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DIVERSITY OF FRAXINUS ORNUS FROM SERBIA AND MONTENEGRO AS REVEALED BY RAPDs

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PCR-RAPD markers revealed individual variation in *F. ornus*. A total of 122 fragments were amplified using 7 primers and of these 97 fragments were polymorphic. The percentage of polymorphic loci was between 53.3% and 74.6% with an average of 63.1%. The mean gene diversity for all populations was 0.30 and the mean Shannon's index was 0.44. Of the total genetic variation 87% was intra-population whilst 13% was inter-population. The Mantel test revealed significant correlation between genetic and geographical distance matrice. Results herein represent the first use of molecular genetic (DNA) markers to characterize genetic variation in *F. ornus* populations. The partition of total genetic variance indicates a relatively restricted population differentiation as expected in outcrossing species. Present and future information on genetic structure and variability in *F. ornus* needs to be incorporated into strategies for the preservation of genetic resources of tree species.

Key words: Manna ash, genetic variance, RAPD

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INTRODUCTION

The existence of genetic diversity within population of forest trees results in enhanced individual fitness, balances various changes originated from the environment and provides forest regeneration and succession processes, which, ultimately, contributes to conservation of natural resources (BOJOVIC *et al.* 2000, BOOTH & GRIME, 2003; RAJORA & PLUHAR, 2003; BOJOVIC *et al.* 2005, NAVASCUES & EMERSON, 2007; BOJOVIC *et al.* 2011; NIKOLIC *et al*. 2011; POPOVIC *et al*. 2012). The main source of biodiversity is based on genetic variation (ULUKAN, 2011) which determines the forest tree species capacity of adaptation to environmental conditions and represents the basis for tree improvement through selective breeding.

In modern research methods, genetic markers give an insight in the amount and distribution of genetic variation within and among populations. In 1990, RAPD markers (randomly amplified polymorphic DNA) were developed independently by two different laboratories (RAHMAN *et al*., 2006). These markers were generated by amplification of random DNA segments with short primers (generally 10-base oligonucleotides) of arbitrary nucleotide sequences, by the Polymerase Chain Reaction (PCR), and they have been extensively used to detect variations of individual (BELAJ *et al.,* 2004), intra-specific (ZHANG *et al.,* 2002), inter-specific (SHASANY *et al*., 2005), between plant populations (ETISHAM-UL-HAQ *et al*., 2001), taxa (ROMAN *et al.,* 2003), and hybrids (TRIEST *et al*., 2000; RAJORA & RAHMAN, 2003). The advantages of these markers over biochemical markers (isozymes) and other molecular genetic markers (RFLP, microsatellites) are that large numbers of samples can be analyzed economically and quickly, that only micro-quantities of material are needed, that the specific DNA fingerprints obtained are independent of ontogenetic expression, that most of the genome can be sampled with a potentially unlimited number of markers, and there is no requirement for prior knowledge of the genome being studied (BARDAKCI, 2001).

However, RAPDs markers also have some disadvantages, the most important being low level of reproducibility and dominant expression, not allowing a clear difference between dominant homozygotes, recessive homozygotes and heterozygotes (NYBOM, 2004). However, these problems of early RAPD analyzes can now be overcome through careful optimization of reaction conditions and improved band scoring procedures. Adequate statistical methods such as analysis of molecular variance (AMOVA) (EXCOFFIER *et al.,* 1992) and Nei's unbiased statistics (NEI, 1978) are now available that circumvent this problem. Despite above mentioned constraints, the use of RAPD markers is recommended for a first appreciation of genetic variation and structure, especially in natural populations of forest trees (DVORAK *et al.,* 2002), and they have been successfully used in the most recent studies (CHENG *et al.,* 2002; HARDY, 2003; BELLETI *et al.,* 2008).

