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Methodology

A new strategy employed for identification of sweet orange cultivars with RAPD markers

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ABSTRACT. We optimized RAPD techniques by increasing the length of RAPD primers and performing a strict screening of PCR annealing temperature to distinguish 60 sweet orange cultivars from the Research Institute of Pomology at the Chinese Academy of Agricultural Sciences. A new approach called cultivar identification diagram (CID) was used to improve the efficiency of RAPD markers for cultivar identification. Thirteen effective primers were first screened from 54 RAPD arbitrary 11-mer primers based on their amplification products and amplified polymorphic bands; they were then used for PCR amplification of all 60 cultivars. All cultivars were manually and completely separated by the polymorphic bands appearing in DNA fingerprints from 13 primers; a CID of the 60 sweet orange cultivars was then constructed. This CID separated all the cultivars from each other, based on the polymorphic bands; the corresponding primers were marked in the correct positions on the sweet orange CID. The CID strategy facilitates the identification of fruit cultivars with DNA markers. This CID of sweet orange cultivars

will be very useful for the protection of cultivar rights and for early identification of seedlings in the nursery industry.

Key words: Sweet orange; RAPD; Cultivar identification; Molecular markers

INTRODUCTION

Sweet orange (*Citrus sinensis*) belongs to the sub-family Aurantioideae. It is one of the most economically important crop plants in the world. Sweet orange is the largest citrus crop among fruit trees, accounting for 70% of total yield. It also includes a number of bud sport cultivars (Fang et al., 2010), and the background of existing resources and cultivated varieties is quite complex. Due to its geographic expansion, sweet orange culture has the problem of different synonyms for cultivars (cultivars having more than one name) and homonyms (different cultivars with the same name).

In the last decades, it has become imperative to manage the large germplasm of sweet orange and to properly identify the different cultivars. Traditional approaches for sweet orange cultivar identification, such as morphological, palynological, cytological, isozyme, etc., have proven to be limited due to scarce information, difficult operability, low stability and reproducibility, susceptibility to environmental influences, and the need for extensive observations in mature plants. DNA-based fingerprinting markers have overcome these limitations and can provide a powerful tool for proper characterization of cultivars.

Although DNA-based molecular markers have been utilized in genetic studies, cultivar characterization and identification of sweet orange, as well as other studies, could provide information about extent of genetic diversity and the separation of individual plants. However, no single reported study, to our knowledge, has been able to identify a large number of sweet orange cultivars. This has resulted in limited referable data, not only for current cultivar identification but also for future study, which is the main problem limiting the practical utility of a DNA marker in plant cultivar identification. The main bottleneck of this situation is that the analysis strategies of DNA fingerprints have not been able to generate some referable information indicating which primer and which polymorphic marker can be used to separate the cultivars to be identified. Obviously, the popular analytical techniques for determining DNA banding patterns, known as cluster analyses, cannot provide an efficient method for separating cultivars or species. Employing a strategy that can distinguish sweet orange cultivars in a reliable, easy, referable, and practical manner is very necessary for the nursery industry, farming industry, protection of plant patents, and the conservation and evaluation of genetic resources related to the sweet orange crop.

Classical approaches for the identification of cultivars have been based on morphological, physiological and agronomic traits. However, these traits have limitations, since they can be easily influenced by the environment and require extensive observation of mature plants. Molecular markers have the advantages of not being affected by the environment and being able to provide a powerful tool for proper characterization of cultivars. In recent years, various DNA-based markers have been developed and used for genetic diversity, fingerprinting studies and determination of the origins of cultivars (D'Onofrio et al., 2009; Melgarejo et al., 2009; Cheng and Huang, 2009; Elidemir and Uzun, 2009; Papp et al., 2010), of which random amplified polymorphic DNA (RAPD) (Williams et al., 1990) markers are useful for cultivar analysis with specific advantages in simplicity, efficiency, and non-requirement of any previ-

ous sequence information. If some optimization of the RAPD technique, by choosing 11-nt primers and strict screening of PCR annealing temperature for each primer, is done before it is employed in fingerprinting plants, RAPD can be a preferred technique in plant cultivar identification. To date, RAPD markers have been popularly used in cultivar identification and genetic relationship analysis of a number of fruit species, such as apricot (Ercisli et al., 2009), pomegranate (Hasnaoui et al., 2010), cherry (Demirsoy et al., 2008), pistachio (Javanshah et al., 2007), and strawberry (Wang et al., 2007). In practice, the powerful DNA markers available for plant identification have not made plant variety identification an efficient, recordable, and easy method as anticipated, which is an awkward challenge facing us today.

