Differentiating Groups of *Abies* Species With a Simple Molecular Marker

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Abstract

The unambiguous identification of closely related species is useful for many practical purposes in forest tree species. For example, international laws require timber identification and the control of the origin of forest reproductive material. In this paper, we present a mitochondrial DNA marker which can be used to differentiate among groups of fir species (*Abies* spp.). Eight Mediterranean and one North American fir species (used as reference) were analysed at the fourth intron of the *NAD* subunit 5 gene. A total of six different haplotypes was identified, one in the American *Abies concolor*, the other five in Mediterranean species. Two different haplotypes were found each in the widespread *A. alba* and in *A. cephalonica*, one haplotype being shared among the two species. A single species specific haplotype was found in the near-eastern *A. cilicica*. The two southwestern species *A. pinsapo* and *A. numidica* shared one haplotype. The fifth haplotype was shared by all remaining eastern Mediterranean firs, *A. cephalonica*, *A. bornmuelleriana*, *A. equi-troiani*, and *A. nordmanniana*. Differences in haplotype sequences were mainly due to large insertions/deletions. Agarose gel electrophoresis thus provides a fast, cheap and reliable diagnosis method for species or species group identification.

Key words: Organelle DNA polymorphism, mitochondrial DNA, *nad5-4*, species differentiation, *Abies* (spec.).

Introduction

Forest tree species are traded worldwide in large quantities as seeds, seedlings, timber or wood products. International laws require that such products should be labelled: e.g. European Council Directive 1999/105/EC on forest reproductive materials or CITES (Convention on the International Trade in Endangered Species) requirements. However, closely related species are often difficult to distinguish morphologically. In forest tree species, this is true for whole plants of the same genus, but also particularly true for other materials such as pollen (recent and fossil), seeds, as well as for all tissues that are fragmented, degenerated or industrially treated (wood products).

There is thus a need to develop fast, low-cost and reliable tools for species identification. Organelle DNA rep-

resents such an opportunity. Genes and non-coding regions of organelle DNA are characterised by low evolutionary rates (review in HEWITT, 2001). Consequently, they have been used as markers for analysing phylogenetic relationships among species (PALMER, 1987) or for range-wide phylogeographic studies within species (review in PETIT and VENDRAMIN, 2005). Additionally organelle DNA is suited for successful analysis of difficult substrates because it is present in multiple copies per cell.

For many closely related plant species, the chloroplast *trnL* intron has served as a suitable marker for species differentiation (GIELLY and TABERLET, 1994). However, a sequence analysis of eight Mediterranean *Abies* species revealed that this region was not variable. Using the universal primer pair by TABERLET et al. (1991), the amplified sequences were 100% homologous (DQ126327–DQ126334). Neither could ISODA et al. (2000) resolve Mediterranean *Abies* species in the intergenic spacer region between the *trnL* and *trnF* genes. However, from inter-specific controlled crosses we had some evidence that variation at the fourth intron of the mitochondrial *NAD* dehydrogenase subunit 5 gene (*nad5-4*) could be species-specific (LIEPELT et al., 2002). Therefore, we chose this region as a candidate DNA region to systematically identify *Abies* species.

In this paper, we describe the haplotypic specificity of the mitochondrial *nad5-4* intron in eight Mediterranean and one North American *Abies* species. Our work was aimed at creating an easy-to-use tool for differentiating species in the genus *Abies*.

Material and Methods

Nine *Abies* species were analysed at the *nad5-4* intron. The *nad5-4* intron had previously been used for a phylogeographic study in *A. alba* (LIEPELT et al., 2002). All results from 100 natural populations with 1061 analysed individuals were included for comparison. From each of the remaining species, 17 to 46 individual trees were studied. The samples originated from a trial where the different species were compared in a common provenance test design. That means, except for *A. alba* they represent mixed samples from a whole region. In the case of *A. alba* samples were collected from natural populations of the entire range (Tab. 1). Genomic DNA was extracted from fresh or dried needles using a miniaturised CTAB protocol (DUMOLIN et al., 1995).

One individual of each species was sequenced and analysed at the entire *nad5-4* intron using the primer pair developed by WU et al. (1998). The PCR products were subjected to direct sequencing, using an automated sequencer (ABI Prism 310, Applied Biosystems, Foster City, CA). The DNA sequences were aligned with the

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Table 1. – List of analysed *Abies* species including the GenBank Accession Nos. of the *nad5-4* sequences.

