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ORIGINAL ARTICLE

Genetic insights into the hybrid origin of *Abies* × *borisii-regis* Mattf.

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Abstract *Abies* × *borisii-regis* Mattf. (King Boris fir) is a taxon endemic to the southern Balkan Peninsula, described as a hybrid between the widespread *A. alba* Mill. (Silver fir) and the Greek endemic *A. cephalonica* Loud (Greek fir). Even though *A.* × *borisii-regis* has attracted much research attention in the past, its origin, geographical distribution and taxonomic status are not fully elucidated and molecular evidence for hybridization is missing. To shed more light on this issue, we analyzed representative populations from these three *Abies* taxa using paternally inherited (chloroplast) and maternally inherited (mitochondrial) DNA markers. Both Silver and Greek fir could be clearly distinguished using mitochondrial markers, while we observed a mixture of maternal lineages in the *A.* × *borisii-regis* populations. In contrast, using chloroplast markers, we could not identify species-specific haplotypes, but a neighbor-joining analysis of population genetic distances revealed two separate clusters for the Silver fir and the Greek fir, while the *A.* × *borisii-regis* populations were placed in intermediate positions. Our results are in agreement with the hypothesis that the *A.* × *borisii-regis* populations investigated are a result of hybridization between *A. cephalonica* and *A. alba*.

Keywords *Abies cephalonica* · *A. alba* · *A.* × *borisii-regis* · Hybrid · cpDNA · mtDNA

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Introduction

The formation of new hybrid plant taxa is of great interest both from a taxonomic point of view (identification of a new taxon) as well as for their ability to occupy intermediate environments and for presenting unique characteristics and properties due to the combination of gene pools of different origin. To that end, hybrids may be environmentally and/or economically superior compared to the parental species.

In plants, hybridization is a common phenomenon and its importance in evolution has been highlighted since the 1960s (Stebbins 1969), and in many successive studies (e.g., Riesenberg and Wendel 1993; Arnold 1997). In conifers, natural hybridization is also common (e.g., Watano et al. 1996; Wang et al. 2001; Semerikova et al. 2011), and the occurrence of hybrids if often due to secondary contacts occurring between previously allopatric species.

The genus *Abies* Mill. (Pinaceae) comprises about 50 species, widely distributed in the northern hemisphere (Farjon and Rushforth 1989). Among them, ten species are

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distributed around the Mediterranean Sea and crossability studies, performed by Kormutak (1985, 2004) and Moulalis (1986), have shown that barriers among the Mediterranean firs are geographic and not genetic. In the same area, a hybrid, *Abies* × *borisii-regis* Mattf. (King Boris fir) has been reported (Mattfeld 1926). It is a taxon endemic to the southern Balkan Peninsula that has attracted much research attention by scientists, but its origin, geographical distribution and taxonomic status are not fully elucidated yet. *A.* × *borisii-regis* was first described by Mattfeld (1926, 1930) as a hybrid between, *A. alba* Mill. (Silver fir, a species widely distributed across Europe) and the Greek endemic fir, *A. cephalonica* Loud (Greek fir). Mattfeld (1930) suggested that this hybrid originated during the glaciations, due to the southwards migration of the former species in the southern Balkan Peninsula and the contact with the latter species, which had its distribution in this area and moved downwards from the mountains during the same period. Later, Turrill (1937) considered as equally possible that *A.* × *borisii-regis* represents an ancestral taxon from which *A. alba* differentiated toward the north and *A. cephalonica* toward the south. *A.* × *borisii-regis* has a restricted distribution in the southern Balkan Peninsula, yet its range is not well-defined in the literature. According to Flora Europaea (Chater 1964) and Flora Hellenica (Christensen 1997), it occurs in central and northern Greece and partially in the Former Yugoslav Republic of Macedonia (FYROM) and Bulgaria. In addition, in its southern distribution in Greece, individuals of *A.* × *borisii-regis* grow together with *A. cephalonica* individuals, while in its northern area of distribution they grow in mixed stands with *A. alba* individuals (Mattfeld 1930; Athanassiadis 1986; Mitsopoulos and Panetsos 1987). Three scattered *A.* × *borisii-regis* specimens, however, have also been reported in Peloponnese (southern Greece), well within the *A. cephalonica* distribution range (Strid and Tan 1997).

Regarding its taxonomic status, currently, in Flora Europaea (Chater 1964), Flora Hellenica (Christensen 1997) and Mountain Flora of Greece (Christensen 1986), *A.* × *borisii-regis* is treated as a hybrid between *A. alba* and *A. cephalonica* and this classification is based mainly on morphological characteristics distinctive of the two species. In particular, *A. cephalonica* is characterized by resinous buds, glabrous twigs, needles with acute or acuminate apex spreading outwards from all around the shoot or curving upwards from the underside of the shoot and grey bark with dark fissures (Christensen 1997). *A. alba,* on the other hand, has non-resinous buds, twigs with short brown pubescence, needles with emarginated apex arranged in two lateral ranks, the lower ones spreading horizontally, and bark with pale grey plates and pale brown fissures (Christensen 1997). Finally, individuals of *A.* × *borisii-regis* show either intermediate or a combination of *A. alba* and *A. cephalonica* characteristics, but they lack diagnostic characters that could facilitate the distinction from the parental species (Chater 1964; Christensen 1986, 1997). Several additional morphological and anatomical characteristics have been investigated by other authors (Fady et al. 1991; Kormutak 1985; Moulalis 1986; Mitsopoulos and Panetsos 1987; Panetsos 1975, 1990, 1992). Some of them have shown a clinal (north–south) variation over the entire *Abies* range in Greece, [e.g., hair density in the twigs, percentage of individuals with marginal resin canals, thickness of the needles, density of stomata in the upper surface of the needles and amount of resin on the buds (Mitsopoulos and Panetsos 1987)]. Based on the analyses of the above traits as well as on terpenes, Mitsopoulos and Panetsos (1987) concluded that the variation observed in Greek fir forests is of secondary origin, due to hybridisation between *A. alba* and *A. cephalonica*.

