Phylogenomic evidence of fire regime changes: the case of a resprouting juniper

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Abstract

The onset of the mediterranean climate, with its characteristic seasonal droughts and increased fire activity, marked a significant shift in the evolution of Mediterranean flora. One trait to adapt to this fire regime shift is the ability to resprout from a lignotuber. We used genotyping-by-sequencing (GBS) data to estimate the origin of lignotuber resprouting in the Iberian *Juniperus oxycedrus* complex and the period when fires—and the Mediterranean seasonality—became sufficiently frequent and stable to favor such plant adaptations. We then infer the demographic consequences of possessing this trait. Results indicate that resprouting and non-resprouting lineages diverged around 6.23 Ma, aligning with key aridification events (e.g., the Messinian Salinity Crisis) followed by the establishment of the mediterranean climate. We propose that fire-adaptive traits, such as lignotuber resprouting, can serve as proxies to estimate fire regime shifts; by doing so, we provide new insights into the assembly of the Mediterranean biome.

Introduction

Past climatic changes likely triggered migrations, extinctions, and adaptive responses (Jablonski 1986; Bradshaw & Holzapfel 2006; Jackson & Overpeck 2010; Thomas 2010; Fritz et al. 2013; Nogués-Bravo et al. 2018). One of the most striking climate changes was the emergence of the mediterranean climate (characterized by a warm, dry, fire-prone season), which replaced a wetter subtropical climate. This shift was associated with the retraction of humid (tropical-like) forests and the rise and expansion of sclerophyllous woody vegetation adapted to seasonal drought and recurrent fires (Keeley et al. 2012).

The timing of the mediterranean climate's onset and the associated plant adaptations has been debated (Tzedakis 2007). Specifically, for the Mediterranean Basin (the Palearctic Mediterranean region), fossil evidence suggests that sclerophyllous vegetation was present as early as the Oligocene (ca. 34–23 Ma), but restricted to arid patches (Axelrod 1975; Palamarev 1989; Kovar-Eder et al. 2006; Postigo-Mijarra et al. 2009). Orbital variations generated intermittent windows of arid conditions during the entire Paleogene and Neogene (Tzedakis 2007). In particular, a significant aridification event occurred during the Messinian Salinity Crisis (ca. 5–6 Ma), when the Mediterranean Sea underwent cycles of partial or near-complete desiccation (Duggen et al. 2003; Jiménez-Moreno et al. 2013). Further intensification of summer drought occurred during the Pliocene Thermal Maximum (mid-Pliocene, ca. 3 Ma), often considered the starting point of the typical mediterranean climate with its characteristic seasonality (Suc 1984; Suc & Popescu 2005;

Barrón et al. 2010; Jiménez-Moreno et al. 2010). Thus, the mediterranean biome (defined here by its characteristic climate, vegetation, and fire regime) likely originated and expanded during the aridification process that occurred between the Oligocene and the Pliocene. In fact, many mediterranean lineages diversified earlier than the Pliocene (Vargas et al. 2018).

As a consequence of this aridification, it is expected that fire activity also increased during this period. However, there is currently a lack of evidence on fire regime changes for this period in paleoecological studies (Suc & Popescu 2005; Carrión & Leroy 2010; Jiménez-Moreno et al. 2010; Postigo-Mijarra et al. 2010). Finding a fire adaptation that originated during this period would provide crucial evidence of the role of fire and the fire regime shift during the assembly of the mediterranean biome. And estimating the timing of such adaptations would offer insight into when fire activity became significant enough to drive the evolution of fire-adaptive traits, a marker of the climate seasonality that characterize the mediterranean biome.

During the aridification that shaped the mediterranean climate and fire regimes, plant species had two alternative pathways for persistence (Pausas & Keeley 2014). The increasing frequency and intensity of fires would have negatively affected resprouting species with small bud banks or those whose resprouting buds were too exposed. One evolutionary pathway, therefore, was to rely on postfire regeneration from a persistent seed bank with fire-released dormancy (postfire seeder species; Pausas & Lamont, 2022). In contrast, some other lineages (e.g., resprouting lineages) increased the number of buds and protected their bud banks by developing resprouting structures, such as lignotubers (postfire resprouters: Paula et al. 2009; Paula et al. 2016; Pausas et al. 2018). Different lineages followed one of these two pathways depending on their evolutionary constraints. For example, species with dry fruits dispersed locally were able to evolve a seed bank of seeds with fire-released dormancy, whereas this was more challenging for species with fleshy fruits dispersed by vertebrates (Herrera 1992; Verdú & Pausas 2013). In such cases, enhancing resprouting became a more efficient strategy for postfire survival. An alternative pathway is to remain fire-sensitive and be relegated to non-fire-prone environments.

