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COMPARATIVE ANALYSIS OF SWEET CHERRY (*P. avium*) GENETIC DIVERSITY REVEALED BY TWO METHODS OF SSR MARKER DETECTION

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*Three previously described highly polymorphic SSR (microsatellite) primer pairs in 126 sweet cherry (*Prunus avium *L.) accessions were tested using two microsatellite marker detection methods: ASCE (automated sequencer capillary electrophoresis) and PAGE (polyacrylamide gel electrophoresis) in screening of sweet cherry germplasm collections. It was determined that ASCE provided more precise genotyping. In the case of PAGE, due to possible manual scoring errors, a higher number of putative alleles was observed, which overestimated the level of polymorphism and hence the genetic diversity. This should be taken into account when comparing results obtained in different investigations. The ASCE detection method generally is considered as expensive, it requires more advanced and expensive equipment, and is available mostly in mid- to high-throughput laboratories. Nevertheless, the detection precision and possibility of complete unification of laboratory protocols among laboratories makes it a very useful tool in the identification and characterisation of sweet cherry breeding material, especially in large, diverse collecions.*

Key words: Prunus avium *L., microsatellites, ASCE, PAGE.*

INTRODUCTION

The importance of evaluation of genetic diversity and identification of plant material in fruit crop breeding is obvious. Therefore, the recognition and measurement of genetic diversity, its nature and magnitude, as well as accurate identification of the breeding material is beneficial or even crucial to the breeding programme. Traditionally, cultivar identification has relied on morphological and agronomic characteristics of the plant material. In fruit crops true identification is difficult since phenotypic characters are generally influenced by the environment and the growth stage of the plant. As a result, long and expensive evaluation during the whole vegetative growth period is required to obtain satisfactory morphological data for genetic diversity and to evaluate heredity.

Various molecular markers can be used to distinguish between accessions and in investigation of genetic diversity and heredity. Markers such as isozymes (Beaver *et al*., 1995; Granger, 1996), restriction fragment length polymorphism (RFLP) (Panda *et al*., 2003), random amplification of polymorphic DNA (RAPD) (Stockinger *et al*., 1996; Gerlach *et al*., 1997; Baranek *et al*., 2006) and amplified fragment length polymorphism (AFLP) (Goulćo *et al*., 2001; Riccardi *et al*., 2002) have been applied to cherry and other *Prunus* species. Simple sequence repeats (SSR or microsatellites) are among the widely used molecular markers in *Prunus* species (Cantini *et al*., 2001; Dirlewanger *et al*., 2002; Wünsch and Hormaza, 2002b; Schueler *et al*., 2003; Bianchi *et al*., 2004; Vaughan and Russell, 2004; Wünsch and Hormaza, 2004; Kacar *et al*., 2005; Ohta *et al*., 2005; Sánchez-Pérez *et al*., 2005; 2006; Baranek *et al*., 2006; Höltken and Gregorius, 2006; Pedersen, 2006). The robustness and reproducibility of these markers to fingerprint horticultural species across national borders and/or in different laboratories, has been shown, for example, by Lamboy and Alpha (1998). SSR have also other advantages over other molecular markers; they are abundant in most genomes, uniformly distributed, multi-allelic and co-dominant. As a result, the polymorphism information content is very high. SSRs are PCR-based, thus requiring little DNA for the amplification.

Polyacrylamide gel electrophoresis (PAGE) with different staining methods and manual band scoring was the first method used in the microsatellite fragment analysis (Cantini *et al*., 2001). The more advanced approach for SSR fragment analysis is automated sequencer capillary electropho-

resis (ASCE) using a genetic analyzer, which has become a standard in mid- to high-throughput laboratories (Aranzana *et al*., 2003; Ahmad *et al*., 2004). This approach is more expensive, and requires more advanced and expensive equipment. Therefore, in many laboratories, still PAGE is utilised for genetic diversity analysis.

In a previous work (Lacis *et al*., 2009b), published data (Cantini *et al*., 2001; Dirlewanger *et al*., 2002; Wünsch and Hormaza, 2002a; Schueler *et al*., 2003; Bianchi *et al*., 2004; Vaughan and Russell, 2004; Wünsch and Hormaza, 2004; Kacar *et al*., 2005; Ohta *et al*., 2005; Höltken and Gregorius, 2006; Pedersen, 2006) was surveyed to determine the level of primer polymorphism and to choose appropriate primers. High variation in the level of polymorphism in sweet cherries was observed using the same markers, as well as different fragment separation and detection methods. Therefore, it was difficult to compare different results and make valid decisions. Due to the different SSR fragment detection methods used it is not valid to combine data from different laboratores and projects for genetic diversity evaluation. A similar problem was encountered in the genotyping of Latvian and Swedish sweet cherry genetic resources, which began using SSR fragment detection by PAGE but later was replaced by ASCE. Unfortunately, comparative investigations on different SSR marker detection methods in sweet cherries (*P. avium* L.) are absent at present. The only available comparison in fruit crops has been made in almond (Sánchez-Pérez *et al*., 2006), which is insufficient to make conclusions about the usability and data transferability in fruit tree genetic diversity and heredity research.

The objective of this work was to compare two microsatellite detection methods (polyacrylamide gel electrophoresis and automated sequencer capillary electrophoresis) in the analysis of sweet cherry genetic resource diversity and accession heredity.