This unclear picture on the origin, geographical distribution and taxonomic status of *A.* × *borisii-regis* is partly due to the lack of distinctive morphological characteristics for this taxon, but also due to the high morphological variability found in various traits (in all three *Abies* taxa occurring in Greece), which makes the systematic identification of *Abies* individuals a difficult task for taxonomists. This high variability was already highlighted by Mattfeld (1930), who characterized *A.* × *borisii-regis* as "populus hybridogenus" [=hybrid swarm (du Rietz 1930)], a term used for highly polymorphic hybrids. The high variability together with the ability to inter-cross led also Stebbins (1950) to suggest that the three *Abies* taxa should be treated as races of a single polytypic species.

High variability, compared to other species in the Mediterranean, in *A.* × *borisii-regis* has been observed also with molecular markers [isozymes, e.g., Fady and Conkle (1993), Scaltsoyiannes et al. (1999), Drouzas (2000), RAPDs, Drouzas (2000) and chloroplast microsatellites (cpSSRs), e.g., Parducci et al. (2001)]. The majority of these studies support a hybrid origin of *A.* × *borisii-regis* through secondary contact between *A. alba* and *A. cephalonica*, while Scaltsoyiannes et al. (1999) suggested that *A.* × *borisii-regis* originated through hybridisation of *A. alba* with an ancient *Abies* progenitor that existed in the larger Aegaeis region (mainly occupied today by the Aegean Sea). New additional molecular studies aiming at elucidating the taxonomic status and the origin of *A.* × *borisii-regis* seem therefore necessary.

Molecular markers are useful tools to reveal the hybrid nature of plant taxa. Conifers offer the opportunity of easily identifying paternal and maternal contributions by using chloroplast (cpDNA) and mitochondrial (mtDNA) markers, which are inherited paternally and maternally, respectively (Wagner 1992; Liepelt et al. 2002). So far however, only *A. alba* has been extensively investigated

using cpDNA and mtDNA markers (e.g., Liepelt et al. 2002, 2009, 2010; Gömöry et al. 2004, 2012), while only a few populations of *A. cephalonica* and *A.* × *borisii-regis* have been investigated and those in studies mainly focusing on other *Abies* species (Parducci et al. 2001; Ziegenhagen et al. 2005; Liepelt et al. 2009, 2010). In the present work, we employed cpDNA and mtDNA markers on nine carefully selected *Abies* populations representative of *A. alba, A. cephalonica* and *A.* × *borisii-regis,* in an attempt to shed more light on the taxonomic status and on the origin of *A.* × *borisii-regis*.

Materials and methods

We sampled 251 individuals from nine *Abies* populations representative of *A. alba, A. cephalonica* and *A.* × *borisii-regis.* We selected the sampling areas based on the information provided in Flora Hellenica (Strid and Tan 1997) and Flora Europaea (Chater 1964). For *A. cephalonica* we sampled four populations: one on Cephalonia island (CEPHALONIA, western Greece), which is the locus classicus of the species, two in Peloponnese (MENALO and CHELMOS, southern Greece) and one on Mt. Parnassos (PARNASSOS, south-central Greece). Three populations of *A.* × *borisii-regis* were sampled in northern Greece: two of them (PIERIA, PINDOS) in areas where only *A.* × *borisii-regis* is currently reported in Flora Hellenica (Strid and Tan 1997) and one in an area within the above-described distribution of *A.* × *borisii-regis* (SMOL-TIMFI). We avoided sampling in areas where *A.* × *borisii-regis* occurs in mixed stands with *A. cephalonica* (e.g., in central Greece) or with *A. alba* (at the northern borders of Greece), as described in the introduction. Finally, because pure *A. alba* forests do not occur within Greece, as representative of this widely distributed species, we selected one population from Slovenia (*A. alba*-SLO) and one from Romania (*A. alba*-ROM). In a previous mtDNA-based study the latter two populations have shown to be representative of the two lineages occurring over the whole range of *A. alba* in Europe (Liepelt et al. 2002). The distribution of the three *Abies* taxa in Greece according to Flora Hellenica (Strid and Tan 1997), and the location of the seven populations sampled within Greece are shown in Fig. 1. The sample size of each population is presented in Table 1.

