

## Efficient identification of ornamental peach cultivars using RAPD markers with a manual cultivar identification diagram strategy

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Genet. Mol. Res. 13 (1): 32-42 (2014)

Received October 31, 2013

Accepted November 30, 2013

Published January 8, 2014

DOI <http://dx.doi.org/10.4238/2014.January.8.2>

**ABSTRACT.** One of the most important uses of DNA markers is cultivar identification. However, no DNA fingerprint analysis strategy is available for making DNA markers helpful in practical plant cultivar identification, especially for the identification of a large number of cultivars. We developed a manual cultivar identification diagram strategy for efficient identification of plant cultivars, from which a cultivar identification diagram (CID) of genotyped plant individuals can be constructed manually. This CID could be used as a reference for quick identification of plant cultivars of interest. We used 11-mer RAPD primers to amplify DNA samples of 32 ornamental peach genotypes; all the cultivars were well distinguished by fingerprints from 6 primers. The utility of this CID was verified by identification of three randomly chosen groups of cultivars among the 32 ones that we selected. This CID generated will be useful for the identification of commercially important ornamental peach cultivars.

**Key words:** Ornamental peach; RAPD; Cultivar identification; Molecular markers

## INTRODUCTION

Peach (*Prunus persica* L.) is one of the most economically important fruit crops. As the center of origin of peaches, China has the most abundant resources of both wild and cultivated varieties (Yuan et al., 2002; Yang et al., 2002), including those with high ornamental value for colorful flower and/or leaves. Ornamental peach trees have also been cultivated in China for thousands of years (Everett, 1967; Hu and Zhang, 2005). The flowers are produced in early spring before the leaves; they are solitary or paired, pink, with 1 or 7 several whorls of petals. All these characteristics make ornamental peach an excellent plant for urban landscapes and gardens. However, scientific research on genetics and molecular biology has only focused on the peach trees cultivated for fruit. This demands more research focused on the genetics and molecular biology of ornamental peach, including accurate identification of ornamental peach varieties and genotypes that are important for the ornamental peach nursery and peach industry.

Traditionally, identification of the plant was based on morphological or physiological aspects. Biochemical markers such as isoenzymes have been used but they have many drawbacks, including the limited number of polymorphisms detected between close cultivars and variations due to the physiological stage. Even though several generations of DNA markers have been developed and used for cultivar identification and genetic analysis, leading to the publication of thousands of papers, no efficient approach has been developed and used to make DNA markers useful in cultivar identification, besides the phylogenetic trees and/or some fingerprints employed in identification on a very small scale. Phylogenetic trees cannot tell us which primer to use and the information to use or refer to for specific cultivar identification, while traditional fingerprinting does not present all the fingerprints together and is thus not ideal for the identification of many cultivars.

Various DNA-based markers have recently been developed and used for studies on genetic diversity, fingerprinting and cultivar origins (D'Onofrio et al., 2009; Melgarejo et al., 2009; Cheng and Huang, 2009; Elidemir and Uzun, 2009; Papp et al., 2010). Random amplified polymorphic DNA (RAPD) markers have been proven to be a reliable marker system for genetic fingerprinting and also in determining the genetic relationships between germplasm collections. RAPD markers have the advantage of being simple, able to detect relatively small amounts of genetic variation and not needing prior information on the genome. The technique has already been successfully applied to estimate genetic relationships in litchi (Ding et al., 2000), apple (Stark-Urnau, 2002a), apricot (Mariniello et al., 2002), Indian cashew (Archak et al., 2003), olive (Belaj et al., 2003), mulberry (Vijayan, 2004), pear (Stark-Urnau, 2002b; Lee et al., 2004), grape (Benjak et al., 2005), fig (Sadder and Ateyyeh, 2006), longan (Yonemoto et al., 2006), and cherry (Demirsoy et al., 2008). The optimization of the RAPD technique, by choosing 11 nucleotide primers and strict screening of PCR annealing temperature before being employed in fingerprinting plants, can make RAPD a preferable technique for use in plant cultivar identification.

