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Full Length Research Paper

A new strategy for complete identification of 69 grapevine cultivars using random amplified polymorphic DNA (RAPD) markers

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DNA marker is a useful technique that has immense power in plant cultivar identification, which used to be the first preferential utility of any newly developed DNA marker technique. However, DNA markers have not been utilized well in the practice of plant identification, for which the bottleneck is no analysis methods available that can make the identification of plants with DNA marker easy, efficient and practical. We developed a novel approach called cultivar-identification-diagram (CID) strategy that can facilitate the utilization of DNA marker in the separation of plant individuals in a much better, efficient, practical, and referable manner. A CID was manually constructed with an intention of providing the polymorphic maker generated from each polymerase chain reaction (PCR) for sample separation. In this study, a total of 69 important grape cultivars cultivated in China were successfully separated with random amplified polymorphic DNA (RAPD) marker through the CID analysis strategy, with only seven 11nt primers being employed. The utilization of the CID of these 69 grapevine cultivars was also verified by identification of two randomly chosen groups of cultivars among the 69. The main advantages of this identification strategy include fewer primers used, and separation of all the cultivars from each other by the corresponding primers marked in the right position on the CID. This grapevine CID can provide information to separate any grapevine cultivars among the 69 studied, which can definitely be of great help in grapevine cultivar identification for cultivar-right-protection and to the grapevine nursery industry in China.

Key words: Grapevine, cultivar identification, new strategy.

INTRODUCTION

The grapevine (*Vitis vinifera* L.) is one of the most economically important crop plants in the world. The primary centre of origin of grapevine is believed to be in some areas between the Caspian and Black Seas (Snyder, 1937), from where it has spread to other areas and is now cultivated in all temperate regions of the world. The ancient culture of grapevine, the vegetative way of propagation, and many breeding programs carried out in the World have led to the development of a large number of cultivars. In addition, the broad geographic expansion of grapevine culture has caused the problem of cultivars with synonyms (cultivars having more than one name) and homonyms (different cultivars mentioned under the same name).

In the last few decades, it has become imperative to find ways of handling the large repertoire of grapevine germplasm and proper identification of the different cultivars. Among the techniques, classical approaches were used to identify cultivars, which were based on morphological, physiological and agronomic traits. However, these traits have limitations as they can be easily influenced by the environment and need extensive observation of mature plants. On the other hand, molecular markers have the unique advantage of not being affected by the environment and can provide a powerful

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tool for proper characterization of cultivars. Though DNA based molecular markers have been utilized in genetic studies, cultivar characterization and identification of grapevine (Bourquin et al., 1992; Fanizza et al., 2000), and the studies could provide the information of genetic diversity levels and the separation of the plant individuals studied, no single report could identify a large number of grapevine cultivars and gave a result for the cultivar identification that is referable now and even in the future. Lack of a method that can give a referable result is the main drawback to practical utilization of DNA marker in plant cultivar identification. The main reason for this situation is that the analysis strategies of DNA fingerprints could not generate some referable information that could readily tell the primer and polymorphic marker to use in separating the cultivars that need to be identified. It is clear that the popular analysis techniques for DNA banding patterns known as cluster analyses cannot efficiently separate cultivars or species. Employing a strategy that can make the verification of grapevine cultivars reliable, easy, referable and practical is very crucial for the grapevine nursery and farming industry, cultivar patent protection, and genetic resource conservation and evaluation.

In recent years, various DNA-based markers have been developed and used for genetic diversity, fingerprinting and cultivar origin studies (Cheng et al., 2009; D'Onofrio et al., 2009; Elidemir et al., 2009; Fang et al. 2006; Melgarejo et al., 2009; Papp et al., 2010). Among the DNA-based markers, random amplified polymorphic DNA (RAPD) (William et al., 1990) marker is useful for cultivar analysis with the advantages of simplicity, efficiency, and non-requirement of any previous sequence information. If optimization of the RAPD technique is done by choosing 11 nt primers and strict screening polymerase chain reaction (PCR) annealing temperature for each primer before RAPD is employed in fingerprinting plants, RAPD can become a preferred technique for use in plant cultivar identification. So far, RAPD marker have been widely 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). Despite their popularity, the powerful DNA markers available for plant identification have not made plant variety identification an efficient, recordable, and easy exercise as anticipated. This presents us with a really awkward situation. This is our moot point to find an alternative.

