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Full Length Research Paper

Employment of a new strategy for identification of *Prunus mume* cultivars using random amplified polymorphic deoxyribonucleic acid (RAPD) markers

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In this study, we applied a new approach of using random amplified polymorphic deoxyribonucleic acid (RAPD) fingerprints to distinguish 64 fruiting mei cultivars based on optimization of RAPD through choosing 11 nt primers and strict screening polymerase chain reaction (PCR) annealing temperature. Results show that this new approach could clearly utilize and record the fingerprints generated from various primers in cultivar identification and a cultivar identification diagram (CID) readily constructed. The CID can make mei cultivar identification efficient just like a periodic table of elements, providing the information needed to separate groups of cultivars as desired. The workability and efficiency of the method were also well verified. To our best knowledge, this new strategy is the most efficient and workable in indentifying plant varieties using deoxyribonucleic acid (DNA) markers, which can be of great help in plant cultivar identification for protection of cultivar rights and for early identification of seedlings in the nursery industry.

Key words: *Prunus mume*, new approach, random amplified polymorphic deoxyribonucleic acid (RAPD), fingerprints, cultivar identification diagram (CID).

INTRODUCTION

Fruiting mei (*Prunus mume* Sieb.et Zucc.) belongs to the sub-family Prunoideae within the larger Rosaceae family and originated in China, it is an important fruit crop in many east Asian countries. In China, fruiting mei has been cultivated for thousands years, and has rich germplasm resources with high-quality cultivars playing a crucial role in mei fruit production. Over the past few decades, cultivation of fruiting mei is proceeded by field seedling propagation. Owing to its natural cross, the genetic background of existing resources and cultivated varieties are quite complex (Chu, 1999). With the development of modern scientific breeding, the parent materials for fruiting mei breeding were progressively more concentrated on fewer superior varieties or strains, which has made varieties selected by man to have quite similar agronomic traits and subsequently making them difficult to distinguish. In addition, homonymity and heteronymity are very common problems in the collection and preservation of fruiting mei germplasm, as they are bothersome in the collection of germplasm resources and their utilization, breeding of new varieties and in the study on genetic backgrounds. This has become a very critical issue that is frequently encountered and thus calls for a way to ensure the accuracy and purity of varieties in scientific research and production. Accurate and rapid identification of fruiting mei varieties is therefore an essential and desirable work for breeders, commercializing companies and for further research.

Traditional approaches for fruiting mei cultivar

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Abbreviations: CID, Cultivar identification diagram; **DNA,** deoxyribonucleic acid; **RAPD,** random amplified polymorphic deoxyribonucleic acid; **PCR**, polymerase chain reaction; **SDS,** sodium dodecyl sulfate; **UV,** ultra violet; **AFLP,** amplified fragment length polymorphism; **SSR,** simple sequence repeat; **SNP,** single nucleotide polymorphism.

identification, such as morphological, palynological, cytological, isozyme etc, have been proven to be limited by low information, difficult operability, low stability and reproducibility, ease of environmental influence, and the need for extensive observations in mature plants. Deoxyribonucleic acid (DNA) based fingerprinting markers have overcome these limitations and can provide a powerful tool for proper characterization of cultivars. In recent years, various DNA molecular markers have been developed and used for the studies on genetic diversity, fingerprinting patterns and origins of the cultivars (Cheng et al., 2009; D'Onofrio et al., 2009; Elidemir et al., 2009; Melgarejo et al. 2009; Papp et al., 2010). Among the available markers, random amplified polymorphic DNA (RAPD) (William et al., 1990) markers seemed to be very useful in plant cultivar identification analysis with superb advantages in simplicity, efficiency, relative ease of performing and non-requirement of any previous sequence information. So far, RAPD markers have been used in the cultivar identification of many fruit species, such as apricot (Ercisli et al., 2009), banana (Gubbuk et al., 2004), pomegranate (Hasnaoui et al., 2010), pear (Schiliro et al., 2001), pistachio (Javanshah et al., 2007; Wang et al., 2010), litchi (Gao et al., 2006), strawberry (Wang et al., 2007) and genetic variation studies.

Even though DNA markers are advantageous, in practice there is no tight link between the marker information and concrete plant varieties, and the markers have not made fruit crop variety identification an efficient, recordable and straightforward task. The main points in this situation are the strategies for analysis of DNA fingerprints which in turn have made utilization of DNA marker in crop and seed identification to fall out of favor in practice. The need to employ new strategies for application of new knowledge and technology to practical agricultural activities is therefore both significant and necessary.

