ANNALS OF
BOTANY
Founded 1887

Early diversification and permeable species boundaries in the Mediterranean firs

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• **Background and Aims** Inferring the evolutionary relationships of species and their boundaries is critical in order to understand patterns of diversification and their historical drivers. Despite *Abies* (Pinaceae) being the second most diverse group of conifers, the evolutionary history of Circum-Mediterranean firs (CMFs) remains under debate.
• **Methods** We used restriction site-associated DNA sequencing (RAD-seq) on all proposed CMF taxa to investigate their phylogenetic relationships and taxonomic status.
• **Key Results** Based on thousands of genome-wide single nucleotide polymorphisms (SNPs), we present here the first formal test of species delimitation, and the first fully resolved, complete species tree for CMFs. We discovered that all previously recognized taxa in the Mediterranean should be treated as independent species, with the exception of *Abies tazaotana* and *Abies marocana*. An unexpectedly early pulse of speciation in the Oligocene–Miocene boundary is here documented for the group, pre-dating previous hypotheses by millions of years, revealing a complex evolutionary history encompassing both ancient and recent gene flow between distant lineages.
• **Conclusions** Our phylogenomic results contribute to shed light on conifers' diversification. Our efforts to resolve the CMF phylogenetic relationships help refine their taxonomy and our knowledge of their evolution.

Key Words: Species delimitation, speciation with gene flow, phylogenomics, RAD-seq, conifers, transposable evolution, *Abies*.

INTRODUCTION

Extant gymnosperms have relatively limited species diversity presumably as a result of low speciation rates and high extinction (Crisp and Cook, 2011). However, recent pulses of radiation have been found in some gymnosperm lineages, challenging the perception that gymnosperm taxa are ancient and barely variable (Davis and Schaefer, 2011). Global climate shifts have been suggested as major drivers of diversification in gymnosperm radiations (Nagalingum *et al.*, 2011). However, other powerful contributors to conifer evolution and diversity are the frequent hybridization events (Isoda *et al.*, 2000; Ru *et al.*, 2016) and bursts of transposable elements (TEs; Nystedt *et al.*, 2013), which could facilitate regulatory, karyotypic or other genetic changes (Oliver *et al.*, 2013). Different TE dynamics can contribute to reproductive isolation and promote species radiation (Serrato-Capuchina and Matute, 2018). Delimiting species boundaries and inferring their evolutionary relationships is critical in order to understand the pace and mode of lineage formation in diversifying taxa, as well as their evolutionary drivers (Barley *et al.*, 2013).

Firs (*Abies*, Pinaceae) are an important component of temperate-cool forests that characterize the boreal regions of the Northern Hemisphere (Farjon and Rushforth, 1989). They represent the second most diverse group of the Pinaceae family and of gymnosperms as a whole (Xiang *et al.*, 2009), consisting of >50 species. Although fir trees have three major areas of distribution,

North America, East Asia and the Mediterranean Basin, evolutionary studies have so far primarily focused on the fir trees outside the Mediterranean Basin (Linares, 2011). Based on paleogeographical data (Linares, 2011), colonization and diversification of firs in the Mediterranean could have been associated with climatic and geological changes during the late Miocene and the Pliocene. Numerous investigations have already been conducted on the genus *Abies* but included only part of the proposed taxa and only single DNA regions or low-resolving molecular markers (see below), hence species delimitation and evolutionary history of all Circum-Mediterranean firs (CMFs) remain debatable.

In the last 200 years, taxonomic classification of firs has undergone numerous revisions, with at least 14 formal classification attempts (Franco, 1950; Liu, 1971; Farjon and Rushforth, 1989). The number of recognized taxa has ranged from six to 12 species and subspecies in different classifications. The most recent taxonomical review (Farjon and Rushforth, 1989) classified CMFs into two groups (section *Abies* and sect. *Piceaster*) and eight species. Section *Abies* comprises *Abies alba* Mill., the most widely distributed fir in Europe; *A. nebrodensis* (Lojac.) Mattei, a critically endangered species (conservation status: CR by IUCN) (Nebrodi mountain range, Sicily, Italy); *A. cephalonica* Loudon (Greece); and *A. borisii-regis* Mattf. (Northern Balkans and Greece), a hybridogenous taxon of *A. alba* and *A. cephalonica* (Bella *et al.*, 2015; Krajmerová *et al.*,

2016). In addition, from the Eastern Mediterranean, Farjon and Rushforth (1989) recognized *A. nordmanniana* (Steven) Spach (Northeast Caucasus and Turkey) as a single taxon. However, *A. nordmanianna* is a complex taxon which other authors (Mattfeld *et al.*, 1925; Liepelt *et al.*, 2010; Linares, 2011) have sub-divided into *A. equi-trojani* (Asch. & Sint. ex Boiss.) Mattf. (Northern Turkey) and *A. bornmuelleriana* Mattf. (Northern Turkey). This sub-division in only partially supported by molecular evidence (Hrivnák *et al.*, 2017). Finally, *A. cilicica* (Antoine & Kotschy) Carrière, inhabiting Lebanon, Syria and Southern Turkey, has been included in the next section by some authors (Franco, 1950; Liu, 1971).

Section *Piceaster* would comprise *A. numidica* de Lannoy ex Carrière (Northern Algeria) and *A. pinsapo* Boiss. (Souterhern Spain and Northern Morocco). However, this last taxon has been sub-divided into three different taxa, i.e. *A. pinsapo* Boiss *sensu stricto* (Southern Spain), *A. marocana* Trab. and *A. tazaotana* Côzar ex Villar (both Northern Morocco), by several authors (Franco, 1950; Arista and Talavera, 1994). Nevertheless, recent studies did not provide molecular support for the Moroccan species distinction (Sánchez-Robles *et al.*, 2014; Terrab *et al.*, 2007).

The inference of macroevolutionary relationships between *Abies* sections has been shown to be mostly robust to coalescent stochasticity of individual phylogenetic markers (Suyama *et al.*, 2000; Xiang *et al.*, 2009, 2015; Semerikova and Semerikov, 2014). However, the attempts to reconstruct the phylogenetic history of CMFs have yielded strongly conflicting signals between nuclear and plastidial markers (Sánchez-Robles *et al.*, 2014; Semerikova and Semerikov, 2014, 2016; Xiang *et al.*, 2015), resulting in a largely unresolved polytomy; inter-specific relationships and divergence times hence remain to date debated. Although many phylogeographical and phenotypic studies have significantly contributed to clarify species delimitation (e.g. Fady *et al.*, 1992; Scaltsoyiannes *et al.*, 1999; Terrab *et al.*, 2007; Bosela *et al.*, 2016; Liepelt *et al.*, 2010; Hrivnák *et al.*, 2017), it is noteworthy that these previous studies did not include all, or even most CMF taxa. In addition, no genome-wide study has been performed to reconstruct a species tree and determine the causes of the observed molecular discordances. Disagreements among classifications of *Abies* are largely attributed to insufficient understanding of phylogenetic relationships within the genus, as well as its morphological complexity (Robson *et al.*, 1993; Xiang *et al.*, 2009). This complexity may be further exacerbated by historical gene flow between several species, especially those that are closely related and in geographical proximity (Scaltsoyiannes *et al.*, 1999; Isoda *et al.*, 2000; Semerikova *et al.*, 2011; Sánchez-Robles *et al.*, 2014; Xiang *et al.*, 2015; Krajmerová *et al.*, 2016). Reproductive barriers in the CMFs can be permeable, as suggested by successful artificial crossings, which is in contrast to the strong barriers existing between North American and Mediterranean firs (Mergen *et al.*, 1962; Alizoti *et al.*, 2011; Kormutak *et al.*, 2013). Secondary contacts among several CMF species during climatic cycles in the Quaternary have been previously suggested (Linares, 2011; Sánchez-Robles *et al.*, 2014). However, the existence of ancient gene flow during the evolution of CMFs has never been tested, probably because of the weak phylogenetic signal of the previously used molecular markers.