MATERIAL AND METHODS

Plant material. Accessions from sweet cherry genetic resources collections at the Latvia State Institute of Fruit-Growing, Dobele (LIFG-Dobele) (58 accessions) and Division of Horticultural Genetics and Plant Breeding at Balsgård, Department of Crop Sciences, Swedish University of Agricultural Sciences (SLU-Balsgård) (68 accessions) were genotyped (Lacis *et al*., 2008; 2009b). The larger part of the LIFG-Dobele collection (all accessions with designation 'PU' and 'U') consists of local sweet cherry accessions acquired by Pēteris Upītis (Lacis *et al*., 2008; 2009b). This material includes both wild accessions and landraces from collection expeditions in Latvia as well as hybrids from the breeding programme of P. Upītis. Unfortunately, information on the origin of these accessions has been lost (Ruisa, 1998). The LIFG-Dobele sweet cherry collection includes also varieties and advanced hybrids created by other Latvian breeders as well some foreign varieties developed in the former USSR (Belarus, Estonia, and Russia). The sweet cherry germplasm collection at SLU-Balsgård was developed to support the long-established Swedish breeding programme. Therefore, it includes local (Scandinavian) material as well as introduced advanced cultivars from Western Europe and North America and a wide diversity of selections developed at the SLU-Balsgård (Trajkovski, 1996).

Isolation of genomic DNA. Young leaves were collected in Dobele and Balsgård during May – June. Total DNA was isolated using a modified CTAB (hexadecyltrimethylammonium bromide) method (Nybom and Schaal, 1990). The leaves were ground to a fine powder in liquid nitrogen, and the DNA was extracted with 7 ml 2% CTAB extraction buffer (2% CTAB, 0.7 M NaCl, 50 mM Tris, pH 8.9, 10 mM EDTA, 0.1% 2-mercaptoethanol) for 1 hour at 60 °C in a water bath. Further DNA isolation was continued according to a published method (Nybom and Schaal, 1990). DNA concentration was estimated using a NanoDrop-1000 spectrophotometer (Thermo Scientific, USA).

PCR analysis. Approximately 50 ng of genomic DNA was used for PCR amplification in a 25 µl reaction containing 1× PCR reaction buffer, 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 2.5 pmol of each primer and 0.6 U *Taq* DNA polymerase (Fermentas, Lithuania). PCR reactions were run in an Eppendorf Mastercycler epgradient thermal cycler (Eppendorf, Germany) with one cycle of 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 90 s at 72 °C, and one cycle of 5 min at 72 °C (Cantini *et al*., 2001). Genotyping was performed using primer pairs PceGA25, PMS3, PMS49 which represent loci with the same designation (Cantini *et al*., 2001).

PCR products were first checked on 1% agarose gels in 1x TAE buffer and visualized by staining with ethidium bromide to test for the presence of PCR products.

The same PCR products subsequently were analysed by polyacrylamide gel electrophoresis (PAGE). DNA products were separated on 6% polyacrylamide gels in a 50 cm Sequi-Gen sequencing system (Bio-Rad, USA), run at 80 W for approximately 2.5 hours and stained with a Silver Sequence staining system (Promega, USA). The bands of amplified DNA were scored visually and size estimated using a 10 bp ladder (Invitrogen, USA) with three ladder lanes per gel: two at the edges and one in the middle of gel. To ensure reproducibility of fragment sizing, each accession per primer sample was scored twice — the same sample of amplified DNA was scores on two separated gels. Genotypes showing a single amplified fragment were considered homozygous for that locus (Callen *et al*., 1993).

Automated sequencer capillary electrophoresis (ASCE) was conducted using the above described PCR protocol and dye labelled primer pairs: PceGA25-FAM, PMS3-NED, PMS49-HEX. Acquired PCR products were first checked on 1% agarose gels as described above and the same samples analysed on an ABI Prism 3100 (Applied Biosystems, USA) genetic analyser.

Data analysis. Heterozygosity, polymorphic information content (PIC), probability of matching genotypes and their discriminating power were calculated using the computer programs GENALEX 6.1 (Peakall and Smouse, 2006) and Arlequin 3.11 (Excoffier *et al*., 2005). AMOVA was performed, using Arlequin 3.11 software (Excoffier *et al*., 2005). The SSR fragments were coded as present (1) or absent (0) and were typed into a computer file as a binary matrix, one for each detection method. The matrices were analysed by FreeTree v. 0.9.1.50 software (Hampl *et al*., 2001). Similarity of data was calculated using the Nei and Li/Dice similarity index (Nei and Li, 1979) and similarity parameters were analysed with UPGMA. Trees were visualized using TreeView software v. 1.6.6. (Page, 1996).

RESULTS

Comparison of two SSR detection methods. Amplification of three markers and SSR fragment detection by ASCE and PAGE approaches were carried out for 126 sweet cherry accessions (Tables 1 and 2). For locus *PceGA25*, 61.1% of alleles were identical using both detection methods, whereas one base-pair difference was found in 26.2% of cases (Table 3). A three base-pair difference between the SSR detection methods was the most common for locus *PMS3* — 47.6%, while identical alleles were found in 4.4% of cases. In the case of locus *PMS49*, the highest proportion was for 1 bp differences (50.4% of cases), following by 3 bp differences — 25%. Completely identical alleles were not found for this locus. Fragment length differences between SSR detection methods were 1 to 8, 1 to 35 and 1 to 36, respectively, for *PceGA25*, *PMS3* and *PMS49*. The mean SSR fragment length difference between detection methods was 3.6 for *PceGA25*, 16.5 for *PMS3* and 12.1 for *PMS49*. Fragment length differences up to three base pairs, which could be assumed as scoring errors, were found in 93.7% (*PceGA25*), 59.9% (*PMS3*) and 75.8% (*PMS49*) of cases.

