1Q	Median	3Q	Max
	-15.2701	-2.0401	-0.1091	1.7764	14.2312

Coefficients:	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.71145	0.16569	4.294	1.89e-05 ***
results$fit.final	0.93412	0.01321	70.718	< 2e-16 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.027 on 1288 degrees of freedom

Multiple R-squared: 0.7952, Adjusted R-squared: 0.795

F-statistic: 5001 on 1 and 1288 DF, p-value: < 2.2e-16

Appendix D: Paco Mayor Population Report

Report R4. Paco Mayor Population

Table SX. Linear mixed models explaining the effect of climate on basal area increment (BAI) after accounting for ontogenetic effects (age model). Selected models are highlighted in bold. Null model considered the effect of BAI as a constant. The best models were selected on the basis of the Akaike Information Criteria corrected by sample size (AICc). K represents the number of variables included in the model plus constant and error terms, Δi is the difference in AICc respect the best model, and Wi is the relative probability to be the best model for the observed data. Taup, Twi, Tsp, Tsu, Tau are standardised temperatures of previous autumn and winter, spring, summer and autumn of current year, respectively. Similar names for standardised precipitation values (Paup, Pwi, Psp, Psu, Pau).

Population	Paco Mayor			
Trees (n)	40			
Data (n)	1249			
Random effect	tree			
	Fixed effects	K	Δi	Wi
	Taup+Tsp+Tsu+Tau+Paup	7	0.00	0.5841
	Taup+Tsp+Tsu+Tau+Paup+Pau	8	0.69	0.4139
	Taup+Tsp+Tsu+Tau+Paup+Pwi+Pau	9	11.46	0.0019
	Taup+Tsp+Tsu+Tau+Paup+Pwi+Psu+Pau	10	21.12	0.0000
	Taup+Tsp+Tau+Paup	6	26.93	0.0000
	Taup+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	11	32.23	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	12	36.61	0.0000
	Tsp+Tau+Paup	5	57.18	0.0000
	Tsp+Tau	4	112.28	0.0000
	Tau	3	208.04	0.0000
	null model	2	255.36	0.0000

Age-effect model

Formula: bai ~ SSlogis(age, Asym, xmid, scal)

Parameters:	Estimate	Std. Error	t value	Pr(>|t|)
Asym	29.499	3.606	8.180	3.98e-16 ***
xmid	108.427	11.454	9.467	< 2e-16 ***
scal	47.560	3.315	14.349	< 2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.968 on 3386 degrees of freedom

Number of iterations to convergence: 6

Achieved convergence tolerance: 2.165e-06

Climate-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
7688.661	7729.663	-3836.33	StdDev:	5.422515	4.900389

Fixed effects: b ~ Taup + Tsp + Tsu + Tau + Paup

	Value	Std.Error	DF	t-value	p-value
(Intercept)	22.788218	2.8085577	1204	8.113851	0
Taup	-0.817381	0.1344593	1204	-6.079026	0
Tsp	1.734068	0.1457326	1204	11.898969	0
Tsu	-0.874123	0.1564326	1204	-5.587855	0
Tau	-1.728029	0.1401647	1204	-12.328565	0
Paup	0.009317	0.0014845	1204	6.276158	0

Correlation:

	(Intr)	Taup	Tsp	Tsu	Tau
Taup	-0.440				
Tsp	0.020	0.016			
Tsu	-0.750	0.017	-0.218		
Tau	-0.366	0.066	-0.304	0.090	
Paup	-0.311	0.285	0.027	0.049	-0.061

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-3.39687981	-0.62733273	-0.03353466	0.57138957	3.75025962

Number of Observations: 1249 Number of Groups: 40

Autocorrelation-effect model

Linear mixed-effects model fit by REML

Data: NULL Random effects: Formula: ~1 | tree

AIC	BIC	logLik		(Intercept)	Residual
6932.646	6953.16	-3462.323	StdDev:	2.228119	3.711565

Fixed effects: c ~ baip

	Value	Std.Error	DF	t-value	p-value	Correlation:
(Intercept)	-6.834324	0.4367304	1208	-15.64884	0	(Intr)
baip	0.473562	0.0162270	1208	29.18351	0	baip -0.538

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-4.34889855	-0.59513962	-0.05238795	0.55449328	5.48211609

Number of Observations: 1249 Number of Groups: 40

Final model

Call: lm(formula = bai ~ results$fit.final)

Residuals:

Min	1Q	Median	3Q	Max
-14.2608	-2.3405	-0.3299	2.1054	19.6496

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.20651	0.23357	5.165	2.79e-07 ***
results$fit.final	0.91165	0.01538	59.272	< 2e-16 ***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.611 on 1247 degrees of freedom

Multiple R-squared: 0.738, Adjusted R-squared: 0.7378

F-statistic: 3513 on 1 and 1247 DF, p-value: < 2.2e-16

Appendix E: Cotatuero Population Report

Linear mixed models explaining the effect of climate on basal area increment (BAI) after accounting for ontogenetic effects (age model). Selected models are highlighted in bold. Null model considered the effect of BAI as a constant. The best models were selected based on the Akaike Information Criteria corrected by sample size (AICc). K represents the number of variables included in the model plus constant and error terms, Δi is the difference in AICc respect the best model, and Wi is the relative probability to be the best model for the observed data. Taup, Twi, Tsp, Tsu, Tau are standardised temperatures of previous autumn and winter, spring, summer and autumn of current year, respectively. Similar names for standardised precipitation values (Paup, Pwi, Psp, Psu, Pau).

Population	Cotatuero			
Trees (n)	24			
Data (n)	818			
Random effect	tree			
	Fixed effects	K	Δi	Wi
	Taup+Paup+Psp	5	0.00	0.6855
	Taup+Tsu+Paup+Psp+Pau	7	3.56	0.1154
	Taup+Paup+Psp+Pau	6	4.01	0.0921
	Paup+Psp	4	4.50	0.0722
	Paup	3	6.03	0.0335
	Taup+Tsu+Paup+Psp+Psu+Pau	8	13.20	0.0009
	Taup+Tsu+Tau+Paup+Psp+Psu+Pau	9	15.65	0.0003
	Taup+Twi+Tsu+Tau+Paup+Psp+Psu+Pau	10	19.11	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Psp+Psu+Pau	11	22.51	0.0000
	Taup+Twi+Tsp+Tsu+Tau+Paup+Pwi+Psp+Psu+Pau	12	35.63	0.0000
	null model	2	64.89	0.0000

Age-effect model

Formula: bai ~ SSlogis(age, Asym, xmid, scal)

Parameters:	Estimate	Std. Error	t value	Pr(>|t|)
Asym	11.4155	0.1264	90.287	<2e-16 ***
xmid	11.2318	0.8153	13.776	<2e-16 ***
scal	8.6686	0.8939	9.698	<2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.309 on 2827 degrees of freedom

Number of iterations to convergence: 6

Achieved convergence tolerance: 4.734e-06

Climate-effect model

Linear mixed-effects model fit by REML

Data: NULL

AIC	BIC	logLik
4725.638	4753.85	-2356.819

Random effects: Formula: ~1 | tree

	(Intercept)	Residual
StdDev:	3.902892	4.053613

Fixed effects: b ~ Taup + Paup + Psp

	Value	Std.Error	DF	t-value	p-value
(Intercept)	-7.663296	1.5359894	791	-4.989159	0.0000
Taup	0.394275	0.1332322	791	2.959305	0.0032
Paup	0.006619	0.0016160	791	4.095944	0.0000
Psp	0.005518	0.0015757	791	3.501964	0.0005

Correlation:	(Intr)	Taup	Paup
Taup	-0.695		
Paup	-0.461	0.157	
Psp	-0.270	-0.112	0.027

Standardized Within-Group Residuals:	Min	Q1	Med	Q3	Max
	-2.8960408	-0.6483620	-0.1200664	0.4791369	4.6761900

Number of Observations: 818

Number of Groups: 24

Autocorrelation-effect model

Linear mixed-effects model fit by REML

Data: NULL

AIC	BIC	logLik
3994.705	4013.523	-1993.353

Random effects: Formula: ~1 | tree

	(Intercept)	Residual
StdDev:	2.845014	2.615651

Fixed effects: c ~ baip

	Value	Std.Error	DF	t-value	p-value	Correlation:
(Intercept)	-7.870441	0.6346779	793	-12.40068	0	(Intr)
baip	0.730635	0.0221934	793	32.92119	0	baip -0.377

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-3.21657073	-0.58431583	-0.04046109	0.48614794	4.79249381

Number of Observations: 818 Number of Groups: 24

Final model

Call: lm(formula = bai ~ results$fit.final)

Residuals:

Min	1Q	Median	3Q	Max
-8.3158	-1.5424	-0.1158	1.2739	12.6071

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.12317	0.21243	0.58	0.562
results$fit.final	0.98857	0.01785	55.39	<2e-16 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

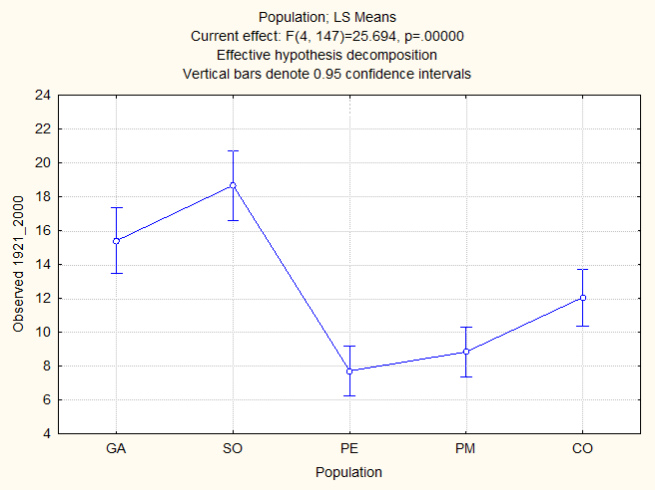
Residual standard error: 2.579 on 816 degrees of freedom

Multiple R-squared: 0.7899, Adjusted R-squared: 0.7896

F-statistic: 3068 on 1 and 816 DF, p-value: < 2.2e-16

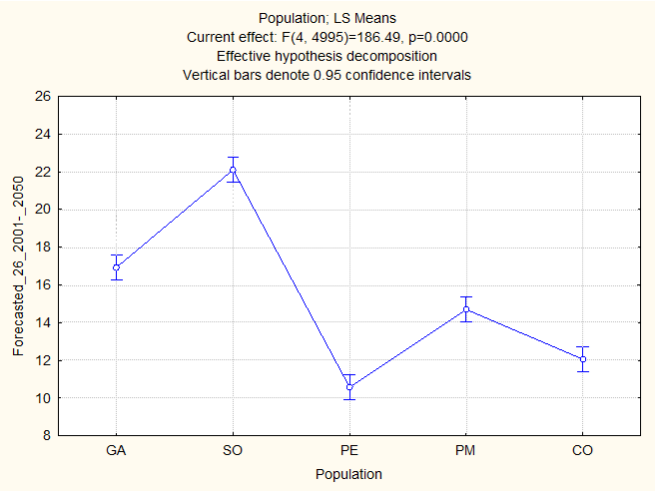
Appendix F: ANOVA results for mean growth (BAI) for the period 1921-2000

Population	{1} 15.406	{2} 18.672	{3} 7.7272	{4} 8.8449	{5} 12.058
GA		0.153969	0.000017	0.000018	0.077109
SO	0.153969		0.000017	0.000017	0.000025
PE	0.000017	0.000017		0.819784	0.001107
PM	0.000018	0.000017	0.819784		0.034474
CO	0.077109	0.000025	0.001107	0.034474	



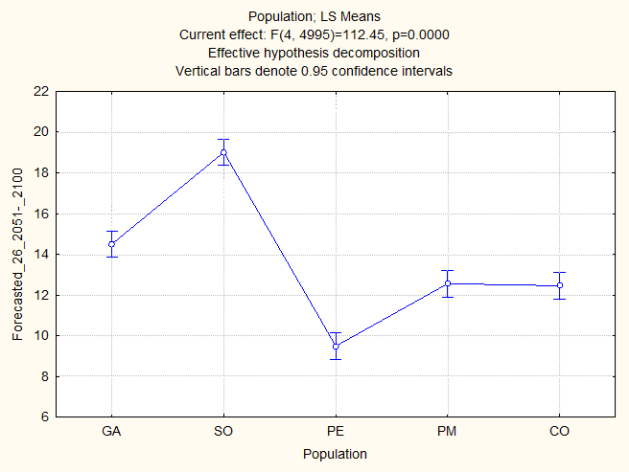
Appendix G: ANOVA results for mean growth (BAI) for the period 2001-2050 under the RCP 2.6 scenario

Population	{1} 16.934	{2} 22.098	{3} 10.576	{4} 14.700	{5} 12.070
GA		0.000017	0.000017	0.000035	0.000017
SO	0.000017		0.000017	0.000017	0.000017
PE	0.000017	0.000017		0.000017	0.012521
PM	0.000035	0.000017	0.000017		0.000017
CO	0.000017	0.000017	0.012521	0.000017	



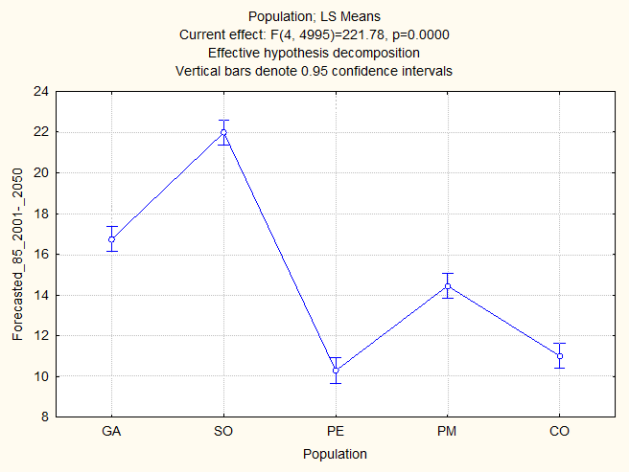
Appendix H: ANOVA results for mean growth (BAI) for the period 2051-2100 under the RCP 2.6 scenario

Population	{1} 14.505	{2} 19.012	{3} 9.4957	{4} 12.556	{5} 12.457
GA		0.000017	0.000017	0.000319	0.000134
SO	0.000017		0.000017	0.000017	0.000017
PE	0.000017	0.000017		0.000017	0.000017
PM	0.000319	0.000017	0.000017		0.999552
CO	0.000134	0.000017	0.000017	0.999552	



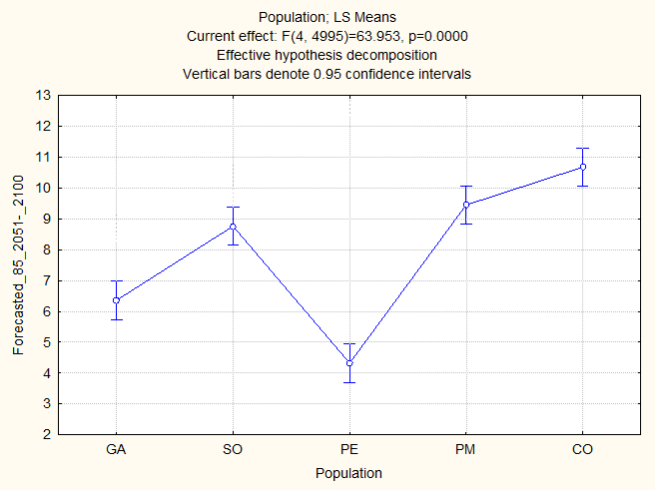
Appendix I: ANOVA results for mean growth (BAI) for the period 2001-2050 under the RCP 8.5 scenario

Population	{1} 16.756	{2} 21.980	{3} 10.290	{4} 14.450	{5} 11.015
GA		0.000017	0.000017	0.000020	0.000017
SO	0.000017		0.000017	0.000017	0.000017
PE	0.000017	0.000017		0.000017	0.490681
PM	0.000020	0.000017	0.000017		0.000017
CO	0.000017	0.000017	0.490681	0.000017	



Appendix J: ANOVA results for mean growth (BAI) for the period 2051-2100 under the RCP 8.5 scenario

Population	{1} 6.3617	{2} 8.7584	{3} 4.3265	{4} 9.4434	{5} 10.669
GA		0.000018	0.000075	0.000017	0.000017
SO	0.000018		0.000017	0.548337	0.000225
PE	0.000075	0.000017		0.000017	0.000017
PM	0.000017	0.548337	0.000017		0.050617
CO	0.000017	0.000225	0.000017	0.050617	



ARTICLE 3

SECTION 2: EPIGENOMICS

ARTICLE 4

Challenges and perspectives in the epigenetics of climate change-induced forests decline

Desafíos y perspectivas en la epigenética del decaimiento de los bosques inducido por el cambio climático

Las especies forestales son altamente vulnerables a los efectos del cambio climático. Como organismos sésiles con largos tiempos de generación, su adaptación a un ambiente en constante cambio podría depender de la epigenética cuando las frecuencias alélicas no puedan cambiar lo suficientemente rápido. Sin embargo, la actual falta de conocimiento en este campo es notable, debido a los muchos desafíos a los que se enfrentan los investigadores al estudiarlo. El gran tamaño de los genomas, la ausencia de genomas de referencia y anotación de calidad y el análisis de grandes cantidades de datos están entre estas dificultades que limitan el entendimiento sobre cómo el cambio climático afecta a las modificaciones epigenéticas en árboles. A pesar de ello, se está empezando a estudiar la relación entre el estrés inducido por el cambio climático y la epigenómica. Las mejoras en las tecnologías de secuenciación de ADN y el creciente número de estudios que abordan este tema ayudarán a ampliar nuestro conocimiento sobre la capacidad adaptativa de los árboles a las condiciones ambientales cambiantes. En este trabajo, se discuten los desafíos y perspectivas en el estudio de la epigenética del decaimiento de los bosques inducido por el cambio climático, con el objetivo de proporcionar una visión general del estado actual del área.

ARTICLE 4

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ARTICLE 4

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Challenges and Perspectives in the Epigenetics of Climate Change-Induced Forests Decline

Isabel García-García[1]†, Belén Méndez-Cea[1]*†, David Martín-Gálvez[2], José Ignacio Seco[3], Francisco Javier Gallego[1] and Juan Carlos Linares[3]*

[1] Departamento de Genética, Fisiología y Microbiología, UD Genética, Facultad de CC Biológicas, Universidad Complutense de Madrid, Madrid, Spain, [2] Departamento de Biodiversidad, Ecología y Evolución, UD Zoología, Facultad de CC Biológicas, Universidad Complutense de Madrid, Madrid, Spain, [3] Departamento de Sistemas Físicos, Químicos y Naturales, Universidad Pablo de Olavide, Seville, Spain

Forest tree species are highly vulnerable to the effects of climate change. As sessile organisms with long generation times, their adaptation to a local changing environment may rely on epigenetic modifications when allele frequencies are not able to shift fast enough. However, the current lack of knowledge in this field is remarkable, due to many challenges that researchers face when studying this issue. Huge genome sizes, absence of reference genomes and annotation, and having to analyze huge amounts of data are among these difficulties, which limit the current ability to understand how climate change drives tree species epigenetic modifications. In spite of this challenging framework, some insights on the relationships among climate change-induced stress and epigenomics are coming. Advances in DNA sequencing technologies and an increasing number of studies dealing with this topic must boost our knowledge on tree adaptive capacity to changing environmental conditions. Here, we discuss challenges and perspectives in the epigenetics of climate change-induced forests decline, aiming to provide a general overview of the state of the art.

Keywords: epigenetics, climate change, forest tree species, abiotic stress, methylation

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***Correspondence:**
Isabel García-García
isabel06@ucm.es
Belén Méndez-Cea
belenmen@ucm.es

†These authors have contributed equally to this work and share first authorship

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CLIMATE CHANGE-INDUCED TREE DIEBACK AND FORESTS DECLINE

Rising temperatures are exacerbating drought stress for trees at global scales, causing events of decline in several species (usually known as forest dieback; Allen et al., 2010). Climatic projections indicate that mean global temperatures are expected to rise at least 1.5°C until 2100 in response to anthropogenic emissions (IPCC, 2018). Such projections of warming indicate the duration and severity of droughts are expected to amplify, probably leading to widespread forest dieback and tree mortality, affecting tree species currently not assumed to be prone to drought stress, for instance in tropical or boreal forests, or already affected by drought stress, such as those of the Mediterranean forests (Allen et al., 2010). Even though several mechanisms have been described as physiological drivers of dieback, including hydraulic failure and carbon starvation (McDowell et al., 2016; Choat et al., 2018), we lack forecasting tools with sufficient predictive power, since the likelihood of drought-induced tree dieback and mortality is not straightforward (Trugman et al., 2021). This mismatch enhances the need to understand the processes that modulate contrasting adaptive

capacity among trees within a given declining forest in order to be able to provide, for instance, adaptive management strategies. Epigenetic processes are known to be able to modulate the patterns of gene expression in plants (Rapp and Wendel, 2005), some being involved in physiological and morphological responses to drought (e. g., Zheng et al., 2013; Liang et al., 2014; Chwialkowska et al., 2016). As drought-induced tree mortality has far-reaching consequences that affect a wide-range of fields, from environmental conservation to climate change mitigation efforts, and it is expected to be more frequent under a climate-change scenario, we need to improve our knowledge about the degree to which epigenetic mechanisms are linked to mortality predictions (e. g., Sow et al., 2018; Amaral et al., 2020; González-Benito et al., 2020).

Trees are capable of coping with short drought periods by avoiding water loss. Thereby, several morphological and physiological adaptations have been developed by forest tree species, for instance variations in tracheids size (Carvalho et al., 2015), reduced leaf area, embedded stomatas, and quick stomata close (Sánchez-Salguero et al., 2015; Kijowska-Oberc et al., 2020). However, long-lasting and severe drought periods are predicted to occur more often, so these strategies might not be enough for their survival (IPCC, 2018).

Tree growth can be used as a marker to study forest tree response to environment alterations, as it is affected by drought stress. Dendrochronology studies have evinced growth decline during drought stress periods, which eventually leads to forest dieback (Camarero et al., 2015; Cailleret et al., 2017). Thus far, drought-induced forests dieback has been observed in several conifer species found in the Mediterranean region, like *Abies pinsapo* Boiss. (Linares et al., 2010), *Abies alba* Mill. (Macías et al., 2006; Camarero et al., 2011, 2015), and *Cedrus atlantica* (Endl.) Manetti ex Carrière (Linares et al., 2011). The Mediterranean basin is considered a climate change hotspot, being one of the most vulnerable areas in the world. As this region was an important refuge in the last glaciation, a large number of endemic and relict species can be found there. Hence, variations in the environment could result in death of these drought-sensitive species which will trigger a huge loss of biodiversity.

In addition, large-distributed forest tree species, like *Pinus sylvestris* L., often live under different environment conditions (Bose et al., 2020), so individuals must show variations that allow them to cope with those circumstances (Camarero et al., 2015; DeSoto et al., 2020). Studying not only endemic species but also largely distributed ones and identifying these differences could be key to understand why some individuals survive while others die (Cailleret et al., 2017).

Surprisingly, there is a lack of data about epigenomic changes in natural populations in spite of their potential role in adaptation to a rapidly changing environment. The most common studies compare individuals under drought conditions with regularly watered individuals, grown in greenhouses (e.g., Gourcilleau et al., 2010; Xu et al., 2018; Li et al., 2020). While this approach allows to accurately control the factors affecting the plants and makes the experiments replicable, it may lack realism, especially when working with long-lived perennial plants due to restriction of their rooting volume, which hinders the extrapolation of the results to natural populations (Gibson et al., 2004). Therefore, studying epigenetic variation in natural populations would be of great interest to understand how trees respond to climate change in the nature.

On summary, as said before, growth of forest tree species is usually slow and the upcoming climate variations will likely be faster than the speed of allele frequencies shift of forest trees, which emphasizes a likely role of epigenetic modifications as a more rapid way of adaptation.

EPIGENETIC MECHANISMS UNDER A CLIMATE CHANGING SCENARIO

Forest trees are sessile organisms which must cope with environment variations since they are not able to migrate to ensure their survival. Hence, they possess other abilities to compensate for their lack of mobility, such as phenotypic plasticity (Petit and Hampe, 2006). Phenotypic plasticity is the capacity to develop phenotypic modifications in response to multiple environmental factors, increasing the probability of surviving (West-Eberhard, 2008). This mechanism is the result of molecular modifications that trigger changes in gene expression. One of the most important processes involved in phenotypic plasticity is epigenetic regulation, which produces changes in genes expression without modifying the DNA sequence (Iwasaki and Paszkowski, 2014). In fact, phenotypic plasticity is one of the main biological processes that allows trees to respond to rapid variations, like climate change (Alberto et al., 2013). Climate change exacerbates drought stress for trees at global scales, causing stand-level growth decline and episodes of mortality (Allen et al., 2010; Anderegg et al., 2013). As drought-induced forests decline and mortality are reported at an unprecedented rate (Jump and Peñuelas, 2005), a particularly urgent scientific challenge is to understand epigenetic mechanics linking an environmental cue, here increasing drought stress, received by adult trees and its resulting effects on the phenotype of coming generations (Alberto et al., 2013).

In particular, conifers, a group of forest tree species which include ecologically and economically important genera like *Pinus*, *Picea*, and *Abies*, seem to be particularly drought-sensitive (McDowell et al., 2016; DeSoto et al., 2020) and prone to a high rate of epigenetic regulation due to their large genome sizes with high amounts of non-coding DNA (Yakovlev et al., 2012). The study of growth patterns in declining conifer forests suggests a lower capacity of resilience in stressed/dying trees. In fact, trees that died after drought were less resilient after previous droughts, relative to surviving conspecific individuals. Specifically, drought-related mortality risk in conifers has been associated with a low capacity to recover after drought (DeSoto et al., 2020), whereas the epigenetic mechanisms involved on this recovery mechanisms remain largely unknown. Here, we hypothesize that epigenetic mechanisms are likely related to the fate of trees that survive after drought, while some conspecifics died.

Three relevant epigenetic mechanisms have been described: DNA methylation, histone modifications and non-coding RNAs

(ncRNAs). Of these, DNA methylation is the most studied one in plants, both model and non-model (Zhang et al., 2018). Two main reasons are responsible for this fact. In one hand, DNA methylation has been reported to be implicated in significant biological processes, like silencing of Transposable Elements (TEs), gene transcription regulation, plant development, and response to biotic and abiotic stress (Zhang et al., 2018). On the other hand, several genomic tools have been developed to easily study at a global level the methylations present in DNA. Therefore, in this review we will pay special attention to this epigenetic mark.

Regarding histone modifications, these epigenetic marks are involved in chromatin compaction, playing a role in determining its state, which can be decondensed (euchromatin) or condensed (heterochromatin). Euchromatin, also known as open chromatin, is characterized by the presence of a high number of genes, which are usually susceptible to transcription as they are accessible to transcription machinery, while heterochromatin, also known as closed chromatin, is characterized by repetitive and silenced sequences (Amaral et al., 2020). Acetylations, methylations, phosphorilations, and ubiquitylations are some of the panoply of modifications that histones can be subjected to Bannister and Kouzarides (2011), creating patterns that are associated to different transcriptional states. Unfortunately, these modifications are numerous and challenging to analyze in trees as standard chromatin immunoprecipitation (ChIP) procedures are hard to apply to woody cells and tissues (Li et al., 2014). Thus, very few studies regarding histone modifications in trees have been published to date.

