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Original scientific paper

GENETIC RELATIONSHIPS AMONG SOME *Pinus, Picea* AND *Abies* SPECIES REVEALED BY RAPD MARKERS

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Studies were undertaken to identify genetic relationships among ten different species of the family Pinaceae through randomly amplified polymorphic DNA (RAPD) markers. Eighteen arbitrary RAPD primers produced 123 fragments of which 107 were polymorphic (87%). The similarity coefficient values varied from 0.34 to 0.67. The highest similarity coefficient was detected between *Pinus wallichiana* and *P. strobus* as well as between *Picea abies* and *P. orientalis*, and the lowest was detected between three *Pinus* species (*P. heldreichii*, *P. peuce* and *P. wallichiana*) and *Picea omorika*. The analysis of RAPD markers confirmed the genetic relationships among species. Genus *Picea* is clearly separated from genus *Pinus* and is closer to genus *Abies* (*A. concolor*) than to genus *Pinus*, what confirms up-to-date numerous comparative-morphological, anatomical, chemotaxonomic and molecular results of these closely related genera. Furthermore, on the basis of our results, pine species from different subgenera - *Pinus* and *Strobus* are clearly separated. This statement is in agreement with contemporary intrageneric classification of the genus *Pinus*.

Key words: Serbian spruce, Bosnian pine, Macedonian pine, RAPD markers

INTRODUCTION

Characterization of the genetic diversity and examination of the genetic relationships within Pinaceae family are important for the sustainable conservation and use of plant genetic

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resources. Traditionally, vegetative anatomy and plant systematics were two common approaches to assess the relationship among them (WANG *et al*., 2009).

Family Pinaceae, with more than 200 extant species, is one of the largest families of conifers. Genera *Pinus* and *Picea* are very large, with more than 100 and 28-56 species, respectively (LISTON *et al.*, 1999; RAN *et al.*, 2006 and refs. cited therein, resp.).

Picea omorika (Panč.) Purkyně (Serbian spruce) is a Tertiary relict and endemite of Balkans, native in South-West Serbia and Bosnia and Herzegovina, which belongs to section *Omorika* (classification of KRÜSSMANN, 1972, after VIDAKOVIĆ, 1991).

Two-needle pine, *Pinus heldreichii* Christ. (Bosnian pine), is a Tertiary relict and subendemite of Balkan Peninsula, native in East Mediterranean and rare in Italy (also known as *Pinus leucodermis*), which belongs to subgenus *Pinus* (classification of LITTLE and CRITCHFIELD, 1969, after VIDAKOVIĆ, 1991).

Five-needle pine, *Pinus peuce* Griseb. (Macedonian pine), is a Tertiary relict and endemite of Balkans, native in Bulgaria, Macedonia, Serbia (Kosovo), Albania and Greece, which belongs to subgenus *Strobus* (classification of LITTLE and CRITCHFIELD, 1969, after VIDAKOVIĆ, 1991).

In investigation of genetic diversity of *Picea* and *Pinus* species different molecular markers were used in population studies (*Picea omorika*, NASRI *et al*., 2008; *Picea* spp., MAGYARI *et al.*, 2011; *P. abies*, GEBUREK, 1999; *P. asperata*, LUO *et al.*, 2005; *P. sitchensis,* GAPARE and AITKEN, 2003; *Pinus heldreichii*, NAYDENOV *et al.*, 2005a; *P. nigra*, RUBIO-MORAGA *et al.*, 2012; *P. sylvestris*, NAYDENOV *et al.*, 2005b), as well as in detection of varieties (*Picea omorika* var. *semidichotomy*, var. *serbica* and var. *nana*, ŠIJAČIĆ-NIKOLIĆ *et al.*, 2000; *Pinus elliottii* var. *elliottii* and *P. caribaea* var. *hondurensis*, SHEPHERD *et al*., 2003), subspecies (*Picea* spp., RUNGIS *et al.*, 2004; *Pinus* spp., BOGUNIĆ *et al.*, 2011), hybrids (*P. sitchensis* x *P. glauca*, HAMILTON *et al.*, 2012; *Pinus elliottii* x *P. caribaea,* DOYLE, 2001), geographic races (*Picea abies* and *P. obovata*, KRUTOVSKII and BERGMANN, 1995), etc.

