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Innovative multiplex and its evaluation for effective genotyping of wild cherry

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Highlights

- We present simple and cost-efficient method of SSR genotyping in cherry species.
- Seven of fifteen previously published primer sequences have been altered in order to produce complementary amplicons in pseudo 15-plex.
- The effectiveness and discriminatory power of established multiplex were verified by SSRs analysis of 48 wild cherry trees.

Abstract

Trees from the family Rosaceae play an important role in forest and agricultural ecosystems. Therefore, they are often an object of interest for both forest and horticultural tree breeders. Here, we present the utilization of an effective microsatellite (SSRs) genotyping method for wild cherry (*Prunus avium* L.) and verified the discriminatory power of the presented multiplex by genotyping 48 genetically distinctive individuals (plus-trees). Concerned loci were previously proven to be cross-compatible among various cultivars of cherry, hence, the method could have a broader utilization beyond to the field of forestry.

Our technique is based on post-PCR processing of 15 polymorphic SSRs loci amplified in three multiplex reactions with fluorescently labeled primers (6-FAM, VIC, PET and NED). All PCR products could be pooled and analyzed simultaneously (pseudo 15-plex). In order to make this approach feasible, we redefined sequences of several primers. Thus, utilizing modified primers provides non-overlapping amplicons of each fluorescent dye.

Keywords *Prunus avium*; SSRs; microsatellites; pseudo 15-plex; one-run genotyping; genetic structure
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1 Introduction

Over the past 50 years, the area of molecular biology has made tremendous progress and an extensive number of genetic markers were developed and further improved. Originally, isoenzyme analyses were applied on sweet cultivars of wild cherry (*Prunus avium* L.), allowing practical application such as identification of various cherry cultivars (Bošković et al. 1997). In the 80's and 90's only few studies paid attention to genetic aspects of the species. Gerlach and Stösser (1997) used Random amplified polymorphic DNA (RAPD) method for cherry cultivar determination. Tavaud et al. (2001) and Zhou et al. (2002) analyzed genetic distances and relationships among sweet cherry cultivars by Amplified Fragment Length Polymorphism (AFLP). Several studies combining different markers, e.g., research published by Clarke et al. (2009) utilized both isoenzymes and microsatellites for linkage map construction. Gulen et al. (2010) took advantage of AFLP and SSR markers to assess genetic relationship among introduced and local sweet cherries. When using microsatellite markers, the mean heterozygosity and detected polymorphism is usually significantly higher than that reported for isoenzymes and other molecular markers such as RFLP and RAPD (Cipriani et al. 1999). Microsatellites or simple sequence repeats (SSRs) are highly polymorphic, codominant and believed to be neutral markers particularly useful for population studies at a localized geographic scale and also for evolutionary scale level (Ganopoulos et al. 2011). They occur with the high abundance in eukaryotic genomes and are therefore generally recognized as genetic markers with the great power of discrimination enabling resolving of unique genetic entities. Despite generally decreasing cost of genotyping this technique is still quite financially demanding. Considerable amount of time, effort, and money can be saved by simultaneous amplification of multiple DNA sequences in a single reaction by a process referred to as multiplex polymerase chain reaction. True multiplexing is accomplished by co-amplification of multiple microsatellites in a single PCR product. Alternatively, PCR products from multiple reactions can be mixed and analyzed as a single product. This procedure is referred to as pseudo-multiplexing or poolplexing (Guichoux et al. 2011).

In our paper, we combined both of the above mentioned approaches and present a pseudo-multiplex composed from 15 polymorphic SSR loci that had been amplified in three multiplex reactions (7-, 4- and 4-plex respectively). In order to employ pseudo-multiplexing, we redefined sequences of several reverse primers. Fluorescently labeled PCR amplicons generated with modified primers provided non-overlapping fragments that could be analyzed in a single run of capillary electrophoresis system. Our technique provides a cost-effective, time and effort saving tool for microsatellite genotyping of wild cherry.