Non-coding RNAs are also rather relevant as an epigenomic mechanism, as they are mobile within and between cells and are not lost during cell divisions, unlike the majority DNA methylations and histone modifications (Yakovlev et al., 2012). They are classified by their length: if they have more than 200 nucleotides, they are called as long ncRNAs (lncRNAs) whereas if they have less than 200 nucleotides, they are called short ncRNAs. These short ncRNAs include micro RNAs (miRNAs), short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs), and they are involved in genomic imprinting, X-chromosome inactivation, repression of gene transcription, and transposon repression (Wei et al., 2016). Therefore, given the elevated content of TEs in conifer genomes, ncRNAs stand as a major epigenetic mark that should be studied in depth.

Lastly, DNA methylation positions itself as a highly relevant epigenetic mark in conifers due to their large content in TEs and its role in silencing them. In addition, there are numerous studies that demonstrate its participation in the control of gene expression in a large number of traits, in plants in general and in conifers in particular, which will be addressed later. DNA methylation is a covalent change which consists in the addition of a methyl group to a cytosine residue, in most cases to its fifth carbon position, and can be, in some cases, inherited by the offspring. In plants, DNA methylation happens not only in CpG sites but also in CHG and CHH contexts, where H can be A, T, or C (Law and Jacobsen, 2010). CpG sites are the most frequent mark in/near protein-coding gene sequences (Ashapkin et al., 2020), especially of those with constitutive expression. Although this methylation is known to affect gene expression, its role is still unclear, as it can lead to both silencing and increased expression (Zemach and Ziberman, 2010). Regarding CHG and CHH sites, different studies have highlighted that they are mainly present in repetitive sequences and TEs, both abundant in tree genomes, and their methylation usually reduces or even blocks DNA transcription, allowing the maintenance of the genome integrity (Zemach et al., 2010a; Bewick and Schmitz, 2017).

It has been proved that DNA methylation is responsible of phenotypic variation in plants (Martin et al., 2009; Mougninot et al., 2021; Zhao et al., 2021). Considering that different types of stress, such as abiotic stress, can trigger methylation, eventually leading to variation of gene expression, this field of research has become a useful tool for understanding how tree species deal with environment variations. Several studies have highlighted that epigenetics seems to act like a memory process to respond to different environmental stresses, which is called "stress memory" (Kinoshita and Seki, 2014). Thus, a new question arises: could it be possible to inherit this stress-induced DNA methylation? Most of the time, epigenetic marks are erased to start over in the progeny, however it is possible for this reset to be incomplete, allowing some marks to pass on to the offspring. Moreover, it is known that some germline cells come from somatic cells in plants, so epigenetic marks that appear in those cells could be passed on to the offspring. Hence, epigenetic marks can be transmitted to the offspring by meiosis in some cases. Studies carried out with *Arabidopsis thaliana* L. (Cervera et al., 2002; Boyko et al., 2010; Lang-Mladek et al., 2010) or *Linaria vulgaris* Mill. (Cubas et al., 1999) evidenced epigenetic modifications related to environment variations can be inherited. However, the mechanisms involved in this process are not well understood to date.

All in all, epigenetic modifications could be a rapid way to achieve short-term adaptations, since epigenetic marks bring new phenotypes which might affect fitness and, thus, the underlying evolutionary processes. Particularly, it could be key for forest trees survival, as they are sessile long-lived organism with long generation times, so the possible beneficial effects of natural selection on them are limited and slow and will soon be surpassed by the effects of climate change.

TECHNIQUES FOR EPIGENOMIC ANALYSIS

Over the years, different techniques have been developed to study DNA methylation. Their first requirement is high-quality DNA, which highlights the importance of selecting an appropriate method for sample conservations during and after each collection, as well as an extraction method as efficient as possible for each type of samples. Once the DNA is obtained, there is a wide variety of methods that can be used to analyze its methylation patters (**Table 1**). The most remarkable ones are shown below.

At the beginning, methods based on antibodies affinity to methylcytosine residues were used. One of them is the well-known methyl-DNA immunoprecipitation (MeDIP) technique,

TABLE 1 | Summary of the advantages and weaknesses of the most relevant DNA methylation techniques and their suitability for species without reference genomes.

Technique	Advantages	Weaknesses	Suitable for species without reference genome
MeDIP-seq	Low initial amount of DNA. Genome-wide scale.	No single base resolution. It does not discriminate methylation contexts. Relative levels of methylation.	No
MRE-seq	Reduction of the complexity of genomes. Single-base resolution.	Low coverage of the genome. Relative levels of methylation.	No
MSAP-seq	Reduction of the complexity of genomes. Useful with large-size genomes.	Low coverage of the genome. Loss of information of CHH and CHG contexts.	Yes
WGBS	It uses entire genomes. Single-base resolution. All contexts are represented. Absolute levels of DNA methylation.	Not suitable for large-size genomes.	No
RRBS	Reduction of the complexity of genomes. Single-base resolution. All contexts are represented.	Low coverage of the genome.	Yes

which captures the methylated DNA fragments with an antibody against methylated cytosines (Thu et al., 2009). Then a new approach of this technique, called MeDIP-seq, was developed (Zhao et al., 2014). The novelty of this method consisted in a final sequencing step based in next-generation sequencing (NGS). Both techniques require a previous DNA sonication step to obtain random DNA fragments. MeDIP-seq is frequently used in combination with MRE-seq (methylation sensitive restriction enzyme sequencing) (Maunakea et al., 2010). MRE-seq consists of a DNA fragmentation using several restriction enzymes which are methylation sensitive followed by a final sequencing step. Although MeDIP-seq and MRE-seq can be used independently, their combination can increase the accuracy of DNA-methylation studies (Li et al., 2015).

Subsequently, an improvement based on NGS allowed to modify the methyl-sensitive amplified polymorphism (MSAP) technique, giving rise to MSAP-seq. Originally, the MSAP technique appeared as a modification of the amplified fragment length polymorphism method (AFLP). This technique was described by Reyna-López et al. (1997) in fungi and then Xiong et al. (1999) adapted it to work with plants. MSAP starts with a genome fragmentation using restriction enzymes with differential sensitivity to cytosine methylation. *Msp*I and *Hpa*II are one of the enzyme isoschizomer pairs used in MSAP. Both recognize the same target 5'-CCpGG-3', but *Hpa*II will not cleave if the inner cytosine residue is methylated. Hence, this pair is used to identify differences in methylation marks between DNA samples in CpG contexts mainly. Following the cleavage step, a PCR amplification is carried out with the aim to obtain an overrepresentation of previously obtained fragments. Then, amplicons are separated and visualized via gel electrophoresis. MSAP-seq replaces this last sept by a sequencing one. This method was first described by Chwialkowska et al. (2016) in barley and was later improved to work with large genome species (Chwialkowska et al., 2017). It allows studies in non-model species with lack of a reference genome. However, restriction enzymes seem to cut less efficiently in CHH and CHG contexts than in CpG islands, which may lead to mistakes in methylation calling, which in turn might imply a loss in methylated states. This could be a remarkable weakness of this technique considering that the larger genome size is, the greater amount of cytosine methylations there is in the other contexts.

Finally, new approaches based on sodium bisulfite treatments have been developed to study DNA methylation, since bisulfite sequencing was developed in 1992 (Frommer et al., 1992). Bisulfite methods are useful to study genome-wide methylation and provide high single-base resolution. These techniques convert unmethylated cytosines to uracils, as methylated cytosines are protected against the deamination effect produced by the bisulfite treatment. After that, uracils are turned into thymines in a PCR amplification. Then, these changes can be easily identified by sequencing. To date, there are two bisulfite techniques: whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS), which is subdivided in three protocols based on RAD-seq (Baird et al., 2008) and genotyping by sequencing (GBS; Elshire et al., 2011) techniques: epiRADseq (Schield et al., 2016), bsRADseq (Trucchi et al., 2016), and epiGBS (van Gurp et al., 2016).

WGBS works with the entire genome, while the RRBS method targets specific regions. In RRBS methylation sensitive restriction enzymes are used to fragment the whole genome, and then DNA fragments are filtered by size. The characteristics of WGBS make it suitable for studies with model organisms with short length genome size and for species with a reference genome (Paun et al., 2019). On the other hand, the genome fragmentation carried out with RRBS methods constitutes a useful tool to reduce the complexity of genomes (Davey et al., 2011), which allows to work with large-size genomes, with or without a reference genome.

Although this review is focused on DNA methylation, here is a brief description of other techniques used to study the remaining epigenetic marks: histone modifications and ncRNAs.

One of the most common techniques to study *in vivo* histone modifications is chromatin immunoprecipitation (ChIP). First, samples are treated with formaldehyde to cross-link the chromatin interactions (protein–protein and protein–DNA). Then, small fragments are obtained by sonication or nuclease digestion. Next, specific antibodies are used to retain the DNA-histone modification complex of interest. Finally, different methods can be carried out to determine these regions such as PCR, real-time PCR, Southern blot, and Western blot. The development of NGS techniques has led to the addition of a final sequencing step to the ChIP protocol, creating

the technique known as ChIP-seq. However, the presence of polysaccharide and phenolic compounds in plants may difficult these experiments, that could turn them into a challenge in some cases (Muhammad et al., 2020).

The most common technique used to study ncRNA sequences is RNA-seq (Wang et al., 2009). Then, sequencing data can be combined with RT-qPCRs to determine expression levels, or with fluorescence *in situ* hybridization (FISH) experiments to localize the ncRNAs in a cellular context, depending on the aim of the study. An *in silico* approach consists in looking for previously described or computationally predicted ncRNAs in the genomic region under study. Several databases of ncRNAs sequences are available online, such as CANTATAdb 2.0[1] (Szcze´sniak et al., 2015) which has over 200,000 lncRNAs from 39 plant species from which 2 are trees. As usual, forest trees are poorly represented in this kind of databases, mainly due to the lack of reference genomes.

IN SEARCH OF A REFERENCE

Forest trees span a huge range of genome sizes, including species with less than 1 Gb, such as the *Populus* genus, to the conifer giga-genomes, whose size varies from ∼6.5 to ∼37 Gb, being ∼15 Gb the most common 1C-value (Ahuja and Neale, 2005). Compared to the ∼0.6 Gb average of most angiosperms (Dodsworth et al., 2015), conifer genomes are remarkably large and, therefore, constitute a challenging work scenario for genomic and epigenomic studies. In addition, a great number of experimental techniques and bioinformatic pipelines are designed work with the 3.3 Gb-long human genome, making it difficult to adapt them to genomes which are on average five times bigger. For instance, techniques aimed at sequencing complete genomes, such as WGBS, might not be suitable for giga-genomes as optimal depth and coverage values will be hard to achieve.

To complicate matters further, approximately 75% of conifer genomes consists of highly repetitive DNA, mainly represented by non-coding DNA, such as mobile elements, along with rDNA repeat units (Ahuja and Neale, 2005). In addition, gene duplications have created large gene families, which are not necessarily found in certain regions but dispersed across the genomes (Kinlaw and Neale, 1997).

On the other hand, luckily, polyploidy is not a common phenomenon in conifers, and it has only been reported in the Cupressaceae family (Ahuja, 2005). All in all, however, the global genetic and, thus, epigenetic landscapes of these tree species do not provide a research-friendly environment.

While many plant genomes are being sequenced and assembled every year due to the improvement of next generation sequencing techniques and their decrease in cost (Mackay et al., 2012), few conifer reference genomes are available. The first gymnosperm giga-genome ever published was that of *Picea abies* (L.) H. Karst. (Nystedt et al., 2013). Since then, six more conifer genomes have been sequenced: *Pinus taeda* L. (Neale et al., 2014), *Picea glauca* (Moench) Voss (Warren et al., 2015), *Pinus lambertiana* Douglas (Stevens et al., 2016), *Pseudotsuga menziesii* (Mirb.) Franco (Neale et al., 2017), *Larix sibirica* Ledeb. (Kuzmin et al., 2019), and *Abies alba* Mill. (Mosca et al., 2019). The N50 statistic is used as an indicator for genome assembly quality. It is defined as the length of the contig that reaches 50% of the total genome length, being the contigs sorted by length in descending order. Therefore, the higher this value is, the better the assembly is, as the median contig size will be longer. The total length of the previously mentioned genomes ranges from ∼12 to ∼32 Gb, with N50 scaffold sizes between ∼5 to ∼2,500 Kb, which shows that further refinement is still needed. Nevertheless, over 45,000 genes have been successfully annotated in each of the cited examples.

The recent upsurge in popularity and number of users of third generation sequencing technologies, also known as long-read sequencing, could provide a substantial boost to the improvement of the quality of the existing reference genomes. Long reads, alone or in combination with short reads, have a high potential for assembling and re-assembling genomes (Amarasinghe et al., 2020). The reads obtained from third generation sequencing methods, such as PacBio single-molecule real-time (SMRT) and Oxford Nanopore, constitute a less fragmented representation of large genomes, facilitating their assembly in longer scaffolds. For instance, the quality of the reference genome of barley (*Hordeum vulgare* L.), which is not a forest tree but whose genome shares some characteristics with the conifer ones, such as being highly repetitive and ∼5 Gb-long, was superior for long-read assemblies than for the one based on short reads, obtaining higher contig N50 values (Mascher et al., 2021).

On the other hand, sequencing transcriptomes is more extensive in conifers, due to their notably reduced size compared to whole genomes, which reduces costs and increases the probability of capturing the majority of the sequences. The One Thousand Plant Transcriptomes Initiative database[2] collects over 1,300 transcriptomes of green plants, of which 76 belong to 72 different conifer species, coming from diverse tissue samples, including leaves, branches, and young shoots. Hardwood Genomics Project[3] and TreeGenes[4] are two other examples of databases specialized in trees that include transcriptomes as well as genomes, annotations and genetic markers.

However, the information coming merely from transcriptome sequencing is clearly insufficient to address the study of epigenetic mechanisms in conifers. Some of the most common epigenomic methodologies, such as identification of differentially methylated regions (DMRs) and differentially methylated promoters (DMPs), need the information of the sequences surrounding the coding regions. Then, the transcripts identified in transcriptomic experiments are useless without a reference to provide their genomic context.

Regarding complete methylomes, it has been described that CpG and CHG methylation is positively correlated with genome size (Ausin et al., 2016; Takuno et al., 2016), and high methylation levels are also known to be characteristic of genomes with a high

[1]http://cantata.amu.edu.pl/

[2]http://www.onekp.com

[3]https://hardwoodgenomics.org/

[4]https://treegenesdb.org/

content of transposable elements, as they constitute the main target of DNA methylation (Baduel and Colot, 2021). The lack of methylation maps of conifers is therefore not surprising, given the vast amount of data that needs to be analyzed to create them. Withal, the Norway spruce (*P. abies*) DNA methylation map was successfully obtained (Ausin et al., 2016). As previously mentioned, the genome of this species is fully sequenced, which highlights the importance of having reference genomes in order to be able to conduct this type of high throughput epigenetic studies.

BIOINFORMATIC TOOLS

Bioinformatic pipelines for the analysis of DNA methylation data obtained after bisulfite treatments consist of four main steps: quality check and read trimming, read mapping, methylation calling, and identification of differentially methylated regions (Wreczycka et al., 2017).

Quality check and trimming of raw reads can be performed with well-known software, including FastQC (Andrews, 2010), and Trimmomatic (Bolger et al., 2014) and Cutadapt (Martin, 2011), respectively. Generally, these steps include removing adapter sequences, filtering out low-quality reads and trimming reads of low-quality bases. Specific bisulfite-sequencing tools are also available, such as BSeQC (Lin et al., 2013), which evaluates quality and automatically trims technical biases that may interfere in accurate methylation calling. For example, it can detect 5' bisulfite conversion failure and remove the reads affected by this bias, which causes artificially high methylation rates at that end of the reads.

Bisulfite conversion rates should also be checked, especially if non-CG methylation levels are the focus of the study. Non-CG contexts, as previously mentioned, play an important role in silencing repetitive sequences and TEs, which are highly abundant in conifer genomes, so their study could be of great interest. In particular, CHH contexts can be quite problematic due to their low methylation levels (Takuno et al., 2016), which require a high bisulfite conversion rate to ensure that they are not underestimated. Thus, a spike-in control, such as unmethylated lambda phage DNA, could be useful to check that the bisulfite conversion is working at optimal levels.

Read mapping is a particularly challenging step. After bisulfite treatment, unmethylated cytosines are converted to uracils and then to thymines in the following PCR amplification, while methylated cytosines are not affected. This creates a need for special reference genomes, modified to take into consideration these nucleotide changes. In addition to the increased mapping searching sequence, asymmetric cytosine to thymine alignments and multiple CpG heterogeneous methylation must be taken into consideration by the mapping software (Xi and Li, 2009). Nonetheless, several bisulfite mapping tools have been developed, such as BSMAP (Xi and Li, 2009) and Bismark (Krueger and Andrews, 2011). Recently, Grehl et al. (2020) compared the efficiency and impact of the mapping results on the identification of DMRs of eight mappers and concluded that these two are the best options, as they showed the highest precision with the shortest run time and lowest memory requirements, respectively.

Both tools also provide methylation level files showing methylation calling status for each cytosine position. The coverage files produced by the Bismark methylation extractor function can be directly used as inputs for DMR-finding programs, such as the popular edgeR package (Robinson et al., 2010), or can easily be edited to fit other tools' input requirements, such as DSS (Park and Wu, 2016) and swDMR (Wang et al., 2015).

In addition to these tools that allow users to perform each step of the analysis separately, others combine all the procedures in one pipeline. Bicycle (Graña et al., 2017) and SMAP (Gao et al., 2015) are some of the recommended all-in-one software for WGBS and RRBS data analysis, respectively (Rauluseviciute et al., 2019).

Despite these apparently wide variety of tool choices, there are several issues to take into consideration when it comes to analyzing high-throughput bisulfite sequencing data. First, the need of a good quality reference genome to perform the read mapping step, which is not a frequent situation, as previously explained. Besides, some tools, like swDMR, require an annotation file as input, whose quality relies on the genome quality itself. For instance, if a DMR falls in a region that is not described, likely due to genome fragmentation caused by large number of scaffolds or small scaffold sizes, this DMR will provide little information regarding its biological meaning. In addition, the amount of Cs that would need to be analyzed will in all probability be quite high in comparison to other non-conifer species. Therefore, the computational requirements in epigenomic experiments on forest tree species are likely to be very demanding, particularly in terms of RAM memory usage, storage space and run time.

Researchers that choose non-bisulfite-based techniques are likely to face the above-mentioned problems derived from genome size and lack of references, as they also include a next-generation sequencing step. Nonetheless, as in the case of bilsulfite-based methods, there are many bioinformatic tools specifically designed to work with this kind of data. The MeDUSA pipeline (Wilson et al., 2012) and Batman software (Down et al., 2008) are suitable for MeDIP-seq data analysis, while the MSEQER pipeline (Chwialkowska et al., 2017) is designed to work with MSAP-seq data.

PREVIOUS STUDIES

As it is said above, studies of epigenetic marks in plants are mainly focused on DNA methylation (Kurdyukov and Bullock, 2016). Nevertheless, the role of histone acetylations in root growth and development (Ma et al., 2016) and in photosynthesis (Li et al., 2017) has been described in different species of poplar, as well as that of miRNAs in growth and phosphorus nutrition (Schönberger et al., 2016). It is worth noting that this kind of studies have been carried out on poplars because they are considered a forest tree model organism. As mentioned above, poplar has a small genome (~0.5 Gb) with a low

proportion of repeated sequences. This genome has been fully sequenced and annotated, so its genomic resources are of high quality. Except in this particular case, histone modifications and ncRNAs remain mostly undescribed in conifer species and represent a major challenge to advance in the knowledge of epigenomics in trees.

The first plant methylome published was that of *A. thaliana* L. (Cokus et al., 2008), and it was developed using a large-scale bisulfite sequencing experiment, while the first conifer one, of *P. abies*, did not come until 2016 (Ausin et al., 2016).

Over the years, some studies have focused on changes in DNA methylation during normal plant development (e.g., Bitonti et al., 2002; Zemach et al., 2010b; Perrin et al., 2020) and heteroblastic development (Hasbún et al., 2016). Others are about phenotypic plasticity and stress memory (e.g., Herrera and Bazaga, 2013; Le Gac et al., 2018). What it is common to the vast majority of them is the use of model species, like *A. thaliana* (e.g., Korotko et al., 2021; Markus et al., 2021), and important commercial species such as crops (e.g., Feng et al., 2010; Wang et al., 2010; Karan et al., 2012; Bhanu et al., 2020; Merce et al., 2020) or fruit trees (e.g., Avramidou et al., 2015; Badad et al., 2021; Rothkegel et al., 2021).

The knowledge about forest tree epigenetics is scarce. Nevertheless, some studies related with epigenetic memory during embryogenesis have been developed on forest trees of species such as *P. abies* (Kvaalen and Johnsen, 2008; Yakovlev et al., 2014, 2016), *P. glauca* (Webber et al., 2005), or *P. sylvestris* (Dormling and Johnsen, 1992), and more studies are coming (e.g., Lafon-Placette et al., 2018; Vanden Broeck et al., 2018).

Furthermore, studies have generally focused on the involvement of DNA methylation in abiotic stresses response and adaptation in forest trees and other plants (**Table 2**). Modifications in response to water have been found in the methylome of *Populus* species (Gourcilleau et al., 2010; Raj et al., 2011), as well as variations in the methylation pattern of *Pinus radiata* D. Don. in response to heat stress (Lamelas et al., 2020). Again, it is remarkable the huge amount of poplar (*Populus* spp.) studies which have been carried out over the years, thanks to the availability of a quality reference genome and its wide phenotypic variation.

Studies in natural populations are of particular importance to understand how epigenetic modifications can contribute to adaptation to the new climatic conditions caused by climate change. One of these studies was carried out with *P. sylvestris* (Alakärppä et al., 2018), which is a widely distributed conifer, likely to show variations in its epigenetic marks as it can be found under quite different environmental scenarios, and a link between local adaptation and environment condition, and modifications in DNA methylation has been suggested. Another work with samples of natural populations of *Populus simonii* Carr. (Ci et al., 2016) was conducted to analyze population epigenetic structure, which showed a substructure population associated to different methylation patterns.

In the literature, several reviews about epigenetics in plants and forest trees can be found (Paun et al., 2019; Amaral et al., 2020; Ashapkin et al., 2020; Zhao et al., 2021). Most of them are focused on the epigenetic response to specific abiotic stresses, such as drought, salt, or heavy metals, and they are useful as an introduction to this challenging field. On top of that, this review should prove valuable to researchers who wish to design an experiment to study methylation, as it covers the whole process: from choosing an appropriate technique to performing the bioinformatic analysis of the data.

TABLE 2 | Brief description of some DNA methylation studies carried out with the main objective of studying plant species response to several abiotic stresses.

Plant species	Technique	Objective	Reference genome	References
Bruguiera gymnorhiza (L.) Lamk.	WGBS	Salt stress response	No	Miryeganeh et al., 2021
Ilex aquifolium L.	MSAP	Epigenetic variability	No	Herrera and Bazaga, 2013
Medicago sativa L. and *Medicago* spp	MSAP	Salt stress response	Yes	Al-Lawati et al., 2016
Morus alba L.	WGBS	Drought stress response	Yes	Li et al., 2020
Quercus ilex L.	MSAP	Drought stress response	No	Rico et al., 2014
Quercus lobata Née	RRBS	Environment response	Yes	Gugger et al., 2016
Eucalyptus globulus Labill.	MeDIP-seq, MRE-seq	Heteroblastic development	Yes	Hasbún et al., 2016
Populus simonii Carr.	MSAP	Various abiotic stresses	Yes	Song et al., 2016
Populus trichocarpa Hook.	WGBS	Drought stress response	Yes	Liang et al., 2014
Populus euphratica Oliv.	WGBS	Salt stress response	No	Su et al., 2018
Populus tremula L. *x Populus alba* L.	RNAi, HPLC, WGBS	Drought stress response	No	Sow et al., 2021
Pinus pinea L.	MSAP	Epigenetic variability	No	Sáez-Laguna et al., 2014

To sum up, epigenomics open a research field to identify some of the processes related with phenotypic plasticity and its study will bring us a better understanding of adaptive forest tree response to a changing environment. Fortunately, substantial efforts are being made to improve DNA methylation techniques to make them suitable and cost-effective for non-model species, specifically with trees or species with large genomes (e.g., Chwialkowska et al., 2017; Pereira et al., 2020; Werner et al., 2020).

CONCLUSION

It is expected that the growth rates of forest tree species become on average slow, while the upcoming climate change trends become faster than the rate of allele frequencies shift of forest trees. This emphasizes the putative role of epigenetic modifications as a fast adaptive mechanism to cope with current warmer and drier climate scenario.

Phenotypic plasticity is the principal strategy to compensate for trees' lack of ability to move when it comes to dealing with rapid environmental variations, such as ongoing climate change. It is based on modifications of genetic regulation which depend

on epigenetic mechanisms such as histone modifications, DNA methylation and non-coding RNAs. Despite the importance of these processes, epigenomic studies in forest tree species are challenging and scarce, mainly due to their complex genomes and lack of references.

Throughout this review, we have gathered enough information to give a general overview about the key points for methylation experiments design when dealing with forest trees. We have also highlighted opportunities for future advances in this framework and the relevance of dedicating efforts to improve the techniques for epigenomic analysis and the bioinformatic tools to make them suitable for large-size genomes.

AUTHOR CONTRIBUTIONS

IG-G and BM-C wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

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ARTICLE 5

DNA methylation analysis in plant giga-genomes: comparison of two bisulfite sequencing techniques

Análisis de la metilación del ADN en giga-genomas de plantas: comparación de dos técnicas de secuenciación por bisulfito

La plasticidad fenotípica es un mecanismo fundamental que permite a las plantas adaptarse a condiciones ambientales cambiantes, como las provocadas por el cambio climático. Las modificaciones epigenéticas, en particular la metilación del ADN, pueden desempeñar un papel clave en este proceso. Sin embargo, este campo sigue en gran medida inexplorado en organismos no modelo con genomas grandes y complejos. Este estudio se centra en el abeto blanco (*Abies alba*), concretamente en una población natural sometida a estrés climático, comparando los resultados obtenidos utilizando dos técnicas diferentes de secuenciación por bisulfito para estudiar los patrones epigenéticos de su giga-genoma. Se extrajo ADN de individuos de *A. alba* decaídos y no decaídos, y se sometió a secuenciación por bisulfito de genoma completo (WGBS) y de representación reducida (RRBS). Se determinaron los niveles generales de metilación en cada contexto (CpG, CHG y CHH) y se identificaron regiones diferencialmente metiladas (DMRs). La técnica WGBS secuenció una mayor cantidad de citosinas, mientras que un mayor número de sitios CpG secuenciados por la técnica RRBS pasaron el filtrado. Ambas técnicas mostraron altos porcentajes de metilación en los contextos CpG y CHG, pero no coincidieron en los CHH, probablemente debido a un posible sesgo de la técnica RRBS hacia los contextos CHH altamente metilados. Solo se identificaron DMRs con los datos de WGBS, pero no pudieron ser anotados funcionalmente. En general, aunque ambas técnicas fueron exitosas en la evaluación de la metilación del ADN, ni WGBS ni RRBS produjeron los resultados deseados en cuanto a la identificación de DMRs. En conclusión, la naturaleza fragmentada de los genomas de referencia disponibles para especies con genomas grandes parece obstaculizar la obtención de resultados significativos a partir de WGBS y RRBS.

