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Full Length Research Paper

Employment of a new strategy for identification of lemon (*Citrus limon L.*) cultivars using RAPD markers

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DNA markers are useful tools that have potential application in crop cultivar identification and fingerprinting besides others. A novel analysis approach called CID strategy was developed that can realize the utility of DNA marker in the separation of plant individuals much better, more efficiently, practical, and referable, in which a Cultivar-Identification-Diagram (CID) was constructed for fingerprinting. In this study, a total of 47 important lemon cultivars cultivated in China were successfully separated with random amplified polymorphic DNA (RAPD) marker analysis. With the analysis using the CID strategy, they could be clearly separated by the fingerprints of 10 RAPD primers. The utilization of the CID of these 47 lemon cultivars was also verified by the identification of two randomly chosen groups of cultivars among the 47. This identification showed that some advantages in fewer primers were used, and all the cultivars could be separated by each other by the corresponding primers marked in the right position on the CID, and this lemon CID could provide the information to separate any lemon cultivars of these 47 which may be of help to the lemon industry in China.

Key words: Lemon cultivar, identification, cultivar-identification-diagram strategy.

INTRODUCTION

The lemon (*Citrus Limon L.*) belongs to the Rutaceae family and is one kind of the small evergreen tree. Lemons ranked the third among the citrus industry in the world, with a total annual production of about 9% in the citrus production. The exact origin of the lemon has remained a mystery, though it is widely presumed that lemons first grew in India, northern Burma and China. The genetic origin of the lemon, however, was reported to be hybrid between sour orange and citron (Gulsen and Roose, 2001). Lemon cultivation is common in China, India, Iran, Brazil, Spain, Italy, Mexico and to some extent in the U.S.A. In China, many lemon varieties popularly cultivated are landraces, and many of them were invariably given local or vernacular names by lemon growers. Scrutiny over the landrace names and their etymology suggests that a given lemon landrace may be named differently in different regions and more than one landrace may share the same name thus causing some problems to breeders, commercial companies and farmers. This calls for an accurate and rapid method for identification of lemon varieties which is also important for cultivar-right-protection. Many studies have utilized molecular markers to examine phylogenetic relationships among *Citrus* and its related genera, including RFLP (Federici et al., 1998), ISSR (Gulsen and Roose, 2001; Fang et al., 1998), RAPD (Nicolosi et al., 2000; Federici et al., 1998), cpDNA sequence (Morton et al., 2003), SSR

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(Barkley et al., 2006). However, the studies on efficient identification of lemon cultivar are limited, since they utilized selected statistical techniques known as cluster analysis to study the banding pattern. These analyses were apparently not able to make identification of the cultivars an easy and referable work, even though they could give the genetic diversity levels and separate individual plants in the final analysis results mostly shown as phylogenetic trees.

Developing a strategy that can make the verification of lemon cultivars reliable, easy, and referable is necessary for nursery industry and growers as a tool for protecting plant patents and providing genetically uniform plants. Classical approaches for identification cultivars were based on morphological, physiological and agronomic traits. However, these traits have limitation since they could be easily influenced by the environment and need extensive observation of mature plants. The molecular markers have the advantages in that they are not affected by the environment and can provide a powerful tool for proper characterization of cultivars. Recent years, various DNA-based markers have been developed and used for genetic diversity, fingerprinting studies and origins of the cultivars (D'Onofrio et al., 2009; Papp et al., 2010; Melgarejo et al., 2009; Cheng and Huang, 2009; Elidemir and Uzun, 2009), of which Random Amplified Polymorphic DNA (RAPD) (William et al., 1990) marker is useful for cultivar analysis with wonderful advantages in simplicity, efficiency and non-requirement of any previous sequence information. If some optimization of RAPD technique by choosing 11 nt primers and strict screening PCR annealing temperature for each primer is done before it is employed in fingerprinting plants, RAPD can be a preferable technique used in plant cultivar identification. So far, RAPD marker have still been popularly used in the 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), strawberry (Wang et al., 2007).