With the strategy of manual cultivar identification diagram (MCID), a new analysis strategy of DNA fingerprints in generating referable information showing specific primers and polymorphic markers that could be used to separate and identify cultivars (Li et al., 2011; Zhang et al., 2011; Zhao et al., 2011; Korir et al., 2012), it is much more feasible to carry out efficient identification of plant individuals. This new way of recording DNA fingerprints of genotyped plants can generate a cultivar identification diagram (CID) to be used as a source of referable information for quick identification of plant and/or seed samples in the future.

Based on the necessity of ornamental peach cultivar identification and the advantages of MCID in applying DNA markers in plant identification, the identification of 32 commercially important ornamental peach cultivars was carried out using RAPD banding patterns.

## MATERIAL AND METHODS

### Plant materials

Young leaves of 32 selected ornamental peach cultivars were collected from the Jiangsu Academy of Agricultural Science, Nanjing, China. The names and main ornamental characters of these cultivars are shown in Table 1, and some representative cultivars are shown in Figure 1.



**Figure 1.** Six representative ornamental peach cultivars. 1-6 = ‘bai hua bi tao’, ‘F2 chui zhi’, ‘Hong fen jia ren’, ‘Fei tao’, ‘Hong shou’, ‘Zhu fen chui zhi’, respectively.

### Genomic DNA extraction

Total genomic DNA of each genotype was extracted from young leaves using the modified cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson, 1980; Fang et al., 2006). The extracted DNA was diluted to a final concentration of 30 ng/ $\mu$ L with 1X TE buffer and stored at -20°C pending use.

### RAPD analysis

In case of RAPD reactions, 54 primers were initially tested with a few genotypes, and only those primers resulting in clear unambiguous banding patterns with all genotypes tested were selected for use in genotyping the full set of cultivars.

Fifty-four 11-mer RAPD primers were used for screening in this study. To increase credibility of the fragments, we used only those primers resulting in clear unambiguous banding patterns. Subsequently, 6 primers (Table 2) that showed well-resolved and reproducible bands were selected to assay all genotypes. Reaction solutions consisted of 2.0  $\mu$ L 10X buffer, 1.2  $\mu$ L 25 mM MgCl<sub>2</sub>, 1.6  $\mu$ L 2.5 mM dNTP, 1.6  $\mu$ L 1.0  $\mu$ M primer, 0.1  $\mu$ L 5 U/ $\mu$ L rTaq Polymerase Dynazyme, and 1  $\mu$ L genomic DNA, making a total volume of 20  $\mu$ L. Amplification reactions were performed based on the standard protocol of Williams et al. (1990), with minor modifications (Wang et al., 2011). PCR was carried out in an Autorisierter Thermocycler (Eppendorf, Hamburg, Germany), programmed as follows: an initial predenaturation step for 5 min at 94°C; then 42 cycles each consisting of a denaturation step for 30 s, an annealing step for 1 min at annealing temperature (Table 2) and an extension step for 2 min at 72°C. Amplification was terminated by a final extension of at 72°C for 10 min. After amplification, the amplified DNA fragments were separated by gel electrophoresis on 1.3% agarose (w/v) (Figure 2) in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 100 V. The gels were stained with 0.5  $\mu$ g/mL ethidium bromide and visualized under ultraviolet light for polymorphic bands among the cultivars. All amplifications were repeated separately at least thrice to obtain reproducible, accurate and clear banding patterns.

### Data analysis

Only clear unambiguous bands in the photographic prints of gels were chosen and scored for cultivar identification. Where some cultivars had a specific band in the fingerprint generated from one primer, they could be separated singly, and those cultivars sharing the same banding pattern were separated into the same sub-group. Based on this strategy, all the ornamental peach cultivars were gradually and completely separated from one another as more primers were employed.

