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MICROSATELLITES AS A POWERFUL TOOL FOR IDENTIFICATION OF OLIVE (*OLEA EUROPAEA* L.) PLANTING MATERIAL IN NURSERIES

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ABSTRACT

Microsatellites were applied to confirm the varietal identity of thirteen olive samples from a private nursery. Allelic profiles of samples were compared to genotyped reference varieties from three olive germplasm collections. Genotyping data of two microsatellite loci (ssrOeUA-DCA3 and ssrOeUA-DCA16) allowed discrimination of ten olive samples and the identification of six olive varieties (Koreneiki, Manaki, Tsounati, Kalamon, Ascolana Tenera, Picual). Mislabelling of two olive accessions (Manzanilla de Sevilla, Coratina) was observed and the lack of reference varieties Nostrana Bitontina and Miloelia hindered the identification of two accessions from the nursery. Accessions denominated as Miloelia and Gaiduroelia were of identical genotype. Different allelic profiles of the Frantoio reference variety were observed from two national olive germplasm collections. Microsatellites were found to be a valuable tool for varietal identity confirmation, in nurseries as well as for screening and managing olive germplasm collections.

Key words: *Olea europaea* L., microsatellites, identification, variety

MICROSATELLITI QUALI STRUMENTI APPROPRIATI PER L'IDENTIFICAZIONE DI MATERIALE DA COLTIVAZIONE DI OLIVI (*OLEA EUROPAEA* L.) IN VIVAI

SINTESI

Microsatelliti sono stati applicati per confermare l'identità delle varietà di tredici campioni d'olivo provenienti da un vivaio privato. I profili allelici sono stati confrontati con le varietà genotipizzate di riferimento derivanti da tre banche genetiche. La genotipizzazione su due locus microsatellitari (ssrOeUA-DCA3 e ssrOeUA-DCA16) ha reso possibile la separazione di dieci campioni di olivo e l'identificazione di sei varietà (Koreneiki, Manaki, Tsounati, Kalamon, Ascolana Tenera, Picual). Gli autori hanno verificato che due dei campioni sono stati contrassegnati erroneamente (Manzanilla de Sevilla, Coratina), mentre causa la mancanza di varietà di riferimento di Nostrana Bitontina e Miloelia non è stato possibile identificare due campioni del vivaio. L'analisi dei campioni delle varietà Miloelia e Gaiduroelia ha evidenziato un genotipo identico. In campioni della varietà di riferimento Frantoio provenienti da due banche nazionali genetiche sono stati riscontrati differenti profili allelici. I microsatelliti si sono rivelati strumenti appropriati per la conferma dell'identità delle varietà provenienti da vivai e per la verifica delle fonti genetiche di olivo provenienti dalle banche.

Parole chiave: *Olea europaea* L., microsatelliti, identificazione, varietà

INTRODUCTION

The olive tree is one of the oldest cultivated fruit species in the Mediterranean Basin and the production of olive oil is of great economic importance for the region. The olive oil industry, nurseries and olive growers are all very interested in an accurate identification system of olive varieties, since a choice of varietal structure significantly contributes to the quality of oil produced. Olive is a vegetatively propagated plant and there are more than 1,000 olive varieties under cultivation, which have originated from selections made by growers over many centuries (Rugini & Baldoni, 2005). The identification of varieties is hindered and uncertain due to the long juvenile stage of the olive tree, the presence of different types and clones and the use of many synonyms and homonyms. Correct identification of olive varieties is important especially in nurseries in which high yielding clones are propagated. To prevent mislabelling of plants, determining the identity of the mother plants is necessary before beginning clone propagation. The management and certification of olive planting material therefore requires the application of a fast and reliable method of identifying cultivated genotypes, particularly at the national and regional levels. The introduction of new methodologies into the olive certification scheme will accelerate and optimise the identification process, by allowing the fingerprinting of each genotype at any developmental stage and independently of environmental factors.

