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Development and Characterization of SSR Markers in Chinese Cherry (*Prunus pseudocerasus* Lindl.)

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Summary

24 simple sequence repeat (SSR) markers were isolated and characterized in *P. pseudocerasus* Lindl. from the cultivar 'Laiyang Short cherry'. To amplify SSR loci primers were developed to flank each repeat region. and they were initially tested on 6 cherry samples using chemiluminescence detection. 18 loci where shown to be polymorphic and the number of alleles detected per locus varied from 2 to 6. 12 loci were chosen for the analysis of 26 cultivars grown in North China using the semi-automatic system ABI PRISM 377. These 12 markers showed a high level of genetic polymorphism with a total of 75 alleles; the number of alleles ranged from 4 to 10 per locus, with an average level of 6.3. The mean expected and observed heterozygosity was 0.743 (range: 0.680–0.845) and 0.829 (range: 0.730–0.930) respectively. The estimated frequency of null alleles showed a positive value for 3 loci, but except for 1 locus, the values were very low. The total value for the probability of identity was 7.01×10^{-13}. Paternity exclusion probability was very high (0.999), sufficiently high to study pollen flow.

Key words. dinucleotide – chemiluminescence detection – DNA-typing – polymorphism – cultivar characterization

Introduction

Chinese cherry (*Prunus pseudocerasus* Lindl.) is grown commercially in China. The numbers of cherry trees and area planted are increasing. Traditional methods for cultivar identification are based on objective descriptions of tree and fruit characteriatics, however, these observations affect the expression of genetic traits. Biochemical markers, such as isozymes, are more consistent and they were used for studying genetic variability in cherry (Granger et al. 1993). Molecular markers based on DNA are stable, detectable in all tissues and independent of environmental or seasonal conditions. They can be used for cultivar identification, diversity analysis, assessment of parentage, patent issues and quality control of rootstock-seed lots. The ability to distinguish cherry cultivars would be greatly enhanced by the use of molecular markers.

Recently, the development of DNA markers has, however, provided a plenty of polymorphism to enable the rapid identification of cultivars and the construction of saturated genetic maps. RAPD, SSR and AFLP markers have been used for the characterization of cherry (Gerlach and Stosser 1998; Downey and Iezzoni 2000; Cantini et al. 2001; Tavaud et al. 2001; Struss et al. 2003), even if they are time-cost and effective, they require an accurate standardization of the analysis to give reproducible patterns and they are difficult to interpret due to their dominant mode of inheritance. In comparison, simple sequence repeats (SSRs) have the potential to provide a more reliable method for DNA fingerprinting because of their high repeatability, high degree of polymorphism and co-dominant inheritance. SSR markers are abundant in most genomes and, generally, are uniformly distributed. They are PCR-based, thus requiring little DNA for the amplification, and every SSR locus is defined by a unique pair of primers, so that, information exchange between laboratories is easy and they are amenable to automation (Sosinski et al. 2000; Yamamoto et al. 2002).

The use of multi-allelic, co-dominant markers for the analysis of heterozygous species is extremely useful since it allows individuals to be uniquely genotyped (Powell et al. 1996). This is particularly important for cultivar identification and to enhance the genetic characterization of germplasm collections and natural populations, permitting effective conservation of plant genetic rescources; in addition molecular markers can be used for phylogenetic studies and linkage analysis.

SSRs has so many above-mentioned advantages, but it also has its disadvantage, that is, it is difficult to acquire the SSR primers.There was much evidence for cross-species applicability of microsatellite sequences, which allows the discrimination of different genotypes within a

fruit species with sequences developed in *Prunus* species (Ai et al. 2007). However, there were problems in the selection of primers, it needed such a great deal of workload and the ratio of the polymorphic primers is very low.

In the present work we report the development and the characterization of 24 SSR markers in *P. pseudocerasus* LindI. These markers are being used for studying the structure of cherry in five regions of China (Shandong, Shanxi, Gansu, Xizang, Liaoning) and the gene flow between populations at different domestication levels (orchard, coppice, naturalized).

Material and Methods

Plant material and DNA isolation

26 *P. pseudocerasus* LindI. individuals from the germplasm cultivated in National Fruit Germplasm Tai' an Cherry Repository were used for testing the SSR loci. The 26 sampled cultivars were: 'Laiyang Short cherry', 'Qianzhaihuang', 'Duiying', 'Qianzhaihong', 'Mahali-9', 'Shanying', 'Maoying', 'YT-97', '8-129', 'Maoying-7', '5-19', 'Taishanhong', '13-33', '8-106', 'Fengshou-100', 'Mahali-1', 'Y2', 'Daqingye', 'Dongbei shanyin', 'Taishanhuang', 'YT00-42', 'ZH-1', 'ZH-4', 'Sichuan Shanying', 'YT-21', 'Laojiazao'. Cultivar 'YT00-9' and 'YT99-2' and their 12 progeny individuals from Liaoning Academy of Agricultural Science were used for testing the segregation of alleles.