The methods showed complete correspondence for several alleles: 178, 195 and 212 (*PceGA25*) and 186 (*PMS3*). Ten cases (six for *PceGA25* and four for *PMS3*) showed the same allele in both detection methods, but the PAGE detection method indicated additional alleles (Table 3). The largest number of additional alleles using PAGE was 17 (189, *PMS3*), followed by 13 (192, *PMS3*) and 11 (140, *PMS49*). Only one additional allele using PAGE was found for 137 (*PMS49*). Mean numbers of additional PAGE alleles were 2.3, 6.5 and 5.7, respectively for *PceGA25*, *PMS3* and *PMS49*.

Identical genotypes between SSR detection methods were found only for one locus (58 cases, int.al. 57 cases for loci *PceGA25* and one — for *PMS3*). Homozygotic genotypes were found for the same samples using both detection methods. In most cases an allele shift between them was typical (Tables 1 and 2). There were some exceptions, when homozigocity was observed only by one detection method: 0236B1, PU-14680 and PU-18426 (PAGE) for *PceGA25*; 0119B1, 1046B1, 1174i, 1239i, 1354B1, 1618B1, 1801C, 2845K, 2862K, 4570D, BPr 37239, Van (ASCE), 0411B1, 2721D, BPr 36781, Kaiser Franz, Zolotaja Loshitskaja, Balzams, PU-14406, PU-14499, PU-14673 and PU-18750 for *PMS3*; 0101B1, 0119B1, 1618B1, BPr 36900, BPr 37230, Hudson, Regina, Schauenburger, Schneiders Späte, Van, Laukgalu, PU-14497, PU-14646, Seglinu (ASCE), 1036B1, 1111B1, 1142D, 1149D, 1174i, 1354B1, 1801C, 2721D, 2845K, Almore, BPr 30165, Hedemora, Mahagony från Näsum, Napoleon (PAGE) for *PMS49* (Tables 1 and 2). The PAGE detection method showed also some alleles different from ASCE detection method, which cannot be explained by a scoring shift, and showed a higher allele number. For example, allele 184 for *PMS3* in accessions Balzams, PU-14499, PU14673 and PU-18750 was found only using PAGE, whereas ASCE for the same genotypes detected allele 189, which corresponds with an allele detection shift 189 (ASCE) to 186 (PAGE). Since ASCE detected homozygotes for these accessions, it is possible that allele 184 is the same as the allele 186 detected by PAGE.

Using ASCE, the highest allele number was found for the loci *PceGA25* and *PMS3* (ten putative alleles), while locus PMS49 had six putative alleles. In contrast, PAGE detected 17, 20 and 13 putative alleles for loci *PceGA25*, *PMS3* and *PMS49*, respectively (Table 4). A large proportion of the putative alleles identified in sweet cherry accessions had frequencies below 0.05 (46% for ASCE and 66% for the PAGE). Frequencies 0.050–0.099 (24 and 31% for ASCE and PAGE, respectively) were found. Significant differences between ASCE and PAGE in allele frequencies were found only for the frequency class below 0.05. There were no alleles in the frequency classes 0.100–0.149 and 0.150–0.199. Similarly, most of genotypes had frequencies under 0.05 (73% for ASCE and 86% for PAGE). Significant differences between ASCE and PAGE in estimated genotype frequencies were found only for the class below 0.05.

Heterozygosity calculated by direct counts for putative loci, identified by each primer pair, ranged 0.444 to 0.786 with a mean value of 0.586 (Table 5). The Polymorphic Information Content (PIC value) ranged from 0.480 to 0.878 with a mean value of 0.721. Values of discriminating power revealed that the most informative locus was *PMS3* for ASCE (0.901) and for PAGE (0.957). Finally, the total discrimination power of all three loci was 0.996 and 0.999 for ASCE and PAGE (Table 5), respectively, which is close to ~ 1.0 obtained using ten primer pairs (Cantini *et al*., 2001; Pedersen, 2006).

Differences between the two detection methods in allele detection were evaluated by AMOVA. The highest variation was found between individuals (63.37 %), the variation among data sets describe 21.64% of variance, and the total within data set variation was 78.37% (Table 6).

Clustering of sweet cherry cultivars. Matrices of genetic similarities was calculated and used to construct dendrograms (Figure 1 and 2). In both cases there were different classification of results: the dendrogram constructed on the

Table 1

PUTATIVE GENOTYPES IDENTIFIED IN ACCESSIONS OF THE LIFG SWEET CHERRY COLLECTION USING THREE SSR LOCI, DETECTED BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

