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Identification of hypothetical duplicate accessions of plums (*Prunus domestica* L.) within the slovene plant gene bank collection using molecular markers

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ABSTRACT

The main goal of the Slovene Plant Gene Bank is preservation, maintenance and evaluation of traditional cultivars and other useful genotypes. The Faculty of Agriculture and Life Sciences houses among other plant materials also numerous accessions of plums (*Prunus domestica L.*). Duplicates among 15 accessions were studied using six microsatellite primer pairs. These microsatellite markers revealed an average of 7.67 alleles per locus, and a range of 4 to 10 different alleles per locus. The genetic distances between studied accessions were calculated using the Dice coefficient to form a dendrogram. The six SSRs were found to be adequate for differentiating among genotypes within the collection. Among the analysed accessions no duplicates were found.

Key words: gene bank, duplicate accessions, *Prunus domestica*, plums, SSR markers

INTRODUCTION

In 1996, the Ministry of Agriculture, Forestry and Food began participating in financing the Slovene Plant Gene Bank Program (SRGB) and its main aims were to maintain, evaluate, regenerate and preserve Slovenian indigenous germplasm such as Slovenian local ecotypes, populations and landraces of agricultural, medicinal and aromatic plants, forest trees and other woody plants from Slovenian forests. The gene bank includes Slovenian currently grown cultivars, abandoned cultivars (cultivars grown in the past), landraces, local populations, clones and lines bred from indigenous plant materials and ecotypes from various natural habitats important for food, and other genotypes important for agriculture and forestry. In the activities of the Slovene Plant Gene Bank Program dealing with agricultural, medicinal and aromatic plants, four institutions are involved: Biotechnical faculty of the University of Ljubljana, Institute for Hop Research and Brewing of Slovenia, Žalec, Faculty for Agriculture and Life Sciences of University of Maribor, and the Agricultural Institute of Slovenia, Ljubljana (Meglic et al. 2014).

The germplasm collection at the Faculty of Agriculture and Life Sciences, University of Maribor, houses among others, accessions of plums (*Prunus domestica* L.). One of the most common problems of gene banks is unintentionally duplication of samples. The reasons could be different local names for the same cultivar, different persons collecting the plant material, collection of the plant materials in different environments (the same cultivar may be phenotypically very different) and/or mistakes during sampling and/or maintenance.

The best way to determine the hypothetical duplicate accessions is through analysis of genetic similarity. In particular, DNA based markers have the advantage of being unaffected by environment. Approaches for the identification of duplicate accessions based on DNA markers are most frequently associated with studies of dendrograms (Lund et al. 2003; Cervera et al. 1998). Microsatellite markers have been used successfully to assess the diversity of wild and clonally propagated fruit plants collections (Gross et al. 2012; Koehmstedt et al. 2011; Laucou et al. 2011). As a result of high levels of allelic diversity within most *Prunus* species (Decroocq et al. 2004; Donoso et al. 2008; Struss et

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al. 2003), relatively few markers are required to differentiate among unique *P. domestica* cultivars. The number of primers sufficient for reliable variety identification depends on the nature and discriminating power of each primer (Tessier et al. 1999) and normally six primer pairs are sufficient for differentiating between genotypes (Zulini et al. 2002).

In the present study, six SSR loci were used to assess genetic diversity among 15 plum genotypes.

MATERIALS AND METHODS

Plant material

The study involve 15 plum accessions (Table 1) which are grown in the national plum germplasm collection which is housed at the estate of the Faculty of Agriculture and Life Sciences, in the vicinity of the University Botanical Garden. The whole plum collection includes 136 accessions originating from various parts of Slovenia.