Random amplified polymorphic DNA (RAPD) markers have been the most widely used molecular marker type in forest trees (WHITE *et al.*, 2007). The RAPD marker system is easy to apply as no prior DNA sequence information is needed for designing PCR primers as is required for other PCR-based genetic marker systems. The small amount of DNA needed is a big advantage of the RAPD technique. The use of RAPD assay to identify genetic variation is preferred over the morphological and biochemical markers since this is completely devoid of environmental effects and of the stage of the experimental material, thus making them highly reliable. Random amplified polymorphic DNA (RAPD) markers have been widely used in the reconstruction of phylogenetic relationships for many organisms and there has been general accordance among the results derived from RAPDs and other techniques. They are abundant in plant genomes and have changed rapidly during evolution as compared with coding DNA sequences. Hence, it is possible to find RAPDs that are specific for individual species, groups of species, or genomes.

The first use of RAPD markers was demonstrated on conifers *Pseudotsuga menziessi* and *Picea glauca* (CARLSON *et al.*, 1991 and TULSIERAM *et al.*, 1992, respectively, after WHITE *et al.*, 2007). Many articles about RAPD markers of *Picea* and *Pinus* species were published up to now (BUCCI and MENOZZI, 1993; HICKS *et al.*, 1998; OSTROWSKA *et al.*, 1998; LIBER *et al.*, 2003; KANT *et al.*, 2006; MONTELEONE *et al.*, 2006; PENG *et al.*, 2007; LUČIĆ *et al.*, 2010, 2011; KURT *et al.*, 2011; etc.). Relationships between species on the basis of RAPD markers were investigated

in a few papers (genus *Picea*, NKONGOLO, 1999; NKONGOLO *et al.*, 2005; genus *Pinus*, ABRAMOVA, 2002; NKONGOLO *et al.*, 2002).

The aim of this study is to analyse RAPD markers among ten species of *Pinaceae* family, as well as to analyse genetic relationship between three endemo-relic species native in Serbia (*Picea omorika*, *Pinus heldreichii* and *P. peuce*) and some other autochtone and allochtone species of the genera *Picea*, *Pinus* and *Abies.*

MATERIALS AND METHODS

For RAPD analysis needles of 10 species of family Pinaceae, collected in Botanical Garden 'Jevremovac' and some Belgrade parks, were used: *Picea omorika* (Panč.) Purkyně (POM), *Picea abies* (L.) Karst. (PAB), *Picea orientalis* (L.) Link. (POR), *Pinus heldreichii* Christ. (PHE), *Pinus nigra* Arn. (PNI), *Pinus sylvestris* L. (PSY), *Pinus peuce* Griseb, (PPE), *Pinus wallichiana* A. B. Jacks. (PWA), *Pinus strobus* L. (PST), and *Abies concolor* (Gord.) Engelm (ACO).

Total genomic DNA was extracted and purified from 0.1 g of needles by CTAB method (BASHALKHANOV and RAJORA, 2008).

Different preliminary experiments were carried out to optimize the factors leading to clear and reproducible amplification products. PCR amplification of genomic DNA was tested with 20 RAPD primers (Genosys Biotechnology, Cambridge, UK; Operon Technologies, Alameda, USA) in two rounds of amplification (WILLIAMS *et al.*, 1990), of which 18 primers gave clear and reproducible bands. List of the primers with sequences is given in Table 1.

The amplification reaction was carried out in 25 µl reaction mixture containing 1x reaction buffer, 2.5 mM $MgCl_2$, 100 µM dNTPs, 0.2 µM of 10-mer primers, 2.5 U of Taq polymerase (Fermentas) and 50 ng of template DNA using a thermocycler PTC-100 (MJ Research).

The amplification profiles followed were: an initial denaturation at 94^{o}C for 2 min followed by 45 cycles at 94^{o}C for 30 sec, 40^{o}C for 1 min and 72^{o}C for 1 min, and final cycle at 72^{o}C for 7 min. The amplified products were separated by electrophoresis on 1.4 % horizontal agarose gels in 0.5 x TBE buffer at 40 mA for 2h, stained with 0,5 µg/µl ethidium bromide, and photographed under UV light. Product sizes were determined using 1 kb DNA ladder (Fermentas).

DNA banding patterns from RAPD gels were converted into binary form, where a 'one' indicates the presence of a specific allele and a 'zero' indicates the absence of that allele. Pairwise comparisons of samples were done to estimate Jacquard's coefficient of similarity.

In order to analyse the relatedness among the species an unweighted pair group arithmetic mean method (UPGMA) cluster analysis was peformed, based on Jacquard's coefficient of similarity, as available in NTSYSpc software package version 2.11a (ROHLF, 2000). Dendrogram was drawn using SAHN clustering method and generated by using TREE display option.