Fraxinus ornus L. (manna ash) is mainly a Mediterranean forest tree species of wide ecological amplitude. In the region of Serbia and Montenegro, large-scale afforestation with *F. ornus* was performed during the fifties of the last century for aimed at preventing erosion and regulating river courses. Because of the deforestation in this region during the past decade, the intensified reforestation with *F. ornus* has again become a necessity. *F. ornus* is also an ornamental tree cultivated on Sicily and Calabria (Italy) for production of edible manna (the substance is a mild laxative and an excellent purgative). In terms of floral biology, *F. ornus* is androdioecious; an extremely rare form of sex expression defined by a simultaneous presence of male and hermaphrodite individuals in a breeding populations. It is a rare and original system, both from theoretical and from empirical points of view.

Until the early 1990s genetic polymorphism studies were rare or non-existant for the majority of European deciduous tree species. Since then the main focus of the studies has been extended to the majority of European deciduous tree species including genus *Fraxinus* (NOWAKOWSKA *et al.,* 2004; PVINGILA *et al.,* 2005) and to date there has been no analyses of *F. ornus* populations. In this study, RAPDs were used to assess the genetic diversity and population structure of natural populations of *F. ornus* from Serbia and Montenegro. This allows the definition of genetically distinct units needed for conservation purposes.

The specific objectives of the present study were: (1) to find PCR-RAPD markers revealing individual variation in *F. ornus* and (2) to analyze the genetic structure of *F. ornus* by evaluating the degree of intra- and inter-population genetic variation.

MATERIALS AND METHODS

Plant material

Five populations of *F. ornus* L. were analyzed, altogether 150 adult trees (with approximately equal share of male and hermaphrodite individuals) (Fig. 1). These are the natural populations which populate the biogeographically contrasted zones of the range in Serbia and Montenegro (TOMIĆ, 2006).

Fig. 1. Map showing the natural range of F. ornus and location of sampled populations from Serbia and Monte Negro. Sampled populations with abbrevaitaons: A = Košutnjak, 44° 46'N et 20° 27'E, altitude: 210 m, soil: calcareous and sandy, climate: continental-Danubian type, association: Quercetum virgilianae prov. (Sarić, 1997); B = Užice, 43° 53'N et 19° 47'E, altitude: 700 m, soil: calcareous, climate: continental, association: Quercetum farnetto-cerris Rud.; C =Pirot, 43° 13'N et 22° 33'E, altitude: 500 m, soil: calcareous, climate: continental, association: Carpinetum orientalis serbicum (Rud.) Jov.; D = Mogren, 42° 19'N et 18° 50'E, altitude: 30 m, soil: calcareous, climate: Mediterranean, association: Orno-cocciferetum H-ic.; E = Bar, 42° 06'N et 19° 08'E, altitude: 80 m, soil: calcareous, climate: Mediterranean, association: Orno-cocciferetum H-ic.

DNA extraction

DNA was extracted from the leaves of adult trees. Approximately 5 g of leaf tissue were ground to fine powder in liquid nitrogen and quartz sand in a mortar and pestle. The powdered tissue was suspended in 21 ml of extraction buffer (0.1 M Tris-HCl pH 8; 0.05 M EDTA pH 8; 0.1 M NaCl; 1% SDS). The homogenate was then incubated at 65 °C for 20 min with occasional mixing. Seven ml of potassium 5 M acetate (pH 9.25) was added and the tube was placed on ice for 20 min. After centrifugation (3500 rpm, 0 °C, 20 min), 1 ml of ammonium acetate 10 M pH 7.5 and 1 volume of isopropanol was added and the sample was placed on ice for 20 min. The pellet obtained after centrifugation (3500 rpm, 0 °C, 10 min) was rinsed with 70% ethanol, dried and then resuspended in 1 ml of 50 mM Tris-HCl, pH 7.5/EDTA 10 mM buffer. The sample was then incubated for 30 min at 37 °C with RNase A (100 µg ml^{-1}) then Tris 10 mM, pH 7.5/EDTA 1 mM was added to give a final volume of 6 ml and this was extracted with a phenol/chloroform mix (1:1 phenol : chloroform). The aqueous layer was removed and the DNA was precipitated by addition of 0.1 volume of 7 M ammonium acetate (pH 7.4) and 1.5 volume of isopropanol. To eliminate the traces of phenol, the DNA pellet was rinsed with 70% ethanol. The final pellet was vacuum dried and dissolved in 1 ml of TE buffer and stored at 4 °C.