No.	Accession name	Loci					
		PceGA25		*PMS3*		*PMS49*	
		ASCE	PAGE	ASCE	PAGE	ASCE	PAGE
1	Agris	161/165	161/164	189/208	186/205	140/140	141/141
2	Aija	161/161	161/161	189/201	186/198	140/142	141/143
3	AM-10-12-6	161/192	161/192	189/198	186/198	140/140	141/141
4	AM-10-6-12	161/208	161/210	189/201	186/198	140/140	141/141
5	AM-24-10-22	154/165	154/164	189/201	186/198	140/142	141/143
6	AM-28-6-7	161/198	161/198	201/214	198/211	140/140	141/141
7	Balzams	161/161	161/161	189/189	184/186	131/140	132/141
8	Brjanskaja Rozovaja	161/192	161/190	201/205	202/205	142/142	143/143
9	Drogans Gelbe Knorpelkirsche	161/161	161/161	189/201	186/198	140/140	141/141
10	Iputj	161/198	161/198	189/214	186/211	142/142	143/143
11	Kati	161/161	161/161	192/192	189/189	142/142	143/143
12	Kompaktnaja	161/165	158/164	189/192	186/189	140/142	141/143
13	Krupnoplodnaja	161/192	161/190	201/205	198/202	140/140	141/141
14	Laukgalu	165/165	164/164	189/198	186/196	140/142	143/143
15	Meelika	165/192	164/188	189/192	186/202	131/142	132/143
16	PU-13314	161/195	161/195	189/208	186/205	140/142	141/143
17	PU-13629	165/192	164/192	189/201	186/196	140/140	141/141
18	PU-13802	161/161	161/161	189/214	186/211	131/140	132/141
19	PU-14406	165/192	166/192	192/192	188/189	140/140	141/141
20	PU-14419	161/161	161/161	189/189	186/186	140/142	141/143
21	PU-14421	161/165	161/164	189/201	188/202	140/140	141/141
22	PU-14423	161/161	161/161	186/201	186/202	140/140	141/141
23	PU-14440	154/165	154/164	205/205	202/202	131/142	132/143
24	PU-14450	161/208	161/210	189/201	186/198	140/140	141/141
25	PU-14497	161/161	161/161	189/201	188/202	140/137	141/141
26	PU-14498	161/161	161/161	189/189	186/186	140/140	141/141
27	PU-14499	161/161	161/161	189/189	184/186	140/142	141/143
28	PU-14646	161/161	161/161	189/189	186/186	142/133	143/143
29	PU-14672	161/161	161/161	189/201	186/198	140/140	141/141
30	PU-14673	161/161	161/161	189/189	184/186	140/140	141/141
31	PU-14680	165/165	164/166	189/201	189/205	140/142	143/145
32	PU-14684	161/165	161/164	186/198	186/198	140/140	141/141
33	PU-18426	165/165	164/166	189/201	186/198	140/140	141/141
34	PU-18463	161/161	161/161	189/205	186/202	140/140	141/141
35	PU-18600	161/161	161/161	189/192	186/189	142/142	143/143
36	PU-18603	165/192	164/188	196/218	196/219	142/142	143/143
37	PU-18619	161/161	161/161	189/189	186/186	140/140	141/141
38	PU-18750	161/208	161/208	189/189	184/186	140/140	141/141
39	PU-19102	161/192	161/192	189/201	186/198	140/140	141/141
40	PU-20035	161/165	161/164	201/208	198/205	140/142	141/143
41	PU-20110	161/161	161/161	189/201	186/198	131/140	132/141
42	PU-20120	161/195	161/195	189/214	186/211	140/142	141/143
43	PU-20299	161/165	161/164	192/208	189/205	140/142	141/143
44	PU-20929	161/192	161/192	189/205	186/202	140/140	141/141
45	PU-20941	161/161	161/161	189/192	186/189	131/140	132/141
46	PU-21019	161/192	161/188	205/208	202/205	142/142	141/141
47	Seglinu	161/161	161/161	189/192	186/189	133/142	145/145
48	Simfonija	161/161	161/161	189/198	186/189	140/142	141/143
49	Skedes	178/192	178/192	189/192	186/189	142/142	143/143
50	Talsu - 1	161/161	161/161	189/189	186/186	133/140	134/141
51	Talsu - 3	161/161	161/161	189/189	186/186	142/142	145/145
52	Talsu Sartais	161/161	161/161	192/208	189/205	140/140	143/143
53	U-251	161/161	161/161	189/205	186/202	140/140	141/141
54	U-253	161/161	161/161	189/189	180/180	142/142	143/143
55	U-254	161/192	161/192	189/205	186/202	140/142	141/143
56	U-300	161/195	161/195	189/192	186/189	140/140	141/141
57	U-371	161/212	161/212	208/218	205/215	140/142	141/143
58	Zolotaja Loshitskaja	161/161	161/161	189/192	196/196	140/142	134/148

Table 2

PUTATIVE GENOTYPES IDENTIFIED IN ACCESSIONS OF THE SLU-BALSGÅRD SWEET CHERRY COLLECTION USING THREE SSR LOCI, DETECTED BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