RESULTS AND DISCUSSION

The RAPD primers were used to reveal the genetic relatedness among ten species belonging to family Pinaceae. From screening 20 RAPD primers, 2 primers failed to amplify products consistently, eighteen primers could produce stable and repeatable bands. A total of 123 DNA bands were detected, 107 of them showed polymorphism (87%). The number of

amplification products produced by a primer ranged from as low as 5 to a maximum of 11, with an average of 6.83 bands per primer. The total number of polymorphic loci detected varied between primers (Table 1).

Certain amplified bands appeared to be common to several species, whereas others were present in some species but absent in others. It could be observed that primer GEN 4-70-7 generated a total of 9 polymorphic bands, including 90% polymorphism and one monomorphic band in the studied species, while one unique band was detected in genus *Picea* and *Abies concolor* (1450bp), and the absence of one band (850bp) was observed in *Pinus peuce* and *Picea omorika*.

Table 1. RAPD primers, nucleotide sequences, number of fragments and level of polymorphism

Primer	Nucleotide sequences (5'-3')	No. of fragments amplified	No. of polymorphic fragments	Polymorphism %
GEN 4-70-7	CTATCGCCGC	10	9	90
GEN 2-80-10	CGCGAACGGC	9	9	100
OPB10	CTGCTGGAC	6	6	100
GEN 4-70-2	GGACCGACTG	6	5	83.3
OPB3	CATCCCCCTG	7	6	85.7
OPB20	GGACCCTTAC	7	6	85.7
OPB17	AGGGAACGAG	5	3	60
OPB1	GTTTCGCTCC	6	6	100
OPB8	GTCCACACGG	7	6	85.7
OPB6	AGGGAACGAG	5	5	100
GEN 1-80-4	CGCCCGATCC	7	6	85.7
OPB2	TGATCCCTGG	5	4	80
GEN 1-70-5	TAGATCCGCG	9	6	66.6
GEN 1-70-10	CAGACACGGC	11	9	81.8
OPB13	TTCCCCCGCT	6	6	100
OPB7	GGTGACGCAG	6	6	100
OPB12	CCTTGACGCA	6	5	83.3
OPB18	CCACAGCAGT	5	4	80

A total of nine polymorphic bands were generated by primer GEN 2-80-10, three bands (580bp, 1250bp, 2250bp) discriminated genus *Pinus* from genus *Picea* and genus *Abies*. One polymorphic band was identified as unique band in *Picea abies* and *P. orientalis* (480bp), while absence of one band (1650bp) was observed in *Pinus peuce*.

The primer GEN 1-80-4 generated six polymorphic bands one of which was unique in *Pinus heldreichi*. One polymorphic band out of six observed by using primer OPB12 discriminates genera Picea and Abies from *Pinus* species. The band of 1650bp was identified as unique band in *Picea omorika* with primer OPB10.

The absence of one polymorphic band characteristic of primer GEN 1-70-5 in *Abies concolor* could be considered as the presence of a negative, unique band (1950bp) of genus *Abies*. The primer GEN 1-70-10 generated a total of 9 polymorphic bands and two monomorphic

bands in studied species. Two unique bands were scored, *Pinus peuce* was characterized by the presence of an unique band (1600b) while genus *Picea* was distinquished by the absence of one band (1250 bp).

All the markers were scored by presence vs. absence of specific amplification products and the data were used to calculate values of genetic distance between all the species studied.

Data illustrated in Table 2 reveal that the highest similarity coefficient was 0.67 between *Pinus wallichiana* and *P. strobus* as well as between *Picea abies* and *P. orientalis*, followed by 0.64 between *Pinus nigra* and *P. sylvestris*. On the other hand, the lowest similarity coeficient was 0.34 between each of the three species, *Pinus heldreichii, P. peuce* and *P. wallichiana* and *Picea omorika*. Genetic similarity coefficient was higher among *Picea* species (0.63) compared with *Pinus* (0.51), as well as in species belonging to subgenus *Pinus* (0.57) than to subgenus *Strobus* (0.53).

The cluster analysis based on genetic distance computed from RAPD data classifies each of 10 genotypes into one of two principal clusters, designated GI, GII. Results are presented in Figure 1. The cluster GI consists of two subclusters, A and B. Subcluster A encompasses 3 species from subgenus *Pinus* and subcluster B contains 3 species from subgenus *Strobus.* Cluster GII includes 3 species from genus *Picea* and one from genus *Abies* (*A. concolor*) loosely linked to them.