DNA extraction

DNA was extracted from fresh, young leaves using the CTAB protocol. To approximately 2-3 cm^2 of fresh leaf tissue, one ml of preheated (68 °C) CTAB extraction buffer (Doyle and Doyle 1987) was added and well homogenized in a mortar with the pestle, and transferred to a 1.5 ml tube. Samples were incubated for 1.5 h at 68 °C in a water bath. After incubation, 600 µL of chloroform:isoamyl alcohol in a 24:1 proportion was added, and the samples were thoroughly mixed. The mixtures were centrifuged at 14.200 gn for 15 min. After centrifugation, the supernatant was transferred to a fresh tube, and the DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 1 volume of ice cold isopropanol and kept at –20°C for 30 min. Samples were again centrifuged at 14.200 gn for 15 min. The pellet was washed in 70% ethanol, air dried and rehydrated in 100 µl of TE buffer. Two separate extractions per plant were performed.The quality of extracted DNA was determined by agarose electrophoresis, and the quantity of obtained DNA was measured using the fluorometer DQ 300 (Hoefer, Holliston, Massachusetts).

SSR markers and PCR

The six previously isolated and characterized SSRs loci (Table 2) for sweet cherry (*P. avium*) were selected (EMPA001, EMPA002, EMPA003, EMPA004, EMPA011, EMPA029) from published papers (Clarke and Tobutt 2003, Guarino et al. 2009, Clarke et al. 2008) and their usefulness in ability to amplify a product also in *P. domestica* were determined. There is a high degree of homology for the SSR loci and transportability of these markers among *Prunus* species (Donoso et al. 2008). For testing the primer pairs, unlabelled primers (Sigma, Germany) were used in PCR. For six selected primers, one primer of each primer pair was labelled with fluorescent dye Cy5 or Cy5.5. The use of different dyes made possible to analyse three PCR products on capillary electrophoresis in the same reaction.

Ten µl of PCR mixture contained 2 ng DNA, 0.5 µl of each primer and 5 µl of Multipleks PCR Plus Kit (Qiagen GmbH, Hilden). PCR conditions were previously optimised for each primer pair considering annealing temperature and number of cycles. For all primers the annealing temperature of 55°C and 30 cycles were used. The polymerase chain reaction (PCR) was performed using a Whatman Biometra T-Gradient thermocycler (Goettingen, Germany). The capillary electrophoresis (Figure 1) of PCR products was performed on Beckman Coulter CEQ8000 according to manufacturer's instructions. The fragment size analysis was done with the in-build software. A fluorescently labelled size marker (Beckman Coulter DNA Size Standard Kit 400 bp) was used as a molecular weight reference.

Data analysis

All unambiguous fragments were scored for the presence (1) or absence (0) of each band. Only clear and reproducible fragments were taken for data analysis. The binary data matrix was used to calculate Dice's similarity coefficients (Dice 1945). Values for Dice's coefficients fall between 0 (there is no common band) and 1 (two genotypes have identical markers, so they are identical). Dice similarity coefficients were calculated using the DARWIN computer package (Perrier and Jacquemond-Collet 2005). For each microsatellite locus, the number of alleles per locus (n), allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E) and probability of identity (PI) were calculated using the 'IDENTITY 1.0' computer program (Wagner and Sefc 1999). The average distance between pairs of accessions was obtained by taking into account microsatellite data, and a neighbor-joining tree was constructed using the DARWIN computer package. A matrix of Dice similarity coefficients was used for assessing relationships among 15 genotypes, using the neighbor-joining algorithm developed by Saitou and Nei (1987).

RESULTS

DNA was successfully isolated from all 15 accessions of studied plums. The smallest concentration of DNA measured was 47.46 µg/ml (sample 15), and the largest amount of DNA was 422.3 µg/ml for sample 10 (data not shown). DNA isolated from fresh leaves was sufficient for further analysis for all accessions.

