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PCR-RFLP analysis of cpDNA in the genus *Abies*

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Abstract We used PCR-RFLP analysis of the chloroplast DNA of the genus *Abies* (family *Pinaceae*), to determine if the method could be employed to detect inter-specific variation in this genus and to study how the variation was distributed in different regions of the genome. Ten different chloroplast DNA regions, consisting of coding and non-coding DNA sequences, were amplified with specific primers in ten different *Abies* taxa. The amplification products were digested with several restriction enzymes. The results showed that the chloroplast genome is highly variable in most of the investigated taxa and contains multiple variable regions that appear to be distributed throughout the whole genome. Species-diagnostic markers were found for four of the ten investigated species. Unexpectedly, intra-specific variation was also detected in four species. It is likely that further studies, including larger sample sizes and/or more powerful methods for the detection of chloroplast DNA variation, will reveal additional variation for this genus.

Key words *Abies* · Chloroplast DNA · Intra-specific variation · Haplotype · PCR-RFLP analysis

Introduction

In recent years, restriction fragment length polymorphism (RFLP) analysis of chloroplast DNA (cpDNA) has proved to be a powerful tool for genetic studies in conifer species, particularly at the inter-specific level (Strauss and Doerksen 1990; Govindaraju et al. 1992; Moran et al. 1992; Wang and Szmidt 1994; Qian et al. 1995; Tsumura et al. 1995; Krupkin et al. 1996). Due to its uniparental inheritance, and to the fact that it does not undergo recombination, the inheritance of the chloroplast genome is linear over generations, changing only by occasional mutations. The slow rate of sequence and structural evolution of this genome (Palmer 1987; Wolfe et al. 1987; Birky 1988) provides opportunities for identifying species-diagnostic markers. However, in the past few years, PCR-amplification of particular cpDNA regions followed by subsequent restriction analyses (PCR-RFLP) has provided evidence for the existence of intra-specific cpDNA variation in some forest tree species, such as *Quercus robur* (Dumolin et al. 1995), *Fagus sylvatica* (Demesure et al. 1996) and *Abies alba* (Ziegenhagen et al. 1995). Furthermore, sequence analysis has revealed a number of polymorphic chloroplast microsatellites (SSRs) in several *Pinus* and *Abies* species (Powell et al. 1995; Cato and Richardson 1996; Vendramin and Ziegenhagen 1997). On the other hand, in some cases there was a complete failure to detect polymorphism in the cpDNA regions investigated (Boscherini et al. 1994; Vicario et al. 1995). Most of these earlier studies have focused on single, or only a few, regions of the chloroplast genome. It therefore remains unclear whether the detected variation is associated with only a few hypervariable hotspots or whether it occurs throughout the whole genome.

The genus *Abies* is complex by comparison with other genera of the family *Pinaceae*. According to Liu (1971) there are 39 species, 23 varieties and eight hybrids of *Abies* growing around the world. All these taxa are native to the cool temperate and boreal regions of the Northern Hemisphere and, similarly to other genera of the *Pinaceae*, they are widely distributed across both eastern and western parts of the world.

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In this paper, we focus on *Abies* species occurring in central and eastern Europe and around the Mediterranean basin, including the relic species *Abies nebrodensis* which perhaps represents the most prominent unsolved taxonomic issue among the taxa of this region. This species has often been treated as merely an insular variant of *A. alba*. Recently, Vicario et al. (1995) used allozymes as well as cpDNA and RAPD markers to assess the genetic relationships between *A. alba* and the relic population of *A. nebrodensis*. Results from allozyme and RAPD analysis showed a clear differentiation between these two species; however, the authors did not detect any differences at the cpDNA level.

Despite the biological and economical importance of the *Abies* species occurring in this region, current knowledge on their relationships is based mainly on morphological, anatomical and biochemical studies (Liu 1971; Mitsopoulos and Panetsos 1987; Farjon and Rushforth 1989; Fady et al. 1992; Fady and Conkle 1993). Little is known about the genetic variation among these *Abies* species at the DNA level (Vicario et al. 1995; Vendramin and Ziegenhagen 1997; Ziegenhagen and Fladung 1997).

The purpose of our study was to determine if the PCR-RFLP method could be conveniently used to detect inter-specific variation in the cpDNA of the genus *Abies* and to study how the variation was distributed in this genome.