2 Materials and methods

Genomic DNA was obtained from post-dormant buds collected during February and March. Initially, 62 wild-cherry trees that represent the entire set of plus-trees clones in a clonal seed orchard were used for evaluation of microsatellite amplification patterns. Later we reduced the number to 48 individuals since some clones proved to be identical, vegetative copies.

Plant material (approximately 150 mg per sample) was frozen in nitrogen liquid and disrupted in an oscillation mill (Retsch MM400). DNA was subsequently extracted using Invisorb Spin Plant Mini Kit (Strateg Biomedical AG) following the manufacturer protocol with minor modifications (extension of lysis phase to 45 minutes for higher DNA yield, addition of 4 µl RNase A (ThermoFisher Scientific, EN0531), and omission proteinase K). DNA was finally eluted into 100 µl of ultrapure water (approx. range of DNA concentrations 15–250 ng μl^{-1}). DNA quantity

and quality was evaluated spectrophotometrically and DNA integrity was confirmed by 1.0% agarose gel electrophoresis with ethidium bromide staining. For consecutive analysis, all DNA samples were diluted to a standardized concentration of 10 ng μl^{-1}. All primers were diluted to a concentration of 10 pmol μl^{-1}. PCR amplification was carried out in Veriti 96-well thermal cycler (Applied Biosystems).

In the initial phase, amplification of 26 selected SSR loci in separate reactions was tested (Supplementary file S1, available at https://doi.org/10.14214/sf.5644). Based on amplification patterns which were obtained by analysis of separate PCR products we finally selected 15 primer pairs which were combined into three multiplex reactions (Table 1) based mainly on assumption of

Table 1. Description of primer sequences, fluorescent labeling and length extension of PCR products amplified by modified primers.

locus name source literature / GenBank accession number	primer sequence (5′–3′)	primer concentration in multiplex (μM)	fluorescent dye (5' F primer)	extension of modified amplicons (bp)
multiplex A				
EMPaS02	F: CTACTTCCATGATTGCCTCAC	0.2	FAM	-
A / AY526619	R: AACATCCAGAACATCAACACAC			
*EMPaS10 long	F: GCTAATATCAAATCCCAGCTCTC	0.2	VIC	127
A / AY526626	R: **CTACTGGCTTGTGTTGTGTG**			
EMPaS11	F: ACCACTTTGAGGAACTTGGG	0.2	FAM	-
A / AY526627	R: CTGCCTGGAAGAGCAATAAC			
UCD-CH11	F: TGCTATTAGCTTAATGCCTCCC	0.3	PET	-
C / NA	R: ATGCTGATGTCATAAGGTGTGC			
UCD-CH31	F: TCCGCTTCTCTGTGAGTGTG	0.2	NED	-
C / NA	R: CGATAGTTTCCTTCCCAGACC			
*PS12A02 long	F: GCCACCAATGGTTCTTCC	0.3	NED	7
E / AB476763	R: **CAACCAAAGCACCAGATGC**			
UDP98-412	F: AGGGAAAGTTTCTGCTGCAC	0.2	VIC	-
B / AY074720	R: GCTGAAGACGACGATGATGA			
multiplex B				
EMPaS05	F: CATGTGCTTTCTCTGCCC	0.2	VIC	-
A / AY526621	R: TCTTCTCAAGCAATTCCCC			
EMPaS06	F: AAGCGGAAAGCACAGGTAG	0.2	NED	-
A / AY526622	R: TTGCTAGCATAGAAAAGAATTGTAG			
*EMPaS12 long	F: TGTGCTAATGCCAAAAATACC	0.2	FAM	99
A / AY526628	R: **GTGGAAGGCCATATTTTCGG**			
*BPPCT 034 long	F: CTACCTGAAATAAGCAGAGCCAT	0.3	PET	99
D / AF374945	R: **GCTCGTAAGATTGCTGGTAGC**			
multiplex C				
*UDP97-403 long	F: CTGGCTTACAACTCGCAAGC	0.3	VIC	76
B / AY074723	R: **CTCACAGACTCGACTCTTCACTTC**			
*PceGA34 long	F: GAACATGTGGTGTGCTGGTT	0.2	NED	100
E / NW007362363	R: **CCCACTTTCATCAAAACAGTGAG**			
*UDP98-411 long	F: AAGCCATCCACTCAGCACTC	0.15	PET	108
B / AY074719	R: **GCTGATGATGACGACGATGATG**			
UCD-CH12	F: AGACAAAGGGATTCTGGGC	0.4	PET	-
C / NA	R: TTTCTGCCACAAACCTAATGG			

