Development of High Quality EST-SSR Markers Without Stutter Bands in Peach and Their Application in Cultivar Discrimination and Hybrid Authentication

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Abstract. Peach (*Prunus persica*) is an important fruit crop worldwide with several thousand cultivars. Cultivar discrimination and hybrid authentication are often required in peach breeding and can be achieved by applying various molecular markers including simple sequence repeat (SSR). In this study a total of 2146 expressed sequence tag (EST)–SSR loci were detected with the 10,737 EST sequences retrieved from the NCBI. A total of 49 EST–SSR markers, including 24 simple ones with a motif comprising of tri-, tetra-, penta-, hexanucleotides, and 25 compound ones, were selected and then primers were designed. Following conventional polymerase chain reaction (PCR) specificity control and sequence authentication, as well as fluorescence-based PCR product size and stutter band evaluation, 37 EST–SSR markers with correct amplification and without stutter band interference were validated. Among them, 14 were polymorphic in 18 closely related peach accessions, with polymorphism information content (PIC) ranging from 0.0994 to 0.3750. The 18 peach accessions can be distinguished using nine polymorphic markers, with the exception of ‘Shangshandayulu’ and ‘Xipu 1’, both being bud sports from ‘Yulu’. The clustering of the accessions as well as the fingerprint profiles supported the authentication of the hybrids. These EST–SSR markers are useful for peach breeding research.

Peach is one of the most commercially important and widely distributed fruit crops with several thousand cultivars and the number is increasing (Li et al., 2013) with enhanced breeding activities. Peach is believed

to be of very low genetic variability with a narrow genetic base (Cipriani et al., 1999). Some cultivars or lines, especially those from the same breeding parents, are close both genetically and morphologically. The fact that peach is self-fertile makes it important to authenticate the origin of selections to avoid any mistakes in peach cultivar pedigrees.

Peach cultivars were traditionally identified morphologically but this approach proved difficult for closely related cultivars. In recent decades, the identification of cultivars has instead been achieved by using various molecular markers. For example, using restriction fragment length polymorphism and random amplified polymorphic DNA (Chaparro et al., 1994; Quarta et al., 1998; Rajapakse et al., 1995). SSR markers have been used more recently in peach as well (Aranzana et al., 2002; Chen et al., 2014;

Cipriani et al., 1999; Dirlwanger et al., 2002; Li et al., 2013; Sosinski et al., 2000; Testolin et al., 2000) because of the advantages of ease of operation, high repeatability, accuracy, allelic diversity, and feasibility of transfer between closely related species (Guichoux et al., 2011).

SSRs are regions of repetitive DNA with certain DNA motifs, normally consisting of one to six nucleotides, repeated several times. SSRs are extensively distributed in eukaryotes. In plants, it can be expected to find at least one SSR locus present in every 10 kb of DNA sequence (Tautz, 1989) and the density was found to be as high as 428.5 per Mb, on average, for eight selected plant species (Cavagnaro et al., 2010). Therefore, numerous SSR loci can be mined to develop SSR markers, especially when genomic or transcriptomic sequencing has been completed. However, for some SSR markers, accurate size identification and allele designation can be difficult due to the presence of stutter bands, or stutter peaks. This problem was encountered in our preliminary study in peach as well as extensively reported in other plants (Caruso et al., 2008; Goulão and Oliveira, 2001; Huang et al., 2000; Jones et al., 1997; Song et al., 2002; Teulat et al., 2000; Wang et al., 2016; Wünsch and Hormaza, 2002). The stutter bands occur as a result of enzyme slippage during amplification and was most common for SSR markers with dinucleotide motifs and the problem is amplified as the repeat number of the alleles increases (Guichoux et al., 2011; Hite et al., 1996). In contrast, markers with motifs consisting of tri- to hexanucleotides, especially when the repeat number is not high, have been shown to be less prone to stuttering problems (Edwards et al., 1991; Goulão and Oliveira, 2001; Song et al., 2002).

In this study, a total of 37 high-quality EST–SSR markers that did not produce stutter bands, consisting of either motifs with at least three nucleotides or compound motifs, were designed and 14 of them were found to be polymorphic in 18 closely related peach accessions, including 10 offsprings from three crosses, studied and therefore useful for peach cultivar discrimination and hybrid authentication.