### Test of the utilization and workability of the CID

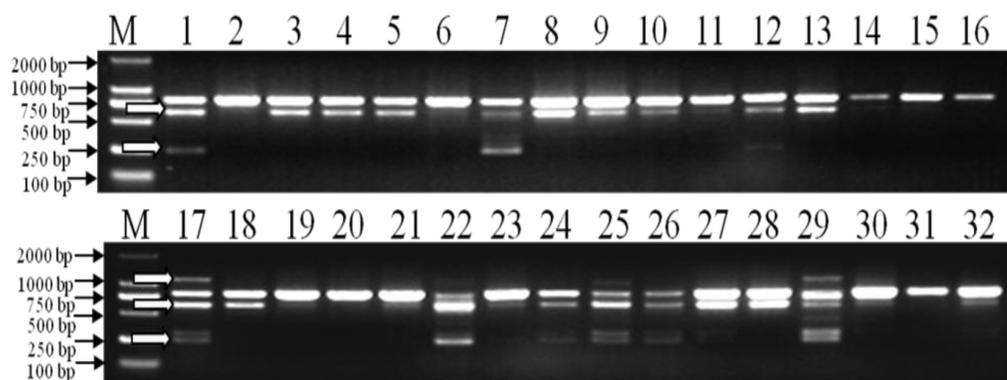
Three groups of ornamental peach cultivars, randomly chosen from the inter- and intra-groups, were used to verify the utilization and workability of the diagram showing the separation of the 32 cultivars. The three groups of cultivars were marked “A”, “B” and “C”, and the corresponding primers to be used for separation of each group were easily picked out from the diagram. If the randomly chosen cultivars could be distinguished accurately and quickly as anticipated on the whole cultivar identification diagram, it would definitely give assurance that the strategy developed and employed in this study is scientific, workable, and efficient, consequently making this method an ideal way to use molecular markers for the identification of fruit crop cultivars and seed samples of field crops. The data from the cultivar separation in the cultivar identification diagram can also be provided in a database for ease of reference.

## RESULTS

### Cultivar identification

To establish a stable and optimistic RAPD system with high reproducibility, longer

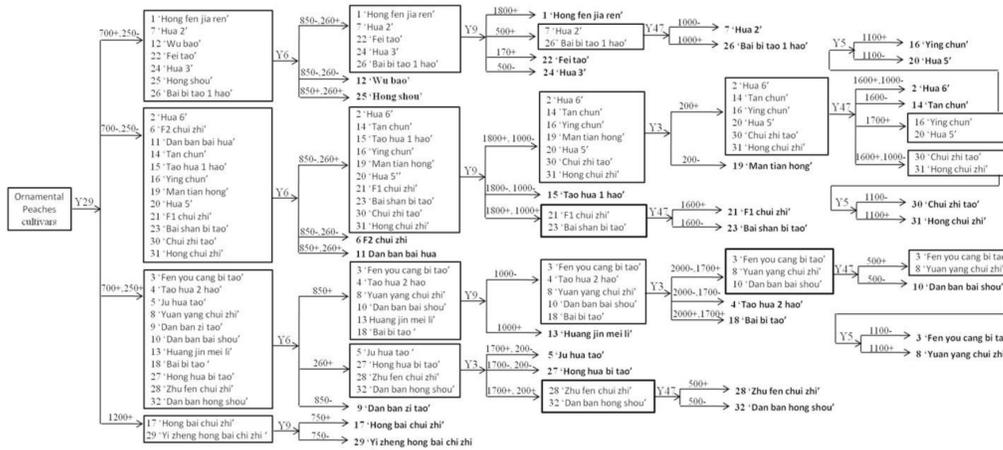
primers (11 nucleotides) were employed and the annealing temperature for each primer was screened based on the quality and reproducibility of banding patterns. The primers were randomly screened from a stock of 54 11-mer primers, and once an optimistic primer that could produce reproducible polymorphic bands was screened, it was further utilized in the identification of the ornamental peach cultivars. By the time the 11th primer was (Table 2) screened and utilized, all 32 ornamental peach cultivars could be successfully identified. An example of the RAPD patterns in the study included that obtained using primer Y29 (Figure 2), which was also the first primer used to amplify the 32 ornamental peach cultivars. The electrophoresis results showed that primer Y29 generated uniform, clear, and reproducible band patterns in 7 ornamental peach cultivars assigned lane number codes 1, 7, 12, 22, 24, 25, and 26 (the lane numbers corresponding to the cultivar names are shown in Table 1). This group of 7 ornamental peach cultivars was easily differentiated from the other two groups by the presence and/or absence of distinct 700- and 250-bp bands. Another group with 2 ornamental peach cultivars was then assigned lane number codes 17 and 29 by the presence of a 1200-bp band; the polymorphic bands with sizes of 700-, 250-, 1200-bp were chosen to separate the 32 cultivars, and the presence and/or absence of the three bands could classify these cultivars into four groups as shown in the diagram (Figure 3). Five other primers (Table 2) were, step by step, screened and chosen to differentiate the ornamental peach cultivars until full separation was achieved as shown in Figure 3. For example, the second primer used (Y6) could further separate the four groups of cultivars, earlier identified by primer Y29, into smaller groups. Other primers were chosen to differentiate the several subgroups of ornamental peach cultivars. The group of 11 cultivars assigned the lane number codes 3, 4, 5, 8, 9, 10, 13, 18, 27, 28, and 32, could be separated with band sizes of about 850 and 260 bp by primer Y6 (Figure 4). Afterwards, the ornamental peach cultivars could still be separated into many more groups, and following this method systematically and utilizing 6 primers, all 32 ornamental peach cultivars could eventually be differentiated from each other.



