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Amplification of fluorescent-labelled microsatellite markers in olives by a novel, economic method

Dunja BANDELJ[1], Jernej JAKŠE[2], Branka JAVORNIK[3]

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ABSTRACT

A novel method for the economic fluorescent labelling of PCR products and its features are described. To investigate the efficiency of the method for amplification of olive microsatellite markers, PCR conditions of three loci were optimised. The results showed that the method can be successfully applied in genotyping studies of olive varieties.

Key words: microsatellite, fluorescent detection, universal M13(-21) primer, olive

IZVLEČEK

NAMNOŽEVANJE FLUORESCENTNO OZNAČENIH MIKROSATELITSKIH MARKERJEV OLJKE Z NOVEJŠO, EKONOMIČNO METODO

Opisana je novejša metoda ekonomičnega fluorescentnega označevanja PCR molekul in nekatere njene posebnosti. Za preučitev uspešnosti metode pri namnoževanju mikrosatelitov oljke smo uporabili tri lokuse, katerih PCR pogoje smo predhodno optimizirali. Rezultati so pokazali, da je metoda lahko uspešno uporabljena v študijah genotipizacije sort oljk.

Ključne besede: mikrosateliti, fluorescentna detekcija, univerzalni M13(-21) začetni oligonukleotid, oljka

[1] Research assistant, B. Sc., SI-5000, Koper, Garibaldijeva 18

[2] Assistant Prof., Ph. D., SI-1111, Ljubljana, Jamnikarjeva 101, P.O. Box 2995

[3] Prof., Ph. D., SI-1111, Ljubljana, Jamnikarjeva 101, P.O. Box 2995

1 INTRODUCTION

Microsatellites, as one of the most popular marker systems, are widely used in plant genetic research for diversity studies, genetic map development, linkage analyses, marker-assisted selection and fingerprinting studies. Their wide usage is based on their good properties: co-dominant nature, high abundance in eucaryotic genomes, robustness, hypervariability, high information content, and amenability to automation (Morgante and Olivieri, 1993; Powell et al., 1996; Weising et al., 1998). Microsatellites are particularly attractive for genotyping individuals because the level of polymorphism detected by them is higher than that detected with any other molecular marker assay (Powell et al., 1996; Russell et al., 1997; Jakše et al., 2001).

There is great interest in developing a robust, reproducible, cheap and high throughput methodology for the genotyping plants of commercial value. The microsatellite amplification protocol is simple once primers for a defined locus have been designed. After amplification of microsatellites by PCR, the products are separated on high resolution polyacrylamide gels by electrophoresis, and detection of amplified alleles is achieved by three different principles: 1) radioactively, 2) silver staining and 3) a laser induced fluorescence detection system. The employment of fluorescent labelled microsatellite primers and laser detection (e.g. automated sequencer) in genotyping procedures significantly improves the throughput and automatisation (Wenz et al., 1998). The use of microsatellites, however, can be costly due to the high price of the fluorescent label, which must be carried by one of the primers in the primer pair. The cost of microsatellite assay increases substantially if a large number of loci have to be tested. In order to overcome this financial difficulty, Schuelke (2000) introduced a novel procedure in which three primers are used for the amplification of a defined microsatellite locus: a sequence-specific forward primer with M13(-21) tail at its 5' end, a sequence-specific reverse primer and the universal fluorescent-labelled M13(-21) primer. To date, the system has been used successfully with tiger snake (Scott et al., 2001) microsatellite markers and could be a valuable tool for genotyping plants.

The objective of this paper is to introduce a simple, less expensive and high-throughput method for amplification and detection of fluorescent-labelled DNA markers and to test its applicability in genotyping olive varieties.

2 MATERIAL AND METHODS

2.1 Plant material

Nineteen varieties from the national olive collection orchard in Strunjan, Slovenia, were included in the molecular analysis. Analysed olive varieties are listed in Table 1.

Table 1 List of varieties and geographical origin of analysed olives.

Variety	Origin
Arbequina	Spain
Ascolana tenera	Italy
Buga	Slovenia
Cipressino	Italy
Črnica	Slovenia
Frantoio	Italy-Tuscany
Istrska belica	Slovenia
Itrana	South Italy
Leccino	Italy-Tuscany
Leccione	Italy-Tuscany
Maurino	Italy-Tuscany
Nocellara del Belice	Italy-Sicily
Pendolino	Italy-Tuscany
Picholine	France
Santa Caterina	Italy-Tuscany
Štorta	Slovenia
Zelenjak	Uncertain
Samo	Slovenia
Athena	Uncertain

2.2 Microsatellite assay

Genomic DNA was extracted from fresh olive leaves by a modified CTAB method following the procedure described by Kump & Javornik (1996).