Materials and Methods

Plant materials. Eighteen accessions of peach [*P. persica* (L.) Batsch], including 14 melting peach accessions and four closely related yellow peach accessions, were used in this study (Table 1). The melting peach accessions consist of four commercial cultivars and 10 elite hybrids from crosses between two of these four cultivars as indicated in Table 1. The hybrids are generated and selected out based on agronomical performances in previous years and hybrid authentication as well as accession discrimination are required. The trees were cultivated at the Fenghua Institute of Honey Peach, Zhejiang province,

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Table 1. List of 18 peach accessions included in this study.

No.	Accession	Note	Parentage/source information
1	Hakurei	Melting peach	Okubo × Feichengtao, a cultivar from Japan
2	Hujingmilu	Melting peach	A cultivar from Wuxi, Jiangsu, China
3	Shangshandayulu	Melting peach	A bud sport from 'Yulu' (a cultivar from Ningbo, Zhejiang, China)
4	Xipu 1	Melting peach	A bud sport from 'Yulu'
5	F1-12	Melting peach	Hujingmilu × Hakurei
6	F1-13	Melting peach	Hujingmilu × Hakurei
7	F2-7	Melting peach	Hujingmilu × Hakurei
8	F2-18	Melting peach	Hujingmilu × Hakurei
9	F3-1	Melting peach	Hujingmilu × Hakurei
10	F3-10	Melting peach	Hujingmilu × Hakurei
11	X1-15	Melting peach	Shangshandayulu × Hujingmilu
12	X2-5	Melting peach	Shangshandayulu × Hujingmilu
13	X2-19	Melting peach	Shangshandayulu × Hujingmilu
14	X5-20	Melting peach	Xipu 1 × Hujingmilu
15	Fenghuang	Yellow peach	A cultivar from Dalian, Liaoning, China
16	Jinxiang	Yellow peach	A cultivar from Shanghai, China
17	Jinxu	Yellow peach	A cultivar from Shanghai, China
18	Jinyuan	Yellow peach	A cultivar with Jinxu as maternal parent

China. Young leaves were collected and subsequently frozen in liquid nitrogen and stored at -80°C until use.

DNA extraction. Genomic DNA was extracted using an improved cetyltrimethylammonium bromide protocol as described by Gawel and Jarret (1991). The DNA was purified with a Mag-MK Plant Genomic DNA Extraction kit [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] following the manufacturers' instructions and quantified according to absorbance at 260 nm.

Development of new EST-SSR markers. MISA (Thiel et al., 2003; <http://pgrc.ipk-gatersleben.de/misa/>) was used to mine SSR markers from GenBank (<http://www.ncbi.nlm.nih.gov/>) with the following coefficients: size of PCR products between 100 and 300 bp; primer length between 18 and 20 bp, annealing temperature $50\text{--}65^{\circ}\text{C}$, GC content around 40% to 70%, optimum 50%; number of repeats no less than five for simple SSR markers consisting of tri to hexanucleotide motifs, and the size between two motifs in a compound SSR marker no more than 100 bp.

SSR-PCR amplification. Conventional, or nonlabeled, SSR-PCR amplification was performed for gene cloning. The PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 ng genomic DNA, 5 pmol of each primer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, and 0.5 units of Taq DNA polymerase (Vazyme Biotech Co., Ltd.). Amplification was performed in 25 μL volumes using an Eppendorf Mastercycler (Eppendorf Scientific, Inc.) with an initial denaturation at 94°C for 5 min followed by 30 cycles of 30 s at 94°C , 30 s at 58°C , 30 s at 72°C , and a final extension of 7 min at 72°C . For fluorescence-based size identification purposes, a fluorescent labeling approach was followed (Schuelke, 2000). The PCR mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 ng genomic DNA, 4 pmol forward primer with M13 tail (M13 universal sequence, 5'-TGTAACGACGGCCAGT-3') at the 5'-end, 5 pmol of downstream

primer, 1 pmol of universal fluorescence dye, either 6-carboxyfluorescein (6-FAM) or 5-hexachloro-fluorescein (HEX), labeled primer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, and 0.5 units of Taq DNA polymerase (Vazyme Biotech Co., Ltd). Amplification was performed in 25 μL volumes using an Eppendorf Mastercycler (Eppendorf Scientific, Inc.) with an initial denaturation at 94°C for 5 min followed by 20 cycles of 30 s at 94°C , 30 s at 58°C , 30 s at 72°C , another 12 cycles of 30 s at 94°C , 30 s at 53°C , 30 s at 72°C , and a final extension of 7 min at 72°C . The primers were synthesized by [Sangon Biotech (Shanghai) Co., Ltd] and the sequences of primers are listed in Table 2.