According to Doyle and Doyle protocol (1990), total genomic DNA was isolated from young cherry leaves using a modified CTAB method.

Isolation, cloning and sequencing of SSR repeats

The SSR library was prepared following the method by Edwards et al. (1996). The chosen approach was to construct and screen a genomic library harbouring small inserts (250–700 bp). The library was enriched with specific sequences so as to have a large set of positives clones and a large number of SSR types.

The seven following oligonucleotides were used to enrich DNA fragments containing SSR $(GA)_{15}$, $(CAA)_{10}$, $(CTG)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(ACT)_{10}$, $(GAC)_{10}$, the enrichment procedure followed the protocol of Edwards et al. (1996).

1 μg of genomic DNA from *P. pseudocerasus* LindI. (cultivar 'Laiyang Short cherry') was digested with *Rsa*I for 2 h at 37 °C. Further, 1 μl Quick T_4 ligase and 1 μg of *Mlu*I adapter (consisting of a 20-mer: 5′ ATCAAGCTTGTGACTACGCA 3′ and a phosphorylated 22-mer: 5′ TAGTACCCTACGAGAGAGCACA 3′) were added to the mix and ligation was allowed to proceed for 2 h at 37 °C.

1 μl of the ligation mix was amplified by PCR in a thermal cycler 9700 (Applied Biosystems), using the 20-mer primer at the following conditions: 3 min at 94 °C; 30 s at 94 °C, 30 s at 51–54 °C, 1 min at 72 °C, 34 cycles; 5 min at 72 °C. After amplification, the sample was phenol-chloroform extracted, precipitated and resuspended in fresh sterile distilled water.

SSR enrichment was carried out by hybridisation with the probes of microsatellite sequences. The enrichment was carried out hybridising for 2 days at 52 °C the ligated denatured DNA in 500 μl of hybridisation buffer (50 % formamide, 6×SSC, 25 mM NaH_2PO_4 pH 7.0, 2.5 % SDS) containing 1 μg of the 20 mer oligonucleotide. Following hybridisation, the filter was washed and then the hybridised DNA was eluted. Finally, 1 μl of the eluted DNA was amplified by PCR using the 20-mer primer following these conditions: 28 cycles at 94 °C for 45 s, 60 °C for 60 s, 72 °C for 90 s with a final step of 72 °C for 7 min. After amplification, the PCR sample was phenol-chloroform extracted, precipitated and finally resuspended in 25 μl of fresh sterile distilled water.

Following this step, the enriched mix was digested with *Mlu*I and purified by Amersham Biosciences S-300 spin column according to the instructions supplied. The sample was vacuum dried and dissolved in sterile distilled water. 1 μl of the enriched DNA was then taken and ligated into 10 ng of a modified pUC 19 vector, containing a *Bss*HII site, for 12 h at room temperature. Ligation products were transformed into competent DH5α.

At this stage of the research only dinucleotide repeats were selected for the isolation of SSR markers. The clones were transferred and replicated onto Hybond N + membranes with a Biomek workstation (Beckman Instruments, Fullerton, CA, USA) to be hybridised with $(CT)_{10}$ and $(CA)_{10}$ radio-active probes for the detection of clones containing (GA)/(TC) and (CA)/(TG) repeats.

70 plasmids from individual positive colonies were extracted using Wizard *Plus* SV Minipreps DNA Purfication System Kit (Promega) and sequenced with a semi-automatic ABI-PRISM 377 sequencer.

Primers design

The identification of SSR loci requires characterization of unique sequences flanking each side of the repeat region to design locus-specific primers for PCR amplification of a given SSR.

The primers were designed so that the size of amplicons were kept between 80 and 300 bp to allow accurate sizing of PCR products. Primers were between 17 to 28 bases in length, with 35–60 % GC content and matching with or very close to annealing temperature for primer pairs.