Three primer pairs for olive microsatellite loci ssrOeUA-DCA3, ssrOeUA-DCA10 and ssrOeUA-DCA16 (Sefc et al., 2000) were synthesised by MWG-Biotech AG. For each primer pair, the sequence of the original published forward primer was 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 fluorescence detection. The sequences of microsatellite primer pairs used in this study are shown in Table 2.

Table 2: Primer sequences of three microsatellite loci and sequence of the fluorescent-labelled universal M13(-21) primer.

Primer	Primers: 5' to 3'
ssrOeUA-DCA3	F: TGTAAAACGACGGCCAGTCCCAAGCGGAGGTGTATATTGTTAC R: TGCTTTTGTCGTGTTTGAGATGTTG
ssrOeUA-DCA10	F: TGTAAAACGACGGCCAGTCGTGACCACCTAAATCCGCCCC R: CTGTCCAGAGCTAAAGGTTTCG
ssrOeUA-DCA16	F: TGTAAAACGACGGCCAGTTTAGGTGGGATTCTGTAGATGGTTG R: TTTTAGGTGAGTTCATAGAATTAGC
Universal M13(-21)	Cy5-TGTAAAACGACGGCCAGT

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. The amplification protocol for loci ssrOeUA-DCA3, ssrOeUA-DCA10, and ssrOeUA-DCA16 according to Bandelj et al. (2002), with some modifications, was performed in a GeneAmp 9700 thermal cycler (Applied Biosystems). The conditions of the two-round PCR amplification were as follows: 94 °C (5 min), then 26 or 30 cycles at 94 °C (30 s) / 50 - 53 °C (45 s) / 72 °C (45 s - 1 min 30 s), followed by 8 cycles at 94 °C (30 s) / 53 °C (45 s) / 72 °C

(45 s -1 min 30 s), 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. The optimal PCR conditions for amplification of microsatellites at each locus are summarised in Table 3.

Table 3: Optimal PCR conditions for three microsatellite loci.

Locus	First round PCR	Second round PCR
ssrOeUA-DCA3	94 ºC/5 min	8 cycles:
	26 cycles:	94 ºC/30 s
	94 ºC/30 s	53 ºC/45 s
	50 ºC/45 s	72 ºC/45 s
	72 ºC/45 s	72 ºC/10 min
ssrOeUA-DCA10	94 ºC/5 min	8 cycles:
	30 cycles:	94 ºC/30 s
	94 ºC/30 s	53 ºC/45 s
	53 ºC/45 s	72 ºC/1 min 30 s
	72 ºC/1 min 30 s	72 ºC/10 min
ssrOeUA-DCA16	94 ºC/5 min	8 cycles:
	26 cycles:	94 ºC/30 s
	94 ºC/30 s	53 ºC/45 s
	52 ºC/45 s	72 ºC/1 min 30 s
	72 ºC/1 min 30 s	72 ºC/10 min

Amplification products were separated on a 0.5 thick and 7.5% polyacrylamide denaturing gel, containing 7 M urea. Electrophoresis was performed on short glass plates, of 80 mm length, using an automated ALFexpressII sequencer (Amersham Biosciences). Separation was done at 1500 V, 60 mA, and 15 W for 360 min at 55 ºC. Fluorescence signals were collected every 1 s and stored in a computer. A fluorescence labelled molecular marker size (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. Allele sizes were determined using the software package ALFwin^{TM} Fragment Analyser 1.01 (Amersham Biosciences).

3 RESULTS AND DISCUSSION

To reduce the cost of automated laser detection of fluorescent-labelled PCR fragments, a novel procedure for amplification of microsatellite markers was described (Schuelke, 2000), in which three primers for a single locus are employed. The basis of the approach is two-round PCR. During the first-round PCR (30 cycles), the forward primer with M13(-21) sequence at its 5' end is incorporated into the PCR products. These products are then the target for the fluorescent-labelled universal M13(-21) primer, which is incorporated during the second-round PCR (8 cycles). The thermocycling conditions are first optimised for annealing the forward primer with its M13(-21) sequence, and later, when the forward primer is used up, the annealing temperature is lowered to facilitate annealing of the universal fluorescent-labelled M13(-21) primer. Incorporation of the fluorescent dye into the PCR product is thus obtained, allowing the laser induced fluorescence detection.