Table 2. Coefficients of similarities among ten conifer species

	PHE	PPE	PNI	PSY	PWA	PST	POM	PAB	ACO
PPE	0.45								
PNI	0.51	0.46							
PSY	0.44	0.50	0.64						
PWA	0.55	0.40	0.52	0.51					
PST	0.49	0.49	0.55	0.49	0.67				
POM	0.34	0.34	0.39	0.46	0.34	0.39			
PAB	0.41	0.36	0.39	0.44	0.44	0.49	0.54		
ACO	0.38	0.42	0.38	0.36	0.36	0.39	0.42	0.42	
POR	0.51	0.43	0.42	0.48	0.48	0.54	0.60	0.67	0.46

On the basis of present results of RAPD markers, genus *Picea* is clearly separated from genus *Pinus* (Fig. 1). Genus *Picea* is closer to genus *Abies* (*A. concolor*) than to genus *Pinus*. Furthermore, pines from subgenera *Pinus* and *Strobus* are separated, too. These results are in accordance with classification based on morfological traits of analysed genera (VIDAKOVIĆ, 1991). Clear separation of *Picea omorika* populations from *Pinus heldreichii* and *P. peuce* populations was also found according to terpene compounds (NIKOLIĆ *et al.*, 2011). In that analysis *P. heldreichii* and *P. peuce* were also separated in more than 90% cases. Furthermore, separation of *Picea omorika* from *Pinus heldreichii* and *P. peuce* populations was also found by a *n*-alkane analyses (NIKOLIĆ *et al.*, 2013).

Picea omorika is clearly separated from two other analysed spruces of section *Eupicea*, *P. abies* and *P. orientalis* (Fig. 1). According to RAPD markers (NKONGOLO, 1999) *P. omorika* was the most similar to *P. jezoensis* (section *Casicta*), *P. glehnii* and *P. mariana* (section *Eupicea*). According to hybridization compatibility, Serbian spruce is more related to *P. orientalis* and some other spruces of subsections *Eupicea* and *Casicta* than to *P. breweriana* (section *Omorika*)

(VIDAKOVIĆ, 1991; LEDIG *et al.*, 2004, and refs. cited therein). According to phylogenetic researches (RAN *et al.*, 2006; ALEKSIĆ *et al.*, 2009), *P. omorika* was related to several spruces of sections *Eupicea* and *Casicta*, and far away from *P. breweriana* (RAN *et al.*, 2006).

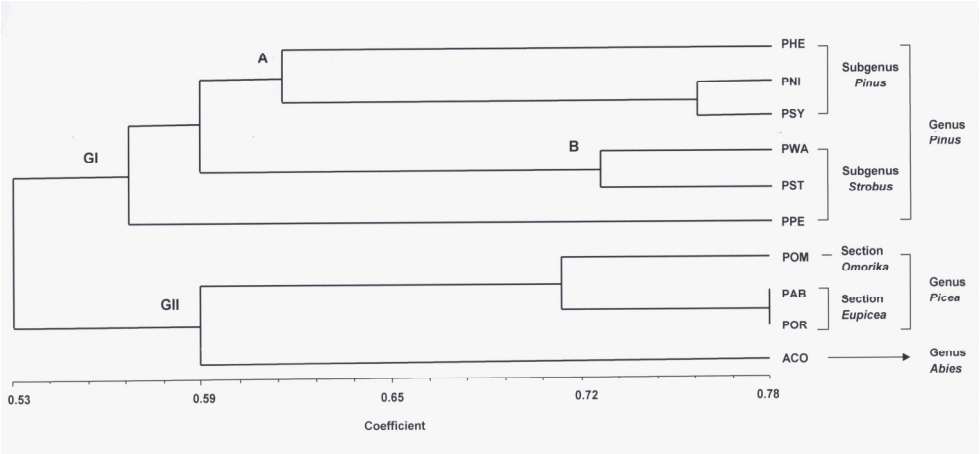

Fig. 1 Relatedness among ten conifer species performed by UPGMA cluster analysis (based on Jacquard's coefficient of similarity)

According to RAPD marker results obtained in our study, the analysed species from subgenus *Pinus* (*P. heldreichii, P. nigra*, and *P. sylvestris*) are clearly separated from those of subgenus *Strobus* (*P. peuce*, *P. wallichiana*, and *P. strobus*) (Fig. 1). Separation of species on these two subgenera was also found in RAPD markers research of NKONGOLO *et al.* (2002) which is in agreement with morphology-based taxonomy (classification of LITTLE and CRITCHFIELD, 1969, after VIDAKOVIĆ, 1991). Furthermore, PALMÉ *et al.* (2009) and KAUNDUN and LEBRETON (2010) confirmed clear separation of two-, three- and five-needle pines. GEADA LÓPEZ *et al.* (2002) also found separation between Eurasian and North American pines.