Materials and methods

Plant material

Fresh needles from documented single adult trees were collected from the Hørsholm Arboretum, Denmark, and the Arboretum of the Institute of Plant Genetics, Nitra, Slovakia. The complete list of the investigated taxa, their geographical distribution and the sample size per taxon are given in Table 1. For comparison, one distant taxon from North America (*Abies concolor* var. *concolor*) was also included in the analysis.

DNA isolation, primer design and DNA amplification

Total DNA was extracted from approximately 50 g of fresh needles with the protocol described by Szmidt et al. (1986). Two types of DNA sample were used for each taxon: composite samples comprised of a mixture of DNAs from all the specimens, and single samples from one single specimen.

Ten pairs of primers specific for the chloroplast genome were employed to amplify the corresponding cpDNA regions from the single and composite DNA samples. The sequences of the primer pairs used and their letter designations are given in Table 2.

Primer pairs *trn*Q-*trn*G, *rpo*C1, *trn*C-*trn*D, *rpl*20-*trn*W, *trn*V-*trn*H, *psb*D-16S and *trn*L-*trn*V were designed based on the complete cpDNA sequence of *Pinus thunbergii* (Wakasugi et al. 1994). Primer pair *trn*F-*trn*T was described in Taberlet et al. (1991), while primer pairs *trn*K and *trn*S-*psb*C were described in Demesure et al. (1995). The exact positions and lengths of the designed primers were chosen using the Primer program, version 0.5 (Whitehead Institute, Cambridge, Mass., USA).

Figure 1 shows a schematic representation of the chloroplast genome of *P. thunbergii* with the approximate locations of the regions amplified by the ten pairs of primers used in this study. The locations of the ten primers were decided in order to amplify different and randomly chosen parts of the genome that included both coding- and non-coding regions.

Primer pair A was used to amplify the region between the two exons of the *trn*K (UUU) gene. Primer pair B was designed to amplify the region between the *trn*Q (UUG) and *trn*G (UCC) genes. This region contains four genes [*psb*K, *psb*I, *psa*M and *trn*S (GCU)], five open reading frames (orf45a, orf45b, orf48a, orf33 and orf59a), and several hundred base pairs (bp) of non-coding DNA. The region amplified with primer pair C includes nearly the whole sequence of the *rpo*C1 gene. This gene is split into two exons by a large intron.

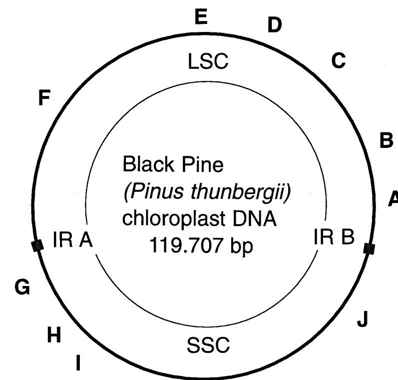

Fig. 1 Schematic representation of the chloroplast genome of *P. thunbergii* with the approximate locations of the ten regions amplified by the primers employed in this study (*LSC* – large, single-copy region, *SSC* – small, single-copy region, *IR* – inverted repeat. The letters designating the primers are explained in Table 2

Table 1 List of the investigated *Abies* taxa with their geographical distribution and sample size (N)

Taxon	Distribution	N
A. alba Mill.	Central and Eastern Europe	6 (26)[a]
A. bornmuelleriana Mattf.	North-western Turkey	4
A. cephalonica var. *cephalonica* Loud.	Greece	5
A. cilicica Carr.	Southern Turkey, Northern Syria	8
A. concolor var. *concolor* (Gord. et Glend.) Lindl.	Western USA, Northern Mexico	7
A. nebrodensis (Lojac) Mattei	Sicily	5 (16)[a]
A. nordmanniana (Stev.) Spach	Caucasus	6
A. numidica De Lann.	Northern Algeria	4
A. pinsapo var. *marocana* Boiss.	Morocco	1
A. pinsapo var. *pinsapo* Boiss.	Southern Spain	5 (15)[a]

