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Analysis of genetic diversity and relationships in waxy rice (*Oryza sativa* L.) using AFLP and ISSR markers

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Abstract

Opaque endosperm is the main phenotypic indicator for waxy rice, but other phenotypic and genotypic variation among waxy rice accessions has largely been ignored. Previous studies showed that wide diversity in starch physiochemical properties exists in both *indica* and *japonica* waxy rices, especially for starch gelatinization temperature (GT) which could be divided into a high- and a low-GT group. In the present study, amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) molecular markers were employed to examine genetic diversity and relationships of 56 waxy rice accessions. A total of 358 AFLP fragments were amplified with five primer combinations, showing a high level of polymorphism (78.3%). A total of 190 ISSR bands were generated with a single primer and a primer pair, showing a very high level of polymorphism (92.2%). The genetic distance matrices obtained from the two sets of markers were significantly correlated ($r = 0.731$, $P = 0.004$). The dendrogram generated with combined AFLP and ISSR markers could clearly differentiate the *indica* and *japonica* groups. Newly released varieties and breeding lines within each subspecies tended to be clustered together, whereas landraces were more distantly placed in the dendrogram. Only one AFLP band was found specific to the *indica* type, while no specific bands were found for starch GT. The implications for the conservation and breeding of waxy rice are discussed.

Introduction

As rice is a pivotal crop in China, collection and conservation of rice germplasm has received increasing attention for sustainable utilization and crop improvement. The success of rice breeding depends largely on the utilization of the broad diversity of rice germplasm, and also on knowledge of its genetics. Waxy rices constitute a large proportion of the landraces (Ying 1993; Zhang 2000). Some are recognized as special germplasm for particular traits and hence special applications such as different colors and fragrances (Zhang 2000). For example, the Chinese national germplasm resource has a collection of 34,663 rice landraces, of which 8963 are red and 235 are black, and about 26% of the red and 97% of the black rice are waxy (Zhang 2000).

A Chinese Academy of Agricultural Science (CAAS) database (searched via http://icgr.caas.net.cn) showed that some rices should be waxy (generally <2% amylose) according to their Chinese name (nuo or nuo dao in Chinese means glutinous or waxy rice), but the amylose content was much greater than 2%. For example, Bayuenuo, Liuxinnuo and Zaohedanuo have amylose contents of 25.7%, 24.3% and 26.8%, respectively. This shows that the names of some

rices do not match the characteristic suggested by the name. The reverse case is also possible where a nonwaxy rice (by nomenclature) is actually waxy (by amylose content). Several reasons may account for this situation, such as cross-contamination with alien pollen and careless handling during seed multiplication, mislabeling and spontaneous mutation. Varietal impurity represents a major problem in germplasm conservation and utilization.

Even for true waxy rices with very low amylose content, DNA sequence analysis of rice *wx* mutants (induced by ethyl methanesulfonate treatment or gamma ray irradiation and a spontaneous *wx* mutant) revealed that the *wx* allele in each mutant originated independently, and is not due to the same mutation event (Inukai et al. 2000). In addition, the starch qualities of waxy rice vary greatly (Juliano and Villareal 1987; Bao et al. 2004). The distinct divergence in waxy rices is in the gelatinization temperature (GT). Waxy rice can be divided into a high- and a low GT group. High-GT waxy rices are harder after cooking and show accelerated staling compared to low-GT rices, but significant differences are also observed among some low-GT pairs (Villareal et al. 1993). The physicochemical properties of waxy rices also differ between the subspecies, *indica* and *japonica*. Hot paste viscosity, cool paste viscosity and flour swelling volume were higher in *indica* than in *japonica* waxy rice (Bao et al. 2004). Clearly, the presence of the waxy gene does not guarantee the same starch properties and end-use quality.