An accurate CMF species delimitation is fundamental to manage biodiversity and forest genetic resources (Scarascia-Mugnozza *et al.*, 2000; Fady *et al.*, 2016; Kavaliauskas *et al.*, 2018; Potter, 2018). Due to a high degree of endemism, geographically scattered distribution and fragmentation by human activities, four CMF taxa are currently included in the IUCN red list as critically endangered or endangered (*A. pinsapo* 'EN', *A. pinsapo* var. *marocana* 'EN', *A. nebrodensis* 'CR' and *A. numidica* 'CR'; Farjon *et al.*, 1993; Farjon and Page, 1999; Esteban *et al.*, 2010). Hence, the clarification and accurate assessment of species status has important conservation implications (Adams *et al.*, 2014; Melville *et al.*, 2014). New species delimitation analyses on high-throughput data have recently allowed the identification of species boundaries in several cryptic species complexes as well as in rapid adaptative radiations, e.g. in some Reptilia (Barley *et al.*, 2013; Grummer *et al.*, 2014), primates (Hotaling *et al.*, 2016) and plants (Jackson *et al.*, 2017; Brandrud *et al.*, 2019). We applied Illumina-based restriction site-associated DNA sequencing (RAD-seq) on all CMF taxa in order to investigate the evolutionary history of this group.

The main goals in the present study were: (1) to explore the origin and phylogenomic relationships of the modern CMFs; (2) to test taxonomic hypotheses about the current diversity of CMFs; and (3) to investigate the existence of inter-specific hybridization and the role of the TEs on the evolutionary history of the group.

MATERIALS AND METHODS

Taxon sampling and genomic data

Leaf material from 40 individual trees (2–4 individuals per taxon) of all recognized CMF taxa (Fig. 1), together with one representative of each of the eight remaining sections of the genus *Abies* were collected at 28 natural sites using the geographic information from the EUFORGEN project (http://www.euforgen.org/) during several field trips in 2015 and 2016 (Supplementary Data Table S1). The samples were preserved in silica gel. Vouchers were deposited in the Herbarium of the University of Seville (SEV). Genomic DNA was extracted using a DNA extraction kit (Qiagen DNeasy Plant Mini Kit) following the manufacturer's instructions. The RAD library was prepared as in Paun *et al.* (2016). Genomic DNA was digested by using high-fidelity *Sbf*I (New England Biolabs) and the resulting fragments were double barcoded. The library was sequenced in a separate lane of an Illumina flowcell HiSeq 2500 at the VBCF NGS Unit (www.vbcf.ac.at/ngs) as 100 bp single-end reads.

RAD-seq catalogue building and SNP detection

Quality filtering and demultiplexing of the library were performed with deML (Renaud *et al.*, 2015). Additionally, the process_radtags.pl program from STACKS ver. 1.29 (Catchen *et al.*, 2011, 2013) was used to remove reads without a restriction cut site. Different empirical tests were performed with the denovo_map.pl program to optimize the parameters used for catalogue
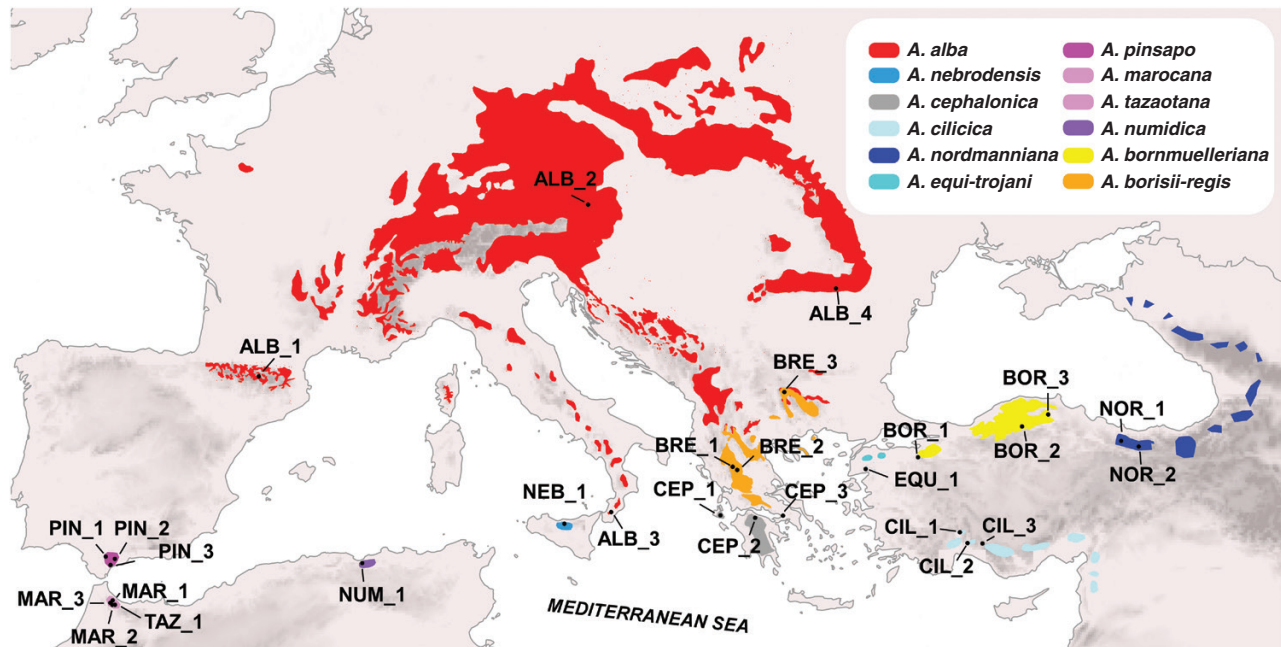
F1G. 1. Sampling map for the Mediterranean firs used in this study. Each population is labelled and species ranges are highlighted with different colours (more information is given in Supplementary Data Table S1). Species distributions are based on the EUFORGEN project (http://www.euforgen.org/) and personal observations.

building. We varied the minimum number of identical reads required for a stack to be formed (i.e. the setting 'm') from three to 11, the number of differences between alleles in a locus when processing one individual ('M') between one and five and the differences between orthologous loci between individuals ('n') from zero to four. The final combination of settings (m = 3, M = 2, and n = 1) was chosen to maximize the number of polymorphic RAD loci with a maximum of nine single nucleotide polymorphisms (SNPs; to avoid pooling paralogues in the same locus) which were present in at least 30 individuals (out of 40, i.e. 75 % of the total). For most of the analyses, we used a reduced data set, 'abies-reduced set', based on the first SNP of each RAD locus (6090 SNPs in total) to avoid linkage disequilibrium and ensure the independence of the loci. For most analyses, we removed the samples of the hybrid *A. borisii-regis* (*A. alba* × *A. cephalonica*; Krajmerová *et al.*, 2016) from the data set to avoid artefacts in the phylogenomic estimates (hereafter referred to as 'abies-reduced-nohyb set'). Finally, some analyses focused on the in-group CMFs only and were performed by excluding the outgroup and *A. borisii-regis* (hereafter the 'med-abies-reduced set').

Phylogenomic analyses and tempo of diversification

Phylogenetic reconstruction of the genus *Abies*, based on the abies-reduced set and the abies-reduced-nohyb set, were conducted by using maximum likelihood (ML) and Bayesian inference (BI). For the ML approach, we used RAxML ver. 8.0 (Stamatakis, 2014) on the CIPRES Web Portal (Miller *et al.*, 2015). The ASC_GTR-GAMMA model as appropriate for a data set of concatenated SNPs and 1000 rapid bootstrap inferences were used to estimate clade confidence. BEAST ver.