Here, we use the *Juniperus oxycedrus* complex (*J. oxycedrus sensu lato*) to date the origin of lignotuber resprouting and infer the period when fires—and the associated mediterranean climate—became frequent and stable enough to select for plant adaptations. Field observations (Paula et al. 2016; Tavsanoglu & Pausas 2018) suggest that some populations of *J. oxycedrus s.l.* do not resprout after fire, while others resprout from a specialized organ named lignotuber. These are belowground burls of ontogenetic origin that accumulate a bud bank for postfire resprouting; they are considered fire adaptations and basically only occur in fire-prone ecosystems (Noble 2001; Keeley et al. 2012; Paula et al. 2016; Pausas et al. 2018; Fig. 1). Given that most juniper species do not resprout, and *J. oxycedrus* is the only needle-leaved juniper (*Juniperus* sect. *Juniperus*; 15 spp. *sensu* Gutiérrez-Larruscain et al. 2024) with this capacity, we consider the acquisition of lignotuber resprouting to be an evolutionary novelty in this lineage. We propose that the emergence of this trait can serve as an indicator of a shift in fire regimes.

Our hypothesis is that during the aridification that gave rise to the mediterranean biome, prickly junipers diverged into two forms: one that survived in non-fire-prone ecosystems, and another that developed the ability to accumulate buds in a belowground lignotuber, allowing it to survive by resprouting in mediterranean fire-prone ecosystems. We further hypothesize that the acquisition of resprouting that allowed postfire survival had consequences in the structure of populations (Segarra-Moragues & Ojeda 2010) as populations of non-resprouters would be more fragmented by Mediterranean fires than those of resprouters that survive fires. To test these hypotheses, we applied genotyping-by-sequencing (GBS) to construct a dated phylogeny of resprouting and non-

resprouting *J. oxycedrus s.l.* populations from the Iberian Peninsula and to analyze their genetic structure.

2. Material and Methods

2.1. Sampling and experiment design

We selected twenty-two wildfires that occurred in the Iberian Peninsula between 2003 and 2021 where *J. oxycedrus s.l.* was present. We visited them and assessed whether they resprouted after fire or not (fire-killed) (R and N populations, hereafter). For R populations, plant material (leaves) were directly collected from resprouts. In the case of N populations, leaf material was collected from unburned individuals located at the edges of the wildfires or within unburned patches inside the wildfire area. To avoid misclassification of R as N plants, populations where both R and N were suspected to coexist were excluded from this study. Leaf material from up to 15 individuals per population was collected and dried in silica-gel for molecular analysis. Additionally, a representative herbarium sheet was prepared from each location (Tab. S1) and several galbuli were collected from different individuals (when possible).

2.2. DNA isolation and Genotyping – Assemblies and Data Processing

Total genomic DNA was isolated from up to 10-11 individuals from each population (231 in total) following the CTAB protocol (Doyle & Doyle 1987) with minor modifications. A Genotyping-by-Sequencing library was carried out following the protocol used in Fernández-Mazuecos et al. (2018; adapted from Elshire et al. 2011, and Escudero et al. 2014). Briefly, 500 ng of DNA from each sample were digested using the Pstl-HF restriction enzyme (New England Biolabs, Ipswich, MA, USA). After ligation to the barcoded and common adapters, 5 µl of each sample were pooled, and the pool was purified using 1:1 AMPure XP beads (Beckman Coulter, Brea,CA, USA). Subsequently, 35 ng of the purified product were used for PCR amplification to enrich the library. After an additional purificacion step with 1:0.8 AMPure XP beads, quality control was performed using a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). The single index library was sequenced by Macrogen (Korea, Seul) in one lane of 150 pb paired end-illumina HiSeq X sequencing (Illumina, Inc., San Diego, CA, USA). Raw data from the 231 individuals were deposited in SRA NCBI database under BioProject PRJNA1180569.