Compared to other main fruit crops, fruiting mei has not been accorded much attention from geneticists and molecular biologists. The available reports about fruiting mei cultivar identification using molecular markers are limited (Shangguan et al., 2009) and mainly employed statistical techniques known as cluster analysis to analyze the banding patterns. However, their results were inadequate to make cultivar identification an easy and referable work, even though they could give the genetic diversity levels and separate the individual plants in the analysis. Employing a strategy that can make optimal use of the advantages of DNA markers for easy identification of fruiting mei cultivars, which is necessary for fruiting-mei nursery and production industry.

It has been established that RAPD could become a preferred technique for use in plant cultivar identification following optimization by choosing 11 nt primers and strict screening of polymerase chain reaction (PCR) annealing temperature for each primer (Li et al., 2010). In this study, we applied a new strategy and successfully identified sixty-four cultivars of fruiting mei using RAPD markers.

This identification could generate a cultivar identification diagram (CID), which can work in the same way as a chemical element periodic table does, providing us with ready information for separating fruiting mei cultivars or varieties as desired. We postulate that the CID strategy can make the identification of more fruiting mei cultivars a practical, efficient, recordable, and referable work and provide a good method for identification in fruiting-mei nursery and production industry in the future.

MATERIALS AND METHODS

Plant materials

Leaf samples of 64 important fruiting mei cultivars (Table 1) were collected from the mei germplasm collection at the Jiangpu Agricultural Research Station, Nanjing Agricultural University, Nanjing, P. R. China. Their background information had previously been documented by Chen (1996) and Chu (1999). The RAPD primers were synthesized by Shanghai Invitrogen Biotechnology Company.

DNA isolation

Young fresh leaves of 64 cultivars (Table 1) were collected for DNA extraction and flash frozen in liquid nitrogen. Each leaf sample was then grinded in liquid nitrogen and the powder stored at -40°C until use. Genomic DNA was isolated from leaves according to the modified sodium dodecyl sulfate (SDS) method (Lin et al., 2001) then purified and checked for quality by electrophoresis on a 0.7% agarose gel. The concentrations and purity were quantified using a Bio-Photometer (Eppendorf). DNA was diluted to 40-70 ng/μL and stored at 40°C until used.

Gradient screening and primer selection

Annealing temperature is a key element of conventional PCR techniques, to ensure high repeatability and stability of RAPD technology, it is essential to perform gradient filter of annealing temperature for different primers. PCR was then performed according to the method of Yu et al. (2009), where the specific reactions were as follows: 10 × Buffer 1.5 μL, 2.5 mmol/L of dNTPs 1.2 μL (Takara Biotechnology Dalian, Co., Ltd., China) 25 mmol/L of Mg^{2+} 0.8μL , 10 pmol/μL primer 0.6 μL, 50 ng/μL template DNA 1μL, 5 U/μL of DNA polymerase 0.08 Ml (Takara Biotechnology Dalian, Co., Ltd., China), then ddH_2O added to a total volume of 15 μL. Amplification reactions were performed in an Eppendorf ™ Thermal cycler, under the following the program: initial denaturation at 94°C for 5 min; 40 cycles with 94°C 30 s, 35~45°C 1 min, 72°C for 2 min, and a final temperature of 72°C under an extension of 10 min. In order to ensure the quality and integrity of PCR products, primers with clear and highly repeated bands were selected based on three consecutive gradients, and also the higher temperature chosen for the appropriate selection.

RAPD amplification

The PCR reaction reagents composed of 2.5 μL 10 × PCR buffer, 2.5 mmol/L Mg^{2+}, 2.5 mmol/L dNTPs, 2.0 U *Taq* polymerase, 50-80 ng DNA template, 10 pmol random primers, and ddH_2O to a final

Table 1. Cultivar name and origin of fruiting mei used in this study.