DNA methylation analysis in plant giga-genomes: comparison of two bisulfite sequencing techniques

Abstract: Phenotypic plasticity is a fundamental mechanism that enables plants to adapt to shifting environmental conditions, such as those induced by climate change. Epigenetic modifications, notably DNA methylation, may play a pivotal role in such process. However, this field remains largely unstudied in non-model organisms with large, complex genomes. Here, we focus on silver fir (*Abies alba*), more precisely on a natural population subjected to climate stress, comparing the results obtained from two different bisulfite sequencing techniques in the study of the epigenetic patterns of its giga-genome. DNA was extracted from non-declining and declining *A. alba* individuals and subjected to whole genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS). General DNA methylation levels in each cytosine context (CpG, CHG and CHH) were determined and differentially methylated regions (DMRs) were identified. WGBS sequenced a higher amount of cytosines, while RRBS had a higher number of CpG sites passing filtering conditions. Both techniques showed high methylation percentages in CpG and CHG contexts, but disagreed on CHH sites, likely due to RRBS having a bias towards highly methylated CHH contexts. Differentially methylated sites were only identified using WGBS data but could not be functionally annotated. All in all, while successful in DNA methylation assessment, neither WGBS nor RRBS produced the desired outcome regarding the identification of DMRs. Thus, the fragmented nature of the available reference genomes for species with large genomes seems to hinder the attainment of meaningful results from WGBS and RRBS.

Key words: DNA methylation; Whole genome bisulfite sequencing (WGBS); Reduced representation bisulfite sequencing (RRBS); Giga-genome; *Abies alba*

1. Introduction

Phenotypic plasticity is the ability of a single genotype to produce different phenotypes when exposed to different environmental conditions (Pigliucci *et al*., 2006). Thus, it can serve as a safeguard against rapid climate variations and aid in quick adaptation (Nicotra *et al*., 2010), which could be a crucial mechanism for species survival to ongoing climate change. In fact, phenotypic plasticity is recognized as a key process through which plants can cope with climate change (Stotz *et al*., 2021). They are able to undergo dynamic physiological and molecular changes to adapt to a changing environment, in which modifications at the epigenetic level are involved (Dar *et al*., 2022), namely DNA methylation, histone modifications and the action of non-coding RNAs (ncRNAs).

DNA methylation, usually understood as an addition of a methyl group onto a cytosine to form 5-methylcytosine (5mC) (Bartels *et al*., 2018), is a widely studied epigenetic mark in plants (reviewed in Zhang *et al*., 2018). DNA methylation is known to play a crucial role in various biological processes, including silencing of transposable elements (TEs), maintaining genome stability, regulation of gene transcription, and stress tolerance (Kumar & Mohapatra, 2021). Specifically, plants methylate cytosines in three different contexts: CpG, CHG and CHH (where H = A, T or G) (Takuno *et al*., 2016). These different contexts are usually studied through bisulfite sequencing, which is considered the gold standard technique to assess DNA methylation (Kurdyukov & Bullock, 2016). Whole genome bisulfite sequencing (WGBS) stands out as the most exhaustive method among these techniques, as it enables the assessment of DNA methylation throughout the genome (Suzuki *et al*., 2018). However, such extensive sequencing might not be necessary given that only certain genomic regions can undergo differential methylation. Thus, a variation of this approach, called reduced representation bisulfite sequencing (RRBS), was also developed. Hence, RRBS uses an enzymatic digestion to reduce the targeted portion of the genome, enriched in CpG content (Beck *et al*., 2022), offering a more cost-effective approach and enhancing sequencing coverage (Kurdyukov & Bullock, 2016).

Employing these techniques to study DNA methylation patterns in non-model species can be specially challenging due to the lack of high-quality references and annotations. This task can become even more complex when dealing with large genomes, such as those of conifers (García-García *et al*., 2022), with sizes ranging from ~6.5 to ~37 Gb, being ~15 Gb the most common 1C-value (Ahuja & Neale, 2005). Indeed, only three species of the Pinaceae family (*Picea abies*, *Pinus tabuliformis* and

Pseudotsuga menziesii), which are among the few conifers with reference genome, have had their methylomes studied so far (Ausin *et al*., 2016; Niu *et al*., 2022; Vu *et al*., 2023). *P. abies* and *P. menziesii* had their reference genomes pre-processed before the bioinformatic analysis in order to reduce the computational costs derived from their highly fragmented state (~9–10 million scaffolds each), being subjected to a merging step or a filtering step to keep contigs with high continuity. On the other hand, *P. tabuliformis* is the only conifer with a chromosome-level reference genome, facilitating any analysis based on it.

Here, we analyze cytosine methylation in silver fir, *Abies alba*, which is the only species of the genus *Abies* with an available reference genome, which is highly fragmented (~37 million scaffolds). In the present study, we focus on a natural silver fir population from the Pyrenees that shows signs of drought-induced forest decline and dieback. As no genetic differentiation was found between declining and non-declining individuals in a previous study (García-García *et al*., 2023), we hypothesized that epigenetic processes could be contributing to the phenotypic diversity observed across vigor classes.

We show results obtained from both whole WGBS and RRBS and compare the ability of these techniques to produce data with enough quality to (1) assess general DNA methylation levels of each methylation context (CpG, CHG and CHH) and (2) allow the identification of differentially methylated regions (DMRs) between declining and non-declining trees in silver fir forests.

2. Materials and Methods

2.1. Plant material

Leaf samples were collected from two declining and two non-declining adult *A. alba* trees from a forest in the Western Spanish Pyrenees (Paco Mayor, 42.71ºN, 0.64ºW) showing signs of dieback. Tree vigor was assessed based on visual estimation of crown transparency (Dobbertin, 2005; González de Andrés *et al*., 2022). In order to minimize potential confounding variables and ensure the reliability of epigenomic analyses, all samples were collected during the same morning under consistent weather conditions (cloud-covered sky, snowing), thereby maintaining environmental uniformity across the experimental set. Two samples per vigor group were used for this pilot study to explore preliminary outcomes before choosing the best sequencing methodology for a large-scale investigation.

2.2. Bisulfite sequencing and data analysis

Total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen®, Germany) and subjected to WGBS and RRBS (enzymatic digestion with *Msp*I and *Taq*I). The obtained raw reads underwent quality and adapter trimming using Trim Galore v0.5.0 (The Babraham Institute) before being mapped to the complete *A. alba* reference genome v1.1, including its ~37 million scaffolds (Mosca *et al*., 2019), using Bismark v0.21.0 (Krueger & Andrews, 2011) with default options for single-end (SE) reads. The SE mode was chosen due to two reasons: (1) the RRBS data was generated through single-read sequencing as RRBS libraries are made of short fragments (50 bp) which do not require paired-end (PE) sequencing to achieve high accuracy levels, and (2) Bismark is a very strict aligner which only reports concordant PE reads with end-to-end mapping, which is acceptable for model organism and high-quality DNA but can be problematic for non-model organisms.

Bismark was then used to perform the methylation calling step, producing files containing information about the methylation state of the sequenced cytosines in each sample, which allowed their general methylation levels in each context (CpG, CHG and CHH) to be calculated. Yates' continuity corrected chi-squared test was used to identify differences between vigor classes using GraphPad Prism v8.2.1 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

Only CpG coverage files were used for subsequent analyses, as CpG sites are usually located near/in genes, having effects on their expression, and they need less computational resources to be studied than CHG and CHH sites. Finally, edgeR v3.34.0 (Robinson *et al*., 2010) was used to identify DMRs, following these steps: filtering (only sites which were not always methylated or unmethylated and had at least 5 counts per sample were kept), normalization, dispersion estimation, negative binomial generalized lineal model (NB GLM) fitting, and performance of a likelihood ratio test with 5% FDR (false discovery rate). Finally, either the available *A. alba* annotation or NCBI's blastn (Altschul *et al*., 1990) was used to identify nearby genomic features.

2.3. Data availability

The datasets for the WGBS and RRBS pilot phases generated and analyzed during the current study are available from the corresponding author on request due to their large sizes.

3. Results and Discussion

The amount of sequenced Cs was approximately 5.5 times higher for the WGBS approach (Table 1), which is an expected outcome, as the sequenced fraction of a genome should be larger using a whole-genome technique than using a reduced-representation technique. However, the number of CpG sites that passed the filtering conditions was higher (~4.5 times) in RRBS sequences (Table 1), as this technique increases the sequencing depth of the targeted regions, making it easier to obtain at least 5 counts per sample. Overall, approximately 16% of the studied cytosines in CpG contexts were sequenced by both techniques (~50% of the regions covered by the RRBS technique were also sequenced by the WGBS technique), with 84.8% of them resulting in the same methylation status, showing a high level of agreement between the two methods and implying they could be fairly consistent and offer reliable outcomes.

Table 1: Comparison of the WGBS and the RRBS results.
Methylation calling, filtering, and differential methylation results obtained from each technique are shown.

	WGBS	RRBS
Methylation calling	~850 million Cs / sample	~150 million Cs / sample
Filtering	10,250 CpG sites	47,674 CpG sites
Differential methylation	14 CpG sites	0 CpG sites

Regarding general DNA methylation levels, both techniques showed high methylation percentages in CpG and CHG contexts (although significant differences were found between the results obtained from WGBS and RRBS data, as shown in Table S1), while they strongly disagree on CHH sites (Figure 1). Methylated CHH islands are likely to appear close to genes with nearby transposable elements (TEs) (Martin *et al.*, 2021), flanking them and playing a role in mitigating their adverse effects in gene expression (Kenchanmane Raju *et al.*, 2019). In addition, conifer species have many ultra-long genes with TE insertions, which create lengthy introns which are likely to be heavily methylated (Li *et al.*, 2023). Therefore, as RRBS targets CpG-rich regions (Beck *et al.*, 2022), which are generally associated with genes (Ashikawa, 2001), the results obtained using this technique could be biased towards highly methylated CHH contexts. Thus, the percentage estimated from the WGBS data could be a more realistic representation of the global levels of methylation in CHH areas in *A. alba* as they were sequenced at random throughout the whole genome.

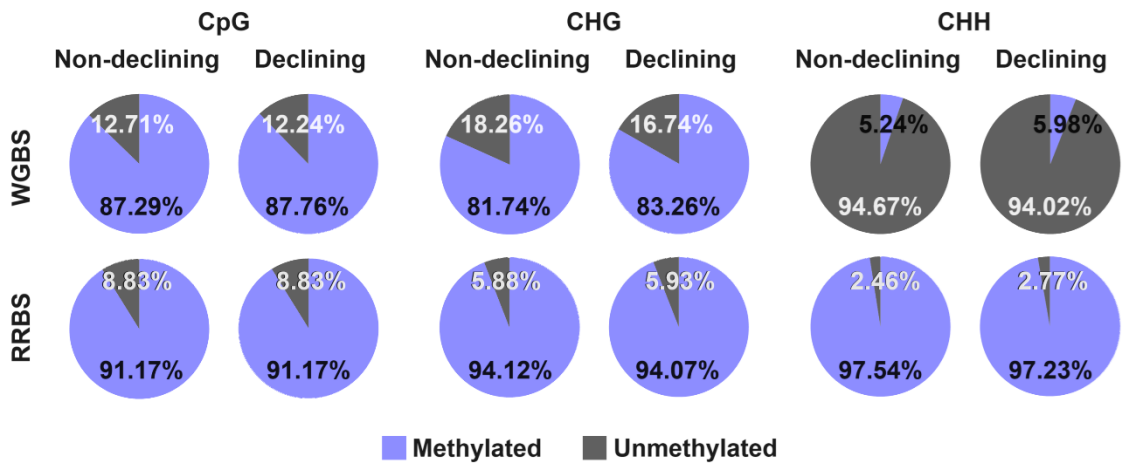

Figure 1: General methylation levels found in each vigor class (non-declining and declining trees) in each context (CpG, CHG, CHH) obtained from WGBS and RRBS data.

Hence, our WGBS results seem to be in line with previous studies that report lower DNA methylation levels in CHH sites than in CpG and CHG contexts (Bartels *et al.*, 2018), while our RRBS results agree with the hypothesis of higher DNA methylation levels within the CHH regions situated in the proximity of genes.

Significant differences between declining and non-declining trees were found in all contexts except for CpG sites when studied through RRBS (Tables S2, S3). However, it is easy to obtain significant results in contingency tests when working with large values (millions of cytosines in each test). For example, the 0.05% difference in methylation in CHG contexts using the RRBS data produces a highly significant result (p-value < 0.0001), which might seem odd, but considering the number of cytosines included in this percentage (~20,000), this outcome does not appear to be illogical. Thus, statistical significance should be combined with the identification of DMRs and their functional annotation to obtain meaningful biological significance about the role of these differently methylated sites in the context of drought-induced decline of silver fir forests.

Despite the higher amount of high quality CpG sites identified in the RRBS data, differentially methylated sites were only found in the WGBS data (Table 1). 14 CpG sites were found to have significant higher methylation levels in the declining group. 13 of these CpG sites are found in the same region (scaffold 5,292,684: 43–148), which constitute a DMR. Unfortunately, this region is part of a 180 bp-long scaffold, which does not contain any annotations and shows no similarity with any of the sequences in the NCBI database. The *A. alba* genome sequence is composed of 37 million scaffolds, sorted by descending size, of which the last 35 million have a length of less than 500 bp.

In other words, instead of having a continuous sequence for each of the 12 chromosomes (2n = 24) present in its genome (Puizina *et al*., 2008), it is fragmented into small pieces. This lack of continuity adds difficulty to the task of finding genes and other genetic structures, making it almost unattainable and highlighting the need of improvement of both the reference genome and its annotation, which is ~3 times more fragmented than those available for other conifers that have had their methylomes studied.

In conclusion, while thousands of DMRs are found in experiments with model species, such as *Arabidopsis thaliana* (Huang *et al*., 2016) and *Lycopersicon esculentum* (Zuo *et al*., 2018), this task is much more complicated in giga-genomes without high quality references. In addition, searching for differential DNA methylation between samples of the same tissue, like the ones used in this pilot study, seems to pose a more complex challenge than comparing different types of cells, likely due to more similar methylation patterns in the same type of sample. As an illustration of this point, several hundreds of DMRs were found in CpG contexts comparing needles with somatic embryogenic cultures in *P. abies* (Ausin *et al*., 2016), while only 83 DMRs were found comparing needles from different trees in *P. menziesii* (Vu *et al*., 2023).

4. Conclusions

Both WGBS and RRBS have been proven to be successful in assessing DNA methylation levels. Special attention should be paid to the context of interest when choosing between WGBS and RRBS, given the differences obtained in the CHH sites. Alternatively, there are other methods that enable the detection of methylation percentages at a lower cost, such as those based on colorimetric quantification via enzyme-linked immunosorbent assay (ELISA), which quantify global DNA methylation, regardless of cytosine context, by measuring levels of 5-methylcytosine (5-mC).

Reference genomes might not be enough to obtain meaningful results from WGBS or RRBS data when they are highly fragmented and not fully annotated, which is, unfortunately, the case of the majority of the plant species with large genomes. Even though differentially methylated cytosines between vigor classes were found, neither of these techniques stands a reliable option to investigate silver fir's giga-methylome yet, as functional annotation of the obtained DMRs is still challenging. Overall, chromosome-level references seem to be fundamental to carry out successful DNA methylation studies in plant species with giga-genomes.

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SUPPLEMENTARY MATERIAL

Table S1: Comparison of methylation levels in each context (CpG, CHG, CHH) obtained from WGBS and RRBS data. Met/Unmet indicates the total number of methylated/unmethylated cytosines found in each context-technique combination. Chi-square value (χ^2) and *p*-value (*p*-val) are shown for each test.

	CpG		CHG		CHH	
	WGBS	RRBS	WGBS	RRBS	WGBS	RRBS
Met	264,873,961	57,224,593	345,397,821	81,838,264	149,729,793	440,610,268
Unmet	37,748,583	5,544,124	73,209,110	5,138,632	2,516,295,088	11,937,258
χ^2; *p*-val	658,614; <0.0001		7,375,289; <0.0001		>2 billion; <0.0001	

Table S2: Comparison of methylation levels in each context (CpG, CHG, CHH) between non-declining (ND) and declining (D) trees obtained from the WGBS data. Met/Unmet indicates the total number of methylated/unmethylated cytosines in each vigor class. Chi-square value (χ^2) and *p*-value (*p*-val) are shown for each test.

	CpG		CHG		CHH	
	ND	D	ND	D	ND	D
Met	130,345,427	134,528,534	168,377,255	177,020,566	68,818,831	80,910,962
Unmet	18,980,629	18,767,954	37,606,507	35,602,603	1,243,672,911	1,272,622,177
χ^2; *p*-val	15,171; <0.0001		165,880; <0.0001		677,970; <0.0001	

Table S3: Comparison of methylation levels in each context (CpG, CHG, CHH) between non-declining (ND) and declining (D) trees obtained from the RRBS data. Met/Unmet indicates the total number of methylated/unmethylated cytosines in each vigor class. Chi-square value (χ^2) and *p*-value (*p*-val) are shown for each test.

	CpG		CHG		CHH	
	ND	D	ND	D	ND	D
Met	24,776,945	32,447,648	35,613,785	46,224,479	190,557,930	250,052,338
Unmet	2,400,851	3,143,273	2,225,970	2,912,662	4,800,932	7,136,326
χ^2; *p*-val	0.09250; 0.7610		77.86; <0.0001		43,510; <0.0001	

SECTION 3: TRANSCRIPTOMICS

ARTICLE 6

De novo transcriptome assembly of the silver fir, *Abies alba* Mill.

Ensamblaje *de novo* de un transcriptoma de abeto común, *Abies alba* Mill.

El abeto común, *Abies alba* Mill., es una especie clave en los ecosistemas forestales de montaña en Europa, que, a pesar de ser una de las pocas coníferas con un genoma de referencia disponible, carecía de información transcriptómica de alta calidad. En este trabajo se presenta un ensamblaje *de novo* del transcriptoma de esta especie, obtenido utilizando datos de secuenciación de lecturas cortas y largas, a partir de tres órganos diferentes: hojas, tallo y raíces. Las muestras se sometieron a diferentes tratamientos, incluyendo frío, calor, sequía y oscuridad, para inducir la expresión del mayor número de genes posible y obtener una representación altamente representativa del transcriptoma del abeto común. El ensamblaje final del transcriptoma obtuvo un valor de completitud del 94,8% según BUSCO, con 539.313 tránscritos ensamblados, de los cuales aproximadamente el 30% fueron anotados funcionalmente. Constituye el transcriptoma de mayor calidad disponible hasta la fecha para *A. alba*, proporcionando una herramienta valiosa para futuros estudios en esta especie.

De novo transcriptome assembly of the silver fir, *Abies alba* Mill.

Abstract: The silver fir, *Abies alba* Mill., is a key species in European mountain forest ecosystems, which, despite being one of the few conifers with an available reference genome, lacked high quality transcriptomic information. Here, we present a *de novo* transcriptome assembly of this species, obtained using both short- and long-read sequencing data, from three different organs: leaves, stem, and roots. Saplings were subjected to different treatments, including cold, heat, drought, and darkness, in order to induce the expression of as many genes as possible to obtain a highly comprehensive representation of the silver fir's transcriptome. The assembled transcriptome shows a completeness value of 94.8%, with 539,313 assembled transcripts, of which approximately 30% were functionally annotated. It constitutes the highest quality transcriptome available to date for *A. alba*, providing a valuable tool for further studies involving this species.

Key words: *De novo* transcriptome, *Abies alba*; Long reads; Short Reads

1. Introduction

How The silver fir (*Abies alba* Mill.), member of the Pinaceae family, is a key species in European mountain forests which holds significant ecological and economic value (Postolache *et al*., 2014). Genetic characteristics of *A. alba* have been extensively investigated, mainly through microsatellites and single nucleotide polymorphisms (SNPs) (e.g. Cvrčková *et al*., 2015; Belletti *et al*., 2017; Scotti-Saintagne *et al*., 2021), and it is one of the few conifers with an available reference genome assembly (Mosca *et al*., 2019), although it is highly fragmented. However, there is still a lack of a high-quality transcriptome, with described transcripts coming only from early developmental stages, namely less than 1-year-old seedlings (Roschanski *et al*., 2016; PRJNA159263 at NCBI) and cotyledons (Behringer *et al*., 2015; PRJNA266095 at NCBI).

Here, we present a highly complete *de novo* transcriptome assembly for *Abies alba*. Transcripts from silver fir saplings were assembled, using RNAseq data from three different organs (leaves, stem and roots) generated through both long- and short-read sequencing technologies. In addition, samples were subjected to different types of conditions, such as drought, heat or darkness, in order to trigger the expression of the highest number of genes to capture the silver fir's transcriptome as complete as possible.

Thus, we aimed to generate a robust transcriptome dataset that can serve as a foundational resource for further genetic studies in *A. alba*. For example, it could be useful for expression studies aimed at elucidating the molecular mechanisms underpinning physiological processes, such as those involved in adaptation and response to abiotic stress (Javaid *et al*., 2022). This is particularly crucial in the context of ongoing climate change, which is posing significant threats to forest ecosystems globally (Hartmann *et al*., 2022) and, specifically, in drought-prone silver fir forests. Increased research efforts should focus on understanding how silver fir forests respond to environmental stress, as decline events are being reported for this tree species across Europe (e.g. Elling *et al*., 2009; Diaci *et al*., 2011; Linares & Camarero, 2012). Consequently, the ultimate aim of this work is to support efforts in preserving silver fir populations in the face of environmental challenges.

2. Material and methods

2.1. Samples and treatments

We used 24 *Abies alba* saplings (3-year-old), obtained from a forest nursery in the Catalan Pyrenees (Spain), to perform contrasting environmental treatments (Table 1), using control growth chambers (CGC) at the Universidad Pablo de Olavide (UPO, Sevilla, Spain). Leaf, stem and root samples were taken from each of the 3 saplings in the 8 groups when their respective treatments ended.

Table 1: Characteristics of the eight treatments samples were subjected to in this study, including information about temperature, relative humidity (RH) and other stressors.

Treatment	Conditions		
	Temperature	RH	Other stressors
1 - Control	26ºC	75%	-
2 - Cold (soft)	4ºC for 48 hours	40%	No light for 48 hours
3 - Cold (extreme)	-20ºC for 2 hours	10%	No light for 2 hours
4 - Heat	45ºC for 2 hours	10%	No light for 2 hours
5 - Drought	26ºC	45% for 2 hours	-
6 - Darkness	26ºC	75%	No light for 48 hours
7 - Hormones	26ºC	75%	Watering with ABA (5mM) for 48 hours
8 - Physical damage	26ºC	75%	Stem and needle pinching for 2 hours

2.2. RNA extraction and sequencing

Total RNA was extracted from 100 mg of each sample (leaves, stem and roots of each of the three individuals subjected to each of the eight treatments) using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA). Quality and quantity were assessed using RNA ScreenTape (TapeStation 2200, Agilent Technologies), with all samples scoring RIN (RNA integrity number) > 7. Samples were then pooled (at least 1000 ng of RNA in each pool, adding the same amount of each sample) as described in Table S1; that is, by organ for long-read sequencing (3 different pools with 24 pooled samples) and by treatment separating organs for short-read sequencing (24 different pools: 8 treatments with 3 pooled samples for each of the 3 organs).

mRNA libraries were constructed and sequenced at Macrogen (Korea): TruSeq Stranded mRNA Library Prep Kit was used to construct the short-read libraries which were sequenced on Illumina NovaSeq X (9 Gb/sample), and PacBio Iso-Seq libraries were prepared for long-read sequencing on PacBio Sequel IIe system (8 Gb/sample).

2.3. Data pre-processing and *de novo* transcriptome assembly

Short raw reads were pre-processed with fastp v0.23.4 (Chen, 2023), in order to remove adapter contamination and low-quality reads (quality < 30; length < 36). Long raw reads were filtered using filtlong v0.2.1 (Wick, 2017), discarding the lowest quality 10% and reads shorter than 1000 nucleotides. Then, three *de novo* transcriptomes were assembled using Trinity v2.15.1 (Grabherr *et al*., 2011) default parameters with the "--long_reads" option; one for each organ separately.

2.4. Non-redundant transcriptome assembly

First, poorly supported transcripts were removed based on their expression level measured in Transcripts Per Kilobase per Million fragments mapped (TPM < 1) using the Trinity scripts *align_and_estimate_abundance.pl*, *abundance_estimates_to_matrix.pl* and *filter_low_expr_transcripts.pl*. Then, SuperTranscripts were obtained using the Trinity script *Trinity_gene_splice_modeler.py*. A SuperTranscript is constructed by collapsing unique and common sequence regions among splicing isoforms into a single linear sequence, creating a sequence that contains the sequence of all exons of a gene without redundancy (Davidson *et al*., 2017). Finally, all three *de novo* transcriptomes were merged and CD-HIT-EST v4.8.1 (Fu *et al*., 2012) was used to create a non-redundant assembly, removing transcripts with >95% of identity. Quality of the obtained transcriptome was assessed using BUSCO v5.5.0, using the Embryophyta lineage.

2.5. Functional annotation

Homology-based functional annotation of the transcripts was carried out using Blast2GO (Götz *et al*., 2008) from OmicsBox v3.2.9. Blast was run with standard sensitivity, the NR database (2024–04–11) filtered for Viridiplantae and keeping 3 hits. InterProScan was then used to annotate proteins with families and domains.

2.6. Transcriptomic profile characterization

subread-align (Liao *et al*., 2013) and featureCounts (Liao *et al*., 2014), both available in the Subread v2.0.6 package, were used to identify transcripts expressed in each organ in order to offer a brief description of their transcriptomic profiles. Transcripts with low counts were removed (<10 counts across the 8 samples included in each organ). GOseq v1.52.0 (Young *et al*., 2010) was then used to obtain a list of counts of GO terms in each organ and treatment. Finally, the web-based server KAAS (KEGG Automatic Annotation Server) (Moriya *et al*., 2007) was used to identify the most represented KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways in each organ and treatment, using single-directional best hit (SBH) and 18 plant species as reference (all available trees and *Arabidopsis thaliana*, because of its highly detailed annotation). Pathways related to processes which do not happen in plants were removed (see Table S2 for more information about the retained pathways in each category).

2.7. Data availability

Raw reads are available at NCBI's Sequence Read Archive (SRA), under the BioProject with accession number PRJNA1159810. The final assembled transcriptome and its annotation are available at Zenodo (10.5281/zenodo.13907293).