SSR-PCR product size identification and sequence authentication. Size identification of fluorescence dye-labeled SSR-PCR products was completed by [Sangon Biotech (Shanghai) Co., Ltd] using capillary electrophoresis in an ABI PRISM 3130 DNA Analyser (Applied Biosystems, Foster City, CA). The authentication of SSR-PCR products was analyzed through conventional gene cloning and sequencing (Sambrook et al., 1989).

Data analysis. A (major allele frequency), N_a (no. of alleles), N_g (no. of genotypes), H_e (expected heterozygosity), H_o (observed heterozygosity), and PIC was calculated with PowerMarker3.25 (Liu and Muse, 2005). The unweighted paired group method using arithmetic averages was used to construct the phylogenetic tree with TreeView3.2 (Page, 1996).

Results

Characterization of peach EST-SSR loci. A total of 10,737 EST sequences were retrieved from the NCBI with a total size of 7.36 Mb. A total of 2146 EST-SSR loci were found with a SSR detection rate of 20% and the distribution density of one SSR locus per 3.43 kb. Near three quarters of the loci detected were simple EST-SSR markers and the remaining consisted of compound SSR markers, defined as the loci located in

a UniGene with less than 100 bases between adjacent loci (Table 3). About two-thirds of simple SSR markers contained dinucleotide motifs and one third trinucleotide motifs. Simple EST-SSR markers containing four to six nucleotides were rare, accounting for only 2% of total loci (Table 3).

Development and validation of EST-SSR markers. A total of 49 EST-SSR markers, including 24 simple and 25 compound repeat sequences, with relatively low repeat numbers of motifs, were selected from the tri-, tetra-, penta-, hexa-, and compound EST-SSRs and primers were designed (Table 2). The simple dinucleotide EST-SSR markers were excluded from this study because of the higher probability of producing stutter bands during PCR amplification.

Conventional SSR-PCR performed using 'Hujingmilu' peach genomic DNA as template produced specific bands of the expected size for 42 EST-SSR markers (Table 2). Sequence analysis of the 42 EST-SSR markers indicated that 40 of these had over 95% nucleotide homology with the target sequence deposited in GenBank (Table 2).

For ease of PCR product size analysis, PCR with fluorescent labeled primers were carried out. For most EST-SSR markers, the addition of an M13 tail to the 5'-end of target-specific primer did not affect the authenticity of the amplification (Fig. 1A). The only exception was PPESTSSR21 where PCR products with an unexpected size were amplified (Fig. 1B). Markers PPESTSSR29 and PPESTSSR46 produced PCR products of the expected size and stutter bands (Fig. 1C). In summary, 37 EST-SSR markers were valid for further analysis and application (Table 2).

Polymorphism of EST-SSR markers. The polymorphism rate of the 37 EST-SSR markers was evaluated using 18 peach accessions (Table 1). It was found that 23 markers did not possess polymorphism for these closely related accessions. A total of 14 polymorphic markers, with PIC ranging from 0.0994 to 0.3750, were developed (Table 4).

Accession discrimination and hybrid authentication with EST-SSR markers. According to the unweighted pair group method using arithmetic average clustering of the results, the 18 peach accessions could all be distinguished, with the exception of 'Shangshandayulu' and 'Xipu 1', both of which are bud sports from 'Yulu' (Fig. 2). These accessions were separated into two groups, with yellow peach types in Group I and melting peach types in Group II, which was divided into subgroups. 'Jinyuan', a progeny of 'Jinxu', clustered together with 'Jinxu' in subgroup I-B. The melting peach accessions were separated into two subgroups, with 'Hujingmilu', 'Hakurei' and their progenies in subgroup II-A and 'Xipu 1', 'Shangshandayulu' and their progenies, with either as maternal parent and 'Hujingmilu' as paternal one, in subgroup II-B. Clustering of progenies with

Table 2. List of primer sequences, repeat motif information, PCR product size, PCR annealing temperature (Ta) and GenBank accession numbers for 49 EST-SSR markers.^z