No.	Cultivar name	Origin	No.	Cultivar name	Origin
1	Koushuu Koume	Japan	33	Taihu3	Jiangsu
2	Zaohuamei	Jiangsu	34	Ruantiaohongmei	Zhejiang
3	Lv'emei	Jiangsu	35	Changnong17	Zhejiang
4	Qijiangxingmei	Sichuan	36	Longyan	Fujian
5	Shuangtaomei	Yunnan	37	Dongshanlimei	Jiangsu
6	Sichuanbaimei	Sichuan	38	Shuangshuodarou	Jiangsu
7	Zhizhimei	Zhejiang	39	Zaohong	Zhejiang
8	Yeliqing	Zhejiang	40	Siyuemei	Hunan
9	Nanhong	Jiangsu	41	Gessekai	Japan
10	Yanhua	Jiangsu	42	Setsudaume	Japan
11	Daqiandi	Jiangsu	43	Shirokaga	Japan
12	Qixingmei	Jiangsu	44	Tougorou	Japan
13	Lizimei	Jiangsu	45	Zonghong	Zhejiang
14	Dayezhugan	Zhejiang	46	Xiyeqing	Zhejiang
15	Gyokuei	Japan	47	Huangxiaoda	Zhejiang
16	Wanhong	Zhejiang	48	Hanakami	Japan
17	Hongmei	Jiangsu	49	Xiaoqingmei	Jiangsu
18	Sichuanghuangmei	Sichuan	50	Jiuzhongmei	Jiangsu
19	Yanzhimei	Fujian	51	Hangzhoubaimei	Zhejiang
20	Daroumei	Fujian	52	Taihu1	Jiangsu
21	Danfenghou	Jiangsu	53	Yourou	Japan
22	Dongqing	Zhejiang	54	Sichuanqingmei	Sichuan
23	Weishangzhong	Zhejiang	55	Koume	Japan
24	Oushuku	Japan	56	Tonglv	Sichuan
25	Henghe	Guangdong	57	Nankou	Japan
26	Xianmimei	Hunan	58	Dalizhong	Guangdong
27	Xiao'ougongfen	Jiangsu	59	Hongding	Zhejiang
28	Shinoume	Japan	60	Yinafenghou	Jiangsu
29	Taoxingmei	Fujian	61	Yunnanzhaoshui	Yunan
30	Momei	Zhejiang	62	Guangdonghaungpi	Guangdong
31	Koushuu Saisyou	Japan	63	Bungo	Japan
32	Gojirou	Japan	64	Pinzimei	Yunan

volume of 25 µL. The amplification reactions were performed following the same steps as mentioned above with each reaction being repeated three times. The amplification products were separated on a 1.5% agarose gel with ethidium bromide (100 mg mL-1), in 1 × TAE buffer for 1 h at 100 V and photographed under ultra violet (UV) light.

Data collection

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 further distinguish the cultivars in each group. As more primers were used, more specific amplified bands were generated and could differentiate all the cultivars separately. Afterwards, the CID, comprising of bands with specific sizes 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.

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

Two groups of cultivars were randomly selected and used to verify the accuracy of this method. The specific primers that could amplify the polymorphic fragments to be used in separating the cultivars could be found easily on the diagram. If the cultivars could be well distinguished as anticipated, this would definitely assure the workability and efficiency of this new approach in the cultivar identification not only for the present work but also for similar work in the future. The PCR reaction was set as earlier described above.