In practice, the powerful DNA markers available for plant identification have not made the plant variety identification an efficient, recordable, and easy work as we anticipated which is a limiting factor. Here, in this study we employed a newly developed analysis strategy that can make the identification of many lemon cultivars a practical, efficient, recordable and referable work. The CID, showing the separation of 47 lemon cultivars generated from the RAPD banding patterns can definitely service the lemon industry well.

MATERIALS AND METHODS

Plant materials

The young leaves of 47 lemon cultivars were collected from the Institute of Pomology at the Chinese Academy of Agricultural Sciences, Chongqing. The name and origin of the cultivars are listed as in Table 1.

Genomic DNA extraction

Total genomic DNA of each genotype was extracted from young leaves using the modified cetyl trimethyl ammonium bromide (CTAB) method (Murray and Thompson, 1980). The extracted DNA was diluted to a final concentration of 30 ng μL^{-1} with 1× TE buffer and stored at -20°C pending use.

RAPD analysis

In case of RAPD reactions, 54 primers were tested with a few genotypes initially and only those primers resulting in clear unambiguous banding patterns with all genotypes tested were selected for use in genotyping. 11-nt RAPD primers were used to screen in this study. In order to increase credibility of the fragments, we used only those primers resulting in clear unambiguous banding patterns. Thus, 10 primers (Table 2) that showed well-resolved and reproducible bands were selected to assay all genotypes, while the others were discarded. Reaction solution were consisted of 2.0 µL 10× buffer, 1.2 µL $MgCl_2$ (25 mM), 1.6 µL dNTP (2.5 mM), 1.6 µL primer (1.0 µM), 0.1 µL rTaq polymerase dynazyme (5 U/µL) and 1 µL of 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 pre-denature step for 5 min at 94°C; then 42 cycles each consisting of a denature step for 30 s; an annealing step for 1 min at annealing temperature (Table 2); an extension step for 2 min at 72°C. Amplification was terminated by a final extension of in 72°C for 10 min.

After amplification, amplified DNA fragments were separated by gel electrophoresis in 1.3% agarose (w/v) (Figure 1) in 1× TAE (0.04 M Tris-acetate, 0.001M EDTA pH 8.0) buffer at 100 V. The gels were stained with 0.5 µg/ml of ethidium bromide and visualized under ultraviolet light. Polymorphic bands among the cultivars were observed from photographs. In order to have reproducible, accurate and clear banding patterns, each amplification reaction was repeated at least thrice separately.

Data analysis

Only clear unambiguous bands in the photographic prints of gels were chosen and scored for cultivar identification. When some cultivars had 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 same way, all the lemon cultivars were step by step completely separated from each other with more primers employed.

Test of utilization and workability of the diagram in cultivar identification

Two groups of lemon cultivars which were randomly chosen from the inter- and intra-groups were used to verify the utilization and workability of the diagram showing the separation of the 47 cultivars. The two groups of cultivars were marked with “A”, “B”, respectively and the corresponding primers to be used for the

Table 1. Name of the materials (commercial cultivar) used in the experiment.

No.	Cultivar	No.	Cultivar
1	‘Limonia’	25	‘Tahiti Lime’
2	‘Lemon’	26	‘Fan(virus-free)’
3	‘Rough Lemon’	27	‘Java Lemon’
4	‘Caijiagoudaningmeng’	28	‘Tuningmeng’
5	‘Eureka Lemon’	29	‘Maganningmeng’
6	‘Genoa Lemon’	30	‘Jiangjincuningmeng’
7	‘Meyer Lemon’	31	‘Fan.5’
8	‘Fino Lemon’	32	‘Veran Lemon’
9	‘FeminelloS_1 Lemon’	33	‘Villafranca Lemon’
10	‘Jinlongdaningmeng’	34	‘Bergamot’
11	‘Red Limonia’	35	‘Volkamer Lemon’
12	‘Fan.1’	36	‘Kaffir Lime’
13	‘Fan.4’	37	‘No.4 Lemon’
14	‘Fan.2’	38	‘Mexican Lime’
15	‘Hekoulaimeng’	39	‘Ichang Lemon’
16	‘Nanchuanningmengyouzi’	40	‘American rough lemon’
17	‘Longfengdaningmeng’	41	‘Mo 84-12’
18	‘Fan.3’	42	‘Lisbonlemon’
19	‘Dana Citron’	43	‘Fem(virus-free)’
20	‘Improved Meyer Lemon’	44	‘2701-1 Limonia’
21	‘Baihuaningmeng’	45	‘Fem(dwarf)’
22	‘Kusaie Lime’	46	‘Fem(no-thorn)’
23	‘Rangpur Lime’	47	‘Fem(4X)’
24	‘Feminellolemon’		