**Figure 2.** DNA banding patterns of 32 ornamental peach cultivars amplified by primer Y29. Lane M = DL2000 plus DNA ladders; lanes 1 to 32 = accession numbers of ornamental peach cultivars listed in Table 1.

**Table 1.** Name of the materials used in the experiment.

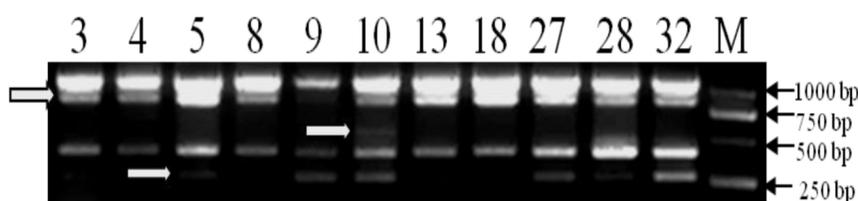
No.	Name	Branch type or tree type	Leaf color	Petal color	Petal type
1	Hongfenjiaren	Straight-stem	Purple	Pink	Double-whirl petal
2	Hua 6	Straight-stem	Green	Pink	Double-whirl petal
3	Fenrousebitao	Straight-stem	Green	Pink	Double-whirl petal
4	Taohuan2hao	Straight-stem	Green	Pink	Double-whirl petal
5	Juhuatao	Straight-stem	Green	Pink	Double-whirl petal
6	F2 chuizhi	Pendulous-stem	Green	Red	Double-whirl petal
7	Hua 2	Pendulous-stem	Green	Pink	Double-whirl petal
8	Yuanyangchuizhi	Pendulous-stem	Green	Mottle	Double-whirl petal
9	Danbanzitao	Straight-stem	Purple	Pink	Single-whirl petal
10	Danbanbaishou	Dwarf	Green	White	Single-whirl petal
11	Dan ban bai hua	Straight-stem	Green	White	Single-whirl petal
12	Wubao	Straight-stem	Green	Mottle	Double-whirl petal
13	Huangjinmeili	Straight-stem	Green	Pink	Double-whirl petal
14	Tanchun	Straight-stem	Green	Pink	Double-whirl petal
15	Taohua1hao	Straight-stem	Green	Red	Double-whirl petal
16	Yingchun	Straight-stem	Green	Pink	Double-whirl petal
17	Hongbaichuizhi	Pendulous-stem	Green	Mottle	Double-whirl petal
18	Baibitao	Straight-stem	Green	White	Double-whirl petal
19	Mantianhong	Straight-stem	Green	Red	Double-whirl petal
20	Hua 5	Straight-stem	Green	Pink	Double-whirl petal
21	F1 chuizhi	Pendulous-stem	Green	Red	Double-whirl petal
22	Feitao	Straight-stem	Green	Red	Double-whirl petal
23	Baihuashanbitao	Straight-stem	Green	White	Double-whirl petal
24	Hua 3	Straight-stem	Green	Pink	Double-whirl petal
25	Hongshou	Dwarf	Green	Red	Double-whirl petal
26	Baibitao 1	Straight-stem	Green	White	Double-whirl petal
27	Honghuabitao	Straight-stem	Green	Red	Double-whirl petal
28	Zhufenchuizhi	Pendulous-stem	Green	Pink	Double-whirl petal
29	Yi zheng chi zhi	Pendulous-stem	Green	Mottle	Double-whirl petal
30	Chui zhi tao	Pendulous-stem	Green	Red	Double-whirl petal
31	Hongchuizhi	Pendulous-stem	Green	Red	Double-whirl petal
32	Dan ban hong shou	Dwarf	Green	Red	Single-whirl petal