SSR analysis revealed 46 polymorphic alleles at 6 microsatellite loci (Table 3). The number of alleles (Table 4) detected per locus ranged from 4 (locus EMPA003) to 10 (locus EMPA011), with an average of 7.67 alleles per locus. The observed heterozygosity ranged between 0.63 (locus EMPA003) and 0.86 (locus EMPA011), with an average of 0.77. The expected heterozygosity ranged between 0.91 (locus EMPA002) and 1.00 (all other loci), with an average of 0.99. The differences between the observed and expected heterozygosity were examined for all investigated loci. At

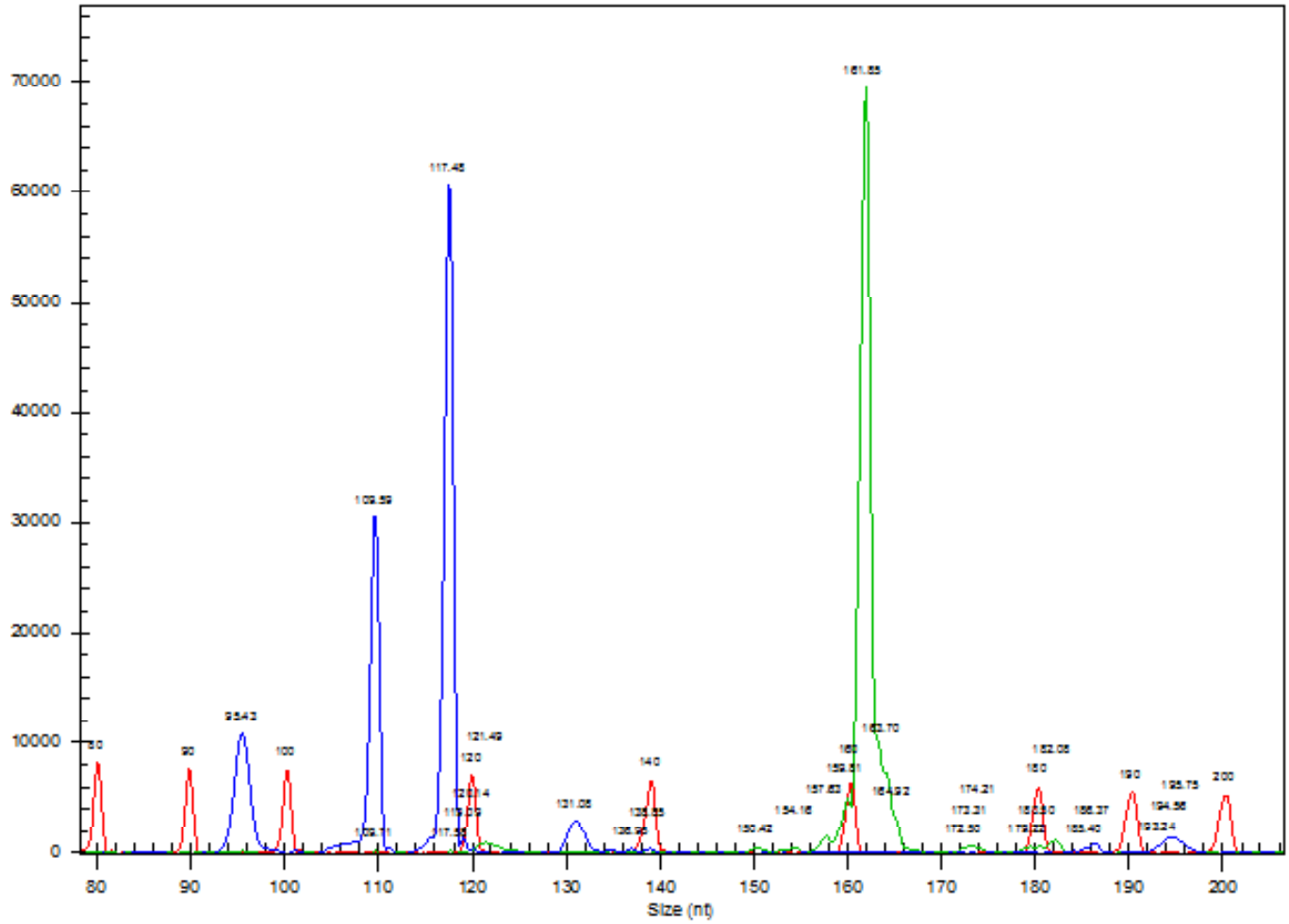

Fig. 1: Fragment lengths (bp) for two SSR loci EMPA004 (green) and EMPA001 (blue) obtained with capillary electrophoresis.

all loci the expected heterozygosity (H_E) was higher than observed (Ho). Allele sizes and allele frequencies are listed in Table 5.