	Species name	No. of sampled trees	GenBank Accession No. (<i>nad5-4</i>)
Med. ²⁾	<i>A. alba</i> ¹⁾	1061	AY147793, AY147794
Med.	<i>A. bornmuelleriana</i>	28	DQ121448
Med.	<i>A. cephalonica</i>	32	DQ121446, DQ121447
Med.	<i>A. cilicica</i>	30	DQ121452
N. Amer. ³⁾	<i>A. concolor</i>	30	AF489282
Med.	<i>A. nordmanniana</i>	46	DQ121449
Med.	<i>A. equi-trojani</i>	17	AF489283
Med.	<i>A. numidica</i>	25	DQ121450
Med.	<i>A. pinsapo</i>	26	DQ121451

¹⁾ Data from LIEPELT et al. (2002): 100 populations, 3–25 individuals per population.

²⁾ Med. = Mediterranean *Abies* species.

³⁾ N. Amer. = North American *Abies* species.

DNASTAR Software SeqMan II (Lasergene, Madison, WI, USA). Insertions/deletions occurred in the same region of the intron that had been diagnostic for two size variants in *A. alba* (LIEPELT et al., 2002). For further analysis we therefore only used the diagnostic primer developed by LIEPELT et al. (2002), with the forward primer 5'-GGACAATGACGATCCGAGATA-3', and the reverse primer 5'-CATCCCTCCCATTGCATTAT-3'. The total reaction volume of 25 µl for the PCR contained 20 ng of template DNA, 1x PCR reaction buffer, 1.6 mM MgCl₂, 0.2 µM of each primer, 0.1 mM of each dNTP and 0.5 U of *Taq* polymerase (Eurogentec, Cologne, Germany). The initial denaturation for 3 min at 94°C was followed by 30 cycles of denaturation (1 min at 92°C), annealing (1 min at 52.5°C) and extension (1 min 20 sec at 72°C), and a final extension step of 8 min at 72°C.

The resulting PCR products were 155 to 341 bp long. For proving within-species consistency of the fragment sizes found by the sequence analysis, we analysed all remaining individuals by high-resolution electrophoresis. A volume of 5–15 µl PCR product was subjected to a highly resolvable 8% polyacrylamide gel electrophoresis for 2.5 hours at 20 V/cm. The highly resolvable gel in combination with the relatively short fragment would have allowed us to detect small fragment length polymorphisms down to about five base pairs difference.

For visualising the results in a geographical context we used the GIS ArcView, v. 3.2 (ESRI, USA).

Results and Discussion

The development of DNA barcodes is gaining more and more importance in species identification e.g., for the application in bioassessments (HEBERT et al., 2003) and commonly starts with sequence analysis of the candidate DNA region. In our case, the *nad5-4* intron sequences of nine *Abies* species were compared (Tab. 1, Fig. 1). The sequences were homologous throughout long stretches of the intron, but exhibited variation in the region that previously had been diagnostic for two size variants in *A. alba* (LIEPELT et al., 2002). This region has

a maximum size of about 200 bp. It is characterised by size variation due to insertions/deletions in repetitive DNA and to a smaller extent by nucleotide substitutions. Fig. 1 gives a schematical alignment of this region. Six haplotypes were detected, five among the Mediterranean species (Figs. 1 and 2). The North American *A. concolor* exhibited another haplotype. Species-specific sequences were found in *A. alba* and *A. cilicica*. These two species differ in length by only two base pairs when comparing *A. cilicica* (haplotype 4) with the western *A. alba* haplotype (1). The two southwestern species *A. pinsapo* and *A. numidica* shared the same sequence which itself is unique. The same holds true for the whole southeastern *Abies* complex, *A. cephalonica*, *A. bornmuelleriana*, *A. equi-trojani*, and *A. nordmanniana*. They share the same sequence which again is clearly different from the other three sequences. The North American *A. concolor* is different from all other species. The Mediterranean *Abies* variants form spatial clusters of species that are geographic neighbors (Fig. 2).

Our results are in good agreement with the morphologically based classification of European fir species

Figure 1. – Species barcode of eight Mediterranean *Abies* species and the North American *A. concolor*. The schematical alignment of the diagnostic *nad5-4* DNA sequence reveals six haplotypes. Abbreviations: *A. ceph.* (= *Abies cephalonica*), *A. nordm.* (= *A. nordmanniana*), *A. bornm.* (= *Abies bornmuelleriana*), *A. equi troj.* (= *Abies equi-trojani*). Black bar = identical sequence, grey bar = missing sequence/deletion, arrow = base substitution.

Figure 2. – a) Geographic distribution of *nad5-4* intron size variation across eight Mediterranean *Abies* species. The haplotypes of Fig. 1 and Fig. 2 are labelled with the same numbers. Geographic distribution of *A. alba* was taken from LIEPELT et al. (2002). Abbreviations: *A. ceph.* (= *Abies cephalonica*), *A. nordm.* (= *A. nordmanniana*), *A. bornm.* (= *Abies bornmuelleriana*), *A. equi-troj.* (= *Abies equi-trojani*). The sizes of the circles do not correlate with sample sizes.

b) Natural distribution ranges of the analysed Mediterranean *Abies* species.