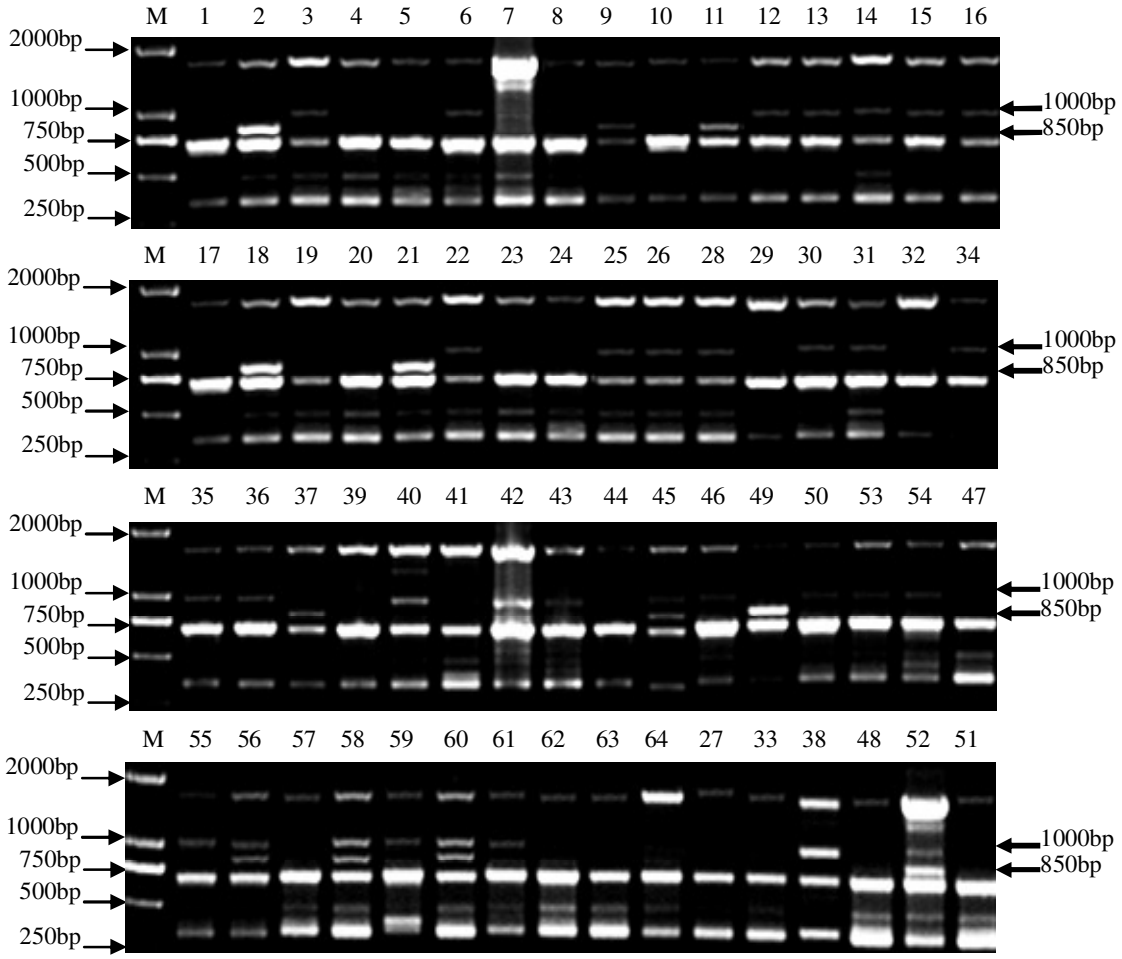

Figure 1. DNA banding patterns of 64 fruiting mei cultivars amplified by primer Y10. M: DL2000 plus DNA ladders; 1-64, accession numbers of fruiting mei cultivars listed in Table 1, same as those in the following Figures.

RESULTS

Optimization of RAPD technology system for fruiting mei

To establish a stable and optimistic RAPD system with high reproducibility, longer primers (11 nt) were employed and annealing temperatures for each primer were screened based on the quality and reproducibility of banding patterns. These two aspects were the key factors influencing the stability of PCR. The primers were randomly screened from a stock of 80 11-mei primers, and once a positive primer that could produce clear, reproducible polymorphic bands was screened, it was utilized further in the identification of fruiting mei cultivars. The fingerprints from the primers screened were polymorphic and stable, with the PCR product size in a range of 200 bp to 3,000 bp (Figures 1 and 2), indicating reliability of the optimized RAPD PCR systems and the fingerprints generated.

Cultivar identification

In cultivar identification, cultivars having a specific band in the fingerprint generated from one primer could be separated singly, while those cultivars sharing a matching banding pattern were separated into the same sub-group. Based on this, all the fruiting-mei cultivars were gradually and completely separated from each other as more and more primers were employed. After the 14th primer (Table 2) was screened and utilized, all the cultivars could be successfully identified.

Of the 14 primers used, primer Y10, whose PCR patterns are shown in Figure 1, was the first to be screened and used in the identification of all the 64 cultivars. The polymorphic bands with sizes of 850 bp and 1000 bp were chosen to separate the 64 cultivars, and the presence and/or absence of the 2 bands could classify these cultivars into 4 groups as shown in the diagram (Figure 1). The first group including 7 fruiting mei cultivars