2.3.1 (Bouckaert *et al.*, 2014) was used for BI and molecular dating using a GTR mutation model with gamma-distributed mutation rates across sites which provides a good approximation of the marginal likelihood at inter- and intra-specific evolutionary levels using highly variable sequences (Jia *et al.*, 2014). The ucld prior was set to a uniform distribution (minimum 0, maximum 1), the tree prior was speciation Yule process and the chain was 20 million Markov chain Monte Carlo (MCMC) steps, logged every 2000 steps. We ran four runs which were concatenated and used to estimate the posterior distributions of topology and divergence time. In a first analysis, we dated the nodes in the genome-wide SNP-based phylogeny by using a relaxed-clock model in BEAST2 and four calibration points. The age of the oldest *Abies* fossil (Schorn and Wesley, 1986) was treated as a minimum age of calibration of the root, by applying a log-normal distribution of 47 million years ago (Ma) to the age of the oldest node. In addition, we used three *Abies* fossils for the calibration of nodes in the ingroup and outgroup. We used the same fossil calibrations as in Xiang *et al.* (2015). Fossil ages were assigned as a uniform distribution for their stratigraphic range (Supplementary Data Table S2). The minimum age of the stems of *Abies* and CMFs were assigned using log-normal prior distributions (47 and 23 Ma, respectively); meanwhile uniform prior distributions (a minimum of 16 Ma and a maximum of 47 Ma) were assigned to the crown node of the clade including sects, *Grandes* and *Oiamel*, and the crown node of sect. *Momi*. In a second independent dating analysis, we used two biogeographical calibration points (six calibration points in total; Supplementary Data Table S2). We used the date of the separation of North Africa from Europe (i.e. the end of the Messinian salinity crisis, the opening of the Strait of Gibraltar and Tyrrhenian Sea; approx. 6 Ma, normal prior

distribution) as a calibration age of the divergence between *A. pinsapo* and *A. marocana*–*A. tazaotana* but also for divergence between *A. nebrodensis* and *A. alba*.

Species delimitation

We compared eight candidate species delimitation models (Fig. 2) by using the Bayesian factor delimitation of species (SNAPP-BFD*; Leaché *et al.*, 2014) as implemented in SNAPP (Bryant *et al.*, 2012). BEAUti v2.3.1 (Bouckaert *et al.*, 2014) was used to set up the analysis file. The null model followed the most recent taxonomic treatment, but considering subspecies as species (11 species in total; delimitation species hypothesis A). Based on previous studies and our phylogenomic results, seven additional hypotheses were tested (Fig. 2). Specifically, we tested that *A. marocana* and *A. tazaotana*, which are locally restricted to Mount Tazaot, together form a single species and that their current distributions are just the result of recent fragmentation (hypothesis B; Sánchez-Robles *et al.*, 2014). In addition, we tested the inclusion of the restricted *A. nebrodensis* in the widespread species *A. alba* (hypothesis C). We also tested a potential clustering of the Turkish taxa (*A. nordmanniana*, *A. bornmuelleriana*

and *A. equi-trojani*) as a single species (hypothesis D). These three taxa have been treated with different taxonomic statuses in several taxonomic revisions (Coode and Cullen, 1965; Farjon and Rushforth, 1989). Finally, we also tested other combinations of these species delimitations (hypothesis E–I). For these analyses, we used the med-abies-reduced set without any missing data. We analysed the alternative tree topologies by using 24 steps of path sampling with 100 000 MCMC steps and 10 000 pre-burn-in steps to estimate marginal likelihoods. Convergence of the parameters (ESS > 200) was checked using Tracer v1.7.1 (Rambaut *et al.*, 2018) after 10 % burn-in removal. Estimated marginal likelihoods for competing models were compared by using Bayes factors (2× loglikelihood difference) to identify the most likely species delimitation. The species tree was subsequently inferred by using the Bayesian multispecies coalescent approach implemented in SNAPP with the same previous parameters (Bryant *et al.*, 2012) and visualized with Densitree 2.01 (Bouckaert, 2010). In addition, we used SVDQuartets (Chifman and Kubatko, 2014) with 100 non-parametric bootstrap replicates in PAUP* version 4.0a147 (Swofford, 2002) to generate a species tree for the best species delimitation.

We also produced a lineage-through-time (LTT) plot, which displays the number of speciation events through time, taking
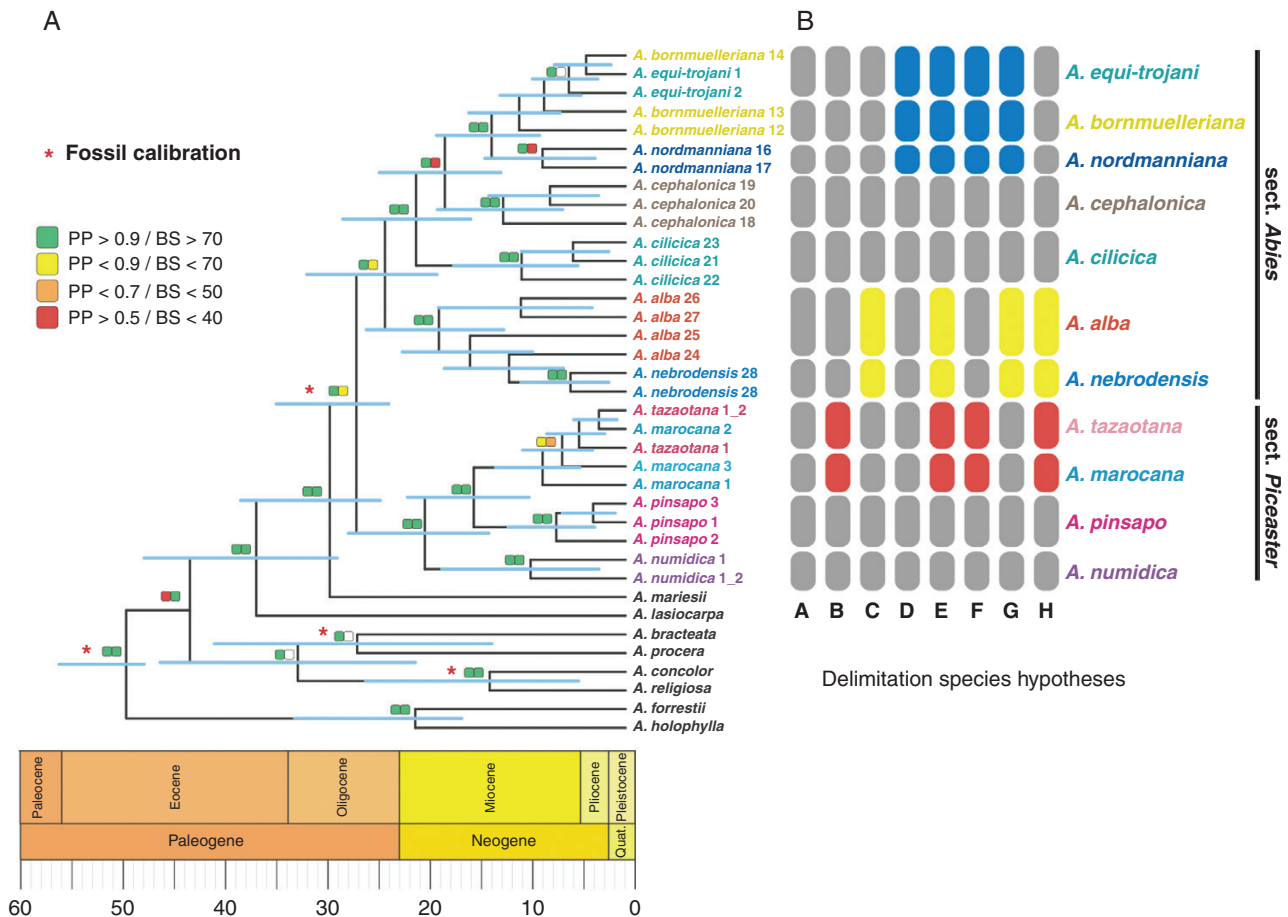

FIG. 2. Phylogenetic reconstructions for the CMFs based on 6090 SNPs. (A) Chronogram built in BEAST2 using six fossil calibration points (asterisks). Posterior probabilities and bootstrap supports from RAxML are colour marked according to the key. (B) Species delimitation hypotheses tested in the BFD* analysis. Each grey cell represents an independent species. Other colours indicate taxa that are merged into a single species.

into account possible extinction events. We calibrated the species trees from SNAPP by enforcing a strict molecular clock using the 'chronos' function in the R library ape (Paradis *et al.*, 2004). As calibration point, we used the 95 % highest posterior density (HPD) node age of the CMFs inferred from the previous genome-wide SNP-based BEAST2 analysis. Additionally, we used the gamma statistic (γ) from chronograms (Pybus and Harvey, 2000) to determine whether a signal of decreased diversification was noticeable.

