A Practical Method for Peach-related Species Identification and Hybrid Analysis Using Simple Sequence Repeat Markers

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ABSTRACT. Cultivated peach (Prunus persica) is an important fruit species worldwide. The wild relatives in Prunus, such as P. mira, P. davidiana, P. kansuensis, P. ferganensis, and P. persica, are valuable for peach breeding, and early and accurate identification of parental and hybrid genotypes is critical. In this study, 20 representative accessions of peach germplasm from the National Germplasm Repository of Peach in China were used to select a set of 18 simple sequence repeat (SSR) markers for accurate species discrimination. Eight unknown peach samples were successfully identified using the SSR panel and species genotype database. Interspecific hybrid genotypes of P. persica × P. davidiana, P. persica × P. kansuensis, and P. persica × P. ferganensis were also analyzed reliably. The markers were amenable to high-throughput fluorescent labeling and capillary electrophoresis (CE) analysis, allowing rapid and efficient species identification. The practical method described in this study will facilitate peach breeding and germplasm management.

Peach is one of the most important fruit species worldwide. China, where peach originated, has the richest resource of species related to cultivated peach. These species contributed to peach domestication history over more than 4000 years, with some cultivars disseminating to Central Asia, the Mediterranean coast, Europe, America, and Japan (Layne and Bassi, 2008; Wang and Zhuang, 2001). The main relatives of cultivated peach, namely P. mira, P. davidiana, P. kansuensis, and P. ferganensis, are found readily in southwestern and northwestern China. Fruit from almost all of the related wild species exhibit poor eating quality, but these species could be valuable as sources of pest and disease resistance traits or as rootstock (Cao et al., 2011; Moing et al., 2003). Increasing numbers of related species are being used to develop new peach cultivars. Efficient and accurate species identification and hybrid analysis are necessary in peach breeding and germplasm management.

A range of practices were used previously to identify peach-related germplasm, including morphology (Wang and Zhuang, 2001), cytology (Guo et al., 1996), palynology (Wang and Zhou, 1990), isozymes (Mowrey et al., 1990), and DNA markers (Cheng et al., 2001; Yu et al., 2004). With the development of sequencing technology, peach genome sequences have become available (Verde et al., 2013), and high-throughput single-nucleotide polymorphism (SNP) tools have been used for large-scale genetic analysis in peach cultivars and accessions (Cao et al., 2014; Verde et al., 2012).

SSR (microsatellites) are tandem repeat DNA sequences with a core unit of 1–6 bps, which are abundant in prokaryotic and eukaryotic genomes and are ubiquitously distributed in both the protein-coding and noncoding regions (Guichoux et al., 2011; Kalia et al., 2011). The high variability of microsatellites usually manifests as different numbers of repeats in the region of the repeated motif, and short insertion/deletion events are also seen (Decroocq et al., 2003). SSR markers are ideal for profiling as they are codominant and can display a large number of alleles per locus. In addition, assaying SSR markers is relatively simple and reproducible and is accomplished using polymerase chain reaction (PCR)-based methods that are amenable to automation (Kalia et al., 2011). SSR markers proved to be highly efficient for genetic analysis in several fruit species, such as grape [Vitis vinifera (Emanuelli et al., 2013)], jujube [Ziziphus jujuba (Ma et al., 2012)], almond [Prunus dulcis (Dangl et al., 2009)], and sweet cherry [Prunus avium (Lacis et al., 2009)]. SSR markers were developed previously in peach and used for genetic diversity assessment (Li et al., 2008), cultivar identification (Chen et al., 2011; Li et al., 2013), trait mapping (Lambert and Pascal, 2011; Liu et al., 2009), and phylogenetic studies (Cheng and Huang, 2009). However, to date, reliable and efficient SSRs for discrimination of peach-related species have not been developed, and no database of species genotypes has been established. A universal database of SSR marker profiles from peach-related species would facilitate peach breeding and germplasm management.