In many food applications, waxy rice is preferred to nonwaxy rice due to its characteristic starch physiochemical properties. To select the desired starch properties for food applications is one of the most important aims in waxy rice breeding. There are few breeding programs particularly focusing on waxy rice in China, though many units at different levels (national, provincial or county level) are engaged in rice breeding. A breeding program in Shaoxing Academy of Agricultural Sciences is uniquely focusing on waxy rice destined for Shaoxing wine (yellow wine) brewing (Wang et al. 1999). Many *indica* breeding lines have been developed but only a few of them are low in GT, which is preferred for brewing. Other important traits such as high-yield potential, and resistance to diseases and pests, should be incorporated into new waxy rice varieties. Poor progress in waxy rice breeding may be due to several reasons. One of the limitations is inadequate knowledge of genetic diversity of the germplasm or narrow diversity of parents used in breeding. Another reason is that waxy rice breeding has received little attention and research on waxy rice is limited especially at the molecular level.

Amplified fragment length polymorphism (AFLP) and inter-simple sequence repeat (ISSR) are efficient DNA fingerprinting methods that do not require prior knowledge of the nucleotide sequence to be analyzed (Zietkiewicz et al. 1994; Vos et al. 1995). One single AFLP or ISSR reaction can simultaneously identify multiple polymorphisms at various loci throughout the genome, depending on the amount of variation between the cultivars being studied. AFLP and ISSR have been widely used for genetic diversity studies in rice (Zhu et al. 1998; Ashikawa et al. 1999; Blair et al. 1999; Fuentes et al. 1999; Qian et al. 2001). However, no study has been reported on the genetic diversity of waxy rice using molecular markers.

We have previously analyzed genetic variation in starch physicochemical properties in waxy rice accessions, showing wide diversities in both *indica* and *japonica* waxy rice (Bao et al. 2004). The objective of present study is to evaluate genetic diversity of waxy rice accessions using AFLP and ISSR molecular markers, to facilitate the use of germplasm in waxy rice breeding.

Materials and methods

Rice materials and DNA isolation

A total of 56 waxy rice accessions (varieties, landraces and breeding lines) were obtained from the rice germplasm center and rice breeding programs in China (Table 1). These accessions represent the provinces of Zhejiang, Guizhou, Henan, Hubei, Jiangsu, Shanxi, Yunnan, Hainan, Jiangxi, Beijing; and accessions 664 and 682 were from Japan. Of the accessions, 34 were *indica* and 22 were *japonica*. All the rice accessions have characteristics of opaque endosperm and have amylose content less than 2% and typical starch pasting viscosity profiles (Bao et al. 2004). These accessions were planted in Hainan in late November, 1999 and

Table 1. List of accessions of waxy rice.

Code[a]	Accessions	Subspecies[b]	Starch GT[c]	Origin[d]
1	Xinguangnuo	J	L	–
2	Cungunuo	I	L	Guizhou
3	Guinuo no. 1	I	L	Guizhou
4	Haocunuo	J	L	Guizhou
5	Huangjinnuo	J	L	Guizhou
6	Jiainuo	I	L	Hubei
7	Aiganyaxuenuo	I	L	Jiangsu
8	Suyunuo	J	L	Jiangsu
9	Accession 664	J	L	Japan
10	Accession 682	J	L	Japan
11	Chunjiangnuo no. 2	J	L	Zhejiang
12	Guixiangsinuo	I	H	Zhejiang
13	Xiangnuo no. 4	J	L	Zhejiang
14	Longqingzixiangnuo	J	L	Yunnan
15	148 nuo	I	L	Zhejiang
16	Zhonghuazixiangnuo	I	L	Beijing
17	Shao 9610	I	H	Zhejiang
18	Zaoxiangnuo	I	L	–
19	Biyunzaonuo	I	H	–
20	Zhenongda 454	J	L	Zhejiang
21	Shaonuo 9617	J	L	Zhejiang
22	T1046	I	H	–
23	Zhenuo no. 2	J	L	Zhejiang
24	Bing 9302	J	L	Zhejiang
25	Zhuyunnuo	I	H	Zhejiang
26	Shaonuo 9144	J	L	Zhejiang
27	Chunjiangnuo 681	J	L	Zhejiang
28	Chunjiangnuo no. 3	J	L	Zhejiang
29	Bing 97252 nuo	J	L	Zhejiang
30	Shaonuo 9714	J	L	Zhejiang
31	Shaonuo 985	J	L	Zhejiang
32	Shaonuo 928	J	L	Zhejiang
33	Linxiangnuoxuan	J	L	–
34	Lishuinuo	I	L	Zhejiang
35	Cinuo 48	I	L	–
36	Dadongnuo	I	L	Zhejiang
37	Shaximonuo	I	L	Shanxi
38	Dian 4	I	L	–
39	Yunanheixiannuo	I	L	Henan
40	Xiangnuo G982280	I	H	Jiangxi
41	Ganxiangnuo	I	H	Jiangxi
42	Yichunnuo 12	I	L	–
43	Zhongyu no. 8	I	L	Beijing
44	Jiangxinuodao	I	L	Jiangxi
45	Youzhizaoxiannuo	I	H	Zhejiang
46	Zaojingnuo	I	H	–
47	Yangfunuo no. 3	I	L	Jiangsu
48	PII 121	J	L	–
49	Shao 9915	I	H	Zhejiang
50	Yuenuo no. 1	I	H	Zhejiang
51	Shao 9924	I	L	Zhejiang
52	Shao 9929	I	H	Zhejiang
53	Shao 9997	I	H	Zhejiang
54	Shao 99102	I	H	Zhejiang
55	Yuenuo 981	I	H	Zhejiang
56	Hainannuodao	I	L	Hainan