In this study, we developed a new strategy, the cultivar identification diagram (CID), and successfully identified 60 cultivars of sweet orange using polymorphic RAPD markers. The separation of 60 sweet orange cultivars, based on RAPD banding patterns, can definitely be of service to the sweet orange industry as well.

MATERIAL AND METHODS

Plant materials

The young leaves of 60 sweet orange cultivars were collected from the Research Institute of Pomology at the Chinese Academy of Agricultural Sciences, Chongqin. The name and origin of the cultivars are listed in Table 1.

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

No.	Cultivar	No.	Cultivar
1	Newhall Navel Orange	31	‘Huapeng 9-11’
2	Robertson Navel Orange	32	‘Yihongcheng’
3	‘Yoshida Navel’	33	‘Zhuashaganpeng’
4	‘Cara red flesh navel orange’	34	‘Wanxiandaetiancheng’
5	‘Fukumoto Navel Orange’	35	‘Anjiangdahongcheng 1hao’
6	‘Tarocco blood orange’	36	‘Jintangcheng’
7	‘Delta’	37	‘Taoyecheng 18hao’
8	‘Xiajin’	38	‘Yinzigan’
9	‘Trovita’	39	‘Eight lanes tiancheng’
10	‘Fengqi’	40	‘Eight lanes tiancheng’
11	‘Suzuki navel’	41	‘Cadenera Sweet Orange’
12	‘Ichang Papeda’	42	‘Hanyuan seedless’
13	‘Late-maturing navel orange’	43	‘Shenghongpigan’
14	‘Zilixiangcheng’	44	‘Kaixian seedless’
15	‘Luohancheng’	45	‘Yunnannanguo’
16	‘Lingyangshaohedahongtiancheng’	46	‘Liuyecheng’
17	‘Thomson’	47	‘Xuegantiancheng’
18	‘Midknight’	48	‘Nanchongdanpei’
19	‘Guangxixiangshuicheng’	49	‘Anyuan C3’
20	‘Egypt honey orange’	50	‘Wuyicheng’
21	‘Washintong Sanquine’	51	‘Zhoupicheng’
22	‘Xianfengcheng’	52	‘Kaixianbopi’
23	‘Zaoshutiancheng’	53	‘Pengan 100 hao’
24	‘Huangbaipitiancheng’	54	‘Qiaobuocheng’
25	‘Xinghuihongcheng’	55	‘Yiancheng’
26	‘Parson brown’	56	‘Chengxi late-maturing’
27	‘Hamlin’	57	‘Jiangbeimeigan’
28	‘Taoyecheng’	58	‘Italy hamlin’
29	‘Cuba taoyecheng’	59	‘Hongmaocheng’
30	‘Toucheng’	60	‘Ziyangxiangcheng’

Genomic DNA extraction

Total genomic DNA of each genotype was extracted from young leaves using the modified cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). The extracted DNA was solubilized and diluted to a final concentration of 30 ng/μL with 1X TE buffer and stored at -20°C until use.

RAPD analysis

In cases 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 further use in genotyping.

Eleven-nucleotide RAPD primers were used for screening. To increase the reliability of the fragments, we used only those primers resulting in clear unambiguous banding patterns. Thus, 13 primers (Table 2) that showed well-resolved and reproducible bands were selected to assay all genotypes, while the others were discarded. The reaction solution consisted of 2.0 μL 10X buffer, 1.2 μL 25 mM $MgCl_2$, 1.6 μL 2.5 mM dNTP, 1.6 μL 1.0 μM primer, 0.1 μL 5 U/μL rTaq Polymerase Dynazyme and 1 μL genomic DNA, making a total volume of 20 μL. Amplification reactions were performed based on the standard protocol of Williams et al. (1990), with minor modification. The PCR was carried out in an Autorisierter Thermocycler (Eppendorf, Hamburg, Germany), programmed as follows: initial predenaturation step of 5 min at 94°C; then, 42 cycles each consisting of a denaturation step of 30 s, an annealing step of 1 min at annealing temperature (Table 2), and an extension step of 2 min at 72°C. Amplification was terminated by a final extension of 72°C for 10 min. After amplification, amplified DNA fragments were separated by electrophoresis on a 1.3% (w/v) agarose gel (Figure 1) in 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffer at 100 V. The gels were stained with 0.5 μg/mL ethidium bromide and visualized under ultraviolet light. Polymorphic bands among the cultivars were observed from photographs. Each amplification reaction was repeated at least thrice to obtain reproducible, accurate and clear banding patterns.