The most informative locus for our set of genotypes was EMPA011, with a probability of identity (PI) of 0.067, and the least informative locus was EMPA003, with a PI of 0.353. The cumulative probability of obtaining identical genotypes using all 6 loci was low (4.77394E-06).

The dendrogram based on microsatellite data (Figure 2) grouped analysed samples in three main clusters. In the Cluster III, there was only one sample named "Drobna plavkica", suggesting that this genotype had a quite different genetic background comparing it with other plum genotypes included in our study.

The second group, Cluster II, included 5 accessions and all belonged to the group "Ringloji". Inside of this cluster the sample number 15 ("Rdečelistni ringlo s krvavo rdečimi plodovi") exhibited the most different germplasm. There were no duplicates in this cluster.

In the first cluster (Cluster I) all other 9 accessions can be found. Sample number 13, which was thought to be from group "Ringlo" showed the most similar genetic background with 'HZW Meschenmoser' in Cluster I. This genotype also showed some morphological differences comparing with other from "Ringlo" group: mesocarp and endocarp could be separated easily and the fruits were bigger in size.

CONCLUSIONS

In the present study, 15 plum accessions from the Slovene Plant Gene Bank were analysed for hypothetical duplicates. The six microsatellite loci which had been used earlier for genetic studies of sweet cherries were successfully applied in genetic evaluation of plums. Our study showed that there were two major clusters of genotypes and one variety ("Drobna plavkica") which was genetically very different and could be considered as separate genotype. Among 15 analysed genotypes, no duplicates were found.This paper presents some preliminary results of an ongoing project aimed to assess all possible duplicates among accession of the plum collection.

