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

Maomao Ding, Ke Wang, and Wenting Wang
The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Quality Improvement, Zijingang Campus, Zhejiang University, Hangzhou 310058, People’s Republic of China

Miaojin Chen and Dajun Wu
Fenghua Institute of Honey Peach, Fenghua 315502, People’s Republic of China

Changjie Xu¹ and Kunsong Chen
Zhejiang Provincial Key Laboratory of Horticultural Plant Integrative Biology, Zijingang Campus, Zhejiang University, Hangzhou 310058, People’s Republic of China

Additional index words. PCR stutter band, slippage amplification, bud sport

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; Dirlewanger 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 Hornaza, 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,
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 | Hujingmilu |
| 15  | Fenghuang | Yellow peach | A cultivar from Dalian, Liaoning, China |
| 16  | Jinxian  | Yellow peach | A cultivar from Shanghai, China |
| 17  | Jinxiu   | Yellow peach | A cultivar from Shanghai, China |
| 18  | Jinyuan  | Yellow peach | A cultivar with Jinxia 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 Gavel 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 co-efficients: size of PCR products between 100 and 300 bp; primer length between 18 and 20 bp, annealing temperature 50–65 °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 MgCl₂, 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 Analyzer (Applied Biosystems, Foster City, CA). The authentication of SSR-PCR products was analyzed through conventional gene cloning and sequencing (Sambrook et al., 1989). The only exception was 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 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 ‘Jinxia’, clustered together with ‘Jinxiu’ 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.

| 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)3                       | 260              | F: TACTGCGGGAAGACAGGGGCTC CCAA R: GAGCAATGATTGAAGGACCA AGT GCC          | 58      | XM_007220467 | P                       | P                      | P                         |
| PPESTSSR2      | (AAAG)3                       | 240              | F: ATGTGTCAGACCTCCGCAGAA R: TCGTGACACCGTCCCTCCTCCA                  | 58      | XM_007203613 | P                       | P                      | P                         |
| PPESTSSR3      | (CCTC)5                       | 259              | F: TCACCTCGAACACCTCTCCCTCTCA R: CAGCTGCTTACCAACCAACCA                | 58      | XM_007222688 | P                       | P                      | P                         |
| PPESTSSR4      | (TTTC)3                       | 174              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_00727482  | P                       | P                      | P                         |
| PPESTSSR5      | (ACA)6N (CAA)6                | 300              | F: TGTTGCTTGGAGAAGGGGACTGA R: ACCCCGACCTCGGCCCTCTTTCTTCTC            | 58      | XM_007222467 | P                       | P                      | P                         |
| PPESTSSR6      | (ATAA)5                       | 215              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | AJ875748     | F                       | /                      | /                         |
| PPESTSSR7      | (CCCT)5                       | 192              | F: ATGTGTCAGACCTCCGCCGAA R: TGGAGGAAGAGGAGGGAGCAGC                   | 58      | DY653960     | F                       | /                      | /                         |
| PPESTSSR8      | (ATCC)5                       | 223              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | DY649221     | P                       | P                      | P                         |
| PPESTSSR9      | (GGCA)2                      | 162              | F: GAGATGAAGCGGCGCAAGAAGGCGA R: ATGCAGGAGCCACCGCTGAGGGAGG             | 58      | DN554956     | F                       | /                      | /                         |
| PPESTSSR10     | (GATG)5                       | 270              | F: GAGATGAAGCGGCGCAAGAAGGCGA R: ATGCAGGAGCCACCGCTGAGGGAGG             | 58      | XM_007205282 | F                       | /                      | /                         |
| PPESTSSR11     | (CAACGG)5                     | 220              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007215646 | P                       | P                      | P                         |
| PPESTSSR12     | (ATG)5                        | 132              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | DN554956     | F                       | /                      | /                         |
| PPESTSSR13     | (ATAA)5                       | 220              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | DY653960     | F                       | /                      | /                         |
| PPESTSSR14     | (ATCC)5                       | 104              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | DY642263     | P                       | P                      | P                         |
| PPESTSSR15     | (GGCA)2                      | 244              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY640145     | P                       | P                      | P                         |
| PPESTSSR16     | (TTC)5                        | 100              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | AJ873677     | P                       | P                      | P                         |
| PPESTSSR17     | (CCTC)5                       | 132              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | DY653960     | F                       | /                      | /                         |
| PPESTSSR18     | (ATG)5                        | 104              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | XM_007222928 | P                       | P                      | P                         |
| PPESTSSR19     | (TGG)5                        | 104              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | XM_007227499 | P                       | P                      | P                         |
| PPESTSSR20     | (TGC)6                       | 105              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | XM_007222318 | P                       | P                      | P                         |
| PPESTSSR21     | (ACC)5                        | 105              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | XM_007218171 | P                       | P                      | P                         |
| PPESTSSR22     | (CGA)5                        | 105              | F: TACAGGGGTTGCGCTGGCTTATA R: GCACTGGATGGGACACCGTGGTTGCG              | 58      | AM291495     | P                       | P                      | P                         |
| PPESTSSR23     | (CCTC)5                       | 108              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY654242     | P                       | /                      | /                         |
| PPESTSSR24     | (AAC)5                        | 109              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007213885 | P                       | P                      | P                         |
| PPESTSSR25     | (GAT)5                       | 112              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY636999     | P                       | P                      | P                         |
| PPESTSSR26     | (TC)12 (CT)7                  | 140              | F: TACAGGGGTTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007209191 | P                       | F                      | /                         |
| PPESTSSR27     | (CT)6N (TC)8                  | 162              | F: TACAGGGGTTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DN553995     | P                       | P                      | P                         |
| PPESTSSR28     | (CT)12N (TC)8                 | 164              | F: TACAGGGGTTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY636994     | P                       | P                      | P                         |
| PPESTSSR29     | (CT)12N (TC)8                 | 180              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007227250 | P                       | F                      | /                         |
| PPESTSSR30     | (CTGT)6N (TC)8                | 181              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007202079 | P                       | P                      | P                         |
| PPESTSSR31     | (CT)12N (ACC)5                | 182              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | XM_007230458 | P                       | P                      | P                         |
| PPESTSSR32     | (CT)12N (TC)5                 | 183              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY640665     | P                       | P                      | P                         |
| PPESTSSR33     | (CT)5 (TC)6                  | 216              | F: GACGGTACGGTGCGCTGTTGCTCA R: ATCGATCCCGAGCCCTGCAACCA               | 58      | DY646029     | P                       | P                      | P                         |