Table 2. Thirteen primers were chosen for further fingerprinting of the 60 sweet orange genotypes.

Primer	Nucleotide sequence (5'-3')	Anneal temperature (°C)
Y10	CTGCTGGGACT	44.4
Y15	AGGGGTCTTGA	44.8
Y17	AGGGGTCTTGG	44.4
Y18	AGGGGTCTTGC	44.4
Y24	GGACCCAACCC	43.8
Y28	GTGTGCCCCAT	44.4
Y34	AAGCCTCGTCT	44.4
Y35	AAGCCTCGTCG	44.4
Y36	AAGCCTCGTCC	44.4
Y41	AGCGTCCTCCG	44.4
Y46	ACGACCGACAT	43.2
Y47	ACGACCGACAG	44.4
Y57	ACCCCCGACTA	44.4

Data analysis

Only clear unambiguous bands were manually scored from photographic prints of gels for each cultivar. We classified these cultivars into different groups according to the fingerprint amplified by each primer. Where some cultivars shared the same band patterns, they were placed into the same group. More primers were then employed to distinguish the cultivars in each group. As more primers were used, more specific amplified bands were generated, which could differentiate all the cultivars separately. Afterwards, the CID, comprising bands of specific size used to separate the cultivars and all the related primers that generated the specific bands, was constructed for the full separation of all the cultivars.

Utilization and feasibility of CID

Two groups of sweet orange cultivars, which were randomly chosen from the inter- and intra-groups, were used to verify the utilization and feasibility of the diagram showing the separation of the 60 cultivars. The two groups of cultivars were labeled A and B, and the corresponding primers to be used for the separation of each group were easily picked out from the CID. If the cultivars could be distinguished as well as anticipated, this would definitely assure the feasibility and efficiency of this new approach in cultivar identification not only for this but also for similar study in the future. PCR was carried out as described above.

RESULTS

Cultivar identification

To establish a stable and optimal RAPD system with high reproducibility, random primers one nucleotide longer (11 nt) were employed and the annealing temperatures for each primer were screened based on the quality and reproducibility of the banding pattern. The primers were randomly screened from a stock of 54 11-nt primers, and once an optimal primer was found that could produce reproducible and clear fingerprints with polymorphic bands, it was further utilized in the identification of sweet orange cultivars. After 13 primers (Table 2) were screened out and utilized, respectively, all the 60 sweet orange cultivars could be successfully identified. An example of the RAPD patterns generated with primer Y34, used to separate the 60 sweet orange cultivars first, is shown in Figure 1. Following this cultivar identification procedure, the other 12 primers (Table 2) were screened step by step and chosen to differentiate the sweet orange cultivars. All 60 cultivars could be completely separated as shown in Figure 2. For easy reading of the CID, all the names of separated sweet orange cultivars were written in bold font. It should be emphasized that only the clear polymorphic bands generated with each primer were used to differentiate the cultivars. The presentation of the sizes and the presence/absence of the polymorphic bands used for cultivar identification in the CID as shown in Figure 2 can make the CID very useful and referable in the identification of sweet orange cultivars in practice.

Utilization and feasibility of CID

The important aim of this study was not just how to use RAPD markers to distinguish