SSR amplification and analysis

The primers were initially tested on 6 DNA samples ('Laiyang Short cherry', 'Qianzhaihuang', 'Mahali-1', 'Dongbei shanyin', 'Qianzhaihong', 'ZH-4') using chemiluminescence detection. PCR amplification of SSR loci was carried out using a reaction mixture (25 μl), consisting of 2.5 μl 10 × buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.0 mM $MgCl_2$, 0.5 μM of each primer, 250 μM dNTP, 0.5 U *Taq*-DNA polymerase and 60 ng template DNA. PCR was carried out in a MJ Research PTC 100 thermal cycler.

Amplification cycles consisted of an initial step of 5 min at 94 °C, followed by 30 cycles of 50 s at 94 °C, 45 s at the annealing temperature (Table 2) of each primer pair, 90 s at 72 °C, with a final extension step of 7 min at 72 °C.

2 μl of each sample were analysed, after denaturation in 2 μl loading buffer (95 % formamide, 20 mM EDTA, 0.05 % bromophenol blue, 0.05 % xylene cyanole) at 94 °C for 3 min on a 50 cm sequencing gel (6 % polyacrylamide 24:1, 8 M urea, 1×TBE). After electrophoresis, the DNA was blotted on a Zeta Probe (Bio-Rad,USA) positively charged nylon membrane and hybridised over-

Table 1. Primer sequence, repeat type and predicted product length of SSR loci isolated in *P. pseudocerasus* (cultivar: 'Laiyang Short cherry'); annealing temperature and number of alleles found analysing six cultivars ('Laiyang Short cherry', 'Qianzhaihuang', 'Mahali-1', 'Dongbei shanyin', 'Qianzhaihong', 'ZH-4'). PTCR = *P. pseudocerasus* (Tai'an Cherry Repository).

SSR name	Primer sequence (5'→3')	Repeat type	Predicted product length (bp)	Optimal annealing temperature (°C)	No. alleles
PTCR1 F	A T G G T G T G T A T G G A C A T G A T G A	IMPERFECT	175	58	4
R	C C T C A A C C T A A G A C A C C T T C A C T	$(AG)_{15}$			
PTCR2 F	A T T C G G G T C G A A C T C C C T	IMPERFECT	224	55	3
R	A C G A G C A C T A G A G T A A C C C T C T C	$(CA)_{23}$			
PTCR3 F	A C C C A C A A A T C A A G C A T A T C C	COMPOUND	204	52	6
R	A G C T T C A G C C A C C A A G C	$(AC)_4TT(TA)_5$			
PTCR4 F	C A T A G G T T C A A A C C A T A C C C G T G	PERFECT	255	52	6
R	C T C A T C T T T G T A G G G T A T A A T A C C	$(AC)_{21}$			
PTCR5 F	A T G G A A G G G A A G A G A A A T C G	PERFECT	195	55	2
R	G T C A T C T C A G T C A A C T T T T C C G	$(CA)_{11}CG(TA)_6$			
PTCR6 F	C A T T T T C T C A T T G T G G C T G C	PERFECT	232	60	2
R	C A C T T G C A C A T C C A A T T A G G	$(AG)_{12}$			
PTCR7 F	T G A T A C C A C C A T C C A A T C T A G C	IMPERFECT	286	58	3
R	T T G C T G G G A C A T G G T C A G	$(CA)_2CT(CA)_6CT$			
PTCR8 F	T G C A T G A G A A A C T T G T G G C	IMPERFECT	200	55	4
R	C C A A G A G C C T G A C A A A G C	$(TC)_{18}$			
PTCR9 F	C A A T T T T A A G C A C A G G G A T C	PERFECT	225	50	2
R	C A A T G T G A A T T G G C C A T C A C	$(TG)_{11}TA(TG)_{13}$			
PTCR10 F	C A C T A T T T T A T C A T G G A C G G	PERFECT	195	50	1
R	C G A A T T G A G A G T T C A T A C T C	$(TG)_{18}$			
PTCR11 F	G G A C G G A C A G A A A T G A A G G T	IMPERFECT	207	50	1
R	C C T T A A C C C A C G C A A C T C C	$(GT)_8(GA)_{17}$			
PTCR12 F	T G A A G G A T G G C T C T G A T A C C	PERFECT	226	54	1
R	A A T T C A T C T A C T T C T T C C T C A A G C	$(CT)_{23}$			
PTCR13 F	T G A A G G A T G G C T C T G A T A C C	IMPERFECT	212	55	2
R	A A T T C A T C T A C T T C T T C C T C A A G C	$(AC)_7$			
PTCR14 F	T G A G C A A G G A T G G A T G A T G A G	IMPERFECT	176	55	2
R	G G T G G T C A T C A T G A C T G C A T C	$(TG)_4T(TG)_{18}$			
PTCR15 F	G A G A A T G C C C A C T T T T G C A	PERFECT	205	50	3
R	G C T C C C T T A T G G T C T C G	$(GA)_6GG(GA)_8$			
PTCR16 F	C T C C T T G A C T T T G A A G T T G C	IMPERFECT	264	58	3
R	C T G A T C G A G A G T A A T A A A G	$(CA)_{12}$			
PTCR17 F	A A G C G G A A A G C A C A G G T A G	PERFECT	104	58	4
R	T T G C T A G C A T A G A A A A G A A T T G T A G	$(AG)_{28}$			
PTCR18 F	G C T A A T A T C A A A T C C C A G C T C T C	PERFECT	144	54	5
R	T G A A G A A G T A T G G C T T C T G T G G	$(CT)_9$			
PTCR19 F	A A G T C A G C A A C A C C A T A T G C	IMPERFECT	118	56	1
R	C C C A C T G T T C A T G A G T T T C T	$(AG)_{20}$			
PTCR20 F	A C C A C T T T G A G G A A C T T G G G	COMPOUND IMPERFECT	282	60	2
R	C T G C C T G G A A G A G C A A T A A C	$(GT)_{19}$			
PTCR21 F	C G A G G T T G T T G T T C A T C A T T A C	COMPOUND	108	50	2
R	G A T C T C A A G T C A A A A G G T G T C	$(CA)_7AA(CA)_5$			
PTCR22 F	T G T G C T A A T G C C A A A A A T A C C	IMPERFECT	132	50	3
R	A C A T G C A T T T C A A C C C A C T C	$(GC)_{18}$			
PTCR23 F	A A G C T C A A T T G G C G T T G C T A	PERFECT	306	58	4
R	C T T G C C T C G A C G G T A T G G T A	$(AC)_{14}$			
PTCR24 F	A C G T A A A A A G C C C T C A A A C C	PERFECT	212	60	3
R	T T G C T T A C G C G T G G A C T A A C	$(GA)_{21}$			