[a] Code used in the present study as shown in figures.
[b] I and J represents *indica* and *japonica*, respectively.
[c] GT: gelatinization temperature of starch, L: low GT; H: high-GT (Bao et al. 2002, 2004).
[d] Only those with known origin (province in China) were listed, but two accessions (No. 9 and No. 10) were from Japan.

one panicle was harvested from each accession in late March 2000. Five seeds per accession were germinated for DNA extraction. Genomic DNA was extracted from fresh young leaves using the CTAB method (Doyle 1991).

AFLP analysis

Amplified fragment length polymorphism analysis was performed as described by Vos et al. (1995) with minor modification (Huang and Sun 1999). Genomic DNA (250 ng) was double-digested at 37 °C overnight in a final volume of 25 μL with *Eco*RI and *Mse*I (5 units each). *Eco*RI (5 pmol) and *Mse* I (50 pmol) adapters were subsequently ligated to the digested DNA fragments with 1 unit of T4 DNA ligase at 20 °C for 2 h. The adapter-ligated DNA was preamplified in a total volume of 25 μL with AFLP primers, each carrying one selective nucleotide. Twenty cycles were carried out at 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s.

The preamplification products were diluted five times, and an aliquot of 1 μL was used as template for selective amplification with *Eco*RI and *Mse*I primers each having three selective nucleotides at the 3′ ends (Table 2). *Eco*RI primers were 5′ end-labeled with fluorescein Cy5 (Amersham Pharmacia Biotech.). The following cycling parameters were used for selective amplification: first cycle at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s, the annealing temperature was then lowered 0.7 °C each cycle during the following 12 cycles, and after the touch down phase of 13 cycles it reached the optimal annealing temperature of 56 °C. The amplification was continued for an additional 23 cycles at 94 °C for 30 s, 56 °C for 30 s and 72 °C for 60 s. All amplifications were carried

Table 2. Total number of amplified bands with each primer combination or a single primer and polymorphism revealed by AFLP and ISSR analyses.

Primers	Total number of bands	Polymorphic bands	Polymorphism (%)
AFLP			
E-AAG/M-CAT	127	96	75.6
E-AGC/M-CTC	75	60	80.0
E-ACT/M-CTA	76	62	81.6
E-AAG/M-CAC	39	26	66.7
E-ACT/M-CTT	41	36	87.8
Total	358	280	78.3
ISSR[a]			
$BDB(AC)_7$	83	77	92.8
$(AG)_8T/BDB(AC)_7$	107	98	91.6
Total	190	175	92.2

[a] B stands for C, G or T; D stands for A, G or T.

out using a PTC-100 thermal cycler (MJ Research Inc.).