3. Results

3.1. Transcriptome assembly

A total of 11,542,621 long reads (~23 Gbp) and 806,809,360 short reads (~118 Gbp) passed the filtering conditions (Tables S3, S4). The *de novo* assemblies yielded ~250,000, ~460,000 and ~888,000 transcripts for leaves, stem and roots samples respectively (Table 2). After filtering by expression, creating SuperTranscripts, merging all three assemblies and clustering by similarity, the final transcriptome assembly retained 539,313 sequences, with a mean length of 501.4 bp (189 minimum – 27,963 maximum) (Table 2). Out of these transcripts, 204,754 had a blast match, 187,086 had an IPS match, and 158,622 were successfully associated with GO terms. The BUSCO analysis indicated that the assembled transcriptome is 94.8% complete (1531 complete BUSCO marker genes), with 0.7% of fragmented BUSCO marker genes (11), and 4.5% of missing BUSCO marker genes (72).

Table 2: *De novo* assembly results for each organ (leaves, stem, roots) before and after each step of data processing, including the final version (merged and non-redundant).

		Leaves	Stem	Roots
Number of sequences	Original	249,236	459,192	887,859
	Filtered	138,380	222,155	385,410
	SuperTranscripts	89,891	168,278	320,266
	Final		**539,313**	
Total length (bp)	Original	217,292,842	326,115,584	513,225,759
	Filtered	128,920,429	158,526,460	214,097,919
	SuperTranscripts	70,689,625	96,573,803	144,697,224
	Final		**270,406,007**	
Average length (bp)	Original	871.8	710.2	578.0
	Filtered	931.6	713.6	555.5
	SuperTranscripts	786.4	573.9	451.8
	Final		**501.4**	

3.2. Transcriptomic profile characterization

The multidimensional scaling (MDS) plot of distances between gene expression profiles shows that treatment pools can be grouped by organ (Figure 1), with leaves having a more diverse expression pattern between treatments than stem and roots. In addition, each organ transcriptomic profile was characterized based on the most represented GO terms and KEGG pathways. There is a high degree of similarity among the 10 most represented GO obtained for leaves, stem and roots (Figure 2), as well as in the number of transcripts involved in the different pathway categories (Figure 3), except for a slight descent in signal transduction in leaves. In fact, 47.9% of the transcripts are shared between organs (Figure 4), with 3.4% of transcripts only present in leaves, 9.9% in stem and 24.7% in roots.

Regarding treatments rather than organs, the percentage of coincidence is similar, with a 40.1% of transcripts common to all treatments, and 2.9%, 3.5%, 2.1%, 2.9%, 6.4%, 4.1%, 2.7% of transcripts only found in treatments 1, 2, 3, 5, 6, 7 and 8, respectively. Treatment 4 (heat) had no exclusive transcripts. Regarding their most descriptive GO terms, results are highly similar to those obtained when grouping by organ (Figure S1). KEGG results show similar numbers of genes in each treatment (Figure S2), with the exception of treatment 2 (cold, 4ºC), with fewer counts in some metabolic pathways and environmental information processing.

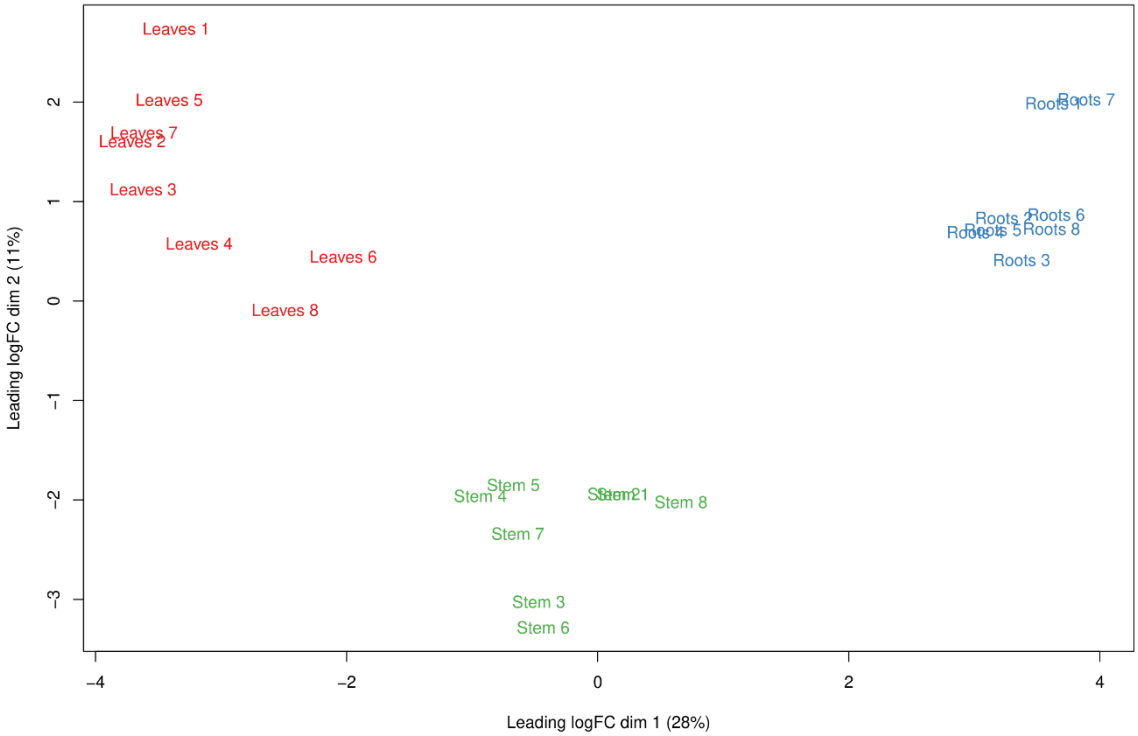

Figure 1: Multidimensional scaling (MDS) plot of distances between gene expression profiles. Leaf samples are shown in red, stem samples are shown in green and root samples are shown in blue. Numbers indicate the treatment.

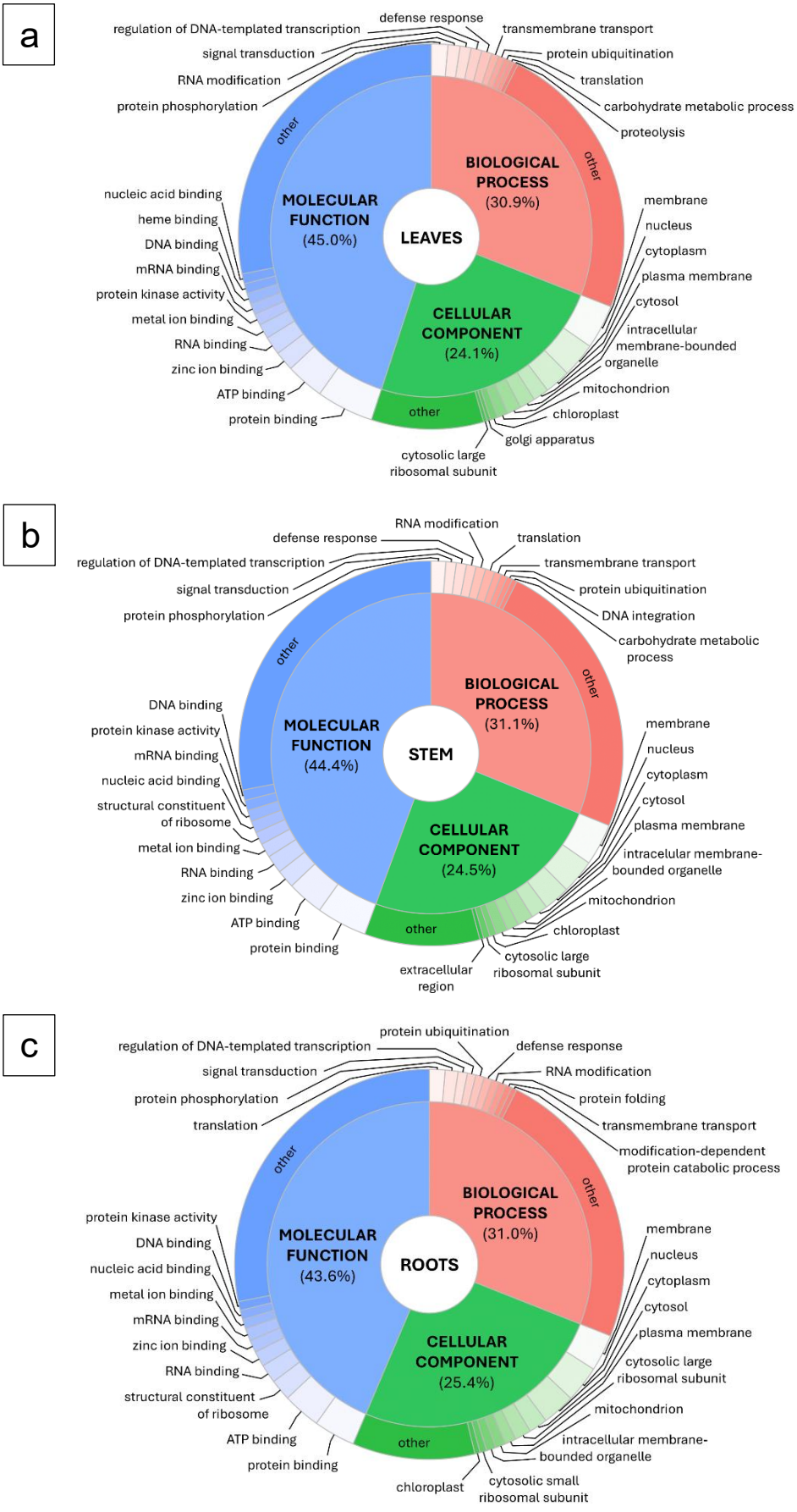

Figure 2: The 10 most represented gene ontology terms from each category in each organ.

a) Leaves. b) Stem. c) Roots.

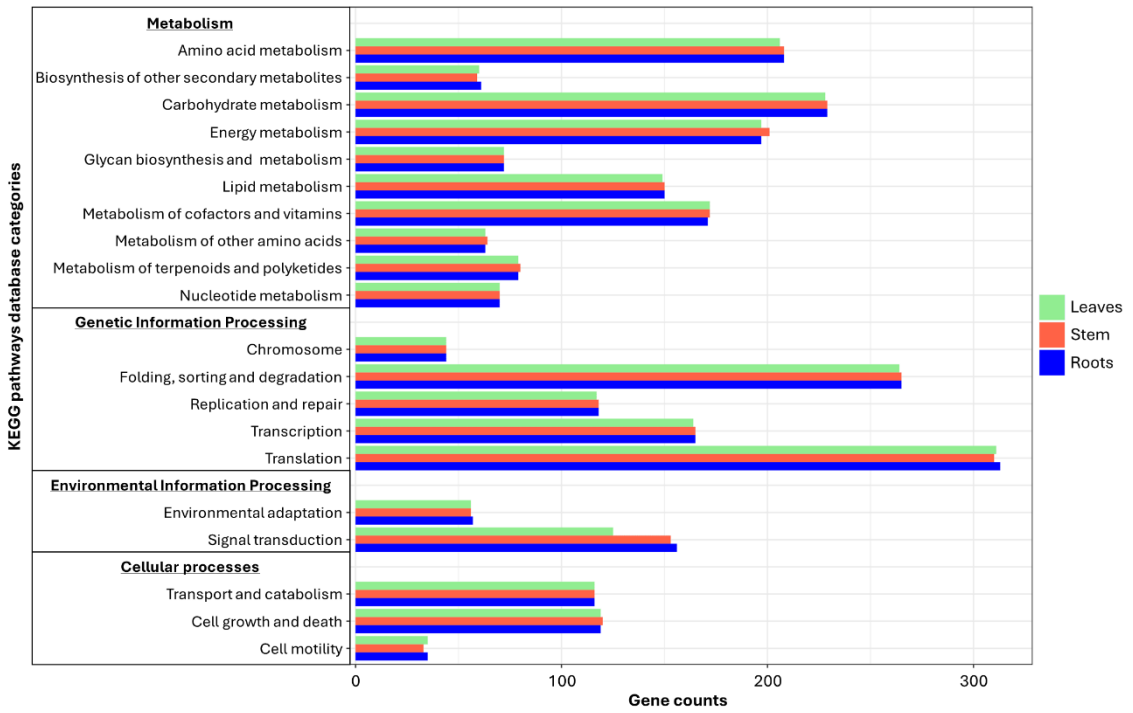

Figure 3: Total number of genes involved in the KEGG pathways included in each category (for more details, see Table S2).

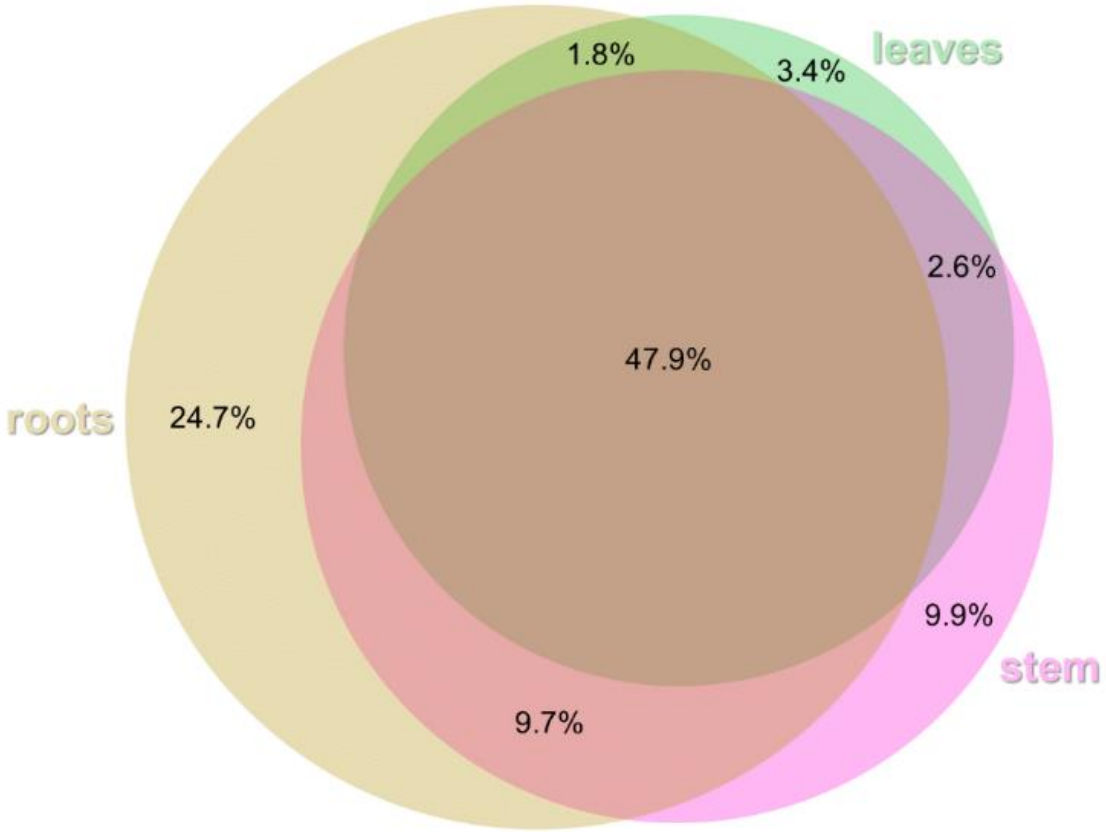

Figure 4: Venn diagram showing coincidences between the expressed transcripts in each organ.

4. Conclusions

Previous transcriptomic data of *A. alba* consisted of 65,535 transcripts (Behringer *et al*., 2015) and 25,149 contigs (Roschanski *et al*., 2016; Roschanski *et al*., 2013), which when they were mapped to the reference genome of the species yielded a 16.4% of completeness according to BUSCO (Mosca *et al*., 2019). Therefore, the here-presented transcriptome, with 539,313 sequences and a completeness value of 94.8%, obtained combining data from short and long-read sequencing, represents a substantial improvement over the previously published data and provides a valuable source of information for future studies on silver fir. In addition, information about organ-specific expression patterns was provided, showing that, even though leaves, stem and roots can be grouped separately based on their transcriptomic profiles, they share common molecular processes and biological functions, which are also displayed across the different treatments samples were subjected to.

5. References

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SUPPLEMENTARY MATERIAL

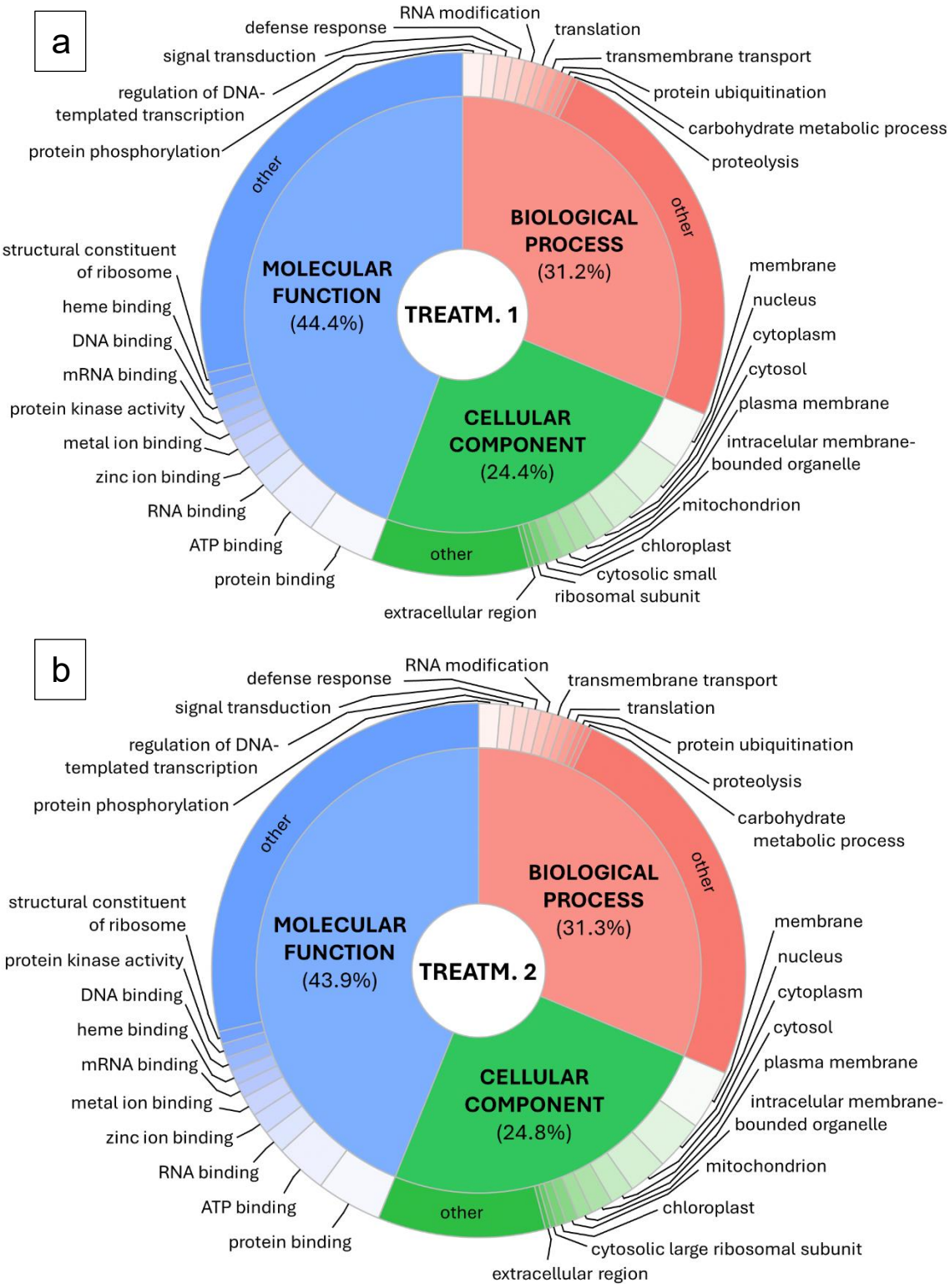
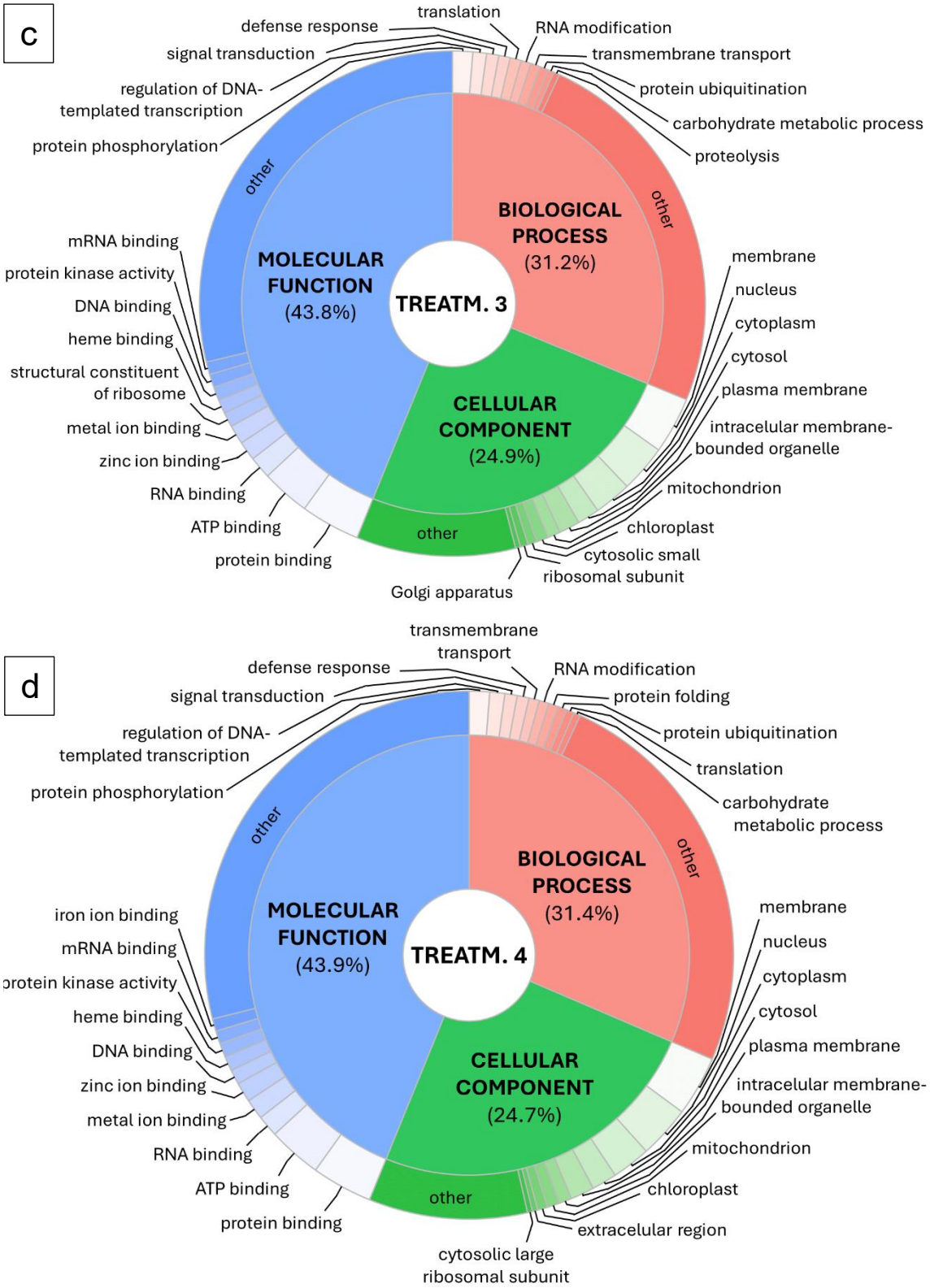


Figure S1: The 10 most represented gene ontology terms from each category in each treatment. a) Treatment 1: control conditions. b) Treatment 2: cold (soft). c) Treatment 3: cold (extreme). d) Treatment 4: heat. e) Treatment 5: drought. f) Treatment 6: darkness. g) Treatment 7: growth hormones. h) Treatment 8: physical damage. [Part 1 of 4]



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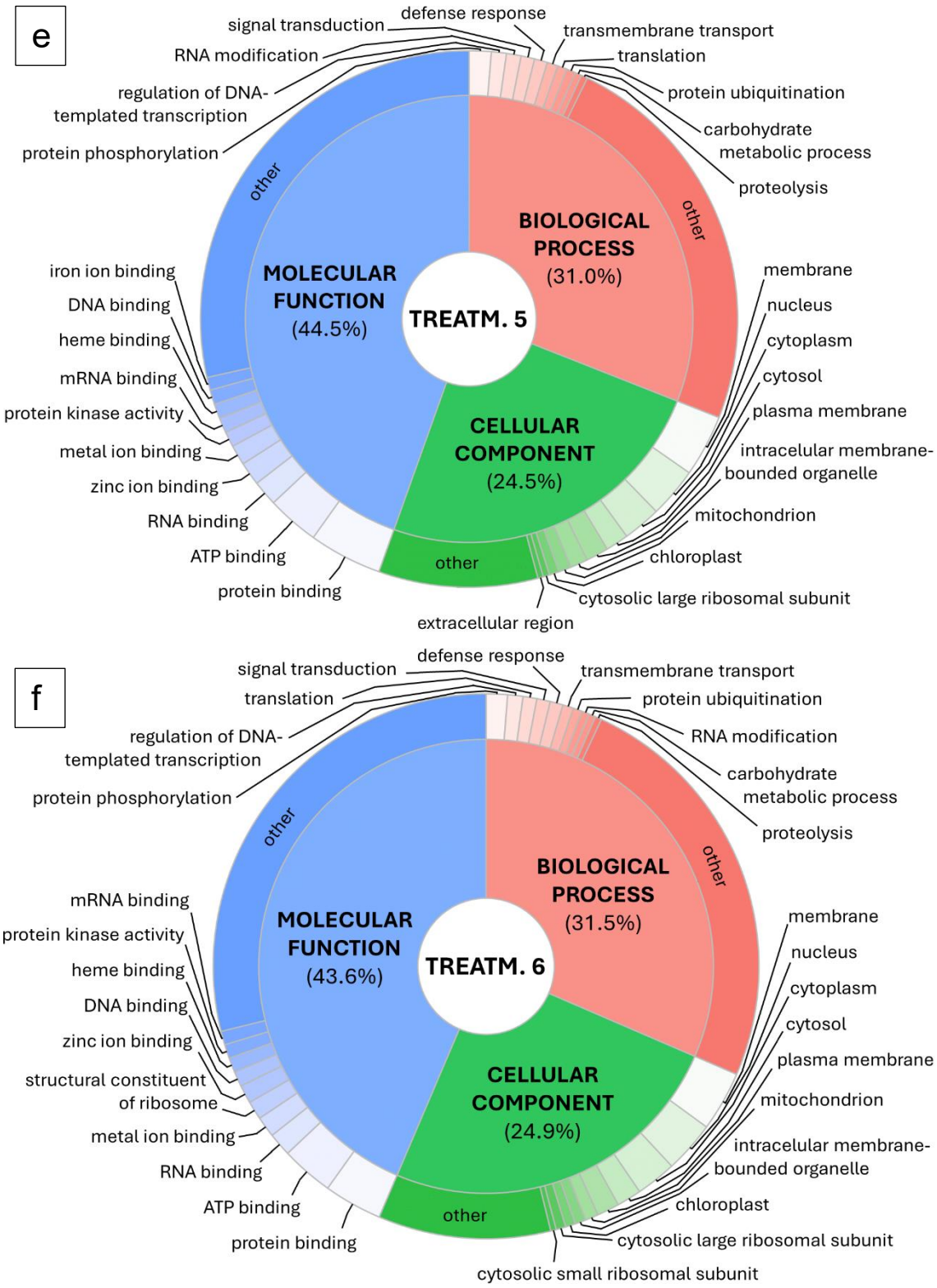