We extracted total genomic DNA from needles using a CTAB method (Doyle and Doyle 1990) with only minor modifications. DNA from the two *A. alba* populations was available from previous studies (Gömöry et al. 2004; Liepelt et al. 2002). We PCR amplified from genomic DNA using different chloroplast and mitochondrial DNA markers. For the cpDNA, we followed the same strategy used in

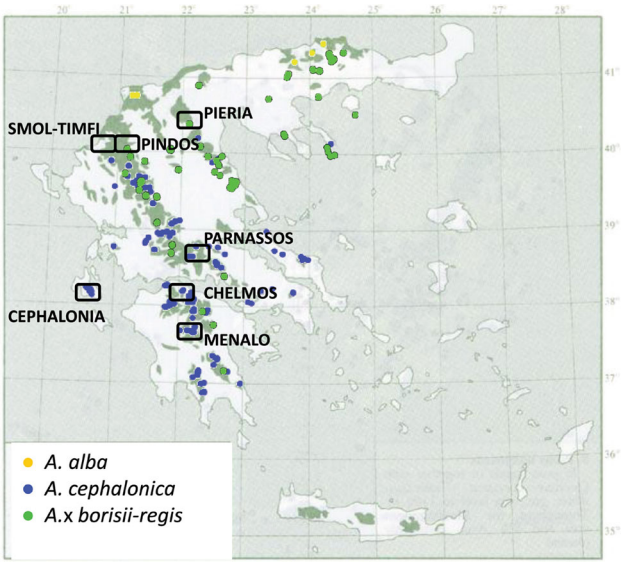

Fig. 1 Current distribution of *Abies alba, A.* × *borisii-regis* and *A. cephalonica* in Greece according to Flora Hellenica (maps taken from "Strid and Tan 1997", adapted and used with permission of Koeltz Scientific Books) and location of the populations studied (shown with *rectangular frames*). The *shaded areas* indicate the mountainous areas in Greece

Liepelt et al. (2010) by employing two slow evolving (PCR–RFLPs) and three fast evolving (SSR, microsatellite) regions. With the PCR–RFLP markers we analyzed the *mat*K (Wang et al. 1999) and *trn*V-*trn*H regions (Parducci and Szmidt 1999), following amplification conditions as in Drouzas (2000). PCR reactions were performed in a volume of 25 µl, containing 30 ng template DNA, 1× PCR reaction buffer, 3 mM $MgCl_2$, 0.4 µM of each primer, 0.15 mM of each dNTP, 1 unit of Taq DNA polymerase (Sigma-Aldrich). The PCR was carried out with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation (1 min at 93 °C), annealing (50 s at 58 °C), and elongation (2 min at 72 °C), and a final extension step of 10 min at 72 °C. The reactions took place on a Mastercycler-ep Gradient S apparatus (Eppendorf, USA). The PCR products were restricted using two fragment/enzyme combinations (*matK*/*Taq*I, *trnV-trnH*/*Hinf*I–*Xmn*I). Both combinations have been previously used to successfully discriminate *Pinus* taxa (Georgolopoulos and Drouzas 2011; Wang et al. 1999). The digestions were carried out in 20 µl final volume, containing at least 500 ng of PCR product, by following the manufacturer's instructions (Takara Bio Inc., Japan; New England Biolabs Inc., UK). The restriction products were separated and scored on a 4 % agarose gel (SeaKem LE, LONZA). The three cpSSR markers employed were: Pt15169 (Vendramin et al. 1996), AAssrCD (Liepelt et al. 2010) and Pt30141 (Liepelt et al. 2001). The amplification procedures followed were as in the original papers. Primers were fluorescence-labeled with

Table 1 The nine *Abies* populations studied with sample size (*N*), and the number of haplotypes detected with cpDNA and mtDNA markers

	Population	Taxon	*N*	Mitotypes	RFLP chlorotypes	SSR chlorotypes
1	MENALO	*A. cephalonica*	25	1	3	15
2	CHELMOS	*A. cephalonica*	30	1	3	20
3	CEPHALONIA	*A. cephalonica*	40	1	3	21
4	PARNASSOS	*A. cephalonica*	30	1	4	18
5	PINDOS	*A.* × *borisii-regis*	28	2	3	17
6	SMOL-TIMFI	*A.* × *borisii-regis*	30	3	3	19
7	PIERIA	*A.* × *borisii-regis*	30	2	4	18
8	Zgornja Velka (*A. alba*-SLO)	*A. alba*	21	1	1	8
9	Anina Caras and Soveja (*A. alba*-ROM)	*A. alba*	17	1	3	15
	Total		251	5	7	66

IRDye®800 (LI-COR® Biosciences, USA) and the products were scored on 6.5 % polyacrylamide gels on an automated sequencer LI-COR® 4200L DNA Analyzer Global Edition IR^2 System. Raw data were extracted by SAGA Application Server and scored using the SagaGT Client Software and the 50–350 Sizing Standard (LI-COR® Biosciences, USA). Occasionally, analyses were repeated at least two times to verify fragment size and increase the accuracy of the analysis. For the mtDNA, we amplified the *nad*5-4 region following procedures described in Liepelt et al. (2002). Size difference was scored via electrophoresis in 2 % agarose gels and samples carrying each variant detected were sequenced using an external DNA Sequencing Service (Macrogen, Korea).