ISSR analysis

A set of simple sequence repeat (SSR) primers was purchased from the Biotechnology Laboratory, University of British Columbia, Canada (UBC primer Kit #9). Analysis of ISSR markers was according to Huang and Sun (2000). Primers 807 [$(AG)_8T$], and 888 [$BDB(AC)_7$] (abbreviations for degenerative base positions: B = C, G or T; D = A, G or T) were 5′ end labeled with fluorescein Cy5 (Amersham Pharmacia Biotech.). Each 20 μL amplification reaction consisted of 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X 100, 2 mM $MgCl_2$, 0.1 mM dNTPs, 2% formamide, 200 nM primers, 0.5 unit of Taq polymerase, and 20 ng of genomic DNA. All amplifications were performed on a PTC-100 thermal cycler (MJ Research, Inc.) under the following conditions: 5 min at 94 °C, followed by 45 s at 94 °C, 60 s at 52 °C, and 60 s at 72 °C for 35 cycles, and 7 min at 72 °C for a final extension.

Electrophoresis and gel analysis

In both AFLP and ISSR analyses, the amplification products were mixed with an equal volume of formamide dye (98% formamide, 10 mM EDTA pH 8.0, 0.1% bromophenol blue and xylene cyanol). After being denatured at 90 °C for 3 min and immediately chilled on ice, 8 μL of the sample was run through a 6% polyacrylamide gel for 6 h under standard sequencing conditions in an ALFexpress™ automated sequencer (Amersham Pharmacia Biotech.). A DNA ladder (ranging in length from 50 to 500 bp) labeled with Cy5 was used to determine the size of the AFLP and ISSR bands. The resulting images were analyzed with the software package DNA Fragment Manager version 1.2 (Amersham Pharmacia Biotech.).

Data analysis

In both AFLP and ISSR analyses, the banding patterns were recorded automatically with the Fragment Manager software package (Amershan Pharmacia Biotech). Each polymorphic AFLP and ISSR band was given a score of 1 for presence or 0 for absence. The data matrix was analyzed using PAUP * 4.0b10 (Swofford 1998) to obtain Nei and Li's (1979) pairwise distances among the accessions. The Mantel test (Mantel 1967) for matrix correspondence was conducted to compute the product-moment correlation (r) between AFLP and ISSR distance matrices. The dendrogram was generated using the unweighted pair-group method with arithmetic means (UPGMA) based on Nei and Li's distance matrix.

Results

Fingerprinting patterns of AFLP and ISSR

Five primer combinations were used to assay the 56 accessions, generating a total 358 AFLP bands ranging in size from 50 to 600 bp, of which 280 were polymorphic (Table 2). The polymorphism averaged 78.3% over primers, ranging from 75.6% with E-AAG/M-CAT to 87.8% with E-ACT/M-CTT. The E-AAG/M-CAT primer combination produced a total of 127 bands, of which 31 bands were shared by all accessions. The E-AAG/M-CAC primer combination produced many fewer bands (39) than E-AAG/M-CAT, although only one nucleotide was different between the primer combinations. This difference could also be seen between the E-ACT/M-CTA and E-ACT/M-CTT combinations. The average number of bands produced for each accession was 228 with five

Table 3. Genetic distance of seven waxy rices showing the shortest and longest distances revealed by AFLP and ISSR bands.

Accession	8	11	25	38	42	49
8 Suyuyuo	–					
11 Chunjiangnuo no 2	0.0288	–				
25 Zhuyunnuo	0.0789	0.0764	–			
38 Dian4	0.0505	0.0478	0.0444	–		
42 Yichunnuo12	0.0483	0.0445	0.0475	0.0102	–	
49 Shao 9915	0.0501	0.0500	0.0468	0.0299	0.0290	–
50 Yuenuo no.1	0.0540	0.0532	0.0440	0.0318	0.0330	0.0124

primer combinations, ranging from 184 in Chunjiangnuo no. 3 to 264 in Shao 9915.