DNA amplification conditions

Amplification was carried out with 100 ng of DNA in a 25 µl total volume, with 2.5 µl of reaction buffer PC2 10X (Ab Peptides), 5 nM of dNTP (200 µM), 50 pM of each primer (2 µM) and 3.75 units of *Klen Taq* DNA polymerase (Ab peptides). Forty one RAPD decamer oligodeoxynucleotide primers were used for screening: 40 (kits G, H with 20 primers in each kit) purchased from Operon Technologies (Alameda, California, USA) and 1 primer (OPO-08) purchased from GIBCO BRL (Custom Primers, Life Technologies, Gaithersburg). The thermocycler program was as follows: preliminary denaturation (3 min at 94 °C) followed by 40 cycles consisting of denaturation (30 sec at 94 °C), annealing (1 min at 35 °C), extension (2 min at 72 °C) and a final extension (5 min at 72 °C). The DNA fragments were separated in 1.5% agarose gels in buffer TAE (Tris-base 40 mM, sodium acetate 20 mM, EDTA 1mM, with pH 7.2 adjusted with glacial acetic acid). Only data from clear, intensely staining unambiguous bands were used for analysis. The reproducibility of an amplified fragment pattern was tested in at least two independent experiments.

Data analysis

DNA profiles were scored as present (1) or absent (0). A fragment (loci) was considered to be polymorphic if the presence and absence of the band were observed and monomorphic if the band was present among all individuals. The analysed loci have the observed frequency less than 1-(3/*n*), where *n* is the number of individuals in analysis (LYNCH & MILLIGAN, 1994). Diverse parameters were calculated: Nei's (NEI, 1973) gene diversity (h), Shannon's information index (LEWONTIN, 1972) (I), the percentage of polymorphic RAPD loci (p), the partition of total genetic variance, Nei's unbiased measures of genetic identity and genetic distance (NEI, 1978). Parameters h, I and p were calculated for each population, as well as the mean value for all populations. The partitioning of total genetic variance was examined by analysis of molecular variance (AMOVA). Partition of molecular variance was tested at two hierarchical levels: among

individuals within populations and among the populations. The number of permutations for significant testing was set at 1000 for analysis. The genetic distance matrix calculated among populations by Nei's unbiased distance estimate (NEI, 1978). Geographic distances among populations were estimated from coordinates obtained using a geographical positioning system. The relationship between the Nei's distance matrix (unbiased estimate; NEI, 1978) and the matrix of geographic distances was analysed using a Mantel test. Significance values were obtained using resampling techniques with 999 permutations. Genetic diversity parameters: h, I, p and Nei's unbiased measures of genetic identity and genetic distance (NEI, 1978) were calculated by the software POPGENE 1.31 (SCHNEIDER *et al.,* 1997), AMOVA by ARLEQUIN 1.1. (YEH *et al.,* 1999) and Mantel test by TFPGA 1.3 (MILLER, 2000).

RESULTS AND DISCUSSION

Results herein represent the first use of molecular genetic (DNA) markers to characterize genetic diversity in *F. ornus* natural populations. Our results indicate that PCR-RAPD markers are sufficently informative and powerful to assess genetic variability in *F. ornus*. RAPDs were shown to be sensitive for detecting individual variation of *F. ornus*. Out of 41 primers tested, 7 resulted in polymorphic banding patterns (Table 1). These primers with arbitrary sequences of 10 nucleotides had G+C contents ranging from 60% to 70%. A total of 122 fragments were amplified and of these 97 fragments were polymorphic. The size of the fragments varied from 0.43 to 2.30 kilobase pairs (Kb). Fig. 2 shows part of the *F. ornus* genetic polymorphism revealed with primer OPG-07.