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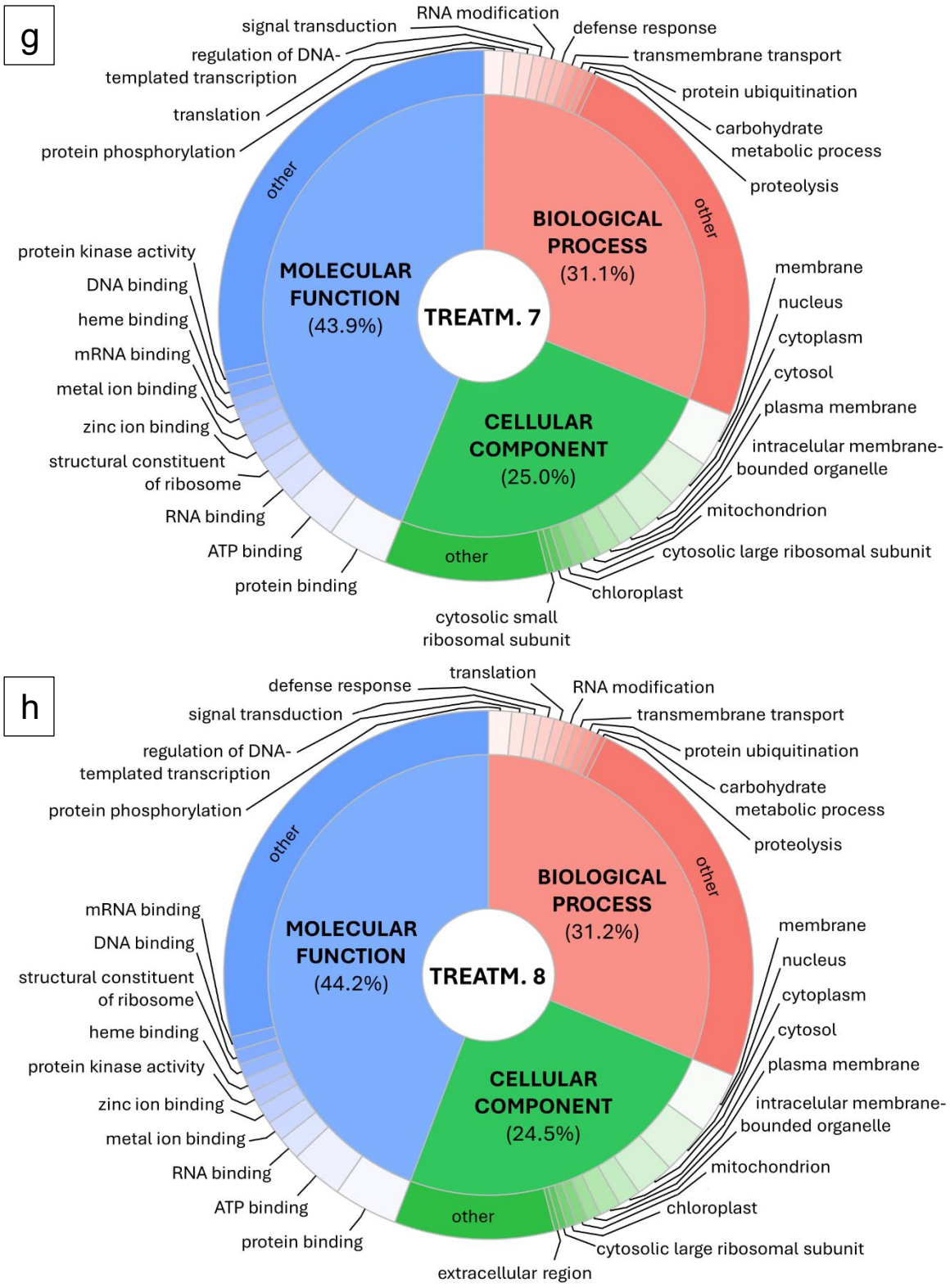

Figure S1: The 10 most represented gene ontology terms from each category in each treatment. a) Treatment 1: control conditions. b) Treatment 2: cold (soft). c) Treatment 3: cold (extreme). d) Treatment 4: heat. e) Treatment 5: drought. f) Treatment 6: darkness. g) Treatment 7: growth hormones. h) Treatment 8: physical damage. [Part 4 of 4]

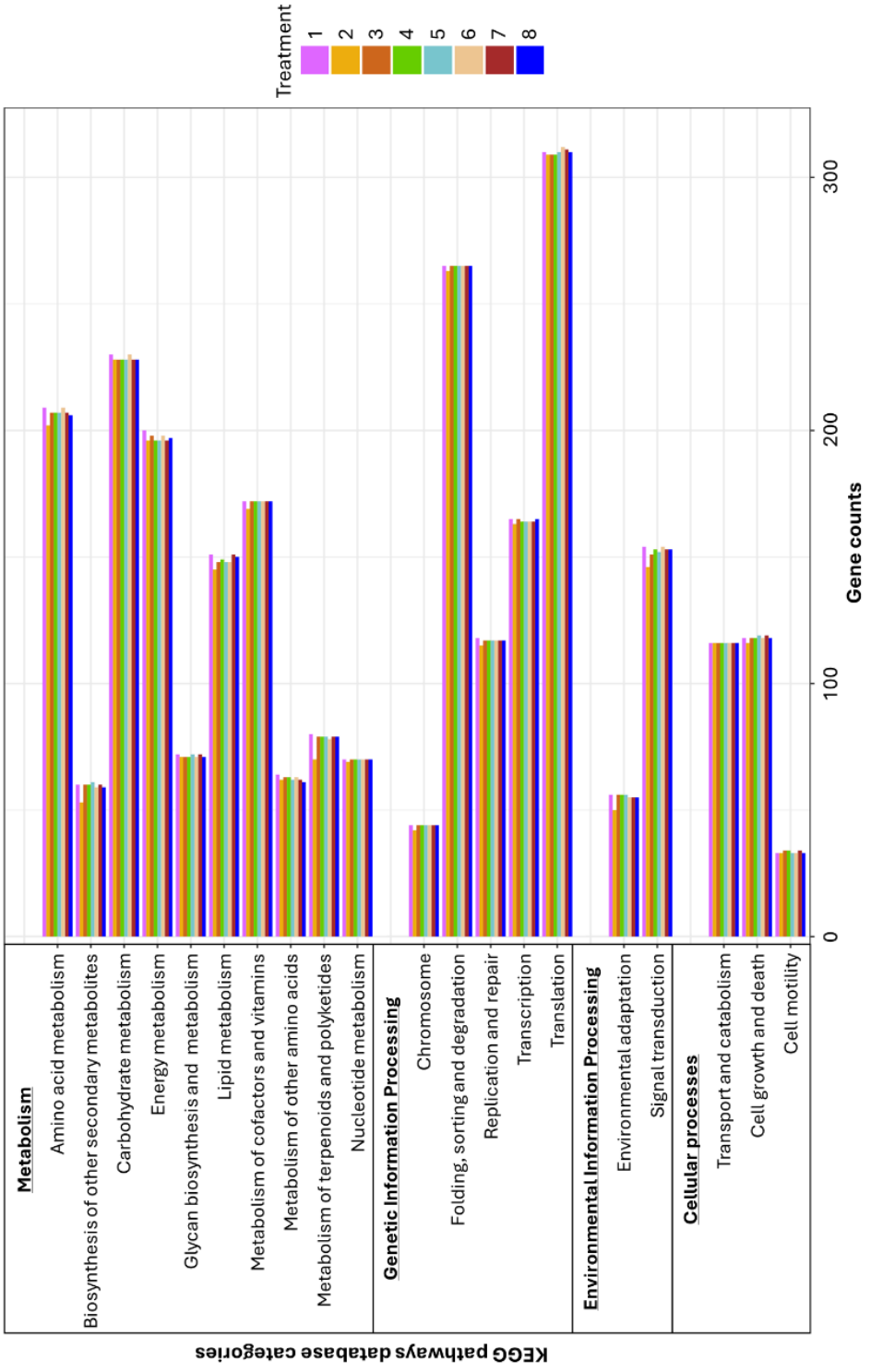

Figure S2: Total number of genes involved in the KEGG pathways included in each category (for more details, see Table S2). Treatment 1 is control conditions, 2 is cold (soft)., 3 is cold (extreme), 4 is heat, 5 is drought, 6 is darkness, 7 is growth hormones and 8 is physical damage.

Table S1: Description of the pools used in this study: samples, treatment and type of sequencing.

Pool	Samples	Treatment	Type of sequencing
Leaves_all	From Leaves 1 to Leaves24	All	Long reads
Leaves_1	Leaves1, Leaves2, Leaves3	Control	Short reads
Leaves_2	Leaves4, Leaves5, Leaves6	Cold (soft)	Short reads
Leaves_3	Leaves7, Leaves8, Leaves9	Cold (extreme)	Short reads
Leaves_4	Leaves10, Leaves11, Leaves12	Heat	Short reads
Leaves_5	Leaves13, Leaves14, Leaves15	Drought	Short reads
Leaves_6	Leaves16, Leaves17, Leaves18	Darkness	Short reads
Leaves_7	Leaves19, Leaves20, Leaves21	Hormones	Short reads
Leaves_8	Leaves22, Leaves23, Leaves24	Physical damage	Short reads
Stem_all	From Stem 1 to Stem24	All	Long reads
Stem_1	Stem1, Stem2, Stem3	Control	Short reads
Stem_2	Stem4, Stem5, Stem6	Cold (soft)	Short reads
Stem_3	Stem7, Stem8, Stem9	Cold (extreme)	Short reads
Stem_4	Stem10, Stem11, Stem12	Heat	Short reads
Stem_5	Stem13, Stem14, Stem15	Drought	Short reads
Stem_6	Stem16, Stem17, Stem18	Darkness	Short reads
Stem_7	Stem19, Stem20, Stem21	Hormones	Short reads
Stem_8	Stem22, Stem23, Stem24	Physical damage	Short reads
Roots_all	From Roots 1 to Roots24	All	Long reads
Roots_1	Roots1, Roots2, Roots3	Control	Short reads
Roots_2	Roots4, Roots5, Roots6	Cold (soft)	Short reads
Roots_3	Roots7, Roots8, Roots9	Cold (extreme)	Short reads
Roots_4	Roots10, Roots11, Roots12	Heat	Short reads
Roots_5	Roots13, Roots14, Roots15	Drought	Short reads
Roots_6	Roots16, Roots17, Roots18	Darkness	Short reads
Roots_7	Roots19, Roots20, Roots21	Hormones	Short reads
Roots_8	Roots22, Roots23, Roots24	Physical damage	Short reads

Table S2: Pathways included in each KEGG pathways category.

Category	KEGG pathway numbers
Amino acid metabolism	00250; 00260; 00270; 00280; 00290; 00300; 00310; 00220; 00330; 00340; 00350; 00360; 00380; 00400
Biosynthesis of other secondary metabolites	00940; 00945; 00941; 00944; 00943; 00946; 00901; 00950; 00960; 00232; 00965; 00966; 00999
Carbohydrate metabolism	00010; 00020; 00030; 00040; 00051; 00052; 00053; 00500; 00520; 00620; 00630; 00640; 00650; 00660; 00562
Energy metabolism	00190; 00195; 00196; 00710; 00720; 00680; 00910; 00920
Glycan biosynthesis and metabolism	00510; 00513; 00514; 00531; 00563
Lipid metabolism	00061; 00062; 00071; 00073; 00100; 00561; 00564; 00565; 00600; 00590; 00591; 00592; 01040
Metabolism of cofactors and vitamins	00730; 00740; 00750; 00760; 00770; 00780; 00785; 00790; 00670; 00830; 00860; 00130
Metabolism of other amino acids	00410; 00440; 00450; 00460; 00470; 00480
Metabolism of terpenoids and polyketides	00900; 00902; 00909; 00904; 00906; 00905; 00908
Nucleotide metabolism	00230; 00240
Chromosome	03082; 03083
Folding, sorting and degradation	03060; 04141; 04130; 04120; 04122; 03050; 03018
Replication and repair	03030; 03410; 03420; 03430; 03440; 03450
Transcription	03020; 03022; 03040
Translation	03010; 00970; 03013; 03015; 03008
Environmental adaptation	04712; 04626
Signal transduction	02020; 04016; 04014; 04310; 04390; 04020; 04070; 04072; 04071; 04024; 04022; 04075
Transport and catabolism	04144; 04146; 04136
Cell growth and death	04110; 04114; 04210; 04216; 04217; 04218
Cell motility	04814; 04810

Table S3: Long reads filtering results, comparing before and after metrics.

	HiFi read bases		Number of HiFi reads		Average read length	
Pool	Before	After	Before	After	Before	After
Leaves_all	8.060 Gbp	7.254 Gbp	4,563,638	3,814,952	1,766 bp	1,901 bp
Stem_all	8.181 Gbp	7.363 Gbp	4,769,816	3,845,637	1,715 bp	1,915 bp
Roots_all	8.812 Gbp	7.931 Gbp	4,706,956	3,882,032	1,872 bp	2,043 bp

Table S4: Short reads filtering results, comparing before and after metrics.

	Read bases		Number of reads		Kept reads
Pool	Before	After	Before	After	
Leaves_1	5.337 Gbp	5.109 Gbp	35,345,669	34,260,830	96.93%
Leaves_2	5.331 Gbp	4.904 Gbp	35,307,038	34,009,664	96.32%
Leaves_3	4.713 Gbp	4.271 Gbp	31,213,322	29,759,211	95.34%
Leaves_4	5.271 Gbp	4.812 Gbp	34,904,525	33,551,470	96.12%
Leaves_5	5.543 Gbp	5.093 Gbp	36,711,801	35,484,308	96.66%
Leaves_6	4.622 Gbp	4.312 Gbp	30,611,158	29,484,223	96.32%
Leaves_7	4.888 Gbp	4.556 Gbp	32,372,253	31,286,631	96.65%
Leaves_8	5.186 Gbp	4.915 Gbp	34,347,308	33,188,979	96.63%
Stem_1	5.581 Gbp	5.300 Gbp	36,959,504	35,861,984	97.03%
Stem_2	4.893 Gbp	4.652 Gbp	32,404,660	31,407,367	96.92%
Stem_3	4.510 Gbp	4.321 Gbp	29,866,275	28,880,144	96.70%
Stem_4	5.968 Gbp	5.703 Gbp	39,526,179	38,373,025	97.08%
Stem_5	5.499 Gbp	5.254 Gbp	36,420,152	35,216,084	96.69%
Stem_6	6.137 Gbp	5.702 Gbp	40,645,102	38,976,000	95.89%
Stem_7	5.229 Gbp	4.972 Gbp	34,631,970	33,469,669	96.64%
Stem_8	5.516 Gbp	5.071 Gbp	36,531,559	34,916,804	95.58%
Roots_1	5.859 Gbp	5.411 Gbp	38,798,557	37,239,809	95.98%
Roots_2	4.972 Gbp	4.763 Gbp	32,929,842	31,944,084	97.01%
Roots_3	4.746 Gbp	4.347 Gbp	31,427,457	29,826,443	94.91%
Roots_4	6.494 Gbp	5.819 Gbp	43,005,530	41,435,716	96.35%
Roots_5	5.156 Gbp	4.909 Gbp	34,142,726	33,091,680	96.92%
Roots_6	4.811 Gbp	4.312 Gbp	31,859,103	30,528,425	95.82%
Roots_7	5.122 Gbp	4.882 Gbp	33,924,696	32,930,602	97.07%
Roots_8	4.980 Gbp	4.612 Gbp	32,981,503	31,686,208	96.07%

ARTICLE 7

Rooting for resilience: transcriptomic, epigenomic and physiologic characterization of the drought stress response of the silver fir (*Abies alba* Mill.)

Enraizando la resiliencia: caracterización transcriptómica, epigenómica y fisiológica de la respuesta al estrés por sequía del abeto común (*Abies alba* Mill.)

Comprender los mecanismos biológicos que subyacen a las respuestas de los árboles a la sequía es crucial para preservar la biodiversidad forestal, ya que el cambio climático está desafiando la capacidad de los árboles sensibles a la sequía para afrontar la escasez de agua. Este trabajo estudia cómo el abeto común (*Abies alba* Mill.) responde al estrés por sequía experimental analizando sus perfiles de expresión génica y de metilación del ADN en diferentes órganos, junto con variables fisiológicas, en condiciones de riego normal, sequía y recuperación. Las raíces mostraron una respuesta transcriptómica más fuerte que las hojas, con 50 veces más genes alterados, lo que revela su valor para evaluar el estrés hídrico en esta especie, que se podría llevar a cabo a través del estudio de la expresión de genes implicados en el transporte de agua en este órgano. Además, los genes relacionados con los brasinoesteroides podrían servir como marcadores de estrés tanto en raíces como en hojas. La sequía también afecta la metilación del ADN, la cual, al igual que las variables transcriptómicas y fisiológicas, comienza a normalizarse una vez que el estrés termina, lo que sugiere cierta resiliencia a la sequía por parte de *A. alba*. Sin embargo, presenta dificultades para mejorar la eficiencia en el uso del agua, lo que aumenta su vulnerabilidad a la sequía en lo relativo al balance de carbono. En conclusión, la resiliencia del abeto común a la sequía parece que dependerá en gran medida tanto de las características del estrés, como la intensidad y duración de los episodios de sequía, como de las características intrínsecas de las plantas, como el sistema radicular y las reservas de carbono.

Rooting for resilience: transcriptomic, epigenomic and physiologic characterization of the drought stress response of the silver fir (*Abies alba* Mill.)

Abstract: Understanding the biological mechanisms underlying tree responses to drought is critical for preserving forest biodiversity, as current global climate change is challenging the ability of drought-sensitive trees to cope with water shortage. In this study, we investigate how silver fir (*Abies alba* Mill.) responds to experimental drought stress by analyzing the gene expression and DNA methylation profiles of different organs alongside physiological variables under well-watered, drought and recovery conditions. Roots exhibited a stronger transcriptomic response than leaves, with 50 times more altered genes, revealing their value for assessing water stress in this species through the expression of genes involved in water transport. In addition, brassinosteroid-related genes can serve as stress markers both in roots and leaves. Drought also affects DNA methylation, which, like transcriptomic and physiological variables, begins to normalize once the stress is over, suggesting some resilience to drought. However, *A. alba* struggles to improve intrinsic water use efficiency, which raises its carbon balance-related vulnerability to drought. Our results suggest that drought resilience in silver fir is strongly dependent on both extrinsic stressors, such the intensity and duration of drought episodes, and intrinsic plants' characteristics, such plant root system and carbon reserves.

Key words: Drought; RNAseq; DNA methylation; Physiology; *Abies alba*

1. Introduction

Current global climate change has triggered fluctuations in temperature, rainfall, and atmospheric vapor pressure deficit, subjecting plants to harsh and extreme climates, which have a negative impact on their morphological, developmental, cellular, and molecular processes (Chaudhry & Sidhu, 2022). Particularly, drought stress is raising serious concerns about the survival of forest tree species, as it can result in reduced growth, higher mortality rates, and even widespread mass mortality (van Mantgem & Stephenson, 2007; McDowell *et al*., 2010; Allen *et al*., 2010). Thus, it is of upmost importance to understand the biological mechanisms behind trees response to drought stress in order to be able to prevent their decline and protect their current distributions and forest biodiversity (Anderegg *et al*., 2013; McDowell *et al*., 2020).

Plants employ various defense mechanisms to cope with drought stress, which include biochemical (e.g. chlorophyll content, hormonal content, secondary metabolites), physiological (e.g. photosynthesis, osmotic balance, transpiration), and morphological changes (e.g. decreased leaf area, increase in root length, changes in growth) (Oguz *et al*., 2022). These responses are driven by molecular mechanisms that are triggered by water deprivation, which can be characterized through transcriptomic analyses. Thus, the classical study of physiological variables can be combined with the study of transcriptomic profiles, which can be used to evaluate the adaptive potential of natural tree populations (Behringer *et al*., 2015), by allowing to identify key genes involved in drought response.

In addition to RNA sequencing experiments, DNA methylation is becoming a field of interest, due to its implication in gene expression regulation (Kumar & Mohapatra, 2021). DNA methylation at cytosine residues is a widely studied epigenetic mark in plants, which is, however, hindered at single-base resolution in some forest species, such as conifers, due to their complex genomes and the lack of high-quality reference genomes (García-García *et al*., 2022). Nonetheless, methods to study global DNA methylation levels are available, offering information about the epigenomes of these organisms, which, in combination with expression data, can be used to identify major processes involved in drought stress response and coping mechanisms.

In this study, we focus on silver fir, *Abies alba* Mill., a conifer species distributed across Europe, where drought events are predicted to increase in frequency and severity in the next decades (IPCC, 2022). The main objective was to characterize the molecular response to drought stress of *A. alba*, both from a genetic and epigenetic perspective, as well as its physiological response. More specifically, we aimed to identify alterations

in the transcriptomic profiles of samples subjected to drought conditions compared to well-watered trees and to samples recovering from said stress, as well as changes in DNA methylation and several physiological variables. In addition to data from leaves, which are the preferred sample for this kind of studies (e.g. Sena *et al*., 2018; Depardieu *et al*., 2021; Xiao *et al*., 2024), we present, for the first time, transcriptomic data obtained from roots, offering a more complete view of the effect of drought on silver fir.

2. Material and methods

2.1. Samples, growth conditions and drought stress experiment

Experimental design was carried out with 3-year-old *A. alba* saplings from the southwestern rear edge of the silver fir distribution range, in the Catalan Pyrenees, obtained from a forest nursery, which were introduced in controlled growth chambers (CGC) under constant conditions of relative humidity (RH = 75%), temperature (T = 27ºC) and an air vapor pressure deficit (VPD = 0.74 kPa), which was calculated using temperature and relative humidity (Cobo-Simón *et al*., 2023), with a photoperiod of 12 hours of light / 12 hours of darkness (light at 600 lux from 7 AM to 7 PM). Net photosynthesis and stomatal conductance were measured weekly by means of a gas exchange system provided with an infrared gas analyzer (IRGA) (LI-COR, LI-6400XT, EEUU), using a conifer chamber (6400-05).

All saplings stayed in the CGC at least for 15 days for acclimation, until the two variables used to monitor them (net photosynthesis and stomatal conductance) showed high and stable values. Then, they were divided into groups and subjected to three different conditions (Figure 1): (1) control group, (2) drought group, and (3) recovery group. The control group grew in an environment with RH = 75%, T = 27ºC and VPD = 0.74 kPa. Samples were taken after 1 month in said conditions, with high, stable net photosynthesis and stomatal conductance values. Drought treatment was applied by reducing RH to 45% (VPD = 2.3 kPa), while temperature was maintained at 27ºC. Samples were taken after 1 month under this stress, with net photosynthesis reaching critical values for survival. Finally, the recovery group was returned to control conditions after the stress for two weeks, when samples were taken.

Leaf (needles), shoot and root samples from at least 4 individuals per group were taken to determine physiological variables when necessary. Leaf and roots samples were taken from 4 individuals per group for RNA sequencing and DNA methylation levels assessment.

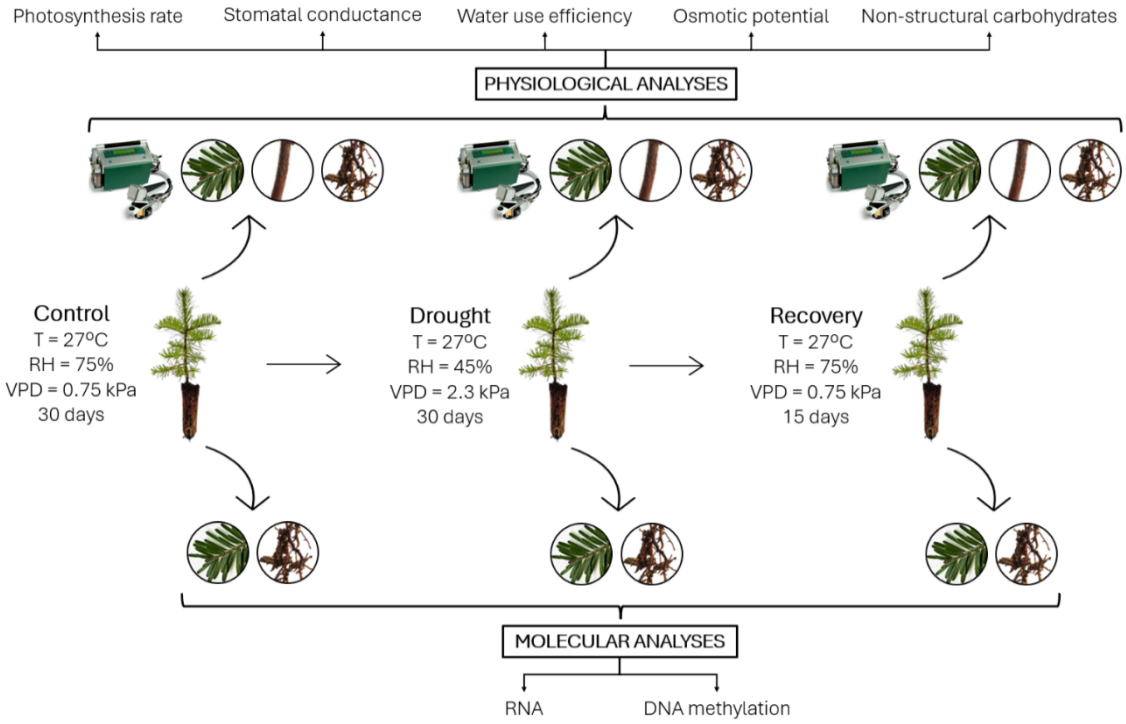

Figure 1: Experimental design of the drought treatment and sampling procedure. T, temperature; RH, Relative Humidity; VPD, Vapor Deficit Pressure.

2.2. Physiological variables

In order to analyze the physiological state of the individuals through the different treatments, different variables were studied. As previously mentioned, net photosynthesis (A) and stomatal conductance (Gs) were monitored through all the experiment. From these two variables, intrinsic water use efficiency (iWUE) was calculated as iWUE=A/Gs. Osmotic potential in needles was measured using a WP4-T dew point potentiometer (Decagon Devices Inc), calibrated with a KCl solution. Non-structural carbohydrates (NSCs), namely total soluble sugars (TSS) and starch, were measured in needles, shoots and roots separately and then summed up, following the resorcinol method (Landhäusser *et al.*, 2018). GraphPad Prism v10.0.0 for Windows (GraphPad Software, Boston, Massachusetts, USA) was used to test differences between treatments, performing unpaired t-tests for parametric variables and Mann Whitney tests for non-parametric variables, after having tested normality through Shapiro-Wilk tests.