Table 2. Allele size and allele frequency of 12 SSR loci studied in 26 cherrys cultivars.

Locus	Allele size (bp)	Allele frequency	Locus	Allele size (bp)	Allele frequency
PTCR1-HEX	179	0.375	PTCR14-NED	156	0.275
	196	0.125		165	0.050
	204	0.150		176	0.025
	212	0.150		179	0.150
	220	0.200		199	0.500
PTCR3-NED	206	0.050	PTCR15-6-FAM	195	0.075
	213	0.500		205	0.150
	215	0.175		210	0.250
	221	0.025		213	0.150
	225	0.025		215	0.025
	229	0.125		217	0.025
	232	0.100		221	0.050
PTCR4-6-FAM	180	0.050	PTCR18-NED	227	0.100
	215	0.400		229	0.200
	217	0.150		130	0.040
	223	0.050		132	0.225
	225	0.025		137	0.125
	231	0.175		141	0.500
	241	0.025		146	0.175
	243	0.025		153	0.025
	247	0.075		117	0.100
	257	0.025		122	0.125
PTCR6-HEX	225	0.275	PTCR19-HEX	124	0.100
	233	0.175		133	0.025
	237	0.025		135	0.525
	247	0.025		137	0.100
	249	0.400		147	0.025
	251	0.100		115	0.050
PTCR8-NED	206	0.275	PTCR22-NED	117	0.350
	221	0.250		123	0.425
	223	0.025		127	0.050
	229	0.300		129	0.050
	235	0.150		133	0.075
PTCR12-HEX	198	0.075	PTCR24-HEX	210	0.375
	218	0.400		212	0.225
	222	0.050		214	0.200
	224	0.425		220	0.200
	234	0.050			

night in 15 ml hybridisation buffer (0.5 M EDTA pH 8.0, 20 % SDS, 1M Na_2HPO_4 pH 7.2) containing 20 pmol of probe. The probes $(GA)_{13}$ and $(GT)_{13}$ were biotin labelled and hybridised at a temperature of 45 °C. Chemiluminescence detection was performed by the phototope-Star Detection Kit (New England Biolabs, Beverly, MA, USA) following the instructions provided by the manufacturer.

26 samples were then analysed at 12 chosen loci using a semi-automatic system in order to evaluate the SSR markers polymorphism. In this case the forward primer were labelled with a fluorochrome (6-FAM, HEX or NED).1 µl of mix containing amplification products of three differently labelled loci, was added to 3 µl of a mix containing 10:2:1 part of formamide, GeneScan-350 ROX size standard and loading buffer (25 mM EDTA, 50 mg mL^{-1} blue dextran). Samples were denaturated at 94 °C for 5 min and analysed on a sequencing gel (4.25 % acrylamide, 1×TBE buffer, 6 M Urea) using a ABIPRISM

377 DNA sequence employing GeneScan software program (Applied Biosytems).

The analyses were repeated three times to show the reproducibility of the results.