Table 2. 10 primers were chosen for further fingerprinting of the 47 lemon genotypes.

Primer	Nucleotide sequence (5’–3’)	Anneal temperature (°C)
Y10	CTGCTGGGACT	44.4
Y17	AGGGGTCTTGA	43.7
Y33	AAGCCTCGTCA	43.8
Y34	AAGCCTCGTCT	44.4
Y35	AAGCCTCGTCG	44.4
Y36	AAGCCTCGTCC	43.7
Y46	ACGACCGACAT	42.8
Y51	TGGTGGCGTTA	44.8
Y54	TGGTGGCGTTC	40.4
Y58	ACCCCCGACTT	42.8

separation of each group were easily picked out from the CID. If these cultivars randomly chosen could be distinguished accurately and quickly as the anticipated results based on the whole CID, we would definitely assure that the strategy developed and employed in this study was scientific, workable, and efficient, which could be the best way to use molecular marker to identify fruit crop cultivars and seed samples. The data of the cultivar separation from this diagram can also be generated into database.

RESULTS

Cultivar identification

To establish stable and optimistic RAPD system with high reproducibility, one nucleotide longer random primers (11 nt) were employed and the annealing temperatures for

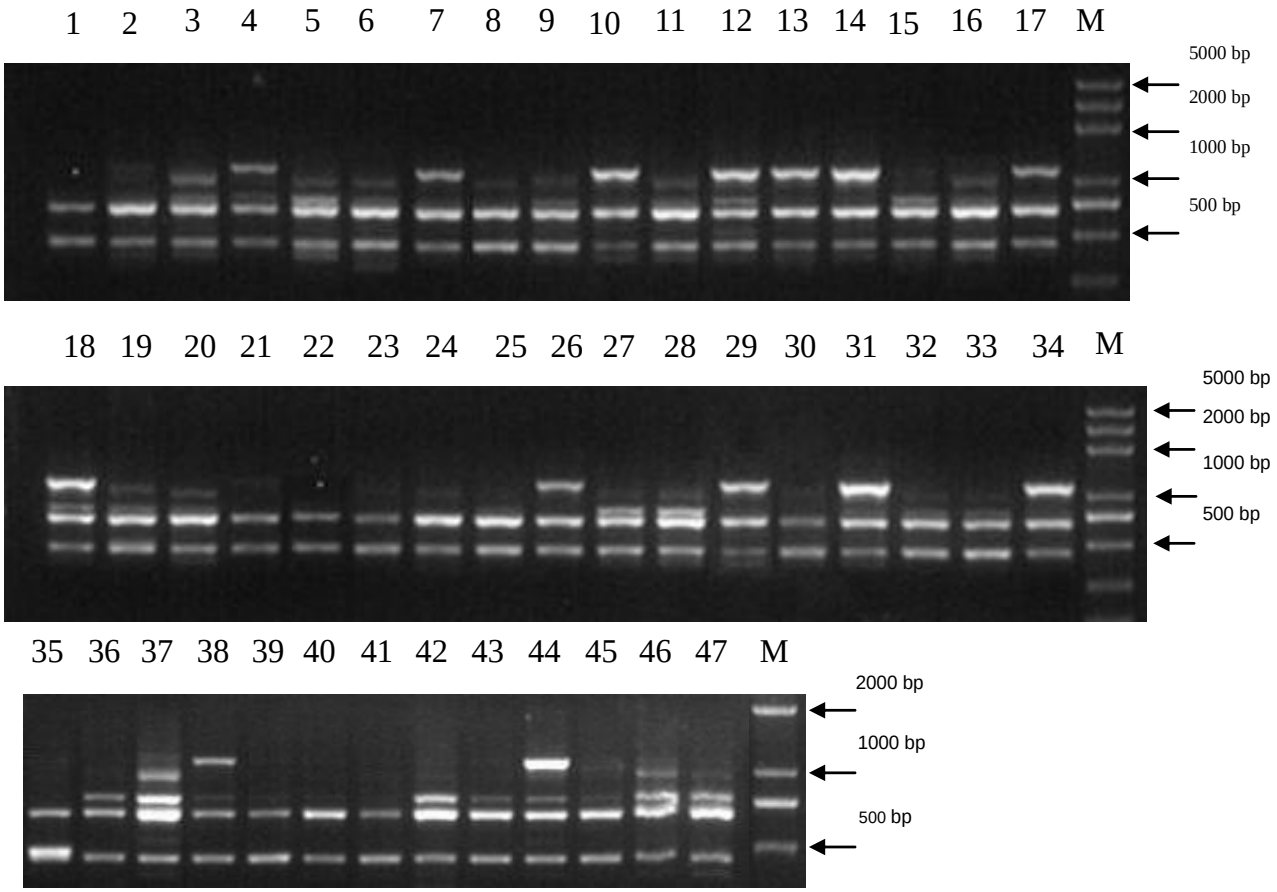

Figure 1. RAPD banding patterns of 47 genotypes within the genus lemon obtained with primer Y33. The white horizontal arrows indicate the specific bands. The lane numbers correspond to the cultivar code in Table 1. M: DL2000 plus marker.

each primer were screened based on the quality and reproducibility of banding pattern. The primers were randomly screened from a stock of 54 11-nt primers, and once an optimistic primer screened that could produce reproducible and clear fingerprints with polymorphic bands, it was further utilized in the identification of lemon cultivars. After 10 primers (Table 2) screened out and utilized, respectively, all the 47 lemon cultivars could be successfully identified. An example of the RAPD patterns generated with primer Y33 used to separate the 47 lemon cultivars first was shown in Figure 1. Following this cultivar identification procedure and with other 9 primers (Table 2) were, step by step, screened and chosen to differentiate the sweet orange cultivars, all the 47 cultivars could be completely separated shown as Figure 2. For easy reading of the CID, all the names of separated lemon cultivars were written in bold font. What need to be emphasized is that only the clear polymorphic bands generated from each primer were used to differentiate the cultivars.

The presentation of the sizes and the presence of the polymorphic bands used for cultivar identification in the CID as shown in Figure 2 can make the CID diagram very useful and referable in the service of sweet orange cultivar identification in practice.

Verification of the cultivar identification result and the workability of the CID

The important aim of this study was not just how to use the RAPD marker to distinguish the 47 lemon cultivars as the most reports before that used to focus on the 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 cultivar-identification-diagram of lemon cultivars with the invention of presence, the information of the polymorphic markers used to separate the lemon cultivars on the CID which made the identification of these lemon cultivars a practical and easy work. This can definitely benefit and