Data analysis

Haplotypes (chlorotypes for cpDNA and mitotypes for mtDNA) were mapped using Arc GIS Explorer Desktop software (ESRI, USA). Chlorotypes were defined separately using PCR–RFLP (*matK/Taq*I, *trnV-trnH/Hinf*I-*Xmn*I) and SSR markers (Pt15169, AAssrCD, Pt30141), and using a combination of the two. We used the cpSSR and cpRFLP haplotypes separately to calculate haplotype frequencies and Nei's genetic distance among populations using GeneAlEx v. 6.41 (Peakall and Smouse 2006), population differentiation (Gst and Nst) using PERMUT v. 1.0 (Pons and Petit 1996) and, based on the haplotypes, pairwise F_{ST} distances between populations and their significance using Arlequin v. 3.5 (Excoffier and Lischer 2010). Analysis of molecular variance (AMOVA) was performed within and among populations and among groups (taxa), taking into account the differences among haplotypes, using Arlequin v. 3.5 (Excoffier and Lischer 2010). Based on the pairwise F_{st} distance matrix, we constructed a neighbor-joining (N-J) dendrogram (Saitou and Nei 1987) of all populations, employing the Neighbor module of PHYLIP v. 3.69 (Felsenstein 2005) and visualized it with TreeView (Page 1996). A principal coordinates analysis was made by GenAlEx v.6.41, also using the pairwise Fst distances as calculated by Arlequin v.3.5. For detecting major genetic discontinuities within the study area, we applied Monmonier's maximum difference algorithm using BARRIER 2.2 (Manni et al. 2004). Finally, we made an attempt to identify the paternal and maternal contribution from the two putative parental species (*A. cephalonica* and *A. alba*) and the presence of diagnostic maternal and paternal markers in the *A.* × *borisii-regis* populations.

A minimum spanning network was constructed for the mitotypes by re-coding the molecular variation. Due to the large size of the insertions/deletions they were treated as single mutations. The network was constructed using the software TCS v.1.21 (Clement et al. 2000) with a fixed 3-step connection limit.

Results

mtDNA

We found five different variants in the *nad*5-4 fragment, with the following fragment sizes (corresponding available GenBank accession numbers in parentheses): m1 339 bp

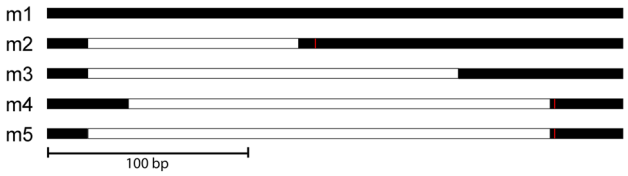

Fig. 2 Schematic view of the mitotype sequence alignment. *Solid bars* indicate identical sequences among samples. *Hollow bars* indicate gaps (insertions/deletions) and *red bars* indicate base substitutions [in m2 C/T, in m4 and m5 (A/T)]

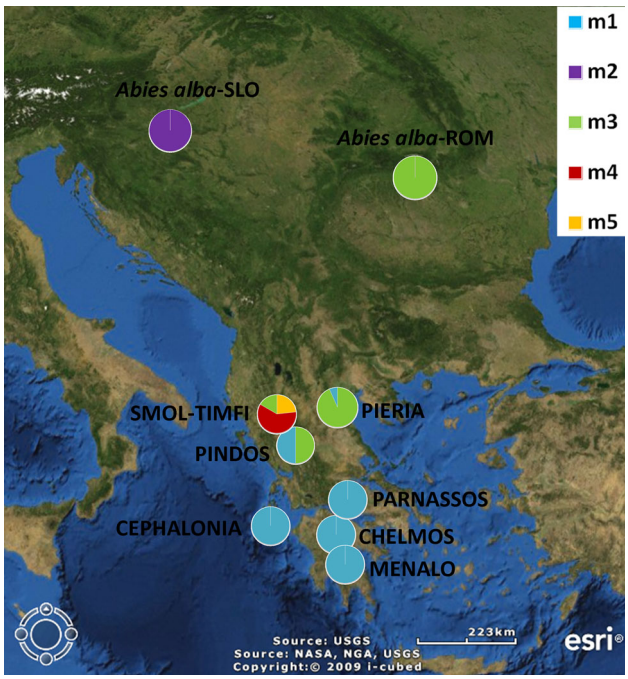

Fig. 3 Distribution of the nad5-4 mitotypes in the nine *Abies* populations studied

(DQ121446), m2 233 bp (AY147793), m3 153 bp (AY147794), m4 127 bp (GU195630) and m5 65 bp (Fig. 2). No mitotype was found in common to all populations or to the three *Abies* taxa (Fig. 3). Mitotype m1 was unique to all the *A. cephalonica* populations, while the two *A. alba* populations showed two distinctive mitotypes (m2 in *A. alba*-SLO and m3 in *A. alba*-ROM). Mitotypes m2 and m3 correspond to the two *A. alba* mitotypes previously identified by Liepelt et al. (2002) and Gömöry et al. (2004, 2012). Two of the *A.* × *borisii-regis* populations (PIERIA and PINDOS) showed the m1 from *A. cephalonica* and the m3 from *A. alba*-ROM, while the third *A.* × *borisii-regis* population (SMOL-TIMFI) showed the m3 and two additional mitotypes (m4 and m5). Mitotype m4 was previously detected in *A.* × *borisii-regis* on Pelister Mountain (FYROM) by Liepelt et al. (2010), while mitotype m5 was recorded here for the first time. The sequence of this

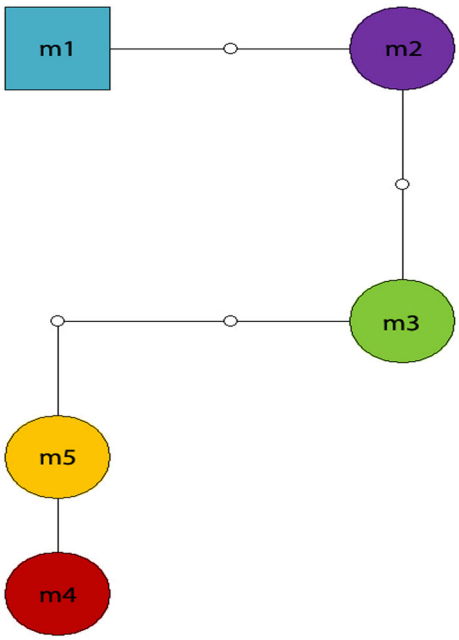

Fig. 4 Minimum spanning network of nad5-4 mitotypes performed with TCS v. 1.21 with a fixed 3-step connection limit. Insertions/deletions of multiple bases were treated as one mutation in a stepwise manner based on the sequence alignment resulting in six variable positions in the sequence alignment

mitotype has been submitted in European Nucleotide Archive (accession number: LK995400)