The NGRPC, located in Zhengzhou (central China), Nanjing (eastern China), and Beijing (northern China), is the most
important germplasm repository of peach, nectarine, and related species in China. The NGRPC preserves original and representative accessions of species related to peach and is the core collection of such species for China and the world. Here, we describe a practical SSR-based method for discriminating species related to peach and for identifying interspecific hybrids. The aims of this study were to 1) screen SSRs and develop a set of markers for efficient identification of peach-related species, 2) establish a universal genotype database for peach-related species using fluorescent-labeled SSR markers, and 3) demonstrate the practical utility of the SSR set for unknown genotype identification and interspecific hybrid analysis.

Materials and Methods

**Plant material.** Twenty-nine *Prunus* samples were used in the study (Table 1). Among them, 20 samples (codes 1–20) were obtained from three NGRPCs (Zhengzhou, Nanjing, and Beijing). These 20 accessions were representative samples of five species (*P. persica, P. davidiana, P. kansuensis, P. ferganensis,* and *P. mira*) that were established on the core collection during initial investigations in China in the last century. Some of these representative accessions were described using classical morphological characteristics, regional origin, and landrace names. Eight samples (codes 21–28) were unknown accessions awaiting species identification, and one sample (code 29) was a cultivar that was used as a maternal parent for hybridization. Three interspecific hybridizations were performed by the Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (ZFRI, CAAS), as follows: *P. persica* (code 29) × *P. davidiana* (code 1), *P. persica* (code 29) × *P. kansuensis* (code 5), and *P. persica* (code 29) × *P. ferganensis* (code 10). Three F$_1$ hybrid populations were obtained and used alongside their parents for hybrid analysis.

**DNA extraction.** Fresh young leaves were collected and rapidly dried at room temperature using solid silica gel pellet desiccant. Genomic DNA was isolated using the modified cetyltrimethylammonium bromide protocol described by Cheng et al. (1997). DNA was quantified using a spectrophotometer (BioPhotometer plus; Eppendorf AG, Hamburg, Germany), and diluted to 20 ng μL$^{-1}$ as template for PCR amplification.

**SSR markers.** Seventy-eight SSR markers (Table 2), previously developed for peach (Aranzana et al., 2002; Dirlewanger et al., 2002; Sosinski et al., 2000; Testolin et al., 2002; Yamamoto et al., 2002), Japanese plum (*Prunus salicina*), almond (*Prunus amygdalus*), and plum (*Prunus domestica*, *Prunus armeniaca*), were tested for discrimination power. Subsequent analysis was performed with fluorescent-labeled primers and CE. The selected SSR primers were labeled with fluorescein on the top of each forward primer discrimination power. Subsequent analysis was performed with fluorescent-labeled primers and CE. The selected SSR primers were labeled with fluorescein on the top of each forward primer.

**PCR amplification.** PCR amplification was performed in 20-μL reaction volumes containing 20 ng of genomic DNA, 10 μL of 2× Taq PCR MasterMix (Tiangen Biotech, Beijing, China), and 0.25 μM SSR primers. Amplification parameters were as follows: 5-min initial denaturation of the template DNA at 94 °C; 35 cycles of 45-s template DNA denaturation at 94 °C, 45-s primer annealing at the appropriate temperature, 45-s primer extension at 72 °C; and a final primer extension at 72 °C for 10 min. PCR products were stored at 4 °C before analysis by electrophoresis. SSR reactions were repeated for each SSR/DNA sample combination at least twice to identify an appropriate annealing temperature and ensure consistent results.

**Data analysis.** The 78 PCR products of 20 representative accessions were analyzed using electrophoresis with 9% polyacrylamide gels. Gels were silver stained according to the protocol described by Bassam et al. (1991). Optimal SSR primers for species identification were selected according to their consistent and reliable amplification patterns and interspecific discrimination power. Subsequent analysis was performed with fluorescent-labeled primers and CE. The selected SSR primers were labeled with fluorescein on the top of each forward primer.

