Genotyping Fruiting Mei (*Prunus mume* Sieb. et Zucc.) Cultivars Using Amplified Fragment-length Polymorphism Markers

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Abstract. We used amplified fragment length polymorphism (AFLP) markers to analyze 14 fruiting mei cultivars from China and Japan. The levels of polymorphism and genetic relationship among cultivars were studied using two types of AFLP primer combinations [*EcoRI* + *Mse* I (E+M) and *EcoRI* + *Taq* I (E+T)] and the combined data from both types of primer combinations (E+M+T). The polymorphism among the cultivars was 57.92% based on E+M primers and 63.04% based on E+T primers. All three dendrograms generated by the three sets of data showed similar relationships among the fruiting mei cultivars. The corresponding main clusters contained the same cultivars and the subgroups correlated closely with the known geographic origins of the cultivars.

Mei (*Prunus mume* Sieb. et Zucc.) originates from China, and is classified into two groups, the fruiting mei and the flowering-mei, according to the different uses by mankind (Lu, 2000). Fruiting mei had been cultivated and consumed in China for more than 7,000 years (Chu and Fang, 2001), however, its economic value was just realized a few decades ago. The high nutrition and medical value of mei fruit and their processed products have made them one of the most healthy foods available. In recent decades, fruiting mei production in China has increased significantly with the increasing market demand around the world. There were 101,642 ha of fruiting mei in China with a yield of 130×10^6 t in 1997. Fruiting mei has developed into one of the top export fruit commodities from China (Chu and Fang, 2001).

China is the center of diversity for fruiting mei germplasm, and there are more than 189 recorded varieties or excellent trees (Chu, 1999). Germplasm evaluation is the prerequisite before use. However, the fruiting mei germplasm has not been fully evaluated and the genetics of fruiting mei have not been well studied. The breeding and improvement program of fruiting mei is still in the early stages. The study of fruiting mei germplasm biodiversity is becoming an important work.

DNA marker techniques have been powerful tools for plant germplasm analysis, and can

be used to evaluate fruiting mei. Randomly amplified polymorphic DNA (RAPD) markers have been used before to study the fruiting mei genetic diversity (Fang et al., unpublished data), however, the results were not reproducible to differentiate fruiting mei cultivars. Amplified fragment-length polymorphism (AFLP) is a polymerase chain reaction (PCR) based markers that have been reliably used for genome mapping and genotyping (Vos et al., 1995). AFLP markers have been used to study the genetic diversity in many fruit crops, such as peach (*Prunus persica* L.) (Dirlewanger et al., 1998), apricot (*Prunus armeniaca* L.) (Hagen et al., 2002; Geuna et al., 2003), sweet cherry (*Prunus avium* L.) (Struss et al., 2001), and plum (*Prunus domestica* L.) (Goulão et al., 2001). Considering the high reproducibility and highly polymorphic nature of AFLP markers (Jones et al., 1997), we chose the AFLP markers to study fruiting mei cultivars, and analyze the genetic diversity among fruiting mei cultivars collected from different regions in China and Japan.

Materials and Methods

Plant materials. Fourteen fruiting mei cultivars (Table 1) were collected from the fruiting mei germplasm collection at the Agricultural Research Station of Nanjing Agricultural University, Nanjing, China. The background information of these cultivars was reported by Chu (1999). Their geographic origins are from Jiangsu to Zhejiang provinces (Z–J) in the northern subtropical fruiting mei cultivation zone in China, from Guangdong to Fujian provinces (G–F) in the southern tropical fruiting mei cultivation zone in China, from Yunnan to Hunan provinces (Y–H) in the

mid-subtropical fruiting mei cultivation zone in China, and from Japan.