Statistical analysis

The number and the frequency of alleles per locus, the expected and observed heterozygosity, the null alleles frequency, the paternity exclusion probability and the probability to find two identical genotypes were calculated with the program Identity 1.0 (WAGNER and SEFC 1999). And the size of alleles were determined using WAVE® System with the ability to obtain reproducible sizing of DNA fragments from injection to injection.

The expected heterozygosity (H_e) was calculated as $H_e = 1-\sum P_i^2$ (NEI 1973) where P_i denotes the frequency of allele i in the cultivars. Observed heterozygosity (H_o) was obtained from direct calculation.

The null alleles frequency was calculated as $r = (H_e-H_o)/(1+H_e)$, following Brookfield (BROOKFIELD 1996), where H_e = expected heterozygosity and H_0 = observed heterozygosity. The paternity exclusion probability was calculated following WEBER and MAY (1989) and the probability to find two identical genotypes was calculated as $PI = 1-\sum P_i^4+\sum\sum(2P_iP_j)^2$ (PAETKAU et al. 1995) where P_i and P_j = frequency of alleles I and j respectively.

Results

A genomic library harbouring small inserts (250–700 bp) was constructed from the cultivar 'Laiyang Short cherry'. The library was screened by hybridisation with SSR probes $(CT)_{10}$ and $(CA)_{10}$. 18 % of the genomic clones from the library $(CT)_{10}$ and 28 % from the library $(CA)_{10}$ were positive.

70 positive clones were sequenced and 46 (66 %) contained repetitive arrays with suitable flanking regions for primers design; the remaining 24 sequences were rejected for being redundant (8 %) for lacking the SSR or due to the proximity of the SSR to the end of the insert.

All primer pairs were pre-screened on 6 individuals in a 6 % polyacrylamide denaturing gel, using a chemiluminescence detection system. 32 primer pairs (70 %) amplified at least 1 allele from all samples, whereas 14 primer pairs failed to amplify. Out of 32 primer pairs, 7 were discarded because they produced genotypes with 3 or more alleles.

The Mendelian type of inheritance was studied among all the alleles, and the segregation of alleles was detected among a small range of progeny (Fig. 1). The results showed that allele segregation, tested in the small progeny, followed the Mendelian type of inheritance for all the loci, but the offsprings of lane 3 and lane 10 inherited the genotype of parent 'YT00-9' while none of parent 'YT99-2', which might result from the apomixis engendered by the stimulation of pollen during the impregnation process, or from the incompatibility of the germ plasm of parent 'YT99-2' with or being excluded totally from the metabolisation of parent 'YT00-9', which consequently lead to the appearance of parents' genotype in hybrid generations.

The SSR of 14 (56 %) out of the 24 selected loci were perfect types; 8 (32 %) were imperfect, 2 (8 %) were compound and 1 (4 %) was imperfect-compound.

18 (72 %) loci out of 24 resulted polymorphic among the 6 individuals. The number of alleles detected per locus varied from 2 to 6: 8 loci showed 2 alleles, 6 loci 3 alleles, 4 loci 4 alleles and the remaining 2 loci 6 alleles (Table 1).

12 (PTCR1, PTCR3, PTCR4, PTCR6, PTCR8, PTCR12, PTCR14, PTCR15, PTCR18, PTCR19, PTCR22, PTCR24) out of the 18 loci were chosen for the analysis on the polymorphism shown in the first stage of the work.

26 DNA samples extracted from cultivars of *P. pseudocerasus* LindI. grown in National Fruit Germplasm Tai'an Cherry Repository were amplified at the 12 loci and analysed on Abi Prism377. They showed at least 1 or 2 alleles for each locus.

The number of alleles found was 75 in total and ranged from 4 to 10 per locus (Table 2), with an average of 6.3. The loci with higher allele number were PTCR4 (10 alleles) and PTCR15 (9 alleles). The largest size range between alleles was 77 bp in PTCR4.

Statistical elaboration of data is reported in Table 2 and 3. The higher allele frequency was 0.525 for allele 135 at locus PTCR19, whereas other alleles were specific for a particular cultivar with allele frequency of 0.025.

