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Full Length Research Paper

Genetic variation within the olive (*Olea europaea* L.) cultivar Oblica detected using amplified fragment length polymorphism (AFLP) markers

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Oblica is the predominant olive cultivar in Croatia, spread widely in all the olive growing regions. Morphological variability within the cultivar is well documented but often it has been attributed to environmental factors rather than to genetic ones. In order to investigate intracultivar variability on the molecular level, olive samples from 4 growing regions in Croatia (South Dalmatia, Middle Dalmatia, Kvarner islands and Iner Dalmatia) were screened for amplified fragment length polymorphisms (AFLP). DNA was extracted from leaf tissue and 9 AFLP primer combinations were used. Genetic distances between individual trees were calculated using Dice similarity coefficient and the dendrogram based on UPGMA cluster analysis was constructed. Preliminary results of AFLP analysis indicate that the cultivar Oblica can be regarded as mixture of clonal variants.

Key words: Olive, amplified fragment length polymorphism, polymorphism.

INTRODUCTION

The olive tree has been cultivated in Croatia for over 2000 years (Miljkovic, 1991). Olive growing region stretches from 42° 24' to 45° 30' N representing the northern boundaries of olive cultivation. Olive growing region in the republic of Croatia has been divided into 6 subregions based on climatic and cultivar classification (Miljkovic et al., 1999). The autochthonous cultivar Oblica makes up 75% of the total number of olive trees in Croatia (Strikic et al., 2007). It is cultivated in all subregions, where it occurs under a series of synonyms (Zec, 1950). The influence of edaphic and climatic factors on olive tree phenotype is well known. The records of morphological and pomological differences among individual trees gave impulse to the studies of intracultivar variability of this valuable olive cultivar. Morphological description has been considerably upgraded by the advent of molecular techniques used for precise genetic characterization (Bassi et al., 2002). DNA-based markers are particularly useful for the correct identification of varieties, due to their independence of environmental conditions and several of them have been successfully applied for olive for example, random amplified polymorphic DNA (RAPDs) (Bogani et al., 1994), amplified fragment length polymorphism (AFLPs) (Angiolillo et al., 1999), sequence characterized amplified regions (SCARs) (Busconi et al., 2006), inter simple sequence repeats (ISSRs) (Hess et al., 2000), single nucleotide polymorphism (SNPs) (Reale et al., 2006) and simple sequence repeats (SSRs) (Poljuha et al., 2008). AFLP marker technology was confirmed to be a powerful tool not only for studying variation between populations of the genus *Olea* as shown by Angiolillo et al. (1999),

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Abbreviations: RAPDs, Random amplified polymorphic DNA; **AFLPs,** amplified fragment length polymorphism; **ISSRs,** inter simple sequence repeats; **SSRs,** simple sequence repeats; **SNPs,** single nucleotide polymorphism; **SCARs,** sequence characterized amplified regions; **CTAB,** cetyltrimethyl ammonium bromide; **PCR,** polymerase chain reaction.

Table 1. Total number of amplified AFLP markers and the number of polymorphic markers.

AFLP primer combination	Total number of amplified AFLP markers	No. of polymorphic markers	% polymorphic markers
P*-AAC/M-CAA	44	11	25.00
P*-AAC/M-CTC	19	8	42.11
P*-AAC/M-CAT	28	8	28.58
P*-AAC/M-CAC	22	6	27.28
P*-AGA/M-CAT	45	17	37.78
P*-AGA/M-CTT	25	1	4.00
P*-ACA/M-CAA	29	9	31.04
P*-ACA/M-CAC	19	6	31.58
P*-ACA/M-CAT	18	5	27.78
TOTAL	249	71	28.52

Table 2. Primer sequences used in AFLP analysis.