Marker	Repeat motif	PCR product (bp)	Primer sequence (5' -3')	Ta (°C)	GenBank	PCR specificity control	Sequence authentication	Size and stutter band check
PPESTSSR1	(GATG) ₅	260	F:TACTGCGGGAAGCAGGTCCCAA R:GAGGATGTGGAAGAAGGCAAGTAGC	58	XM_007220467	P	P	P
PPESTSSR2	(AAAG) ₅	240	F:ATGTGTCTCAGACCTCCGCCCGAA R:TTCCGAGATCCGTTTCCGGCCA	58	XM_007202582	P	P	P
PPESTSSR3	(CCTC) ₅	259	F:TCCACCTCGAACCTCTCTCCTCA R:CAGCTGCTTACCCACCAACCCA	58	XM_007203613	P	P	P
PPESTSSR4	(TTTC) ₅	174	F:GACGGTACGGTGCATGGTGCTA R:ATCGATCCCCCGTCTGCAACCA	58	XM_007222688	P	P	P
PPESTSSR5	(ACA) ₆ N ₇₉ (CAA) ₆	300	F:TGTGCTTGGAAGGAGGGGCTGA R:ACCCCTCCATCTTGGGCTTCT	58	XM_007227482	P	P	P
PPESTSSR6	(ATAA) ₅	215	F:TAGAGGGGTGGCTGGCCTTAGA R:GCACGTGATGGGAACCTGAGTTCCG	58	AJ875748	F	/	/
PPESTSSR7	(CCCT) ₅	192	F:AGGGGAAAAAGGGAAGGAGCCCA R:TCCGGCTCCCAATTTTGGCCC	58	DY653960	F	/	/
PPESTSSR8	(ATCC) ₅	223	F:TCGACACACCAGCAGAGAGACCA R:GCGAGAAATGCACCGCTGAGGA	58	DY649221	P	P	P
PPESTSSR9	(GGCAA) ₇	162	F:GGATGAAGCGCGCAAGAAACGC R:ATGCCCCGACCTTGGCTTACCT	58	DN554956	F	/	/
PPESTSSR10	(GGTGAA) ₅	270	F:GGGATACAGGGGTAGTGGGGTGT R:TTCCGGTTCGGCGTCTGAGATT	58	XM_007205282	F	/	/
PPESTSSR11	(CAACGG) ₅	220	F:CAATGGCAGTGGCGGTGGTCTT R:ATTGGTGCCCAACATGTCCGCC	58	XM_007215646	P	P	P
PPESTSSR12	(TCATCT) ₆	132	F:CACCTGCACCGTTGACTTGCGG R:AATGGGTTGAAAGGCATTCTTCTCT	58	DN553995	P	P	P
PPESTSSR13	(TAGAGG) ₇	220	F:AGCCGCACAGGAACAGGACTCT R:TCTGGGGCTGCCCTCATAATCTT	58	DY653540	P	P	P
PPESTSSR14	(GATAGG) ₅	104	F:ATTCGAGCTGGCTCGGCATCTG R:ACTCGTTCCTTTCCCTTCCCT	58	DY642263	P	P	P
PPESTSSR15	(GGACAG) ₅	244	F:ACGCCACCTTCTGTGAAAGCG R:TCGATTCCGATCCCGGTCCCTT	58	DY640145	P	P	P
PPESTSSR16	(TTC) ₅	100	F:TCTCCAGCATCAGCATTTTCAACCGC R:TGTGGAAGCAGAAGAGGAACCCGT	58	AJ873677	P	P	P