[a] Sample size analysed with the primer pair *trn*S-*psb*C

Primer pair D was designed to amplify the region between the *trn*C (GCA) and the *trn*D (GUC) genes, which includes six open reading frames (orf 29, orf 88, orf 44c, orf58, orf73a and orf87), the *psb*M gene, and several hundred bp of non-coding DNA. Primer pair E was designed to amplify the region between the *rpl*20 and the *trn*W (CCA) genes. This region contains four genes [*rps*18, *rpl*33, *psa*J and *trn*P (UGG)], one open reading frame (orf69), and several hundred bp of non-coding DNA. The region amplified with primer pair F includes the region separating the *trn*V (UAC) and *trn*H (GUG) genes, involving six open reading frames (orf64a, orf56b, orf66, orf67a, orf133c and orf57c) as well as several hundred bp of non-coding DNA. Primer pair G was used to amplify the spacer separating the *trn*F (GAA) and *trn*T (UGU) genes. It includes a large non-coding region, the *trn*L (UAA) gene, and the open reading frame orf51a. Primer pair H was used to amplify the region between the *trn*S (UGA) and the *psb*C genes. These two genes are separated by the open reading frame orf49b. Primer pair I was designed to amplify the region between the *psb*D and the 16S rDNA genes. This region includes five open reading frames (orf42d, orf77, orf42e, orf82 and orf64b), the *trn*T (GGU) gene, and several hundred bp of non-coding DNA. Finally, the DNA sequence amplified by primer pair J includes the inter-genic spacer separating the *trn*L (UAG) and the *trn*V (GAC) genes. The region contains two genes [*trn*P (GGG) and *rpl*32] and three open reading frames (orf44d, orf40f and orf115).

The PCR reaction mix contained 20–30 ng of template DNA, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml of BSA, 0.15 μM of each the primers, 150 μM of each dNTP (Pharmacia) and 1 unit of *Taq* Polymerase (Pharmacia), in a total volume of 25 μl. PCR-amplification was performed at 94°C for 4 min for initial denaturation, followed by 35 cycles at 94°C for 1 min, 55°–60°C (depending on the primer pair) for 1 min, 72°C for 2 min and 50 s, and was terminated by 10 min at 72°C. To confirm successful amplification and to determine the size of the amplified fragments, 3.5 μl of the PCR products were separated by electrophoresis in an 0.8% agarose gel, in 0.5 × TBE buffer. The DNA fragments were visualised by UV fluorescence after staining with ethidium bromide.

In the first screening, we amplified DNA samples from all the ten *Abies* taxa listed in Table 1, with six pairs of primers (*trn*K, *trn*C-*trn*D, *trn*V-*trn*H, *trn*F-*trn*T *psb*D-16S and *trn*L-*trn*V).

In the second screening, we continued the analysis with an additional four pairs of primers (*trn*Q-*trn*G, *rpo*C1, *rpl*20-*trn*W and *trn*S-*psb*C). Based on the results from the first screening, we restricted the number of taxa to those occurring exclusively around the Mediterranean basin and closer to *A. nebrodensis*, namely A. *alba*, *A. cephalonica* var. *cephalonica*, *A. nebrodensis*, *A. numidica* and *A. pinsapo* var. *pinsapo*.

Furthermore, to allow more detailed analysis of the region between the *trn*S and the *psb*C genes that showed intra-specific polymorphism in *A. alba* (Ziegenhagen et al. 1995; Ziegenhagen and Fladung 1997), we increased the sample sizes of the taxa for which additional material was available (*A. alba*, *A. nebrodensis* and *Abies pinsapo* var. *pinsapo*) (see Table 1).

DNA digestion

After amplification, the PCR products were digested with several restriction enzymes among the following: *Hin*fI, *Msp*I, *Taq*I, *Alu*I, *Hae*III, *Rsa*I, *Hpa*II, *Mva*I, *Scr*FI, *Sau*3AI, *Cfo*I, *Bcl*I, *Bgl*II and *Dde*I. Two microliters of the total PCR product were digested in a total volume of 10 μl following the manufacturer's instructions (Boehringer, Mannheim). The digested cpDNA fragments were then separated on 6% non-denaturing polyacrylamide gels, in 1 × TBE buffer, in a vertical PROTEAN TM-II (BioRad) apparatus. The gels were run at a constant current of 25 mA, for about 4–5 h. The digested products were visualised by the silver-staining method (Bassam et al. 1991; Klinkicht and Tautz 1992). The size of individual fragments was estimated by comparison with a molecular-size standard (1-kb ladder, Gibco BRL Inc.) using the algorithm developed by Schaffer and Sederoff (1981).