In order for the procedure to be successfully performed, some important facts should be taken into consideration (Schuelke, 2000): 1) the fluorescent labelled M13(-21) universal primer and sequence specific reverse primer should be used in equimolar amounts, 2) the amount of modified sequence specific forward specific primer should not exceed one-fourth the amount of the reverse primer, so that the universal primer can take over when the forward primer is used up, 3) the second-round PCR (the last eight PCR cycles) should be run with an annealing temperature of 53 ºC (the

annealing temperature of the universal fluorescent-labelled M13(-21) primer is 53 ºC).

The main advantage of the method is that the universal fluorescence-labelled M13(-21) primer can be used for amplification of all loci included in the microsatellite assay, so fluorescence-labelling of one primer is no longer necessary, which reduces the cost of analysis. In the case of the ALFexpressII sequencer, where Cy5 dye is used for labelling DNA fragments in the analysis of ten microsatellite loci, the costs can be reduced by half as compared with the classical method.

To test the efficiency of the method in amplification of microsatellite markers in olives, three published loci ssrOeUA-DCA3, ssrOeUA-DCA10 and ssrOeUA-DCA16 (Sefc et al., 2000) were chosen. Our previous studies (Bandelj et al., 2002; Bandelj et al., 2004) showed that these 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.

For the amplification of microsatellite markers by the novel procedure, the original published sequence of the forward primer was modified according to Schuelke (2000). On the author's recommendation, the PCR mixture contained equimolar amounts of fluorescent-labelled M13(-21) universal primer and sequence specific reverse primer, and one-fourth the amount of sequence specific reverse primer. The optimal two-round amplification thermocycling profiles were determined for each locus independently. The optimisation of the first round PCR conditions mainly comprised adjustment of the primer annealing temperature, an increase of cycle number from 26 to 30 and a prolongation of the annealing and extension time. At locus ssrOeUA-DCA10, weak amplification of long alleles in contrast to short ones was observed. Wattier et al. (1998) explained this problem of short allele dominance as a consequence of competition between short and long alleles, which leads to preferential amplification of short alleles. The problem was only partly overcome by changing the amplification conditions, since the two longest alleles could not be amplified. The protocol for the second round PCR was performed as described by Schuelke (2000) with a modification for loci ssrOeUA-DCA10 and ssrOeUA-DCA16, whereby the extension time was prolonged from 45 s to 1 min 30 s.

The PCR products of the three microsatellite loci were separated and visualised on an ALFexpressII sequencer. The separation of microsatellite markers by sequencing apparatus was found to be very suitable, since the detection of alleles was performed automatically using a computer software package and there was no need for manual scoring of the data. Allele sizes were determined by the fragment sizing software Fragment Analyser 1.01, with the help of a fluorescence labelled molecular marker size as an external standard. The smiling effect was eliminated by identification of internal standards.

At three loci, all microsatellite alleles were successfully amplified (Figure 1), with the exception of the two above mentioned alleles of the ssrOeUA-DCA10 locus. Allele sizes were 18 bp longer than those determined by silver staining (Bandelj et al., 2002), when genotyping the same set of olive varieties was performed. The differences are due to modification of the forward specific primer by adding 18 bp of the M13(-21) sequence. Comparison of allele sizes determined by silver staining and

fluorescent detection showed that in some cases two bases longer alleles were determined by silver staining. Differences in allele sizes are frequently reported in literature and can be explained by different electrophoresis and detection systems (Weber, 1990; Bowers et al., 1996; Kline et al., 1997; Kozjak et al., 2003).

In conclusion, the reported method can be successfully used for amplification of microsatellites in plants. In genotyping studies, where many samples are analysed, this procedure reduces the overall costs, which is of great importance with large scale projects.

