RESTRICTION SITE POLYMORPHISM IN *psb*C GENE OF SOME *ABIES* AND *PINUS* SPECIES [1]

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ABSTRACT

Genetic structure of silver fir (*Abies alba* Mill.) populations in Slovakia was analyzed using a PCR-RFLP approach. Based on the original finding of ZIEGENHAGEN and FLADUNG (1997), the variation in the cpDNA region consisting of coding and intergenic spacer sequences of the *trn*S and *psb*C genes was tested in 285 individuals representing 8 populations of the species. The two *Hae* III haplotypes have accordingly been recognized. The first haplotype was characterized by 3 restriction fragments as compared with the 4 restriction fragment-pattern of the second haplotype. The proportion of the two haplotypes varied considerably in individual populations but the prevalence of the first haplotype was observes in the East Slovakian populations Zboj and Palota. The opposite figure was characteristic for the populations in the middle and northern parts of Slovakia with a prevalence of individuals exhibiting second haplotype.

Keywords: *Abies, Pinus*, cpDNA, polymorphism

INTRODUCTION

Of the chloroplast genes commonly used in phylogenetic studies of gymnosperms the *psb*C gene was reported to be of increased evolutionary relevance. According to ZIEGENHAGEN and FLADUNG (1997) its *Hae* III restriction profiles exhibit not only interspecific but also intraspecific variation, the latter being characteristic for *Abies* species only. The 5 restriction patterns recognized among 5 different species of the gymnosperms were characterized by 1-3 restriction fragments of variable size. Individual variants of restriction patterns were proved to be due to insertion/deletion within the flanking regions of the *trn*S gene [tRNA-Ser (UGA)] and adjacent *psb*C gene (*ps* II 44 kDa protein) (ZIEGENHAGEN *et al.* 1995). Of the 7 *Abies* species studied, the species *A. nordmanniana*, *A. cephalonica*, *A. grandis* and *A. numidica* shared a one-fragment pattern, the pair of species *A. homolepis* and *A. pinsapo* a three-fragment pattern, whereas the species *A. alba* exhibited both the above pattern variants (ZIEGENHAGEN & FLADUNG 1997). However, only *A. alba* with combined pattern variants was represented in the experiment by the 220 individuals. In the remaining 6 species only one individual of each was subjected to restriction analysis. The same was true of the 4 *Pinus* species compared all of which shared a three-fragment restriction pattern. It is reasonable to believe that involvement of additional individuals of a given species may shed more light on the nature of the *trn*S-*psb*C/*Hae* III restriction profiles in *Abies* and *Pinus* species. Therefore, a comparative study was made at the population and individual levels aiming in description of the extent of both inter- and intraspecific variations in the respective gene region in 5 *Abies* and 4 *Pinus* species.

MATERIAL AND METHODS

The list of species subjected to restriction analysis of chloroplast DNA (*cp*DNA) together with their taxonomic position, geographic distribution and origin of sampled trees is given in Table 1.

Total DNA was extracted from 0.5 g of fresh needles using protocol by MURRAY & THOMPSON(1980). The flanking regions between the genes *trn*S [tRNA-Ser(UGA)] and *psb*C (*ps* II 44 kDa protein) was amplified by a pair of primers of the sequences 5`-GGT CGT GAC CAA GAA ACC

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Table 1. Taxonomic status and geographic distribution of the species investigated. Latin names according to LIU 1971 (*Abies*) and PILGER 1926 (*Pinus*).

Latin name	Section	Distribution	Sample origin
A. alba Mill.	*Abies*	centr., south. Europe	Slovakian populat.
A. nordmanniana (Stev.) Spach	*Abies*	Caucasus, Turkey	Denmark – seed orchard
A. concolor (Gord. et Glend) L.	*Grandes*	western USA	Denmark – seed orchard
A. lasiocarpa (Hook). Nut	*Balsameae*	northern Canada	Denmark – seed orchard
A. koreana Wils.	*Elate*	Korean peninsula	Denmark – seed orchard
P. sylvestris L.	*Eupitys*	Europe, Asia	Slovakia – Pezinok
P. mugo Turra	*Eupitys*	Pyrenees, Alps, Carpathians	Slovakia – High Tatras
P. nigra Arn.	*Eupitys*	south. Europe, Crimea, Asia Minor	Slovakia – Pezinok
P. cembra L.	*Cembra*	Alps, Carpathians	Slovakia – High Tatras