In this study, we employed a newly developed analysis strategy that can make the identification of many grape cultivars a practical, efficient, recordable, and referable work, in which a cultivar identification diagram (CID) generated from the RAPD banding patterns was constructed manually. The invention of this strategy was to show the polymorphic bands used to separate the cultivars on the CID, which was different from the results from the popular cluster analysis of computer work. The CID showing the separation of 69 grape cultivars can definitely be of valuable service to the grape industry in China.

MATERIALS AND METHODS

Plant materials

Young leaves of 69 important grapevine cultivars grown in China were collected from the Institute of Pomology, Jiangsu Academy of Agricultural Sciences, Nanjing- China. The names and origins of these cultivars are shown in Table 1.

Genomic DNA extraction

Total genomic DNA of each genotype was extracted from young grapevine 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} with1xTE buffer and stored at -20°C pending use.

RAPD analysis

In case of RAPD reactions, 54 random primers, synthesized by the company Generay Biotechnology in Shanghai, China, 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 for screening in this study. In order to increase credibility of the fragments, we used only those primers resulting in clear unambiguous banding patterns. As a result, 7 primers (Table 2) that showed well-resolved and reproducible bands were selected to assay all genotypes, while the others were discarded. Reaction solutions consisted of 2.0 µl (10 x 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) (10 x buffer, $MgCl_2$, dNTP and rTaq (TaKaRa, Japan) 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 modifications. 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 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 1xTAE (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 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.

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.

Table 1. Name and origin of the grapevine materials used in the experiment.

No.	Cultivar	Origin	No.	Cultivar	Origin
1	'Tano Red'	Japan	36	'Xiang fei'	China
2	'Xi yang hong'	China	37	'Ruby Okuyama'	Brazil
3	'Ji xiang'	China	38	'Ruby Seedless'	USA
4	'Xin nong le'	Japan	39	'Delaware(4×)'	Japan
5	'Ju mei gui'	China	40	'Da mei gui xiang'	China
6	'Yong you 1 hao'	China	41	'Autumn Black'	USA
7	'Feng hou'	China	42	'Yotomi Rosa'	Japan
8	'Da li liu yue zi'	China	43	'White Malaga'	※
9	'Campbell'	USA	44	'Italia'	Italy
10	'Hojyu'	Japan	45	'Bolgar'	Turkey
11	'Kyohō'	Japan	46	'CentennialSeedless'	Turkey
12	'Ryūhō'	Japan	47	'Red Globe'	USA
13	'Benizuihō'	Japan	48	'Yang ge er'	Russia
14	'Zui jin xiang'	China	49	'Manicure Finger'	Japan
15	'Summer Black'	Japan	50	'Queen of Vineyard'	Hungary
16	'Takasumi'	Japan	51	'Christmas Rose'	USA
17	'Gui xiang yi'	China	52	'Rizamat'	Russia
18	'Honey Red'	Japan	53	'Huang jin xiang'	Japan
19	'Jing feng wu he'	※	54	'Jing yu'	China
20	'Irsay Oliver'	Hungary	55	'Xin ma te'	Jepan
21	'Green Mountain'	USA	56	'Fantasy Seedless'	USA
22	'New-Muscat'	Jepan	57	'Superior Seedless'	USA
23	'Hei gui xiang'	China	58	'Saperavi'	Georgia
24	'Na duo er'	Hungary	59	'Hong ji xin'	China
25	'Ji la er'	※	60	'Victoria'	Roumania
26	'R775'	※	61	'87- 1'	China
27	'Felekey'	※	62	'Jing zao jing'	China
28	'Lzunishiki'	Japan	63	'Peala of Csaba'	Hungary
29	'Blck Rose'	USA	64	'Ju xing'	China
30	'Hong mu na ge'	China	65	'Jing xiu'	China
31	'Pannuoniya'	※	66	'Jing ya'	China
32	'Melissa Seedless'	USA	67	'8611wu he zao hong'	China
33	'Otilia'	Roumania	68	'Himrod Seedless'	USA
34	'Yello Italia'	Italy	69	'Venus Seedless'	USA
35	'Ke la ba ma ke'	※			

"※" unknown origin.