Genetic structure and recent admixture

To assess the genetic structure of CMFs, we performed a Bayesian MCMC approach with STRUCTURE ver. 2.3.4 (Pritchard *et al.*, 2000). Ten independent runs were carried out for the data set for values of *K* ranging from 1 to 13. The number of iterations was 500 000 with a burn-in of 100 000 steps. STRUCTURE analyses were performed for all the *Abies* species in the Mediterranean area, as well as separately for each section (sect. *Abies* and sect. *Piceaster*, with *K* ranging from one to ten and one to eight, respectively). STRUCTURE HARVESTER software (Earl and VonHoldt, 2011) was used to determine the optimal number of clusters (*K*) implementing the Evanno method. The graphical representation of STRUCTURE results was generated by using CLUMPAK (Kopelman *et al.*, 2015). Additionally, fineRADstructure and RADpainter ver. 0.1 (Malinsky *et al.*, 2018) were used to infer the shared ancestry among individuals. The estimated haplotype co-ancestry matrix was used to infer recent admixture and to build a tree by using default parameters. For this analysis, we removed, as recommended, a sample with more missing data (A. equi-trojani_94_1) than the rest.

Divergence with gene flow

As popular approaches such as STRUCTURE (Pritchard *et al.*, 2000) and ADMIXTURE (Alexander *et al.*, 2009) are only appropriate for detecting gene flow over relatively short evolutionary time scales and may have limited power when only a few individuals per population are available (Soraggi *et al.*, 2018), we applied an ABBA–BABA approach (Green *et al.*, 2010) to the SNP matrix. This method distinguishes the contribution of incomplete lineage sorting from introgression by calculating imbalances in alternative tree topology frequencies under a four-taxon scenario (three ingroup taxa and one outgroup). In short, for a particular SNP with an ancestral ('A') allele and derived ('B') allele, the alternative patterns 'ABBA' and 'BABA' should be equally frequent under a scenario of incomplete lineage sorting without gene flow. *D*-statistic and *Z*-score calculations were performed with the R package evobiR (Blackmon and Adams, 2015), which measures signals of alternative phylogenetic asymmetry and the proportion of the genome that is shared between two taxa due to admixture, respectively (Durand *et al.*, 2011). ABBA/BABA tests were performed on the abies-reduced set including heterozygous sites by generating pseudohaploid sequences (Green *et al.*, 2010). One thousand bootstrap iterations were used to measure the s.d. of the *D*-statistic (Eaton and Ree, 2013). Based on previous

studies (Sánchez-Robles *et al.*, 2014) and our STRUCTURE results, we investigated gene flow among *A. alba* (sect. *Abies*) and *A. numidica* and *A. pinsapo* (sect. *Piceaster*). Using the approach proposed by Martin *et al.*, (2013) and the previously estimated species relationships, we performed several ABBA–BABA tests with different candidate species to distinguish between admixture in different time periods. Briefly, if gene flow was only ancient (e.g. prior to *A. nebrodensis* divergence from *A. alba*), then *A. alba* and *A. cilicica* should both be equally admixed with *A. numidica* and *A. pinsapo*. However, if gene flow was more recent (i.e. after *A. nebrodensis* divergence), then *A. alba* should be more admixed with *A. numidica* and *A. pinsapo* than with *A. cilicica*. We performed analyses for all combinations of pairs of conspecific individuals from candidate admixers (*A. alba*, *A. nebrodensis*, *A. cilicica*, *A. numidica* and *A. pinsapo*) using *A. lasiocarpa* as outgroup.

Genomic divergence in CMF

Additionally, to infer the contribution of TEs to genomic divergence of CMF species, the presence of TEs was checked in raw reads using the approach proposed by Trucchi *et al.* (2017). We randomly extracted 100 000 single reads from each individual, which were inspected for TEs by using RepeatMasker ver. 4.0.6 against the RepBase TE database (Kohany *et al.*, 2006) of Viridiplantae ver. 2016-08-29. Each individual sample was analysed independently and the relative abundances (i.e. TEs per 100 000 reads) of different TEs (Gipsy, Copia, LINEs and DNA transposons) were compared between sections by using Poisson-based generalized linear models (GLMs) in the software R.

RESULTS

The 100 bp Illumina RAD-seq for 40 DNA samples (1.7 million reads on average per sample) representing virtually all recognized CMF taxa (Fig. 1; Supplementary Data Text S1; Supplementary Data Table S3) produced in the final STACKS catalogue a total of 1 585 024 RAD loci with an average coverage per sample (± s.d.) of 81.3 ± 25.9 reads per locus. The demultiplexed data have been deposited in the NCBI Short Reads Archive (BioProject ID PRJNA563575). We found a high variability in the percentage of missing RAD loci among samples, ranging from 4.9 to 61.2 % (mean ± s.d. 12.84 ± 10.24 %; Supplementary Data Table S3) but this pattern was not biased towards particular taxa ($F_{19,20}$ = 1.593; $P = 0.155$). After retaining only polymorphic RAD loci that were present in at least 30 individuals and had a maximum of nine SNPs per locus, we obtained a final data set with 6090 RAD loci and a total of 23 931 SNPs. However, for further analyses, we retained only one SNP per RAD locus (6090 unlinked SNPs in total).

Genome-based phylogeny and species delimitation

The ML and BI resulted in robust and generally consistent topologies with two highly supported [bootstrap support (BS) = 1.0 and posterior probablity (PP) = 99] lineages within the CMFs (Fig. 2A). These main lineages corresponded to the

two previously recognized sections in the Mediterranean, sect. *Abies* and sect. *Piceaster*. In the *Piceaster* lineage, *A. numidica* (occurring in Algeria) is an early-diverged clade (BS = 1.0 and PP = 99), whereas *A. pinsapo* (Spain) and *A. tazaotana* + *A. marocana* (North-West Morocco) are sister clades. Individuals of *A. tazaotana* and *A. marocana* appeared intermixed. In the *Abies* section, two highly supported clades were shown. The first one included *A. alba* and *A. nebrodensis*. The second clade included species from Greece, *A. cephalonica*, and an inner clade of Turkish taxa, *A. cilicica*, *A. nordmanniana*, *A. bornmuelleriana* and *A. equi-trojani*. As expected in a bifurcating tree, the individuals of the hybrid taxon *A. borisii-regis* clustered with either parental species, *A. alba* and *A. cephalonica* (Supplementary Data Fig. S1).

For the Bayesian species delimitation analysis in SNAPP, the species hypothesis with the highest likelihood suggested that all previously recognized taxa (either at the species or at the subspecies level) in the Mediterranean should be treated as independent species, with the exception of *A. tazaotana* and *A. marocana* [maximum likelihood estimation (MLE) = –6500.7 for distinct, hypothesis A; MLE = –6440.5 for the two taxa grouped as one species, hypothesis B; Bayes factor value = 120.4 representing decisive evidence, Supplementary Data Table S4].