In order to investigate the acquisition of resprouting and its monophyly in the *J. oxycedrus* group, a first dataset was created (hereafter M48 – Macroevolutionary Dataset; n = 48). M48 included one specimen per studied population (n = 22) together with representative samples of *Juniperus* and related genera (n = 26) taken from Gutiérrez-Larruscain et al. (2024). M48 was assembled using *Cupressus sempervirens* L. as reference genome (GeneBank ID: GCA_028749045.1) throughout the software Ipyrad v0.9.92 (Eaton & Overcast 2020) as described in Gutiérrez-Larruscain et al. (2024).

The second dataset aims to deal with population structure and gene-flow in N and R juniper populations (hereafter S234 – Structure Dataset; n = 234). S234 included all the sequenced R and N sampled specimens (n = 231) plus one sample of each *J. macrocarpa, J. maderensis* and *J. cedrus* taken from Gutiérrez-Larruscain et al. (2024). S234 dataset was *de novo* assembled using the seven steps of the software Ipyrad.

Lastly, two datasets were generated to calculate population genetic indices separately in each N and R juniper populations. For that, two independent *de novo* assemblies were carried out (N96 and R135; Nonresprouter and Resprouter datasets, with n = 96 and n = 135, respectively) in each N and R group as described above for S234.

All raw FASTQ files were assembled on a High Performance Computer (Drago – CSIC). Several values of the Ipyrad parameters M (minimum sample counts per locus) and C (clustering threshold) were tested up to a total of 117 different assemblies (36 *de novo* assemblies for each S234, N96 and R135 datasets plus 9 *reference* assemblies for the M48 dataset). Information regarding the number of row reads, parameters used in each assembly, number of final loci, length of the alignments, total number of SNPs (Single Nucleotide Polymorphism) and PIS (Parsimony Informative Sites) for the selected assemblies were summarized in Tab. S2. Additionally, a graphical summary with the parameters obtained in each assembly is provided in supplementary material (Fig S1).

2.3. Phylogenetic Inference and molecular dating

Maximum likelihood phylogenetic reconstruction was performed using aligned matrices obtained from the Ipyrad assembly for the M48 and the S234 datasets. The phylogenetic analyses were carried out using the software IQ-TREE multicore version 2.2.2.7 (Minh et al. 2020). A partition model was implemented using the best-fitting substitution model obtained by *ModelFinder* algorithm (Kalyaanamoorthy et al. 2017) available in IQ-TREE (Chernomor et al. 2016). Branch support was estimated by 1000 iterations of ultrafast bootstrap (UFBoot; Hoang et al. 2018) and 1000 iterations of SH-like approximate likelihood ratio test (SH-aLRT; Guindon et al. 2010).

Molecular dating based on Relative Rate Framework (RRF) was conducted using RelTime (Tamura et al. 2012, 2018) analysis implemented in MEGA11 (Molecular Evolutionary Genetic Analysis version 11; Tamura et al. 2021) as described in Gutiérrez-Larruscain et al. (2024). We employed as input the tree topology obtained in the Maximum Likelihood analysis (M48) and the calibration points described in Gutiérrez-Larruscain et al (2024).

2.4. Population Structure, Gene-flow and Population genetics indices

Variant Call Format (VCF) matrices obtained from S234, N96 and R135 datasets Ipyrad assemblies were filtered using the software VCFtools 0.1.16 (Danecek et al. 2011). We excluded positions with a minimum allele frequency of < 0.01, minimum quality score of < 13, a minimum read depth of < 5, and a minimum distance between SNPs > 1000. Position with a level of missing data > 10% were also excluded, and indels were removed. These filtering parameters yielded 1779 high-quality SNPs for the S234 matrix out of a possible 202008 sites; 1925 SNPs from 100363 and 1931 SNPs from 122728 possible sites for the N96 and R135 datasets, respectively. We used these filtered matrices for the following downstream analysis.