| Code | *Prunus* species | Source | Characteristic |
|------|----------------|--------|---------------|
| 1    | *P. davidiana* | NGRPC, Zhengzhou | Red flower |
| 2    | *P. davidiana* | NGRPC, Zhengzhou | White flower |
| 3    | *P. davidiana* | NGRPC, Nanjing | Red flower |
| 4    | *P. davidiana* | NGRPC, Beijing | White flower |
| 5    | *P. kansuensis* | NGRPC, Zhengzhou | Red root |
| 6    | *P. kansuensis* | NGRPC, Zhengzhou | White root |
| 7    | *P. kansuensis* | NGRPC, Nanjing | |
| 8    | *P. kansuensis* | NGRPC, Beijing | Red root |
| 9    | *P. ferganensis* | NGRPC, Zhengzhou | Kashi no.1 |
| 10   | *P. ferganensis* | NGRPC, Zhengzhou | Kashi no.2 |
| 11   | *P. ferganensis* | NGRPC, Nanjing | Datianren |
| 12   | *P. ferganensis* | NGRPC, Beijing | |
| 13   | *P. mira* | NGRPC, Zhengzhou | Linzhi, Tibet, China |
| 14   | *P. mira* | NGRPC, Zhengzhou | Aha, Sichuan, China |
| 15   | *P. mira* | NGRPC, Nanjing | |
| 16   | *P. mira* | NGRPC, Beijing | |
| 17   | *P. persica* | NGRPC, Beijing | Hubei, China |
| 18   | *P. persica* | NGRPC, Beijing | Guizhou, China |
| 19   | *P. persica* | NGRPC, Beijing | Sichuan, China |
| 20   | *P. persica* | NGRPC, Beijing | Hebei, China |
| 21   | unknown | ZFRI, CAAS | Chance seedling |
| 22   | unknown | ZFRI, CAAS | Chance seedling |
| 23   | unknown | ZFRI, CAAS | Chance seedling |
| 24   | unknown | ZFRI, CAAS | Chance seedling |
| 25   | unknown | ZFRI, CAAS | Chance seedling |
| 26   | unknown | ZFRI, CAAS | Chance seedling |
| 27   | unknown | ZFRI, CAAS | Chance seedling |
| 28   | unknown | ZFRI, CAAS | Chance seedling |
| 29   | *P. persica* | ZFRI, CAAS | Cultivar (Dajiuobao) |

Twenty-nine samples including 20 representative accessions of five species (codes 1–20) in *Prunus*, eight unknown samples awaiting species identification (codes 21–28), and one cultivar (code 29).

National Germplasm Repository of Peach in China (NGRPC), Zhengzhou Fruit Research Institute (ZFRI), Chinese Academy of Agricultural Sciences CAAS).
Results

SSR AMPLIFICATION AND SELECTION. The diversity of the 78 SSRs was assessed among the 20 representative peach samples of five species (P. persica, P. davidiana, P. kansuensis, P. ferganensis, and P. mira) from NGRPC. Sixty loci were eliminated from further analysis for the following reasons (Table 3). Alleles could not be scored for nine primer pairs: three primer pairs failed to amplify fragments, three amplified more than two random fragments, and three loci were difficult to score due to poor amplification. Alleles at an additional 51 loci could be scored but were not sufficiently discriminatory. Of these, six loci were monomorphic for all 20 accessions; 36 loci exhibited low interspecific polymorphism, usually due to some very high-frequency alleles; and nine loci exhibited high intraspecific polymorphism (Fig. 1B). Eighteen loci were selected for further testing. All of the 18 loci were consistently amplifiable, had only one or two alleles at a locus in every specific accession, and exhibited high interspecific polymorphism and low intraspecific polymorphism (Fig. 1C).
DEVELOPMENT OF A PEACH-RELATED SPECIES GENOTYPE DATABASE USING FLUORESCENT-LABELED SSR MARKERS.

