S. Bojović, P. Heizmann, P. Pavlović, M. Mitrović, L. Djurdjević & M. Barbero

Genetic and geographical relationships of manna ash populations from Serbia

Abstract

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The objectives of this study were to reveal DNAs (RAPD) individual variation and to analyze the genetic and geographical diversity of *Fraxinus ornus* populations from Serbia. PCR-RAPD variation was examined in three natural populations (90 trees) and the data analysed with genetic softwares (POPGENE, Arlequin and TFPGA) for obtaining parameters of genetic diversity and structure. PCR-RAPD markers reveal individual variation in *F. ornus*. A total of 122 fragments were amplified using 7 primers and of these 77 (63%) fragments were polymorphic. The mean gene diversity for all populations was 0.27 and the Shannon's index was between 0.38 and 0.42. 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.

Introduction

RAPD markers are already accepted in the assessment of genetic and geographical relationships natural populations of forest trees (Khasa & Dancik 1996; Schierenbeck & al. 1997). *Fraxinus ornus* L. (manna ash) is a mainly a Mediterranean forest tree species of wide ecological amplitude. Its wide tolerance of various soils makes it a species of choice for the afforestation of limestone barrens and for soil conservation. In the region of Serbia large-scale afforestation with *F. ornus* was performed during the fifties of the last century for preventing erosion and for 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. The genetic analyses are rare or non-existing for the majority of European deciduous tree species (i.e. *F. ornus*).

The objectives of the present study were to analyze the genetic structure of *F. ornus* by evaluating the degree of intra- and inter-population genetic variation.

Material and methods

Plant material

Altogether 90 adult trees of *F. ornus* L. from natural populations of biogeographically contrasted zones in Serbia were analyzed: Košutnjak (44° 46'N, 20° 27'E): 210 m asl, calcareous and sandy soil, continental-Danubian type of climate, ass. *Fraxino orni-Quercetum virgilianae* Gajić (Tomić 2006); Užice (43° 53'N, 19° 47'E): 700 m asl, calcareous soil, continental climate, ass. *Quercetum frainetto-cerridis* Rudski (Tomić 2006); Pirot (43° 13'N, 22° 33'E): 500 m asl, calcareous soil, continental climate, ass. *Syringo-Carpinetum orientalis* (Rudski) Mišić (Tomić 2006) (Fig. 1).

Fig. 1. The natural range of *F. ornus* and location of sampled populations from Serbia: **A** Košutnjak, **B** Užice, **C** Pirot.

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

The 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). DNA amplification was repeated at least twice, and only reproducible and unambiguous fragments were scored. 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).

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 genetic diversity parameters: Nei's (Nei 1973) gene diversity (h), Shannon's information index (Lewontin 1972) (I), the percentage of polymorphic RAPD loci (p) and Nei's unbiased measures of genetic identity and genetic distance (Nei 1978) were calculated by the software POPGENE 1.31 (Schneider & al. 1997). The partition of total genetic variance, AMOVA by ARLEQUIN 1.1. (Yeh & al. 1999) and Mantel test by TFPGA 1.3 (Miller 2000).

Results

A total of 122 fragments were amplified using 7 primers and of these 77 (63%) fragments were polymorphic. The mean gene diversity for all populations was 0.27 and the Shannon's index was between 0.38 and 0.42. The percentage of polymorphic loci was relatively similar across populations, ranging between 53.3% (A) and 59.8% (C), with an average of 63.1% (Tab. 1). The range of variation in gene diversity (h) between the *F. ornus* populations was from 0.25 to 0.28. The mean gene diversity for all populations was 0.27. The relative degree of diversity in each population as measured by Shannon's index varied from 0.38 (C) to 0.42 (A). The mean Shannon's index for five populations was 0.40.

Our results indicate that 12% of the total genetic diversity is attributable to the differences among populations and 88% is within-population variation (FST in the molecular variance analysis = 0.12, $P<0.05$, tested using 1023 random permutations). All pair wise 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 analyzed by the Nei's unbiased genetic distances (Nei 1978) among populations (Tab. 2). The highest genetic distance was found between the A and C (0.08) and the lowest between the B and C (0.01). The dendogram based on Nei's (1978) unbiased genetic distance matrix reveals a distinct grouping structure among populations (Fig. 2). The Mantel test revealed significant correlation between genetic and geographical distance matrice ($r=0.7$, $P<0.05$).

Table 1. Five *F. ornus* populations: h - gene diversity (Nei 1973), I - Shannon's diversity measure, % p - polymorphic RAPD loci, (s. d.) - standard deviations, MV - meanean values.