DNA extraction and AFLP reactions. Total genomic DNA was isolated from young leaves using a modified C-TAB method (Murray and Thompson, 1980). The quality and quantity of the DNA samples were checked by 0.8% agarose gel and Genequant II RNA/DNA calculator (Pharmacia Biotech, Cambridge, UK). The 14 cultivars were genotyped by AFLP generated by two different enzyme combinations: *EcoRI* + *Mse* I (E+M) (Kashkush et al., 2001) and *EcoRI* + *Taq* I (E+T) (David et al., 2001). For the digestion and ligation reaction, double-stranded *EcoRI*, *Mse* I and *Taq* I adapters (Table 2) were prepared by first synthesizing oligonucleotides corresponding to each strand. Two strands of each adapter were mixed in equimolar amounts and annealed at 95 °C for 3 min followed by being kept at room temperature for 5 min. Digestion of the genomic DNA and ligation of the adapters were carried out simultaneously for the *EcoRI* + *Mse* I enzyme combination at 37 °C for 2 h. The digestion–ligation reaction contained 0.50 µg DNA, 10 units *EcoRI*, 12 units *Mse* I, 1.2 units T4-DNA ligase, 5 pmole *EcoRI* adapter, 50 pmol *Mse* I adapter, 0.5 M NaCl, 5 µg bovine serum albumine (BSA), and 1 µL $10 \times$ DNA ligase buffer in a final volume of 10 µL adjusted with ddH₂O. For digestion with *EcoRI* + *Taq* I, genomic DNA (0.5 µg) was carried out at 65 °C for 1.5 h with 5 units *Taq* I in a volume of 25 µL buffer containing 10 mM TRIS-acetate pH 7.5, 10 mM MgCl₂, 50 mM potassium acetate, 5 mM dithiothreitol, 1 mM ATP and 50 ng·µL⁻¹ BSA. The reaction was cooled to 37 °C, supplemented with 15 µL of buffer containing 10 units *EcoRI* and incubated at 37 °C for an additional 2 h. For adapter ligation, 10 µL of restriction-ligation buffer, containing 50 pmol of *Taq* I, 5 pmol of *EcoRI* I adapters, 0.5 mM ATP and 1.2 units of T4-DNA ligase was added, and the reaction was incubated in 37°C for 3 h. The adapter-ligated DNA was diluted (1:10) and preamplified with AFLP primers (Table 2), each having one selective nucleotide at its 3' end. The preamplified DNA was diluted (1:20) and an aliquot was used for selective PCR amplification with the selective primer (Table

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

Cultivar	Origin
Bungo	Japan
Dalimei	G–F ²
Gaotian Bungo	Japan
Gessekai	Japan
Hongding	Z–J
Jijiangxingmei	Y–H
Kaiti	Z–J
Koshukoume	Japan
Longyanmei	G–F
Nanhong	Z–J
Pinzimei	Y–H
Rantiaohongmei	Z–J
Tonglu	Y–H
Xiaoqing	Z–J

²G–F = Guangdong to Fujian provinces of China; Z–J = Zhejiang to Jiangsu provinces of China; and Y–H = Yunnan to Hunan provinces of China.

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Table 2. Sequences of AFLP adapters and primers used in the study. The underlined are the core sequences of the related primers. Primers are named E, M and T, respectively for *EcoR* I, *Mse* I and *Taq* I primers. The preamplification primers contain the core sequence with an extra nucleotide added at the 3' end: E-A, M-C and T-A. The selective primers are similar to the preamplification primers with the addition of two more nucleotides.

Enzyme	Adapters	Preselective primer	Selective primer
<i>EcoR</i> I	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'	5'-GACTGCGTACCAATTCA-3'	5'-GACTGCGTACCAATTCA NN-3'
<i>Mse</i> I	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'	5'-GATGAGTCCTGAGTAAC-3'	5'-GATGAGTCCTGAGTAAC NN-3'
<i>Taq</i> I	5'-GACGATGAGTCCTGAC-3' 3'-TACTCAGGACTGGC-5'	5'-GATGAGTCCTGACCGAA-3'	5'-GATGAGTCCTGACCGAA NN-3'