**Figure 3.** Cultivar identification diagram of the ornamental peach cultivars by 6 primers. The numbers above each horizontal line in the diagram were the size (in bp) of the polymorphic bands used to separate the cultivars following the line. (+) = presence of the polymorphic band; (-) = absence of it. The cultivars name in bold fonts are those that were separated.

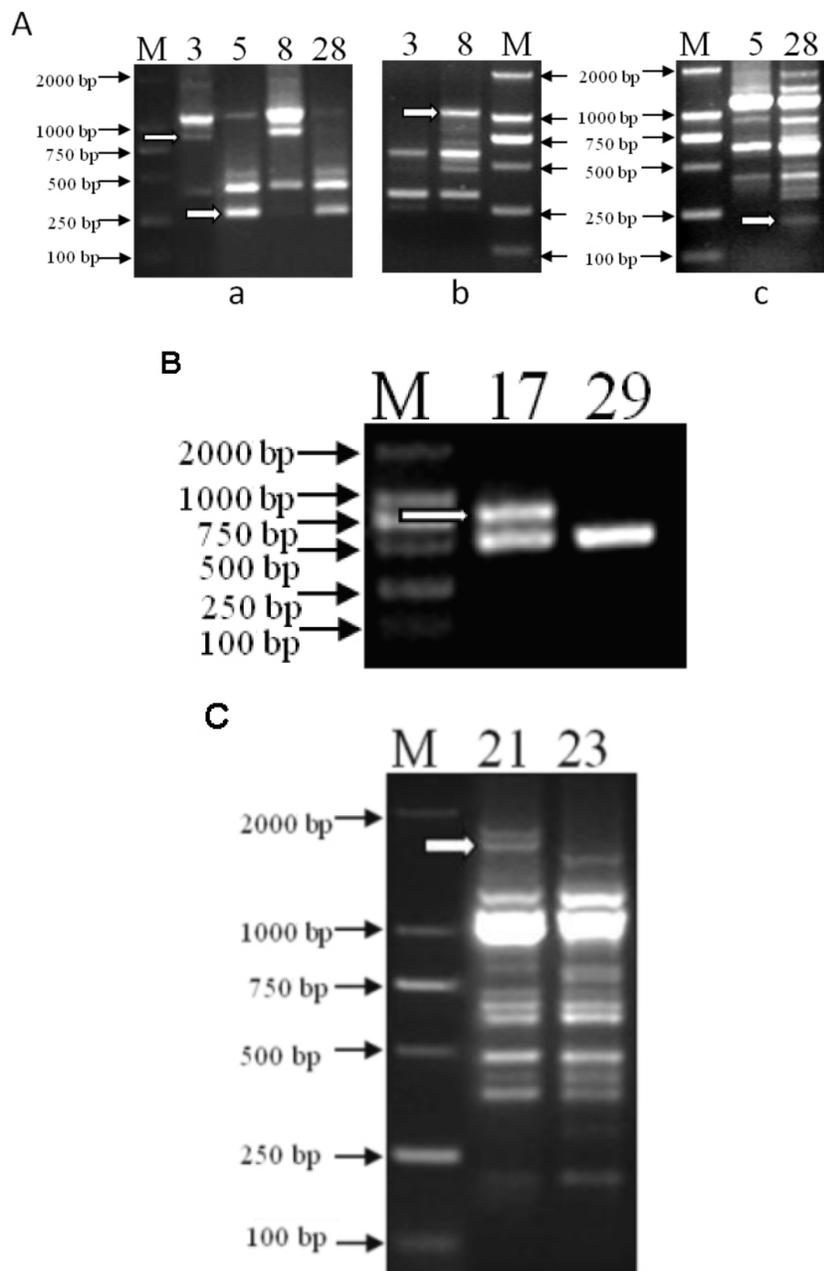
**Table 2.** Six primers chosen for further fingerprinting of the 32 ornamental peach genotypes.