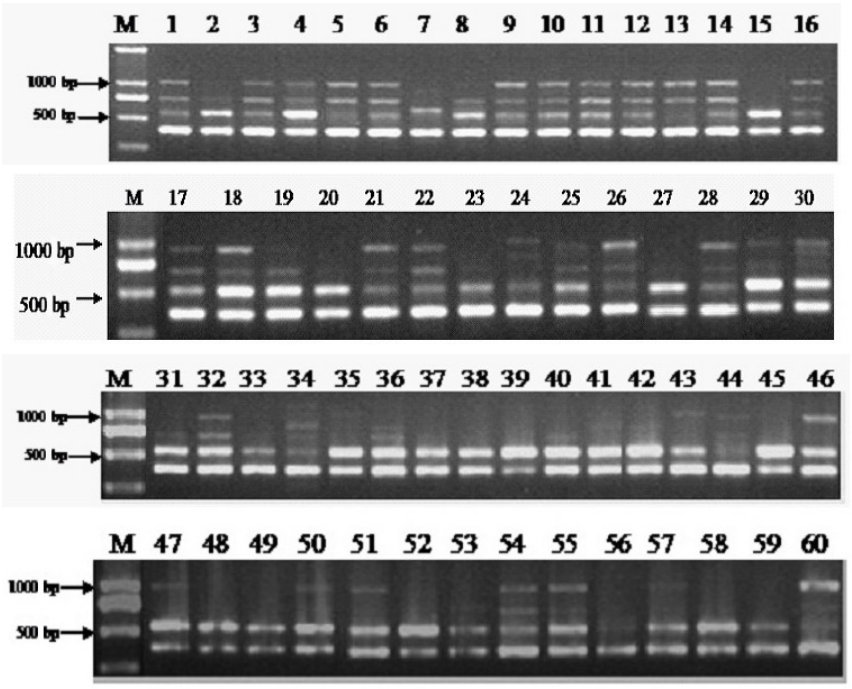

Figure 1. DNA banding patterns of 60 sweet orange cultivars amplified by primer Y34. *Lane M* = DL2000 plus DNA ladders; *lanes 1-60* = accession Nos. of sweet orange cultivars listed in Table 1.

60 sweet orange cultivars, since most previous reports have focused on genetic analysis and presence of some phylogenetic trees without referable information for practical plant sample identification. The more interesting and more important purpose of our study was to generate a referable CID of sweet orange cultivars with the intention of presenting the information of the polymorphic markers used to separate the sweet orange cultivars in the CID. This made the identification of these sweet orange cultivars practical and easy. This can definitely be of benefit and service to the sweet orange nursery industry and protection of cultivar rights. If there were a need to identify some of the sweet orange cultivars studied here, the required primers could be easily determined and the target polymorphic PCR product chosen using the CID for further identification. Therefore, it was necessary to verify the utilization, feasibility and efficiency of the sweet orange CID, for which two groups of cultivars, group A comprising 'Jintangcheng', 'Taoyecheng 18hao', 'Yinzigan', and 'Eight lanes tiancheng' and group B comprising 'Robertson Navel Orange', 'Yoshida Navel' and 'Cara red flesh navel orange', were randomly chosen from the inter- and intra-groups in the CID to be used for verification. From the location of these cultivars in the CID, it was easy to find the primers to be used to separate them.

Obviously, primers Y28, Y34, Y47, Y57 were those that could be used to separate the two groups of cultivars chosen: Y57 could be found to be the primer separating 'Jintangcheng' of four cultivars in group A, and Y28 was the other primer separating 'Yinzigan' and 'Eight lanes tiancheng'; Y34 could separate 'Robertson Navel Orange' first and Y47 could separate