Pinus heldreichii has a large distance from two other analysed species of subgenus *Pinus*, section *Pinus*, subsection *Sylvestres* (*P. nigra* and *P. sylvestris*) (Fig. 1). In investigation of seed protein similarity it was found that *P. leucodermis* had divider position between Mediterannean pines (SCHIRONE *et al.*, 1991), being closely related to those of subsection *Pinaster* (after classification of GERNANDT *et al.*, 2005). It was confirmed in taxonomic and phylogenetic researches of *P. heldreichii* (WANG *et al.*, 1999; GERNANDT *et al.*, 2005). These conclusions are opposite of classification of LITTLE and CRITCHFIELD and statement of KLAUS (1989) who regarded that, according to morphology, *P. heldreichii* is more closely related to *P. nigra*, *P. sylvestris* and some other pines from subsection *Sylvestre*s.

Results of RAPD marker analyses show large distance of *Pinus peuce* from *P. wallichiana* and *P. strobus* (all from subgenus *Strobus*) (Fig. 1). *Pinus peuce* also has the largest distance from all investigated pines. According to successful hybridization, *P. peuce* is related to *P. wallichiana*, *P. strobus*, *P. flexilis*, and *P. monticola* (all from subsection *Strobi*) (VIDAKOVIĆ, 1991 and refs. cited therein). According to morphological traits *P. peuce* is the most related to *P.*

wallichiana (KLAUS, 1989). Recent genetic investigations denied these statements (WANG *et al.*, 1999; LISTON *et al.*, 1999) and pointed to related links between *P. peuce* and endemic *P. krempfii*, from subgenus *Ducampopinus* (WANG *et al.*, 1999; GERNANDT *et al.*, 2005), and furthermore, to close relationship of *P. peuce* with *P. strobus* (SCOTT, 2004; TSUTSUI *et al.*, 2009) and some pines of subsections *Strobi* (GERNANDT *et al.*, 2005; TSUTSUI *et al.*, 2009) and *Parrya* (SCOTT, 2004). According to RAPD markers (ABRAMOVA, 2002), *Pinus peuce* had closer relationship with some North American species from subsection *Strobi* than to Asian ones.

Polymorphism in random amplified polymorphic DNA (RAPD) markers was high and sufficient in distinguishing each of the species. Despite the fact that in this work small number of taxa was analysed, using RAPD markers, it could be concluded that given results provided sufficient information to discriminate between genera *Pinus*, *Picea* and *Abies*, subgenera *Pinus* and *Strobus* of the *Pinus*, and sections *Omorika* and *Eupicea* of the *Picea* genus. *Abies concolor* was closer to genus *Picea* than to genus *Pinus*. These results are in agreement with morphology-based classifications. Relic and endemic *Picea omorika*, *Pinus heldreichii* and *P. peuce* were the most distant from their analysed relatives. RAPD markers strictly separated *Picea omorika* (section *Omorika*) from two spruces of section *Eupicea*. Distances of *Pinus heldreichii* and *P. peuce* from other analysed two- and five-needle pines were also very high. Our results confirm the statement of NKONGOLO *et al.* (2002) that RAPD analysis is a reliable method of determining genetic relationships within a conifer group.

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GENETIČKI ODNOSI IZMEĐU NEKIH *Pinus, Picea* I *Abies* VRSTA UTVRĐENI POMOĆU RAPD MARKERA

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Izvod

Prikazana su proučavanja sa ciljem utvrđivanja genetičke veze 10 različitih vrsta familije Pinaceae putem nasumično umnoženih polimorfnih DNA (RAPD) markera. Osamnaest proizvoljnih RAPD prajmera produkovalo je 123 fragmenata od kojih 107 polimorfnih (87%). Vrednosti koeficijenta sličnosti su varirale od 0.34 do 0.67. Najviši koeficijent sličnosti je detektovan između *Pinus wallichiana* i *P. strobus,* kao i između *Picea abies* i *P. orientalis*, a najniži između tri vrste roda *Pinus* (*P. heldreichii*, *P. peuce* i *P. wallichiana*) i *Picea omorika*. Analiza RAPD markera potvrdila je genetičku vezu između vrsta. Rod *Picea* se jasno razdvojio od roda *Pinus* i bliži je rodu *Abies* (*A. concolor*) nego rodu *Pinus*, što potvrđuje dosadašnje brojne uporedno-morfološke, anatomske, hemotaksonomske i molekularne rezultate ovih blisko srodnih rodova. Nadalje, na osnovu naših rezultata, jasno se razdvajaju analizirane vrste borova iz različitih podrodova - *Pinus* i *Strobus*, što je, takođe, u saglasnosti sa savremenom intrageneričkom klasifikacijom roda *Pinus*.

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