2.3. RNA extraction, sequencing and differential expression analysis

RNA was extracted from 100 mg of each organ and sample (12 roots + 12 leaves in total) using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA). ScreenTape (TapeStation 2200, Agilent Technologies) was used to measure quantity and quality (RNA integrity number > 7) of the extractions, which were then sent to Macrogen (Korea) for sequencing on Illumina NovaSeq X, yielding 9 Gb/sample. Raw reads were cleaned with fastp v0.23.4 (Chen, 2023), removing adapter contamination and low-quality bases and reads (quality < 30; length < 36). The retained high-quality reads were mapped to the *Abies alba* transcriptome (García-García, 2025) using subread-align (Liao *et al*., 2013) and gene counts were obtained using featureCounts (Liao *et al*., 2014), both available in the Subread v2.0.6 package. Transcripts with low counts (<15 per group) were removed, counts were normalized and differentially expressed genes (DEGs) were identified with edgeR v3.42.4 (Robinson *et al*., 2010). Leave and root samples were analyzed separately, making comparison between each pair of groups (drought-control; drought-recovery; recovery-control). FDR correction was applied and those genes with a q-value < 0.05 were considered DEGs.

MapMan4 (Schwacke *et al*., 2019) and Mercator4 (Lohse *et al*., 2014) were used to visualize how DEGs are distributed across general plant metabolic processes, with 89.36% of the genes used by these programs being successfully assigned to *A. alba* transcripts. In addition, GOseq v1.52.0 (Young *et al*., 2010) was used to get a general description of biological processes of the identified DEGs through an over-representation analysis (ORA), using the custom database for the *A. alba* transcriptome (García-García, 2025). An FDR correction was applied and GO terms with a q-value < 0.05 were considered significantly overrepresented. Finally, DEGs with the following gene ontology (GO) terms were selected as candidate genes for drought stress response in *A. alba*: water transport (GO:0006833), water channel activity (GO:0015250), response to water (GO:0009415), and response to water deprivation (GO:0009414). In addition, based on the GO over-representation analysis, genes associated with the following terms related to brassinosteroids were also selected: brassinosteroid biosynthetic process (GO:0016132), and brassinosteroid homeostasis (GO:0010268).

2.4. Global DNA methylation levels analysis

Global DNA methylation levels of each sample were assessed using the *MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit* (Epigentek, USA). It consists of a colorimetric quantification of global DNA methylation status by specifically

measuring levels of 5-methylcytosine (5-mC) in an ELISA-like reaction. Each sample was measured 4 times to ensure consistency, using 100 ng of genomic DNA, which was extracted using with the *DNeasy Plant Mini Kit* (Qiagen®, Germany) from 100 mg of lyophilized leaves / roots. Mean values were calculated, and DNA methylation percentages were calculated following the manufacturer instruction. Differences between groups (control, drought, recovery) were tested through t-tests after checking for normality with a Shapiro-Wilk test in GraphPad.

2.5. Data availability

Raw RNA sequences are available at NCBI's Sequence Read Archive (SRA), under the BioProject with accession number PRJNA1170242.

3. Results

3.1. Physiological variables

Under control conditions, photosynthetic rate was 7.24 (± 0.57) µmol $CO_2/m^2 \cdot s$, which was significantly reduced to 2.79 (± 0.29) µmol $CO_2/m^2 \cdot s$ in samples subjected to drought and showed an intermediate value of 4.84 (± 0.55) µmol $CO_2/m^2 \cdot s$ in the recovery group, yielding significant differences with both control and drought conditions (Figure 2a).

Stomatal conductance under control conditions was 37.93 (± 3.03) mmol $H_2O/m^2 \cdot s$, significantly dropped to 15.08 (± 3.25) $H_2O/m^2 \cdot s$ in the drought group and raised to 22.47 (± 2.67) $H_2O/m^2 \cdot s$ in the recovery group, which only showed significant differences with the control group, but not with the drought treatment (Figure 2b).

Regarding water use efficiency, there were no significant differences across treatments, with values of 193.6 (± 8.7) µmol CO_2/mol H_2O, 233.1 (± 33.8) µmol CO_2/mol H_2O and 220.6 (± 22.9) µmol CO_2/mol H_2O in the control, drought and recovery group respectively (Figure 2c). Similarly, statistical analyses revealed no significant differences in osmotic potential between groups (Figure 2d). Samples exhibited a mean of -2.86 (± 0.11) MPa in the control treatment, -3.27 (± 0.32) MPa under drought conditions and -2.94 (± 0.28) MPa in the recovery group.

Finally, the analysis of NSCs revealed significant differences across treatments in TTS, with a marked drop from 835.9 (± 22.5) mg/gDW to 439.7 (± 23.1) mg/gDW in drought conditions compared to the control group, which then increased to 616.9 (± 20.9) mg/gDW (Figure 2e). Regarding starch, the control and drought groups showed similar values, with 228.0 (± 7.3) mg/gDW and 234.2 (± 17.4) mg/gDW respectively, with a significant decrease in the samples subjected to recovery (179.8 ±13.9 mg/gDW) compared to control conditions (Figure 2f). Results were similar when testing each organ separately (Figure S1).

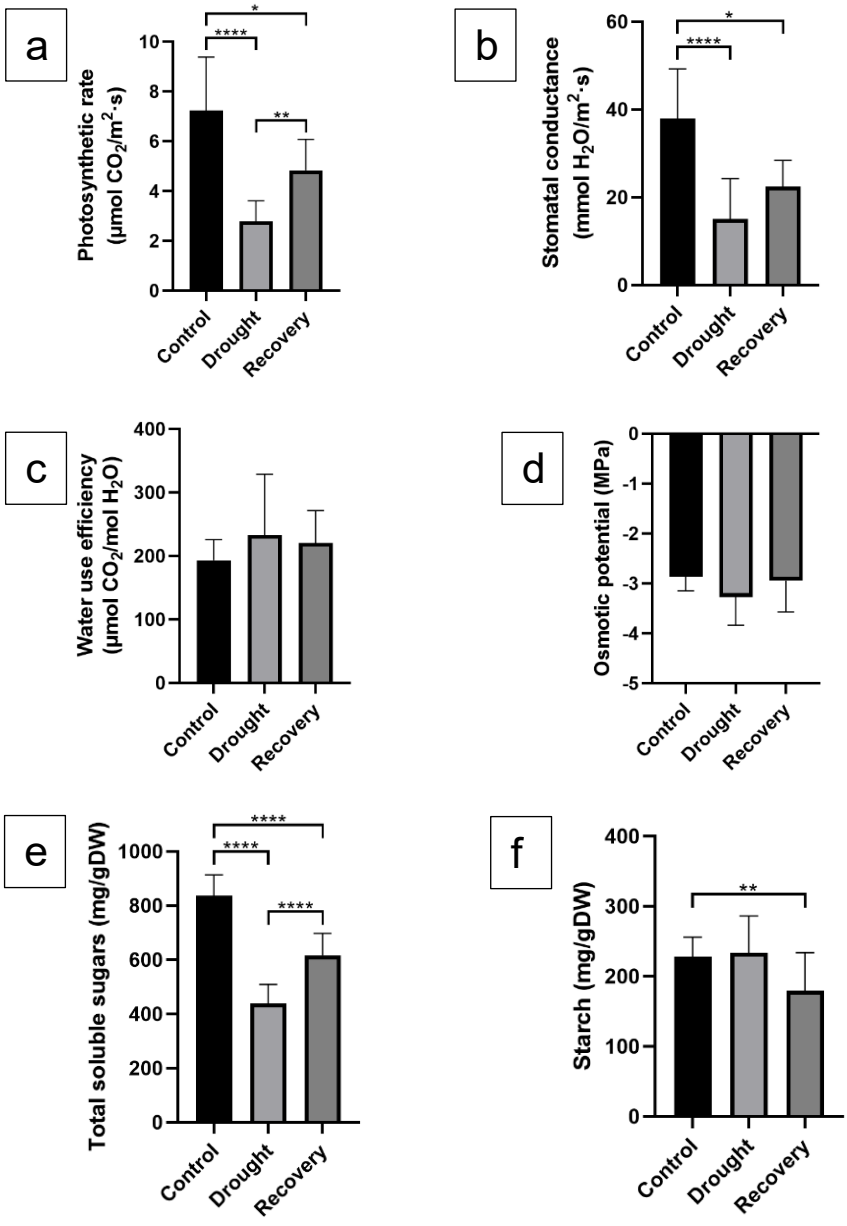

Figure 2: Physiological analyses results, indicating differences between groups (control, drought, recovery). *p*-value significance codes: 0 '****' 0.0001 '***' 0.001 '**' 0.01 '*' 0.05. a) Photosynthetic rate (n = 27). b) Stomatal conductance (n = 27). c) Water use efficiency (n = 27). d) Osmotic potential (n = 14). e) Total soluble sugars (n = 36). f) Starch (n = 39).

3.2. Differential expression analysis

On average, >96% of the reads passed the trimming filters (Table S1), which were then mapped to the reference transcriptome and filtered based on their counts, resulting in 41,768 expressed transcripts in leaves and 53,803 in roots.

The multidimensional scaling (MDS) plot of distances between gene expression profiles shows a mixed response in leaves (Figure 3a) while samples can be grouped by treatment in roots (Figure 3b). As a result, a higher number of DEGs were identified in roots (Figure 4). Leaves subjected to drought showed 27 upregulated and 49 downregulated transcripts compared to the control group (Figure 4a; Table S2), 41 upregulated and 64 downregulated transcripts compared to the recovery group (Figure 4b; Table S3), and no differences were found between recovery and control treatments (Figure 4c). On the other hand, roots subjected to drought showed 3,044 upregulated and 3,015 downregulated transcripts compared to the control group (Figure 4d; Table S4), 1,191 upregulated and 1,496 downregulated transcripts compared to the recovery group (Figure 4e; Table S5), and no differences were found between recovery and control treatments (Figure 4f). More than half of the DEGs identified in leaves is part of the DEGs identified in roots (Figure 5a), just like there is a high degree of coincidence between the transcripts altered by drought compared to the control and recovery groups both in leaves (Figure 5b) and roots (Figure 5c).

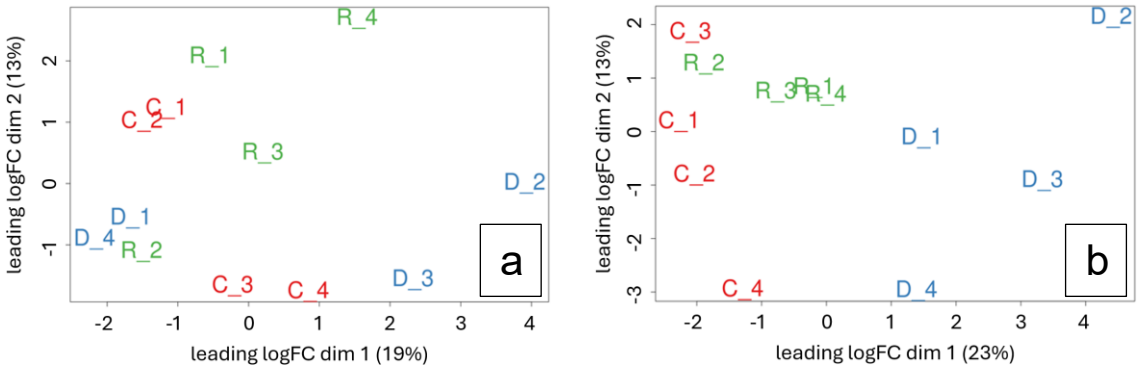

Figure 3: Multidimensional scaling (MDS) plot based on distances between gene expression profiles in leaves (a) and roots (b). C stands for control (in red), D for drought (in blue) and R for recovery (in green).

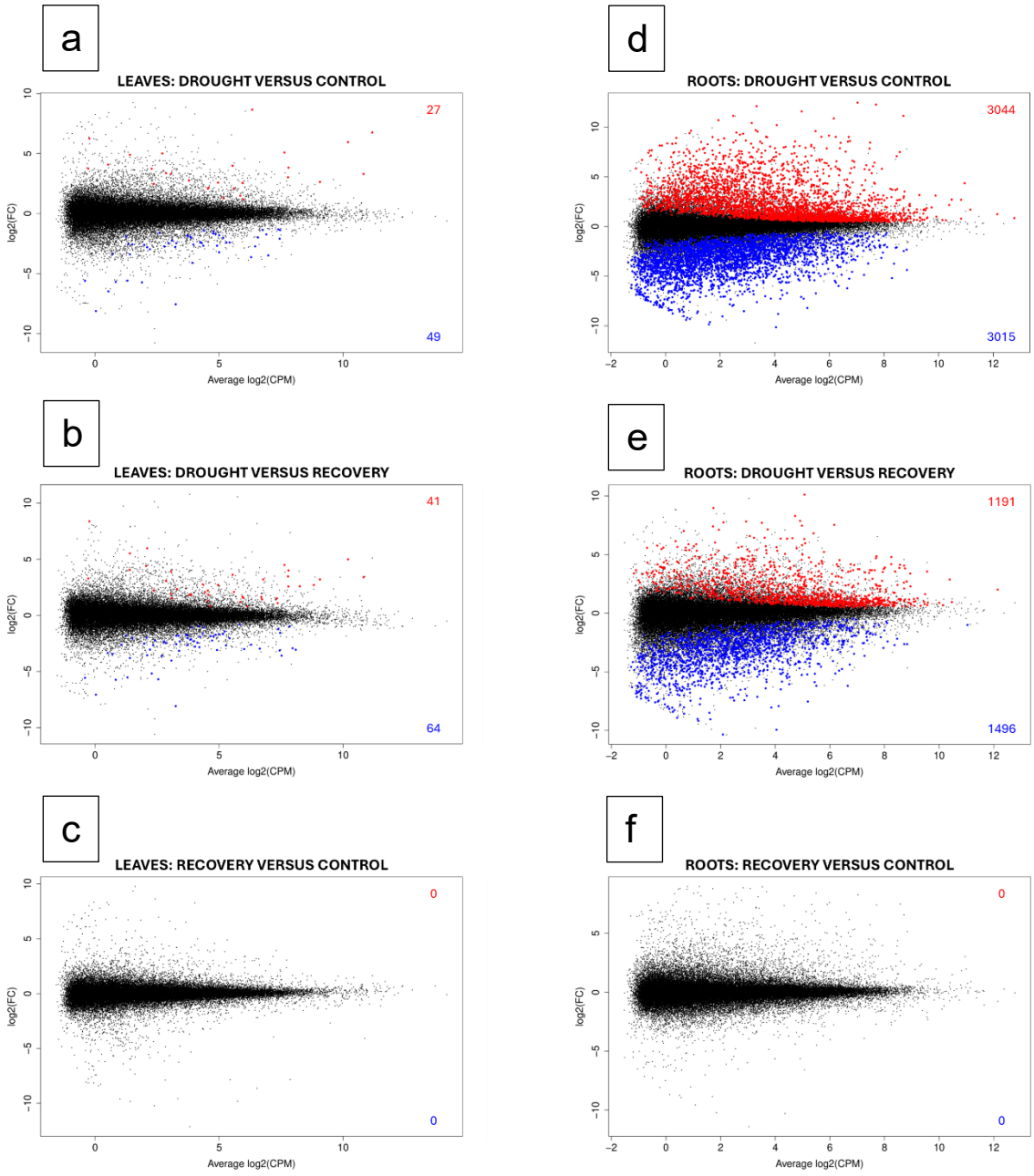

Figure 4: Mean-difference (MD) plot of expression data. a) Leaves subjected to drought compared to leaves in control conditions. b) Leaves subjected to drought compared to leaves in recovery. c) Leaves recovering from drought stress compared to control leaves. d) Roots subjected to drought compared to roots in control conditions. e) Roots subjected to drought compared to roots in recovery. f) Roots recovering from drought stress compared to control roots.

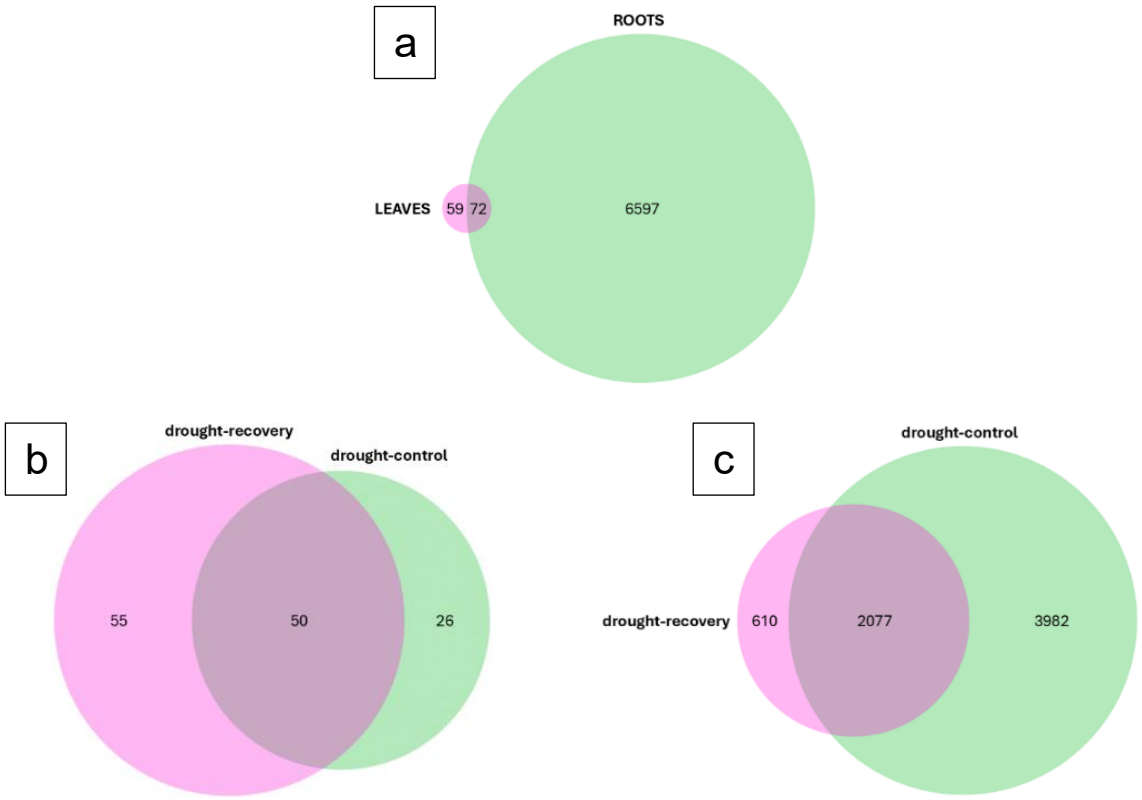

Figure 5: Venn-diagrams showing coincidences in DEGs between different groups. a) Leaves versus roots. b) Drought conditions compared to recovery versus drought compared to control conditions in leaves. c) Drought conditions compared to recovery versus drought compared to control conditions in roots.

Regarding metabolic pathways in which DEGs can be found, leaves show less altered metabolic pathways (Figure 6a, b) than roots (Figure 6c, d), as expected by the number of DEGs found in each organ. Processes related to cell wall are downregulated, as well as terpenoid metabolism, lipid metabolism and nutrient uptake, while carbohydrate and amino acid metabolism processes show upregulation, along with energy production (OPP pathway and TCA cycle). In agreement with this, the GO over-representation analysis (Figures 7–9, Tables S6–S9) reveal that drought causes an increase in transcripts involved in response to stimulus (e.g. response to water, abscisic acid-activated signaling pathway) and some metabolic processes (e.g. different amino acid and carbohydrate metabolic processes), while it decreases the expression of genes related to several metabolic processes (e.g. involving brassinosteroids, sterols, lipids and some secondary metabolites, such as terpenoids), cellular organization (e.g. cell wall biogenesis) and developmental processes. Both leaves and roots present similar GO results.

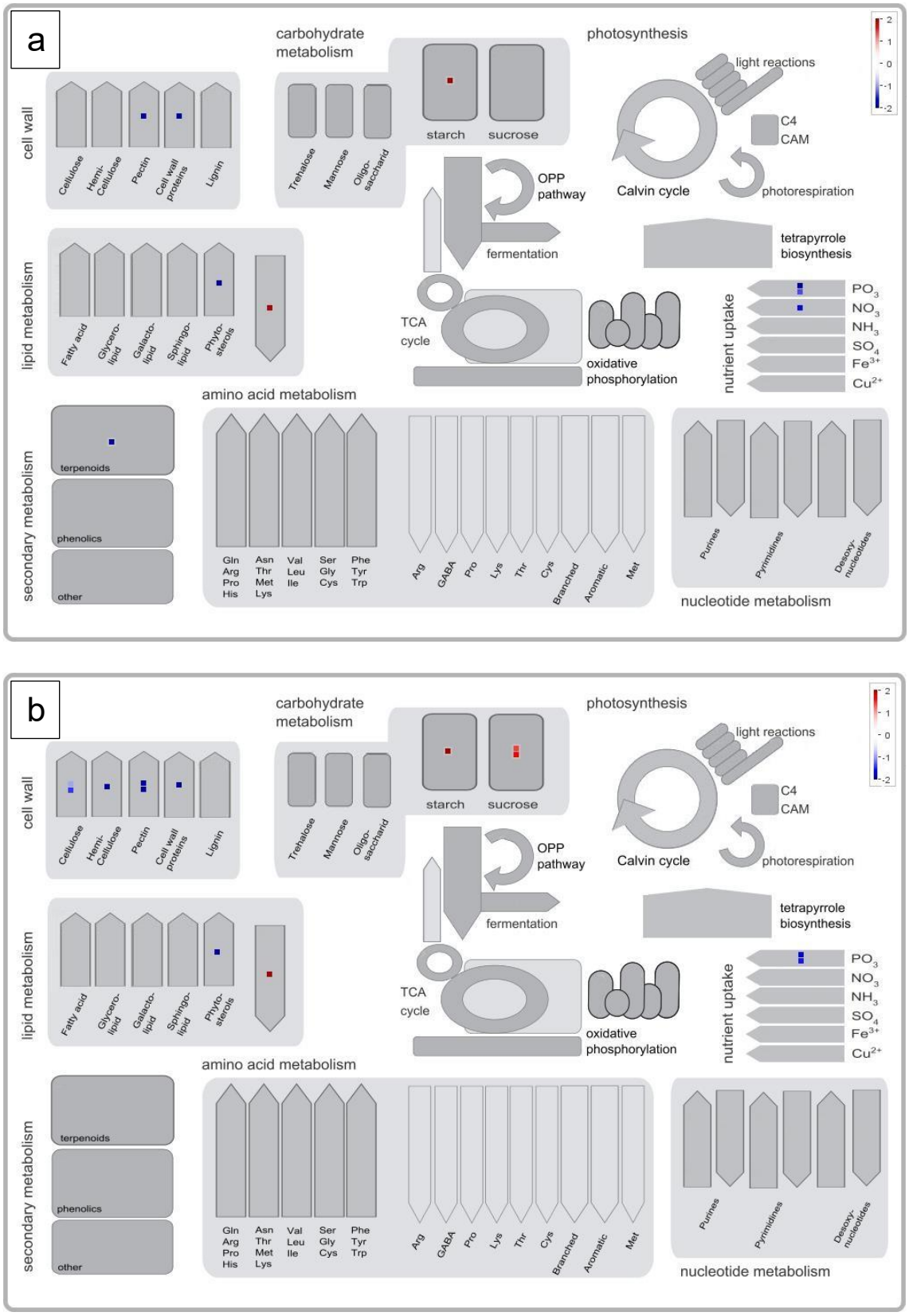

Figure 6: Overview of metabolic processes with DEG genes. Scale indicates fold change compared to control conditions. Photosynthesis has been removed from roots as it does not occur in this organ. a) Drought versus control in leaves. b) Drought versus recovery in leaves. c) Drought versus control in roots. d) Drought versus recovery in roots. [Part 1 of 2]

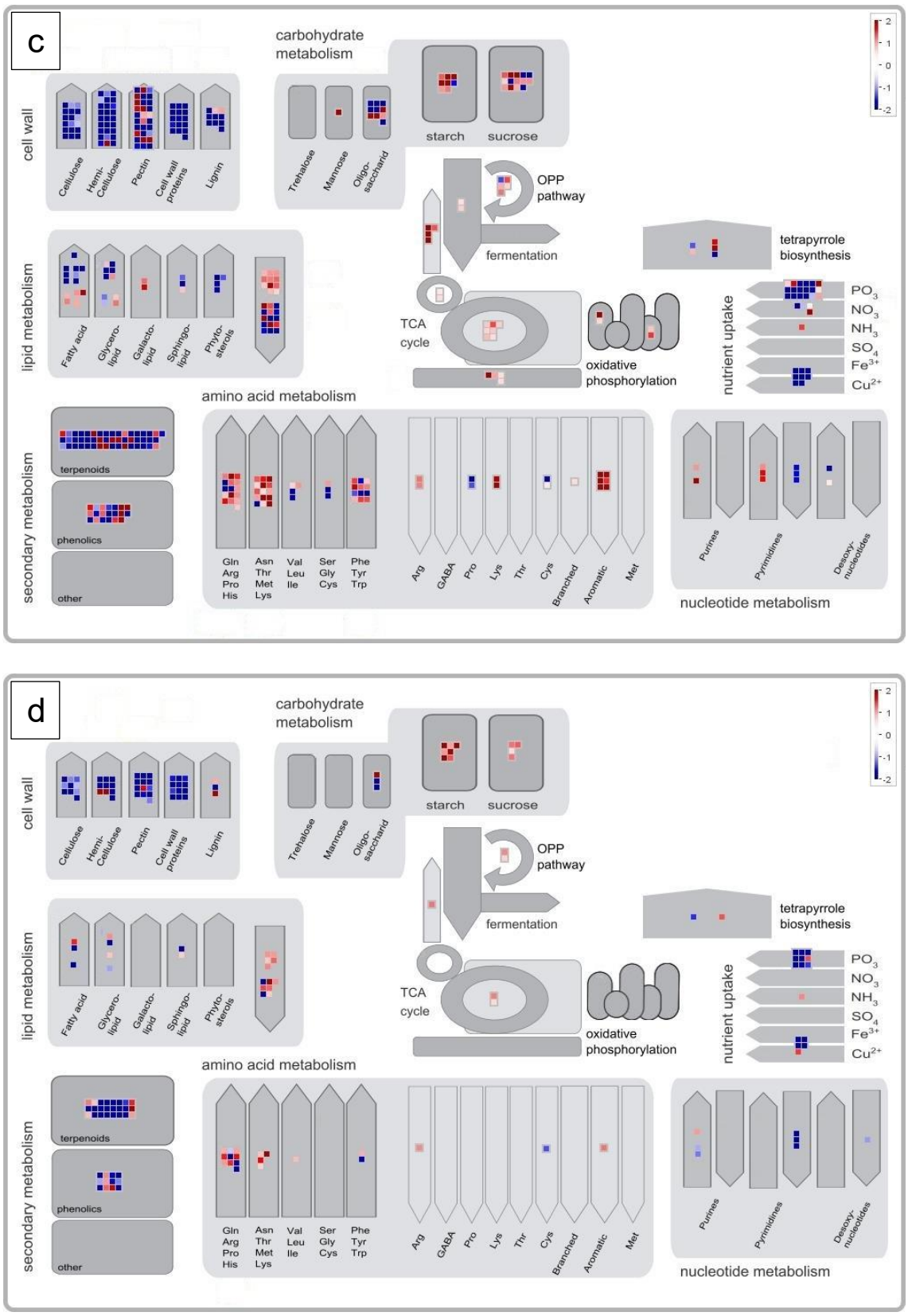

Figure 6: Overview of metabolic processes with DEG genes. Scale indicates fold change compared to control conditions. Photosynthesis has been removed from roots as it does not occur in this organ. a) Drought versus control in leaves. b) Drought versus recovery in leaves. c) Drought versus control in roots. d) Drought versus recovery in roots. [Part 2 of 2]