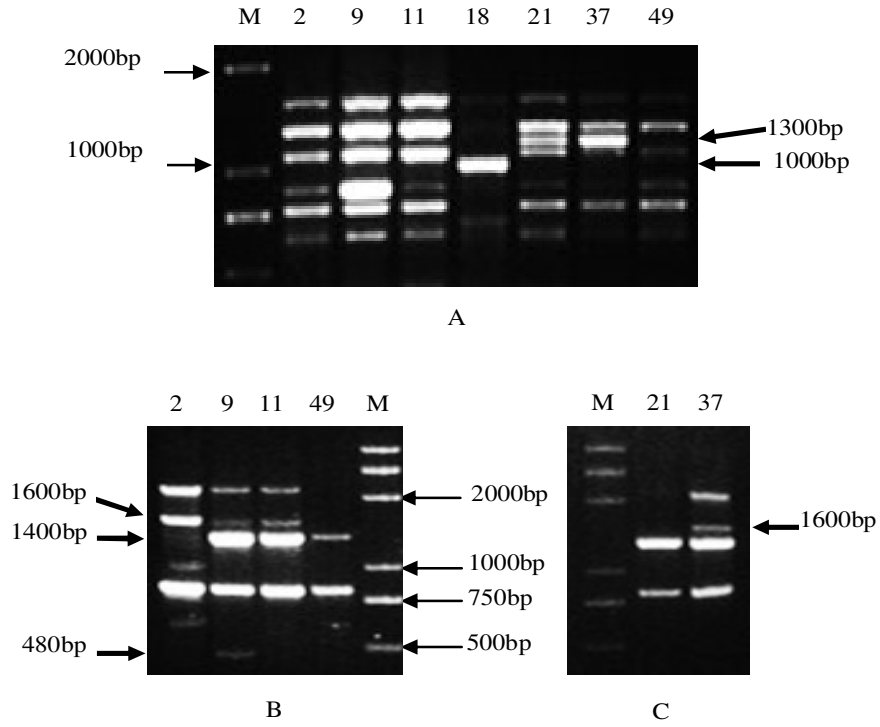

Figure 2. DNA banding patterns of 7 fruiting mei cultivars in the first group **A** from the separation by primer Y40 and two sub-groups **B, C** separated by primer Y29. M: DL2000 plus DNA ladders. The bands in different size as annotated were the specific ones used in separating the fruiting mei cultivars.

was picked out by the presence of an 850 bp band, and could be further separated into 3 subgroups based on the presence or/and absence of 1,000 bp and 1,300 bp bands amplified by primer Y40 (Figure 2A). Of the 3 subgroups, the cultivar 'Sichuanhuangmei' was first identified as a single-cultivar group by the presence of the 1,000 bp PCR product. The 4 cultivars ('Nanhong', 'Xiaoqingmei', 'Zaohuamei', 'Daqiandi') in the 2nd subgroup were distinguished by the absence of both the 1,000 bp and 1,300 bp bands, then further identified by polymorphic bands of 1,600 bp, 1,400 bp, 480 bp from primer Y29 as shown in the diagram (Figure 2B). The members of the 3rd subgroup containing 'Danfenghou' and 'Dongshanlimei' were differentiated well by the 1,600 bp polymorphic band also amplified by Y29 (Figure 2C). The result indicates that the 7 fruiting mei cultivars in the first group from the separation by primer Y10 were successfully identified. There was an intimate connection between the specific bands, primers used, and the cultivars identified, which is important information that could be utilized in cultivar identification of these 7 cultivars even in future. Similarly, the other 3 groups of cultivars from the separation by primer Y10 were fully separated by several primers, and the related information was included in Figure 3. Eventually, all the 64 fruiting cultivars were successfully identified by the joint use of 14 different primers, and the flow diagram of the amplification would make the identification of these 64 cultivars an efficient, referable, and easy task in the fruiting mei industry, which is supported by the close connection observed between the specific bands, primers used, and the cultivars identified. Our strategy could definitely realize the power of DNA markers in plant identification using DNA markers, whereby the identification result is also more readable and recordable than the plant identification work reported previously.

Verification of the cultivar identification results and workability of the CID

This work did not just aim at generating a diagram like the work of cluster analysis for some cultivars, but we also seeking to ensure the diagram generated should be referable and workable for the identification of mei cultivars in practice even in future. From the results above, this strategy could also make DNA markers more applicable for plant variety identification. However, verification of the utilization, workability and efficiency of the diagram in cultivar identification was necessary, for which 7 groups of cultivars including 'Sichuanbaimei' and 'Dongshanlimei', 'Hongmei' and 'Momei', 'Hongding' and

Table 2. Summary of primers used in the study.

Primer No.	Primer (5'→3')	Annealing temperature (℃)
B3	GTCCACACGGG	43.7
C3	GGACTGGAGTG	43.7
D4	GTCAGAGTCCC	44.8
D5	GTCAGAGTCCT	44.4
E3	GTGGCATCTCG	42.8
Y6	GTTTCGCTCCC	43.7
Y10	CTGCTGGGACT	44.4
Y15	AGGGGTCTTGA	40.4
Y22	GGACCCAACCT	40.4
Y29	GTGTGCCCCAG	43.7
Y30	GTGTGCCCCAC	40.4
Y33	AAGCCTCGTCA	44.8
Y40	AGCGTCCTCCT	40.4
Y54	TGGTGGCGTTC	43.7

'Yinafenghou', 'Koushuu Koume' and 'Zaohong', 'Yanhua' and 'Taoxingmei', 'Changnong17' and 'Setsudaume', 'Gyokuei' and 'Shinoume' were randomly chosen and used in the verification. From the location of these cultivars in the CID, it was easy to find that primers Y10, Y33, D4, Y29 and Y15 were definitely those to be used in separating these seven groups of cultivars. The PCR results of these 7 groups of cultivars using the corresponding primers were same as those anticipated (Figure 4).