PPESTSSR17	(CTC) ₅	103	F:CGTTGTGTCTGCCGGAACCGGA R:CGAACAAATCGACCGACGCCGA	58	DY636273	P	P	P
PPESTSSR18	(ATG) ₅	104	F:TTGGTGGGGGCTGAGAGGGTTA R:ACCTGTGCCACCTCCCAATCA	58	XM_007222928	P	P	P
PPESTSSR19	(TGG) ₅	104	F:GCATGGGCGAAACATTCGCGTC R:AATAGCACCATCCCGCGGTA	58	XM_007227499	P	P	P
PPESTSSR20	(TGC) ₆	105	F:CCCCACTTTGTGGTTGCTGTTGC R:AGCAGCAGCATCAGTTTCAGCA	58	XM_007218171	P	P	F
PPESTSSR21	(ACC) ₈	105	F:ACAGCAGCATCAGCAGCAGCAA R:ATTGTTCTGAGGCGGCGGAGGA	58	AM291495	P	P	P
PPESTSSR22	(CGA) ₅	105	F:ACCAAAACACCTCTTGGCCCTC R:TGGCAGCAATGTAGCTGAGGCG	58	DY654242	P	P	P
PPESTSSR23	(CTT) ₆	108	F:AGACGCAGACATGGCTGAACCC R:CACCGGTGAACCGAACCAGCTT	58	XM_007213885	P	P	p
PPESTSSR24	(AAC) ₉	109	F:ACTTTCAGTGTGCCCTTGCCG R:TGTTGTTGGAGGTCGTCGAGGC	58	DY636999	P	P	P
PPESTSSR25	(GAT) ₅	112	F:TCAGCTAGAGCATGATGGGCAGGA R:TGATGATCGCCCACTACCGCCT	58	XM_007209191	P	F	/
PPESTSSR26	(TC) ₁₃ A(CT) ₇	140	F:TTCCCGTTCAATGCCGTGCCCTC R:AGTCGATGCTGAACCTCCGCT	58	DN553995	P	P	P
PPESTSSR27	(CT) ₆ N ₄₆ (TC) ₈	162	F:TGCCTCCACCACCTCTCACTCT R:CCACTCCCCGACAGTGCTAGTTA	58	DY636694	P	P	P
PPESTSSR28	(CT) ₁₂ N ₄₀ (TC) ₈	164	F:TCTCAATGTCTGCCTCAACCACCC R:TGGTGGCTCTTGATTGGCCGTG	58	XM_007227250	P	P	F
PPESTSSR29	(CT) ₂₂ N ₃₈ (CT) ₆	180	F:GCAACCTCTCTCCCAAAACCA R:TGAGAGAGAGCGAGAGTGACCGT	58	XM_007202079	P	P	P
PPESTSSR30	(CTGT) ₅ N ₉ (TC) ₇	181	F:TCTGTATCCGTCACGCTCTCT R:GAAGGCCGCGACAACGAAAGA	58	XM_007203458	P	P	P
PPESTSSR31	(CT) ₇ N ₉₃ (ACC) ₅	182	F:AGCCAGCCCTGCTCTCTCAACT R:TGTGGCCACTTTCCGACAGGGA	58	DY640665	P	P	P
PPESTSSR32	(CTCTTC) ₅ N ₇₂ (TCA) ₅	187	F:ACCCATTGAAGCTGGAGAAGCTC R:GTCGTTTGGAGGCGGCCATGA	58	DY646029	P	P	P
PPESTSSR33	(CT) ₈ T(TC) ₆	216	F:CCTCCGTTGACATCATTTACACACACC R:TTTTTGGAGGGAAGGGCCGGGA	58				