Classical approaches for discriminating among olive varieties are based on phenotypic observation and description of morphological markers. The limitations of these markers, which hamper their use in a reliable identification process, are: strong influence of environmental factors and cultivation conditions, subjective evaluation of markers, and the time required for the analysis. By developing biochemical markers, structural gene products such as isozymes were first successfully applied in varietal identification (Trujillo *et al.*, 1995). More recently, PCR-based techniques have been introduced and the first RAPD markers have been used for the molecular characterization and discrimination of olive varieties (Ganino *et al.*, 2006). AFLPs have also been reported as an applicable marker system in the varietal identification process and these markers seems to be more suitable for genotyping olive varieties (Belaj *et al.*, 2003), but the development of microsatellite markers in olives (Rallo *et al.*, 2000; Sefc *et al.*, 2000; Carriero *et al.*, 2002; Cipriani *et al.*, 2002) has provided an improved approach for varietal identification (Bandelj *et al.*, 2002; Lopes *et al.*, 2004). Compared with other DNA fingerprinting techniques, microsatellites are extremely polymorphic, and they have the potential for providing unique fingerprints for each distinct genotype, a useful means of identifying different varieties.

The aim of this paper was to test the applicability of microsatellite markers in identifying olive trees belonging to a set of Greek, Spanish and Italian olive varieties and to confirm the genetic identity of the mother plants in the nursery for propagation purpose.

MATERIALS AND METHODS

Plant material

Thirteen olive samples from Greece (Koreneiki 1, Manaki, Kalamon, Miloeliá, Tsunati, Gaiduroeliá, Koreneiki 2), Spain (Manzanilla de Sevilla, Picual) and Italy (Frantoio Fs-17, Nostrana Bitontina, Coratina, Ascolana Tenera) included in a genotyping analysis were provided by a private nursery. Olive varieties used in the analysis as 'reference' for varietal identification and comparison were obtained from the World Olive Germplasm Bank of Cordoba (Spain), the Plant Protection Institute of Thessaloniki-Agricultural Research Station of Khalkidiki (Greece) and the national olive collection of Strunjan (Slovenia). Olive accessions and reference varieties used in microsatellite analysis are listed in Table 1.

DNA isolation and amplification of microsatellites

Olive DNA extraction by a modified CTAB method and amplification of microsatellites with fluorescence-based detection was performed as previously reported by Bandelj *et al.* (2004a). Two primer pairs for olive microsatellite loci ssrOeUA-DCA3 and ssrOeUA-DCA16 (Sefc *et al.*, 2000) were redesigned by adding an universal M13(-21) tail (5'-TGT AAA ACG ACG GCC AGT-3') to their 5' ends (Schuelke, 2000). The third, universal M13(-21) primer was labelled with Cy5, allowing incorporation of fluorescence dye into the PCR fragment and its subsequent detection. Amplification reactions were carried out in a total volume of 10 µl, containing 20 ng genomic DNA, 1X supplied PCR buffer (Promega), 0.2 mM of each dNTP, (Roche), 0.25 unit of *Taq* DNA polymerase (Promega), 0.5 µM of sequence-specific reverse primer, 0.5 µM of fluorescence labelled universal M13(-21) primer and 0.125 µM of forward primer with M13(-21) tail. Amplification was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) and the conditions of the two-round PCR amplification were as follows: 94 °C (5 min), then 26 cycles at 94 °C (30 s) / 50 °C (ssrOeUA-DCA3) or 52 °C (ssrOeUA-DCA16) (45 s) / 72 °C (45 s), followed by 8 cycles at 94 °C (30 s) / 53 °C (45 s) / 72 °C (45 s (ssrOeUA-DCA3), 1 min 30 s (ssrOeUA-DCA16)), and a final extension step at 72 °C for 10 min. The PCR products were denaturated by adding an equal volume of formamide loading dye (98% formamide, dextran blue 5 mg/ml) and by heating at 94 °C for 4 min. Amplification products were separated on a 7.5% polyacrylamide denaturing gel, containing 7 M

urea. Electrophoresis was performed on short glass plates of 80 mm separating length, using an automated ALFexpressII sequencer (Amersham Biosciences). Fluorescence signals were collected every 1 s and stored in a computer. A fluorescence labelled molecular size marker (Cy5 Sizer 50–500; Amersham Biosciences) comprising 10 fragments in the size range of 50 to 500 bp was used as an external size marker. An in-house amplified and labelled PCR fragment from a plasmid template of the known size (100 or 150 bp) was added to each sample as an internal standard. Allele sizes were determined using the software package ALFwin™ Allele Locator 1.01 (Amersham Biosciences).