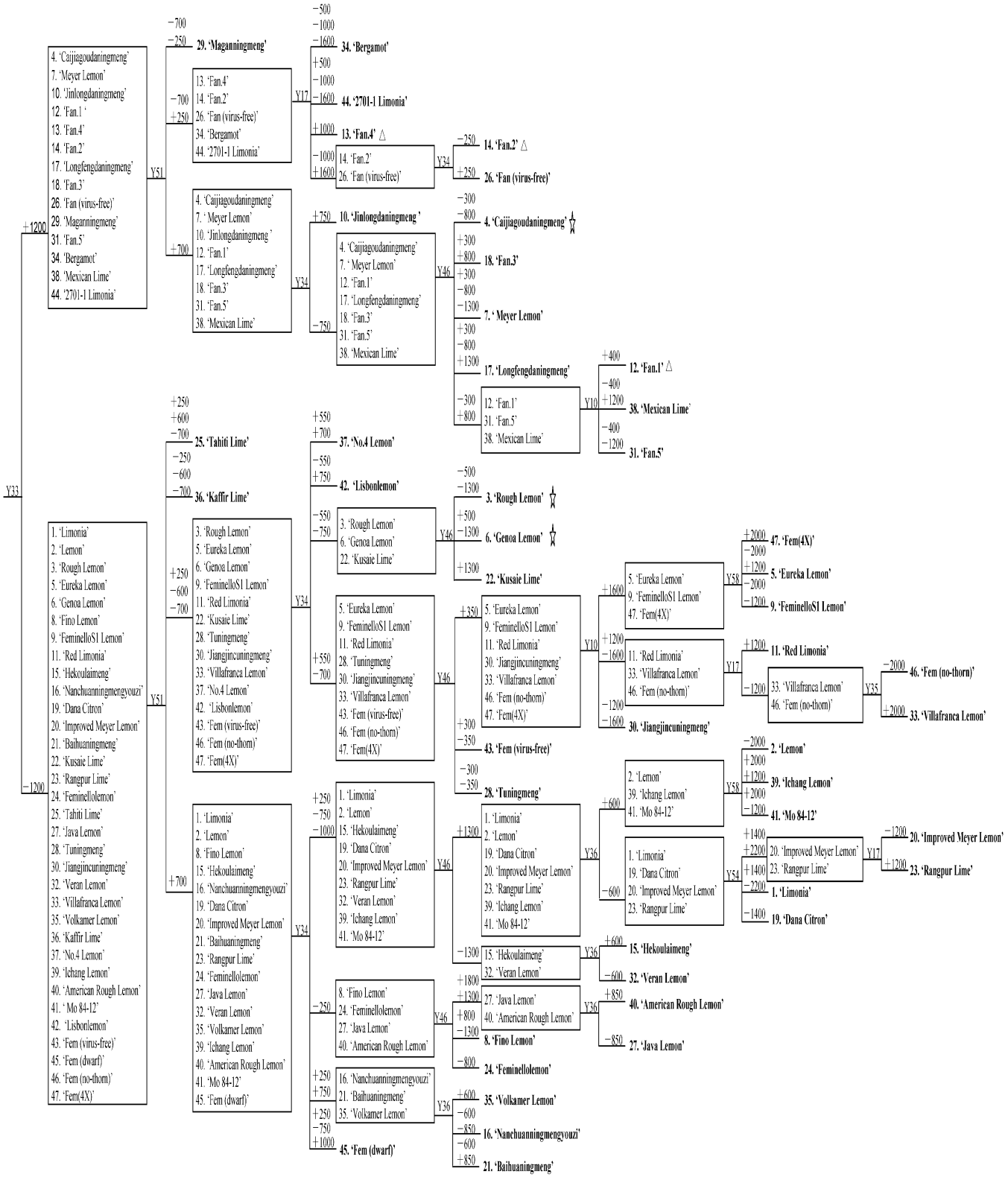

Figure 2. Classification of 47 lemon cultivars by the DNA fingerprints of 10 RAPD primers (Table 2). Note: The lane number in the figure mean the size of the band, units is bp. "+" mean have this band; "—" mean have not this band ; '☆' and '△' mean the cultivars was used for the validation of the workability of the cultivar identification diagram; the cultivars name in bold fonts mean those were separated.

