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Fingerprinting and variety identification of rice (*Oryza sativa* L.) based on simple sequence repeat markers

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Abstract

Fingerprinting with molecular markers allows precise, objective and rapid variety identification. In this study, simple sequence repeat (SSR) markers were used to fingerprint the forty-eight main commercial rice cultivars under cultivation at present in Zhejiang Province, China, and identify *indica* and *japonica* subspecies including eight groups of closely related cultivars. Eighteen of the thirty-two SSR primer pairs were polymorphic and generated a total of 42 distinct reproducible bands with an average of 2.33 bands per primer pair. 41 (97.6%) of the 42 bands amplified were polymorphic. The number of polymorphic bands detected with each primer pair ranged from 1 to 4 with an average of 2.28 per primer pair. The polymorphic information content (PIC) values of each primer pair ranged from 0.10 to 0.50 with an average of 0.31. The UPGMA cluster analysis separated the 48 cultivars into two major groups. The first major cluster consisted of the 34 *indica* cultivars, and the leaving 14 *japonica* cultivars formed the second major cluster. Primer pair RM249 or RM250 was able to differentiate between *indica* and *japonica* subspecies, respectively. Most of the closely related cultivars were identified with the fingerprinting based on the polymorphic SSR primer pairs, apart from cultivars 'Xiushui 09' and 'Xiushui 114'. The results revealed a narrow genetic diversity among the forty-eight main commercial rice cultivars in Zhejiang Province of China, and combination of SSR with other marker systems might be a potential strategy for fingerprinting database development and authentification of rice cultivars in further study.

Keywords: Cultivar identification; Genetic diversity; *indica*; *japonica*; SSR; Type classification.
Abbreviation: CTAB-Hexadecyl trimethyl ammonium Bromide; EDTA-Ethylene Diamine Tetraacetic Acid; SSR-inter-simple sequence repeat; UPGMA- unweighted pair-group method with arithmetic means.

Introduction

Rice (*Oryza sativa* L.), which has two cultivated subspecies, *indica* and *japonica*, is one of the leading food crops in the world and staple for more than half the world's population (Ohtsubo and Nakamura, 2007). High-quality seeds and elite cultivars play a crucial role in its production. However, since new cultivars normally arise from hybridizations between members of an elite group of genetically similar parents, the amount of genetic variability among newly developed cultivars is likely to become even smaller (Rahman et al., 2009), which makes it more difficult to unambiguously distinguish cultivars from the others with morphological characteristics and isozyme electrophoresis patterns because of influences by environmental factors. Fingerprinting with molecular markers allows precise, objective and rapid cultivar identification, which has been proved to be an efficient tool for crop germplasm characterization, collection and management. Simple sequence repeat (SSR) markers have been widely used for genetic analysis and cultivar identification because of their abundance, co-dominance inheritance, high polymorphism, reproducibility, and ease of assay by polymerase chain reaction (PCR) (Kuleung et al., 2004; Xie et al., 2011). Application of SSR markers on plant cultivar identification have been reported, such as grape (Dangl et al., 2001), potato (Coombs et al., 2004), rape (Louarn et al., 2007), rice (Rahman et al., 2009), almond (Dangl et al., 2009), apple (Moriya et al., 2011), wheat (Zhu et al., 2011) and so on. Cultivar identification and classification of rice require fingerprinting. When large numbers of cultivars are involved, the fingerprinting work can be costly in terms of laboratory consumables, labor and time. As a consequence, it is essential to build the fingerprinting database of the main commercial cultivars in the market for rapid and unambiguous cultivar identification with the number of newly similar cultivars increasing yearly. What's more, type classification of *indica* and *japonica* rice germplasm is extremely important for the utilization of heterosis between *indica* and *japonica* subspecies in rice cultivar improvement. SSR markers have been used in genetic analysis and fingerprinting of different rice accessions (Thomson et al., 2007; Lu et al., 2010; Rampant et al., 2011). Rice is the first major grain crop of Zhejiang Province, China, which supports about 82 % of the grain yield and occupies an important strategy place. Nowadays, one of the problems which limit the development of rice production is the cultivar mixing in seed market of Zhejiang Province. Fingerprinting the commercial rice cultivars based on molecular markers is a crucial measure for unambiguous and quick identification of similar or closely-related cultivars. However, no study on fingerprinting of the main commercial rice cultivars under cultivation at present in Zhejiang Province of China was reported up to now. This investigation is conducted to fingerprint the forty-eight main commercial rice cultivars under cultivation now in Zhejiang Province of China based on SSR markers.