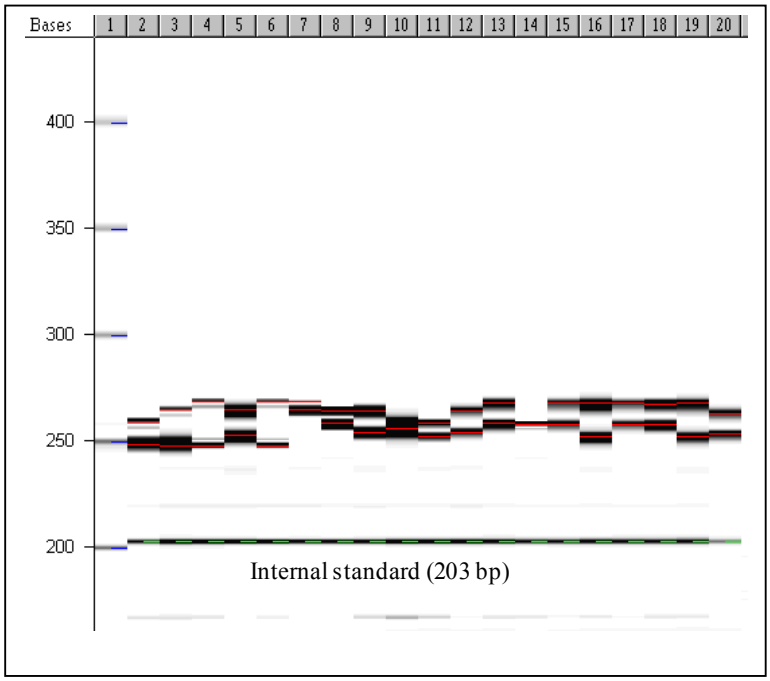

Figure 1: Electrophoregram of 19 olive varieties genotyped at locus ssrOeUA-DCA3: 1-Size marker (50 - 500 bp), 2-Arbequina, 3-Ascolana tenera, 4-Samo, 5-Štorta, 6-Picholine, 7-Santa Caterina, 8-Nocellara del Belice, 9-Itrana, 10-Cipressino, 11-Frantoio, 12-Buga, 13-Zelenjak, 14-Leccione, 15-Athena, 16-Črnica, 17-Leccino, 18-Pendolino, 19-maurino, 20-Istrska belica. Allele sizes include the additional 18 bp due to the M13(-21) tail.

4 REFERENCES

Bandelj, D., Jakše, J., Javornik, B. 2002. DNA fingerprinting of olive varieties by microsatellite markers. Food Technology Biotechnology 40, 3: 185-190.

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

Bowers, J.E., Dangl, G.S., Vignani, R., Meredith, C.P. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome 39: 628-633.

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

Kline, M.C., Duewer, D.L., Newall, P., Redman, J.W., Reeder, D.J., Richard, M. 1997. Interlaboratory evaluation of short tandem repeat triplex CTT. Journal of Forensic Science 42: 897-906.

Kozjak, P., Korošec-Koruza, Z., Javornik, B. 2003. Microsatellite analysis by automated fluorescent detection using ALFexpress aparatures: A case of grapevine analysis. Zb. Bioteh. Fak. Univerza v Ljubljani, Kmetijstvo 81, 1: 47-55.

Kump, B., Javornik, B. 1996. Evaluation of genetic variability among common buckwheat (*Fagopyrum esculentum* Moench) populations by RAPD markers. Plant Science 114: 149-158.

Morgante, M., Olivieri, A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. The Plant Journal 3: 175-182.

Powell, W., Morgante, M., Andre, C., Hanafey, M., Vogel, J., Tingey, S., Rafalski, A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. Molecular Breeding 2: 225-238.

Russell, J.R., Fuller, J.D., Macaulay, M., Hatz, B.G., Jahoor, A., Powell, W., Waugh, R. 1997. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. Theoretical Applied Genetics 95: 714-722.

Schuelke, M. 2000. An economic method for the fluorescent labelling of PCR fragments. Nature Biotechnology 18: 233-234.

Sefc, K.M., Lopes, M.S., Mendonça, D., Rodrigues Dos Santos, M., Laimer Da Câmara Machado, M., Da Câmara Machado, A. 2000. Identification of microsatellite loci in olive (*Olea europaea* L.) and their characterization in Italian and Iberian olive trees. Molecular Ecology 9: 1171-1173.

Scott, I.A.W., Hayes, C.M., Keogh, S., Webb, J.K. 2001. Isolation and characterization of novel microsatellite markers from the Australian tiger snakes (Elapidae: *Notechis*) and amplification in the closely related genus *Hoplocephalus*. Molecular Ecology Notes 1: 117-119.

Weber, J.L. 1990. Informativeness of human $(dC\text{-}dA)_n\cdot(dG\text{-}dT)_n$ polymorphisms. Genomics 7: 524-530.

Weising, K., Winter, P., Hüttel, B., Kahl, G. 1998. Microsatellites markers for molecular breeding. Journal of Crop Production 1: 113-143.

Wattier, R., Engel, C.R., Saumitou-Laprade, P., Valero, M. 1998. Short allele dominance as a source of heterozygote deficiency at microsatellite loci: experimental evidence at the dinucleotide locus Gv1CT in *Gracilaria gracilis* (Rhodophyta). Molecular Ecology 7: 1569-1573.

Wenz, H.M., Robertson, J.M., Menchen, S., Oaks, F., Demorest, D.M., Scheibler, D., Rosenblum, B.B., Wike, C., Gilbert, D.A., Efcavitch, J.W. 1998. High-precision genotyping by denaturing capillary electrophoresis. Genome Research 8, 3: 69-80.