The mean expected heterozygosity (H_e) was 0.743 (range: 0.680–0.845). The mean observed heterozygosity (H_o) was 0.829 (range: 0.730–0.930), The locus PTCR22 showed the largest difference with H_e = 0.845 and H_o = 0.930.

The estimated frequency of null alleles showed positive values for 3 loci among 12 SSR markers, precisely for PTCR3 (0.103), PTCR6 (0.003), PTCR18 (0.009).

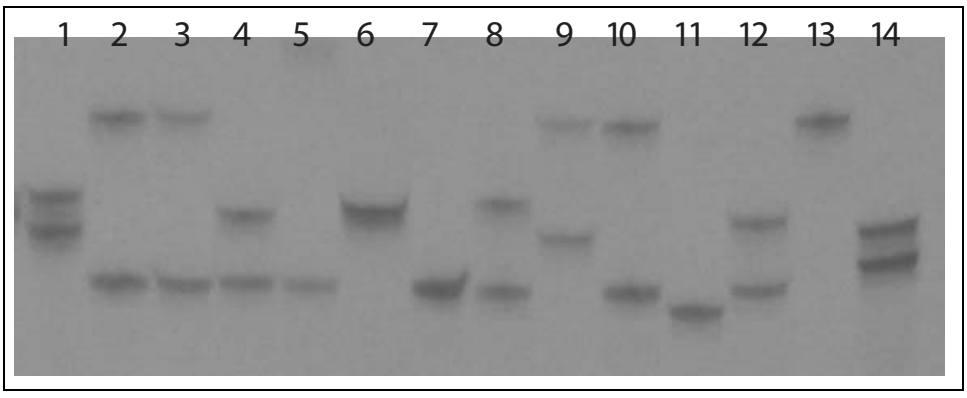


Fig. 1. Allele segregation in progeny plants detected by RTCR8 primer. 1: parental genotype 'YT99-2'; 2: parental genotype 'YT00-9'; 3 to 14: progeny individuals.

Table 3. Expected and observed heterozygosity, estimated frequency of null alleles, paternity exclusion probability and probability of identity of 12 SSR loci studied in 26 cherry cultivars.

Locus	Heterozygosity expected (H_e)	Heterozygosity observed (H_o)	Frequency of Null alleles	Paternity exclusion probability	Probability of identity
PTCR1-HEX	He: 0.692	Ho: 0.940	−0.106	0.452	0.150
PTCR3-NED	He: 0.840	Ho: 0.810	0.103	0.555	0.089
PTCR4-6-FAM	He: 0.806	Ho: 0.800	−0.015	0.640	0.140
PTCR6-HEX	He: 0.744	Ho: 0.750	0.003	0.485	0.210
PTCR8-NED	He: 0.758	Ho: 0.850	−0.026	0.510	0.174
PTCR12-HEX	He: 0.777	Ho: 0.820	−0.001	0.489	0.283
PTCR14-NED	He: 0.683	Ho: 0.900	−0.114	0.544	0.225
PTCR15-6-FAM	He: 0.710	Ho: 0.800	−0.064	0.652	0.193
PTCR18-NED	He: 0.685	Ho: 0.760	0.009	0.449	0.241
PTCR19-HEX	He: 0.694	Ho: 0.860	−0.076	0.495	0.096
PTCR22-6-FAM	He: 0.845	Ho: 0.930	−0.011	0.510	0.177
PTCR24-HEX	He: 0.680	Ho: 0.730	−0.099	0.614	0.230

The total paternity exclusion probability was 0.9999; this index was high for PTCR6 (0.640) and PTCR15 (0.652), with a mean value of 0.497 (range: 0.449–0.652).

The probability of identity (P*I*) changed from 0.089 for PTCR3 to 0.283 for PTCR12, the mean value was 0.181. According to frequency results the total probability of identity was 7.01×10^{-13}.

Discussion

The most frequently occurring SSR dinucleotide repeats in plants are (AT) with (CT)/(AG) and (CA)/(TG) as the second and third most frequent ones (Lagercrantz et al. 1993; Thomas and Scott 1993; Wang et al. 1994). However due to the difficulties in screening for (AT), autocomplementary oligonucleotides, using hybridisation techniques, we decided to develop SSR markers containing (CT)/(AG) and (CA)/(TG) repeats. We found that there was a prevalence of (CA)/(TG) positive clones (42.1 %) on (CT)/(AG) clones (23.2 %).

From the initial screening of 70 positive clones we designed 46 (66 %) primer pairs, 24 out of the tested 46 primer pairs amplified an expected number of alleles per genotype, 7 were discarded because they showed extra bands and 14 failed to amplify probably due to mismatches in the annealing region or to artifact inserts originated during the enrichment procedure.