The 18 SSR markers were used in fluorescent PCR and CE analysis, and a genotype database was generated for 20 representative accessions based on the 18 SSR markers (Table 4), then the genotype database for five species was generated based on the 18 SSR markers and 20 representative accessions (Table 5). The discrimination ability of 18 loci in the database varied and ranked from high (BPPCT010) to low (BPPCT023). The first five markers had high discrimination power and could be useful for the generation of a standard profile for species identification. Marker BPPCT010 can discriminate five species, and any marker of CPPCT30, BPPCT032, UDP96-005, and UDP96-013 can be used to discriminate two species (Table 5). Adoption of the other markers for species identification would facilitate data sharing and help to correct for variation in data analysis between laboratories. The species P. kansuensis was the most easily identified among the five related species, and was one of the species that could be discriminated with most of the 18 selected SSR loci. The species P. mira, P. davidiana, P. ferganensis, and P. persica can also be discriminated by their unique loci (Table 5).

IDENTIFICATION OF UNKNOWN SAMPLE SPECIES. All the allele sizes of the eight unknown peach samples (codes 21–28) and 21 known accessions (codes 1–20, code 29) are presented in Table 4. A dendrogram of 29 samples was established (Fig. 2). Eight anonymous samples were included in three species clusters: samples 21, 22, and 23 were in the P. davidiana cluster; samples 24, 25, and 26 were in the P. ferganensis cluster; and samples 27 and 28 were in the P. persica cluster. As expected, sample 29, which was a P. persica cultivar, was found in the P. persica cluster. It was therefore possible to identify species by analysis with 18 SSR loci and phylogenetic comparison with the 20 known representative samples.

INTERSPECIFIC HYBRID ANALYSIS. Early identification of hybrids is desirable in peach cross breeding, and the selected 18 SSR markers can be used for hybrids analysis at the seedling stage. The F1 hybrids share one allele with each of their parents on each locus, and progeny genotype(s) can therefore be predicted and proved by their parental genotypes. For example, only one genotype occurs in the F1 hybrid on the locus of BPPCT010 in the hybridization of P. persica × P. ferganensis (Fig. 3A), two genotypes on the locus of UDP96-005 in the hybridization of P. persica × P. kansuensis (Fig. 3B), and four genotypes on the locus of UDP96-005 in the hybridization of P. persica × P. davidiana (Fig. 3C).

Discussion

Peach cultivars can be easily distinguished using a unique DNA fingerprint (Li et al., 2013) or by molecular identity (Chen et al., 2011), because commercial peach clones are propagated by grafting. Peach-related species are wild resources in a mixed

Table 5. Simple sequence repeat (SSR) genotype database for five peach-related species in Prunus based on 18 SSR markers and 20 accessions.

| SSR marker   | P. davidiana | P. kansuensis | P. ferganensis | P. mira | P. persica |
|--------------|--------------|---------------|----------------|---------|-----------|
| BPPCT010     | 111/121/129  | 139/141       | 131            | 117     | 127       |
| CPPCT30      | 165/169/171/175 | 221          | 191/193        | 151/191 | 191/193   |
| BPPCT032     | 173/175/181/183 | 190          | 200            | 163     | 194/200/202|
| UDP96-005    | 134/138/154/156/158/164/175 | 130/132       | 158/164/175    | 122     | 156/173   |
| UDP96-013    | 149/151      | 122           | 179            | 179/224/226/261 | 163/179  |
| pchgsms2     | 145/151/155/161/171 | 181/185       | 155            | 126     | 155/157   |
| CPPCT33      | 142/144/146/148 | 161          | 152            | 137     | 142/148/150/152 |
| UDP97-401    | 100/106/110  | 98            | 121/123        | 121/123/125/172 | 119/121  |
| CPPCT16      | 175/195/197/199 | 179          | 193            | 183     | 185/193/205 |
| BPPCT001     | 132          | 130           | 154/160        | 146/152 | 152/156/162|
| pchgsms1     | 194/208      | 200           | 191            | 183/191 | 191/193   |
| pchgsms4     | 141/155/173  | 165/169       | 173            | 150/165/173 | 173/175  |
| UDP96-001    | 108/110/139  | 106           | 125            | 86/96/104 | 117/119/125 |
| UDP98-412    | 94/98/106/109/115/117/121 | 113          | 125            | 86/92   | 113/119/125/127 |
| BPPCT034     | 205/213/228/236/240 | 207          | 226/238        | 224/238/246 | 224/238  |
| pchgsms3     | 182/186/194  | 169/175       | 204/206        | 198/200/204/206 | 175/182/190/204 |
| BPPCT025     | 147/153/171/179/185 | 183/185      | 175            | 175/175/181/191/193 | 171/187/189/191/193 |
| BPPCT023     | 179/211/215/225/227/229 | 215          | 181            | 181/215 | 181/211   |