The single SSR primer BDB$(AC)_7$ produced a total of 88 bands ranging from 200 to 600 bp, of which 77 bands were polymorphic (Table 2). The double SSR primers $(AG)_8$T/BDB$(AC)_7$ produced more bands than the single primer BDB$(AC)_7$ used alone (Table 2). As shown in Huang and Sun (2000), many of the bands amplified with double primers were shorter than those amplified with a single primer, i.e., more bands less than 200 bp were produced with the double primers $(AG)_8$T/BDB$(AC)_7$. The average polymorphism of the total ISSR markers was 92.2%, much higher than AFLP polymorphism. The average number of bands generated for each accession in ISSR analysis was 105, ranging from 80 bands in Bing 97252 nuo to 124 bands in 148 nuo.

Genetic distances and cluster analysis

The AFLP and ISSR bands were used to determine the genetic distances among accessions. It was shown that the genetic distance matrices for AFLP and ISSR data sets were significantly correlated ($r = 0.731$, $P = 0.004$). In order to obtain more accurate genetic distance estimates, combined analysis was carried out using all the AFLP and ISSR bands together. The resulting Nei–Li distance matrix containing all accessions is not presented. Only seven accessions that have the longest or shortest pairwise genetic distances are listed in (Table 3). The shortest genetic distance was found between Dian 4 and Yichunnuo 12 (0.0102), and the second shortest genetic distance was between Shao 9915 and Yuenuo no. 1 (0.0124). Zhuyunnuo had the longest genetic distance with Suyunuo (0.0789), and it also had the second longest distance with Chunjiangnuo no. 2 (0.0764).

The Nei–Li distance matrix based on combined AFLP and ISSR data was used to generate a dendrogram showing genetic relationships among the accessions (Figure 1). An unambiguous classification into *indica* and *japonica* groups was obtained. According to its Chinese name, Zaojingnuo is an early japonica rice, but it was placed into the *indica* group with both AFLP and ISSR markers. Using a subspecies-specific microsatellite for starch branching enzyme I (Bao et al. 2002), Zaojingnuo has been confirmed to be an *indica* rice. A possible reason for this was admixture in the collection taken for this accession, or that this accession had been given an erroneous name.

In the *japonica* group (the top clade in Figure 1), the currently released varieties and breeding lines, such as Shaonuo 9617, Shaonuo 985, Shaonuo 9714, Shaonuo 928, Shaonuo 9144, Chunjiangnuo 681, Chunjiangnuo no. 3 and Bing 97252 nuo were clustered together using both AFLP and ISSR markers, indicating that these rices are closely related or have low diversity. Other germplasm accessions such as Xinguangnuo, Huangjinnuo and Haocunuo collected from the Germplasm Center of the Chinese National Rice Research Institute (CNRRI) were grouped together; this cluster also included three varieties and one breeding line: Chunjiangnuo no. 2 and Xiangnuo no. 4 were released by CNRRI, Zhenongda 454 was released by Zhejiang University, and Bing 9302 was a breeding line from Jiaxing Academy of Agricultural Sciences in Zhejiang. PII 121 and Suyunuo showed less similarity to most other *japonica* rices.

Similarly, the recently released breeding lines in the *indica* group (the bottom clade in Figure 1), such as Shao 9915, Yuenuo no. 1, Shao 9924,