Table 1. Attributes of the seven selected RAPD primers

primer	nucleotide sequence 5' to 3'	% GC content	number of main fragments*	number of polymorphic fragments	fragment size range (Kb)
OPG-07	GAACCTGCGG	70	18	14	0.57-2.75
OPG-11	TGCCCGTCGT	70	19	15	0.47-1.10
OPG-20	TCTCCCTCAG	60	18	16	0.51-1.05
OPH-07	CTGCATCGTG	60	11	6	0.42-0.83
OPH-10	CCTACGTCAG	60	14	12	0.51-1.30
OPH-11	CTTCCGCAGT	60	25	22	0.34-1.55
OPH-15	AATGGCGCAG	60	17	12	0.46-2.30
total			122	97	

* amplified fragments that were not repeatable were excluded

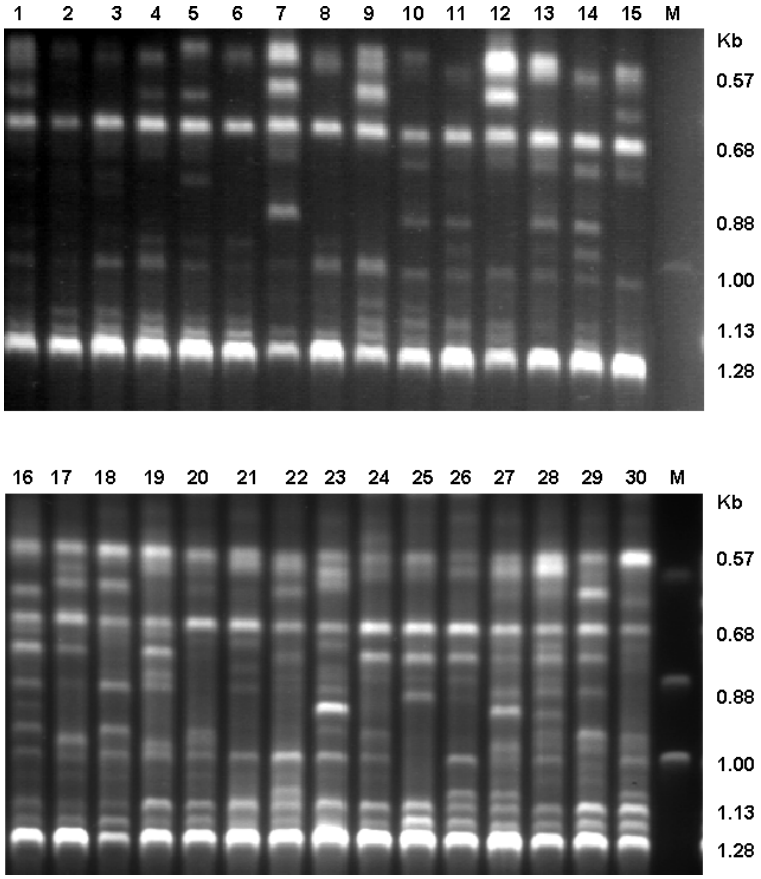

Fig. 2. RAPD amplification products of DNA isolated from *F. ornus* using primer OPG-07. Population from Košutnjak (A) (30 trees, lane 1-30), lane M is a size marker (Smart Ladder, Eurogentec).

The percentage of polymorphic loci was relatively similar across populations, ranging between 53.3% (A) and 74.6% (D), with an average of 63.1% (Table 2). The range of variation in gene diversity (h) between the populations was from 0.25 to 0.34, with the mean gene diversity for all populations 0.30. The relative degree of diversity in each population as measured by Shannon's index varied from 0.38 (C) to 0.51 (E). The mean Shannon's index for five populations was 0.44.

Our results indicate that 12.6% of the total genetic diversity is attributable to the differences among populations and 87.4% is within-population variation (FST in the molecular variance analysis = 0.13, $P<0.05$, tested using 1023 random permutations).

Table 2. Nei's (1973) gene diversity (h), Shannon's diversity measure (I) and percent polymorphic RAPD loci (% p) for five F. ornus populations. Standard deviations (s.d.) are given in parentheses. Mean values averaged over all populations.