Population structure analysis was carried out using the software STRUCTURE (Pritchard et al. 2000) at two levels. The first level included all the N and R populations (S234 dataset) to detect admixture events between N and R individuals. The second level consisted in two independent analyses, one for each N and R groups to address the genetic structure underlying each group (N96 and R135 datasets). For each analysis, we explored several K values (ranging from 1 up to the number of populations of each dataset) by running 20 independent analyses of 150000 MCMC iterations where the first 50000 were discarded. Optimal K value (Fig. S2) was assessed by the Evanno test (Evanno et al. 2005) using the StructureHarvester python script (Earl& vonHoldt 2012). Summarized matrices with the information of each run for the optimal K values were permutated using CLUMPP v1.1.2 (Jakobsson & Rosenberg 2007) then, the genetic membership matrices were plotted using a R custom script over the topology obtained from the S234 phylogenetic analysis.

FineRadStructure pipeline (Malinsky et al. 2018) was employed to estimate coancestral relationships between samples from the S234. This method implements a Markov Chain Monte Carlo algorithm to infer patterns of population structure based on haplotype linkage information.

First, we used the “RADpainter” utility to get the haplotype file from the filtered VFC matrix and the coancestry matrix. Next, we used the “finestructure” module to assign individuals to groups and to construct a tree describing their relationships. The results were visualized using the “fineRADstructurePlot.R” script.

For each of the two datasets at group level, N96 and R135, genetic variation statistics were calculated within populations using the R packages “adegenet v2.1.10” (Jombart 2008; Jombart & Ahmed 2011), “poppr v2.9.6” (Kamvar et al. 2014; Kamvar et al. 2015), “PopGeneReport v3.1” (Adamack & Gruber, 2014) and “hierfstat v0.5.11” (Goudet & Jombart 2022). Specifically we computed the following statistics: number of P_A (Private alleles), values of A_R (Allelic richness), observed heterozygosity (H_O), gene diversity within populations (H_S) population-specific fixation index (F_{ST}) and inbreeding coefficient (F_{IS}). Mean values of nucleotide diversity (π) for each locus at the population level were calculated using VCFtools. Significant differences in the means of these parameters between the two groups at population level were tested using a t-test. At group level (N and R), H_O, H_S, F_{ST}, F_{IS}, total gene diversity (H_T), diversity among populations (D_{ST}) and Jost's D population differentiation index (D_{EST}) and corrected H_T’, D_{ST}’ and F_{ST}’ were calculated following Nei (1987). Statistical differences between parameter values for the two groups were tested using a permutation approach with 10000 replicates.

2.5. Biometric analysis

In order to evaluate trait differences between N and R groups and validate the taxonomic characters typically used in identification keys of the *J. oxycedrus* group, we performed a biometric analysis. The following characters were measured using an electronic caliper: leaf maximum length (LML), leaf maximum width (LMW) and galbulus diameter (DIM). Ten measures of each trait were taken from a representative individual from each population, except for DIM, where several galbulus from different individuals were collected (when possible). Variables were log-transformed and a generalized linear mixed model (GLMM) was fitted using the R package “lme4 v1.1.35.5” (Bates et al. 2015) for each morphological trait (response variable), setting the group (species) as fixed effect and the population of origin (n= 22) as a random effect.

3. Results

3.1. Resprouting characterization, phylogenetic reconstructions and molecular dating

Nine populations were characterized as N and thirteen as R. They show a distinct geographic distribution: N populations are distributed in the central and western-northwestern regions of the Iberian Peninsula, while R populations are located in the northeastern, eastern and southern parts of the Iberian Peninsula (Fig. 2). Most R populations occur in warm, dry, fire-prone shrublands, whereas N populations are found inland (e.g., on the Iberian Plateau and adjacent mountains) at higher altitudes with colder conditions and often in rocky outcrops.

Both groups of N and R individuals were recovered in two independent clades with full branch support (Fig. 2). The N individuals were recovered in the same clade as individuals previously identified as *J. badia* (*i.e.* MAR1, MAR2, ROBCC, TUN and VILZA; Gutiérrez-Larruscain et al., 2024), while the R individuals were recovered together with two specimens previously identified as *J. oxycedrus* (*i.e.* MONCS and TORVA; Gutiérrez-Larruscain et al., 2024). Therefore, the R and N forms of *J. oxycedrus s.l.* are termed hereafter *J. oxycedrus* and *J. badia*, respectively; both traditionally considered within the variability of *J. oxycedrus* in regional floras. The sister relationship between the clade including the Macaronesian junipers (*J. cedrus* and *J. maderensis;* Fig. 2) and *J. badia*, further supports the taxonomic independence of both species (*J. oxycedrus* and *J. badia*).