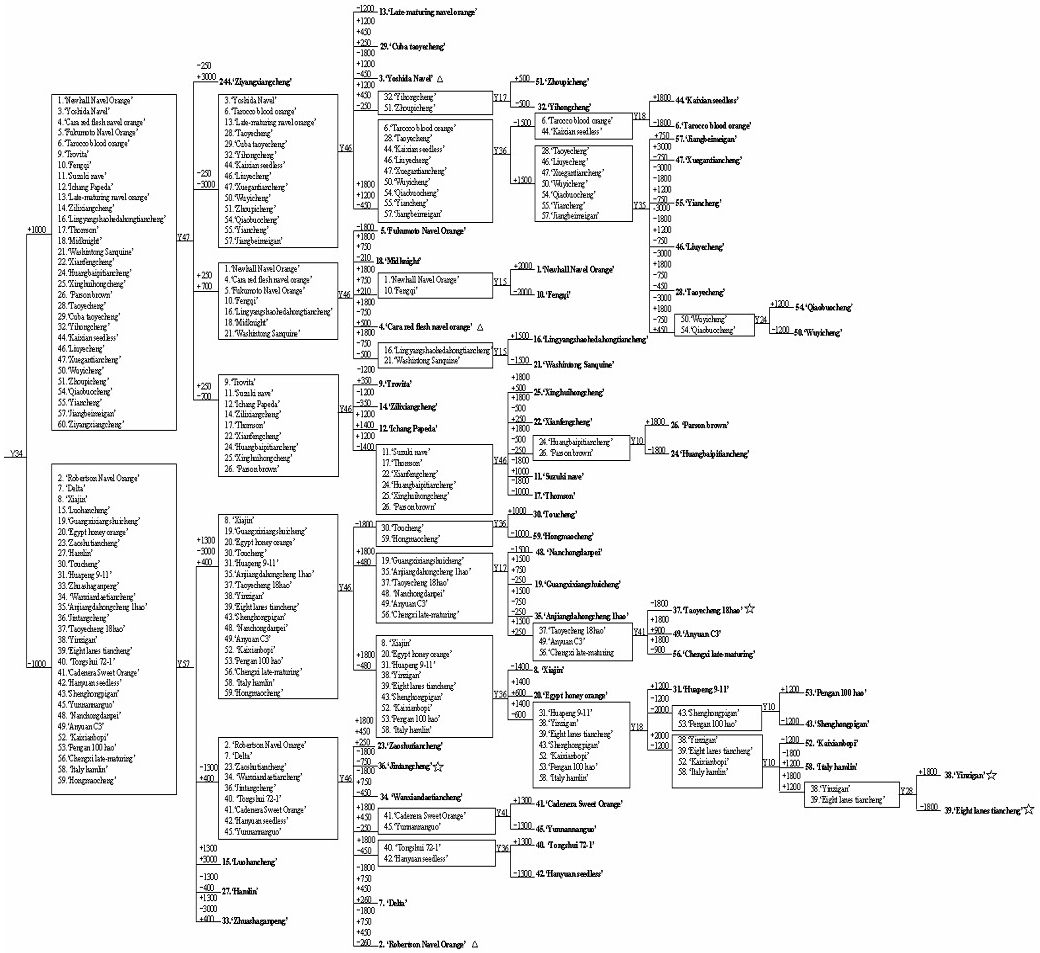

Figure 2. Classification of 60 sweet orange cultivars by the DNA fingerprints of 13 RAPD primers (Table 2). The lane number in the figure means the size of the band in bp. (+) = band present; (-) = band not present. Triangles and stars mean cultivars used for the validation of the workability of the cultivar identification diagram. Cultivar names written in bold are those of separated sweet orange cultivars.

the other two. The corresponding polymorphic bands to be used for the separation can also be found on the CID. After the validation of the identification of the 2 groups of cultivars, the PCR results could definitely show the information as anticipated in that all cultivars in these 2 groups were distinguished using the CID. It was clear that primers Y57 and Y28 could separate group A cultivars based on the banding patterns shown in Figure 3A: 'Jintangcheng' was first identified of the 4 cultivars with a band of 1300 bp in size from primer Y57; 'Yinzigan' and 'Eight lanes tiancheng' were separated with a band of about 1800 bp from primer Y28. The group B cultivars, including 'Robertson Navel Orange', 'Yoshida Navel' and 'Cara red flesh navel orange' could be separated with the primers Y34 and Y47, for which the banding patterns are shown in Figure 3B. This validation of the separation of the two randomly chosen groups of cultivars not only indicated that this sweet orange CID strategy was definitely feasi-