Only 8 % (2 clones) of the 24 SSR sequences contained a compound repeat type. The observed number is in agreement with other studies; in fact only 7 % of di- and trinucleotide soybean repeats are compound (Akkaya et al. 1995) while none was found during development of 30 *Arabidopsis* dinucleotide markers (Bell and Ecker 1994).

From the average length of (CT)/(AG) and (CA)/(TG) motifs we expected a high polymorphism, as asserted by Weber (1990); among 24 loci, 18 (72 %) were polymorphic after chemiluminescence detection. We did not observe polymorphism for loci with less than 5–8 repeats as it was observed and demonstrated in human SSR by Armour et al. (1994).

In order to define a set of SSR sequences useful for the fingerprinting and mapping of cherry, we choose 12 loci for the analysis of 26 cultivars. At first, loci with 3 or more alleles, detected using chemiluminescence, were selected; later some loci with only 2 alleles were considered, to check if their polymorphism could be useful for studying a larger number of samples; indeed locus PTCR8 showed 5 alleles and PTCR19 showed 7 alleles; so also loci with 2 alleles at the first step of analysis may be useful for fingerprinting.

The polymorphic level was good, considering that we analysed 26 genotypes from a small geographic area. This fact reduced the probability of finding a higher allelic diversity which can be expected when further individuals will be studied. Most of the 12 loci can be rated as highly informative, also for the value of heterozygosity.

The occurrence of null alleles was already pointed out by Callen et al. (1993) as a possible problem associated with the use of SSR markers. If undetected, a null allele will merely result in that individual was scored as a homozygote resulting in loss of information. The presence of a null allele at an appreciable frequency can be suspected when there was observed heterozygosity. For 2 loci (PTCR6, PTCR18) a positive value for null allele frequency was found but very low, thus not giving conclusive indication about the issue; only 1 locus (PTCR3) showed a high value of null allele frequency (0.103) and can be regarded as a locus with a possible occurrence of null alleles, although homozygote excess may not always be obviously attributable to the presence of non amplifying alleles (O'Reilly and Wright 1995), The negative value for the null allele index, found in most loci, on the contrary, could associate the homozygotes with unfavourable characteristics, eliminated by man selection of cultivated germplasm.

The ability of genetic markers to study pollen flow is represented by the paternity exclusion probability, which is the parameter used to describe the chance of correctly identifying pollen donors (Brant et al. 1999). This value

was particularly high at loci PTCR15 (0.652) and PTCR4 (0.640) respectively and the very high combined probability of paternity exclusion (0.999) also indicates that this set of markers will be useful in parentage studies even in situations when both parental individuals are homozygous.

The range of variability makes it possible to select loci for specific applications. For example markers with only a few alleles are well-suited for population genetic studies (Schueler et al. 2006). The more variable SSR loci however are ideal for determination of parentage, pedigree analysis and for genome mapping (Sosinski et al. 2000; Dirlewanger et al. 2002; Struss et al. 2003).

In this study we described 24 SSR markers developed for *P. pseudocerasus* LindI. We began to evaluate in details 12 markers; among these 12 loci, 7 are particularly interesting considering their good polymorphism and are possible to be used for the study of gene flow and genetic mapping.

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References

Ai, C.X., L. Xin, L.S. Zhang, H.R. Wei, K.J. Yuan, Q.R. Sun, S.N. Jin and Q.Z. Liu 2007: Phylogenetic relationships among cherry genotypes using microsatellite markers. J. Fruit Sci. **24**, 460–465.

Akkaya, M.S., R.C. Shoemaker, J.E. Specht, A.A. Bhagwat and P.B. Cregan 1995: Integration of simple sequence repeat DNA markers into a soybean linkage map. Crop Sci. **35**, 11439–11445.

Armour, J.A.L., R. Neumann, S. Gobert and A.J. Jeffrevs 1994: Isolation of human simple repeat loci by hybridization selection. Hum. Mol. Genet. **3**, 599–605.

Bell, C.J. and J.R. Ecker 1994: Assignement of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genonics **19**, 137–144.

Brant, B., J. Witherspoon, A. Granger and G. Collins 1999: Identification of pollen donors for sweet cherry cultivars 'Stella' and 'Summit' by isozyme analysis. Aust. J. Exp. Agr. **39**, 473–477.

Brookfield, J.F.Y. 1996: A simple new method for estimating null allele frequency from heterozygote deficiency. Mol. Ecol. **5**, 453–455.