Primer	Primer sequences
*Pst*I* + ACA	Cy5GAC TGC GTA CAT GCA GAC A
*Pst*I* + AGA	Cy5GAC TGC GTA CAT GCA GAG A
*Pst*I* + AAC	Cy5GAC TGC GTA CAT GCA GAA C
*Mse*I* + CAA	GAT GAG TCC TGA GTA ACA A
*Mse*I* + CAC	GAT GAG TCC TGA GTA ACA C
*Mse*I* + CAT	GAT GAG TCC TGA GTA ACA T
*Mse*I* + CTT	GAT GAG TCC TGA GTA ACT T
*Mse*I* + CTC	GAT GAG TCC TGA GTA ACT C

but also for characterising intraspecific variation among cultivated accessions of *Olea europaea* L. subsp. *europaea*. In order to investigate intracultivar variability of cultivar Oblica on the molecular level, olive samples from different growing regions in Croatia were screened for AFLP polymorphisms. These studies will facilitate clonal selection of Oblica cultivar adding much to the conservation, evaluation and increased use of olive genetic resources in Croatia.

MATERIALS AND METHODS

Plant material

12 trees of Oblica cultivar grown in different agroclimatic conditions were selected (Table 1): Krk, Punat 1 and Punat 2 - northern Adriatic; Banici, Slano 1 and Slano 2 - southern Dalmatia; Sestanovac, Blato na Cetini and Trnbuse - Inner Dalmatia and Kastel Novi, Kastel Stari 1 and Kastel Stari 2 from the olive collection of the Institute for Adriatic Crops in split in mid-Dalmatia.

AFLP procedure

Genomic DNA was extracted from young leaves in the Laboratory of Plant Biotechnology and Breeding, University of Ljubljana, by a modified cetyltrimethyl ammonium bromide (CTAB) method (Kump and Javornik, 1992). The AFLP procedure with fluorescence-based detection was performed as previously reported by Bandelj et al. (2004). Genomic DNA was restricted with PstI and MseI enzymes and linked to PstI and MseI adaptors. Restricted and ligated DNA was then pre-amplified using PstI and MseI primers with one selection nucleotide. Selective amplification was performed on the pre–amplified fragment mixture using a total of 9 primer combinations that had 3 selective nucleotides (P-AAC/M-CAA, P-AAC/M-CTC, P-AAC/M-CAT, P-AAC/M-CAC, P-AGA/M-CAT, P-AGA/M-CTT, P-ACA/M-CAA, P-ACA/M-CAC and P-ACA/M-CAT) (Table 2). Pre-amplification was carried out in a GeneAmp 9700 thermal cycler (Applied Biosystem) using 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. Selective amplification was then carried out using the following temperature profile: 13 cycles of 94°C for 30 s, 65°C for 30 s with a decrease of annealing temperature of 0.7°C per cycle and 72°C for 1 min, followed by 23 cycles at the annealing temperature of 56°C. Amplified and denatured polymerase chain reaction (PCR) products were separated in a 7.5% polyacrylamide denaturing gel containing 7 M urea. Electrophoresis was performed on automated ALFexpresssII sequencer (Amersham Bioscience) and AFLP markers were finally analised using the software package ALFwin^{TM} fragment analyser 1.01 (Amersham Bioscience).

Data analysis

AFLP results were scored for presence (1) and absence (0) of amplified fragments. Pairwise genetic similarities were calculated using Dice similarity coefficient (Dice, 1945; Neil and Li, 1979). Dendrogram was constructed from the resultant matrix via the unweighted pair group method with the arithmetic averages algorithm (UPGMA) method using the NTSYS computer package (Rohlf, 1998).

