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Natural hybridization in the genus *Abies*: II. Mitochondrial variation in the hybridogenous complex *Abies alba – A. borisii-regis – A. cephalonica*

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Abstract

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Twenty nine fir populations originating from the putative zones of interspecific hybridization in southern Balkans were studied using a mitochondrial *nad5-4* gene marker. The populations were classified to three taxa based on their geographical distribution and an overall taxonomical assessment at the population level based on needle and twig morphology: *Abies alba* Mill., *Abies cephalonica* Loud. and *Abies borisii regis* Mattf. Three haplotypes were found: 230 bp in Calabrian *A. alba* populations, 150 bp in Bulgarian and Macedonian *A. alba* populations, and 341 bp in *A. cephalonica* populations. Populations from central and northern Greece, classified as *A. borisii regis*, shared the 150 bp and 341 bp haplotypes with their closest neighbours, whereby haplotype frequencies changed clinally along the latitudinal gradient. This geographical distribution of mtDNA haplotypes supports the hypothesis that *A. borisii regis* represents a relatively recent hybrid swarm.

Keywords

Abies alba Mill., *Abies borisii regis* Mattf., *Abies cephalonica* Loud., hybrid swarm, hybridization zone

Introduction

Hybridization has long been considered a lapsus of nature. Interspecific hybrids have traditionally been supposed to be sterile or apomictic and restricted to specific sites. This prejudice was based on the experience with mammals, where interspecific hybrids are mostly sterile. In trees, some emblematic hybrids actually do occur in specific environments. For example, the occurrence of hybrid swarms of two widely distributed pine species *Pinus mugo* and *P. sylvestris* is restricted to peat bogs and similar sites (Kormuťák et al., 2008, Staszkiewicz and Tyszkiewicz, 1969). Nevertheless, with the advent of molecular methods it was demonstrated that hybridization is quite common in plants. Almost one-quarter of plant taxa has probably been involved in hybridization (Mallet, 2005).

In the former Czecho-Slovakia, there has been a long tradition in the study of interspecific hybridization within the genus *Abies* (Kantor and Chira, 1972; Kormuťák, 1985; Greguss, 1984; Janeček and Kobliha, 2007), which focused not only on obtaining artificial hybrids, but also on the processes of spontaneous hybridization. Taxonomy of the genus is an issue of controversies. The state-of-the-art taxonomy comprises 59 species organized in two subgenera and 14 sections (Farjon, 2010). However, many taxa recognized as separate species are considered hybrids or subspecies by different authors (see Farjon and Rushforth, 1989). There are several hybrid zones within the distribution range of the genus, e.g., *A. procera* × *A. lasiocarpa* in North America, *A. sibirica* × *A. nephrolepis* in East Asia or *A. cephalonica* × *A. nordmanniana* (= *A.* ×*bornmuelleriana*) in Turkey (Klaehn and

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Wiesnieski, 1962). From the European perspective, the most interesting hybrid zone is located in the southern Balkans, at the contact of the ranges of two basal species: *A. alba* Mill. and *A. cephalonica* Loud. The hybridogenous taxon was named *A. borisii regis* Mattf. (Mattfeld, 1925). The range of this taxon is fragmented, as it is restricted to high-elevation sites. In contrast to *A. alba*, which is a typical climax species, *A. borisii regis* is capable to colonize free sites close to the upper tree limit. On the lower distribution limit, it forms mixtures mainly with beech (*Fagus sylvatica*). According to the original Mattfeld's description, the taxon differs from both putative parental species mainly by vegetative-organ traits. This may be the reason for a high uncertainty in the delimitation of its range: according to, e.g., Liepelt et al. (2010) it covers the whole central and northern Greece, Macedonia and southern Bulgaria, on the other hand, only the populations in the north Pindos mts. and Thessalia were considered *A. borisii-regis* by Fady (1993).

The origin of *A. borisii-regis* is similarly unclear as its distribution. Mattfeld (1930) proposed two mutually exclusive hypotheses (with several subvariants): either *A. borisii-regis* represents an ancient taxon from which *A. alba* and *A. cephalonica* developed, or it is a product of hybridization between *A. alba* and *A. cephalonica*. Both versions clearly suggest that all three taxa represent a single evolutionary branch, either bifurcated (eventually multifurcated) or reticulated.