Populations	h (s.d.)		I (s.d.)		% p
A	0.280	(0.182)	0.418	(0.254)	53.28
B	0.271	(0.200)	0.399	(0.283)	54.10
C	0.254	(0.194)	0.381	(0.269)	59.84
D	0.339	(0.143)	0.508	(0.179)	74.59
E	0.343	(0.157)	0.509	(0.199)	73.77
Mean	0.297	(0.175)	0.443	(0.237)	63.11

Except among the populations D and E, all pairwise FST values derived from AMOVA were significant ($P<0.05$) when individual pairs of populations were compared. The geographic structuration of the diversity can be analysed by the Nei unbiased genetic distances (NEI, 1978) among populations (Table 3). The highest genetic distance was found between the C and E (0.09) and the lowest between the D and E (0.01). The dendogram based on Nei's unbiased genetic distance matrix reveals a distinct grouping structure among populations (Fig. 3). The Mantel test revealed significant correlation between genetic and geographical distance matrice ($r=0.63$, $P<0.05$).

Table 3. Coefficients of genetic distances (Nei's unbiased estimate; Nei, 1978) between five populations of F. ornus.

Population	A	B	C	D
A				
B	0.0304			
C	0.0860	0.0120		
D	0.0862	0.0403	0.0457	
E	0.0446	0.0497	0.0888	0.0098

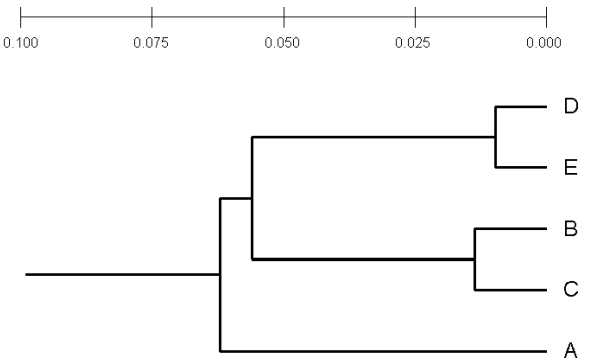

Fig. 3. UPGMA dendrogram based on Nei's genetic distance (Nei 1978, unbiased estimate, Table 3). Population codes are indicated in Fig. 1.

Our RAPD-based AMOVA studies show that most genetic variation in *F. ornus* is distributed within populations rather than between them, indicating a relatively restricted population differentiation as expected in outcrossing species. Of the total genetic variation, 87% was within population and 13% was among population variation. BLUMENRÖTHER *et al*. (2001) observed that species with large geographic ranges (e.g. *Pinus sylvestris*) tend to show little genetic differentiation among populations within regions, but greater differentiation among populations derived from different glacial refugia. Our data seems to confirm a similar pattern holds in *F. ornus* with RAPD markers in the continuous distribution in the region of Serbia and Montenegro.

Outcrossing woody plants generally show high levels of within population genetic diversity with RAPD markers. Overview of patterns of genetic variation within and among plant populations by RAPDs shows that widespread, outcrossing species should have a high proportion of the total genetic variation within populations and a low proportion among populations. It seems that the deciding factors in the distribution of the genetic variability are the method of reproduction and distribution (RAO and HODGKIN, 2002). In the case of *F. ornus,* the share of within populations variability is higher than in the species with discontinuous distribution, and lower than in the species with continuous distribution, with high outcrossing rates, which suggests that the method of reproduction and the distribution also affect the distribution of the genetic variability in *F. ornus* (Table 4). *F. ornus* expresses a constant tendency to expand, which is confirmed by its wide range of distribution. Also, it is an andro-dioecious plant and hence is mainly out-crossing, there is also a potential for inbreeding (DOMMEE *et a*l., 1999), and after BUSSEL (1999) inbreeding decreases the within populations variability.

The genetic distance tended to increase with increasing geographical distances between populations. Significant relationship was found between geographical and genetic distance among populations ($r = 0.63$, $P<0.05$). This is an indication that isolation by distance is the process accounting for the distribution of genetic variation among populations within the region. The greatest value of Nei's unbiased genetic distance (NEI, 1978) among the study populations of *F. ornus,* C and E is 0.089. This value is considerably lower than the value of the highest genetic distance reported by PVINGILA *et al*. 2005 for *Fraxinus excelsior* (0.209). Also, the range of variation in NEI's (1973) gene diversity (h) between the *F. ornus* populations was (0.254-0.343) and it is similar to those reported by PVINGILA et al. (2005) for *Fraxinus excelsior* (0.210-0.365) and by ISHIDA and HIURA (2002) for *Fraxinus lanuginosa* (0.22-0.34).