Results

Polymorphic information analysis of SSR primer pairs

Thirty-two published SSR primer pairs were initially screened

Table 1. Resolving power value of polymorphic primers.

Primer	Number of amplified bands	Number of polymorphic bands	Number of band patterns	Polymorphic Information content (PIC)
RM1	3	3	5	0.21
RM11	2	2	2	0.39
RM13	2	2	2	0.39
RM16	4	4	3	0.37
RM17	2	2	3	0.10
RM19	2	2	3	0.21
RM21	2	2	3	0.39
RM206	3	3	5	0.16
RM210	2	1	2	0.12
RM211	2	2	2	0.18
RM212	2	2	2	0.50
RM214	2	2	3	0.33
RM225	2	2	2	0.41
RM228	2	2	3	0.22
RM249	2	2	2	0.41
RM250	2	2	2	0.41
RM276	3	3	5	0.48
RM280	3	3	3	0.38
Total	42	41	—	—
Average	2.33	2.28	2.88	0.31

Fig 1. Amplification profiles of primer pair RM210 from 48 rice cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 48 correspond to the rice cultivars studied as list in Table 1. The capital letters A ~ B represent the two different types of banding pattern.

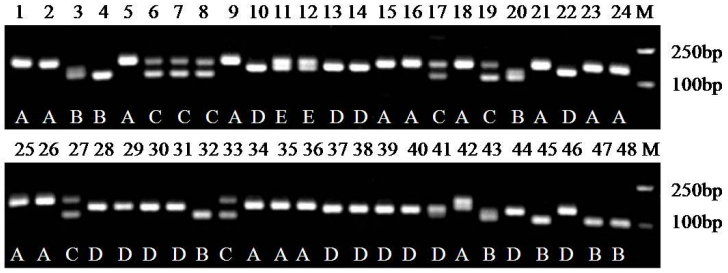

Fig 2. Amplification profiles of primer pair RM276 from 48 rice cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 48 correspond to the rice cultivars studied as list in Table 1. The capital letters A ~ E represent the five different types of banding pattern.

against the 48 commercial rice cultivars widely cultivated in Zhejiang Province, China. Eighteen polymorphic SSR primer pairs were chosen for variety identification and generated a total of 42 unambiguous bands with an average of 2.33 bands per primer pair. 41 (97.6%) of the 42 bands amplified were polymorphic. The number of polymorphic bands detected with each primer pair ranged from 1 (primer pair RM210) to 4 (primer pair RM16) with an average of 2.28. The number of band patterns produced by each primer pair ranged from 2 (primer pair RM210, Fig 1) to 5 (primer pair RM276, Fig 2) with an average of 2.88. The polymorphic information content value of each SSR primer pair ranged from 0.10 to 0.50 with an average of 0.31 (Table 1).

DNA fingerprinting database and cultivar identification based on SSR analysis

An elementary DNA fingerprinting database of the main 48 commercial rice cultivars was successfully constructed based on the eighteen polymorphic SSR primer pairs. The database of rice cultivars achieved in this study was open and could be expended as the number of new additional rice cultivars and