Primer	Nucleotide sequence (5'-3')	Annealing temperature (°C)
Y-3	GTTTCGCTCCA	44.7
Y-5	GTTTCGCTCCG	43.7
Y-6	GTTTCGCTCCC	44.4
Y-9	CTGCTGGGACA	41.7
Y-29	GTGTGCCCCAG	42.8
Y-47	ACGACCCGACAG	43.7

**Figure 4.** DNA banding patterns of 11 ornamental peach cultivars in group from the separation of primer Y6. Lane M = DL2000 plus DNA ladders. The bands pointed with larger arrows were those employed to separate the cultivars.

### Test of the utilization and workability of the CID

The important aim of this study was not only to use the RAPD marker to distinguish the important 32 ornamental peach cultivars as done by most earlier reports focusing on the utilization of DNA marker in identifying plant cultivars, but also to generate a referable ornamental peach CID that could be used in practical identification of some of these cultivars in the future for the nursery industry as well as in cultivar-right-protection. With these two aims in mind, it was critical to verify the utilization, workability and efficiency of the diagram in cultivar identification. To undertake this, three groups of cultivars composed of ‘Fen you cang bi tao’, ‘Ju hua tao’, ‘Yuan yang chui zhi’, and ‘Zhu fen chui zhi’, ‘Hong bai chui zhi’ and ‘Yi zheng hong bai chi zhi’, ‘F1 chui zhi’ and ‘Bai shan bi tao’, which came from the inter- and intra-groups in the CID, were randomly chosen and used for the verification exercise. From the location of these cultivars in CID, it was easy to find the primer to use in separating them, which were the primers Y6, Y5, Y3, Y9, and Y47. Clearly, the PCR results agreed with those anticipated in the CID, in that all cultivars in these three groups were separated accordingly. The first group according to Figure 5 (A, B, C) could be validated with the three primers Y6 Y5 and Y3. The PCR results showed that four ornamental peach cultivars could first be separated into two groups by primer Y6 with a band of about 850 and 260 bp. One group composed of ‘Fen you cang bi tao’ and ‘Yuan yang chui zhi’, could be further separated with primer Y5 by a 1100-bp band. The other group of ‘Ju hua tao’ and ‘Zhu fen chui zhi’, could then be divided by the use of primer Y3 with a band of 200 bp. ‘Hong bai chui zhi’ and ‘Yi zheng hong bai chi zhi’ could be separated with a specific band of about 750 bp with primer Y9 as in the CID. Another group of ‘F1 chui zhi’ and ‘Bai shan bi tao’ was divided by primer Y47 with a band of about 1600 bp. By this procedure, all three groups were successfully identified with a suitable combination of primers. This test therefore proved the availability, workability and efficiency of this method in ornamental peach cultivar identification.