Three groups 'Sichuanbaimei' and 'Dongshanlimei', 'Hongmei' and 'Momei', 'Hongding' and 'Yinafenghou' could be identified by primer Y10 with 2 bands of 1,000 bp and 850 bp (Figure 4A). Figure 4B shows that 'Koushuu Koume' and 'Zaohong' could be separated by primer D4 with the 2500 bp band while 'Yanhua' and 'Taoxingmei' could be separated by primer Y33 with 380 bp (Figure 4C); 'Changnong17' and 'Setsudaume' were distinguished by primer Y29 (Figure 4D) while 'Gyokuei' and 'Shinoume' were separated by primer Y15 (Figure 4E), with the specific bands of 1400 bp and 1800 bp, respectively. Clearly, identification of the 7 groups of cultivars using the five specific primers as anticipated indicated the usability of this CID and that separation of all the 64 cultivars could also be verified to be powerful.

DISCUSSION

The need to demystify science by developing strategies of applying new knowledge and technology to practical activities is both necessary and significant. DNA marker is a powerful technique that can be used to identify plant cultivars and species, with several generations of DNA markers having been developed and used to cultivar identification (Chiu et al., 2010; Saker et al., 2006), genetic analysis (Baysal et al., 2010; Bhau et al., 2009; Boronnikova et al., 2007; Silvestrini et al., 2008). Thousands of papers on utilization of DNA marker have also been published. Despite all these, DNA markers have not been easily used in genotyping plants. In fact, the situation is much more serious than anticipated, with the question of whether DNA markers can be well and easily used in identification of plant varieties yielding a negative response. No efficient approach has been applied to use DNA markers easily and efficiently in *Prunus* plant cultivar identification except where phylogenetic tree clusters or some fingerprints were employed. Obviously, the clusters formed in phylogenetic trees cannot tell us which information can be used for the identification of the *Prunus* plant samples desired while the later cannot present all the fingerprints of many cultivars together for the identification. The main reasons for these weaknesses can be due 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 employed in this study can use DNA markers efficiently to distinguish the cultivars as desired. It has the advantages of less cost, timeliness and objectivity among others. The strategy can realize the power of DNA markers in plant cultivar identification activities and can use the polymorphic bands of each primer gradually to distinguish every species and individual plant, from which a cultivar identification diagram for further identification of these cultivars can be finally constructed for practical application. Although the method does not accurately reflect the genetic relationships of the cultivars being identified, the earlier the separation form the rest the greater the genetic distance distance between it and the others theoretically. This