Following this criteria, all the grape cultivars were gradually completely separated from each other as more primers were employed.

Test of utilization and workability of the diagram in cultivar identification

Two groups of grape 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 69 grape cultivars. The two groups were marked with "A", "B", respectively, and the corresponding primers to be used for the separation of each group could easily be searched and picked out from the CID. If these randomly chosen cultivars could be distinguished accurately and quickly as anticipated based on the whole CID, we would definitely assure that the strategy developed and employed in this study was scientific, workable, and efficient, making it to be probably the best way to employ molecular marker in the identification of fruit crop cultivars and field and vegetable crop seed samples. The data of the cultivar separation from this diagram can also be generated into a readily referable database.

RESULTS

Cultivar identification

To establish a stable and optimistic RAPD system with

Table 2. Seven primers chosen for further fingerprinting of 69 grape genotypes.

Primer	Nucleotide sequence (5'–3')	Anneal temperature
Y-3	GTTTCGCTCCA	39.2
Y-5	GTTTCGCTCCG	44.8
Y-23	GGACCCAACCG	42.8
Y-41	AGCGTCCTCCG	43.7
Y-46	ACGACCGACAT	42.8
Y-51	TGGTGGCGTTA	44.8
Y-60	ACCCCCGACTC	41.7

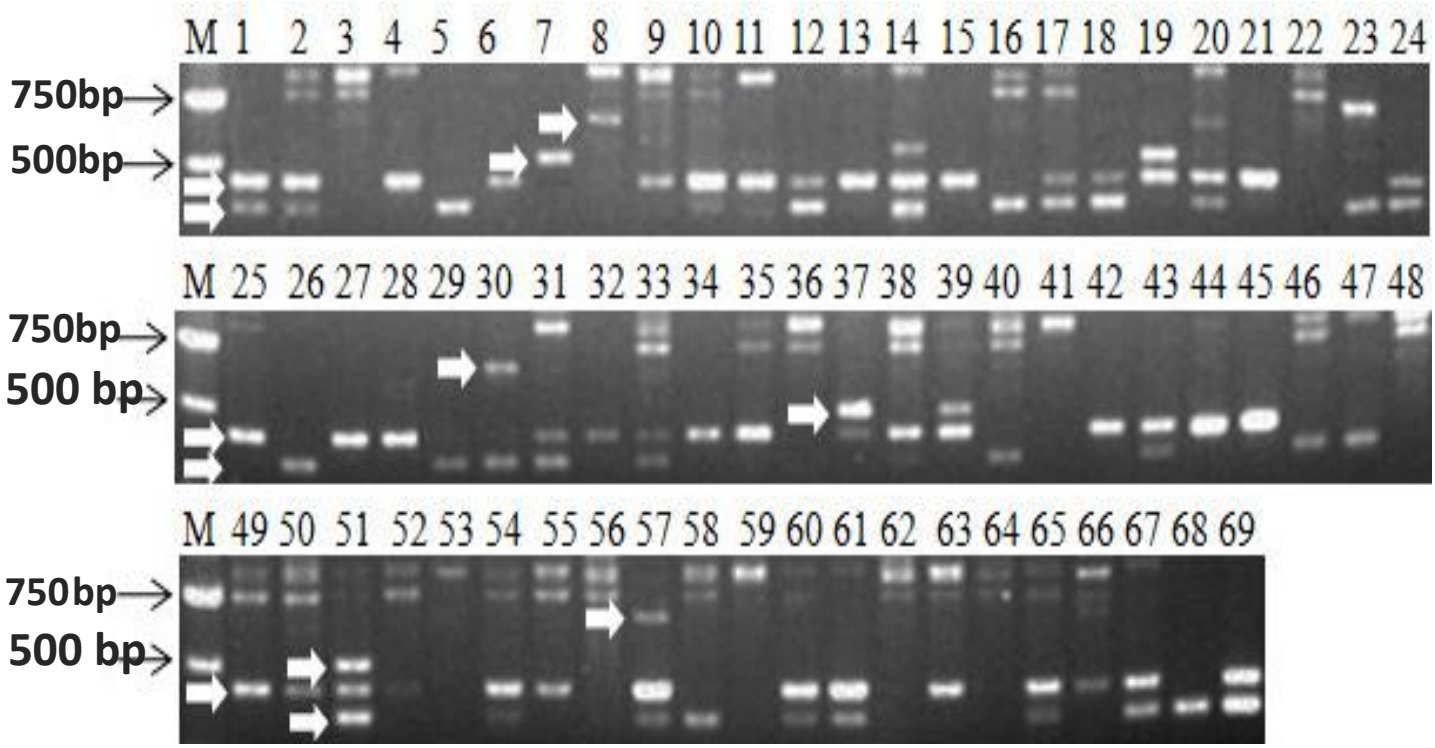

Figure 1. RAPD banding patterns of 69 genotypes within the genus *V. vinifera* obtained with primer Y5. The white horizontal arrows indicate the specific bands. The lane numbers correspond to the cultivar code in Table 1. M: DL2000 plus marker.

high reproducibility, one nucleotide longer random primers (11 nt) were employed and the annealing temperatures for each primer were screened based on the quality and reproducibility of the banding pattern. These primers were randomly screened from a stock of 54 11-nt primers, and once an optimistic primer that could produce reproducible and clear fingerprints with polymorphic bands was screened, it was further utilized in the identification of grape cultivars.

After 7 primers (Table 2) were screened out and utilized, all the 69 grape cultivars could be successfully identified. An example of the RAPD patterns generated with primer Y5, used as the initial pri-mer in separating the 69 grapevine cultivars is as shown in Figure 1. Primer Y5 could divide all the 69 grapevine cultivars into 10 subgroups with PCR bands of different sizes as shown in Figure 1. Three cultivars namely 'Feng hou', 'Da li liu yue zi' and 'Superior Seedless' were sepa-rated into single-cultivar groups indicating they could be separated out of all the other cultivars at this initial step. Following this cultivar identification procedure the remaining 6 primers (Table 2) were step by step screened and chosen to differentiate the grapevine cultivars, eventually leading to complete separation of the 69 cultivars shown in Figure 2. For easy reading of the CID, all the names of separated grapevine cultivars were written in bold font. It should be emphasized that only the clear polymorphic bands generated from each primer were used to