No.	Accession name	Loci					
		PceGA25		*PMS3*		*PMS49*	
		ASCE	PAGE	ASCE	PAGE	ASCE	PAGE
1	0101B1	165/165	164/164	192/214	188/205	140/142	118/118
2	0119B1	165/165	164/164	189/192	184/184	140/142	116/116
3	0205B1	165/165	164/164	189/205	186/196	140/142	143/145
4	02076i(0206i)	198/198	198/198	189/205	186/202	131/140	138/145
5	0236B1	165/165	164/166	192/214	188/209	140/142	143/145
6	0402B1	165/165	164/164	189/192	184/188	140/142	143/145
7	0411B1	165/165	161/161	189/189	193/209	127/140	141/143
8	0956B1	165/165	161/161	192/208	209/215	140/142	143/145
9	1036B1	154/161	154/161	189/192	193/193	140/140	141/143
10	1046B1	154/161	151/161	189/192	184/184	140/140	143/143
11	1111B1	161/161	161/161	189/192	193/219	140/140	141/143
12	1115i	161/195	161/195	189/192	189/189	142/142	118/118
13	1117D	165/198	164/198	192/205	202/186	140/140	143/143
14	1126D	165/165	164/164	189/192	163/163	140/140	143/143
15	1137D	165/165	164/164	189/214	177/184	140/140	116/116
16	1142D	165/165	164/164	189/214	193/202	140/140	141/143
17	1149D	198/198	198/198	189/192	184/188	140/140	143/145
18	1156i	161/192	161/192	189/189	188/188	140/140	116/116
19	1166D	198/198	198/198	189/208	158/177	140/140	143/143
20	1174i	161/198	161/198	205/214	193/193	140/140	128/148
21	1176D	198/198	198/198	189/208	186/202	140/140	143/143
22	1182i	161/165	161/164	189/205	186/196	140/140	143/143
23	1223D	165/198	164/198	189/189	175/188	140/140	143/143
24	1239i	161/161	161/161	189/192	186/186	140/142	143/145
25	1354B1	161/161	161/161	208/214	205/205	140/140	141/145
26	1616B1	161/165	161/164	192/192	193/193	127/140	128/148
27	1618B1	165/165	164/164	192/205	184/184	131/140	143/143
28	1801C	165/198	161/198	189/192	193/193	140/140	141/145
29	2102C	165/165	164/164	189/196	158/167	127/140	134/143
30	2118C	154/165	154/164	192/192	193/193	127/140	128/148
31	2721D	154/165	154/164	189/189	186/188	140/140	128/148
32	2845K	161/192	154/192	189/192	186/186	140/140	141/143
33	2862K	161/195	164/195	189/192	193/193	140/140	104/104
34	2984K	192/208	192/208	192/201	188/198	140/140	116/116
35	4570D	165/198	166/198	192/214	198/198	131/140	141/143
36	Almore	161/161	164/164	189/205	186/198	140/140	138/141
37	Bigarrå fröplanta från E. Petterss	165/195	164/195	192/201	163/173	140/142	118/118
38	BPr 21409	161/168	161/168	189/214	189/209	140/142	116/116
39	BPr 30165	161/198	161/198	189/214	189/198	140/140	141/143
40	BPr 36781	165/198	164/198	189/189	202/219	140/140	118/118
41	BPr 36900	161/198	161/190	189/208	163/177	131/140	116/116
42	BPr 37230	165/198	161/198	192/192	215/215	140/142	118/118
43	BPr 37231	165/195	161/195	208/208	198/198	140/142	143/145
44	BPr 37239	165/198	166/198	189/192	184/184	127/140	130/143
45	Fryksas	165/168	164/170	189/208	173/186	140/140	143/143
46	Gårdebo	165/165	164/164	189/208	158/177	140/140	118/118
47	Hedelfinger	165/198	164/198	189/214	186/211	140/140	141/141
48	Hedemora	161/195	161/195	189/192	215/219	140/140	141/143
49	Heidi	165/165	164/164	208/192	158/173	140/140	143/143
50	Heinrich's Riesen	165/168	164/170	192/208	188/205	140/140	143/143
51	Hudson	161/192	161/192	189/201	186/198	140/142	122/122
52	Kaiser Franz	161/198	161/195	189/189	188/205	140/140	143/143
53	Lambert	154/165	151/158	189/214	186/211	140/142	143/145
54	Lapins	165/195	161/195	192/214	188/211	140/140	141/141
55	Mahagony från Näsum	165/168	164/170	192/205	163/175	140/140	141/143
56	Merpet	165/198	164/198	189/205	186/202	140/140	141/141
57	Napoleon	161/161	161/161	189/189	186/186	140/140	143/145
58	Regina	165/195	164/195	189/192	198/202	140/142	122/122
59	Schauenburger	161/161	161/161	189/214	186/211	131/140	116/116
60	Schmidt	165/192	164/188	189/192	186/188	127/140	104/118
61	Schneiders Späte	165/165	164/164	189/201	186/198	131/140	118/118
62	Starking Hardy Giant	165/165	164/164	192/201	188/198	140/142	141/143
63	Stella	165/165	164/164	208/214	186/189	140/142	141/143
64	Sture Holmberg	168/198	170/198	196/208	167/177	140/142	143/145
65	Sunburst	165/195	161/195	192/214	188/211	140/142	141/143
66	Van	195/195	195/195	189/192	163/163	131/140	116/116
67	Vit Spansk	165/198	164/198	192/192	186/186	140/140	118/118
68	Vitaby	161/192	161/190	189/189	184/184	140/140	143/143

Table 3

SSR ALLELES DETECTED IN 126 SWEET CHERRY ACCESSIONS BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND THE CORRESPONDING ALLELES DETECED IN THE SAME ACCESSION BY POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

Loci								
PceGA25			*PMS3*			*PMS49*		
ASCE	PAGE	No. of cases	ASCE	PAGE	No. of cases	ASCE	PAGE	No. of cases
1	2	3	4	5	6	7	8	9
154	151	2	186	186	2	127	118	1
	154	5	189	158	3		128	2
161	154	1		163	3		130	1
	158	1		173	1		134	1
	161	97		175	1		141	1
	164	3		177	1	131	116	3
165	158	1		180	2		118	1
	161	9		184	10		132	6
	164	60		184	1		138	1
	166	6		186	63		141	1
168	168	1		188	7		143	1
	170	4		189	4	133	134	1
178	178	1		193	6		143	1
192	188	4		202	2		145	1
	190	3		205	1	137	141	1
	192	11		209	1	140	104	3
195	195	13		215	1		116	11
198	190	1		219	1		118	10
	195	1	192	158	1		122	2
	198	23		163	4		128	2
208	208	2		184	4		134	1
	210	2		186	5		138	1
212	212	1		188	11		141	83
				189	12		143	50
				193	7		145	5
				196	1		148	4
				198	2	142	116	2
				202	1		118	5
				209	1		122	2
				215	2		141	2
				219	2		143	30
			196	167	2		143	3
				196	1		145	12
			198	189	1		148	1
				196	1			
				198	2			
			201	173	1			
				196	1			
				198	16			
				202	4			
				205	1			
			205	175	1			
				184	1			
				193	1			
				196	2			
				198	1			
				202	11			
				205	1			
			208	173	1			

Table 3 (continued)

1	2	3	4	5	6	7	8	9
				177	4			
				186	2			
				198	2			
				202	1			
				205	3			
				205	6			
				215	1			
			214	184	1			
				189	1			
				193	1			
				198	2			
				202	1			
				205	2			
				209	2			
				211	9			
			218	215	1			
				219	1			

base of the PAGE data distinctly separates 15 groups of sweet cherry accessions based on genetic similarities above 0.30 (Figure 1), whereas the dendrogram constructed from ASCE data showed only four accesion groups at the same genetic similarity level (Figure 2). The higher number of groups based on genotyping by PAGE is explained by the variability of alleles estimated by this method.