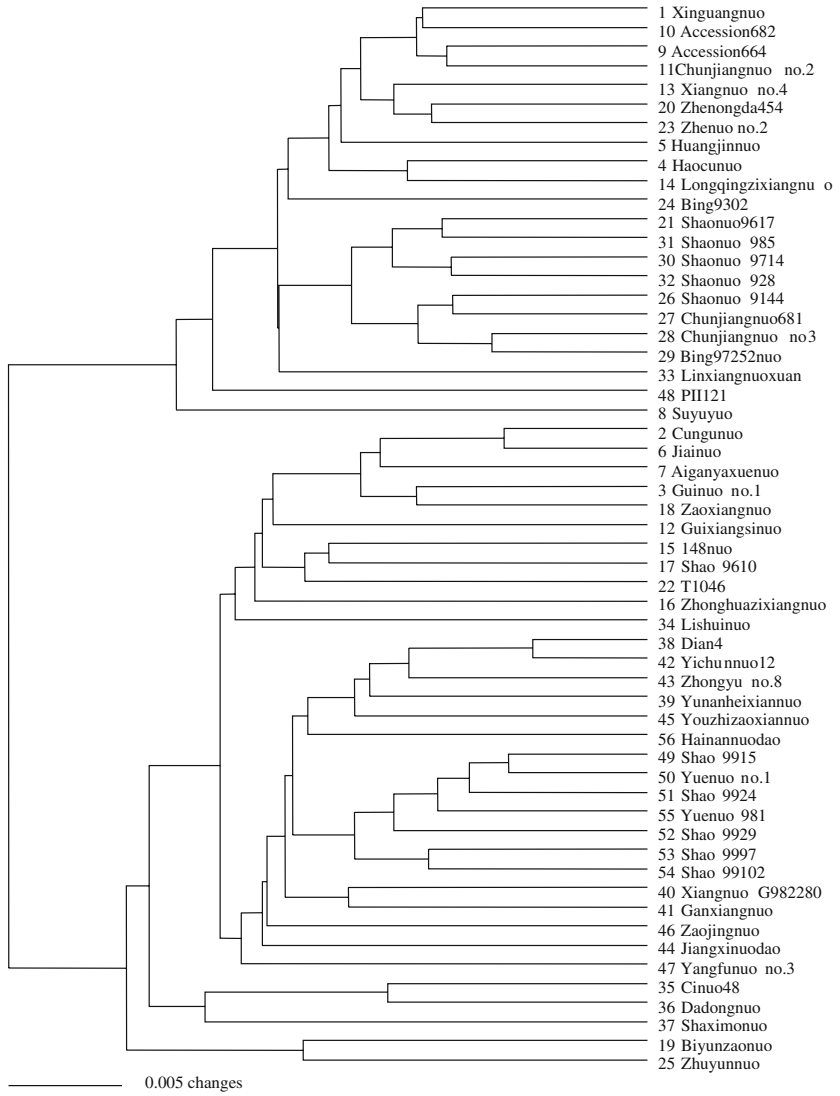

Figure 1. UPGMA dendrogram generated with AFLP and ISSR bands based on Nei and Li's (1979) distance matrix.

Yuenuo 981, Shao 9929, Shao 9997, Shao 99102, also clustered together. These breeding lines and varieties were collected from Shaoxing Academy of Agricultural Sciences. Shao 9610, a breeding line selected in earlier years, was more distant to those released in recent years. The landraces such as Cungunuo, Jiainuo and Aiganyaxuenuo were clustered in the same subgroup as Shao 9610. Cinuo 48, Dadongnuo and Shaximonuo were clustered together to form a small subgroup from the above two indica subgroups, while Biyunzaonuo and Zhuyunnuo were clustered in another subgroup distant from all other *indica* rice.

Specific markers for different groups

The cluster analysis performed for the AFLP and ISSR data easily differentiated *indica* and *japonica* as distinct subspecies. However, only one *indica*-specific AFLP marker of 170 bp in length was found generated with the E-AAG/M-CAC primer combination (Figure 2), whereas no specific ISSR markers were found for each subspecies group. Waxy rice could also be divided into high- and low-GT group (Bao et al. 2002, 2004), but neither AFLP nor ISSR markers were found to be specific for the two GT groups.