RESULTS

To confirm the identity of olive trees that have been chosen as mother plants for propagation of cuttings in a

Tab. 1: List of olive accessions and reference varieties included in genotyping analysis, and observed genotypes (allele sizes in bp) at loci ssrOeUA-DCA3 and ssrOeUA-DCA16. The letters indicate the olive GenBank (S = Standard, Gr = Greece, Sp = Spain, SI = Slovenia). Legend: n = number of amplified alleles; n_G = number of observed genotypes; n_{UG} = number of unique genotypes per locus.
Tab. 1: Seznam akcesij oljk in referenčnih sort, vključenih v genotipizacijsko analizo, ter opaženi genotipi (dolžine alelov v bp) na lokusih ssrOeUA-DCA3 in ssrOeUA-DCA16. Črke označujejo gensko banko oljk (S = Standard, Gr = Grčija, Sp = Španija, SI = Slovenija). Legenda: n = število namnoženih alelov; n_G = število opaženih genotipov; n_{UG} = število edinstvenih genotipov na posameznem lokusu.

No.	Olive accession / reference variety	Samples and ref. genotypes (Gen Bank and register number)	ssrOeUA-DCA3	ssrOeUA-DCA16
1	Koreneiki1	*	237:237	147:151
2	Koreneiki2	*	237:237	147:151
3	Koreneiki S-Sp	Cordoba, R218	237:237	147:151
4	Koreneiki S-Gr	Thessaloniki, Greece	237:237	147:151
5	Kalamon	*	230:251	125:127
6	Kalamon S-Sp	Cordoba, R105	230:251	125:127
7	Kalamon S-Gr	Thessaloniki, Greece	230:251	125:127
8	Frantoio Fs-17	*	230:241	151:155
9	Frantoio S-Gr	Thessaloniki, Greece	241:247	147:155
10	Frantoio S-SI	Strunjan, Slovenia	235:241	151:157
11	Manaki	*	241:243	151:155
12	Manaki S-Gr	Thessaloniki, Greece	241:243	151:155
13	Manzanilla de Sevilla	*	237:247	125:176
14	Manzanilla de Sevilla S-Gr	Thessaloniki, Greece	243:251	155:176
15	Manzanilla S-Sp	Cordoba, R21	243:251	155:176
16	Picual	*	237:247	127:155
17	Picual S-Gr	Thessaloniki, Greece	237:247	127:155
18	Picual S-Sp	Cordoba, R662	237:247	127:155
19	Ascolana Tenera	*	230:247	127:155
20	Ascolana Tenera S-SI	Strunjan, Slovenia	230:247	127:155
21	Coratina	*	237:243	151:157
22	Coratina S-Sp	Cordoba, R79	237:241	151:174
23	Tsunati	*	230:241	125:147
24	Tsounati S-Gr	Thessaloniki, Greece	230:241	125:147
25	Gaiduroelia	*	247:251	155:182
26	Gaiduroelia S-Gr	Thessaloniki, Greece	241:251	125:127
27	Nostrana Bitontina	*	230:251	155:176
28	Miloelia	*	247:251	155:182
		n	7	9
		n_G	13	11
		n_{UG}	5	3

* Samples for identification

private nursery, two published loci, ssrOeUA-DCA3 and ssrOeUA-DCA16 (Sefc *et al.*, 2000), were chosen. Previous studies (Bandelj *et al.*, 2004b; Lopes *et al.*, 2004) have shown that these two microsatellite loci are extremely powerful in genotyping olive varieties, due to their high polymorphic information content (PIC value), low probability of identity (PI value) and high effective number of alleles. Microsatellites were successfully amplified, revealing a total of sixteen alleles in all twenty-eight olive samples. Allele sizes were accurately determined with the help of a fluorescent labelled molecular size marker as an external standard and by identification of an internal standard. The size range of the detected alleles was similar to the range reported by Lopes *et al.* (2004) and La Mantia *et al.* (2005).

The number of observed genotypes at locus ssrOeUA-DCA3 was 13, while 11 genotypes were found at locus ssrOeUA-DCA16 (Tab. 1). Comparison of DNA profiles of reference varieties from Greece, Spain and Slovenia was performed and identical genotypes of the same variety from Greece or Spain were observed in four olive varieties (Koreneiki, Kalamon, Manzanilla de Sevilla, Picual). Reference variety Frantoio from Slovenia and Greece showed different allelic patterns at two loci, which could be only explained as mislabelling of plants in the collection, since seven different alleles were found in two accessions at the analysed loci (data not shown). The mislabelling probably occurred in Greece, because the genotyping results of Frantoio variety in Slovenia (Bandelj *et al.*, 2002) and Spain (De la Rosa *et al.*, 2004) showed the same allelic profiles at other loci (ssrOeUA-DCA4 and ssrOeUA-DCA9).