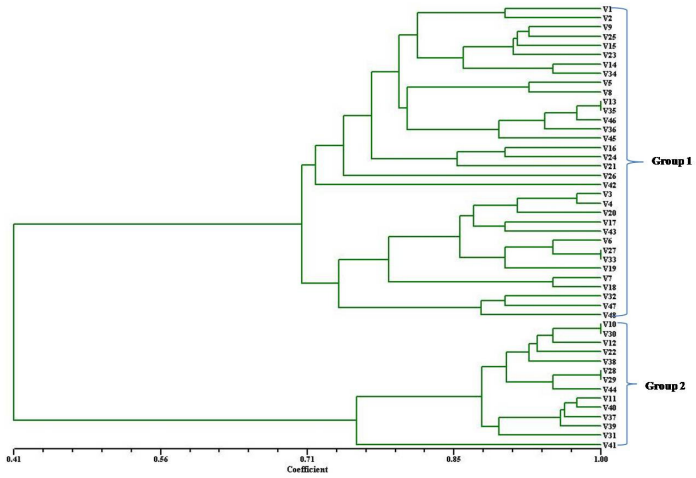

Fig 3. Dendrogram of 48 rice cultivars resulted from UPGMA cluster analysis based on SSR analysis. Note: Group 1 consisted of 34 rice cultivars, which were all belong to *indica*; Group 2 contained the 14 *japonica* rice cultivars.

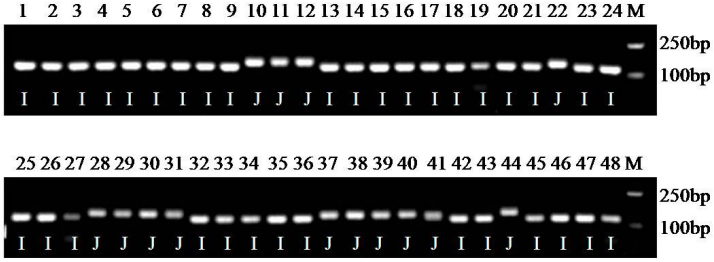

Fig 4. Amplification profiles of primer pair RM249 from 48 rice cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 48 correspond to the rice cultivars studied as list in Table 1. The capital letter I represented banding pattern for *indica*, and J for *japonica* rice cultivars.

molecular markers (systems) increasing. Eight groups of closely related rice cultivars were firstly identified based on the fingerprinting data. Among the eight groups of closely related rice cultivars, seven groups were able to be unambiguously distinguished based on the DNA fingerprinting data. However, two closely related cultivars, Xiushui 114 and Xiushui 09 (female parent of Xiushui 114) can not be identified.

UPGMA cluster of the 48 rice cultivars based on SSR analysis

The UPGMA cluster constructed from SSR analysis separated the 48 cultivars into two major groups at genetic similarity coefficient value of 0.68 (Fig 3). Cultivars □You 623 and Yongyou 9 were the two extremes in the dendrogram. The first major group consisted of the 34 *indica* cultivars, and the 14 *japonica* cultivars formed the second major cluster, which showed that SSR molecular markers might be potential to identify *indica* from *japonica* rice cultivars or germplasm. In this study, primer pair RM249 (Fig 4) or RM250 (Fig 5) was able to successfully distinguish the *indica* and *japonica* rice cultivars, respectively.

Discussion

This is the first try to fingerprint the forty-eight main commercial rice cultivars under cultivation at present in Zhejiang Province of China using SSR markers, and the fingerprinting data obtained can be used for cultivar identification in practice. SSR markers have been successfully applied in fingerprinting of rice cultivars as well as their parental lines for cultivar identification and genetic purity assessment, which proved to be a reliable technique (Nandakumar et al., 2004; Sundaram et al., 2008; Rahman et al., 2009). However, there was no report on identification of closely-related rice cultivars. In this study, the elementary fingerprinting database of the forty-eight main commercial rice cultivars under cultivation at present in Zhejiang Province of China was built using SSR markers, which has been used for identification of closely related cultivars. The results of this study showed SSR markers have a high and potential discriminating ability in rice cultivar identification. Xiushui 09 is the female parent of Xiushui 114 (Xiushui09×Xiushui123), which led to the difficulty in their discrimination. While, cultivars Yongxian 57 and Yongxian 69 sharing the same origin (Jiayu143×G95-40-3) were identified with primer pair RM17 (Fig 6). Therefore, a suitable SSR primer pair for differentiation between Xiushui 09 and Xiushui 114 might be required for further screening. The polymorphism information content (PIC) refers to the value of a marker for detecting polymorphism within given genotypes. In previous study, SSR revealed a valuable diversity among rice accessions (Thomson et al., 2007). However, no PIC value of each SSR primer pairs analyzed in this study was above 0.50, which revealed a narrow genetic diversity among the forty-eight main commercial rice cultivars in Zhejiang Province of China. Due to the narrow genetic diversity of rice cultivars in Zhejiang Province, the molecular differences among cultivars may exist in a few specific traits or genes. Therefore, developing molecular