(Continued on next page)
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
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),

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

| Marker     | A | N_a | N_g | Ho  | He  | 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 |
| PPESTSSR33 | 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 |

A = major allele frequency; N_a = number of alleles; N_g = Number of genotypes; Ho = observed heterozygosity; He = expected heterozygosity; PIC = polymorphism information content; EST = expressed sequence tag; SSR = simple sequence repeat.

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.
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.

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

| Marker           | Accession | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|------------------|-----------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|
| PPESTSSR11*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR13*      |           | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR14       |           | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR15*      |           | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR20       |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR24*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR28       |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR30*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR32*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR43*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR44       |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |
| PPESTSSR48*      |           | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |    |

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

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

*See list of accessions in Table 1.

---

### Literature Cited

Aranzana, M.J., J. Garcia-Mas, J. Carbó, and P. Arús. 2002. Development and variability analysis of microsatellite markers in peach. Plant Breed. 121:87–92.

Caruso, M., C.T. Federici, and M.L. Roose. 2008. EST-SSR markers for asparagus genetic diversity evaluation and cultivar identification. Mol. Breed. 21:195–204.

Cavagnaro, P.F., D.A. Senalik, L.M. Yang, P.W. Simon, T.T. Harkins, C.D. Kodira, S.W. Huang, and Y.Q. Weng. 2010. Genome-wide characterization of simple sequence repeats in cucumber (Cucumis sativus L.). BMC Genom. 11:569.

Chen, C.X., D.J. Werner, D. O’Malley, and R.R. Sederoﬀ. 1994. Targeted mapping and linkage analysis of morphological, isozyme, and RAPD markers in peach. Theor. Appl. Genet. 87:805–815.

Chen, C.X., B. Fock, R.W. Okie, F.G. Gmitter, S. Jung, D. Main, T.G. Beckman, and B.W. Wood. 2014. Genome-wide characterization and selection of expressed sequence tag simple sequence repeat primers for optimized marker distribution and reliability in peach. Tree Genomes 10:1271–1279.

Cipriani, G., G. Lot, W.G. Huang, M.T. Marrazzo, E. Peterlunger, and R. Testolin. 1999. AG/CT and AG/CT microsatellite repeats in peach [Prunus persica (L) Batsch]: Isolation, characterisation and cross-species amplification in Prunus. Theor. Appl. Genet. 99:65–72.

Dirlewanger, E., P. Cosson, M. Tavaud, M. Aranzana, C. Poizat, A. Zanetto, P. Arús, 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 (Prunus avium L.). Theor. Appl. Genet. 105:127–138.

Edwards, A., A. Civitello, H.A. Hammond, and C. T. Caskey. 1991. DNA typing and genetic mapping with trimeric and tetratermed tandem repeats. Am. J. Hum. Genet. 49:746–756.

Graul, N.J. and R.L. Juvik. 1991. A modified CTAB DNA extraction procedure for Musa and Ipomoea. Plant Mol. Biol. Rpt. 9:262–266.

Goulão, L. and C.M. Oliveira. 2001. Molecular characterisation of cultivars of apple (Malus × domestica Borkh.) using microsatellite (SSR and ISSR) markers. Euphytica 122:81–89.