The mitotype network (Fig. 4) exhibited that the mitotypes observed only in the *A.* × *borisii-regis* populations (m4, m5) are closely related and most likely derived from m3, which represents the eastern maternal lineage of *A. alba*.

cpDNA

The two fragment/enzyme combinations *trnV-trnH*/*Hinf*I-*Xmn*I and *matK*/*Taq*I yielded six and two alleles, respectively, which, in combination, gave seven different chlorotypes (herein RFLP-chlorotypes, hr1–hr7 in Fig. 5, where the distribution of all RFLP-chlorotypes in the nine *Abies* populations studied is presented). In the population *A. alba*-SLO, we obtained amplification only in three individuals all of which exhibited the same RFLP-chlorotype (hr1). Because *trn*V-H amplifications were successful in all *Abies* individuals, and because all *A. alba*-SLO individuals gave successful amplification in other regions, our failure to amplify the *trn*V-H fragment in 18 *A. alba*-SLO individuals may be attributed to sequence variation in the primer-binding sites. RFLP-chlorotypes hr1, hr6 and hr7 were found in common to all populations and hr1 showed the highest frequency. Only one RFLP-chlorotype (hr2) was shared between two taxa: *A. cephalonica* and *A.* ×

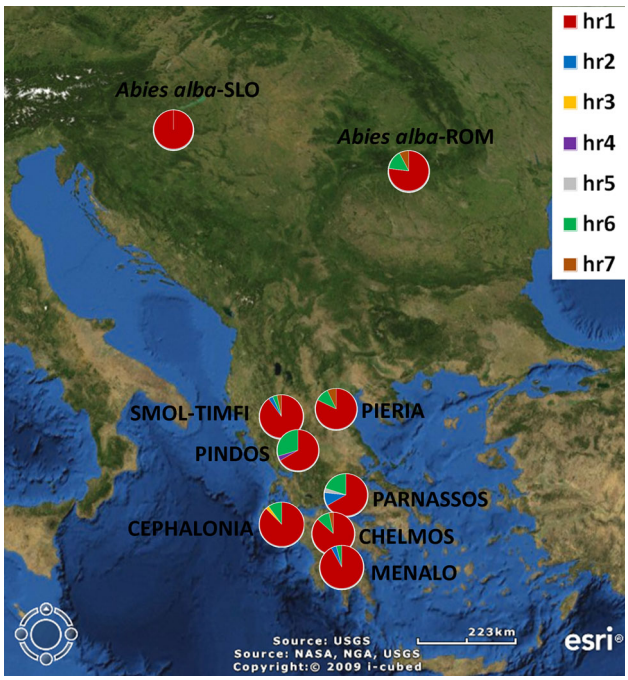

Fig. 5 Distribution of the PCR–RFLP chlorotypes in the nine *Abies* populations studied

borisii-regis. AMOVA analysis revealed that most of the molecular variation was distributed within populations (97.60 %), while no variation among taxa was found. The N-J dendrogram showed no meaningful grouping of the populations, since representative populations of each taxon were grouped together (results not shown). N_{st} value (=0.044) was higher than the G_{st} (=0.041) but the difference was not statistically significant, suggesting an absence of phylogeographic structure in the nine *Abies* populations.

The chloroplast microsatellite analysis at the loci Pt15169, AAssrCD and Pt30141 revealed four, five and 16 alleles, respectively, which yielded 66 different chlorotypes (herein SSR chlorotypes, hs1–hs66 in Fig. 6, where the distribution of all SSR chlorotypes in the nine *Abies* populations studied is presented). As many as eight SSR chlorotypes were shared among all three *Abies* taxa, while no SSR chlorotype was found in common to all populations. *A.* × *borisii-regis* shared four SSR chlorotypes with *A. alba,* and twelve with *A. cephalonica,* while only one was found in common between *A. cephalonica* and *A. alba.* The population from the island of Cephalonia (CEPHALONIA) exhibited the highest number of SSR chlorotypes (21), while *A. alba*-SLO showed the lowest (8). AMOVA analysis revealed that 90.06 % of the variation was within populations, while variation among populations was 3.69 % and among taxa 6.25 %. N_{st} (0.097) was significantly higher ($p = 0.01$) than G_{st} (0.032), suggesting a phylogeographic structure in the whole range. Results from the BARRIER analysis are presented in Fig. 6 and show two major genetic barriers separating *A. alba*-SLO and population PARNASSOS (*A. cephalonica*) from the rest of the populations. Additional minor barriers are separating *A. alba*-ROM and population PIERIA (*A.* × *borisii-regis*) from the rest. The differentiation of *A. alba*-SLO was also evident from the pairwise F_{st} values, shown in Table 2. We found high F_{st} values (>0.23) between *A. alba*-SLO and the rest of the populations (including *A. alba*-ROM), while *A. alba*-ROM was significantly different only from two *A. cephalonica* populations (CEPHALONIA and MENALO). Due to these results, we ran an additional AMOVA analysis without *A. alba*-SLO and found a higher level of variation within populations (98.53 %) and a lower level among populations and taxa (0.70 and 0.77 %, respectively). The N-J dendrogram based on SSR markers grouped all *A.* × *borisii-regis* populations and the population PARNASSOS (*A. cephalonica*) in an intermediate position between *A. cephalonica* and *A. alba* populations (Fig. 7). Similar results were obtained with the principal coordinates analysis (data not shown).