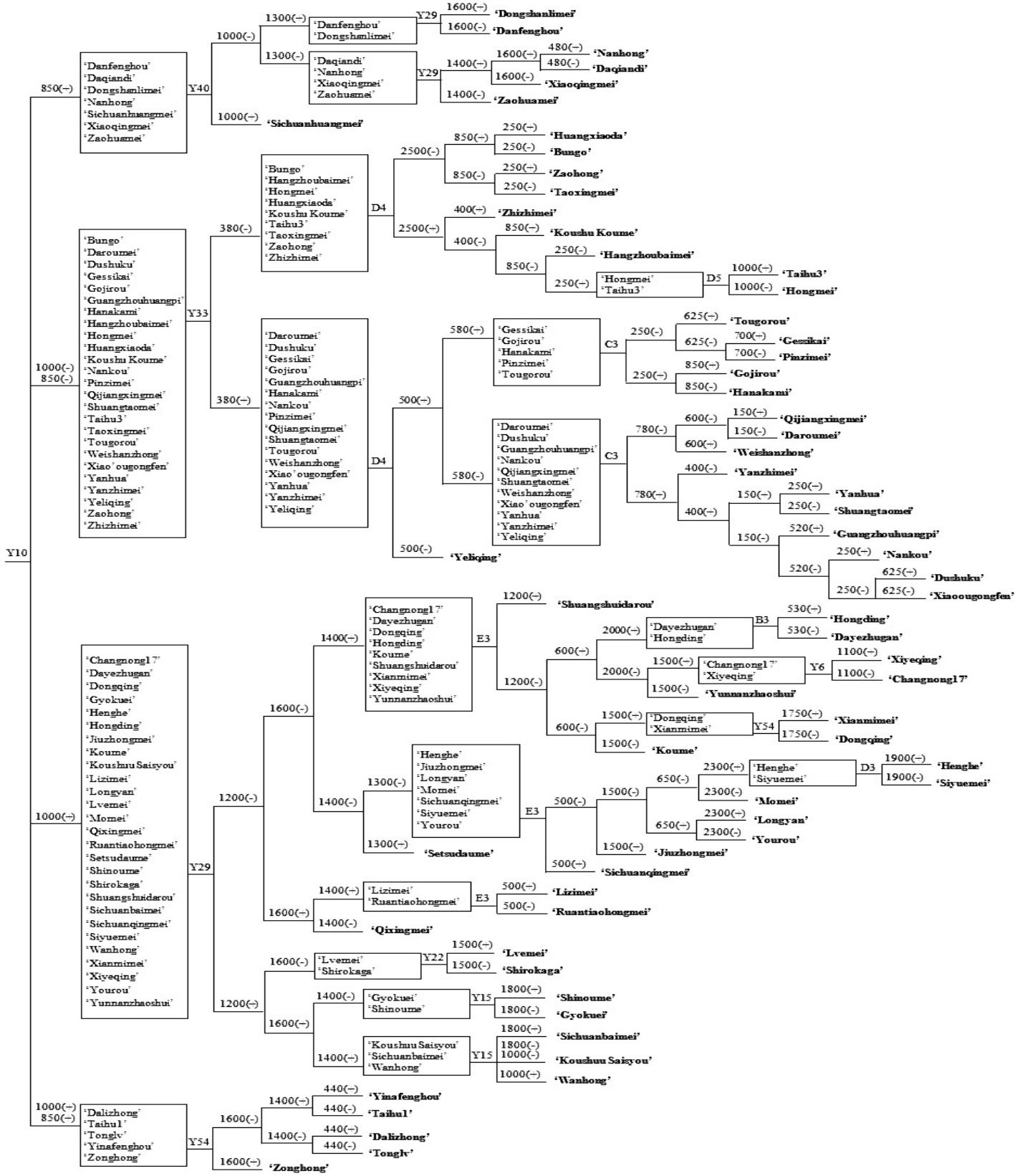

Figure 3. Results of identification of fruiting mei cultivars by 14 primers and detailed fingerprints. All the numbers marked in this chart indicate different sized fingerprints, and the unit is "bp"; (+), presence of bands; (-), absence of bands.