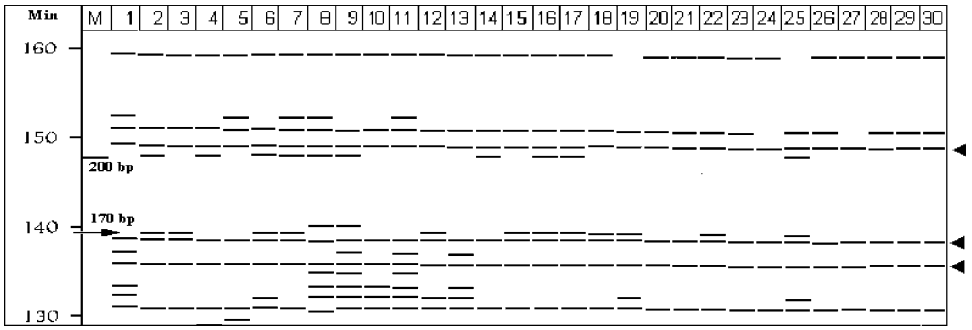

Figure 2. The *indica* specific AFLP marker of 170 bp generated with E-AAG/M-CAC combination (arrow mark on the left). Arrow marks on the right show monomorphic bands in both *indica* and *japonica* rice. M = marker, 1 = Xinguangnuo, 2 = Cungunuo, 3 = Guinuo no. 1, 4 = Haocunuo, 5 = Huangjinnuo, 6 = Jiainuo, 7 = Aiganyaxuenuo, 8 = Suyunuo, 9 = Accession 664, 10 = Accession 682, 11 = Chunjiangnuo no. 2, 12 = Guixiangsinuo, 13 = Xiangnuo no. 4, 14 = Longqingzixiangnuo, 15 = 148 nuo, 16 = Zhonghuazixiangnuo, 17 = Shao 9610, 18 = Zaoxiangnuo, 19 = Biyunzaonuo, 20 = Zhenongda 454, 21 = Shaonuo 9617, 22 = T1046, 23 = Zhenuo no. 2, 24 = Bing 9302, 25 = Zhuyunnuo, 26 = Shaonuo 9144, 27 = Chunjiangnuo 681, 28 = Chunjiangnuo no. 3, 29 = Bing 97252 nuo, 30 = Shaonuo 9714.

Discussion

China is currently focusing on conservation, exploitation and utilization of major crop germplasm including rice, wheat, maize and soybean (http://icgr.caas.net.cn/973). Studies on genetic diversity of wild rice in China have been undertaken (Qian et al. 2001). However, there has been relatively little research on waxy rices. The opaque endosperm is usually regarded as a phenotypic marker for waxy rice, and other phenotypic and genotypic differences among waxy rices have often been neglected. As explained earlier, due to various reasons, some waxy landraces have lost the opaque endosperm character and been classified as nonwaxy rice in the database maintained at the CAAS. On the other hand, even among those carrying the waxy gene, their starch physicochemical properties display wide diversity, especially for starch gelatinization temperature. Additionally, waxy rice breeding is carried out on a limited scale, and correspondingly research on waxy rice is scarce. Therefore, to augment present genetic knowledge and to support breeding practices, genetic diversity studies on waxy rice are very important.

The present study showed that inter-subspecies differences could be clearly revealed by AFLP and ISSR markers as well as some of the intra-subspecies relationships (Figure 1). Our results clearly indicate that many of newly released varieties and new breeding lines tend to be clustered together, and are more distant from those landraces collected from the germplasm center. These results have implications for germplasm conservation as well as for waxy rice breeding. For germplasm conservation, the phenotypic and genetic variation, other than only the opaque endosperm, in waxy rice should not be neglected during conservation. For waxy rice breeding, more distantly related parents (such as landraces) could be introduced into breeding programs, and inter-subspecies hybridization could also be employed to increase genetic diversity.

Only one AFLP marker was *indica* specific, and this marker could be cloned and converted to a sequence characterized amplified region marker (Cho et al. 1996; Ashikawa et al. 1999). However, our method using fluorescence detection in the automated sequencing system does not allow cutting of the band from the gel, because DNA is leaching out after fluorescence detection. We are interested in finding a starch GT specific marker, but none of the 455 markers was GT specific. GT specific markers could improve early selection efficiency in waxy rice breeding programs.

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