Our results also confirmed that RAPDs are sufficiently informative and powerful to assess genetic variability in *F. ornus*. The advantages of this method (non-expensive, simple, quick, uses specific DNA fingerprints and, therefore, correlate directly with genomic DNA structure) prevail over its disadvantages (mainly dominant markers, not allowing a clear difference between dominant homozygotes, recessive homozygotes and heterozygotes (LEE *et al.* 2002). In many cases, including the results reported herein, RAPD markers can open the "research runways" to other, more informative markers (VAN TIENDERNEN *et al.,* 2002). These more informative, but more difficult to use and more expensive markers, can be used in more specific and deeper analyses.

Table 4. Comparison of the variability component in different plant species estimated from RAPD patterns with the AMOVA procedure.

species	distribution	population size	% wp	% ap	(I)	reference
Protium glabrum	continuous	3 (15-19 trees)	100			Schierenbeck *et al.*, 1997
Inga thibaudiana	continuous	3 (30 trees)	99.1	0.9		Schierenbeck *et al.*, 1997
Populus tremuloides	continuous	249 trees	97	-	0.65	Yeh *et al.*, 1995
Saxifraga oppositifolia	continuous	10 (189 individuals)	95	5		Gugerli *et al.*, 1999
Dendropanax arboreus	continuous	5 (62 trees)	96.8	3.2		Schierenbeck *et al.*, 1997
Fraxinus ornus	continuous	5 (150 trees)	87.4	12.6	0.44	this study
Araucaria araucana	restricted area	13 (195 trees)	87.2	12.8	0.65	Bakessy *et al.*, 2002
Fitzroya cupressoides	restricted area	89 trees	85.6	-	0.54	Allnutt *et al.*, 1999
Chamaecyparis taiwanensis	discontinuous	2 (60 trees)	85.3	14.7	0.41-0.45	Hwang *et al.*, 2001
Hippophae rhamnoides ssp. rhamnoides	discontinuous	10	85	15		Bartish *et al.*, 1999
Chamaecyparis formosensis	discontinuous	2 (60 trees)	84.9	15.1	0.27-0.39	Hwang *et al.*, 2001
Prunus mahaleb	discontinuous	7 (144 trees)	82.0		0.14-0.21	Jordano and Godoy, 2000
Pinus halepensis	discontinuous	9 (225 trees)	50.6	13.6		Gómez *et al.*, 2001
Saxifraga paniculata	discontinuous	30	43.6	15.4		Reisch *et al.*, 2003

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DIVERZITET VRSTE FRAXINUS ORNUS IZ SRBIJE I CRNE GORE PROCENJENA RAPD METODOM

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Izvod

U radu su korišćeni PCR-RAPD markeri radi procene individualnih varijacija kod vrste *F. ornus*. Ukupno 122 fragmenta je amplificirano korišćenjem 7 prajmera, i među njima je bilo 97 polimorfnih fragmenata. Procenat polimorfnih lokusa se kretao između 53.3% i 74.6% sa prosečnom vrednošću od 63.1%. Srednji diverzitet gena za sve ispitivane populacije je iznosio 0.30, dok je srednji Shannon's index imao vrednost 0.44. Od totalne genetičke varijabilnosti 87% pripada intra-populacionoj varijabilnost, a 13% inter-populacionoj. Mantel test je pokazao značajne korelacije između matrica genetičke i geografske distance. Rezultati ovog rada predstavljaju prvu upotrebu molekularno genetičkih (DNA) markera u cilju određivanja genetičke varijabilnosti populacija *F. ornus*. Odnosi unutar ukupne genetičke varijabilnosti ukazuju na relativno ograničenu populacionu diferencijacije u odnosu na vrednosti koje su očekivane kod stranooplodne vrste. Na osnovu ovih kao i budućih informacija koje se odnose na genetičku strukturu i varijabilnost vrste *F. ornus* potrebno je kreirati strategije za očuvanje genetičkih resursa drvenastih vrsta.

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