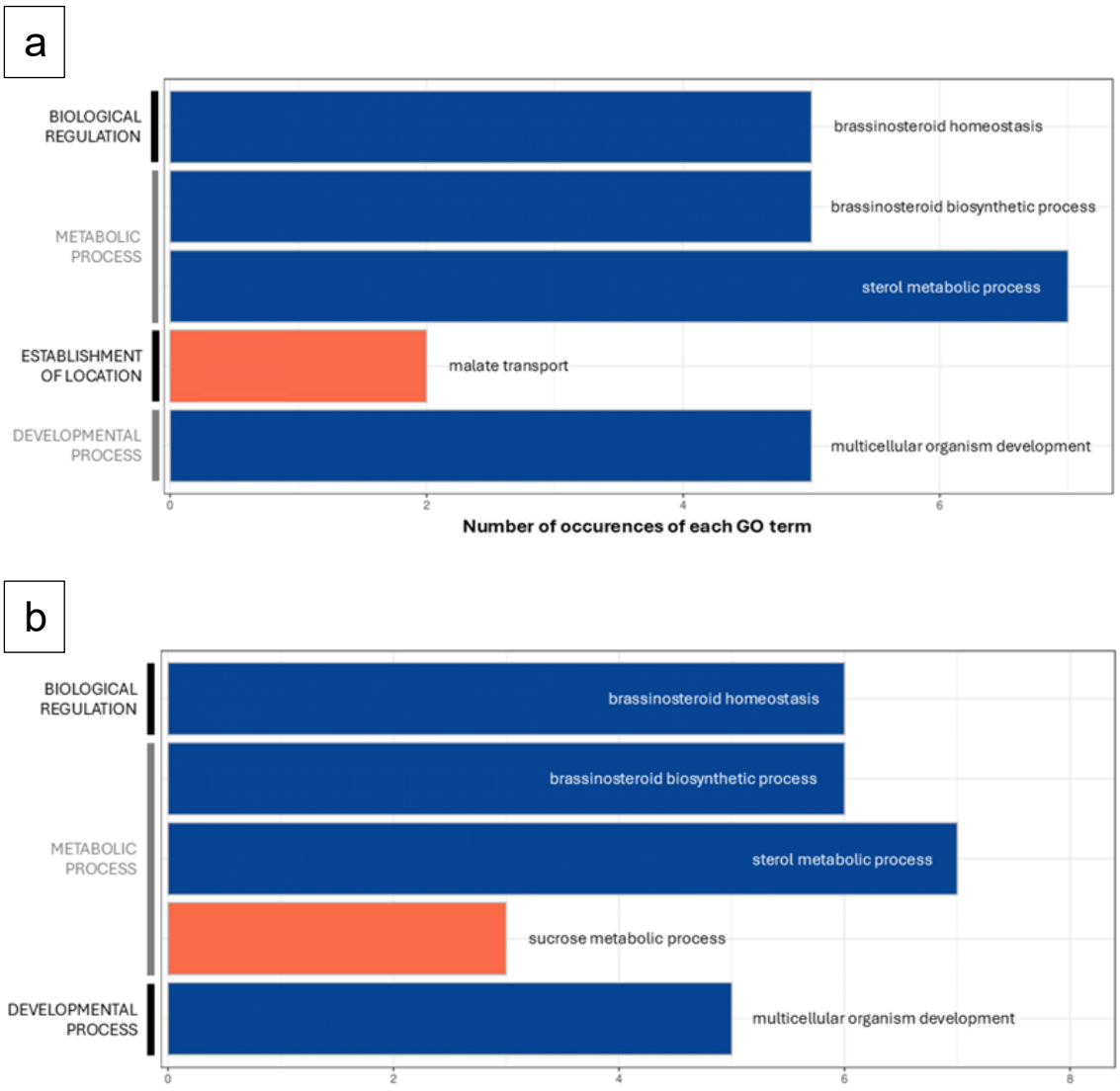

Figure 7: Overrepresented gene ontology (GO) terms in DEGs identified in leaves. Red indicates the term is significatively overrepresented in upregulated transcripts; blue indicates the term is significatively overrepresented in downregulated transcripts. a) Significative GO terms in drought compared to control conditions. b) Significative GO terms in drought compared to the recovery group.

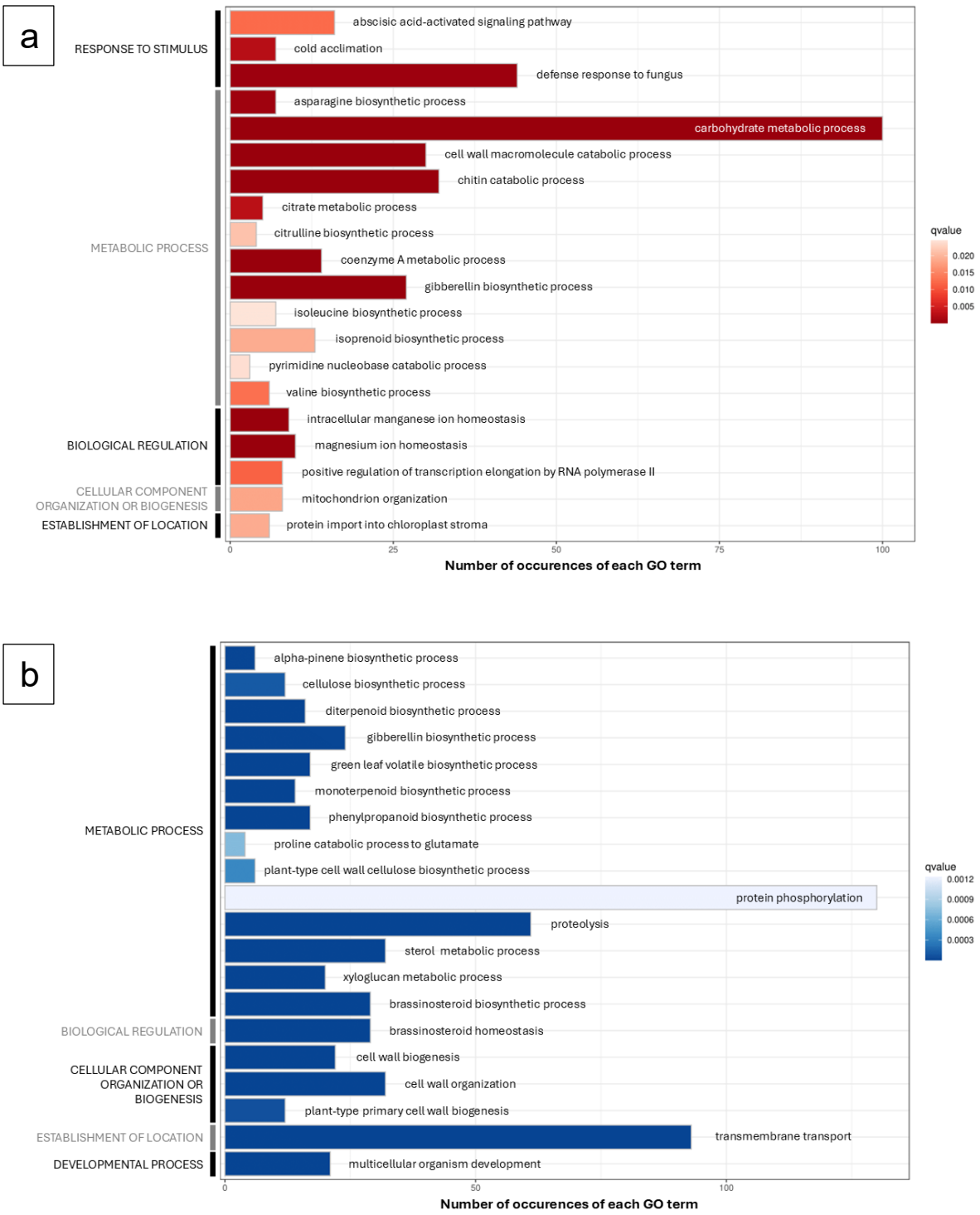

Figure 8: The 20 most overrepresented gene ontology (GO) terms in upregulated (a) and downregulated (b) transcripts identified **in roots subjected to drought compared to control conditions**.

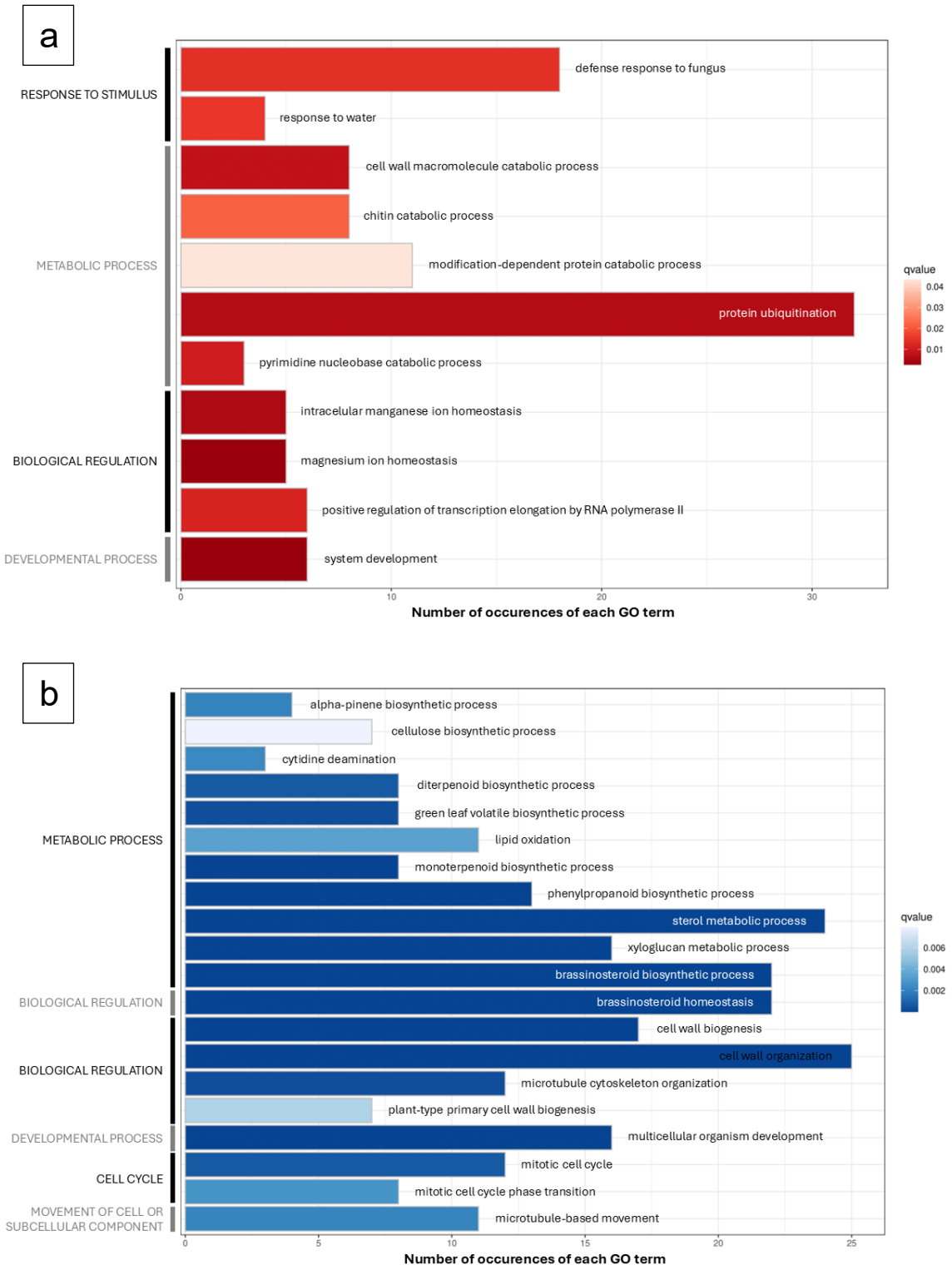

Figure 9: The 20 most overrepresented gene ontology (GO) terms in upregulated (a) and downregulated (b) transcripts identified **in roots subjected to drought compared to the recovery group.**

Finally, all transcripts with GO terms related to water processes were selected as candidates to potentially play an important role in response to drought stress and represented on a heatmap (Table S10): 7 downregulated and 13 upregulated (Figure 10a). Among them are major intrinsic proteins (MIPs), which are a superfamily of integral membrane proteins that includes aquaporins, whose function is to facilitate flow of water molecules across cellular membranes (Forrest & Bhave, 2007). These transcripts are only differently expressed in root samples, but not in leaves, which did not show any alterations in genes with GO terms related to water caused by drought stress. Consequently, transcripts associated with brassinosteroids (Table S11) were also proposed as candidate genes for drought diagnosis in this species, being significantly downregulated in both leaves and roots (Figure 10b). These 5 transcripts encode for proteins belonging to the CYP family (cytochrome P450), which are essential for brassinosteroid biosynthesis and homeostasis (Nomura & Bishop, 2006).

3.3. Global DNA methylation levels analysis

As a first approximation to study the role of epigenetic regulation in response to drought stress in *A. alba*, DNA methylation levels were measured. Leaves had 1.605% (± 0.178), 0.298% (± 0.084) and 1.047% (± 0.120) of methylated DNA in the control, drought and recovery groups respectively. Roots had 0.6094% (± 0.047), 0.090% (± 0.014) and 0.209% (± 0.011) of methylated DNA in the control, drought and recovery groups respectively (Figure 11). Significant differences were found between each pair of conditions (higher in the control group, lower when subjected to drought, and intermediate under recovery) both in leaves and roots (Figure 11), being more pronounced in roots, which also show higher percentages of DNA methylation than leaves.

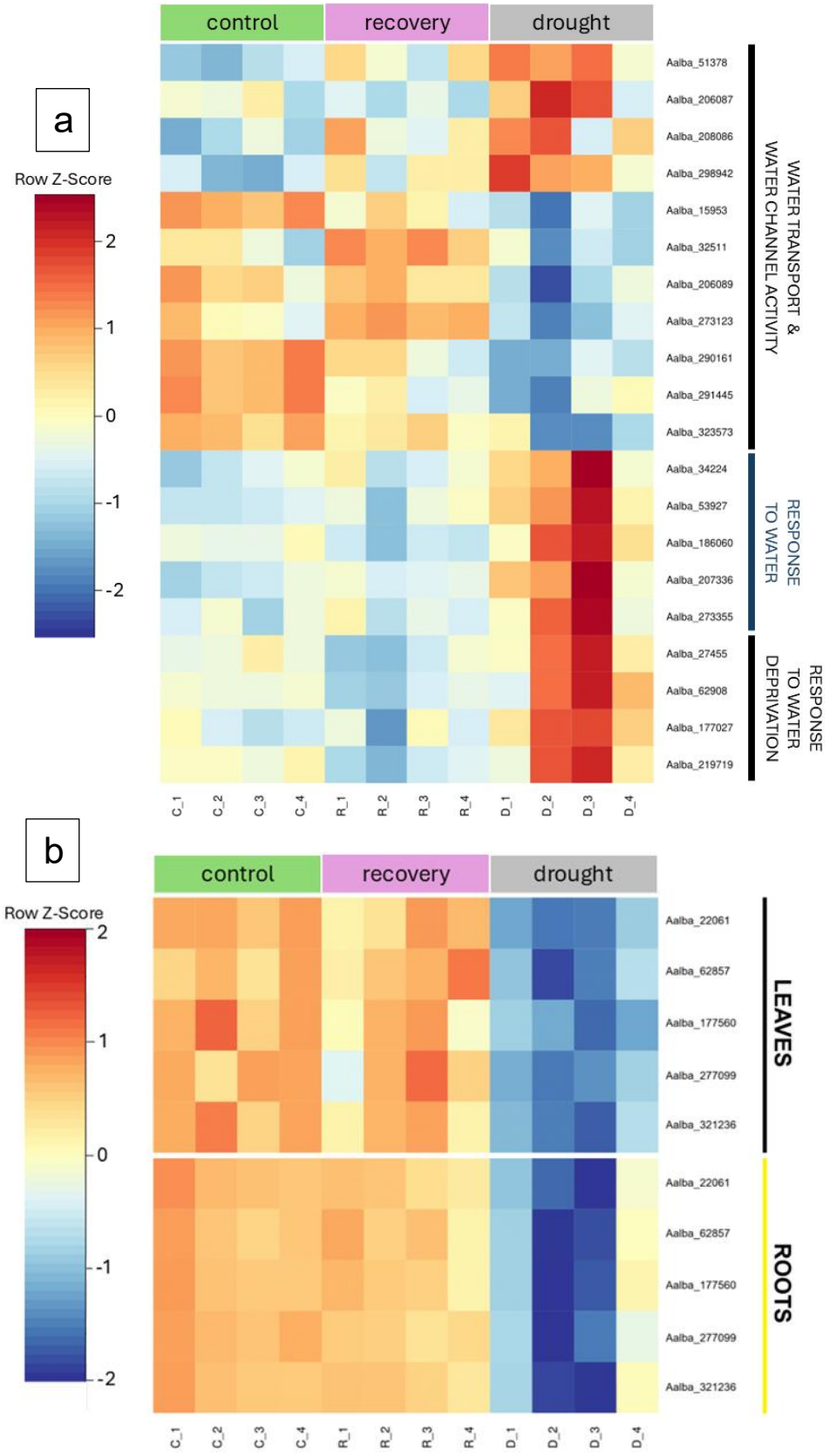

Figure 10: Heatmap comparing the expression of DEGs related to water transport and water channel activity, response to water, and response to water deprivation, in the control (C_), recovery (R_) and drought (D_) groups, in root samples (a) **and comparing the expression of DEGs related to brassinosteroids** in the control (C_), recovery (R_) and drought (D_) groups, in leaf and root samples (b).

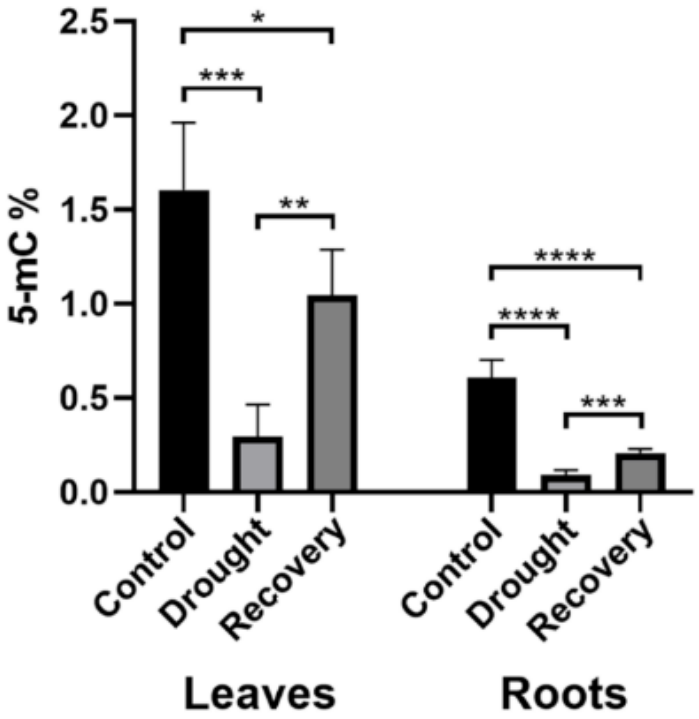

Figure 11: Global DNA methylation levels (5-mC %) in the control (n = 4), drought (n = 4) and recovery (n = 4) groups in leaves and roots. *p*-value significance codes: 0 '****' 0.0001 '***' 0.001 '**' 0.01 '*' 0.05.

4. Discussion

Climate change is shortening rainy seasons while enhancing intense rainfall over brief periods, leading to both drought and flood events (Trenberth, 2011). These changing environmental conditions trigger complex responses in plants, involving morphological, physiological, and molecular mechanisms, such as modulation of transcriptomic regulation (Singh *et al*., 2019), which may differ between species (Benny *et al*., 2019). In this study, we focused on the first scenario, investigating the effects of short-term drought stress on *Abies alba* by combining molecular data (RNA sequencing and DNA methylation quantification) and physiological variables (such as intrinsic water use efficiency, needle osmotic potential and non-structural carbohydrates).

We found that roots show an intense transcriptomic response while leaves do not (Figure 4). The number of transcripts whose expression was altered was 50 times higher in roots than in leaves. These results are in line with roots being the first organ to detect drought in plants, which react by undergoing morphological, anatomical, and genetic changes, transferring then the information to other organs (Kalra *et al*., 2023). Furthermore, ~10,000 more transcripts were expressed in roots, which displayed lower DNA methylation levels than leaves (Figure 11), which agrees with the existence of

tissue-specific methylation patterns and the generally accepted dogma that DNA methylation suppresses gene expression (Wan *et al*., 2015). Thus, our results show that roots undergo more changes than leaves, making them an interesting option for further studies of adaptation to water shortage in silver fir populations, putting the spotlight on the processes happening underground, which are much less investigated than how the aboveground parts of tress respond to drought (Brunner *et al*., 2015).

Nevertheless, some of the pathways and biological functions modified by drought in roots were shared by leaves (Figure 5), as revealed by the pathways and GO analyses (Figures 6–9). Cell wall metabolism is among the affected biological processes in both organs, with the biosynthesis of its components, such as cellulose, downregulated by drought, which could be affecting cell wall integrity and cell turgor pressure (Kesten *et al*., 2017). This response was also seen in black spruce (*Picea mariana*) saplings exposed to water shortage, which showed a decrease in cell wall thickness (Balducci *et al*., 2015). Brassinosteroids also show downregulation caused by drought. These steroidal plant hormones regulate various plant growth and developmental processes, such as cellular expansion and proliferation, and play a role in response to abiotic stresses (Fariduddin *et al*., 2014), conferring resistance to drought (Krishna, 2003). In conifers, exogenous brassinosteroids have been used to improve germination, development and somatic embryogenesis (Pullman *et al*., 2003; Kuneš *et al.,* 2016; Fan *et al*., 2021). However, the role of endogenous brassinosteroids under drought stress remained undescribed in conifer species. It has been proposed they antagonize abscisic acid, which is involved in promoting drought stress responses (Nolan *et al*., 2020). Therefore, the obtained downregulation of brassinosteroids (Figures 7, 8b and 9b) would be in line with the upregulation of ABA signaling pathway (Figure 8a) and the downregulation of developmental processes (Figures 7, 8b and 9b). In addition, it has been reported that brassinosteroids increase the accumulation of starch and soluble sugars (Liu *et al*., 2022), which agrees with the latter showing a significant reduction under drought conditions (Figure 2e) along with the observed downregulation of brassinosteroids. Regarding starch, no significant differences were observed between control and drought conditions while there was a significant decrease when trees were returned to control conditions for recovery (Figure 2f). This could be due to the different functions of NSCs, which in general serve as storage of excess photosynthesis products for tree processes during periods of stress, but specifically, TSS are used for immediate response while starch is reserved for long-term use (He *et al*., 2020). Thus, it is possible that the extent or severity of the applied stress did not reach the point where starch needs to be mobilized during the stress, although it was mobilized during recovery.

On the other hand, processes related to carbohydrates are upregulated in both leaves and roots (Figures 6, 7b, 8a and 9a), as well as those related to amino acids and the TCA cycle in roots (Figures 6c, 6d, 8a and 9a). These results agree with previously described plant responses to drought: carbohydrate metabolism is coordinated with amino acid degradation to supply carbon skeletons for the TCA cycle, which likely helps to maintain energy balance during drought stress adaptation, facilitating recovery once the stress is over (Diniz *et al*., 2020).

These transcriptional alterations could be used to assess the level of drought stress in natural populations by measuring expression levels. We propose a set of genes related to water transport (Table S10) as candidates for drought stress diagnosis in *A. alba*, whose expression was affected (Figure 10a). However, they are only altered in roots, requiring, thus, the use of roots as samples, which are less accessible than leaves. Therefore, we propose another set of genes (Table S11), due to their role in brassinosteroid metabolism and their marked downregulation in both organs(Figure 10b).

The above-described transcriptional changes happen only under drought conditions, while there are no differentially expressed genes between control and recovery samples (Figure 4c and f). Furthermore, some altered processes compared to control conditions start to return to normal when compared to the recovery group (Figures 6c and 6d). Therefore, these findings reveal the existence of some degree of resilience to drought in *A. alba*, which is able to return to its state before the stress. Even though there are epigenetic differences between control and recovery groups (Figure 11), they represent an intermediate state between control and drought conditions, indicating that the trees are slowly returning to their original state, affirming the tree's resilience once water becomes available after the stress. Photosynthetic rate and stomatal conductance also present this behavior (Figures 2a and b).

Regarding osmotic potential, we obtained almost steady values across the three treatments (Figure 2d), which, along with a decrease in TSS (Figure 2e), agrees with *A. alba* being an isohydric species (McDowell *et al*., 2008; Bachofen *et al*., 2020), as they present a drought avoidance strategy. That is, they close their stomata when they sense drought stress, limiting the loss of water at the cost of restricting carbon uptake and further production of carbohydrates (Mayer *et al*., 2020) and leading to little osmotic adjustment (Nolan *et al*., 2017). However, there is no significant increase in iWUE (Figure 2c), which implies that photosynthetic rate has been reduced proportionally to stomatal conductance instead of responding with a more moderate reduction to improve iWUE, making the species, therefore, vulnerable to drought-induced carbon starvation.

All things considered; *A. alba* seems to be sensitive to drought but able to recover once the stress ends. Silver fir's sensitivity to drought had already been described through tree ring analysis (Battipaglia *et al*., 2009), as well as its resilience (Mihai *et al*., 2021). Growth patterns also indicate that this species may thrive under non-extreme drought (Bottero *et al*., 2021), as long as annual precipitation is not too low (<850 mm) and summer drought is not too extreme (>3 months) (Walder *et al*., 2021). Thus, this study provides molecular and physiological evidence to support that severity and duration of drought episodes will be key factors for silver fir forests' survival, challenging the trees' ability to regulate their carbon reserves. Further, intrinsic tree characteristics, such as the root system and total carbon reserves, may be critical for coping with drought stress.