Callen, D.F., A.D. Thompson, Y. Shen, H.A. Philips, R.I. Richards and J.C. Mulley 1993: Incidence and origin of "null" alleles in the $(AC)_n$ microsatellite markers. AM. J. Hum. Genet. **52**, 922–927.

Cantini, C., A.F. Iezzoni, W.F. Lamboy, M. Boritzki and D. Struss 2001: DNA fingerprinting of tetraploid cherry germplasm using simple sequence repeats. J. Amer. Soc. Hort. Sci. **126**, 205–209.

Dirlewanger, E., P. Cosson, M. Tavaud, M. J. Aranzana, C. Poizat, A. Zanetto, P. Arus and F. Laigret 2002: Development of microsatellite markers in peach [*Prunus persica* (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry. Theor. Appl. Genet. **105**, 127–138.

Downey, S.L. and A.F. Iezzoni 2000: Polymorphic DNA markers in black cherry (*Prunus serotina*) are identified using sequences from sweet cherry. J. Amer. Soc. Hort. Sci. **125**, 76–80.

Doyle, J.J. and J.L. Doyle 1990: Isolation of plant DNA from fresh tissue. Focus **12**, 13–15.

Edwards. K.J., J.H.A. Barker, A. Daly, C. Jones and A. Karp 1996: Microsatellite libraries enriched for several microsatellite sequences in plants. Biotech. **20**, 758–760.

Gerlach, H. and R. Stosser 1998: Sweet cherry cultivar identification using RAPD-derived DNA-fingerprints. Acta. Hort. **468**, 63–69.

Granger, A.R., G.R. Clarke and J.F. Jackson 1993: Sweet cherry cultivar identification by leaf isozyme polymorphism. Theor. Appl. Genet. **86**, 458–464.

Lagercrantz, U., H. Ellegren and L. Anderson 1993: The abundance of various polymorphic microsatellite motifs differ between plants and animals. Nucl. Acid Res. **21**, 1111–1115.

Nei, M. 1973: Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. **70**, 3321–3323.

O'Reill, Y.P. and J.M. Wright 1995: The evolving technology of DNA fingerprinting and its application to fischeries and aqua culture. J. Fish Biol. **47**, 29–55.

Paetkau, D., W. Calvert, I. Stirling and C. Strobeck 1995: Microsatellite analysis of population structure in Canadian polar bears. Mol. Ecol. **4**, 347–354.

Powell, W., G.C. Macharay and J. Provan 1996: Polymorphism revealed by simple sequence repeats. Trends Plant Sci. **1**, 215–222.

Schueler, S., A. Tusch and F. Scholz 2006: Comparative analysis of the within-population genetic structure in wild cherry (*Prunus avium* L.) at the self-incompatibility locus and nuclear microsatellites. Mol. Ecol. **15**, 3231–3243.

Sosinski, B., M. Gannavarapu, L.D. Hager, L.E. Beck, G.J. King, C.D. Ryder, S. Rajapakse, W.V. Baird, R.E. Ballard and A.G. Abbott 2000: Characterization of microsatellite markers in peach [*Prunus persica* (L.) Batsch]. Theor. Appl. Genet. **97**, 1034–1041.

Struss, D., R. Ahmad and S.M. Southwick 2003: Analysis of sweet cherry (*Prunus avium* L.) cultivars using SSR and AFLP markers. J. Amer. Soc. Hort. Sci. **128**, 904–909.

Tavaud, M., A. Zanetto, F. Santi and E. Dirlewanger 2001: Structuration of genetic diversity in cultivated and wild cherry varieties using molecular markers. Acta. Hort. **546**, 263–269.

Thomas, M. R. and N.S. Scott 1993: Microsatellite repeats in grape vine reveal DNA polymorphism when analysed as sequence tagged sites (STSs). Theor. Appl. Genet. **86**, 985–990.

Wagner, H.W. and K.M. Sefc 1999: Survey of plant short tandem DNA repeats. Theor. Appl. Genet. **88**, 1–6.

Wang, Z., J.L. Weber, G. Zhong and S.D. Tanksrey 1994: Survey of plant short tandem DNA repeats. Theor. Appl. Genet. **88**, 1–6.

Weber, J.L. and P.E. May 1989: Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. **44**, 388–397.

Weber, J.L. 1990: Informativeness of human (dC-dA)n (dG-dT)n polymorphisms. Genomics **7**, 524–530.

Yamamoto, T., T. Kimura, Y. Sawamura, T. Manable, K. Kotobuki and T. Hayashi 2002: Simple sequence repeat for genetic analysis in pear. Euphytica **124**, 129–137.

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