AC-3` and 5`-GGT TCG AAT CCC TCT CTC TC-3` (DEMESURE *et al.* 1995). The reaction mixture for PCR consisted of 20 ng of template DNA, 67 mM Tris-HCl pH 8.0, 16.6 mM ammonium sulphate, 2 mM $MgCl_2$, 10 mM β-mercaptoethanol, 4.4 μg/ml bovine serum albumin, 200 mM of each four dNTP, 1 unit of *Taq* polymerase (BRL, Life Technologies, GmbH, Eggenstein, Germany) and 0.6 μM of each primer in a total volume of 25 μl. The following cycles and temperature were used during PCR: 94 °C for 4 min. followed by 35 cycles at 95 °C for 1 min., 57 °C for 1 min. and 72 °C for 2 min. Last strand elongation (72 °C) was allowed an additional 10 min. The size of PCR products was estimated electrophoretically separating 3 μl of the respective product in 0.8 % agarose, in 0.5× TBE buffer. The DNA fragments were visualized by UV fluorescence after staining with ethidium bromide (0.25 $\mu l \cdot ml^{-1}$ staining solution).

The PCR products were digested with *Hae* III restriction enzyme using 5 μl of the respective product and 5 enzyme units in a total volume of 20 μl. Digestion was performed overnight according to the manufacturer`s instructions (Boehringer, Mannheim). Total digestion volume was loaded onto the gel. The generated *cp*DNA fragments were separated on 8 % non-denaturing polyacrylamide gels, in 1× TBE buffer, using vertical electrophoretic apparatus. The gels were run at a constant current of 25 mA, for about 4–5 hours. Separated fragments were visualized by UV fluorescence as in case of PCR products. The size of individual fragments was estimated by comparisom with a molecular size standard.

RESULTS

The primers used have efficiently amplified the corresponding *cp*DNA region in both *Abies* and *Pinus* species. The approximate size of the PCR products was determined to range about 1600 bp in *Abies* and 1500 bp in *Pinus* (Fig. 1). No variation in size of PCR products was observed between individual taxa of investigated genera.

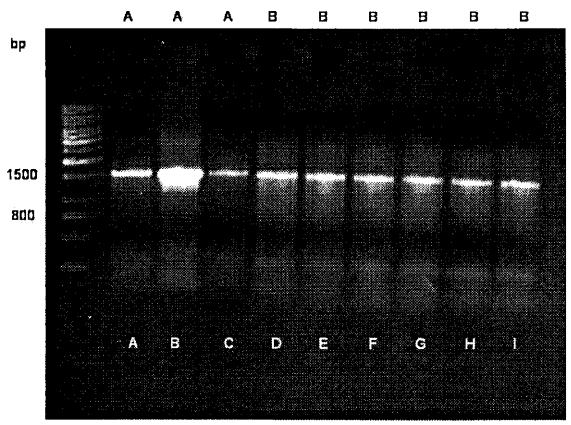

Figure 1. *Abies* (A) and *Pinus* (B) PCR products. A – *A. alba*, B – *A. concolor*, C – *A. koreana*, D, E – *P. nigra*, F – *P. mugo*, G, H. – *P. sylvestris*, I – *P. cembra*, M – molecular size standard, 1 kb ladder (Gibco BRL, Life technologies).

At the species level, there were revealed as many as 4 *Hae* III restriction patterns of *cp*DNA within the genus *Abies* referred to here as haplotypes A1-A4 and 2 restriction patterns within the genus *Pinus* referred accordingly to as P1 and P2 haplotypes. The profiles of these haplotypes described in terms of number and size of generated fragments are given in Table 2.

Among species investigated so far, *A. alba* was the only exception exhibiting individual variation. Within a total number of 285 individuals represent-

Table 2. Structure of A1-A4 and P1-P2 haplotypes.