Fig. 2. Dendrogram of 29 accessions in Prunus resulting from the unweighted pair-group method with arithmetic means (UPGMA) cluster analysis based on the similarity coefficient obtained from simple sequence repeat (SSR) data. Twenty-nine accessions (codes 1–29) are divided into five clusters corresponding to five species (P. davidiana, P. kansuensis, P. ferganensis, P. mira, and P. persica).
population that are commonly propagated by seed. We fully considered the sample origin, amount, and representativeness in this study. The 20 accessions used to screen for optimal SSRs all came from NGRPC, and different type and origin accessions were used in *P. davidiana* (red flower and white flower), *P. kansuensis* (red root and white root), *P. mira* (in Tibet and in Sichuan) and *P. persica* (in Huibei, Guizhou, Sichuan, and Hebei). Four accessions is a small sample; however, they were the recommended representative accessions from the three NGRPCs and should be considered as a starting point to which data from additional accessions and alleles can be added. Eight unknown accessions were tested for species identification, and three known interspecific hybrid populations were tested for hybrid analysis in this study.

Nine to ten representative loci were initially proposed in each *Prunus* linkage group; thus, a total of 78 SSRs were used as candidates. No more than 20 SSRs for validation were proposed prescreening with 20 representative accessions, resulting in 18 SSRs being finally selected on the basis of their amplification reliability and discrimination power. Some highly polymorphic SSRs from previous publications, such as BPPCT020 (Chen et al., 2011) and CPPCT22 (Bouhadida et al., 2011), were excluded from the current SSR panel. Use of loci with relatively few alleles simplified germplasm identification and increased efficiency of identification. Three known interspecific hybrids were used to evaluate transferability between species of the SSRs panel, and the results were in accord with their real identities.

A basic genotype database was established based on the 18 selected SSRs and 20 representative accessions, which should be useful for the identification of unknown *Prunus* accessions using dendrogram construction and cluster analysis. Fluorescent labeling and CE analyses improved the efficiency and accuracy of species identification in the current study. As allele size validation can be affected by primer tailing fluorescence chemicals, multiplexing PCR, and PCR product analysis (agarose gel, polyacrylamide gel, or capillary systems), SSR screening streamlining and database development for unknown samples identification and hybrid analysis using the same FAM-labeled forward primers, single-multiplexing PCR, and automatic CE analysis were used in this study. In fact, the relative range of allele sizes for five species on each locus is independent on the method used, which can be used as reference for species identification (Table 5). Eight unknown samples were successfully identified by the ZFRI, CAAS, which demonstrated the reliability of the genotype database and the SSRs panel.

Some peach-related species have disappeared from their original habitats in recent years due to climate change, overexploitation, pollution, and agricultural land reclamation (Xing et al., 2015; Zhong, 2008). On the other hand, accessions of species related to peach are preserved less frequently than those of cultivated peach, which are preserved at NGRPC. The practical genotyping developed in this study for five peach-related species will facilitate investigation and preservation of further wild accessions and allow the breeding potential of peach-related species to be fully realized. Peach-related species identification will be further enhanced by the availability of the peach genome sequence (Verde et al., 2013), the express sequence tag SSR database (Chen et al., 2014), microsatellite maps for the whole peach genome, and functional mining of genes associated with SSRs (Dettori et al., 2015; Zalapa et al., 2012).

**Conclusions**

In this study, 20 representative accessions at NGRPC were used to select for 18 SSRs, which were then successfully used for the efficient genotyping of five peach-related species...
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