Origin and evolution of Mediterranean firs

The topology of the species tree constructed with SNAPP, based on the best hypothesis according to Bayesian factor delimitation of species (BFD*), was mostly congruent with the concatenated SNP trees (Fig. 3), but the main lineages evolved comparatively early. The *Piceaster* lineage (including *A. numidica*, *A. pinsapo* and *A. marocana–A. tazaotana*) showed the same internal relationships, whereas the Turkish lineages (including *A. nordmanniana*, *A. bornmuelleriana* and *A. equi-trojani*) diverged rapidly from one another. Additional support for the CMF relationships came from the species tree inferred using SVDQuartets, whose topology was highly supported (Supplementary Data Fig. S2). This species tree, again,

supported the *Abies* and the *Piceaster* sections as monophyletic, with 100 % BS. Whereas the SVDQuartets tree was largely congruent with inferences from concatenated data sets, the species tree differed in some relationships, especially for deep divergence events.

Dating the diversification of CMFs is key to understanding the putative contribution of climatic fluctuations and geological events around the Mediterranean Basin to the evolution of CMF. The estimated divergence times using BEAST and several fossil records are shown in Fig. 2. The chronogram was congruent with an early divergence of the lineages of sect. *Abies* and sect. *Piceaster* around the late Oligocene (95 % HPD: 23.1–34.1 Ma). Miocene divergence times for all current CMF lineages are clearly inferred, with speciation times pre-dating by tens of millions of years the Messinian salinity crisis (5.59–5.33 Ma; Krijgsman *et al.*, 1999) and establishment of the the Mediterranean climate (approx. 3.2 Ma; Suc, 1984). For the *Piceaster* lineage, the split of *A. numidica* and *A. pinsapo–A. marocana–A. tazaotana* was estimated at 13.3–27.1 Ma and at 9.4–21.3 Ma for that of *A. pinsapo* and *A. marocana–A. tazaotana*. For the lineage of sect. *Abies*, an Oligocene–Miocene split of *A. alba* and the eastern lineage was estimated (18.4–31.1 Ma). The eastern lineage diverged in the early Miocene, with 15.1–27.6 Ma for the split of *A. cilicica* and 12.2–24.0 Ma for that of *A. cephalonica*. The Turkish species were the last to diverge, around 8.4–18.5 Ma. Additionally, the speciation between *A. nebrodensis* and *A. alba* occurred around 9.1–21.8 Ma. These estimates were robust to additional calibration points derived from geological events, such as the opening of the Strait of Gibraltar and Tyrrhenian Sea after the Messinian salinity crisis (Supplementary Data Table S5). Nevertheless, this secondary dating analysis (including geological events) invariably showed shorter ranges for the HPD estimates. Additional dating analyses using other tree prior speciation processes (i.e. birth–death and Bayesian skyline; not shown) provided similar estimates.

We produced an LTT plot, which displays the number of speciation events through time, taking into account extinction events (Fig. 3). The CMF species have accumulated early and quickly in the history of the group. The gamma statistic was further employed to explore the acceleration of
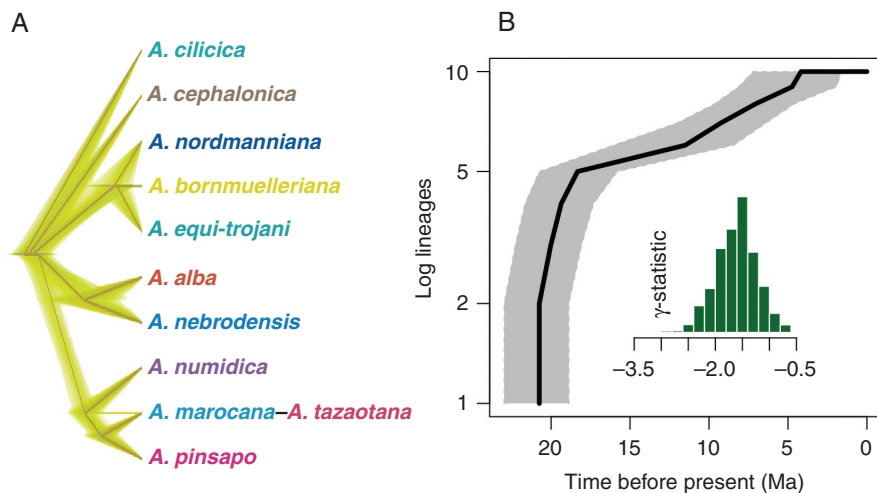

Fig. 3. SNAPP species cladogram for the best species delimitation hypothesis (A). Lineage through time plot and distribution of γ-statistics for the SNAPP trees (B).

speciation rate over the history of CMFs. The distribution of the gamma values on the 2276 SNAPP species trees rejects a constant speciation rate; γ (mean ± s.d.) = −1.61 ± 0.38; *P*-values < 0.05 (Fig. 3). The negative gamma values revealed an early and rapid accumulation of branching times near the root, indicative of a decelerated diversification. The shift in the number of speciation events occurs quickly in the group, with the highest number of speciation events estimated at the boundary between the Oligocene and the Miocene. The rapid divergence and expansion in the Tertiary and a relative speciation stasis in the Quaternary were congruent with the lack of differences in *Abies* fossil records along the latitude (*t*-test, *t* = −0.384, d.f. = 85.268, *P* = 0.702) or longitude (*t* = 1.299, d.f. = 90.376, *P* = 0.197) between these two epochs (Supplementary Data Fig. S3).

Genomic divergence between fir species and evidence for ancient hybridization during the speciation process

The rapid CMF radiation was congruent with low genomic divergence among species. More than half of the SNPs (51.8 %) represented shared polymorphism between at least two CMF species. Heterozygosity was similar between species, ranging from 0.04 to 0.08 with an average of 0.055 ± 0.002 ($\chi^2_{11,20}$ = 16.13, *P* = 0.134). However, the *Piceaster* section showed a significantly lower heterozygosity (0.049 ± 0.005) than *Abies* (0.058 ± 0.003; $\chi^2_{1,20}$ = 6.35, *P* = 0.01; Supplementary Data Fig. S4). The pairwise $\bar{F}_{ST}$ values between species ranged from 0.04 to 0.53 (Supplementary Data Table S6).