The split (Fig. 2, Tab. S3) between the resprouting clade (*J. oxycedrus*), and the non-resprouting clade (*J. badia* + *J. cedrus* + *J. maderensis*) was dated to 6.23 Ma (95% confidence interval, CI: 4.25, 9.12). The crown clade of *J. oxycedrus* populations was dated to 3.75 Ma (CI: 2.37, 5.94), while the crown clade of *J. badia* populations was dated to 3.54 Ma (CI: 2.24, 5.56).

In most cases, the recovered intraspecific relationships (Fig. 3) do not support monophyletic populations nor do they reflect any clear geographical pattern. Exceptions are three populations (NUNCC, PORNI and VILLE; Fig. 3) in *J. badia,* and one (ESTMA; Fig. 3) in *J. oxycedrus,* which are recovered in independent clades.

3.2. Population structure and genetic diversity

The Evanno test identified as optimum models three genetic groups for the R135 dataset, five for the N96 and two for the S234 (Fig. S2). Looking at the two genetic groups of all populations dataset (S234), one lineage is predominantly represented in *J. badia* (J1; Fig. 3) and residually in *J. oxycedrus* samples, while the other is almost restricted to *J. oxycedrus* samples (J2; Fig. 3). Some genetic lineages (B1, B3, B4 and B5; Fig. 3) are primarily associated with samples from the westernmost distributed populations (Fig 2; VILZA, NUNCC, VILLE and PORNI) whereas lineage B2 is represented in all *J. badia* populations (Fig. 3). Certain genetic lineages (O1 and O2) are represented in almost all the *J. oxycedrus* populations, whereas O3 is widely represented in samples from the southernmost *J. oxycedrus* population (Fig. 2: ESTMA).

The coancestral relationship analysis identified two distinct groups corresponding to the two forms, *J. badia* and *J. oxycedrus* (Fig. S3). There was minimal population structure within each taxon, except for a few *J. badia* populations and a small number of *J. oxycedrus* individuals, suggesting greater genetic isolation in these few cases. Some individuals within species exhibited higher shared ancestry with individuals from other populations than with those from the same population, indicating cases of high gene flow and weak population structure.

Overall population genetics parameters (Tab. 1) indicate that *J. oxycedrus* presents a higher genetic diversity within populations (H_S) but lower genetic diversity among populations (D_{ST}). Fixation index and Jost's D ($F_{ST,}$ D_{EST}) indicates higher genetic differentiation in *J. badia* populations, along with higher inbreeding coefficient (F_{IS}). At the population level (Fig. 4, Tab. 1, Tab. S4), *J. badia* populations show higher values for number of private alleles (P_A), whereas *J. oxycedrus* populations presented higher allelic richness (A_R) and nucleotide diversity (π). All populations from both species (Tab. S4) presented higher H_S than H_O values, suggesting a tendency towards an excess of homozygosity, a pattern corroborated by F_{IS} values (Tab. S4).

3.3. Morphological differences

Analysis of deviance (type II Wald χ^2 tests) shows that leaf width and galbulus diameter were significantly larger in *J. badia* than in *J. oxycedrus* ($\chi^2 = 4.04$, df= 1, $p < 0.043$ and $\chi^2 = 20.90$, df= 1, $p < 0.00001$; respectively), although their values largely overlap (Fig. 4).

4. Discussion

The onset of the climatic seasonality that characterize the Mediterranean Basin has traditionally been estimated through paleopalynological and marine paleoclimate records (Suc, 1984; Suc & Popescu, 2005; Barrón et al. 2010; Jiménez-Moreno et al. 2010). They indicate that the late Pliocene (around 3 Ma) marked a period of major reduction in subtropical taxa, coinciding with the expansion of sclerophyllous vegetation more adapted to xeric conditions. In contrast to the humid subtropical climate that preceded it, the mediterranean climate had frequent droughts, dry summers, and recurring wildfires (Keeley et al. 2012). This shift likely created new selective pressures that

favored traits for postfire survival like lignotuber resprouting (Paula et al. 2016; Pausas et al. 2018). Although resprouting is a ubiquitous trait in many environments and lineages, lignotuber resprouting is mostly restricted to fire-prone ecosystems and widely recognized as an adaptation to recurrent high-intensity fires (Noble 2001, Pausas et al. 2018, Keeley & Pausas 2022). Thus, dating the origin of this trait may provide an indicator of the fire regime shift and an independent source of information on the origin of the mediterranean fire-prone biome (Lamont & He 2017).