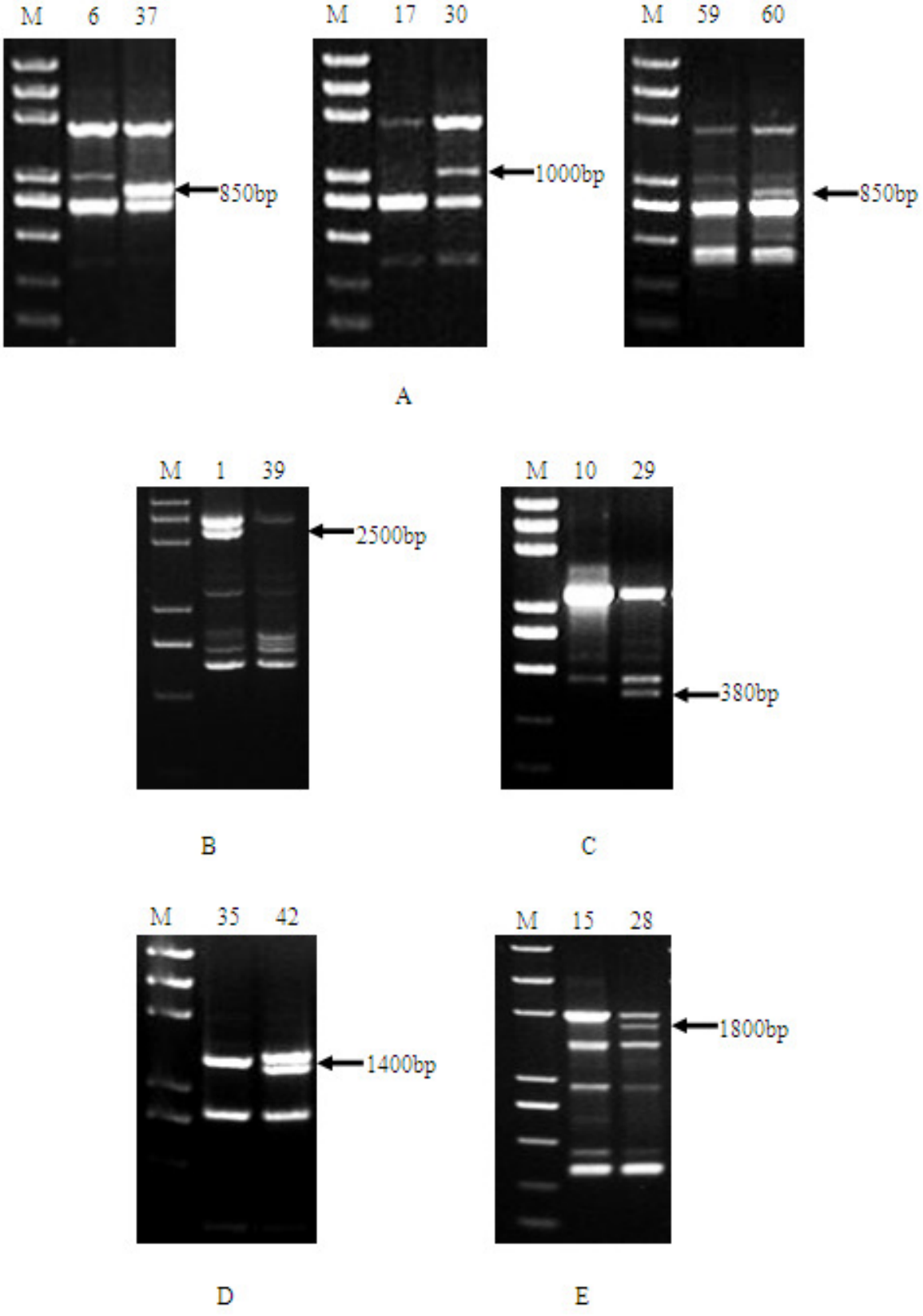

Figure 4. Results from verification of the separation of two randomly selected cultivars by corresponding primers. M: DL2000 plus marker; Number: Accession numbers and names of these cultivars used as listed in Table 1.

strategy definitely does help greatly in plant cultivar identification for cultivar-right-protection, and early identification of seedlings in nursery industry. To improve the efficiency and utilization of this strategy in fruit crop industry, the identification of more commercial and important cultivars in production was chosen.

Fruiting mei has been cultivated in China for more than 7000 years according to the historical records, and now it is also widely cultivated in Japan. In terms of economic importance, the processed products of fruiting mei which include salted mei, mei wine and juice have high nutritional and medicinal value and are consumed in a number of countries, including China, Japan, and Korea, and have long played an important role in human diet and health (Chu, 1999). For better development of the mei research and industry, identification of fruiting mei cultivars and germplasm resources is indispensable. Molecular markers have been used to study genetic analysis of variety relationships and organisms including fruiting mei cultivars using RAPD (Shimada et al., 1994), amplified fragment length polymorphism (AFLP) (Fang et al., 2005a; Fang et al., 2006), simple sequence repeat (SSR) (Kyohei et al., 2008), single nucleotide polymorphism (SNP) (Fang et al., 2005b; Li et al., 2010). However, no efficient strategy that can apply DNA markers easily on fruiting mei variety identification had been earlier reported. The important aim of this study was not just how to use the RAPD marker to distinguish the 64 fruiting mei cultivars, which focuses on the utilization of DNA fingerprints in identifying plant cultivars, but to also provide a new strategy to properly utilize DNA marker in the separation of fruiting mei cultivars which could also be considered as a universal strategy used in distinguishing other plant and seed samples.

In this study, 14 RAPD primers were used to distinguish all the 64 fruiting mei cultivars by the practical CID strategy. Actually, large numbers of fruiting mei cultivars could not be distinguished at the same time with a single RAPD primer. However, CID strategy employed here could obviously make full use of the polymorphic bands to identify fruiting mei cultivars efficiently. The informative CID (Figure 2) of the fruiting mei cultivars is the key point as it can tell us the primer or primers that can be used to separate specific fruiting mei cultivars. Basically, any 2 cultivars can be distinguished with one RAPD primer. If new fruiting mei cultivars are released, the set of 14 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. But if the set of primers used originally, such as the 14 in this study cannot provide clear separation of the new cultivars, additional primers need to be screened and used to separate these new cultivars. With the identification information of the new cultivars, a larger CID can be formed. That is to say, less work is needed to disjoin one or several new cultivars. In addition, the verification results of the CID can confirm the practical importance of fruiting mei cultivar identification using this method, which is workability and accuracy as anticipated. Therefore, the present study provides a reliable method for identification of fruiting mei varieties. We believe that the strategy employed here deserves to be utilized not only in fruiting mei but in the whole agricultural industry in China.

This strategy overcomes the notion that DNA markers could not easily be used in practical plant separation since most analysis results from work using DNA markers were not practically referable and workable for future use. This CID can work like the periodic table of elements and has advantages of referability, showing us clear information for separating the varieties as desired. Fewer primers can be efficiently used and all cultivars contained can be separated easily by PCR with the corresponding primers easily found on the diagram. It is the first strategy that makes fruiting mei cultivar identification much more applicable, efficient, easier and direct in practice, even though it requires one or more PCR reactions. It will not only provide valuable information and theoretical scientific basis on identification of cultivars, genetic diversity cultivar introduction and genetic improvement on the molecular level, but also be essential in grant of protection to all the new varieties through distinctness, uniformity and stability (DUS) testing (Lu et al., 2009). Therefore, the strategy we used not only can make DNA marker more applicable for other plants, but even in seed sample and animal identification, and it can be a fundamental requirement to enforce intellectual property protection for plant breeders' and farmers' rights (Staub et al., 1996; Wang et al., 2009).

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