When different restriction patterns were observed between the composite and the single samples from the same taxon, the analysis was repeated on all the specimens to confirm the presence of intra-specific variation.

Results

The ten pairs of primers used in the present study successfully amplified the corresponding cpDNA regions in all the *Abies* taxa investigated. The amplified products were all similar in size to the corresponding regions calculated from the cpDNA sequence of *P. thunbergii* (Table 2), ranging from 1500 bp for the

Table 2 DNA sequences of the primer pairs used in the present study (letter designations in parentheses), and size of the corresponding PCR products (in base pairs) based on the chloroplast DNA sequence of *P. thunbergii*

Primer pair	Sequence	Size
(A) cp-*trn*K	5′-GGG TTG CCC GGG ACT CGA AC-3′ 5′-CAA CGG TAG AGT ACT CGG CTT TTA-3′	2560
(B) cp-*trn*Q-*trn*G	5′-TCG AAC CTC CGA ATA ACA GG-3′ 5′-CGC ATC GTT AGC TTG GAA G-3′	3017
(C) cp-*rpo*C1	5′-TCG ATT GAA ACG AGT ACG ACC-3′ 5′-CAC TGG AGG GCC AAT ACC TA-3′	2854
(D) cp-*trn*C-*trn*D	5′-ACT GCA AAT CCT CCA TCC C-3′ 5′-CTC AAT TGG TTA GAG TAC CGC C-3′	2051
(E) cp-*rpl*20-*trn*W	5′-TTT TCG AAC TGC TAA CCA ACG-3′ 5′-ACC TAC GGC ATC AGG TTT TG-3′	2191
(F) cp-*trn*V-*trn*H	5′-GCT CAG CAA GGT AGA GCA CC-3′ 5′-CTT GGT CCA CTT GGC TAC GT-3′	2539
(G) cp-*trn*F-*trn*T	5′-ATT TGA ACT GGT GAC ACG AG-3′ 5′-CAT TAC AAA TGC GAT GCT CT-3′	1477
(H) cp-*trn*S-*psb*C	5′-GGT TCG AAT CCC TCT CTC TC-3′ 5′-GGT CGT GAC CAA GAA ACC AC-3′	1561
(I) cp-*psb*D-16S	5′-CCA CAA AAA CGA AAC GGT CT-3′ 5′-ACT AAC TAA TCA GAC GCG AGC C-3′	2084
(J) cp-*trn*L-*trn*V	5′-CTG CTT CCT AAG AGC AGC GT-3′ 5′-TTG ACA TGG TGG AAG TCA TCA-3′	2234

regions amplified with primer pairs *trn*F-*trn*T and *trn*S-*psb*C, to 3100 bp for the region amplified with primer pair *trn*Q-*trn*G. The total length of the amplified regions was 21.5 kbp, which accounts for 18% of the total length of the *Abies* chloroplast genome, assuming an average length of 120 kbp (Szmidt et al. 1986; Wakasugi et al. 1994). No visually detectable variation was observed among the undigested PCR products from the ten *Abies* taxa, following separation on agarose gels.

In total we analysed 63 fragment/enzyme combinations and detected 106 different cpDNA variants (hereafter referred to simply as variants) among the ten *Abies* taxa studied. The differences among the variants detected with the same fragment/enzyme combination ranged between 5 and 165 bp (the variant observed in *A. alba* was taken as a reference). Two exceptions were found in *A. concolor* var. *concolor* with the fragment/enzyme combinations *trn*C-*trn*D/*Taq*I and *trn*L-*trn*V/*Hin*fI (+875 bp and −375 bp respectively). Furthermore, we found no substantial differences in size between the original undigested fragments and the sums of the sizes of the digested fragments.

Detailed information on the variants detected in this study, and the taxa in which each single variant occurred, can be obtained upon request from the senior author or retrieved via *anonymous ftp* at the following address: *fisher.genfys.slu.se* (directory:/*pub*/*data*/).

Figure 2 shows the restriction fragment patterns of the two variants detected by the fragment/enzyme combination *trn*V-*trn*H/*Msp*I from the ten *Abies* taxa analysed (the 1600-bp fragment is not shown).

When we combined all the variants observed in each specimen over all the amplified regions, we identified ten different haplotypes in the first screening with six primers (h_1–h_{10}), and eight different haplotypes in the second screening with ten primers (h_{11}–h_{18}). The types of haplotypes found in the *Abies* taxa analysed in the two screenings are given in Tables 3 and 4.