5. Conclusions

Molecular and physiological analyses were combined to describe the drought stress response of *A. alba*. Roots show an intense transcriptomic response compared to leaves, positioning them as a valuable organ for water stress assessment in this species. While genes related to water transport can be used to evaluate the level of stress caused by drought in this species using root samples, genes related to brassinosteroids can be used in both root and leaves samples, as they are markedly downregulated in both organs. DNA methylation levels are also affected by drought but start to return to their original state once the stress is over, just like gene expression patterns and physiological variables, suggesting that silver firs have some degree of resilience to water shortage. However, it fails to increase its water use efficiency when closing its stomata, challenging its carbon balance and making it a drought-sensitive species. Therefore, the severity and duration of drought episodes will be critical factors in the survival of silver fir forests, as well as some of their inherent traits, such as their root system and total carbon reserves.

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SUPPLEMENTARY MATERIAL

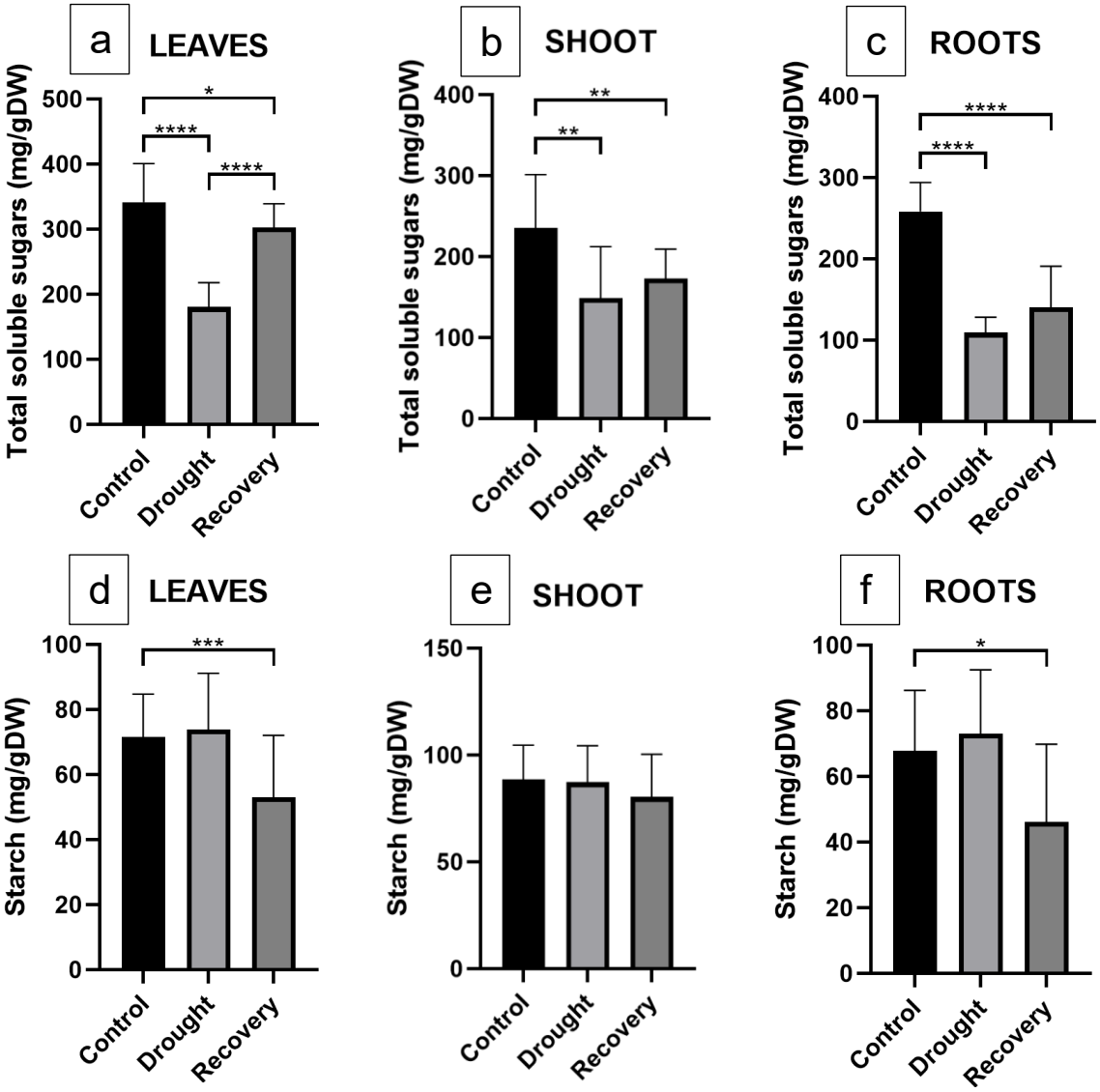

Figure S1: Total soluble sugar results separated by tissue (36 samples each) [leaves (a), shoot (b), and roots (c)] **and starch results separated by tissue** (39 samples each) [leaves (d), shoot (e), and roots (f)]. *p*-value significance codes: 0 '****' 0.0001 '***' 0.001 '**' 0.01 '*' 0.05.

Table S1: Read filtering results, comparing before and after metrics.

			Total length (Gbp)		Number of reads		Kept reads
Sample	**Tissue**	**Group**	Before	After	Before	After	
C_1	Leaves	Control	6.521	6.205	43,184,984	41,513,299	96.13%
	Roots		6.073	5.778	40,220,413	38,722,870	96.28%
C_2	Leaves		5.199	4.757	34,432,600	33,168,742	96.33%
	Roots		6.025	5.742	39,901,848	38,435,357	96.32%
C_3	Leaves		6.053	5.785	40,085,460	38,614,793	96.33%
	Roots		5.109	4.908	33,835,051	32,735,897	96.75%
C_4	Leaves		5.245	4.807	34,735,240	33,628,404	96.81%
	Roots		5.610	5.387	37,151,970	36,047,212	97.03%
D_1	Leaves	Drought	5.677	5.248	37,595,394	36,174,199	96.22%
	Roots		5.374	4.999	35,591,535	34,230,125	96.17%
D_2	Leaves		6.015	5.546	39,832,163	38,448,220	96.53%
	Roots		4.869	4.512	32,242,254	30,991,362	96.12%
D_3	Leaves		5.694	5.272	37,706,866	36,339,537	96.37%
	Roots		4.926	4.570	32,620,607	31,257,428	95.82%
D_4	Leaves		5.183	4.625	34,327,118	32,793,522	95.53%
	Roots		4.938	4.573	32,700,696	31,502,884	96.34%
R_1	Leaves	Recovery	4.621	4.228	30,603,709	29,568,020	96.62%
	Roots		5.223	4.837	34,589,615	33,182,361	95.93%
R_2	Leaves		4.734	4.339	31,348,481	30,197,893	96.33%
	Roots		4.558	4.361	30,185,809	29,139,470	96.53%
R_3	Leaves		5.658	5.414	37,467,639	36,332,817	96.97%
	Roots		5.941	5.384	39,344,213	37,856,378	96.22%
R_4	Leaves		5.593	5.068	37,039,820	35,619,977	96.17%
	Roots		6.297	5.980	41,702,175	39,915,467	95.72%

Table S2: Differentially expressed genes (DEGs) in leaves subjected to drought compared to control conditions.

Due to its extensive size, Table S2 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S3: Differentially expressed genes (DEGs) in leaves subjected to drought compared to the recovery group.

Due to its extensive size, Table S3 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S4: Differentially expressed genes (DEGs) in roots subjected to drought compared to control conditions.

Due to its extensive size, Table S4 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S5: Differentially expressed genes (DEGs) in roots subjected to drought compared to control conditions.

Due to its extensive size, Table S5 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S6: Over-represented GO terms in DEGs in leaves subjected to drought when compared to control conditions.

Due to its extensive size, Table S6 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S7: Over-represented GO terms in DEGs in leaves subjected to drought when compared to recovery conditions.

Due to its extensive size, Table S7 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S8: Over-represented GO terms in DEGs in roots subjected to drought when compared to control conditions.

Due to its extensive size, Table S8 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S9: Over-represented GO terms in DEGs in roots subjected to drought when compared to recovery conditions.

Due to its extensive size, Table S9 has not been included in the main body of this thesis. However, it is available at: https://github.com/igargar/RNAseq_Aalba/blob/main/Aalba%20drought%20supplementary%20tables.xlsx

Table S10: Description, source organism and accession number of the selected transcripts related to water.

Transcript	Description	Source organism	Accession number	Ontology term/s
Aalba_15953	Major intrinsic protein (MIP)	*Picea sitchensis*	ABK22546	water transport; water channel activity
Aalba_27455	Pentatricopeptide repeat-containing protein DOT4, chloroplastic isoform X2	*Cryptomeria japonica*	XP_057825740	response to water
Aalba_32511	Aquaporin TIP	*Picea sitchensis*	ABK22410	water transport; water channel activity
Aalba_34224	NA	*Picea sitchensis*	ABK22729	response to water deprivation
Aalba_51378	Major intrinsic protein (MIP)	*Picea sitchensis*	ABK23750	water transport; water channel activity
Aalba_53927	Dehydrin-like protein (Dhn4)	*Picea abies*	ABU89744	response to water deprivation
Aalba_62908	Contains dehydrin region	*Picea sitchensis*	ABK21645	response to water
Aalba_177027	Contains dehydrin region	*Picea sitchensis*	ABK21645	response to water
Aalba_186060	Uncharacterized protein LOC131063907	*Cryptomeria japonica*	XP_057853867	response to water deprivation
Aalba_206087	Probable aquaporin (similar to *Arabidopsis thaliana* probable aquaporin PIP2.8)	*Picea abies*	AAX92683	water transport; water channel activity
Aalba_206089	Major intrinsic protein (MIP)	*Picea sitchensis*	ABK21046	water transport; water channel activity
Aalba_207336	NA	*Picea sitchensis*	ABK22729	response to water deprivation
Aalba_208086	Major intrinsic protein (MIP)	*Pnius taeda*	AFG66783	water channel activity; water transport
Aalba_219719	Contains dehydrin region	*Picea sitchensis*	ABK21645	response to water
Aalba_273123	Probable aquaporin TIP	*Picea sitchensis*	ABK22410	water transport; water channel activity
Aalba_273355	NA	*Picea sitchensis*	ADE77959	response to water deprivation
Aalba_290161	Major intrinsic protein (MIP)	*Musa troglodytarum*	URE35445	water channel activity
Aalba_291445	Major intrinsic protein (MIP)	*Ceratopteris richardii*	KAH7433945	water transport; water channel activity
Aalba_298842	Major intrinsic protein (MIP)	*Picea sitchensis*	ABK23750	water transport; water channel activity
Aalba_323573	Probable aquaporin TIP	*Picea sitchensis*	ABR17761	water transport; water channel activity

Table S11: Description, source organism and accession number of the selected transcripts related to brassinosteroids.

Transcript	Description	Source organism	Accession number	Ontology term/s
Aalba_22061	CYP720B4	*Picea sitchensis*	ADR78276	brassinosteroid homeostasis; brassinosteroid biosynthetic process
Aalba_62857	CYP720B4	*Picea sitchensis*	ADR78276	brassinosteroid homeostasis; brassinosteroid biosynthetic process
Aalba_177560	CYP720B4	*Picea sitchensis*	ADR78276	brassinosteroid homeostasis; brassinosteroid biosynthetic process
Aalba_277099	Contains CYP90-like region	*Picea sitchensis*	ACN40968	brassinosteroid homeostasis; brassinosteroid biosynthetic process
Aalba_321236	CYP720B7	*Picea sitchensis*	ADR78276	brassinosteroid homeostasis; brassinosteroid biosynthetic process

DISCUSSION

Nearly half of the Earth's terrestrial surface is covered by forests [143], with many tree species serving as keystone organisms that provide habitat, food, and mutualistic relationships with different animals, fungi, microorganisms, and other plants, as well as offering numerous ecosystem services and resources for human use [144]. Thus, as evidence of global warming and its impacts on ecosystems continues to increase rapidly [2], there is growing interest in forecasting the future of trees and forests. Can natural populations of forest trees adapt or change their distribution ranges quickly enough to keep pace with the rapid changes in climate? Will their genetic diversity enable them to adapt swiftly, despite their long generation times? The present doctoral thesis delved on these issues focusing on silver fir, *Abies alba*, as case study, integrating knowledge from various fields of genetics and ecology.

Even though meaningful results could be extracted from the explored omic data types, working with sequencing information obtained from this species has been challenging. Its large genome size (~18 Gb), high fraction of repetitive regions (~80%) and highly fragmented state of its reference genome (~37 million scaffolds) [26] led to different difficulties. Firstly, the interpretation of the bisulfite sequencing results was hindered by the lack of detailed annotation. Although differentially methylated cytosines between declining and non-declining trees were identified, their functional annotation was not possible, so their biological functions could not be inferred. Even though both of the tested bisulfite sequencing methods were successful in assessing global methylation levels, other more cost-effective methods exist, such as colorimetric ELISA targeting 5-methylcytosines, which was the preferred protocol for the rest of epigenomic experiments included in this research. Although it does not provide single-base resolution data, it can effectively estimate global DNA methylation, whose changes could indicate that major epigenomic processes are happening. Further, computational costs were too demanding to align the genomic data using the complete reference genome, so a shortened version had to be used, consisting only of the first ~70,000 scaffolds, which are the longest and best annotated ones. In spite of this, 350 Gb of RAM memory were necessary to carry out the mapping step and not every identified SNP could be related to described genes. The identification of SNPs was also limited by the characteristics of the silver fir genome, resulting in a low number of variants (~4,000) compared to species with smaller genome sizes, such as *Populus tremula* (~16,000 SNPs in a <0.5 Gb-long genome) [145]. Lastly, the annotation of the *de novo* assembled transcriptome was also constrained by the lack of described sequences in databases (not only those of *A. alba* but of conifer species in general), resulting in ~70% of transcripts with unknown functions, which accounts for ~370,000 undescribed sequences. Nevertheless, every obstacle that emerged was

gradually addressed until results of interest for the study of adaptation to climate in *A. alba* were obtained.

Regarding evolutionary adaptation, the first requirement for selection to act is the existence of genetic variability [146]. Both neutral and adaptive genetic diversity in Pyrenean silver fir forests were explored, concluding that the adaptive set, comprising SNPs associated with environmental variables, is more informative for evolutionary studies. Many of these SNPs also showed signs of selection, which usually leads to an evolutionary reduction of genetic variability [147], as it was seen in several of the studied populations. Nevertheless, the predominance of foreign relatives in coldspots of forest decline, where decline processes have not started yet, contrasted with the dominance of native relatives in hotspots, where aridity is higher and trees are already dying, suggests that all trees, regardless of their health status, can grow in coldspots, thanks to their less demanding conditions. Hence, coldspots could act as a refuge for silver fir's genetic diversity and, thus, adaptive potential, for at least some time, until climate becomes unfavorable for the species there too. The severity and duration of drought episodes will likely be a decisive environmental factor in creating unfavorable climatic conditions for *A. alba*, as shown by the transcriptomic results, which indicate that it is able to recover from short-term drought stress but its limited water use efficiency increases the likelihood of carbon starvation. Global DNA methylation results also pointed to some degree of resilience to drought, as well as the existence of epigenomic differences between declining and non-declining trees. Therefore, both types of adaptive responses to climate stress were seen in silver fir: phenotypic plasticity, in the form of modulation of gene expression through epigenetic processes, and evolutionary selection, in the form of genomic changes occurring in the different natural populations in the Pyrenees.

The establishment of a direct relationship between the epigenomic and genomic responses to environmental variations would have been of great interest, but it was not possible. Nevertheless, our results led to the formulation of a hypothesis that requires further investigation: adaptive changes might occur in rear-edge silver fir forests following a step-by-step pattern. We propose that epigenetic processes would play a key role in *A. alba*'s initial response to stressful environmental conditions through phenotypic plasticity, while genetic changes through selection may happen later to achieve long-term adaptation to severe long-term climate stress. This hypothesis was formulated based on the following results: two hotspots showed differences in DNA methylation levels between declining and non-declining trees but no genetic differences, while the third hotspot, where environmental conditions have been stressful for longer, presented the opposite behavior, with genetic differences between declining and non-declining

trees but no differences in DNA methylation. Thus, the first two populations would be facing relatively recent environmental stress that could be triggering an epigenetic response, which is line with what was seen in the drought experiment included in the transcriptomic part of this thesis; resilient trees reduce their DNA methylation levels under stress, just like non-declining trees show lower levels than declining trees, which might not be able to respond. However, in the hotspot subjected to harsher conditions for a longer period of time, these non-responsive trees might already be dead, potentially explaining the absence of detectable epigenetic differences between declining and non-declining trees at the present time. Then, the declining process might be modulated by genetic characteristics, in line with the observed differences between declining and non-declining trees. Therefore, both types of responses would be necessary to achieve long-term adaptation. Nonetheless, as previously stated, further studies are needed in order to confirm or refute this hypothesis, as the link between both responses cannot be stated from our results. As transgenerational studies, which could add valuable information to this matter, are challenging in tree species due to their long generation times, more populations should be studied in order to verify if this pattern is recurrent across the silver fir range.

While epigenetic modifications seem to be a key mechanism for rapid response and can then return to their pre-stress state, as supported by the transcriptomic section of this doctoral thesis, environmental signals and stresses can also trigger lasting changes in epigenetic modifications, which might be heritable [148]. However, as mentioned above, transgenerational studies to prove epigenetic inheritance are difficult to perform in tree species. Nevertheless, forest trees offer an advantage that short-lived plants do not: information about their past responses to climatic conditions remains stored in growth rings. Secondary growth patterns allow us to understand and use the past to predict the future. Records about how past environmental stresses affected populations, such as documented long drought periods, allow us to model expected responses under similar conditions in the future. Tree-ring studies are also useful to evaluate climate sensitivity, that is, to confirm long-term past inferences of climate variables significantly related to growth decline. For instance, we demonstrate the coincidence of reduced tree-ring widths under warmer conditions and declines in the effective population sizes inferred through genomic data during warmer periods, such as the Mid-Brunhes event (MBE) around 0.45 million years ago [149]. Thus, combining genetic and physiological prediction models can greatly help to evaluate vulnerability of tree species to climate change and to design effective conservation plans.

A standardized list of indicators has already been proposed in order to conduct a successful assessment of forests' status and be able to establish measures to promote adaptation to climate change [150], which includes, for example, climatic factors and functional traits related to eco-physiology. Although it already included some genetic indicators, such as heritability and evolutionary potential, and genetic diversity, the here-presented results indicate the necessity to add more genetic parameters (Table 1): genomic, epigenomic, and transcriptomic indicators.

Table 1: Standardized list of indicators of forests' status. Newly added parameters are underlined.

Indicator	Description
Drought	Months with Standardized Precipitation-Evapotranspiration Index < 0
Extreme weather	Days < 0ºC (April-July), and > the 95% percentile
Windstorms	Days with wind speed > 100 km/h
Snowstorms	Timber volume resulting from storm damage
Wildfires	Frequency and affected area
Regeneration	Number of seedlings and saplings
Resilience and resistance	Measurements derived from changes in growth
Growth	Basal area increment (BAI)
Reproduction	Number of seeds
Mortality	Percentage of dead individuals, loss of BAI
Decline	Percentage of defoliation
Structure	BAI/density and BAI variation
Mixed forest diversity	Tree species richness, number of endangered species…
Management	Management strategies
Social use	Number of studies, number of visitors
Distribution range	Location in the distribution range
Changes in distribution	Probability of extinction under climate change according to species distribution models
Adaptive traits	Carbon isotopes
Heritability and evolutionary potential	Provenance experiments, genetic offset under climate change
Genetic diversity	Allelic richness, heterozygosity, adaptive markers
Epigenomic marks	Global DNA methylation level
Transcriptomic profile	Expression levels of genes involved in stress response

So, from the same DNA extraction, we can obtain both DNA sequencing data, which can provide information to assess several indicators (namely genetic offset, heterozygosity, and adaptive markers), and DNA methylation information, which are related to stress levels.

Regarding the indicators that can be obtained through DNA sequencing, calculating the genetic offset provides information about the vulnerability to future climatic conditions through the desired projected area, which indicates what areas are likely to be more vulnerable [e.g. 151–153] and, thus, would need prompt action. Then, heterozygosity and its differences between health, age or population cohorts offers a view on adaptive potential, as reductions in genetic diversity might lead to difficulties in the adaptation of populations to the changing environment [154]. Finally, the identification of molecular markers associated with environmental variables indicates what factors are more relevant in the tree's relationship with its environment [e.g. 155–157]. On the other hand, the use of DNA methylation levels to assess stress might not be straightforward, as they are not only dependent on the presence or absence of stress but also on the environment itself, changing throughout the year [158] and making them unique to each location and restricting the possibility of setting a general threshold to interpret the results. Therefore, even though DNA methylation can and should definitely be used as an indicator, genomic resources for tree species should be first improved in order to be able to use single-base resolution techniques that can identify specific genes with a clear response to each stress, such as drought, to use as genetic markers to evaluate stress levels. Lastly, if an RNA extraction is performed, expression levels of key genes related to stress response can be assessed and directly related to stress levels. This can be achieved either through RNA sequencing or quantitative PCR.

Consequently, with the evaluation of all the proposed genetic tools already available for the Pyrenean silver fir forests, the question emerges as to what their state is and which management strategy would be most effective in preserving the species' current distribution in the region. We see that changes in genetic diversity are occurring, with reductions in younger generations compared to older trees in some populations. Besides, declining individuals showed a reduction in DNA methylation levels compared to non-declining, in line with the reduction identified in stressed samples compared to those under control, favorable conditions. These observations seem to indicate that climatic stress is already affecting silver fir populations in the Pyrenees, which is supported by the finding that rapid changes towards warmer conditions in the past led to reductions in effective population size.

Furthermore, genetic offset calculations depict an east-to-west gradient, with greater changes in genetic composition being necessary in the western-central region of the Pyrenees in order to adapt to the predicted environmental conditions under climate change, which is in line with the predictions based on growth patterns and field observations that report more severe and widespread decline in western silver fir forests

[122]. In addition, the transcriptomic response to drought in combination with the physiological analysis revealed that resilience to drought stress could be surpassed by its sensitivity to such stress if it lasts long enough to lead to carbon starvation [159, 160]. Therefore, the long generation time of this species hinders its survival in the Pyrenees, since its adaptation to the rapidly changing conditions will be compromised if its pace of adaptation lags behind the speed of climate change. Moreover, drought periods are predicted to increase in frequency and duration in the area [132], which will challenge their resistance to water shortage. Consequently, silver fir populations in the Pyrenees are at risk of local extinction, especially in the western-central range. More populations within their distribution in the area should be studied to obtain more information about their response to climate change and current state. Particularly, eastern populations could be of great interest, as they were not included in the present study and seem to be likely to face less challenges to adapt to the new changing environmental conditions.

Overall, the outlook for Pyrenean silver forests appears bleak, with a considerable trend towards their disappearance from the region. Therefore, what steps can be taken to preserve the current distribution of *A. alba* in the Pyrenees? The following recommendations are proposed:

- Gene flow between forests should be maintained to avoid habitat fragmentation and the loss of potentially beneficial variants in small groups of isolated individuals. Pollen and seeds in this species are large, which limits their dispersal beyond 40 meters from the forest edge [161]. Consequently, the construction of wide roads, hiking trails and ski slopes should be avoided or minimized.
- Regarding management strategies, the effect of logging has also been explored in this study. The only population that has not been managed in recent decades is the only one that does not exhibit a decline in predicted secondary growth under the high-emission scenario. Thus, the chosen management strategy can impact population dynamics, particularly under more arid climate scenarios. Consequently, intensive logging activities should be avoided in the Pyrenean silver forests in order to reduce their risk of decline.
- A demographic rescue to increase population size once it starts to decline is not recommended, as environmental conditions are already harsh and will be worse in the future, so neither local nor introduced trees would be likely to survive.

Some argue that preserving all existing biodiversity is still an attainable goal, while others contend that biodiversity loss is inevitable regardless of our conservation efforts, and that we should prioritize deciding what to save, why, and where [162]. Based on the information presented, the case of silver fir forests in the Pyrenees could be categorized under the second scenario. The species will still exist across extensive areas in Central Europe, so maybe conservation efforts should be put on other trees affected by climate change (e.g. the Spanish fir, *Abies pinsapo*, which a climate-relict fir species, endemic to the South-West of the Iberian Peninsula). Nevertheless, their genetic diversity could be beneficial for other populations once climate change effects turn their environmental conditions challenging for them, and, therefore, should be conserved, which can be achieved through seed banks and botanical gardens.

Lastly, the next steps and future implications of this investigation should be addressed. Regarding the epigenomic section, the development of a chromosome-level reference genome for *A. alba* should be a priority, which would not also enhance the knowledge about epigenetic marks but also provide a foundation for genomic and transcriptomic studies. Further, in order to deepen into how adaptive changes occur in silver fir forests, populations from areas across all the silver fir distribution should be sampled and studied. As mentioned above, this could also lead to the identification of a link between the epigenomic and genomic responses to environmental changes, which could greatly contribute to the investigation of adaptation in natural populations. Finally, the candidate genes to evaluate response to drought obtained from the transcriptomic experiment should be explored in detail, designing primers for quantitative PCR amplification and defining expression thresholds to differentiate between non-stressed trees, stressed trees, and trees undergoing a recovery process. Besides, protocols that enable the use of root samples need to be developed and optimized, as this tissue could provide relevant information to complement the already existing knowledge obtained from classical studies in leaves.

CONCLUSIONS

Based on the here-presented results, the following conclusions have been drawn:

1. The complexity of forest tree genomes (large size and high percentage of repetitive regions) and the limited availability of high-quality reference genomes and annotations make omic studies in *Abies alba* scarce and challenging.
2. Changes in adaptive genomic diversity and DNA methylation are present between declining and non-declining trees, indicating a molecular basis underlying the ongoing decline in silver fir forests.
3. Coldspots of forest decline may serve as temporary refuges for the genetic diversity and adaptive potential of silver fir in the face of climate change.
4. Genome-environment associations are abundant in the studied populations, including some that display signs of selection, pointing out the strong influence of environmental factors, such as soil and climate, on the silver fir genome.
5. Genomic and dendrochronological modelling and past demography indicate that climate change is currently a threat to the survival of silver fir forests in the Pyrenees.
6. Neither whole genome nor reduced representation bisulfite sequencing are currently recommended techniques for investigating the giga-methylome of *A. alba*. Alternatively, a colorimetric ELISA can be used to assess DNA methylation by measuring global 5-methylcytosine levels.
7. The here-assembled *A. alba* transcriptome represents a substantial improvement over previously published data and provides a valuable source of information for future research on silver fir.
8. Roots show a more intense transcriptomic response to vapor pressure deficit than leaves, making them a valuable organ for drought stress assessment in *A. alba*.
9. Genes related to water transport or brassinosteroid metabolism could be used as indicators to evaluate drought stress in *A. alba*.
10. Even though *A. alba* behaves as a drought-sensitive species, DNA methylation levels tend to revert to their original state once the stress ends, just like gene expression and physiological variables, suggesting that it has some degree of resilience to drought.

CONCLUSIONS

In conclusion, this doctoral thesis successfully achieved its primary objective of **deepening the understanding of tree adaptation to environmental conditions and their response to climate variability**. By integrating genomic, epigenomic, transcriptomic, soil, climate and dendrochronological data, this research provides a multidisciplinary perspective that provides valuable knowledge that could ultimately be used to design strategies for managing silver fir forests in a changing climate and evaluating their vulnerability.

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