Fragment bp	Haplotype					
	A1	A2	A3	A4	P1	P2
830					#	
800						#
750	#	#	#	#		
740	#					
550					#	
500		#	#			#
450				#		
320						
200					#	#
190		#	#	#		
100					#	#
80	#	#	#	#		
50			#	#		

ing 8 populations of the species in Slovakia the A1 and A2 haplotypes were revealed (Fig. 2). Individual populations differed in their proportions but in general no tendency with regard to geographic cline was observed. It follows from the data presented in Table 3 that in the eastern Slovakian populations Zboj and Palota the A1 haplotype prevails equally as in the population Kamenec of western Slovakia. The reverse is true of the A2 haplotype predominance in the population Močiar of western Slovakia and in the populations Kežmarské Žľaby and Krompachy of eastern part of the country.

As to other species, some degree of similarity with *A. alba* exhibited only *A. nordmanniana* both of which shared A1 haplotype and a common taxonomic position within the section *Abies*. The remaining *Abies* species deviated in this respect. *A. lasiocarpa* of the section *Balsameae* possessed A3 haplotype, whereas *A. concolor* of the section *Grandes* and *A. koreana* of the section *Elate* A4 haplotype. A slight differentiation between the two last mentioned haplotypes is based on the differential presence of 500 bp fragment in the former and 450 bp fragment in the latter (Table 2).

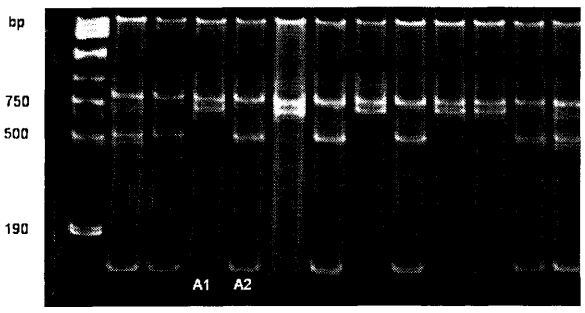

Figure 2. *trn*S/*psb*C/*Hae*III restriction profiles of *A. alba* individuals with two haplotypes recognized.

Essentially similar restriction patterns were characteristic also for the group of species *P. sylvestris*, *P. mugo* and *P. nigra* of the section *Eupitys* all of which exhibited P1 haplotype. Small deviations from *Abies* restriction profiles were due to the variations in size of individual fragments. The only exception was in this group the species *P. cembra* of the section *Cembra* possessing P2 haplotype (Fig. 3). Summarrily the pertinence of investigated species to individual haplotypes is given in Table 4. It is apparent from the data presented here that except for *A. alba* no variation in the *trn*S-*psb*C/*Hae* III restriction pattern was observed between individual trees of investigated species.

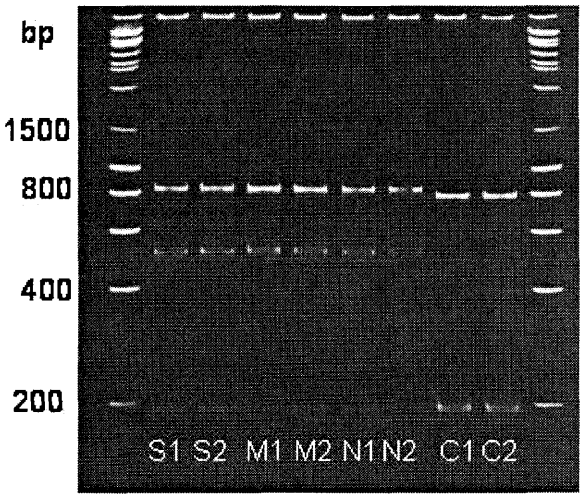

Figure 3. *trn*S/*psb*C/*Hae*III restriction profiles of *P. sylvestris* (S1, S2), *P. mugo* (M1, M2), *P. nigra* (N1, N2) and *P. cembra* (C1, C2) individuals with two haplotypes recognized. M – SmartLadder (Euro-gentec).

Table 3. *Hae* III haplotype proportions in *A. alba* populations in Slovakia.