The Bayesian assignment of genetic structure (Fig. 4) showed, again, two main clusters (i.e. best partition *K* = 2
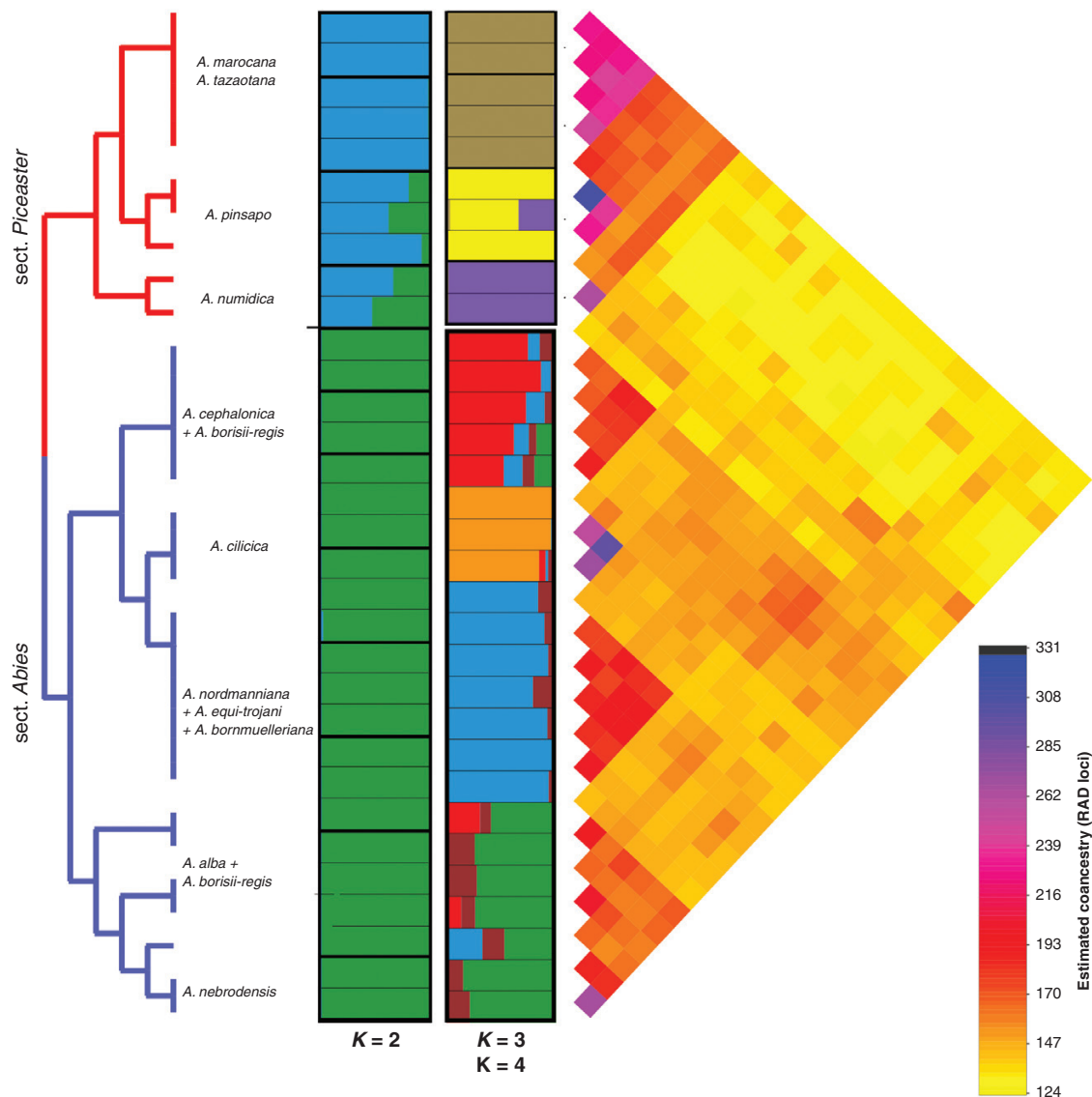

FIG. 4. Different types of evidence of recent gene flow in the CMFs. Hierarchical structure among and between CMF samples estimated by fineRADstructure. Results of the Bayesian assignment analysis (STRUCTURE) for CMFs (and independently for each section). Haplotype co-ancestry matrix based on 6090 SNPs with individual *A. equi-trojani_94_1* removed due to excessive missing data. The colour in the heat map corresponds to the number of haplotypes imported from a donor genome to a recipient genome. High levels of haplotype co-ancestry among distantly related species (e.g. *A. numidica* and *A. alba*) suggest secondary contacts.

following the Evanno method; *LK* = 50984.8, Δ*K* = 789.2; Supplementary data Fig. S5a) corresponding to the two taxonomic sections. Although most samples showed a high assignment probability to just one section, admixture signals appeared in several individuals (Fig. 4) of *A. numidica* and *A. pinsapo*, which showed intermediate probability of belonging to sect. *Abies*. Additional sub-optimal groupings were *K* = 5 and *K* = 7 (Supplementary Data Fig. S5a), which did not show clear taxonomical patterns. Within sections, the genetic structure generally mirrored the species tree (Fig. 4). For sect. *Piceaster*, *K* = 3 was the best partition (Supplementary Data Fig. S5b), corresponding to *A. numidica*, *A. pinsapo* and *A. marocana* + *A. tazaotana*. For sect. *Abies*, *K* = 5 partition was selected (Supplementary Data Fig. S5c). The first group corresponded to *A. cephalonica* individuals and the second one included *A. cilicica* individuals. *Abies alba* and *A. nebrodensis* together supported a third cluster, whereas the last group encompassed

A. nordmanniana, *A. equi-trojani* and *A. bornmuelleriana*. High haplotype co-ancestry levels among the accessions of *Abies* section *Piceaster* were estimated with RADpainter (Fig. 4). In addition, and consistently with recent hybridization between the species of both sections, we observed high levels of haplotype co-ancestry among distantly related species (e.g. *A. numidica* and *A. alba*) as estimated with RADpainter (Fig. 4).

Additionally, we tested if ancestral gene flow was also compatible with these admixture signals. ABBA–BABA tests (Patterson's *D*-statistic tests; Fig. 5; Supplementary Data Table S7) supported an ancient divergence with gene flow among species from the two CMF sections. Significant negative *D*-statistics support a notable ancient gene flow between *A. alba* and two species of sect. *Piceaster*, *A. numidica* and *A. pinsapo*, but also between *A. cilicica* and the sect. *Piceaster* species. In addition, higher absolute admixed values (*Z*-statistics) were found for comparisons including *A. nebrodensis* (as opposed to
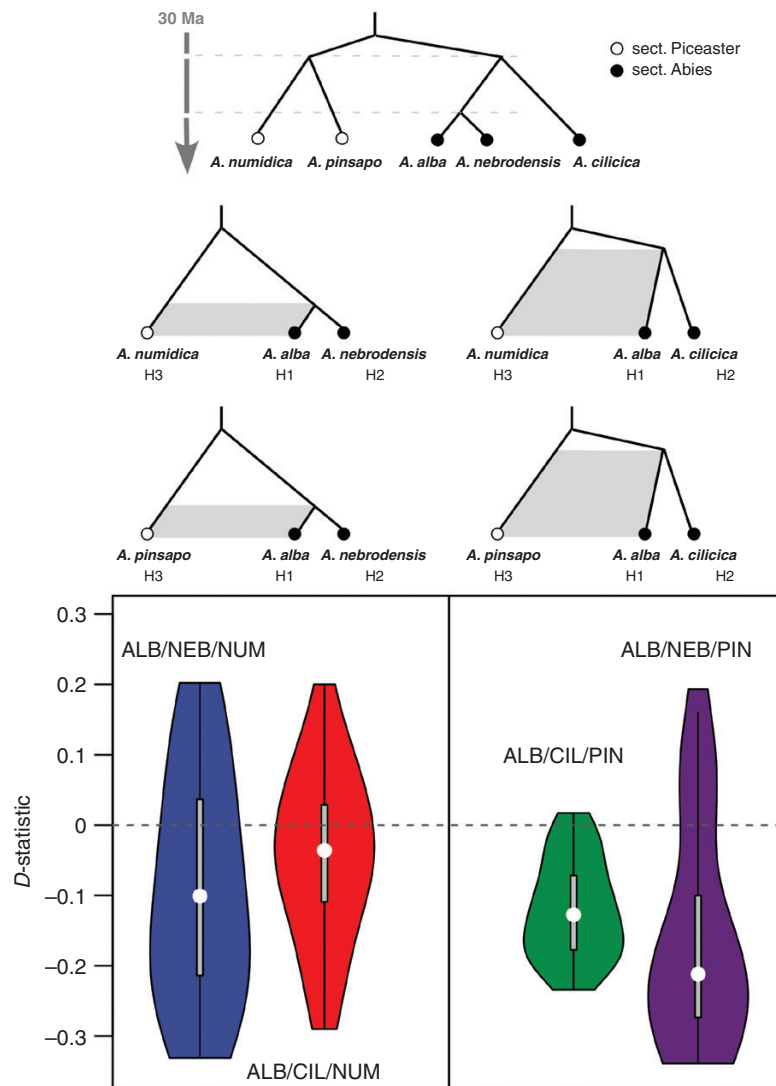

FIG. 5. Hypothetical schemes of introgression between species of *Abies* sect. *Abies* and *A.* sect. *Piceaster* at different time periods. Violin plots show the distribution of the *D*-statistics (ABBA–BABA tests) resulting from testing different individuals from each species. Detailed information about the significance of the *D*-statistics for each test is provided in Supplementary Data Table S7. Values different from zero denote introgression between species. ALB, *Abies alba*; CIL, *A. cilicica*, NEB, *A. nebrodensis*; NUM, *A. numidica*; and PIN, *A. pinsapo*. *Abies lasiocarpa* was used as outgroup.