2) combinations of *EcoR* I + *Mse* I or *EcoR* I + *Taq* I having three selective nucleotides at their 3' ends. In the selective PCR, *EcoR* I primer was end-labeled with γ -³³P-ATP. The reaction conditions for preselective PCR, selective PCR, and primer label are the same as those reported by Kashkush et al. (2001). E+M primer combinations were used for the study: E-ACT/M-CAT, E-ACT/M-CAG, E-AAC/M-CTT, E-AAC/M-CTA, E-AGG/M-CAA, E-AGG/M-CAG; and six E+T primer combinations were also used: E-ACT/T-AAG, E-ACT/T-AAT, E-AAC/T-AAA, E-AAC/T-ATG, E-AGG/T-ATA, E-AGG/T-ATT. The selective PCR products were diluted 1:1 with 20 μ L of formamide dye (98% formamide, 10 mM EDTA, bromophenol blue, xylene cyanol), denatured and separated on 5% denaturing polyacrylamide (20:1 acrylamide: bis, 7.5 M urea, 1 $\times$ Tris borate EDTA-TBE buffer) standard sequencing gel (43 cm long) at 110 W for 1.5 h after 40 to 50 min of prerun in the same conditions. The gel was then dried for 1 h at 80 °C and exposed to X-ray film (Kodak) for about 50 h at -80 °C.

Data analysis. The clear and reliable bands for each sample from all primer combinations were scored as present (1) or absent (0) across all genotypes. The 1/0 data matrix was used to analyze the genetic relationships of the fruiting mei cultivars. The analysis was conducted in two stages: a) calculation of genetic distance and b) cluster analysis and drawing of a phylogenetic tree. For the individual score analysis, one hundred bootstraps and genetic difference estimates, expressed as genetic distance by Nei index (Nei and Li, 1979), were calculated using the PhylTools software (Buntjer, 2000). Cluster analysis by Neighbor Joining method (Saitou and Nei, 1987) was conducted with NEIGHBOR and CONSENSE software (PHY-LIP package) (Felsenstein 1993). A consensus tree was drawn using the TreeView software (Page, 1996). For the AFLP frequency analysis, one hundred bootstraps were done using SEQBOOT software (PHY-LIP package), and the Nei genetic distance values were calculated using GENDIST software (PHY-LIP package). The cluster analysis and the tree were done as described above.

Results

Characterization of fruiting mei AFLP fingerprinting. This is the first study of fruiting mei using AFLP markers. We used six selective primer combinations of E+T or E+M. These two enzyme combinations were used to identify 14 different fruiting mei cultivars. Each primer combination could generate a clear fingerprint. The examples of AFLP band patterns of these

two enzyme combinations produced using E-AAG + M-CAT and E-ACT + T-AAT primer combinations are presented in Fig. 1A and B, respectively. Only the clearly delineated bands within the range of 50 to 300 bp were used for analysis. The AFLP primer set combinations, total number of bands generated by each primer set, number of polymorphic bands detected, and percentage of polymorphic bands used in the study of fruiting mei is shown in Table 3. Six selective primer combinations resulted in a total of 240 (E+M) and 230 (E+T) bands and 57.92% and 63.04% were polymorphic, respectively. The average band number per lane of each cultivar derived from E+M (23.16) was lower than that (24.17) derived from E+T. From the high band polymorphism and the clear

AFLP fingerprints, these two AFLP primer combinations can both be efficiently used to study fruiting mei germplasm.

Genetic relationship among fruiting mei cultivars. In the cluster analysis based on 139 AFLP polymorphic markers derived from E+M primer sets, most of the 14 cultivars were grouped according to their geographic origins. Two main clusters were found (shown as A1 and A2 in Fig. 2). All the cultivars from Z-J are in cluster A1 containing three subgroups with low bootstrapping values. The exception is 'Pinzimei', from Y-H geographically, which was grouped together with two Z-J originated cultivars ('Rantiaohongmei' and 'Hongding') in a small cluster with non-significant bootstrap. 'Koshukoume', a Japanese cultivar,

Fig. 1. 1A and 1B were AFLP band patterns of 14 fruiting mei cultivars. (A) The primer combination was E-ACT + M-CAT. (B) The primer combination was E-ACT + T-AAT.