Populations	h (s. d.)		I (s. d.)		% p
Košutnjak	0.280	(0.182)	0.418	(0.254)	53.28
Užice	0.271	(0.200)	0.399	(0.283)	54.10
Pirot	0.254	(0.194)	0.381	(0.269)	59.84
MV	0.268	(0.192)	0.399	(0.269)	55.74

Table 2. Five *F. ornus* populations: coefficients of genetic identity (above diagonal) and genetic distances (below diagonal) (Nei 1978).

Population	Košutnjak	Užice	Pirot
Košutnjak	0	0.9700	0.9176
Užice	0.0304	0	0.9881
Pirot	0.0860	0.0120	0

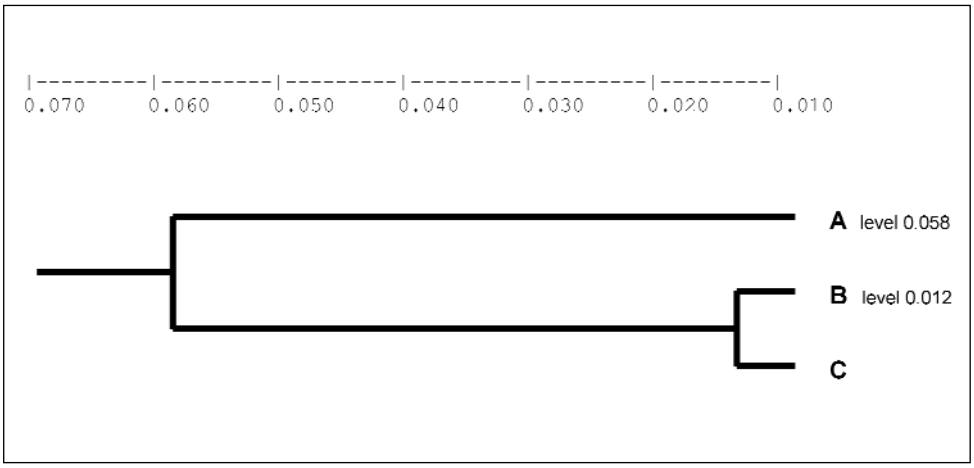

Fig. 2. UPGMA dndrogram based on Nei's genetic distance (Nei 1978): **A** Košutnjak, **B** Užice, **C** Pirot.

Discussion and conclusion

Our results indicate that PCR-RAPD markers are sufficiently informative and powerful to assess genetic variability in *F. ornus*. RAPDs were shown to be sensitive for detecting individual variation of *F. ornus*. A total of 122 fragments were amplified using 7 primers and of these 77(63%) fragments were polymorphic. The mean gene diversity for all populations was 0.27 and the Shannon's index was between 0.38 and 0.42. The partition of total genetic variance indicates a relatively restricted population differentiation as expected in outcrossing species.

Müller-Starck & al. (1992) in his review of results of isoenzymes studies on genetic variation in various coniferous and angiosperm tree species, observed that species with large geographic ranges (such as *Picea abies*, *Pinus sylvestris*, *Fagus sylvatica*) tend to show little genetic differentiation among populations within regions, but greater differentiation among populations derived from different glacial refuge. Our data seems to confirm a similar pattern holds in *F. ornus* with RAPD markers in the continuous distribution in the region of Serbia. Of the total genetic variation, 87% was within population and 13% was among population variation.

The significant relationship between geographical and genetic distance among populations is an indication that isolation by distance is the process accounting for the distribution of genetic variation among populations within the region.

In conclusion, our results indicate that RAPDs are sufficiently informative and powerful to assess genetic variability in *F. ornus*. RAPD markers can open the 'research runways' to other more informative markers. These more informative, but more difficult to use and more expensive markers, can be used in more specific and deeper analyses. However, in the first phase of the assessment of genetic variation in forest tree populations, the use of RAPD markers is quite satisfactory.

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Addresses of the authors:
Srdjan Bojović, Pavle Pavlović, Miroslava Mitrović, Lola Djurdjević, Department of Ecology, Institute for Biological Research "Sinisa Stankovic", D. Stefana 142, 11060 Belgrade, Serbia.
Philippe Heizmann, Reconnaissance Cellulaire et Amélioration des Plantes, UMR 9938, CNRS, INRA, Ecole Normale Supérieure de Lyon, 46 allée d'Italie, 69 364 Lyon cedex 07, France.
Marcel Barbero, Faculté des Sciences et Techniques de St-Jérôme, Institut Méditerranéen d'Ecologie et de Paléoécologie, Case 461, Av. Escadrille N. Niemen 13 397 Marseille, Cedex 20, France.