(Continued on next page)

Table 2. (Continued) List of primer sequences, repeat motif information, PCR product size, PCR annealing temperature (Ta) and GenBank accession numbers for 49 EST-SSR markers.^z

Marker	Repeat motif	PCR product (bp)	Primer sequence (5'-3')	Ta (°C)	GenBank	PCR specificity control	Sequence authentication	Size and stutter band check
PPESTSSR34	(TCC) ₆ N ₃₆ (TC) ₁₆	223	F:TCCCCACACCTCGTTCTCTCT R: CGCGATGCATTGGGATTGGCG	58	DY641046	P	P	P
PPESTSSR35	(CTG) ₅ N ₇ (TGC) ₅	239	F:TGTGCACAAAAGGACTGGGAAGC R:TGGTCTCTTCTGTGGCAGCTCCT	58	DY636787	P	P	P
PPESTSSR36	(CTC) ₅ N ₄₅ (CTC) ₅	246	F:ATCTCTGGGGAGGACGAGGAGT R:TCCGGTGCAGGGGGTAGCTTTT	58	DY654291	F	/	/
PPESTSSR37	(CAA) ₆ CCAAG(GCA) ₆	254	F:CCCCAATGTCTGCCACAGCACT R:TGAGTTTGCACCACCACCCCT	58	DN556015	P	P	P
PPESTSSR38	(CAG) ₅ N ₁₅ (CAG) ₅	259	F:TATCCGGCGGGCGGCTATGGTAA R:TGGAGGCAATCTCGCCAGTGGA	58	XM_007222224	P	P	P
PPESTSSR39	(AGT) ₅ GGC(AGA) ₅	265	F:AGCTTGTAAGCTAGAGGGGTGGCT R:AGCACGTGATGGGAACCTGAGT	58	XM_007218692	F	/	/
PPESTSSR40	(AGA) ₅ GGC(AGG) ₅	265	F:TCGTACGCGCAGTGAGGGAGAA R:CCAGGCGCACTGGAATAACGA	58	DY642760	P	F	F
PPESTSSR41	(TC) ₁₁ N ₈₅ (CTT) ₆	265	F:TCCCACGACCACAACCCACCAT R:GCGCCCTTTACAGCAGGCAAGT	58	DY637098	P	P	P
PPESTSSR42	(CT) ₁₀ A(TC) ₁₀	266	F:TTGGAGTCCGCCGGAAGTGTCT R:ACTCTCTCTGTCAAGAGTTTCGCAGC	58	AM289347	P	P	P
PPESTSSR43	(AAG) ₆ N ₁₇ (GA) ₇	272	F:AGAGGCAGGCAGCTTAGCTTTGT R:TGAGAGCAACCTCAGCCTCGCA	58	XM_007223746	P	P	P
PPESTSSR44	(AAT) ₅ (ATT) ₆	278	F:AGGTGGTTGGTGGATGGTGTGT R:TTTGGACACGGGTCAAGGCACG	58	XM_007204588	P	P	P
PPESTSSR45	(GA) ₁₃ N ₆₃ (AGA) ₅ (AGC) ₅	279	F:ATTTTGCCAGTTCCGAAGCCG R:ATCGGAAGACGAAACGCGCCTG	58	DY636204	P	P	P
PPESTSSR46	(TA) ₁₁ (GA) ₂₂	282	F:AGCGCTCAAAAGATACTGTGTGTATGT R:AGAAGGCCATCCCATTGCCGT	58	DY652709	P	P	F
PPESTSSR47	(TC) ₇ (AC) ₆	284	F:TGCAAACCCACCCTGGTCTTCT R:TCAACTTAAGCATGCCATCCATCACA	58	DY642088	P	P	P
PPESTSSR48	(CTC) ₇ N ₃₅ (CTC) ₅	285	F:TCCAAGCTCCACCATGGCGTCT R:TCGGCGTTGAAGGGGACGTAGT	58	XM_007215618	P	P	P
PPESTSSR49	(TGA) ₅ N ₁₀ (GAC) ₈	286	F:GGACGACGACGATGTGGACGAA R:TTCCTCATCCTCTGCACGCCCT	58	XM_008225187	F	/	/

PCR = polymerase chain reaction; EST = expressed sequence tag; SSR = simple sequence repeat.

^zP, F and / in right three columns indicates passed, failed, and not analyzed, respectively.

Table 3. Constitution of 2146 EST-SSR loci derived from the EST sequences deposited in GenBank.

Motif	No. of repeats										Total (%)
	6	7	8	9	10	11	12	13	14	>14	
AG/CT	96	64	41	35	23	17	19	25	27	141	488 (22.74)
GA/TC	79	46	31	22	14	16	15	13	15	118	369 (17.19)
Other 2-nt	45	21	16	10	7	7	13	6	6	6	137 (6.38)
Total	220	131	88	67	44	40	47	44	48	265	994 (46.32)
AAG/AGA	5	6	7	8	9	10	11	12	13	14	46 (2.14)
AGC/CAA	31	7	2	2	0	2	1				34 (1.58)
CAG/CAT	16	11	3	3	1			1	1		47 (2.19)
CCA/CTC	20	7	5	11	2						30 (1.4)
CTG/CTT	19	4	6	1							57 (2.66)
GAA/GCT	29	6	4	8	5	5					44 (2.05)
TCT/TTC	22	15	3	3		1					44 (2.05)
Other 3-nt	21	19	1	2							232 (10.81)
Total	124	60	21	11	8	3	3	2		1	534 (24.88)
4-nt	5	6	7	8	9						20 (0.93)
5-nt	18	2									9 (0.42)
6-nt	4	2	1		2						12 (0.56)
Total	7	3	2								41 (1.91)
Compound	29	7	3		2						577 (26.89)

EST = expressed sequence tag; SSR = simple sequence repeat.

either parent was observed (Fig. 2). The authentication of hybrids was supported by the fingerprint profiles where the SSR bands of progenies were covered by at least one parents (Table 5).