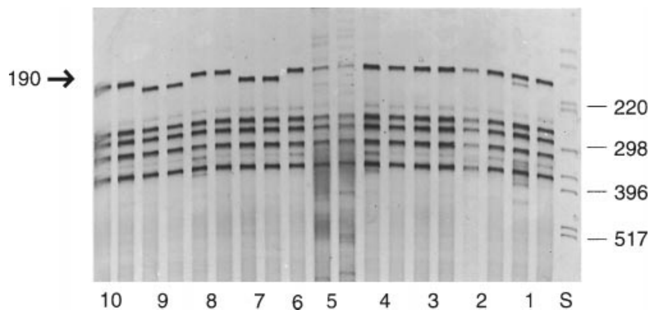

Fig. 2 Restriction fragment patterns of cpDNA from ten *Abies* taxa detected by the fragment/enzyme combination *trn*V-*trn*H/*Msp*I (size in base pairs; the 1600-bp fragment is not shown). Note the 190-bp fragment present in the single and composite samples of *A. numidica* and *A. nebrodensis* and in the composite sample of *A. alba*. *Lanes 1–10* are single and composite DNA samples of: *1 A. alba*, *2 A. bornmuelleriana*, *3 A. cephalonica* var. *cephalonica*, *4 A. cilicica*, *5 A. concolor* var. *concolor*, *6 A. pinsapo* var. *marocana* (single sample only), *7 A. nebrodensis*, *8 A. nordmanniana*, *9 A. numidica*, *10 A. pinsapo* var. *pinsapo*. *S* = 1-kb standard

The *trn*S-*psb*C region

The results from the analysis of the *trn*S-*psb*C region were concordant with those of Ziegenhagen et al. (1995) and Ziegenhagen and Fladung (1997). As expected, after digestion of the region with *Hae*III, we detected two variants in *A. alba* evenly present among the 26 individuals analysed. Surprisingly, despite the small sample size analysed for *Abies cephalonica* var. *cephalonica* and *Abies numidica* (five and four individuals respectively), both of the variants were found in these taxa. In contrast, in the larger samples of *A. nebrodensis* and *Abies pinsapo* var. *pinsapo* (16 and 15 individuals respectively), we detected only one of the two variants. However, the sizes of the fragments in the two variants (760, 690, 80 bp and 760, 500, 190, 80 bp) were slightly different from those reported by Ziegenhagen et al. (1995) (740, 740, 110 bp and 740, 555, 230, 110 bp).

Table 3 Chloroplast DNA haplotypes found in ten *Abies* taxa with six pair of primers

Taxon	Haplotypes									
	h_1	h_2	h_3	h_4	h_5	h_6	h_7	h_8	h_9	h_{10}
A. alba	X	X	X	X	X					
A. bornmulleriana	X									
A. cephalonica var. *cephalonica*				X	X					
A. cilicica	X									
A. concolor var. *concolor*									X	X
A. nebrodensis						X				
A. nordmanniana	X									
A. numidica						X				
A. pinsapo var. *marocana*								X		
A. pinsapo var. *pinsapo*							X			

Table 4 Chloroplast DNA haplotypes found in five *Abies* taxa with ten pair of primers

Taxon	Haplotypes							
	h_{11}	h_{12}	h_{13}	h_{14}	h_{15}	h_{16}	h_{17}	h_{18}
A. alba	X	X	X	X	X			
A. cephalonica var. *cephalonica*				X	X			
A. nebrodensis						X		
A. numidica						X	X	
A. pinsapo var. *pinsapo*								X

Discussion

The present PCR-RFLP analysis detected diagnostic haplotypes for four of the ten investigated species, namely *A. concolor* var. *concolor*, *A. numidica*, *A. pinsapo* var. *marocana* and *A. pinsapo* var. *pinsapo*, thereby supporting the utility of this method for detecting inter-specific cpDNA variation in the genus *Abies*. However, due to the intra-specific variation detected in some species, we could not carry out a phylogenetic analysis of our data. Typically, in phylogenetic studies, inter-specific comparisons of cpDNA variation have involved from one to few individuals per species analysed (Strauss and Doerksen 1990; Wang and Szmidt 1994; Tsumura et al. 1995; Krupkin et al. 1996; Ziegenhagen and Fladung 1997). The intra-specific cpDNA variation detected in our investigation suggests that, at least in this genus, phylogenetic studies would require an analysis of a much larger number of individuals.