The allelic profiles of the thirteen olive samples from the commercial nursery were then compared with profiles of reference varieties from olive germplasm collections. Genotyping data allowed discrimination of ten olive samples, but the identity of only six olive varieties (Koreneiki, Manaki, Tsounati, Kalamon, Ascolana Tenera, Picual) was confirmed.

Samples Koreneiki 1 and Koreneiki 2 showed the same allelic patterns at both loci, thus indicating genetic homogeneity of the analysed trees. Identical genotypes were also observed in Miloelia and Gaiduroelia samples. These two samples could be the same variety, but they did not match the reference variety Gaiduroelia from Thessaloniki. To confirm their identity, more microsatellite loci should be characterized.

Mislabelling was also found of sample Manzanilla de Sevilla, one of the main Spanish varieties, widely distributed in Andalusia and Catalonia. Previous fingerprinting analyses of this variety by AFLP and RAPD markers have shown the existence of intravarietal polymorphism (Belaj *et al.*, 2004). Cipriani *et al.* (2002) assigned these differences to somatic mutations occurring during the process of olive vegetative propagation or to mislabelling of plants in collections.

Another mislabelling case was observed in the Coratina sample, which was characterised by a unique genotype [237:243] at locus ssrOeUA-DCA3. Two samples, Nostrana Bitontina and Miloelia, could not be identified in this work, owing to the lack of standard varieties in olive germplasm collections. These two varieties are probably known at local level only, or their denomination could be uncertain.

Fs-17, an Italian clone of Frantoio obtained by breeding programme and selected for high productivity and very early oil formation, did not match two standards (Fontanazza *et al.*, 1998) as expected. Three accessions of Frantoio shared only two alleles (241 and 151 bp) of eight detected.

DISCUSSION AND CONCLUSIONS

The need for an accurate discriminating system for olives is often reported in the literature, since the identification of olive varieties is important for management of olive germplasm collections, as well as variety protection and certification of propagated plants in nurseries. The introduction of DNA fingerprinting techniques has provided a high resolution system of discriminating and, compared to other DNA based markers, microsatellites are reported as being particularly attractive for genotyping plants, because the level of polymorphism detected is much higher than that detected with any other molecular marker assay (Jakše *et al.*, 2001). Microsatellite marker systems have been reported to be a valuable tool in genotyping olive varieties. The amplification of three microsatellite loci were sufficient to discriminate among nineteen olive varieties in Slovenia (Bandelj *et al.*, 2002) and the high discriminatory capacity of microsatellite markers has also been reported by Rallo *et al.* (2000), Cipriani *et al.* (2002), Lopes *et al.* (2004) and La Mantia *et al.* (2005).

The high level of mislabelling plants observed in this work confirmed the need to establish a reliable identification system in olives. The greatest challenges in the varietal identification process are: to establish an effective and low cost method for analyzing plants, and to obtain results that are comparable among different laboratories and countries. The choice of a microsatellite marker system seems to be suitable, since highly informative microsatellite loci have been identified and microsatellite genotyping results are comparable and easily exchangeable among laboratories. The confusion in naming olive varieties, different clonal selections of economically important olive varieties and the presence of vast number of synonyms and homonyms supports and demands the establishment and availability of a worldwide genotyping database of reference olive varieties based on microsatellite data. The allelic profiles of some important olive varieties from national collections published in this work could be used by other research

groups to check the identity of varieties presented here with varieties of the same name in other locations.

In conclusion, microsatellites could be successfully used for confirmation of the varietal identity of olive trees in nurseries, as well as for screening and managing olive germplasm collections.

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MIKROSATELITI KOT PRIMERNO ORODJE ZA IDENTIFIKACIJO SADILNEGA MATERIALA OLJK (*OLEA EUROPAEA* L.) V DREVESNICAH

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POVZETEK

Mikrosatelite smo uporabili za potrditev identitete sort trinajstih vzorcev oljk iz zasebne drevesnice. Alelne profile vzorcev smo primerjali z genotipiziranimi referenčnimi sortami iz treh genskih bank. Genotipizacija na dveh mikrosatelitskih lokusih (ssrOeUA-DCA3 in ssrOeUA-DCA16) je omogočila ločitev desetih vzorcev oljk in identifikacijo šestih sort (Koreneiki, Manaki, Tsounati, Kalamon, Ascolana Tenera, Picual). Pri dveh vzorcih (Manzanilla de Sevilla, Coratina) smo ugotovili napačno označitev, nerazpoložljivost referenčnih sort Nostrana Bitontina in Miloelia pa je preprečila identifikacijo dveh vzorcev iz drevesnice. Vzorca sort Miloelia in Gaiduroelia sta imela identičen genotip. Pri referenčni sorti Frantoio smo ugotovili različne alelne profile vzorcev iz dveh nacionalnih kolekcij. Mikrosateliti so se pokazali kot primerno orodje za potrjevanje identitete sort v drevesnicah kot tudi za pregled in upravljanje genskih virov oljk v kolekcijah.