The combined PCR–RFLP/SSR analysis revealed a larger number of haplotypes (88 instead of 66) but it did not increase the resolution, as their distribution among populations and taxa was very similar to the one obtained with the cpSSR markers only (data not shown).

Distribution of genetic lineages in *A.* × *borisii-regis*

Of the five observed mitotypes, m1 and m3 were considered as diagnostic markers for assessing the status of the *A.* × *borisii-regis* populations since they did not occur in *A. alba* or, respectively, *A. cephalonica* (Fig. 3).

The large number of chlorotypes found compared to the number of individuals investigated did not allow us to infer paternal lineages or diagnostic chlorotypes. Out of the 87 *A.* × *borisii-regis* individuals analyzed, carried mitotype m1 representative of the *A. cephalonica* maternal lineage, while 25 exhibited m3 attributed to the maternal lineage of *A. alba.* The rest of the individuals showed m4 or m5, which, at the moment, cannot be assigned to either *A. cephalonica* or *A. alba,* and were only detected in *A.* × *borisii-regis*.

Discussion

Our aim with this study was to shed more light on the complex history and the taxonomic status of *A.* × *borisii-regis*. For this purpose, we used a combination of molecular markers from the mitochondrial and chloroplast genomes, which allowed us to study geographic patterns of maternal mitochondrial lineages and paternally inherited cpDNA variation among the three studied

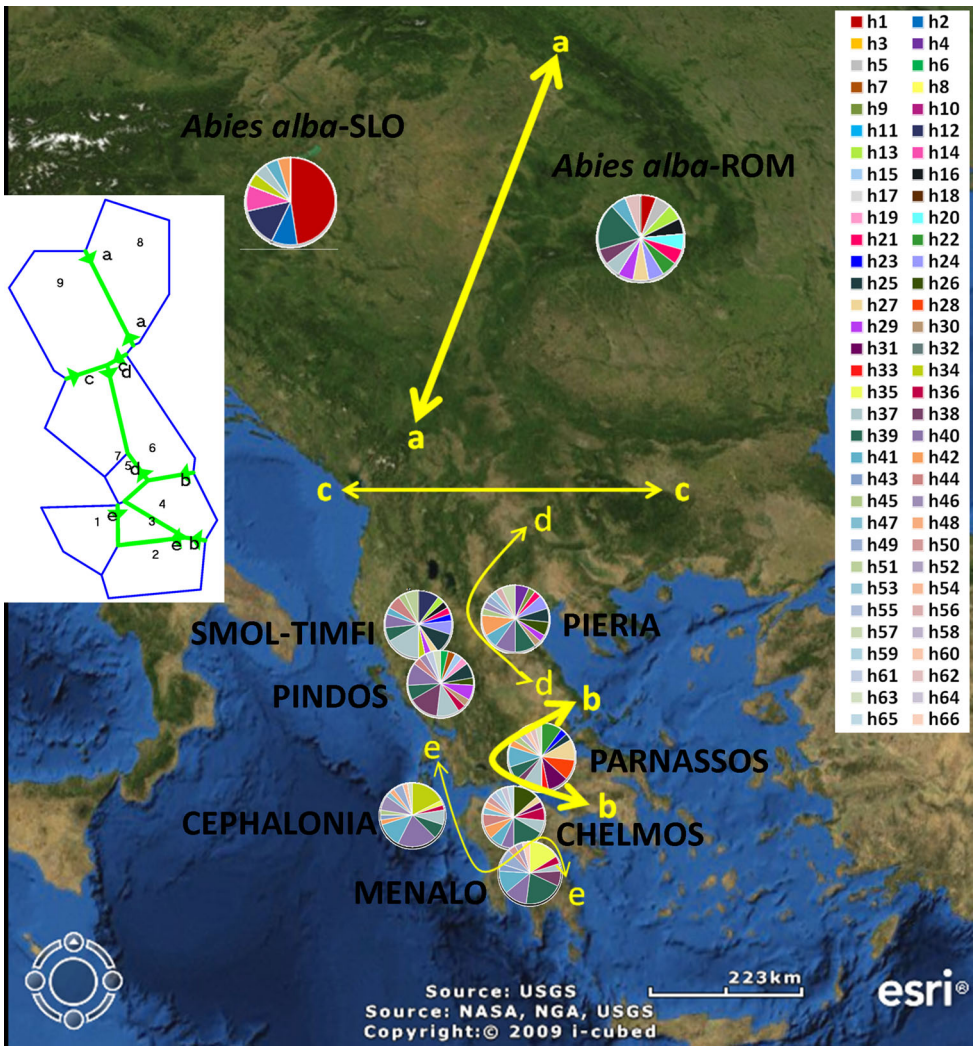

Fig. 6 Distribution of the SSR chlorotypes found in this study in the nine *Abies* populations and the genetic barriers as identified by the BARRIER software (*yellow lines*, the width of which corresponds to their importance)