* – modified loci are tagged with suffix long, sequences of redefined reverse primers are highlighted in bold; NA – sequences not published in GeneBank database. Source literature: A – Vaughan and Russel 2004, B –Testolin et al. 2000 (Schueller et al. 2006), C – Struss et al. 2003, D – Dirlewanger et al. 2002, E – Downey and Iezzoni 2000.

dimer absence. All forward primers from selected subset of markers were 5'-fluorescently labeled (6-FAM, VIC, PET, NED) as well as LIZ fluorescently labeled GMC-GT 500 dye size standard (ThermoFisher Scientific) in order to enable processing by five-color fluorescent system Genetic Analyzer 3500 (Applied Biosystems).

For the final multiplex PCR arrangement, reverse primers of seven loci, namely EMPaS10, PS12A02, EMPaS12, BPPCT 034, UDP97-403, PceGA34 and UDP98-411 were modified. An application of this approach was enabled owing to nucleotide sequences stored in the GenBank database (Suppl. file S2). Suitability of newly designed sequences was checked via the web interface of Primer3 software (Untergasser et al. 2012). PCR conditions of established multiplex reactions were initially optimized with the Multiplex PCR kit (Qiagen). Nevertheless, it required intricate temperature touchdown steps in each multiplex (not shown). A substantial simplification was further achieved by replacement of the Multiplex PCR kit with the Type-it Microsatellite PCR kit (Qiagen). As a result, PCR conditions were established as uniform for all three multiplexes. Namely, starting with an initialization for 15 min at 95 °C followed by 40 cycles of denaturation by 95 °C for 30 s, annealing by 60 °C for 45 s, elongation by 72 °C for 30 s and a final elongation step took 30 min at 72 °C. Each multiplex PCR mixture consist from 1µl DNA, 4.5 µl 2x Type-it Multiplex PCR Master Mix, appropriate volume of primers (Table 1) fill up with RNase-free water to the total reaction volume of 10 µl. For allele length analysis, 2 µl of each PCR product (multiplex A, B, C) was mixed together and vortexed. After that, 1 µl of this mixture, 12 µl of formamide solution and 0.2 µl DNA ladder was blended. The pseudo-multiplex product was denatured at 95 °C for 5 min and rapidly cooled on ice for 5 min. Amplified fragments were separated on Genetic Analyzer 3500 (Applied Biosystems) and data were evaluated in GeneMarker® V 2.4.0 software.

Multiloci data in numerical format were processed using software Cervus (Kalinowski et al. 2007). The following parameters in the data set were calculated: number of alleles per locus (k), expected (H_e) and observed (H_o) heterozygosity, Polymorphic Information Content (PIC, Botstein et al. 1980) and evaluation of Hardy-Weinberg equilibrium. An estimation of null alleles was processed via Micro-Checker (van Oosterhout et al. 2004). Fixation indices (F_{IS}) and effective number of alleles (N_e) were generated by GenAlEx 6.5 (Peakall and Smouse 2012).

3 Results

From the initial set of 26 markers, 24 loci showed scorable and polymorphic pattern of amplification with the PIC of 0.630±0.110. We excluded those with lower PIC (generally bellow 0.62, Suppl. file S1) and/or with an inferior quality of amplification pattern. Sixteen markers with clear amplification and the average-above PIC (0.686±0.070), namely EMPaS02, EMPaS05, EMPaS06, EMPaS10, EMPaS11, EMPaS12 (Vaughan and Russell 2004), UCD-CH11, UCD-CH31, UCD-CH12 (Struss et al. 2003), UDP97-403, UDP98-411, UDP98-412 (Testolin et al. 2000), BPPCT 040, BPPCT 034 (Dirlewanger et al. 2002), PS12A02, PceGA34 (Downey and Iezzoni 2000) were identified for further optimization.