Ključne besede: *Olea europaea* L., mikrosateliti, identifikacija, sorte

REFERENCES

Bandelj, D., J. Jakše & B. Javornik (2002): DNA fingerprinting of olive varieties by microsatellite markers. Food Technol. Biotechnol., 40, 185-190.

Bandelj, D., J. Jakše & B. Javornik (2004a): Amplification of fluorescent-labelled microsatellite markers in olives by a novel, economic method**.** Acta Agric. Slov., 83, 323–329.

Bandelj, D., J. Jakše & B. Javornik (2004b): Assessment of genetic variability of olive varieties by microsatellite and AFLP markers. Euphytica, 136, 93–102.

Belaj, A., Z. Satović, G. Cipriani, L. Baldoni, R. Testolin, L. Rallo & I. Trujillo (2003): Comparative study of the discriminating capacity of RAPD, AFLP and SSR markers and their effectiveness in establishing genetic relationship in olive. Theor. Appl. Genet., 107, 736–744.

Belaj, A., L. Rallo, I. Trujillo & L. Baldoni (2004): Using RAPD and AFLP markers to distinguish individuals obtained by clonal selection of 'Arbequina' and 'Manzanilla de Sevilla' olive. HortSci., 39, 1566–1570.

Carriero, F., G. Fontanazza, F. Cellini & G. Giorio (2002): Identification of simple sequence repeats (SSRs) in olive *(Olea europaea* L.). Theor. Appl. Genet., 104, 301–307.

Cipriani, G., M. T. Marrazzo, R. Marconi, A. Cimato & R. Testolin (2002): Microsatellite markers isolated in olive *(Olea europaea* L.) are suitable for individual fingerprinting and revealing polymorphism with ancient cultivars. Theor. Appl. Genet., 104, 223–228.

De la Rosa, R., C. M. James & K. R. Tobutt (2004): Using microsatellites for paternity testing in olive progenies. HortSci., 39, 351–354.

Fontanazza, G., F. Bartolozzi & G. Vergari (1998): Olivo Fs-17. Riv. Fruttic. Ortofloric., LX, p. 61.

Ganino, T., G. Bartolini & A. Fabbri (2006): The classification of olive germplasm – A review. J. Hortic. Sci. Biotechnol., 81, 319–334.

Jakše, J., K. Kindlhofer & B. Javornik (2001): Assessment of genetic variation and differentiation of hop genotypes by microsatellite and AFLP markers. Genome, 44, 773–782.

La Mantia, M., O. Lain, T. Caruso & R. Testolin (2005): SSR-based DNA fingerprints reveal the genetic diversity of Sicilian olive *(Olea europaea* L.) germplasm. J. Hortic. Sci. Biotechnol., 80, 628–632.

Lopes, M. S., D. Mendonça, K. M. Sefc, F. Sabino Gil & A. Da Câmara Machado (2004): Genetic evidence of intra-cultivar variability within Iberian olive cultivars. HortSci., 39, 1562–1565.

Rallo, P., G. Dorado & A. Martin (2000): Development of simple sequence repeats (SSRs) in olive tree *(Olea europaea* L.). Theor. Appl. Genet., 101, 984–989.

Rugini, E. & L. Baldoni (2005): *Olea europaea* Olive. In: Litz, R. E. (ed.): Biotechnology of Fruit and Nut Crops. CABI Publishing, Oxfordshire, p. 404–428.

Sefc, K. M., M. S. Lopes, D. Mendonça, M. Rodrigues dos Santos, M. Laimer da Câmara Machado & A. da Câmara Machado (2000): Identification of microsatellite loci in olive *(Olea europaea* L.) and their characterization in Italian and Iberian olive trees. Mol. Ecol., 9, 1171–1173.

Schuelke, M. (2000): An economic method for the fluorescent labelling of PCR fragments. Nat. Biotechnol., 18, 233–234.

Trujillo, I., L. Rallo & P. Arús (1995): Identifying olive cultivars by isozyme analysis. J. Am. Soc. Hortic. Sci., 120, 318–324.