Table 2 Population pairwise F_{st} values between the studied populations based on the SSR-chlorotype data

CEPHALONIA	MENALO	CHELMOS	PARNASSOS	PINDOS	PIERIA	SMOL-TIMFI	*A. alba*-SLO	*A. alba*-ROM	
0.0000									CEPHALONIA
−0.0114	0.0000								MENALO
0.0039	−0.0057	0.0000							CHELMOS
0.0465	0.0646	0.0159	0.0000						PARNASSOS
0.0082	0.0272	−0.0091	0.0131	0.0000					PINDOS
0.0265	0.0407	0.0017	0.0018	−0.0008	0.0000				PIERIA
0.0126	0.0381	0.0006	0.0064	−0.0228	−0.0091	0.0000			SMOL-TIMFI
0.3399	0.3673	0.3213	0.3357	0.3014	0.2977	0.2910	0.0000		*A. alba*-SLO
0.0463	0.0580	0.0114	−0.0032	−0.0086	−0.0082	−0.0126	0.2320	0.0000	*A. alba*-ROM

taxa. We carefully sampled representative populations of all three taxa, growing within the recognized ranges of *A. cephalonica, A.* × *borisii-regis* and *A. alba*, based on previous taxonomic identification (Chater 1964; Christensen 1997).

Distribution of mitotypes

Analysis of the mtDNA showed that *A. cephalonica* and *A. alba* are each characterized by different maternal lineages (mitotypes), whereas a mixture of mitotypes was

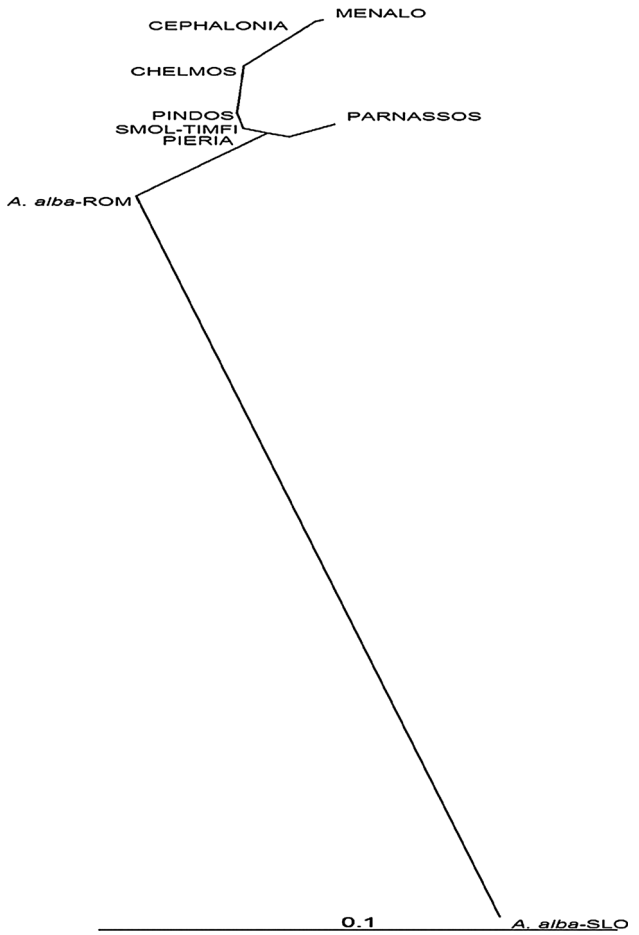

Fig. 7 N-J dendrogram of the nine *Abies* populations studied based on the SSR chlorotypes

present in the three *A.* × *borisii-regis* populations. In two of them we observed mitotypes of the two putative parent species *A. alba* and *A. cephalonica*, while in the third population (SMOL-TIMFI located in north-western Greece) we found a mitotype from *A. alba* and two new mitotypes (m4, m5). This is probably due to the biogeographic history of north-western Greece. The pollen fossil record identified a glacial refugium in an adjacent area (Ioannina plateau; Tzedakis 1993; Tzedakis et al. 2002). Thus, the two new mitotypes (m4, m5) might represent refugial lineages, which did not contribute to re-colonization after the last glaciation but persisted locally. This region was identified as a glacial refugium for another temperate tree species, *Fagus sylvatica* (Magri et al. 2006), where unique, locally restricted cpDNA haplotypes have been identified in a range-wide study conducted in Greece by Hatziskakis et al. (2009). The mitotype m4 has been previously found further north in a nearby *A.* × *borisii-regis* population from Pelister Mountain (FYROM) by Liepelt et al. (2010), while the mitotype m5 is recorded here for the first time.

Because mtDNA is transmitted by seeds (not able to move large distances), our finding of a mixture of different maternal lineages in the PIERIA and PINDOS suggests that *A. alba* and *A. cephalonica* were/are co-occurring in the studied regions. In addition, as Silver fir and Greek fir have been successfully crossed in artificial experiments (e.g., Kormutak 2004), the co-occurrence of *A. alba* and *A. cephalonica* in the same region implies that hybridization between the two species may have occurred. However, given the repetitive nature of the DNA sequence at the variable sites and its uniparental mode of inheritance, we cannot totally rule out the alternative hypothesis that the presence of the two mitotypes is due to ancient polymorphism.