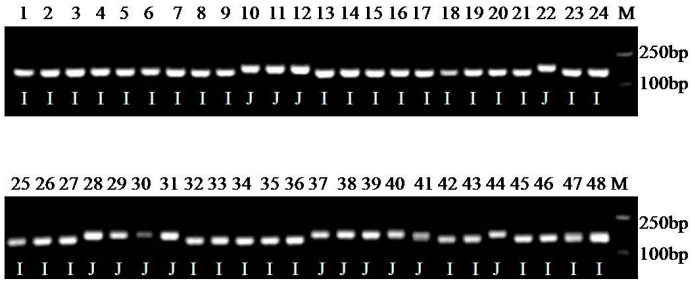

Fig 5. Amplification profiles of primer pair RM250 from 48 rice cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 48 correspond to the rice cultivars studied as list in Table 1. The capital letter I represented banding pattern for *indica*, and J for *japonica* rice cultivars.

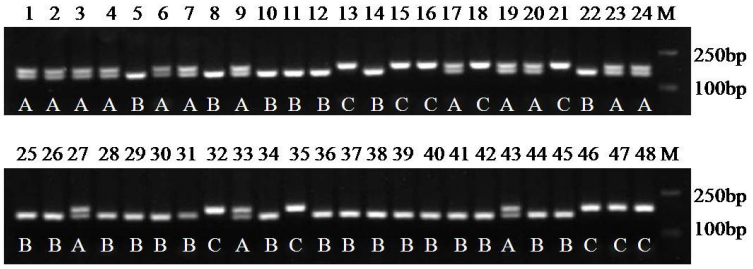

Fig 6. Amplification profiles of primer pair RM17 from 48 rice cultivars. M is DL 2000 DNA molecular marker. The numbers of lanes 1 to 48 correspond to the rice cultivars studied as list in Table 1. The capital letters A ~ C represent the three different types of banding pattern.

markers based on the specific traits or genes might be a potential method for rice cultivar identification in the further study. What's more, as the primary disadvantages of SSR markers, high cost and intense research effort required for their development have limited their application in fingerprinting and identification of rice cultivars to some extent. Except for screening more published SSR primer pairs for effective application, combining SSR markers with the other marker systems, such as inter-simple sequence repeat (ISSR) and single nucleotide polymorphism (SNP) markers, for fingerprinting rice cultivar will be another way to solve the problem. The DNA fingerprinting database of rice cultivars achieved in this study can be expended as the number of additional cultivars and molecular markers (systems) increasing. Utilization of heterosis between *indica* and *japonica* subspecies is currently one of the main ways in *Japonica* rice cultivar improvement. *Japonica* rice germplasm has a narrow genetic diversity which has greatly limited its cultivar improvement. The alternate strategy of transferring desirable genes from *indica* rice cultivars by conventional breeding methods has not been very successful because of progenies associated with high sterility, poor plant type, and linkage drag (Jeung et al., 2005). The practice shows that the intermediate materials with wide compatibility can solve the above problem to a certain extent, but the differentiation degree measure of the intermediate materials has become a new problem. Therefore, a rapid and effective genotype identification of breeding materials and differentiation degree measure of *indica* and *japonica*, is extremely important to speed up rice cultivar improvement. There were several reports on rice cultivar classification. Jeung et al (2005) used three marker systems, RAPD, ISSR, and AFLP for fingerprinting 14 rice genotypes consisting of seven temperate *japonica* rice cultivars, three *indica* near-isogenic lines, three *indica* introgression lines, and one breeding line of *japonica* type adapted to high-altitude areas of the tropics with cold tolerance genes. In this study, cluster analysis based on SSR analysis can classify the *indica* and *japonica* rice cultivars. The result showed that SSR markers were potential to distinguish *indica* from *japonica*, which might be of great utility in the effective application of heterosis between *indica* and *japonica* rice germplasm. However, only two SSR primer pairs were able to clearly discriminate the *indica* and *japonica* rice cultivars in this study, more SSR primer pairs might be screened and chosen for accurate fingerprinting and identification of *indica* and *japonica* rice germplasm, which might be deserved for further study.