A. cilicica comparisons), indicating additional, recent admixture events. Remarkably, *A. alba* showed higher admixture with *A. pinsapo* than with *A. numidica*.

Furthermore, a significant divergence between genomic landscapes of the two *Abies* sections was also mediated by differences in TE activity. We found significant differences between both *Abies* sections (Fig. 6) in the relative amount of DNA transposons ($\chi^2 = 27.5$, $P < 0.001$), Copia ($\chi^2 = 324.5$, $P < 0.001$), Gipsy ($\chi^2 = 447.5$, $P < 0.001$) and LINE ($\chi^2 = 3.3$, $P < 0.1$) within our RAD-seq data. Interestingly, the relative abundances of some TE classes also showed a strong correlation with the latitude of the samples (Supplementary Data Fig. S6).

DISCUSSION

To our knowledge, this research represents the first genome-wide evolutionary study in firs and the most intensive sampling including all CMF taxa. Although some limitation of RADseq for outlier scans has been suggested and debated (Tiffin and Ross-Ibarra, 2014; Lowry *et al.*, 2016; but see Catchen *et al.*, 2017), this study highlights the advantages of applying RAD-seq to obtain SNPs for phylogenetic studies in taxa with giant genomes, such as in the genus *Abies*. Furthermore, the combined use of phylogenetic methods and population genomic methods (such as STRUCTURE) have contributed to reveal the complex evolutionary history of Mediterranean firs, clarifying not just their phylogenetic relationships but also the recent and old secondary contacts.

Phylogeny of the Mediterranean *Abies*

Our dated phylogenomic tree corroborated the monophyly of all Mediterranean firs, as previously suggested (Suyama *et al.*, 2000; Semerikova and Semerikov, 2014, 2016; Xiang *et al.*, 2015). However, our molecular analyses, for the first time, clearly separated CMFs into the two previously classified sections, *Abies* and *Piceaster* (Farjon and Rushforth, 1989). Previous studies using classical markers have been

unable to clarify the phylogenetic relationships of these taxa. The monophyly of the *Piceaster* section, including the south-western species *A. numidica*, *A. marocana*, *A. tazaotana* and *A. pinsapo*, was additionally supported by other molecular markers (Ziegenhagen *et al.*, 2005; Sánchez-Robles *et al.*, 2014). However, the taxonomic status of the Moroccan species has been controversial; sometimes they were considered as mere varieties of *A. pinsapo* (Farjon and Rushforth, 1989), sometimes as subspecies (Govaerts, 1995) or distinct species (Maire, 1952). The species delimitation analysis suggested that the Moroccan *Abies* species must be classified as different species from *A. pinsapo*, but that they should not be split into independent species. This result is congruent with the genetic distinctiveness of *A. pinsapo* from A. *marocana–A. tazaotana* and the lack of genetic structure among populations of the latter by using amplified fragment length polymorphism (AFLP) and plastid markers (Terrab *et al.*, 2007; Sánchez-Robles *et al.*, 2014). Both Moroccan taxa were previously separated (Sánchez Cózar, 1946) on the basis of morphological characters (mainly leaves, cones and bracts) but, because of their close genetic similarity, and based on finer morphological studies (Sekiewicz *et al.*, 2013) as well, we propose to redefine their taxonomic status as a single species. Based on these result, we update the current taxonomy of CMFs (Supplementary Data Text S1).

In the *Abies* section, the results from the phylogenetic reconstructions and the species delimitation hypothesis test support the identity of *A. nebrodensis*, endemic to the Madonie range of northern Sicily (Italy), as a species derived from an ancestor of *A. alba*, the most widely distributed species inhabiting central Europe. This taxonomical status is supported by differences in traditional nuclear and plastid markers (Vicario *et al.*, 1995; Parducci and Szmidt, 1999; Parducci *et al.*, 2001a) and refute its classification as a subspecies or a variety of *A. alba*, or its alleged hybrid origin from *A. alba* and *A. numidica* (Landry, 1984; Parducci *et al.*, 2001b). A close relationship between these two latter species from different sections was previously suggested by Sánchez-Robles *et al.* (2014) based on AFLP markers, but our results refute this hypothesis. Furthermore, the relationships
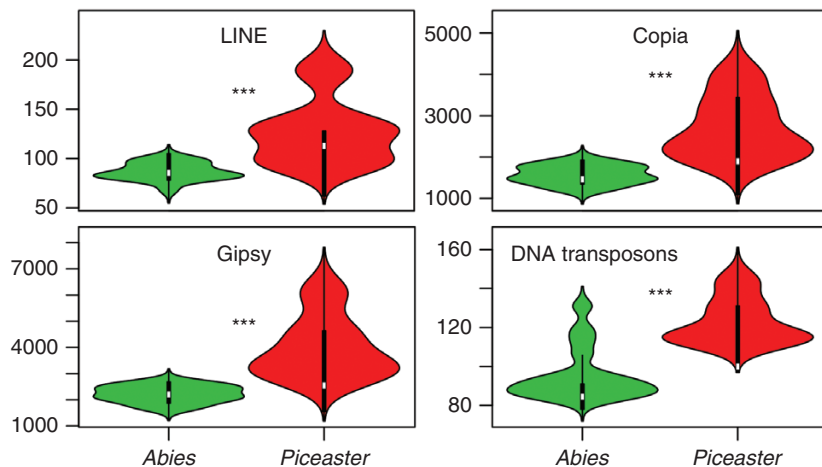

FIG. 6. Violin plots of the relative abundance of different transposable element families per individual of the two Mediterranean *Abies* sections. Asterisks denote statistically significant differences at $P < 0.001$ (Poisson generalized linear model).

between *A. alba*, *A. cilicica* and *A. cephalonica* have been resolved here for the first time. The two latter species (plus the *A. nordmanniana* complex clade) formed a deeply diverged sister lineage to *A. alba*. In this clade, an early divergence of *A. cilicica* first occurred, whereas the Greek *A. cephalonica* and the Eastern Turkish clade diverged later. Although *A. cilicica* was previously wrongly assigned to the *Piceaster* section (Franco, 1950; Liu, 1971), our data rather support a phylogenetic relationship with sect. *Abies*. In addition, the monophyly of the Greek *A. cephalonica* and the Eastern Turkish *Abies* species (i.e. the *A. nordmanniana* complex) was previously highlighted by their shared mitochondrial haplotype (Ziegenhagen *et al.*, 2005). Their genetic distinctiveness was further visible in our STRUCTURE results. The *A. nordmanniana* complex formed a single cluster, but the BFD* analysis suggested the existence of three different species: *A. nordmanniana sensu stricto*, *A. equi-trojani* and *A. bormulleriana*. A recent study (Hrivnák *et al.*, 2017) on the *A. nordmanniana* complex resulted in partially congruent phylogenetic relationships with our species delimitation hypothesis. However, the differentiation between the sister species *A. equi-trojani* and *A. bormulleriana* was unclear (Hrivnák *et al.*, 2017). Finally, the clarification of CMF taxonomy has predictable positive effects on conservation. New CMF classification could help authorities to focus conservation efforts (especially for the Moroccan and Turkish firs).