**Figure 5.** Result of cultivars selected randomly by the corresponding primers. Lane M = DL2000 plus marker. **A.** Fingerprint obtained with three primers used to separate the first group of cultivars selected: “a”, “b”, and “c” obtained with the primers Y6, Y3, and Y5, respectively. **B.** Fingerprint obtained with the primer Y9 used to separate the group ‘Hong bai chui zhi’ and ‘Yi zheng hong bai chi zhi’. **C.** DNA fingerprint obtained with the primer Y47 used to separate the group ‘F1 chui zhi’ and ‘Bai shan bi tao’.

## DISCUSSION

China is an important agricultural country in the world and has abundant plant resources, which makes distinguishing of plant cultivars or varieties an important task for plant scientists. One of the ultimate purposes of plant science is to serve agriculture, and the means of practical application of new biological techniques in agricultural production are very important. Ornamental peach is an important plant for urban landscapes and gardens. Along with frequent exchange of resources between regions and the lack of management of varietal resources, background knowledge of some of these cultivars used is unclear. The name confusion, especially in nursery stock markets, is a real problem as myriad false claims of inferiority or superiority of a cultivar are not backed by any evidence. This can adversely affect future production of desired cultivars. The need to demystify science by developing strategies of applying new knowledge and technology to practical activities to solve the above problem is therefore both necessary and significant. DNA markers are a powerful technique that can be used to accurately identify plant cultivars and species, with several generations of DNA markers having been developed and used for cultivar identification (Saker et al., 2006; Chiu et al., 2010) and genetic analysis (Boronnikova et al., 2007; Silvestrini et al., 2008; Bhau et al., 2009; Baysal et al., 2010). Thousands of papers on this subject have also been published. Despite this, DNA markers have not been readily used for the genotyping of plants. In fact, the situation is much dire than anticipated, with the question of whether DNA markers can be adequately and easily used in the identification of plant varieties yielding a negative response. No efficient approach has been developed to employ DNA markers easily and efficiently in plant cultivar identification except where phylogenetic tree clusters or some fingerprints were utilized. Apparently, clusters formed in phylogenetic trees cannot tell us which information can be used for identification of the desired plant samples while fingerprinting cannot present all the fingerprints from many cultivars together for identification. The main reasons for these weaknesses can be attributed to the fact that no analysis could connect the information of DNA fingerprints with cultivars in an easy, clear and readable way. The new approach we developed to utilize DNA markers in distinguishing cultivars is both an efficient and practical one, providing less cost, quick work and clear aims, among other benefits. This strategy can realize the power of DNA markers in plant cultivar identification, and can use the polymorphic nature of each primer, gradually distinguishing and charting every species. Although the method does not accurately reflect the genetic relationship of the cultivars/species, theoretically, the first cultivars/species to be separated will have the widest genetic distance between them. The reverse of this holds true. This method definitely does help greatly in plant cultivar identification for purposes of the protection of cultivar rights, and early identification of seedlings in the nursery industry. To improve the efficiency and utilization of this strategy in the fruit crop industry, more commercially important cultivars in production were chosen for identification. If there are one or more new cultivars that need to be separated and the identification result put in the diagram, the whole set of the primers used to run the additional DNA samples can be used and a larger diagram with the identification information of the new cultivars formed. If the set of primers used originally, such as the 6 primers in this study cannot work well in the separation of new cultivars, new primers need to be screened and used. This study is not difficult or time-consuming and it deserves to be utilized in agriculture.

This is the first report on using RAPD primers in sequence to identify ornamental peach cultivars. The reliability of this identification result was also verified. In this study, the

experimental verification yielded satisfactory results. Therefore, this experiment confirms the possibility of utilizing DNA markers even in plant species that have a highly heterozygous genome, without requiring a genetic linkage map and/or any DNA sequence information to distinguish the cultivars. The MCID strategy is not just a simple theoretical model but a truly handy one as it makes DNA markers more applicable for plant variety identification in practice.

## ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#30871681) and the China Agriculture Research System (#CARS-31).

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