Regarding the third *A.* × *borisii-regis* population (SMOL-TIMFI), the mitotypes m4 and m5 are probably derived from m3 according to the sequence alignment and the minimum spanning network. This and their locally restricted distribution suggest that they might constitute refugial lineages, which remained at the rear edge of *A. alba*. Thus, the hybrid origin of this population cannot be confirmed with the applied set of markers. If the above hypothesis concerning the origin of the mitotypes holds true, *A. alba* was therefore dominant in this area. Hybridisation, however, might have occurred via pollen.

The distribution of m3 is also indicative of the distribution of *A. alba*. Since this species-specific mitotype was found exclusively in the *A. alba*-ROM population [as well as in all the eastern European *A. alba* populations by Liepelt et al. (2002)], its presence at a high frequency in the three *A.* × *borisii-regis* populations, from northern Greece, suggests that Greece may have served as a refugium for *A. alba* during the glaciations. This is further supported by the occurrence of the locally restricted derived mitotypes m4 and m5. The presence of a refugium in northern Greece has been proposed in many studies for different tree species (e.g., Magri et al. 2006; Petit et al. 2002; Taberlet et al. 1998), including *A. alba* (Konnert and Bergmann 1995).

Distribution of chlorotypes

Our choice of using cpDNA markers with different rates of evolution (PCR–RFLP and SSR) allowed us to investigate both ancient and modern gene flow in the *Abies* populations. The more slowly evolving PCR–RFLP markers exhibited an overall low structure within our dataset. A similar picture was observed with PCR–RFLP markers in a previous study based on several European fir species by Liepelt et al. (2010) and was interpreted as the persistence of ancestral polymorphisms in the modern populations. We interpret these results in a similar way and conclude that these markers did not provide conclusive evidence on hybridization and gene flow occurring among *Abies* species.

A different picture emerges, however, from the more polymorphic and faster evolving cpSSR markers. Although far from being diagnostic, cpSSR variation was more informative than PCR–RFLP alone. The high differentiation detected between the two *A. alba* populations with both N-J and Barrier analyses, was not unexpected, as the *A. alba*-SLO population belongs to the western maternal lineage of *A. alba,* while *A. alba*-ROM belongs to the eastern lineage (Liepelt et al. 2002, 2010). Population PARNASSOS (*A. cephalonica*) was also clearly distinguished from the rest of the populations. The status of this population has been intensely discussed in the literature in the past due to the presence of non-typical morphological traits in *Abies* individuals on this mountain. This has led various plant taxonomists to identify different *Abies* taxa on Parnassos mountain (*A. cephalonica* f. *parnassica*, *A. apollinis*, *A. cephalonica* var. *apollinis*) (Christensen 1986, 1997). Similarly, Liu (1971) suggested that the fir population from Parnassos mountain together with the *A. equitrojani* (from Ida mountain in Asia Minor, Turkey) should be classified as *A. cephalonica* var. *graeca*, as opposed to the other Greek fir populations, which should instead be regarded as *A. cephalonica* var. *cephalonica*. High genetic polymorphism and unique cpPCR-RFLP alleles have been recorded on this mountain by Drouzas (2000). Based on the above, the divergence of PARNASSOS population may be the result of long-term isolation with no genetic bottlenecks.

Among the remaining populations we observed no strong geographic pattern of genetic differentiation with the paternally inherited cpDNA markers. This might be interpreted as evidence of high levels of gene flow among the species and would thus be supportive of a hybrid origin of *A.* × *borisii-regis*.

Combining evidence from cpDNA and mtDNA markers

The combined evidence from cpDNA and mtDNA markers provided useful information to assess the status of *A.* × *borisii-regis* in our study. Based on the distribution of the mitotypes and the chlorotypes in the *A.* × *borisii-regis* populations, two of them were shown to be hybridogenous (PIERIA and PINDOS) due to the contact between *A. cephalonica* and *A. alba*. Since in *A. cephalonica* populations there is no evidence of admixture by seeds (mtDNA), the zone of contact between the two species could be geographically located north of the *A. cephalonica* populations we analyzed.

Overall, our conclusions, based on results from both organelle genomes, are therefore in favor of a hybrid origin of *A.* × *borisii-regis*. Nevertheless, additional analyses including also the nuclear genome and more densely sampled populations are necessary to provide conclusive evidence on the hybrid origin of this taxon and to elucidate the ongoing processes occurring in this hybrid zone.

Implications for conservation

The three *Abies* taxa analyzed in this study share the same number of chromosomes, $n = 12$ (Mergen and Burley 1964) implying that the outcome of hybridization between *A. alba* and *A. cephalonica* did not involve ploidy changes in *A.* × *borisii-regis* (homoploid hybrid). A homoploid hybrid of the genus *Pinus*, *P. densata*, has shown a great potential to migrate and adapt to new environments and an ability to form stabilized populations (e.g., Wang et al. 2001, 2011; Gao et al. 2012). Here, we identified two highly variable populations of hybrid origin. We also identified an additional highly polymorphic population in a region close to a glacial refugium, i.e., the Ioannina plateau. In our opinion, all three populations deserve special attention for conservation purposes, since the genetic constitution of these polymorphic *Abies* populations may contain useful genetic variation necessary to cope with future environmental challenges in this area. The *A.* × *borisii-regis* individuals may enjoy several potential genetic benefits compared to the parental species, since individuals with a history of hybridization usually have greater possibilities of coping with changing environments (Wang et al. 2001). Therefore, the *A.* × *borisii-regis* populations may provide valuable genetic resources and should be studied for their suitability as reproductive material for reforestation and for breeding purposes.

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