DISCUSSION

The sweet cherry genotypes described using SSR markers (Tables 1 and 2) will contribute to plant genetic resources information systems. Detailed accession description is crucial for search of duplication, harmonisation of collections, development of common plant genetic resources collections and research activities. The available genotyping information can be used for the sweet cherry accession comparison, synonym identification, and detection of different clones of old widely grown cultivars.

Both evaluated SSR detection methods (ASCE and PAGE) showed high variability, and they are efficient and able to

Table 4

PUTATIVE ALLELES IDENTIFIED IN 126 SWEET CHERRY ACCESSIONS USING THREE SSR LOCI, DETECTED BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

Loci	Putative alleles	
	ASCE	PAGE
PceGA25	154, 161, 165, 168, 178, 192, 195, 198, 208, 212	151, 154, 158, 161, 164, 166, 168, 170, 178, 188, 190, 192, 195, 198, 208, 210, 212
PMS3	186, 189, 192, 196, 198, 201, 205, 208, 214, 218	158, 163, 167, 173, 175, 177, 180, 184, 186, 188, 189, 193, 196, 198, 202, 205, 209, 211, 215, 219
PMS49	127, 131, 133, 137, 140, 142	104, 116, 118, 122, 128, 130, 132, 134, 138, 141, 143, 145, 148

Table 5

COMPARISON OF THREE MICROSATELLITE LOCI CHARACTERISTICS DETECTED BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

Method	SSR marker	Data set chatacreristics*											
		N	Na	Ne	Ng	Nh	I	Ho	He	PIC	F	Ds	Dp
ASCE	*PceGA25*	126	10	3.652	20	57	1.585	0.548	0.726	0.726	0.246	0.887	0.88
	PMS3	126	10	3.991	26	27	1.704	0.786	0.749	0.749	0.048	0.908	0.901
	PMS49	126	6	1.922	9	70	0.914	0.444	0.480	0.480	0.073	0.693	0.688
	Mean values	126	8.7	3.188	18.3	51.3	1.401	0.593	0.652	0.652	0.122	0.829	0.823
	Total											0.997	0.996
PAGE	*PceGA25*	126	17	3.922	31	54	1.821	0.571	0.745	0.745	0.233	0.901	0.894
	PMS3	126	20	8.224	52	33	2.513	0.738	0.878	0.878	0.16	0.965	0.957
	PMS49	126	13	3.98	20	72	1.744	0.429	0.749	0.749	0.428	0.869	0.862
	Mean values	126	17.7	5.375	34.3	53	2.026	0.579	0.791	0.791	0.274	0.912	0.904
	Total											0.999	0.999

* N – cample size, Na – No. alleles, Ne – No. effective alleles, Ng – No. genotypes, Nh – No. homozygous plants, I – information index, Ho – observed heterozygosity, He – expected heterozygosity, PIC – gene diversity at locus, F – fixation index, Ds – discrimination power at the locus in the sample, Dp – discrimination power at the locus in the population

Table 6

SUMMARY OF AMOVA ANALYSIS BASED ON THE COMPARISON OF THREE MICROSATELLITE LOCI DETECTED BY AUTOMATED SEQUENCER CAPILLARY ELECTROPHORESIS (ASCE) AND POLYACRYLAMIDE ELECTROPHORESIS (PAGE)

Locus	Source of variation								Fixation indice (FST)
	between detection methods				among accessions within detection methods				
	SSD	d.f.	Va	% variation	SSD	d.f.	Vb	% variation	
PceGA25	9.853	1	0.03763	9.25	185.373	502	0.36927	90.75	0.09249
PMS3	22.282	1	0.08680	17.52	205.111	502	0.40859	82.48	0.17521
PMS49	48.613	1	0.19169	38.34	154.774	502	0.30831	61.66	0.38337
Total	80.748	1	0.31612	22.54	545.258	502	1.08617	77.46	0.22543

Va, Vb, FST P-value = 0.00000 +/- 0.00000

resolve allelic variation among sweet cherry accessions at a very fine scale (Tables 1 and 2). The acquired data allowed evaluation of the used SSR detection methods in accuracy and usability in genetic resources characterisation. Several base pair shifts for most accessions (Table 3) were observed (38.9%, 95.4% and 100%, respectively for *PceGA25*, *PMS3* and *PMS49*). Small 1 to 3 bp shifts (26.2%, 47.6% and 75.4%, respectively, for *PceGA25*, *PMS3* and *PMS49*) could be assumed as scoring errors. These differences in the PAGE genotyping data can be explained by errors in sizing of fragments, which is not as accurate as automated genotyping methods, mainly due to the presence of ladder-like stutter alelle patterns (Wünsch and Hormaza, 2002a), as well as by manual band length detection and gel running environment conditions. Allelic differences between SSR detection methods caused differences in the number of putative alleles and and SSR marker characteristics (number of alleles, effective alleles, genotypes, homozygous plants; information index, observed heterozygosity, expected heterozygosity, PIC — gene diversity at locus, fixation index, discrimination power at the locus in the sample, discrimination power at the locus in the population) (Table 5). In this regard, SSR marker characteristics showed higher values in the case of the PAGE in comparison with ASCE, as a higher number of putative allele was suggested. Using PAGE in microsatellite detection, some discrepancies in sweet cherry accession pedigree also was observed, whereas the ASCE data set was more in accordance with reported pedigree data (Lacis *et al*., 2009b). Allele diferences caused by manual scoring errors of the PAGE detection method were shown also by AMOVA (Table 6): moderate variation explained by detection methods (22.54%) with highest variation found for *PMS49* (38.34%) following by *PMS3* (17.52%) and *PceGA25* (9.25%).