The minimum number of EST-SSR markers required for separation of these accessions, excepting ‘Shangshandayulu’ and ‘Xipu 1’, was reduced to nine, based on the recorded SSR product sizes (Table 5).

Discussion

Application of EST-SSR markers in peach accession discrimination. SSR markers were present in both transcribed and non-transcribed sequences, and were named as EST-SSRs and genomic SSRs, respectively. Historically, genomic SSRs were developed earlier, from the late 1980s, and EST-SSRs became predominant from the era of next-generation sequencing (Guichoux et al., 2011). For peach, the development of the first set of genomic SSRs was reported in the closing years of the last century (Cipriani et al., 1999; Testolin et al., 2000) while most EST-SSR literature began to appear 5 years later with the availability of ESTs deposited in GenBank (Chen et al., 2014; Vendramin et al., 2007; Xu et al., 2004).

Here, with the public EST information from NCBI, 37 EST-SSR markers were developed. Since the genetic background of the accessions applied in this study is narrow, especially, 10 hybrids from three crosses were included. Therefore, the 14 markers possessing polymorphism for these closely related accessions are expected to be generally valid for other peach accession discrimination and hybrid authentication as well. Besides, though 23 of these were not polymorphic applied to the 18 closely related peach accessions, these markers can be useful

Fig. 1. Representative electropherogram profiles of fluorescence-labeled simple sequence repeat products of (A) correct size and without stutter band interference, (B) wrong size, or (C) with stutter band interference.

Table 4. Characteristics of the 14 polymorphic EST-SSR markers validated with 18 peach accessions.

Marker	A ^a	N _a	N _g	H _o	H _e	PIC
PPESTSSR15	0.5000	2	3	0.5556	0.5000	0.3750
PPESTSSR32	0.5000	2	3	0.4444	0.5000	0.3750
PPESTSSR43	0.5000	2	3	0.3333	0.5000	0.3750
PPESTSSR34	0.7222	3	3	0.5556	0.4151	0.3490
PPESTSSR11	0.6944	2	2	0.6111	0.4244	0.3343
PPESTSSR48	0.6944	2	3	0.3889	0.4244	0.3343
PPESTSSR13	0.8056	2	2	0.3889	0.3133	0.2642
PPESTSSR24	0.8333	2	2	0.3333	0.2778	0.2392
PPESTSSR30	0.8611	2	2	0.2778	0.2392	0.2106
PPESTSSR33	0.8889	2	2	0.2222	0.1975	0.1780
PPESTSSR20	0.9167	2	2	0.1667	0.1528	0.1411
PPESTSSR28	0.9167	2	3	0.0556	0.1528	0.1411
PPESTSSR44	0.9167	2	2	0.1667	0.1528	0.1411
PPESTSSR14	0.9444	2	2	0.1111	0.1049	0.0994
Mean	0.7639	2.0714	2.4286	0.3294	0.3111	0.2541

^aA = major allele frequency; N_a = number of alleles; N_g = Number of genotypes; H_o = observed heterozygosity; H_e = expected heterozygosity; PIC = polymorphism information content; EST = expressed sequence tag; SSR = simple sequence repeat.

for other studies when accessions with broader biodiversity are being studied. One of the main advantages of EST-SSRs over genomic SSRs is the high levels of transferability, i.e., the EST-SSRs can be transferred across closely related species (Guichoux et al., 2011). Therefore, the EST-SSRs developed here are likely to be easily transferred to other *Prunus* species as found with the cases described by Vendramin et al. (2007).

Discrimination between bud sports, or clones, with molecular markers remains a challenging task for breeding research. As described by Monte-Corvo et al. (2001), none of the five types of molecular markers, including SSRs, succeeded in identification of nine 'Rocha' pear clones. Similarly, four types of molecular markers, including SSRs, failed to detect genetic variations between 'Shatangju'

and its sport 'Wuzishatangju' (Huang et al., 2012). In peach, with 26 SSR markers, several sports as well as two pairs of cultivars were not successfully discriminated (Testolin et al., 2000). Here, with the 37 EST-SSR markers, it was not possible to discriminate Shangshandayulu and Xipu 1 peach accessions, two bud sports from 'Yulu', suggesting more EST-SSR markers are needed. Alternatively, other molecular markers like interretrotransposon amplified polymorphisms, inter-SSRs, sequence-related amplified polymorphism, and sequence-specific amplification polymorphism, successful cases of which have been reported previously, can be tried (Huang et al., 2012; Sun et al., 2015; Zhao et al., 2010).