(FARJON and RUSHFORTH, 1989). Thereafter, the two western Mediterranean species *A. pinsapo* and *A. numidica* (section *Piceaster*) are differentiated from the eastern Mediterranean species (section *Abies*). *A. bornmuelleriana* and *A. equi trojani* were treated as sub-species to *A. nordmanniana* by FARJON and RUSHFORTH (1989) and as hybrid species by other authors (MATTFELD, 1926; FLOUS, 1936). In this study, these species shared the same haplotype with *A. nordmanniana* and *A. cephalonica*, suggesting close genetic relationships between all four species and not allowing further differentiation at least at the marker locus. The most widespread species *A. alba*, which is distributed across central Europe, exhibited two haplotypes, one being unique and the other shared with *A. cephalonica*. Since we dealt with only one region and large insertion/deletions in repetitive DNA we did not perform a phylogenetic analysis. The length differences obviously do not relate to the phylogenetic relationships among the species analysed. The intraspecific variants of *A. alba*, for example, differ more in length than some other European and the North American species.

In the parapatric species *A. alba* and *A. cephalonica*, a considerable amount of hybridisation seems to have taken place. The map reveals that about one quarter of the analysed trees that were morphologically described as *A. cephalonica* exhibited the eastern haplotype of

A. alba. These trees are maternal descendants of *A. alba* although they may have regained the phenotype of *A. cephalonica* by repeated backcrosses. Artificial crosses suggest weak reproductive barriers among the Mediterranean *Abies* species as opposed to strict barriers between Mediterranean and North American fir species (KORMUTAK et al., 2002; KORMUTAK, pers. communication).

The variation found in the mitochondrial *nad5-4* intron seems to be highly conserved and may be as old as the divergence of the Mediterranean *Abies* species. TSUMURA et al. (1998) studied the geographical distribution of mtDNA variation in five Japanese *Abies* species. In contrast to the mainly allopatric Mediterranean *Abies* species, mtDNA haplotypes were shared among most species. Accepting natural hybridization as a common mechanism (PETIT et al., 2003) this may be explained by the sympatry of the Japanese species. Due to the allopatric distribution of Mediterranean *Abies*, our marker with low evolutionary rates and transmission by seeds, is suited to differentiate species or species groups of interest. This requirement is not fulfilled by paternally inherited cpDNA variants as several variants occur within *Abies* species and many of them are shared among species (PARDUCCI and SZMIDT, 1999; KORMUTAK et al., 2004; LIEPELT et al., unpublished data).

Most mitochondrial haplotypes presented here can be easily identified using agarose gels. Discrimination between the western *A. alba* and the *A. cilicica* haplotypes, however, requires sequencing efforts.

This molecular marker holds several interesting practical features. As the fragment is short-sized and occurring in many copies per cell, it is suited for analyses of woody or other tissues containing degenerated DNA. Therefore, it could also be highly useful for paleobotany and archaeology as well as for forensic applications.

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Evolution of Genome Size in Conifers

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Abstract

Conifers are the most widely distributed group of gymnosperms in the world. They have large genome size (1C-value) compared with most animal and plant species. The genome size ranges from ~6,500 Mb to ~37,000 Mb in conifers. How and why conifers have evolved such large genomes is not understood. The conifer genome contains ~75% highly repetitive DNA. Most of the repetitive DNA is composed of non-coding DNA, including ubiquitous transposable elements. Conifers have relatively larger rDNA repeat units, larger gene families generated by gene duplications, larger nuclear volume, and perhaps larger genes, as compared to angiosperm plants. These genomic components may partially account for the large genome size, as well as variation in genome size, in conifers. One of the major mechanisms for genome size expansion and evolution of species is polyploidy, which is widespread in angiosperms, but it is rare in conifers. There are only a few natural polyploids in one family of conifers, Cupres-

saceae. Other conifers, including well-studied pines, are nearly all diploids. Whether ancient polyploidy has played a role in the evolution of genome size in conifers still remains an open question. The mechanisms that account for the variation and evolution of genome size in conifers are addressed in this review.

Key words: genome size, conifers, pines, polyploidy, paleopolyploidy, duplicate genes, repetitive DNA, retrotransposons, introns, genome evolution.

Introduction

Conifers, belonging to the order Coniferales, are the largest and the most diverse group of cone-bearing gymnosperms that are distributed widely, albeit unevenly, throughout the world (FARJON, 1998). While conifers mostly dominate the temperate zone forests in the northern hemisphere, they are rather scattered in the southern hemisphere. They are wind-pollinated, highly heterozygous, and long-lived trees with vegetative phases extending from one to several decades, and even centuries in the case of bristle cone pine (*Pinus longaeva*). Conifers are economically the most important plant group of wood and fiber crops. Fossil records suggest

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