Origin and evolution of the Mediterranean firs

The information on the phylogenetic relationships uncovered in this study, combined with our knowledge of the geographical distribution of the species and the fossil record, can provide insights into the origin and evolution of Mediterranean firs. In short, the dated monophyletic origin of the Mediterranean firs and their close relationships with eastern Asian sections *Momi* and *Pseudopicea* (Xiang *et al.*, 2015) would suggest a single colonization event by a CMF ancestor from Asia in the late Eocene–Oligocene. This scenario is consistent with the estimated date for the Mediterranean crown group in previous studies on the genus *Abies* (Aguirre-Planter *et al.*, 2012; Xiang *et al.*, 2015). It is also noteworthy that the global expansion and diversification of firs have been dated during the Eocene as well, in agreement with dispersed fossils all over the Northern Hemisphere (Xiang *et al.*, 2007, 2015). The global climate cooling down through the Eocene–Oligocene (Zachos *et al.*, 2008) period could have favoured the geographical expansion of firs. In a relatively short time, as supported by the fossil records (Cavagnetto and Anadón, 1996), the CMF ancestor could have reached the westernmost region of the Mediterranean Basin (i.e. the Iberian Peninsula).

With regard to the speciation sequence in the Mediterranean firs, a previous hypothesis suggests a slow diversification along the Miocene–Pliocene, culminating with the rise of the modern CMF species after the Messinian climate crisis and the Pliocene marine transgression (Linares, 2011). Contrary to expectations, our conservative molecular dating and the speciation rate analyses suggest a much earlier diversification of the Mediterranean firs in the late Oligocene–Early Miocene, which is congruent with the radiation of the Mesoamerican firs (Aguirre-Planter *et*

al., 2012). The finding of rapid diversification in CMFs is particularly striking given that it concerns one of the most important components of temperate-cool forests in Eurasia and it has been widely studied. Our molecular dating method was carefully designed to incorporate the uncertainty associated with paleontological data and it was robust to different settings (including some biogeographical events). Understanding why CMFs have diversified during the Miocene requires a reconsideration of paradigms concerning patterns of diversification in the Mediterranean basin, as previously suggested by Vargas *et al.* (2018). Quaternary climatic cycles have been evoked as a main factor of diversification, but the new evidence suggests a secondary role providing secondary contacts and posterior isolation.

Our reconstruction of past evolutionary events suggests that during the late Oligocene–Early Miocene, the archaic Mediterranean *Abies* would have split into two groups, corresponding to the two sections *Piceaster* and *Abies*, perhaps in each extreme of the Mediterranean Basin. In the western region, the ancestor of *A. numidica*, *A. pinsapo* and *A. marocana* (i.e. sect. *Piceaster*) would remain genetically isolated, and in the central–east region would have differentiated the archaic *Abies* sect. *Abies* species. Additionally, the latter species would have quickly split into two lineages, one for proto-*A. alba* and another one for the ancestor of all eastern species.

Additionally, our LTT plot and gamma statistics analysis showed signals of a rapid speciation burst shortly after the CMF expansion in the early Miocene. These results pre-dated by tens of million years the previous hypothesis about CMF diversification. Interestingly, the divergence between *Ephedra* and disjunct *Cedrus* species in the Mediterranean has been estimated to the Miocene (Qiao *et al.*, 2007; Ickert-Bond *et al.*, 2009) as well. Provided CMFs, *Cedrus* and *Ephedra* species share a similar biogeographic history in the Mediterranean region, our study supports the idea that living Mediterranean gymnosperms are not relicts and they have originated by an early Neogene pulse of diversification (Davis and Schaefer, 2011) together with many Mediterranean angiosperms (Vargas *et al.*, 2018). The likely reason behind this pulse of speciation in the Mediterranean flora could be attributed to the global climate change during the Miocene. After the temperature and rainfall decreased during the Eocene–Oligocene, the temperature rose slightly through the Neogene until reaching in the Miocene a climatic optimum (15–17 Ma; Zachos *et al.*, 2001). The elevated temperature connected to a global CO_2 increase (Zachos *et al.*, 2008) could have aggravated the effects of increased aridity and caused extinctions and geographic isolation in the CMFs (Rundel *et al.*, 2016).

Furthermore, we found differential dynamics in TEs between the two sections, which is in contrast to the slow and steady rate of accumulation of TEs found otherwise in gymnosperms (Nystedt *et al.*, 2013). However, a recent study (Voronova *et al.*, 2017) suggests that some retrotransposon elements have been established, diverged and expanded after separation of the Pinaceae family. In spite of the genome similarity between fir species, differences in TEs could have profound effects on gene expression (Bennetzen and Wang, 2014) and possibly might contribute to adaptative radiation and morphological diversity in CMFs. The role of climatic stress in the activation of TEs has been widely assessed (Capy *et al.*, 2000) and may

additionally support the role of the climate in the speciation of CMFs (Kaplan and Guy, 2005; Krasensky and Jonak, 2012; George *et al.*, 2015; Sánchez-Salguero *et al.*, 2017).

Evidence of early divergence with gene flow and secondary contacts in the Mediterranean firs

Generally, our study revealed signs of admixture between CMF species of both sections but also within sections, causing potential conflicts between gene trees, but also when constructing a bifurcating species tree. In spite of the morphological differentiation between CMF species, a lack of reproductive isolation was evidenced by artificial inter-specific crossing experiments (Mergen *et al.*, 1962; Kormutak *et al.*, 2013). The multiple locations of *A. borisii-regis* in the multiple phylogenomic analysis and the low pairwise F_{ST} values support its recent hybridogenous origin due to multiple introgression events between *A. alba* and *A. cephalonica*. This pattern of genomic variation is consistent with a previously found genomic cline (Krajmerová *et al.*, 2016) and suggests that *A. borisii-regis* is an unstabilized hybridogenous taxon. Furthermore, the existence of recent gene flow between other CMF species was also apparent from multiple lines of evidence. The signals of admixture in the STRUCTURE analysis and the high haplotype coancestry are congruent with other previously suggested secondary contacts, most probably during the Pliocene–Pleistocene, between *A. alba*, *A. pinsapo* and *A. numidica* (Parducci *et al.*, 2001*a*; Sánchez-Robles *et al.*, 2014). Based on chloroplast simple sequence repeat (cpSSR) analysis, Sánchez-Robles *et al.* (2014) suggested ancient secondary contacts among these species as no recent admixture signals were found using AFLP markers. In our study, we uncovered the importance of this ancient gene flow among species from the two main lineages in the evolution of the CMFs. The process of fast speciation in CMFs involves alternate periods of partial isolation and secondary contacts among divergent species as a likely consequence of climatic oscillations.

Conclusions

In summary, our modern phylogenomic approach sheds light on the diversification of the Circum-Mediterranean firs. Our efforts to resolve the phylogenetic relationships among the different lineages have refined their taxonomy and our knowledge of their evolution. An unexpectedly early pulse of speciation in the Oligocene–Miocene boundary is here documented, predating by millions of years previous hypotheses, revealing a complex evolutionary history encompassing both ancient and more recent gene flow between distant lineages. Different transposable element dynamics could have contributed to the genome divergence of Circum-Mediterranean firs.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Text S1: taxonomic revision. Table S1: population details of the samples included in the study. Table S2: fir fossil calibrations and geological events used in this study. Table S3: SNP screening by using the STACKS software for all the studied individuals. Table S4: marginal likelihoods and Bayes factor values for the alternative species delimitation hypothesis. Table S5: mean ages and 95 % HPD heights for the major nodes of the CMF chronogram using two additional geographic events for calibration. Table S6: Circum-Mediterraneam *Abies* pairwise F_{ST} estimates from 6090 SNPs. Table S7: details of individual ABBA–BABA tests for ancient gene flow among *Abies* species from sections *Abies* and *Piceaster*. Figure S1: maximum likelihood phylogenetic tree of CMFs including the hybrid *A. borisii-regis*, based on 6090 SNPs. Figure S2: SVDQuartet species tree. Figure S3: geographic pattern of *Abies* fossil records along latitude and longitude in the Tertiary and the Quaternary periods. Figure S4: violin plots of heterozygosity per individual of the two Mediterranean *Abies* sections. Figure S5: summarized results from STRUCTURE. Figure S6: relationship between DNA transposons and latitude.

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