Our aim was to enable analysis of all amplified fragments into one single sequencing run. In order to do that, the length of some amplicons needed to be altered. We altered seven unlabeled reverse primers (Table 1, altered primers have been labeled with suffix *long*) in order to modify length of PCR amplicons. During the optimization process locus BPPCT040 was omitted from the final primer selection while it did not show apparent amplification under the final and unified PCR conditions. All modified primers in the pseudo-multiplex generated complementary, i.e. non-overlapping fragments of the same fluorescent dye (Table 2).

Table 2. Size ranges of PCR products in pseudo 15-plex.

fluorescent dye	primer 1	primer 2	primer 3	primer 4
PET	UCD-CH11	UCD-CH12	UDP98-411 long	BPPCT 034 long
size range (bp)	144–168	176–200	268–284	294–330
NED	UCD-CH31	PS12A02 long	EMPaS06	PceGA34 long
size range (bp)	124–136	157–179	204–228	239–273
FAM	EMPaS11	EMPaS02	EMPaS12 long	
size range (bp)	66–108	134–148	224–248	
VIC	UDP98-412	EMPaS05	UDP97-403 long	EMPaS10 long
size range (bp)	113–133	162–174	194–224	281–312

The entire set of 15 primer pairs produced scorable PCR amplicons. Automatic pre-scoring was done in the software GeneMarker with the final manual adjustment. The revealed allelic dropout was generally very low across all loci, with one missing value at UCD-CH12 and one at PceGA34. All of 15 microsatellite loci are polymorphic in the tested population of wild cherry superior genotypes with the number of alleles per locus ranging from 4 to 15 (mean 7.400 ± 2.746) in the dataset with the mean PIC of 0.685 ± 0.072 (Table 3). The loci did not show the distortion of the Hardy-Weinberg equilibrium, fixation index (F_{IS}) did not significantly excess or lack heterozygosity. None of loci evinced significant evidence for null alleles as evaluated using Monte Carlo algorithms implemented in Micro-Checker. Effective number of alleles (N_e) gained 3.776 ± 0.917.

Table 3. Summary evaluation of amplified loci.

locus name	k	N_e	H_o	H_e	PIC	F_{Null}	F_{IS}
EMPaS02	7	3.294	0.542	0.604	0.568	0.0663	0.132
EMPaS10 long	7	3.393	0.614	0.684	0.653	0.0281	0.055
EMPaS11	4	2.678	0.795	0.777	0.737	0.0273	0.069
UCD-CH11	8	2.491	0.771	0.731	0.679	–0.0232	–0.044
UCD-CH31	7	2.898	0.795	0.826	0.799	0.07	0.109
PS12A02 long	10	4.697	0.53	0.592	0.556	0.0201	0.021
UDP98-412	6	4.133	0.723	0.792	0.757	0.0333	0.066
EMPaS05	4	3.643	0.687	0.804	0.778	–0.0027	–0.005
EMPaS06	6	3.371	0.735	0.832	0.805	0.0206	0.023
EMPaS12 long	6	3.401	0.639	0.718	0.671	–0.0933	–0.180
BPPCT 034 long	8	5.789	0.699	0.75	0.702	0.0365	0.068
UDP97-403 long	9	4.820	0.759	0.745	0.703	0.0513	0.106
PceGA34 long	15	4.807	0.744	0.882	0.867	0.0048	0.006
UDP98-411 long	5	3.294	0.602	0.645	0.593	–0.0693	–0.137
UCD-CH12	9	3.934	0.775	0.855	0.834	–0.0101	–0.027
EMPaS02	7	3.294	0.778	0.792	0.76	0.0663	0.132
mean	7.400	3.776	0.708	0.729	0.685	0.011	0.017
SD	2.746	0.917	0.081	0.065	0.072	0.046	0.088
SE	0.709	0.237	0.021	0.017	0.019	0.012	0.023

k – number of detected alleles
N_e – effective number of alleles
H_o, H_e – observed and expected heterozygosity
PIC – polymorphic information content
F_{Null} – potential occurrence of null alleles
F_{IS} – fixation index