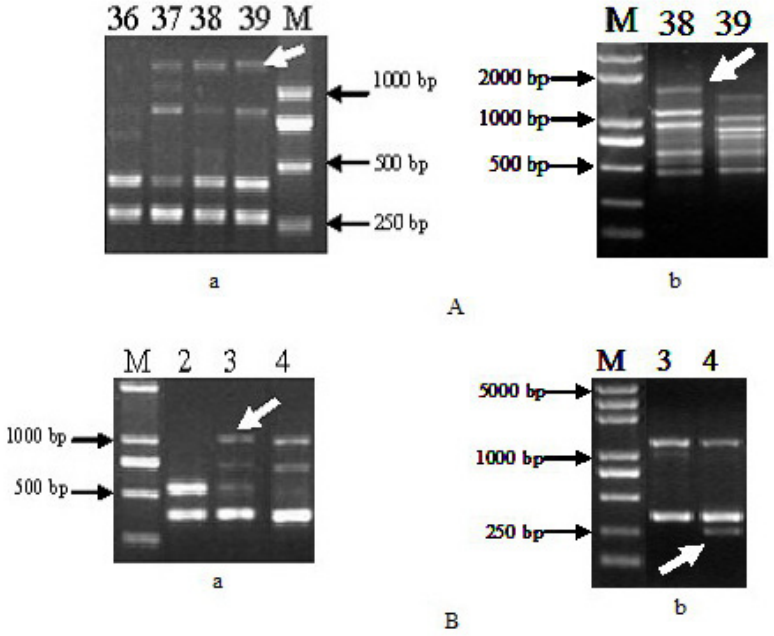

Figure 3. Result of cultivars selected randomly by the corresponding primers. White arrows indicate the specific bands. Lane numbers correspond to the code in Table 1. *Lane M* = DL2000 plus marker. **A.** DNA banding patterns obtained with two primers used to separate the first group of cultivars marked in Figure 2 by squares. "a" obtained with the primer Y57, "b" obtained with the primer Y28. **B.** DNA banding patterns obtained with the primer Y34 used to separate the group "B", which was marked in Figure 2 by squares. "a" obtained with the primer Y34, "b" obtained with the primer Y47.

ble, efficient, referable, and practicable, but also showed us how to use this CID to better serve the sweet orange industry and research on sweet orange genetic resources. Another requirement that should be mentioned is that the data of this cultivar separation from this diagram can also be put into a database for future use *in silico*.

DISCUSSION

The use of DNA markers is a powerful technique that has great potential and utility in the identification of plant cultivars and species. Although several generations of DNA markers have 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), and although thousands of related papers have been published, it does not mean they have been easily used in genotyping. In fact, the situation is more serious than anticipated. No effective approach has been developed to use DNA markers easily and efficiently in plant cultivar identification, except where phylogenetic tree clusters or some fingerprints were employed. The new approach of CID that we employed in this study allowed DNA markers to be more efficiently and practically utilized in distinguishing plant cultivars, which seemed to facilitate the efficient use of the primers and was easily operated. The CID gener-

ated can be very referable information for sweet orange cultivar identification. This strategy would enhance the power of DNA markers in plant cultivar identification activities and use the polymorphic bands of each primer to gradually distinguish each species and individual plant, from which a CID can be finally constructed for practical use. Although the method does not accurately reveal their genetic relationships, theoretically the first cultivar to be separated out indicates that there is the greatest genetic distance between it and the other cultivars studied. This method can be of great help in plant culture for purposes of protection of cultivar rights, cultivar identification, and early identification in nursery industry.

In this study, 13 RAPD primers were sufficient for distinguishing all 60 sweet orange cultivars, providing a diagrammatic representation for future reference in sweet orange cultivar identification. It is very convenient and easily operated by users. Although a single RAPD primer used to be unable to distinguish quite a number of sweet orange cultivars at the same time, the new CID strategy in this study could obviously make the use of most of the polymorphic PCR bands into an efficient identification of the sweet orange cultivars, which overcomes the impossibility of the cluster analysis previously employed in plant identification. The informative CID (Figure 2) of the sweet orange cultivars is the key result that can tell us which primer or primers can be used to separate which sweet orange cultivars. Basically, any two cultivars can be identified with one RAPD primer. In practice, if more new sweet orange cultivars are released, the set of 13 primers can be used to run the DNA samples of the new cultivars and the PCR banding patterns can inform us as to where to position the new cultivars in the CID. If all 13 primers cannot distinguish the 60 original sweet orange cultivars from the new ones, some new primers should be screened and used to separate them and position them in the CID, from which the separation of new cultivars could generate a larger CID. It seems that not much work needs to be done for the separation of one or several new cultivars. The verification of the feasibility and accuracy of the CID as anticipated can confirm the practical importance of this method for sweet orange cultivar identification. We believe that this separation of the sweet orange cultivars and the new strategy employed here can definitely be of importance to the sweet orange industry in China.

This study can initiate new research for the efficient application of DNA markers even in the identification of other plant and seed samples, which are important in plant germplasm conservation, protection of cultivar rights, provision of genetically uniform seedlings in production, and seed industry. This plant CID has shown some advantages in that fewer primers are required. Now, all cultivars included can be easily separated and in the future using PCR with the corresponding primers that are found without difficulty in the diagram. The CID information can be transferred to a database *in silico* and then shared by scientists and farmers all over the world. It is not just a simple diagram and it can make DNA markers more applicable for plant cultivar identification in practice. We have now initiated the same study on the most important fruit cultivars grown in China to aid the protection of cultivar rights, nursery industry, and conservation of genetic resources. We think this new method can be used to design the CIDs for each crop species, and the CID generated can work like a periodic table of the elements, providing us with the information for separating the cultivars of interest.

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