Our phylogenetic reconstruction (Fig. 2) points to a single evolutionary origin of resprouting in the *J. oxycedrus* clade from non-resprouting (fire-killed) ancestors. The divergence between the resprouting (*J. oxycedrus*) and the sister non-resprouting junipers (*J. badia+J. cedrus+J. maderensis*) is estimated to have occurred between approximately 6.23 Ma (late Miocene), with a crown age for the *J. oxycedrus* populations of around 3.75 Ma (mid-Pliocene). The time of the split of the R and N (6.23 Ma) is the most likely origin of the resprouting trait (Lamont et al. 2019); a conservative approach would consider the mid-stem-to-crown point (5 Ma). That is, during the late Miocene, junipers likely experienced a strong selective pressure by frequent fires. That points to fire as an evolutionary pressure intrinsically linked to the mediterranean biome that likely arose during the Miocene (e.g., Messinian Salinity Crisis; 6-5.3 Ma) and early Pliocene.

Our phylogenomic evidence of fire regime shift during the Miocene-Pliocene is also consistent with age of the Mediterranean clade *Cistus+Halimium+Tuberaria* (11 to 5.3 Ma, i.e. stem to crown age; Guzmán & Vargas 2009), a clade with most species having fire-released seed dormancy (Pausas & Lamont, 2022). Although the origin of the Palearctic mediterranean climate is often considered around 3.6 Ma (Suc, 1984; Suc & Popescu, 2005), it is plausible that pockets with mediterranean-like conditions emerged earlier at drier sites, and spread through the landscape as aridification intensified (Tzedakis 2007; Keeley et al. 2012; Vargas et al. 2018). Our results suggest that fires (and the associated climate seasonality) were recurrent and significant enough to select for specialized fire-resistant traits since about 6 Ma. By using relevant traits rather than diversification rates, we assess the post-Messinian temporal gap observed in many mediterranean lineages by Fiz-Palacios & Valcárcel (2013). Indeed, the absence of significant juniper extinctions over the past 6 Ma, as indicated by a sequential branching pattern between the stem and crown nodes of the *J. oxycedrus* complex phylogeny (Fig. 2), supports the notion of sustained evolutionary success since acquisition of lignotuber resprouting. The initial phases of the Mediterranean biome may have gone unnoticed in the paleobotanical records, perhaps because the paleoecological records were usually preserved in wetlands and this may create a bias against dry and fire-prone ecosystems.

The different dynamics associated with non-resprouting and resprouting habits under a mediterranean fire-prone environment (i.e., postfire mortlity and survival, respectively) generated contrasted genetic diversity profiles and population structure between *J. badia* and *J. oxycedrus*. Despite its broad geographical distribution in the Iberian Peninsula, *J. badia* does not occur at fire-prone low elevations —like *J. oxycedrus*— and is more abundant on rocky outcrops and nutrient-poor soils, where competition with broadleaved trees may be attenuated. These microhabitats have reduced biomass production and shorter and milder drought seasons, which leads to low fire-proneness, effectively acting as pyro-refugia. As a result, fire activity restricts the non-resprouting *J. badia* from other, more fire-prone habitats, which, over time, has led to their spatial isolation. This isolation is reflected in the genetic data (Fig. 4, Tab. 1, Tab. S4), with *J. badia* populations exhibiting higher levels of private alleles (P_A), which are indicative of long-term isolation and limited gene flow. The fragmented and isolated nature of these pyro-refugia is also reflected by the distinct genetic groups (Fig. 3) linked to isolated populations.