4 Discussion

Microsatellite primers initially selected for our study were published for wild cherry DNA amplification in 2000 by Downey and Iezzoni (PS12A02, PceGA34) and Testolin et al. (UDP97-403, UDP98-411, UDP98-412), in 2002 by Dirlewanger et al. (BPPCT034), in 2003 by Struss et al. (UCD-CH11, UCD-CH31, UCD-CH12) and in 2004 by Vaughan and Russel (EMPaS02, EMPaS05, EMPaS06, EMPaS10, EMPaS11, EMPaS12) respectively. Primer UDP 97-403 was developed in *Prunus persica* (L.) Batsch by Cipriani et al. (1999). Besides these publications describing primers and its utilization for the first time, many other studies based on some of these primers have been published, e.g. Jarni et al. (2012); Jolivet et al. (2012, 2013); Vaughan et al. (2007); Stoeckel et al. (2006); Ganopoulos et al. (2011); Höltken and Gregorius (2006); Avramidou et al. (2010); Mariette et al. (2007) and Sharma et al. (2015). In these studies, the average number of genotyped loci varied in total around 10 SSRs markers. Microsatellite fragments were amplified usually into three or four multiplex reactions with products being analyzed for each multiplex separately. Therefore, utilizing of our optimized panel of primers suitable for pseudo-multiplex could save significant expenses in the step of fragmentation analysis. When proposing new reverse primers from the DNA sequence database, we took into account general guidelines for primer development such as optimal primer length, primer melting and annealing temperatures, GC content and GC clamp or 3′end stability (Untergasser et al. 2012). Interestingly, we found out that even some of the original and frequently capitalized primer pairs did not follow these common recommendations. To address this, we relaxed selection criteria and preferred primer sequences selection that generate appropriate (i.e. complementary) length of PCR product size. Experienced this, an evaluation study for efficiency and reliability of newly established primer pairs seemed to be necessary.

Originally, we composed pseudo-multiplex with 16 markers (an additional locus BPPCT 040 long). When optimized PCR protocol for uniform PCR conditions, this locus did not evinced apparent amplification. Moreover, the length difference between amplicons of BPPCT 040 long and EMPaS02 labelled with the same fluorescent dye was the shortest in our dataset, made up of only 7 nucleotides. In order to eliminate the potential risk of allelic overlapping and simplify the protocol as much as possible, we omitted this locus from the final optimization. Based on detected size ranges of PCR amplicons marked with the same fluorescent dye, it does not seem improbable that some individuals from diverse populations of wild cherry might carry alleles which will cause overlapping between amplicons belonging to the different loci. In our case, the shortest distances between amplicons oscillated around 10 bp, namely for PET fluorescent dye UCD-CH11 and UCD-CH12 (length difference of 8 bp), for NED UDP98-411 and BPPCT 034 long (10 bp), for 6-FAM EMPaS06 and PceGA34 long (11 bp) (Table 2). Nevertheless, in the scenario of potential longitudinal allelic overlapping, the manual genotyping adjustment could easily eliminate this issue, since each locus evinced the specific amplification pattern.

The amplification reliability of newly composed primer pairs was controlled by genotyping of identical DNA samples using modified and originally published primers. The products differ only by length of amplicons. These findings correspond with our expectations, since PCR amplicons (alleles) obtained by redesigned primers are fully compatible with amplicons generated by its original form after adjustment of the length extension (PCR products' elongation in Table 1).

5 Conclusions

Newly established pseudo-multiplex aims to simplify and decrease cost of microsatellite genotyping. Complementary amplicons generated by this innovative method of primer redesigning ensures that all products of PCR amplification can be mixed (pooled) and analyzed together. Our results prove the high discriminatory power of established pseudo-multiplex. By doing that, we verified that our approach is an economically meaningful alternative to previously published protocols, especially when genotyping large numbers of individuals, such as transformation of half-sib progeny test into full-sib populations when speeding up the traditional breeding cycle.

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Total of 28 references.

Supplementary files

S1.xlsx,
S2.pdf,
available at https://doi.org/10.14214/sf.5644