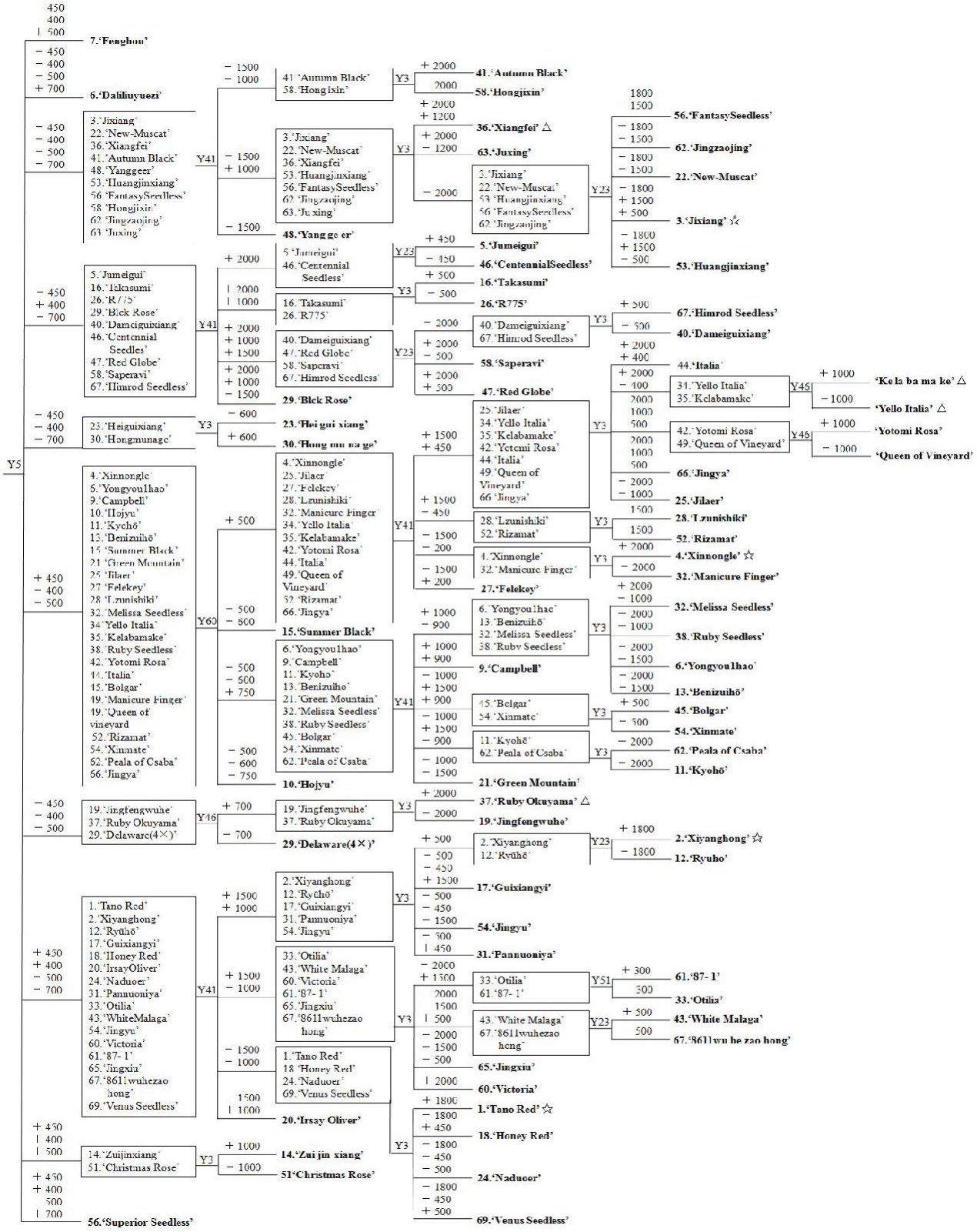

Figure 2. Classification of 69 grape cultivars by the DNA fingerprints of 7 RAPD primers (Table 2).The lane number in the figure mean the size of the band, units is bp. "+"mean have this band; "—" mean lack this band; '☆'and '△'mean the cultivars were used for validation of workability of the cultivar identification diagram; The cultivar names in Bold fonts mean those was separated.

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 Fig. 2 can make the CID diagram very useful and referable in practical grapevine cultivar identification services.

Test of the utilization and workability of the diagram in cultivar identification

Although an important aim of this study was to find a technique of utilizing RAPD marker to distinguish 69 grapevine cultivars, a much more interesting and important purpose was to generate a referable CID of grapevine cultivars with the invention of presenting the information of the polymorphic markers used to separate the grapevine cultivars on the CID, thus making the identification of these grapevine cultivars practical and easy work. Compared to our method, most of the earlier reports focused on the genetic analysis and presence of some phylogenetic trees without referable information for practical plant sample identification. Our finding can definitely benefit and service the grapevine nursery industry and facilitate cultivar-right-protection.