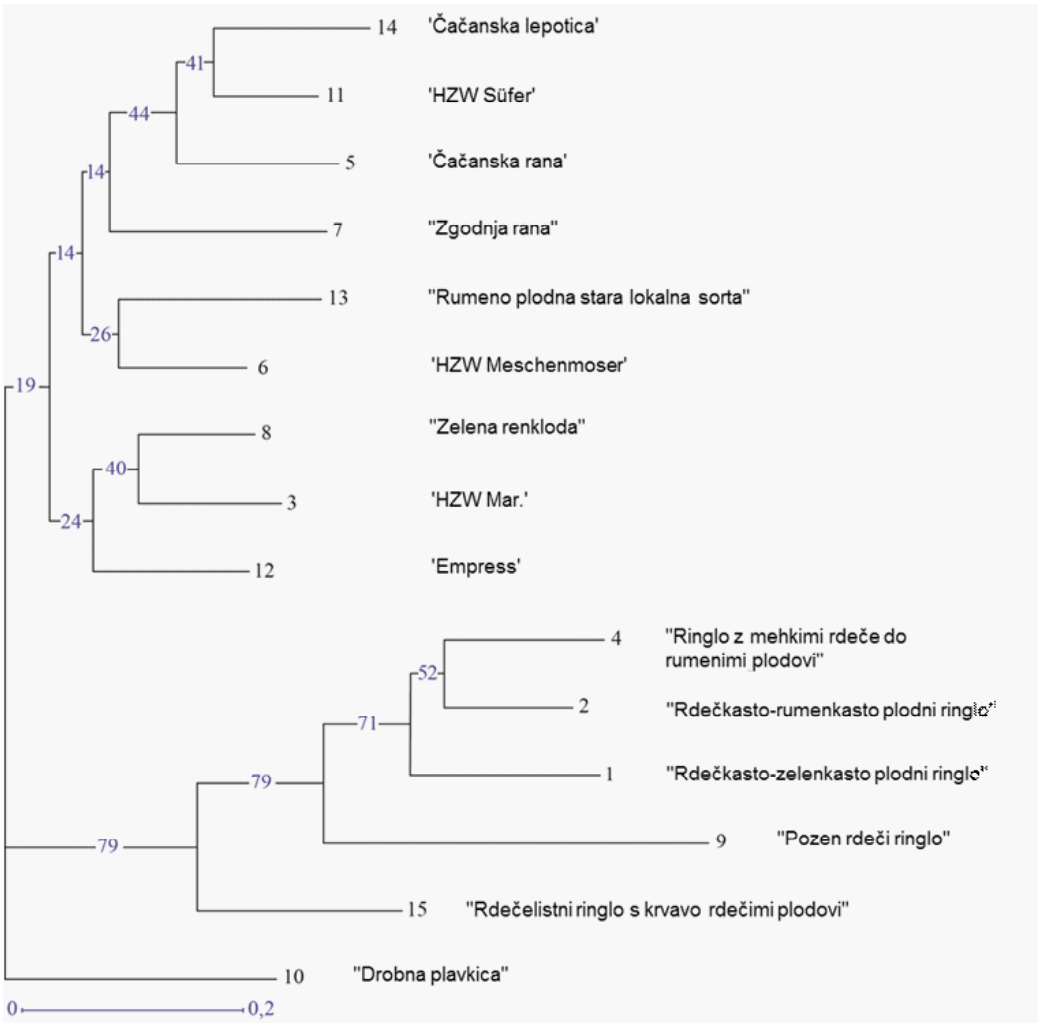

Fig. 2: Neighbour-joining dendrogram of 15 plum accessions relationships obtained from SSR data.

Table 1: Plant material obtained from the Slovene Plant Gene Bank.

Sample	Accession number	Name
1	3568	“Rdečkasto-zelenkastoplodniringlo”
2	3569	“Rdečkasto-rumenkastoplodniringlo”
3	3570	'HZW Mar. '
4	3571	“Ringlo z mehkimirdeče do rumenimiplodovi”
5	3567	'Čačanska rana'
6	3572	'HZW Meschenmoser'
7	3573	“Zgodnjatemna”
8	3574	“Zelenarenkloda”
9	3575	“Pozenrdečiringlo”
10	3576	“Drobnaplavkica”
11	3577	'HZW Schüfer'
12	3578	'Empress'
13	3579	“Rumenoplodnastaralokalnasorta”
14	3580	'Čačanskalepotica'
15	3581	“Rdečelistniringlo s krvavordečimiplodovi”

Table 2: Repeat motifs, primer sequences, and references for six SSR loci used in the study.

Locus	Repeat motif	Sequence (5'→ 3')	Reference
EMPA001	$[AG]_4GGGT[AG]_{26}$	F: GCTCTGCTGCTTCAACCATT R: TTCCCAACACACTTACCCC	Clarke and Tobutt 2003
EMPA002	$[AG]_{13}$	F: TGACAGGTCATCATACCATTTG R: CAGGATTAAGCATTGCAAATTA	Clarke and Tobutt 2003
EMPA003	$[AC]_8$	F: AGCCATTCTGAAAAGGTGGA R: GCATTCAGCCAACAAAATCA	Clarke and Tobutt 2003
EMPA004	$[GA]4AA[GA]4AA[GA]_{15}$	F: TACGGTAGGCTTCTGCAAGG R: TTGGCAGGTTCTGTTCACAT	Clarke and Tobutt 2003
EMPA011	$[AG]_{16}$	F: TGTGCTCACTCTCTGCTGCT R: TGTGTGGGTTCACAGTCTCC	Clarke and Tobutt 2003
EMPA029	$[CT]_{17}$	F: GCTGCTGATTGTCTGTGGTC R: CAAACCCCTCTTTCTTCCAC	Clarke et. al. 2008

Table 3: Allele sizes (bp) for 15 analysed samples on six loci: EMPA001, EMPA002, EMPA003, EMPA004, EMPA011 and EMPA029.