On the other hand, *J. oxycedrus* populations exhibit far less genetic structure (Fig. 3, Fig. S3), which is explained by the continuity of their distribution across the landscape. The ability of *J. oxycedrus* to resprout after fire enable it survive multiple fires and form more continuous

populations, allowing gene flow across larger areas, and thus maintaining some genetic homogeneity. The limited population structure observed in *J. oxycedrus* indicates that gene flow remains high, with only minor exceptions (e.g., ESTMA population, genetic group O3; Fig. 3). The higher gene flow in *Juniperus oxycedrus* populations is indicated by greater allelic richness (A_R), nucleotide diversity (π), observed heterozygosity (H_O), and gene diversity within populations (H_S), suggesting reduced genetic drift (Fig. 4). The higher levels of genetic diversity within populations (H_S) and the low F_{ST} levels in *J. oxycedrus* compared to *J. badia* (Tab. 1) further highlight the role of fire driving genetic diversity and low differentiation in the resprouting species. These differences are consistent with those observed between resprouting and non-resprouting populations of South African *Erica* species based on microsatellite markers (Segarra-Moragues & Ojeda 2010).

In summary, lignotuber resprouting in *J. oxycedrus*, enables population persistence across fire cycles, granting population connectivity, reducing genetic differentiation and maintaining higher within-population diversity in a fire-prone scenario (Pausas & Keeley, 2009; Pausas et al. 2018). In contrast, *J. badia*, a non-resprouter confined to fire refugia, shows higher genetic isolation due to fragmented populations, greater reliance on seed recruitment, and limited gene flow, leading to higher genetic differentiation and more exclusive alleles.

The two forms within the *Juniperus oxycedrus* group are phylogenetically distinct taxa (Fig. 2) and exhibit different population dynamics (Table 1). Morphologically, the resprouting form (*J. oxycedrus* s.s.) possesses a lignotuber, and adults are typically multi-stemmed, in contrast to the non-resprouting form (*J. badia*), which usually grows as a single-stemmed tree. The phylogenetic distinction between resprouting and non-resprouting populations (as two monophyletic groups) supports a strong genetic basis for this trait, rather than phenotypic plasticity. Thus, the complex development of a lignotuber could constitute a relevant taxonomic character (as in *Eucalyptus*, Gosper et al., 2019); however, this trait is not always evident in the field, and less so in herbarium specimens. In contrast, there are some other traits related to leaf and galbule sizes between the two juniper forms that show considerable overlap (Fig. 4). The two taxa also occupy distinct distribution areas. *Juniperus oxycedrus* is primarily found in warm coastal lowlands of the eastern Iberian Peninsula (Fig. 2), while the non-resprouting *J. badia* occurs primarily inland, generally at higher altitudes in colder environments (e.g. Iberian Plateau). This spatial and environmental segregation is consistent with other fire traits; for instance, pine serotiny and the prevalence of fire-released seed dormancy are higher in warmer coastal areas than inland of eastern Iberia (Hernández-Serrano et al. 2013, Verdú & Pausas 2007). Interestingly, resprouting forms of junipers appear to be absent from northern Africa, a region where needle-leaved junipers only occur at relatively high altitudes and correspond to *J. badia* (JG Pausas, personal observations). In northern Africa, the ecological niche of *J. oxycedrus* (warm lowlands) seems to have been occupied by *Tetraclinis articulata*, another member of the Cupressaceae family, which also possesses a resprouting lignotuber (Tavsanoglu & Pausas, 2018). Several floras report *J. oxycedrus* in northern Africa, but these records need verification based on the current knowledge of this species complex.

Resprouting is an ancestral trait in many angiosperms (Pausas & Keeley 2014), but this does not prevent the existence of non-resprouting lineages where resprouting is a recent acquisition of a few species. This is the case of *J. oxycedrus*, and probably, of resprouting in other gymnosperm lineages (e.g., *Pinus*, He et al 2012). Overall we show the potential of using fire-adaptive traits as proxies for predicting fire regime and climate shifts as in the case of the onset of the mediterranean climate. By tracing the evolutionary acquisition of resprouting in *J. oxycedrus*, we provide a novel indicator of an intensification of fire regimes during the late Miocene. It is also an example of trait and demographic divergence driven by fire regime changes (Keeley et al. 2011; Lamont et al. 2019; Keeley & Pausas 2022). The divergence between resprouting and non-resprouting clades indicates

that by about 6 Mya, fire regime had become sufficiently frequent and intense to select for lignotuber resprouting. At that time, the mediterranean biome may have been widespread.