In contrast to other applications of *PceGA25*, *PMS3* and *PMS49* SSR loci in sweet cherries (Kacar *et al*., 2005; Pedersen 2006), the level of polymorphism in our investigation was more close to that of sour cherry (*P. cerasus* L., 2n = 4x = 32) (Cantini *et al*., 2001), which has naturally higher polymorphism due to tetraploidity. This was observed for both SSR detection methods, probably due to the high number of genotypes evaluated and wide genetic diversity. In other investigations only ten widely grown sweet chery cultivars (Kacar *et al*., 2005) or six sweet cherry genotypes from three cultivars (Pedersen 2006) were evaluated. The detected allele range in all cases was comparable with our investigation (Table 4):

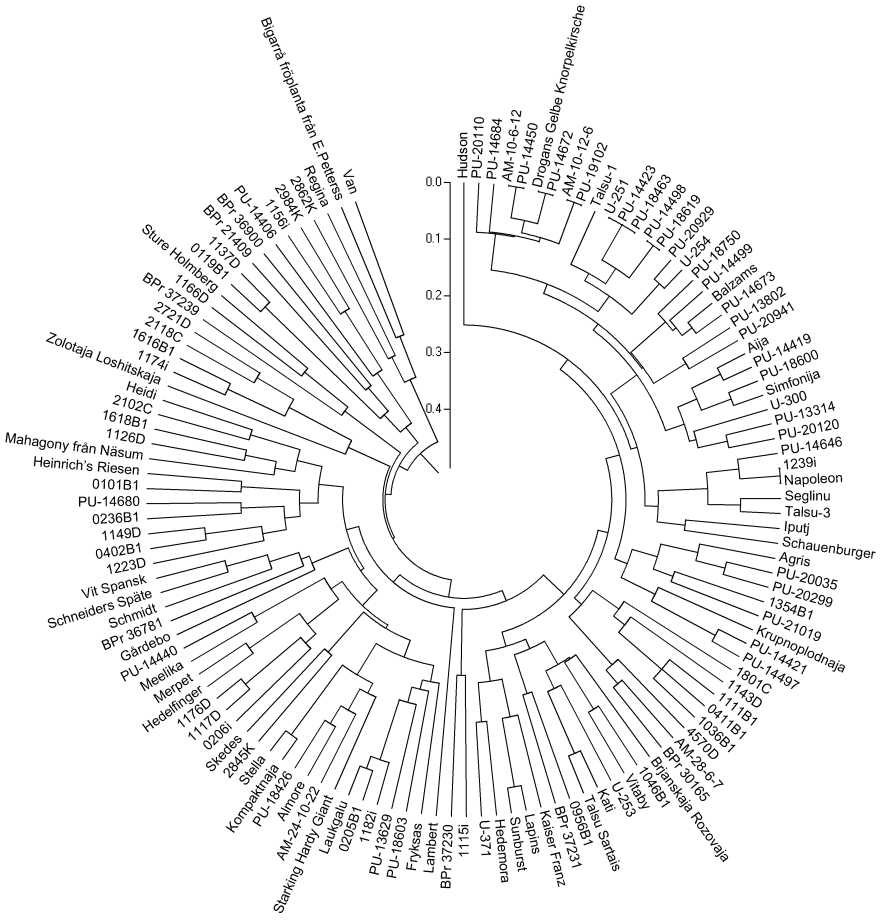

Fig. 1. Dendrogram of genetic similarity among analysed sweet cherry accessions constructed on the basis of PAGE data.