Sample	EMPA 001 (bp)		EMPA 002 (bp)		EMPA 003 (bp)		EMPA 004 (bp)		EMPA 011 (bp)		EMPA 029 (bp)	
1	110	/	165	181	169	177	162	164	195	/	130	151
2	110	/	157	/	169	177	162	164	175	195	130	135
3	110	118	155	171	165	173	162	164	185	/	138	/
4	110	/	157	/	169	177	162	164	195	/	134	141
5	110	118	155	163	165	173	162	/	199	201	136	143
6	110	118	151	165	165	173	162	/	185	/	136	/
7	110	118	151	167	165	173	162	/	181	201	142	/
8	110	118	155	163	165	173	162	164	175	185	130	/
9	106	118	159	/	169	177	164	164	195	/	136	153
10	110	118	151	157	165	173	162	/	175	189	124	133
11	110	118	153	167	165	173	162	/	185	/	136	143
12	110	118	151	155	165	173	162	/	175	185	134	141
13	110	118	165	171	165	173	162	/	185	201	130	149
14	110	118	153	159	165	173	162	/	189	201	136	143
15	110	118	147	/	169	173	162	/	175	195	130	145

Table 4: Parameters of genetic variability calculated for different microsatellite loci for 14 plum genotypes: number of alleles (n), observed (H_0), and expected (H_e) heterozygosity, and probability of identity (PI).

Locus	n	H_0	H_e	PI
EMPA001	8	0.748971	1	0.132822
EMPA002	9	0.760355	0.909091	0.129158
EMPA003	4	0.628889	1	0.353435
EMPA004	7	0.798186	1	0.128038
EMPA011	10	0.857639	1	0.066979
EMPA029	8	0.842975	1	0.077522
Average	7.666667	0.772836	0.984849	4.77394E-06

Table 5: Allele sizes (bp) and allele frequencies (in paranthesis) of 15 plum genotypes at six microsatellite loci.

Allele/locus	EMPA001	EMPA002	EMPA003	EMPA004	EMPA011	EMPA029
A	118 (0.444)	155 (0.038)	162 (0.467)	164 (0.286)	124 (0.042)	133 (0.091)
B	147 (0.037)	157 (0.038)	164 (0.033)	175 (0.238)	130 (0.208)	135 (0.091)
C	151 (0.148)	159 (0.038)	173 (0.367)	181 (0.048)	134 (0.083)	141 (0.182)
D	153 (0.074)	163 (0.077)	177 (0.133)	185 (0.190)	136 (0.208)	143 (0.273)
E	155 (0.111)	165 (0.423)	/	189 (0.048)	138 (0.042)	145 (0.091)
F	157 (0.074)	167 (0.077)	/	195 (0.143)	142 (0.042)	149 (0.091)
G	159 (0.037)	169 (0.192)	/	199 (0.048)	185 (0.083)	151 (0.091)
H	165 (0.074)	171 (0.077)	/	/	189 (0.042)	153 (0.091)
I	/	181 (0.038)	/	/	195 (0.083)	/
J	/	/	/	/	201 (0.167)	/
Allele number	8	9	4	7	10	8

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Identifikacija morebitnih duplikatov med akcesijami sliv (*Prunus domestica* L.) znotraj Slovenske rastlinske genske banke z uporabo molekulskih markerjev

IZVLEČEK

Slovenska rastlinska genska banka skrbi za ohranjanje, vzdrževanje in vrednotenje tradicionalnih kultivarjev in drugih uporabnih genotipov rastlin. Fakulteta za kmetijstvo in biosistemske vede skrbi med drugim tudi za številne akcesije sliv (Prunus domestica L.). Med 15 izbranimi akcesijami, smo s pomočjo šestih mikrosatelitskih lokusov želeli poiskati morebitne duplikate. Z mikrosatelitskimi markerji smo v povprečju namnožili 7.67 alelov na lokus in dobili od 4 do 10 različnih alelov na posamezen lokus. Za računanje genetskih oddaljenosti med proučevanimi akcesijami smo izračunali Dice koeficient in izrisali dendrogram. Šest mikrosatelitskih lokusov je zadostovalo za razlikovanje med akcesijami. Med preučevanimi akcesijami nismo našli duplikatov.

Ključne besede: genska banka, podvojene akcesije, *Prunus domestica*, slive, mikrosatelitski markerji