Populations	Location	Haplotype		Total number of individuals
		A1	A2	
Zboj	East Slovakia	21	13	34
Palota	East Slovakia	19	8	27
Krompachy	East Slovakia	22	31	53
Kežmarské Žľaby	East Slovakia	14	21	35
Staré Hory	Middle Slovakia	12	12	24
Zuberec	Middle Slovakia	14	18	32
Močiar	Western Slovakia	16	25	41
Kamenec	Western Slovakia	27	12	39

Table 4. Pertinence of *Abies* and *Pinus* species to *trn*S-*pbs*C/*Hae* III haplotypes according to number of sampled individuals.

Species	Haplotypes					
	A1	A2	A3	A4	P1	P2
A. alba Mill.	145	140				
A. nordmanniana (Stev.) Spach	28					
A. concolor (Gord. et Glend.) Lindl				5		
A. lasiocarpa (Hook). Nut			9			
A. koreana Wils.				20		
P. sylvestris L.					28	
P. mugo Turra					14	
P. nigra Arn.					28	
P. cembra L.						14

DISCUSSION

Owing to conservative character of chloroplast genome, *cp*DNA markers were considered initially as good indicators of genetic divergence of distant taxa but insensitive for detection of differentiation at the intraspecific level (PALMER 1987). Restriction site analysis of *cp*DNA has accordingly been looked upon as the most popular technique in plant systematics for phylogenetic reconstruction below the family level, preferentially at the genera and species levels (LISTON 1992, SOLTIS *et al.* 1992). However, in spite of these opinions WAGNER *et al.* (1987) demonstrated that certain regions of conifer *cp*DNA appear as "hot spots" showing intraspecific variation. In particular, it was true of *Pinus contorta* and *Pinus banksiana* populations exhibiting increased frequency of novel *cp*DNA variants in hybrid populations. In Europe, no results on intra- or interpopulational genetic variation in European tree species using DNA markers were published until 1992 (MÜLLER-STARCK *et al.* 1992). The first report illustrating unequivocally the presence of intraspecific variation in *Abies* has appeared in 1994 when TSUMURA *et al.* (1994) demonstrated a gradual cline along latitude and longitude in 7 natural populations of *A. mariesii* in Japan. Using heterologous probes *pCS7* and *pCS10* of *Cryptomeria japonica cp*DNA the authors proved the existence of two types of variants in *pCS10/Hind* III, *pCS7/Hind* III and *pCS7/Bgl* II probe-enzyme combinations. Still other species of the region exhibiting intra- and interpopulation variation was *A. sachalinensis* with variable *cox* II gene of mitochondrial DNA and *atp* A gene of chloroplast DNA. Based on 3 phenotypes of the *cox* II/*Bam* HI and on 2 phenotypes of the *atp* A/*Eco* RI probe-enzyme combinations, HAYASHI *et al.* (2000) were able to discriminate between 19 populations of the species in Japan and to prove distinct differentiation of the southern populations in this context.

Presented results on *trn*S-*psb*C/*Hae* III variation in *A. alba* seem to be only the third example in the series illustrating intraspecific variation in *Abies* described originally by ZIEGENHAGEN & FLADUNG

(1997). It is difficult to predict the potential of this flanking region for *A. alba* population study. The number of investigated populations was too small to show some tendency in species' variation and had not covered all the species' habitats on geomorphologically heteromorphous territory of Slovakia. Like *A. mariesii* and *A. sachalinensis*, *A. alba* is also unique in possessing variable spacer region *trn*S-*psb*C differring in this respect not only from *Pinus* species but also from *A. nordmanniana*, *A. concolor*, *A.lasiocarpa* and *A. koreana* all of which lack the variation in the respective region oftheir *cp*DNAs. This corroborates the opinion expressed by PARDUCCI & SZMIDT (1999) that *Abies* species may be even more heterogenic in their *cp*DNAs than other coniferous species. Therefore, the basic question of plant systematics and evolutionary biology concerning the frequency and taxa in which the presence of intraspecific variability in *cp*DNA has been observed remains still open in *Abies*.

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