Key points for obtaining high-quality EST-SSR markers without interference from stutter bands. As described in Introduction section, the stutter band interference for

SSR-PCR product size identification occurs frequently. This was also encountered in our preliminary studies with some peach SSR markers reported previously. As shown in Figure 1, such interference makes the correct allele designation difficult (Guichoux et al., 2011).

Stutter bands occur as a result of enzyme slippage during amplification (Hite et al., 1996; Guichoux et al., 2011). Occurrence of stutter bands has been related to the features of some markers themselves, including the number of nucleotides in the motif and the number of repeats, as well as PCR amplification conditions and changes in reaction conditions, such as the use of new-generation polymerase, changes in PCR temperature regimes, and reduction in amount of template DNA, have been proposed to reduce stuttering (Guichoux et al., 2011; Hite et al., 1996; Olejniczak and Krzyzosiak 2006; Seo et al., 2014). Some of these were tested in our preliminary studies and it was found that the application of high-fidelity Pfu DNA polymerase, the application of purified template DNA, and the reduction in amount of template DNA did not obviously reduce the stuttering interference (data not shown).

The most effective way to avoid the stuttering problem we found is the selection of suitable SSR motifs. As the interference was especially serious with dinucleotide motifs and exaggerated as the repeat number increased (Edwards et al., 1991; Guichoux et al., 2011; Hite et al., 1996), in this study simple EST-SSRs with dinucleotide motifs were excluded for marker development. Most selected markers were free of stutter bands, except for two compound EST-SSRs, PPESTSSR29 and PPESTSSR46 (Fig. 1),

Fig. 2. Dendrogram for 18 peach accessions derived by the unweighted pair group method using arithmetic average cluster analysis based on 14 polymorphic expressed sequence tag-simple sequence repeat markers.

Table 5. Digital fingerprint of 18 peach accessions from 14 EST-SSR markers.

Marker	PCR	Accession ^y																	
	product (bp)	1	2	3/4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
PPESTSSR11 ^z	233	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	239	0	1	0	0	0	1	0	1	0	1	1	1	1	1	1	1	1	
PPESTSSR13*	233	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	239	0	0	1	0	0	0	0	0	0	1	0	1	0	0	1	1	1	
PPESTSSR14	117	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	
	123	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
PPESTSSR15*	257	0	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	
	263	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	0	
PPESTSSR20	112	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	
	124	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
PPESTSSR24*	116	1	0	0	1	1	1	0	1	0	0	0	0	0	0	1	0	0	
	125	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
PPESTSSR28	179	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	
	183	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	
PPESTSSR30*	202	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	204	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	
PPESTSSR32*	200	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	
	206	1	0	1	0	1	0	0	0	0	1	1	1	1	1	1	1	1	
PPESTSSR33	231	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	241	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	
PPESTSSR34*	242	0	1	0	0	0	1	0	1	1	1	1	1	1	0	0	1	0	
	248	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	
PPESTSSR43*	262	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	289	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1	
PPESTSSR44	291	1	1	0	1	1	1	1	1	1	0	0	1	0	1	1	1	1	
	294	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	
PPESTSSR48*	297	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
	295	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	
	298	1	0	1	0	1	1	1	0	0	0	0	0	0	1	0	1	0	

EST = expressed sequence tag; SSR = simple sequence repeat; PCR = polymerase chain reaction.

^zThe markers suffixed with * indicates the nine ones that allow discriminating the 18 accessions.

^ySee list of accessions in Table 1.

both of which possessed two dinucleotide motifs with a high number of repeats of around 30 (Table 2). Therefore, other similar compound SSRs should be excluded.

In conclusion, 37 high quality, i.e., without stutter band interference, EST-SSR markers have been developed and successfully applied for peach cultivar discrimination and hybrid authentication. Besides, it was observed that selection of suitable SSR markers, simple ones with motifs containing at least three nucleotides and compound ones with low number of repeats for each motif, dinucleotide motif allowed, is the most effective way to avoid the stuttering problem encountered during SSR-PCR amplification.

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