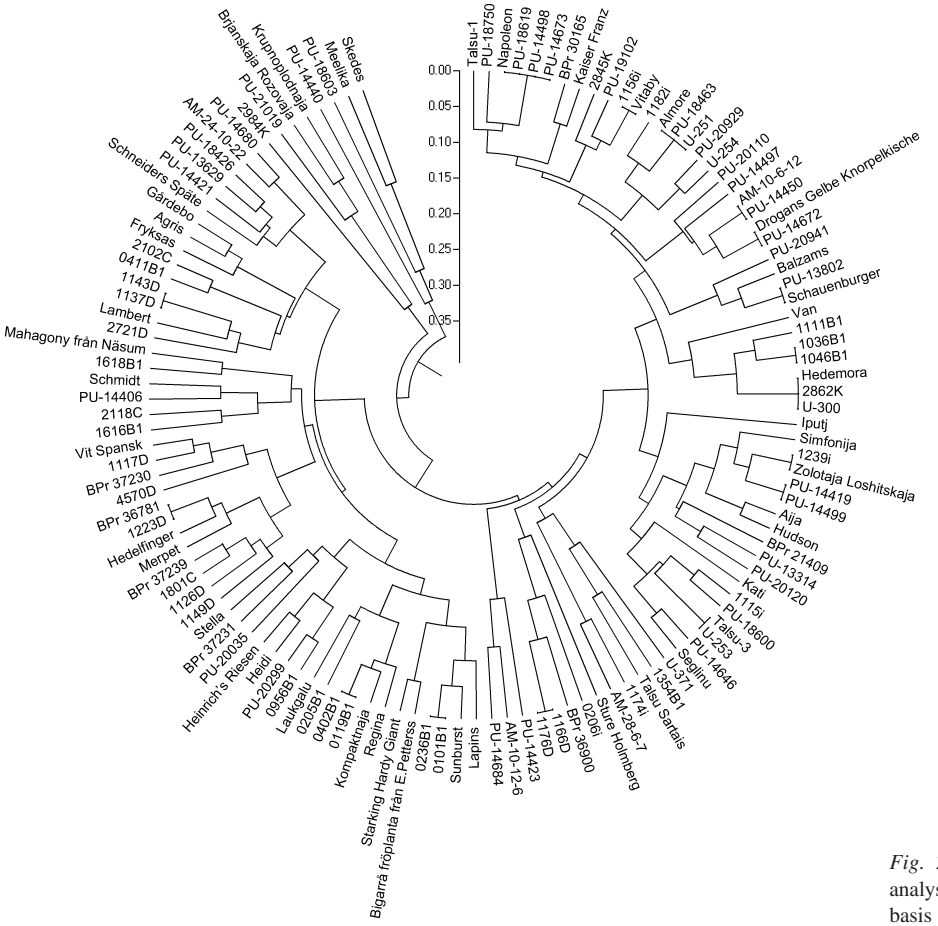

Fig. 2. Dendrogram of genetic similarity among analysed sweet cherry accessions constructed on the basis of ASCE data.

- *PceGA25* – 5 alleles (range 158-207: 158, 160, 192, 195, 207); PMS3 – 4 alleles (range 185–210: 185; 186, 200, 210); PMS49 – 1 (142) (Kacar et al., 2005);

- *PceGA25* — 4 alleles (range 137-195: 137, 163, 164, 195); PMS3 — 8 alleles (range 173-210: 173, 176, 183, 189, 190, 191, 192, 210); PMS49 — 5 alleles (range 133-145: 133, 138, 143, 144, 145) (Pedersen 2006).

Pedersen (2006) who used the PAGE detection method recorded alleles 163, 164 for *PceGA25*; 189, 190, 191 for *PMS3* and 143, 144, 145 for *PMS49* as common, which could be a result of ladder-like stutter band pattern presence. A similar situation was observed also in our data using the same detection method. Microsatellite marker detection by ASCE eliminated this effect, as increased detection resolution did not record a group of putative alleles with 1 bp difference. This indicates possible overestimation of putative allele number and polymophism using PAGE.

The DNA fingerprinting data obtained by different detection methods showed slightly different phylogenetic UPGMA dendrograms (Figure 1 and 2). There were no common groups in the dendrograms for both detection methods. Since clustering is a sensitive analysis, greatly affected even by small differences in sample allele composition, even a few bp shifts in the accession genotypes can cause significant changes in the clustering. The overestimated genetic diferences could be also a reason for troublesome accession group determination and underestimated genetic relatedness (Figures 1 and 2). Therefore, the applied SSR detection method should be taken into account in the comparison and analysis of genetic heredity data. In principle, the data acquired by different detection methods cannot be directly compared as this can lead to inaccurate or even incorrect conclusions. Confirmation of estimation of genetic diversity by ASCE has been made also by additional molecular (Lacis *et al*., 2009b) and morphological (Lacis *et al*., 2009a) characterisation of the same plant material.

Comparison of two SSR allele detection methods (ASCE and PAGE) showed that more precise genotyping can be acquired by ASCE. The results did not show any ladder-like stutter allele patterns, frequent for PAGE and had higher correspondence with known pedigree. Therefore, this SSR allele detection method is suitable for cultivar genotyping and identification purposes. In case of PAGE detection, due to possible application specificity and manual scoring errors, a higher number of putative alleles was detected, which led to overestimation of the polymorphism level, and higher genetic diversity. This should be taken into account when comparing results obtained by different methods. However, this method is still useful for characterisation of sweet cherry collection and evaluation of general genetic diversity. Until now, ASCE was considered as an expensive SSR detection method, which requires more advanced and expensive equipment, and is available mostly for mid- to high-throughput laboratories. However, the detection precision, possibility of complete unification of protocols among laboratories and exclusion of environmental and human factors, make it a very useful tool in the identification and characterisation of sweet cherry plant material, especially in large, diverse collecions, as well as compilation and comparative analysis of research data.

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DIVU SSR MARĶIERU NOTEIKŠANAS METOŽU SALĪDZINĀJUMS SALDO ĶIRŠU (*P. avium*) ĢENĒTISKĀS DAUDZVEIDĪBAS NOTEIKŠANAI

Pētījumā veikta divu mikrosatelītu marķieru noteikšanas metožu — ASCE (automātiskā ģenētiskā analizatora kapilārā elektroforēze) un PAGE (poliakrilamīda gēla elektroforēze) — salīdzināšana, izmantojot trīs iepriekš aprakstītus polimorfiskus SSR (mikrosatelītu) praimeru pārus 126 saldo ķiršu (*Prunus avium* L.) paraugiem. Pētījumā konstatēts, ka precīzākus genotipēšanas rezultātus iespējams iegūt, izmantojot ASCE noteikšanas metodi. PAGE metodei raksturīgas fragmentu noteikšanas kļūdas, jo darbs tiek veikts manuāli, līdz ar to rezultātos tiek pārvērtēts alēļu skaits, polimorfisma un ģenētiskās daudzveidības līmenis, kas būtu jāņem vērā, salīdzinot un analizējot dažādus pētījumu rezultātus. ASCE fragmentu garuma noteikšanas metode parasti tiek uzskatīta par samērā dārgu, jo nepieciešams moderns un dārgs aprīkojums, kas pieejams galvenokārt tikai vidējās un lielās laboratorijās. Tomēr šī metode tās precizitātes dēļ, un ar iespēju izmantot standartizētus starplaboratoriju protokolus, ir ļoti noderīgs līdzeklis saldo ķiršu materiāla identificēšanai un raksturošanai, it īpaši lielās un daudzveidīgās kolekcijās.