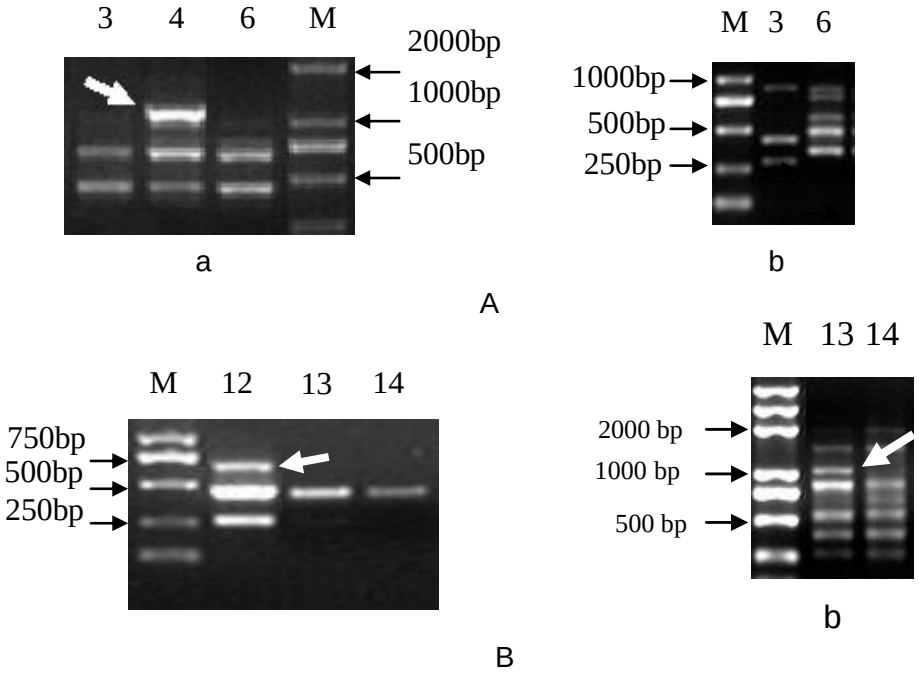

Figure 3. Verification result of cultivars selected randomly by the corresponding primers. The white horizontal arrows indicate the specific bands. The lane numbers correspond to the code in Table 1. M: DL2000 plus marker. “A” was the DNA banding patterns obtained with two primers used to separate the first group cultivars which marked are in Figure 2 by “☆”. “a” obtained with the primer Y33, “b” obtained with the primer Y46. “B” was the DNA banding patterns obtained with the primer Y51 used to separate the group “B” which was marked in Figure 2 by ‘△’. “a” obtained with the primer Y51 and “b” obtained with the primer Y17.

service lemon nursery industry and cultivar-right-protection. When you want to identify some lemon cultivars among those studied here, you can easily locate the primers and chose the target polymorphic PCR product on the CID for the further identification. Therefore, the verification of the utilization, workability and efficiency of the lemon CID was necessary, for which two groups of cultivars including group “A” which comprises ‘rough lemon’, ‘Caijiagoudaningmeng’ and ‘Genoa lemon’ and group “B” comprises ‘Fan.1’, ‘Fan.4’ and ‘Fan.2’ were randomly chosen from the inter-and intra-groups in the CID to be used for the verification. From the location of these cultivars in CID, it was easily to find the primers to be used to separate them.

Obviously, primers Y17, Y33, Y46, Y51 were those that could be used to separate the two groups of cultivars chosen: Y33 could be found to be the primer separating ‘Caijiagoudaningmeng’ out of three cultivars in group A, and Y46 was the other primer separating ‘rough lemon’ and ‘Genoa lemon’; Y51 could separate ‘Fan.1’ first and Y17 could separate the other two. The corresponding polymorphic bands to be used for the separation could also be found there. After the validation of the identification of the two groups of cultivars, the PCR results could definitely show the information as anticipated in that all cultivars in these two groups were disjoined as the result in CID. It was clear that primers Y33 and Y46 could separate the group “A” cultivars from the banding patterns shown in Figure 3A: ‘Caijiagoudaningmeng’ were first identified out of the three cultivars with the band of 1200 bp in size from primer Y33, respectively; ‘rough lemon’ and ‘Genoa lemon’ were separated with band about 500 bp from primer Y46. The group “B” cultivars including ‘Fan.1’, ‘Fan.4’ and ‘Fan.2’ could be separated with the primer of Y17 and Y51, of which the banding patterns were show as Figure 3B. This validation of the separation of the two randomly chosen groups of cultivars could not only indicate that this lemon CID strategy was definitely workable, efficient, and referable, and practicable, but could also show us how to use this CID for better service in the lemon industry and research on lemon genetic resources.

Other need to be mentioned is the data of this cultivar separation from this diagram can also be generated into database for future use *in silico*.