Guichoux, E., L. Lagache, S. Wagner, P. Chausmeil, P. Léger, O. Lepais, C. Lepoittevin, T. Malassa, E. Revardel, F. Salin, and R.J. Petit. 2011. Current trends in microsatellites genotyping. Mol. Ecol. Resour. 11:591–611.

Hite, J.M., K.A. Eckert, and K.C. Cheng. 1996. Factors affecting fidelity of DNA synthesis during PCR amplification of d(G-A)n.d(C-T)n microsatellite repeats. Nucleic Acids Res. 24:2249–2243.

Huang, J.F., Y.H. Qin, H.X. Miao, C.Y. Zhang, Z.X. Ye, and G.B. Hu. 2012. Molecular marker analysis of ‘Shatangju’ and ‘Wuzishanju’ mandarin (Citrus reticulata Blanco). Afr. J. Biotechnol. 11:15501–15509.

Huang, S.W., B.X. Zhang, D. Milbourne, L. Cardle, G.M. Yang, and J.Z. Guo. 2000. Development of pepper SSR markers from sequence databases. Euphytica 117:163–167.

Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. van Driel, G. Bedemeijer, B. Vosman, M. Mathews, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestrì, A. Malcovci, N. Marmioli, R. Aert, G. Volckaert, J. Rueda, R. Linacero, A. Vazquez, and A. Karp. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. Mol. Biotechnol. 3:381–390.

Li, Y., W. X.Q. Meng, J.H. Jing, J.C. Liu, R.J. Ma, L.R. Wang, K. Cao, Z.J. Shen, L. Niu, J.B. Tian, M.J. Chen, M. Xie, P. Arus, Z.S. Gao, and M.J. Aranzana. 2013. Peach genetic resources: Diversity, population structure and linkage disequilibrium. BMC Genet. 14:84.

Liu, K. and S.V. Muse. 2005. PowerMarker: An integrated analysis environment for genetic marker analysis. Bioinformatics 21:2128–2129.

Monte-Corvo, L., L. Goulão, and C. Oliveira. 2001. ISSR analysis of cultivars of pear and suitability of molecular markers for clone discrimination. J. Amer. Soc. Hort. Sci. 126:517–522.

Olejnizcak, M. and W.J. Krzyzioziak. 2006. Genotyping of simple sequence repeat factors implicated in shadow band generation revisited. Electrophoresis 27:3724–3734.
Page, R.D.M. 1996. TreeView: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357–358.

Quarta, R., M.T. Dettori, I. Verde, A. Gentile, and Z. Broda. 1998. Genetic analysis of agronomic traits and genetic linkage mapping in a BC1 population using RFLPs and RAPDs. Acta Hort. 465:51–59.

Rajapakse, S., L.E. Belthoff, G. He, A.E. Estager, R. Scorza, I. Verde, R.E. Ballard, W.V. Baird, A. Callahan, R. Monet, and A.G. Abbott. 1995. Genetic linkage mapping in peach using morphological, RFLP and RAPD markers. Theor. Appl. Genet. 90:503–510.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York, NY.

Sosinski, B., M. Gannavarapu, L.E. Hager, G.J. Beck, C.D. King, S. Ryder, W.V. Rajapakse, R.E. Baird, and A.G. Ballard. 2000. Characterization of microsatellite markers in peach (Prunus persica (L.) Batsch). Theor. Appl. Genet. 101:421–428.

Sun, J.M., H. Yin, L.T. Li, Y. Song, L. Fan, S.L. Zhang, and J. Wu. 2015. Evaluation of new IRAP markers of pear and their potential application in differentiating bud sports and other Rosaceae species. Tree Genet. Genomes 11:25.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res. 17:6463–6471.

Testolin, R., T. Marrazzo, G. Cipriani, R. Quarta, I. Verde, M.T. Dettori, M. Pancaldi, and S. Sansavini. 2000. Microsatellite DNA in peach (Prunus persica L. Batsch) and its use in fingerprinting and testing the genetic origin of cultivars. Genome 43:512–520.

Teulat, B., C. Aldam, R. Trehin, P. Lebrun, J.H.A. Barker, G.M. Arnold, A. Karp, L. Baudouin, and F. Rognon. 2000. An analysis of genetic diversity in coconut (Cocos nucifera) populations from across the geographic range using sequence-tagged microsatellites (SSRs) and AFLPs. Theor. Appl. Genet. 100:764–771.

Zhao, G.L., H.Y. Dai, L.L. Chang, Y. Ma, H.Y. Sun, P. He, and Z.H. Zhang. 2010. Isolation of two novel complete Ty1-copia retrotransposons from apple and demonstration of use of derived S-SAP markers for distinguishing bud sports of Malus domestica cv. Fuji. Tree Genet. Genom. 6:149–159.