Several previous studies employing large sample sizes failed to detect any intra-specific cpDNA polymorphism in different *Pinus* species (Boscherini et al 1994; Wang and Szmidt 1994). Thus, it is striking that, with ten pairs of primers, we found as many as five different haplotypes in a sample of only six *A. alba* individuals, and two different haplotypes in *A. cephalonica* var. *cephalonica*, in *A. concolor* var. *concolor* and in *A. numidica* (five, seven and four individuals respectively). This result suggests that cpDNA in the genus *Abies* may be more polymorphic than in other conifers. Unfortunately, due to the limited sample sizes, we could not quantify the level of variation observed in each species.

Most of the recent studies on conifer species harbouring simple sequence repeats (SSRs) have focused on only few polymorphic regions of the chloroplast genome (Powell et al. 1995; Cato and Richardson 1996; Vendramin and Ziegenhagen 1997). It is, therefore, unclear whether the polymorphism detected in those studies is associated with only a few isolated hotspots or rather occurs in various regions of the genome. To provide information on this subject we analysed several different and randomly chosen regions of the genome. We were able to detect polymorphism in virtually each investigated region. This result suggests that in *Abies* the cpDNA contains multiple variable regions which appear to be distributed throughout the whole genome. In the absence of sequence information about the regions analysed in the present study, we cannot determine whether they represent microsatellite regions similar to those recently described in *Abies* by Vendramin and Ziegenhagen (1997).

Most of the cpDNA variation observed in our study was due to small length-differences among the variants, ranging from 5 to 165 bp, while only two variants, detected by the fragment/enzyme combination *trn*S-*psb*C/*Hae*III, could be attributed to a point mutation. This result is concordant with other studies, which also suggest that most observed cpDNA variants are due to length mutations (Hipkins et al. 1994).

The pattern of cpDNA variation detected in our study suggests a number of preliminary hypotheses about the genetic relationships among the ten *Abies* taxa analysed. *A. alba*, *Abies bornmuelleriana*, *Abies nordmanniana*, *A. cephalonica* var. *cephalonica* and *Abies cilicica*, which grow in relatively adjacent regions, harboured the same group of haplotypes. On the other hand, taxa growing in scattered and isolated areas, in Sicily (*A. nebrodensis*), in North Africa (*A. numidica* and *A. pinsapo* var. *marocana*), on the Iberian Peninsula (*A. pinsapo* var. *pinsapo*) and in North America (*A. concolor* var. *concolor*), were characterised by a different group of haplotypes. It therefore appears that the two groups of taxa are genetically differentiated. Furthermore, our present results reveal that *A. alba* and *A. nebrodensis* differ at the cpDNA level since in both screenings they harboured different haplotypes. The results also show there is a close relationship between *A. nebrodensis* and *A. numidica* and suggest that the two species may have been in contact in the past since, although geographically isolated, they share the same haplotype in both screenings. Further studies employing cladistic methods and larger samples per taxon are required to verify our present hypotheses.

The PCR-RFLP method is convenient for surveying many samples and for investigating many cpDNA regions simultaneously. Compared with the conventional restriction fragment analysis that employs DNA hybridisation, PCR-RFLP analysis on polyacrylamide

gels is more sensitive for the detection of small-length differences (5–200 bp) among the digested fragments. Moreover, small length-differences are easier to detect than point mutations, since they do not require the modification of a particular restriction site in order to become apparent. Indeed, in the present study most of the cpDNA variation detected among the variants was due to small length-differences rather than to point mutations.

However, in a PCR-RFLP analysis, artificial variation may also be created by the presence of DNA heteroduplexes. In the presence of these heteroduplexes the sum of the sizes of the digested fragments will substantially exceed the size of the original undigested fragment (Wang et al. 1996). However, our results show that with only the two exceptions found in *A. concolor* var. *concolor* (+875 bp and −375 bp), the differences involved were far too small to be attributable to the presence of heteroduplexes. Furthermore, the exceptions mentioned above seem to be characteristic of the taxon itself and we believe that, due to the large sizes of the undigested amplified products, this difference could not be detected after separation on an agarose gel.

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