DISCUSSION

DNA markers are a type of powerful technique that has the potential and powerful utility in identification of plant cultivars and species. Though, several generations of DNA markers have been developed and used to cultivar identification, genetic analysis, and thousands of papers related have been published, it does not mean they have been easily used in genotyping. In fact, when we plan to use DNA markers to identify some plant varieties efficiently and easily in practice, the situation does not seem to be so. Till now, no efficient approach has been developed to use DNA markers easily in cultivar identification except that the phylogenetic trees or the fingerprints of several cultivars were employed to show the separation of plant samples of which the former used to be derived from cluster analysis. The new approach of CID we employed in this study can enable DNA markers to be more efficiently and practically utilized in distinguishing plant cultivars which seemed to be able to use the primers efficiently and could be easily operated. The CID diagram generated can be a very referable information for the lemon cultivar identification. This strategy can realize the power of DNA markers in plant cultivar identification, use the polymorphic bands better from each primer screened and gradually distinguish the individual samples, and chart the identification results informatively and clearly.

Although, the method does not accurately reflect the genetic relationship of the plant cultivars, theoretically, the first cultivar to be divided out of use to be farther genetically to the others and those later to be identified might be genetically closer. This method can do great help in plant cultivar identification for cultivar-right-protection, cultivar identification and early identification in nursery industry. China is a leading agricultural country in the world and has plenty of plant genetic resources which makes the differentiation of plant samples an important task. Lemon is also an important horticultural crop and has been cultivated in a large scale in Chongqing provinces of China in recent years. Despite its importance, little work has been reported on efficient cultivar identification and genetic diversity of lemon. At present, the phenomenon where a name might be used for various lemon cultivars or a lemon cultivar has different names in different production regions is quite common in China. Development of the lemon industry calls for related enhancement of lemon research and production. Therefore, scientific identification of lemon cultivars and germplasm resources is essential, since it can be of much help to genetic resource conservation and utilization as well as plant variety protection. In this study, only 10 RAPD primers could be enough to be employed to distinguish all the 47 lemon cultivars. It is very convenient and easily operated for the users. Although, single RAPD primer used to be unable to distinguish quite a number of lemon cultivars at the same time, the new CID strategy in this work could obviously make most of the polymorphic PCR bands in an efficient identification of the lemon cultivars which overcome the impossibility of the cluster analysis employed before in the plant identification.

The informative CID diagram (Figure 2) of the lemon cultivars is the key result that can tell us which primer or primers can be used to separate which lemon cultivars. Basically, any two cultivars can be identified with one RAPD primer. In practice, if more new lemon cultivars are released, the set of 10 primers can be used to run the DNA samples of the new cultivars and the PCR banding patterns can let us know where to position the new cultivars in the CID. If all the 10 primers can not disjoin the 47 original lemon cultivars with the new ones, some new primers should be screened and used to separate them and position them on the CID, from which the separation of new cultivars can generate a larger CID. It seems that not much work needs to do for the separation of one or several new cultivars. The verification of the workability and accuracy of the CID as the anticipated can confirm the practical importance of this lemon cultivar identification. We can believe that this separation of the lemon cultivars and the new strategy employed here can definitely be significant to the lemon industry in China. This study can initiate new work of efficient application of DNA markers even in the identification of other plant and seed samples which are important in the plant genetic germplasm conservation, cultivar-right-protection, provision of genetically uniform seedlings in production and seed industry. This CID plant cultivar identification showed some advantages in that fewer primers can be efficiently used, all cultivars included can be separated now and in the future easily by the PCR with the corresponding primers easily found on the diagram.

The CID information can be transferred to database *in silico* and made to be shared by the scientists and farmers all over the World. It is not just a simple diagram; it can make DNA markers more applicable for plant variety identification in practice. Now, we have initiated the same work on most important fruit crop cultivars cultivated in China for service to cultivar-right-protection, nursery industry and genetic resource conservation. We think this new method can be used to draw the CIDs for each organism species, and the CID generated can work as a chemical element periodic table, providing us the information for separating the cultivars you planed.

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