Materials and methods

Plant materials

The seeds of forty-eight (thirty-four *indica* and fourteen *japonica*) rice cultivars (Supplementary Table 1) used in the study were collected from Seed Management Station of Zhejiang Province, China. The materials contain eight groups of closely related cultivars, (1) you 623 and you 906; (2) e Fufengyou 11, Y Liangyou 1, Fengliangyou 1, Fengliangyouxiang 1, Liangyoupeijiu and Yangliangyou 6; (3) Jiayou 1 and Jiayou 2; (4) Jiayu 280, Yongxian 15, Yongxian 57, and Yongxian 69; (5) Xieyou 1429 and Xieyou 9308; (6) Xiushui 09, Xiushui 114 and Xiuyou 5; (7) Yongyou 3, Yongyou 5, Yongyou 6, Yongyou 8 and Yongyou 9; (8) Zhongzheyou 1 and Zhongzheyou 8. The pedigree information of the cultivars was obtained from the website of China Rice Data Center (http://www.ricedata.cn/variety/).

Methods

Genomic DNA extraction

Genomic DNA was extracted from young leaves of the seedlings according to the modified CTAB method (Zhu et al.,

2010). The concentrations and quality of the obtained genomic DNA samples were estimated on an ultramicrospectrophotometer ND-2000 (Nanodrop, USA). Finally, all the genomic DNA samples were diluted to a final concentration of 20 ng•μL^{-1} with 1×TE buffer (10 mM Tris-HC1, pH 8.0; 1 mM EDTA) and stored at -20 ^{o}C for further use.

SSR-PCR amplification

A total of thirty-two rice SSR primers (Supplementary Table 2) covering all the 12 chromosomes were used in this study. Primers were obtained from the SSR panel in the rice Gramene database (http://www.gramene.org/markers/microsat/ssr.html). Primers were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). SSR polymerase chain reaction (PCR) amplification was conducted in a 20 μL volume containing 40 ng of genomic DNA, 1 U *Taq* DNA polymerase, 2.5 mM Mg^{2+}, 0.20 mM dNTPs and 0.2 μM of each primer. The PCR protocol consisted of an initial denaturation at 94 ^{o}C for 4 min, followed by 40 cycles of 94 ^{o}C for 45 s, annealing for 45 s at 56 ^{o}C, 72 ^{o}C for 1 min, and a final extension step of 72 ^{o}C for 10 min. All PCR reactions were carried out in a thermal cycler C1000 (Bio-Rad, USA). PCR products were separated on 1.5% agarose gels, stained with GelRed^{TM} (Biotium, USA) and photographed under UV light using Image Lab^{TM} software Version 2.0.1 (Bio-Rad, USA).

Data analysis

The band profiles were scored only for distinct, reproducible bands as present (1) or absent (0) for each SSR primer pair. Jaccard's similarity coefficient values were calculated and dendrograms based on similarity coefficient values were generated using unweighted pair-group method with arithmetic means (UPGMA) by the NTSYSpc 2.10e software (Rohlf, 2000). The polymorphism information content (PIC) value of SSR markers was calculated using the following formula (Anderson et al., 1993):

$$PIC = 1 - \sum_{i=1}^{k} p_i^2$$

Where k is the total number of alleles (bands) detected for one SSR locus and p is the proportion of the cultivars or genotypes containing the allele (band) in all the samples analyzed.

Conclusion

In this study, an elementary DNA fingerprinting database of the 48 main commercial rice cultivars under cultivation in Zhejiang Province of China was built using eighteen SSR primer pairs, which could be expended as the number of additional cultivars and molecular markers (systems) increasing. Dendrogram of SSR markers divided the 48 cultivars into two major clusters, 34 *indica* cultivars, and the 14 *japonica* cultivars. It suggested SSR markers were potential to distinguish *indica* from *japonica*, which might be deserved for further study.

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