The knowledge of variation patterns in cytoplasmic genes with maternal inheritance could substantially contribute to the elucidation of gene exchange among parental and hybridogenous taxa. Such genes are transferred exclusively by seeds and thus allow making inferences about migration (Taberlet et al., 1998). In the Pinaceae family, mitochondrial DNA is maternally inherited (Mogensen, 1996). Liepelt et al. (2010) studied the variation at the mitochondrial *nad5-4* locus in Mediterranean firs, and found variation within *A. alba* as well as its differentiation from *A. cephalonica*. Their study also included one population of *A. borisii regis*, even located in southern Macedonia (FYROM), where occurrence of this taxon is disputable. In any case, such a small sample is insufficient to resolve phylogenetic relationships. Therefore, our study focused on the broader transition zone between *A. alba* and *A. cephalonica* in more detail based on substantially larger sample size. As phylogenetic proximity was found in other species between southern Balkans and southern Apennine peninsula (Gömöry et al., 1999, Musacchio et al., 2006), we also included Calabrian *A. alba* populations. The objectives were (i) describing geographical trends of mtDNA variation in the transition zone and (ii) making inference about the origin of *A. borisii regis*.

Materials and methods

We sampled indigenous silver fir populations from Bulgaria, Macedonia, Greece and Calabria (Italy) (Table 1). Taxonomical determination of the samples at the tree level was impossible, because the sampled trees often represented morphological transitions among morphotypes described by Mattfeld (1930) or Fady (1993). However, at the population level, the prevailing morphotypes were in accordance with a preliminary classification based on distribution ranges according to Fady (1993). Therefore, for operational purposes, the samples from the Peloponesos and the Kefalonia and Euboia islands were initially classified as *A. cephalonica*, Bulgarian and Macedonian (FYROM) populations as *A. alba*, and the populations from the central and northern Pindos and Thessalia as *A. borisii-regis*.

Twigs with 2nd-year needles were collected from approx. 30 trees per population and dried in plastic bags with silica gel until the analysis. Total genomic DNA was extracted from the needles using a modified CTAB protocol following Doyle and Doyle (1987). DNA concentration was measured spectrophotometrically.

The assessment of the mtDNA variation followed Liepelt et al. (2002) with slight modifications. The PCR mixture contained 1 × PCR buffer (Invitrogen, Frankfurt a.M., Germany), 1.75 mM $MgCl_2$, 0.2 µM forward and reverse primer (for primer sequences, see Liepelt et al., 2002), 0.2 µM each dNTP, 0.2 unit *Taq* DNA polymerase and 25 ng of template DNA. The cycle profile consisted of an initial denaturation at 94 °C for 3 minutes, followed by 30 cycles of denaturation at 93 °C for 1 min, annealing at 52.5 °C for 1 min, and extension at 72 °C for 1 min 20 sec, and a final extension step at 72 °C for 8 min. The amplified fragments were separated by electrophoresis in a 1.2% agarose gel for 2 hours at 4.5 V/cm (8 µl of each PCR product).

Haplotypic diversity was calculated according to Pons and Petit (1995) using the program HaploDiv (http://www.pierroton.inra.fr/genetics/labo/Software/Haplodiv/index.html). As the evolutionary history of mitochondrial haplotypes under study cannot be reconstructed from allele sizes, measures for unordered alleles were used. For the estimation of haplotypic diversity, unbiased estimates following Nei (1987) were used. The distribution of haplotypic diversity along the latitudinal gradient was modelled using the Gaussian curve (procedure NLIN, SAS 2009):

$$h = h_{\max} \frac{e^{-[l-c)/\sigma]^2}}{\sigma\sqrt{2\pi}},$$

where h_{max} is the maximum attainable diversity (height of the peak), l is the population latitude, c is the latitude

Table 1. Geographical coordinates and mitochondrial haplotype frequencies of the analyzed populations within the *A. alba*/*A. cephalonica* complex