To identify some grapevine cultivars among those studied here, one can easily locate the primers and chose the target polymorphic PCR product on the CID for further identification. To confirm this ease, verification of utilization, workability and efficiency of the grapevine CID was necessary. For this, two groups of cultivars namely group “A” which comprises ‘Tano Red’ ‘Xiyanghong’ ‘Jixiang’ and ‘Xinnongle’ and group “B” comprising of ‘Yello Italia’ ‘Ke la ba ma ke’ ‘Xiang fei’ and ‘Ruby Okuyama’ were randomly chosen from the inter-and intra-groups in the CID and used for the verification. From the location of these cultivars in CID, it was easy to find the primers to use in separating them. Evidently, primers Y5, Y41 and Y46 could be used to separate the two chosen groups of cultivars, with primer Y5 separating ‘Jixiang’ and ‘Xinnongle’ out of four cultivars in group A, while primer Y41 separated the other two cultivars ‘Tano Red’ and ‘Xiyanghong’. Y5 could also separate ‘Xiang fei’ and ‘Ruby Okuyama’ out first and Y46 could separate the other two. The corresponding polymorphic bands to be used for the separation could also be found there. After the validation of 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 separated just as the result in CID. It was clear that primers Y5 and Y41 could separate the group “A” cultivars from the banding patterns as shown in Figure 3A: ‘Jixiang’ and ‘Xinnongle’ were each first identified out of the four cultivars by the 450 and 400 bp bands from primer Y5, respectively; ‘Tano Red’ and ‘Xiyanghong’ were separated by a band about 1500 bp from primer Y41. The group “B” cultivars, com-prising‘Yello Italia’, ‘Ke la ba ma ke’, ‘Xiang fei’, and ‘Ruby Okuyama’, could also be separated with the primers Y5 and Y46, where the banding patterns are shown in Figure 3B. This validation of the separation of the two randomly chosen groups of cultivars not only indicates that this grapevine CID strategy was clearly workable, efficient, referable and practicable, but could also show us how to use this CID for better service in the grape industry and research on grapevine genetic resources. It is also worth mentioning that the data on cultivar separation from this diagram can also be generated into database for future use *in silico*.

DISCUSSION

DNA markers are a powerful technique that has a potentially powerful application in identification of plant cultivars and species. Though several generations of DNA markers have been developed and used in cultivar identification and genetic analysis, as well as thousands of related papers published on the subject, it does not imply DNA markers have been easily used in genotyping which can service the identification of the cultivars. Usually, attempts to use DNA markers to identify some plant varieties efficiently and easily in practice have always been proved futile. Till now, no efficient approach have been developed to use DNA markers easily in cultivar identification except where the phylogenetic trees or fingerprints of several cultivars were employed to show the separation of plant samples, of which the phylogenetic trees being derived from cluster analysis cannot tell us how to use them to separate the plant samples and the fingerprints cannot work well in the identification of many plant individuals. The new approach of CID employed in this study can enable DNA markers to be utilized more efficiently and practically in distinguishing plant cultivars, as the information contained in the generated CID diagram can be referred to for plant cultivar identification. Even though the key invention of this new strategy is just to connect the polymorphic markers closely with the separation of some cultivars at each step, this strategy can utilize 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, in theory the first cultivar to be divided out could be farther genetically from the other cultivars while those identified later might be genetically closer. This method can be quite helpful in plant cultivar identification for cultivar-right-protection, cultivar identification, and early identification in the nursery industry.

China is an important agricultural country in the World and has plenty of plant resources, thus making the differentiation of plant samples an important and urgent