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Table 1. Gobal population genetics parameters (upper part) and population level summary (lower part; mean and standard deviation from Tab. S4 values) based on 136 R and 96 N individuals within 13 and 9 populations, respectively. H_O: observed heterozygosity; H_S: gene diversity within populations; F_{ST}: population-specific fixation index and F_{ST}' its correction; F_{IS}: inbreeding coefficient; H_T: total gene diversity and H_T' its correction; D_{ST}: diversity among populations and D_{ST}' its correction; D_{EST}. Jost's D population differentiation index; P_A: number of private alleles; π: mean values of nucleotide diversity; A_R:. allelic richness. *P*-values were computed based on a 10000 permutation test for global parameters (Fig. S4) and using a t-test for population level parameters (Tab. S4).

	R (*J. oxycedrus*)	**N (*J. badia*)**	**P-value**
H_O	0.133	0.110	0.8
H_S	0.165	0.142	**< 0.0001**
F_{ST}	0.036	0.050	**< 0.0001**
$F_{ST'}$	0.039	0.056	**< 0.0001**
F_{IS}	0.195	0.206	**< 0.0001**
H_T	0.171	0.149	0.05
$H_{T'}$	0.172	0.150	0.98
D_{ST}	0.006	0.007	**< 0.0001**
$D_{ST'}$	0.007	0.008	**< 0.0001**
D_{EST}	0.008	0.010	**< 0.0001**
P_A	15.8 ±14.4	77.6 ±25.1	**< 0.0001**
π	0.164 ±0.014	0.141 ±0.008	**< 0.0001**
A_R	2774.84 ±86.4	2657.443 ±57.4	**< 0.01**

Figure Captions

Fig. 1. Post-fire resprouting in *Juniperus oxycedrus* from a belowground bud bank of ontogenetic origin (lignotuber). Photos by J. G. Pausas.

Fig. 2. Time-calibrated phylogenetic reconstruction (M48 dataset) and distribution map of sampled *Juniperus* populations. Green and orange color represents non-resprouting (*J. badia*) and resprouting (*J. oxycedrus*) populations, respectively. Node bars in the phylogram indicate confidence intervals of the dated nodes, with age estimates taken from the RelTime analysis shown alongside. Numbers above branches correspond to branch support values: SH-like approximate likelihood ratio test (SH-aLRT) on the left and Ultrafast Bootstrap (UFBoot) on

the right. Letters below the branches refer to calibration points based on Gutiérrez-Larruscain et al. (2024). Labels on the map indicate the location of each sampled population.

Fig. 3. Phylogenetic reconstruction for all studied *Juniperus* populations (S234). Node colors represent UFBootstrap and SH-aLRT support values higher than 90. The inner rings depict the optimal K=2 structure analysis for the entire group (S234 dataset). The outer rings represent two independent analyses, with optimal models of K=5 for *J. badia* (N96) and K=3 for *J. oxycedrus* (R135). The color of the labels indicated the two forms, *J. badia* (green) and *J. oxycedrus* (orange).

Fig. 4. Box-plots summarizing the population genetic parameters for the studied *Juniperus* populations: number of Private alleles (P_A), Allelic richness (A_R), mean values of nucleotide diversity (π); The three last box-plot represent the results of the biometric study between all individuals of both groups. A t-test was performed to assess the statistical significance of differences between groups (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s. not significant). Results of the ANOVA performed on the generalized linear mixed models for biometric measurements (* > 0.05, ** > 0.01, ** > 0.001, n.s. no significance).

Fig. S1. Graphical summary of the explored assemblies. A) S234, B) N96, and C) R135 *de novo* assembled datasets: the graphs display output variables obtained at each step of the Ipyrad pipeline, where several values for the most influential parameters in the assemblies performance were explored. These parameters include the clustering threshold parameter (represented as c), and the minimum number of samples in the assembly sharing a locus (m). D) M48 *reference* based assembled dataset exploring different values of m. Red points represents the total number of loci recovered in each assembly and the numbers under each box-plot indicate the percentage of missing data and the number of parsimony informative sites.

Fig. S2. Mean estimated LnP values for the explored K groups and results for the Evanno tests in the R135 (upper panel, orange), N96 (middle panel, green) and S235 (lower panel, purple) datasets.

Fig. S3. Heatmap showing shared coancestry between individuals from the S234 dataset.

Fig. S4. Permutation test for the global population genetic parameters based on 10000 permutations.