Population	Country	Classification[1)]	Sample size	Longitude [°]	Latitude [°]	nad 5-4 haplotype frequency		
						150 bp	230 bp	341 bp
Serra San Bruno	IT	A	37	16.3506	38.5736		1.000	
Gariglione	IT	A	38	16.5919	39.0859		1.000	
Aspromonte	IT	A	39	15.8434	38.1683		1.000	
Rilski monastir	BG	A	30	23.3416	42.1323	1.000		
Bansko	BG	A	30	23.4592	41.8012	1.000		
Borovec	BG	A	30	23.6137	42.2631	1.000		
Slavjanka	BG	A	30	23.6406	41.4180	1.000		
Yundola	BG	A	30	23.8589	42.0620	1.000		
Ribaritsa	BG	A	30	24.3337	42.8211	1.000		
Trigrad	BG	A	30	24.3828	41.5995	1.000		
Devin	BG	A	30	24.3951	41.7416	1.000		
Paranesti	BG	A	30	24.4877	41.5093	1.000		
Pamporovo	BG	A	30	24.6945	41.6503	1.000		
Asenovgrad	BG	A	30	24.8511	41.9219	1.000		
Zhenda	BG	A	30	25.1556	41.7910	1.000		
Kirkovo	BG	A	30	25.3716	41.2861	1.000		
Mavrovo	MK	A	30	20.8074	41.7052	1.000		
Pelister	MK	A	30	21.1041	41.0632	1.000		
Olympos	GR	B	30	22.2733	40.1861	1.000		
Anilio	GR	B	30	21.1807	39.7534	1.000		
Pertouli	GR	B	30	21.4857	39.5555	1.000		
Tymfristos	GR	B	30	21.9099	38.9114	0.033		0.967
Rentina	GR	B	22	21.9743	39.0657	0.318		0.682
Komi Evoia	GR	C	11	24.2262	38.6565			1.000
Taygetos	GR	C	10	22.2000	37.1000			1.000
Tithorea	GR	C	8	22.6630	38.5715			1.000
Vytina	GR	C	30	22.1951	37.6500			1.000
Veria	GR	C	30	22.5566	37.1875			1.000
Kefalonia	GR	C	12	20.6238	38.1590			1.000

[1)]A, *A. alba*; B, *A. borisii-regis*; C, *A. cephalonica*; re-classification based on Fady (1993).

where haplotypic diversity attains maximum (center of the peak), and σ is the standard deviation (width of the peak).

Results and discussion

The marker is located within the fourth intron of the mitochondrial NAD dehydrogenase subunit 5 gene (*nad5-4*). As this is a non-coding region, there is a potential for large re-structurations without any effects for fitness. Length differences between alleles are enormous. Liepelt et al. (2002) found in a rangewide study of *A. alba* two variants of the amplified fragment, differing by an 80 bp insertion/deletion. In a later study covering Mediterranean fir species, Ziegenhagen et al. (2005) identified in *A. cephalonica, A. nordmanniana* and their transitional taxa another, very long allele (341 bp). Liepelt et al. (2010) further found another low-frequency allele in their *A. borisii regis* population.

Our results were partially in concordance with these findings (Table 1). In Calabrian populations, exclusively the haplotype characteristic for the western *A. alba* lineage (230 bp) was found. The Bulgarian and Macedonian populations of *A. alba* contained purely the haplotype with allele size of 150 bp (eastern *A. alba* lineage sensu Liepelt et al., 2002). In spite of technical problems associated probably with damaged samples and leading to reduced sample size, all trees originating from populations were initially classified as

A. cephalonica contained solely the 341 bp haplotype. This is in contrast with the findings of Ziegenhagen et al. (2005), who found in their *A. cephalonica* material a mixture of 150 bp and 341 bp alleles. Nevertheless, their material was not sampled in situ but taken from a provenance test, which may already be a source of technical errors. Moreover, they did not exactly specify the location of the analyzed provenances, but they seem to be identical with those used in the study of Liepelt et al. (2010). If so, geographical coordinates indicate that the *A. cephalonica* provenance must have been located somewhere close to Tymfristos, which means that it should be classified as *A. borisii regis* rather than *A. cephalonica*.

Our analyzed populations initially classified as *A. borisii regis* were heterogeneous. The northern ones contained only the eastern *A. alba* haplotype (150 bp), whereas two southernmost populations Tymfristos and Rentina were mixed and contained predominantly the *A. cephalonica* allele (341 bp), with a lower proportion of the 150 bp allele. This means that *A. borisii regis* shares the mitochondrial haplotype with the geographically most proximate populations of *A. alba* and *A. cephalonica*, respectively.

As mentioned in the Introduction, Mattfeld (1930) formulated two hypotheses about the origin of *A. borisii regis*: this taxon is either an ancient one, from which *A. alba* and *A. cephalonica* diverged, or a recent one, product of ancient or recent hybridization of both parental taxa, but anyway younger than they from the evolutionary point of view. In the former case, *A. borisii regis* is expected to be more diverse than the other two taxa and to contain all haplotypes present in the whole taxonomical complex. This proved to be not true, the allele 230 bp was not found in any of the Balkan populations. Of course, a more complicated scenarios are thinkable, where the western *A. alba* haplotype occurred through insertion after *A. alba* diverged from the ancestral population close to *A. borisii regis* or got lost from *A. borisii regis* through genetic drift. Such scenarios are, however, less likely. First, the locus under study is rather conservative, as shown by the studies of Ziegenhagen et al. (2005) and Liepelt et al. (2010). Second, mitochondrial variation within *A. borisii regis* could be geographically structured even if it was an ancestral taxon, but there would be no reason for a concordance of this geographical structure with that of daughter species. The divergence of *A. alba* and *A. cephalonica* must have been an ancient event; the separation of mitochondrial lineages within *A. alba* must have appeared at latest during the Eemian (Liepelt et al., 2009), which means that the separation of species must have occurred earlier. It is utterly improbable that a latitudinal cline in haplotype frequencies that we observed within *A. borisii regis* could have persisted over several glacial/interglacial cycles.