Table 3. AFLP primer set combinations, total number of bands generated by each primer set, number of polymorphic bands detected, and percentage of polymorphic bands used in the study of fruiting mei.

Primer sets	Total no. of bands	No. of polymorphic bands	Percent of polymorphism
E-ACT+M-CAT	37	25	67.57
E-ACT+M-CAG	30	19	63.33
E-AAC+M-CTT	52	25	48.08
E-AAC+M-CTA	26	12	46.15
E-AGG+M-CAA	47	27	57.45
E-AGG+M-CAA	48	31	64.58
Total	240	139	
Average	40	23.16	57.92
E-ACT+T-AAT	50	41	82.0
E-ACT+T-AAG	37	32	86.49
E-AAC+T-AAA	30	15	50.0
E-AAC+T-ATG	32	16	50.0
E-AGG+T-ATA	35	19	54.29
E-AGG+T-ATT	46	22	47.83
Total	230	145	
Average	38	24.17	63.04

Fig. 2. Unrooted phylogenetic trees generated based on *EcoR* I + *Mse* I markers (A), *EcoR* I + *Taq* I markers (B), and the combined of *EcoR* I + *Mse* I and *EcoR* I + *Taq* I (C). A1 and A2, B1 and B2, and C1 and C2 were used to name the two main clusters in A, B, and C, respectively.

is another exception because it is clustered individually as a subgroup in cluster A1. In cluster A2, two of the three subgroups are the mixture of cultivars with different origins, such as 'Jijiangxingmei' (Y-H) grouped with two Japanese cultivars ('Gaotian Bungo' and 'Bungo'), and 'Tonglu' (Y-H) grouped with 'Dalimei' and 'Longyanmei' (G-F). In the analysis, some cultivars having the same origin were grouped with significant bootstraps: 'Nanhong', 'Xiaqing' and 'Kaiti'; 'Rantiaohongmei' and 'Hongding'; 'Gaotian-fenghou' and 'Bungo'; 'Tonglu', 'Dalimei', and 'Longyanmei'.

The dendrogram generated from E+T primer set data (145 polymorphic markers) showed two main clusters similar to those derived from E+M data. In the main cluster B1 from E+T, there are only two separate subgroups with the same cultivars as those in the A1 from E+M. In this dendrogram, 'Pinzimei' and 'Koshukoume' are clustered with 'Nanhong', 'Xiaqing' and 'Kaiti' and with 'Rantiaohongmei' and 'Hongding', respectively. In another main cluster (B2), 'Jijiangxingmei' is grouped

with 'Gessekai' with a significant bootstrap value, while 'Tonglu' separated from 'Dalimei' and 'Longyanmei'.

In the E+T dendrogram, except for 'Tonglu', which separated from 'Dalimei' and 'Longyanmei', the same small groups clustered as those in the E+M dendrogram: 'Nanhong', 'Xiaqing' and 'Kaiti'; 'Rantiaohongmei' and 'Hongding'; 'Gaotian Bungo' and 'Bungo'; 'Dalimei' and 'Longyanmei'. The bootstrapping values of the nodes of these groups are also significant. Results based on the data of 284 polymorphic markers obtained from the combination of E+T and E+M markers (E+T+M) generated the most distinct clusters in the unrooted trees. All the bootstraps were significant ($\geq 50\%$). This E+T+M dendrogram was more similar to that derived from E+M data than that from E+T data. Thirteen cultivars were located in similar subgroups as shown in E+M and E+M+T dendrograms. Only the 'Gessekai' was clustered individually as a subgroup in C2 (E+M). However, some bootstrap values in the E+M dendrogram were lower than 50%.