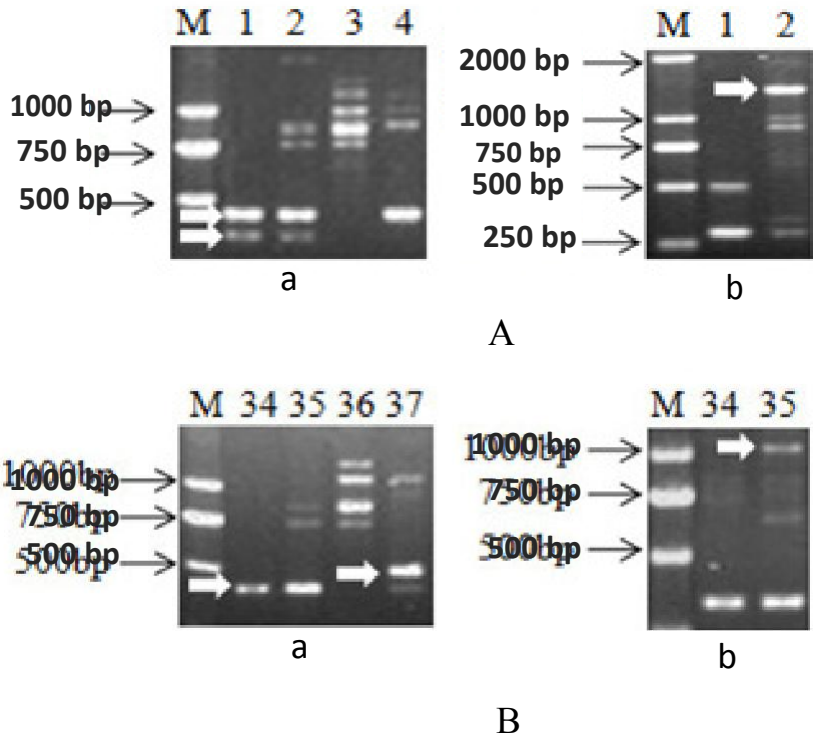

Figure 3. Verification result of cultivars selected randomly by the corresponding primers. The white horizontal arrows indicate the specific bands. The lane numbers correspond to the code in Table 1. M: DL2000 plus marker. “A” was the DNA banding patterns obtained with two primers used to separate the first group cultivars which are marked in Figure 2 by “☆”. “a” obtained with the primer Y5, “b” obtained with the primerY41. “B” is DNA banding patterns obtained with the primer Y5 used to separate the group “B” which was marked in Figure 2 by ‘△’. “a” obtained with the primer Y5, “b” obtained with the primer Y46.

task. Grapevine is an ancient horticultural plant and is widely cultivated in most countries of the World. Its berries have been used for wine and juice production, as table grapes, raisins, and more recently for leaf, seed, and skin extracts by the nutritional and cosmetic industries (Iriti et al., 2006; Monagas et al., 2006). At present, the phenomenon that a name might be used by various grape cultivars or a cultivar has different names in different production regions often happens in China. It is therefore fundamental to identify grapevine cultivars for conservation and research on genetic resources, nursery industry as well as plant variety protection.

In this study, only 7 RAPD primers were sufficient to distinguish all the 69 grape cultivars using the developed technique. It is very convenient and easy to operate. Usually, a single RAPD primer could not simultaneously distinguish quite a number of grapevine cultivars. However, the new CID strategy employed in this work could obviously make the most of the polymorphic PCR bands for efficient identification of the grapevine cultivars, which in turn overcomes the drawbacks of the cluster analysis employed earlier in plant identification. The informative CID diagram (Figure 2) of the grape cultivars is the key result that can tell us which primer or primers can be used to separate which grape cultivars. Basically, any two cultivars can be identified with one RAPD primer. In practice, if more new grapevine cultivars are released, the set of 7 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 7 primers can not disjoin the 69 original grapevine cultivars with the new cultivars to be identified, some new primers should be screened and used to separate and position the new cultivars on the CID, with separation of new cultivars generating a larger CID. It seems that not much work is needed for the separation of one or several new cultivars. The verification of the workability and accuracy of the CID as anticipated can confirm the practical importance of this grapevine cultivar identification. We believe that this separation of grapevine cultivars and the new strategy employed here can definitely be significant to the grape industry in China.

This study can initiate new work on efficient application of DNA markers even in the identification of other plant

and seed samples, which are important in plant genetic germplasm conservation, cultivar-right-protection, provision of genetically uniform seedlings in production, and the seed industry. This CID plant cultivar identification exhibited advantages where fewer primers can be efficiently used and all cultivars included can be separated now and in the future easily by PCR with the corresponding primers easily found on the diagram. The CID information can be transferred to database *in silico* and made available to 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. Currently, 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 also postulate that this new method can be used to draw the CIDs for each organism species, and the CID generated can work in the same way as a chemical element in periodic table does, providing us with ready information for separating the cultivars or varieties as desired.

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