The within-population diversity component is close to zero when all populations are considered ($h_S = 0.018$; Table 2). As already mentioned, the intron under study is conservative, each variant was probably formed by a single mutation event. The range of the genus *Abies* in the Mediterranean area has very probably always been highly fragmented during the Pleistocene, so except the recent hybrid zones, opportunities for gene flow by seeds were extremely rare. Therefore, recent populations are predominantly formed of individuals belonging to a single genetic lineage only. In the absence of mixed populations, no wonder that differentiation is almost complete ($G_{ST} = 0.965$). Of course, our sampling does not completely and regularly cover the species' ranges (especially for *A. alba*), but previous studies (Liepelt et al., 2002; Gömöry et al., 2004) demonstrated that mixed populations are restricted to very narrow hybrid zones in the north-western Balkans and the Ukrainian Carpathians. This means that a more complete and more regular sampling would bring the same outcome.

Table 2. Haplotypic diversity and differentiation measures within the *A. alba*/*A. cephalonica* complex (mean ± S.D.)

Diversity component	All populations	*Abies borisii regis*
h_S	0.018 ± 0.016	0.103 ± 0.089
h_T	0.519 ± 0.076	0.443 ± 0.141
G_{ST}	0.965 ± 0.029	0.766 ± 0.073

Within *A. borisii regis* itself, differentiation is smaller, but most diversity resides among, not within populations (Table 2). Haplotype frequencies exhibit a latitudinal cline, nevertheless, an extremely narrow one. Consequently, the peak of the diversity distribution along the latitudinal gradient is narrow as well, peak width is only 0.113°, which represents 12.49 km (Fig. 1).

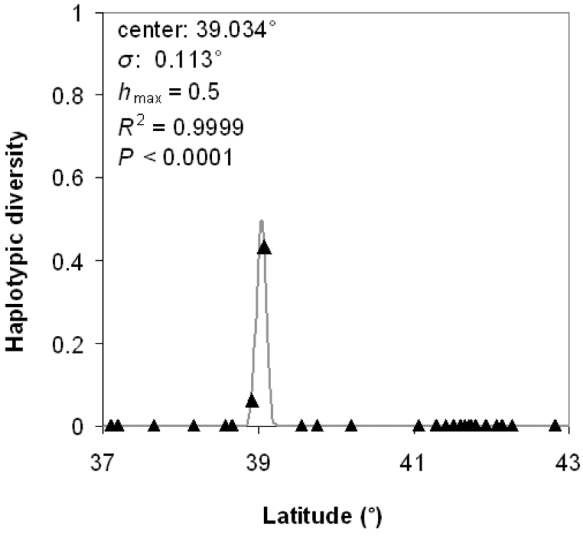

Fig. 1. Distribution of haplotypic diversity along the latitudinal gradient fitted to the Gaussian function.

All known facts support thus the latter Mattfeld's (1930) hypothesis, namely that *A. borisii regis* is a hybridogenous taxon, a product of a relatively recent bidirectional hybridization. Hybrids among phylogenetically close fir species are generally fertile (Klaehn and Winieski, 1962), so that *A. borisii regis* may represent a hybrid swarm. However, nuclear markers are necessary for the assessment of the extent of introgression. In any case, whatever concept of *A. borisii regis* distribution is adopted, the taxon contains both the eastern *A. alba* and the *A. cephalonica* haplotype, distributed along a geographical gradient connecting the ranges of both parental species. This pattern can most plausibly be explained by restricted seed migration from opposite sources.

As artificial hybrids between *A. alba* and *A. cephalonica* generally exhibit hybrid vigour in growth and belong to the most prospective ones (Greguss, 1984), the natural hybrid *A. borisii regis* may be interesting from the point of view of commercial forestry. Silver fir in central Europe suffers from periodically appearing syndrome of fir decline with unknown etiology (Larsen, 1986). *A. borisii regis* may become an option for the replacement of silver fir. Nevertheless, transplanting experiments are needed to verify whether this option is viable.

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