Discussion

AFLP fingerprinting. The AFLP markers generated a large number of bands with a few primer combinations, allowing efficient identification of unknown accessions, and establishing genetic relatedness among them with high certainty. AFLP markers were even more useful in identifying potential duplicate cultivars (Barker et al., 1999; Karp, 2000). In this study, we report the first use of AFLPs markers for fingerprinting and evaluating genetic relationships of fruiting mei cultivars. The E+M primer combination generated more bands (240) than the E+T primer combination (230). The percentage of polymorphic bands from E+M and E+T primer combinations were 57.92% and 63.04%, respectively. This high polymorphism may be due to the reasons that most fruiting mei cultivars develop from seedlings, and the genetic difference among these fruiting mei cultivars is relatively obvious. Both types of AFLP primer combinations can be efficiently used to study fruiting mei germplasm.

Genetic relationships among cultivars. Both AFLP primer combinations allowed the evaluation of genetic diversity of the screened fruiting mei cultivars. Because of the difference between the digestion sequences of *Mse* I and of *Taq* I, which means E+M and E+T detect different genetic polymorphisms, using the combined data of these two primer combinations will be more representative of the whole genome. Compared to the dendrograms from E+T and E+M, the grouping obtained in the E+T+M dendrogram improved with a larger number of markers, and this E+M+T phylogenetic tree revealed higher bootstrap values. This is consistent with what Schut et al. (1997) reported that the combination of genetic similarity estimates of both primer combinations could reduce the effect of their independent inaccuracies. Therefore we relied more on the E+T+M AFLP data for an explanation of genetic relationships among the accessions. Consequently, more AFLP markers are better for higher resolution of fruiting mei cultivars.

Compared to the dendrograms constructed based on E+M+T data, those from E+T and E+M represented more similar relationships among cultivars that were closely correlated with the known origins. The relationships of cultivars in the subgroups of the dendrograms generated by E+T and E+M+T data are somewhat different. 'Gaotian Bungo' and 'Bungo' are separated from 'Jijiangxingmei' and 'Gessekai' in the E+T dendrogram, but all four cultivars are clustered together in the same subgroup in the E+M and E+M+T dendrograms.

All 14 cultivars were grouped into two main clusters in the three dendrograms, and the corresponding clusters contained the same seven cultivars. The fact that all the Z-J originated cultivars ('Nanhong', 'Rantiaohongmei', 'Xiaqing', 'Hongding', and 'Kaiti') were grouped together in the same main cluster plus 'Pinzimei' (Y-H) and 'Koshukoume' (Japan) showed that all the Z-J originated cultivars

were closely related and the latter two cultivars might have originated from the Z-J region. Other main clusters in the dendrograms contained the same seven cultivars, however, more overlapping occurred between some cultivars of different geographic groups. The subgroup data showed that 'Tonglu' (Y-H), 'Dalimei' (G-F), and 'Longyanmei' (G-F); 'Jijiangxingmei' (Y-H), 'Bungo' (Japan), 'Gessekai' (Japan), and 'Gaotian Bungo' (Japan) were the two groups having closer genetic relationships. Those overlapping between the Y-H and the G-F or Japan originated cultivars might be due to germplasm exchange or movement that happened previously.

Studies of other plants found the similar results between the genetic clustering and their geographic origins. Hurtado et al. (2001) used the restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD), and AFLP markers to separate sixteen apricot cultivars into different clusters and the clustering were very similar to their known geographic origins. Sanz-Cortés et al. (2001) reported that there was a correlation between the clustering of olive cultivars based on genetic similarity from RAPD marker analysis and their geographic origins in the Valencia region of Spain.

In conclusion, the markers generated by both the *EcoR* I + *Mse* I and *EcoR* I + *Taq* I primer sets proved very effective in distinguishing the cultivars in this study. The dendrogram constructed from the genetic similarity values agreed fairly well with the origin information. Thus, these markers can be used for DNA fingerprinting and estimation of the genetic relatedness in fruiting mei cultivars. The combination of these two AFLP primer sets could be used to uncover the genetic distance and present the phenetic relationships of fruiting mei of different geographic origins.

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