RESULTS AND DISCUSSION

A total of 249 AFLP markers were analyzed, of which 71 were polymorphic (28.5%). The number of amplified fragments varied from 18 (P-ACA/M-CAT) to 44 (P-AAC/M-CAA) with an average of 27.6 fragments per primer combination. The average percentage of polymorphism ranged from 4% for P-AGA/M-CTT to 42% for P-AAC/M-CTC primer combination (Table 1). Detection range was from 50 to 500 bp (not shown). Genetic similarity was calculated using Dice similarity coefficient and the den-

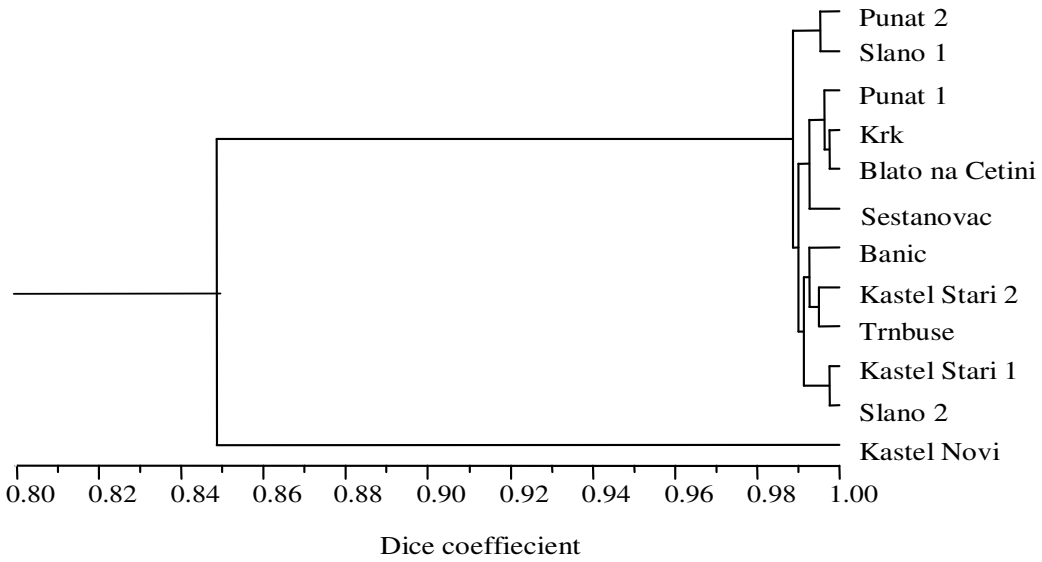

Figure 1. UPGMA dendrogram based on dice similarity matrix between samples of olive cv. Oblica.

drogram based on UPGMA cluster analysis was constructed (Figure 1). As shown by the dendrogram, the sample from Kastel Novi was the most distant from other olive samples divided at the similarity index value of 0.85 from the rest of the studied samples. The results suggest that the sample from Kastel Novi does not belong to Oblica group even though some authors assigned it there. The rest of samples showed similarity coefficient ranging from 0.9850 to 0.9975 (Figure 1). Resta et al. (2002) obtained a similarity index of 0.97 for Leccino cultivars from different growing regions. However, no polymorphism has been found within the cultivars Ogliarola Gargancia and Ogliarola Barese. Our studies showed intracultivar variabilty within the limits of clonal variability. Uzun et al. (2009) reported citrus clonal variability at the level 0.00 to 0.20, while Ozkaya et al. (2006) found the differences among cultivars in the range of 45 to 75%. They also showed an intracultivar variability of trees grown in the same and different agroclimatic conditions. The samples Krk, Punat 1 and Punat 2 from the northern Adriatic growing regions showed the highest intragroup variability and those from Sestanovac, Blato na Cetini and Trnbusi from Iner Dalmatia the lowest. Using RAPD markers, Lave et al. (1999) recorded the polymorphism of the olive cultivar, Nabali Baladi, grown both in the same and different agroclimatic conditions.

Our study showed intracultivar polymorphism in Oblica grown both under the same and different agroclimatic conditions. Obtained polymorphism did not exceed the limits of clonal variability. This study may provide the basis for further clonal selection of this cultivar and detection of the origins and association between cv. Oblica, some other cultivars and wild olive ecotypes. These are, at the same time, the first results of the utilization of molecular markers in olive studies in Croatia.

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