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A Comparative Study of SSR Diversity in Chinese Major Rice Varieties Planted in 1950s and in the Recent Ten Years (1995-2004)

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Abstract: Forty pairs of SSR markers were used to compare the genetic diversity changes in 151 Chinese major rice varieties planted in 1950s and in the recent ten years. Of 40 SSR loci, 39 were found to be polymorphic while one locus (RM479) monomorphic. A total of 213 alleles were identified from the 39 polymorphic loci. The average number of alleles per locus (*Na*) was of 5.5, ranging from 2 to 11. Nei's gene diversity index (*He*) varied drastically among loci from 0.309 at RM174 to 0.869 at RM418, with an average value of 0.649. There existed significant difference in SSR allelic diversity between indica and japonica subspecies, and indica had more variation than japonica both in *Na* and *He*. By comparison with the genetic changes in *Na* and *He*, it was revealed that the varieties planted in 1950s had more alleles and higher *He* than those in the recent ten years both for indica and japonica rices. The difference between two subspecies for *Na* was significant in a tendency over time (indica: $z = 2.677$, $P = 0.007$; japonica: $z = 3.441$, $P = 0.001$), but not significant for *He* (indica: $z = 1.471$, $P = 0.141$; japonica: $z = 1.932$, $P = 0.053$). Analysis of molecular variance (AMOVA) indicated that there existed significant difference ($P < 0.05$) in genetic variation between the two periods, of which more genetic variation was contributed by indica (*Fst* = 0.050) and japonica (*Fst* = 0.082) subsets. Using locus-by-locus AMOVA procedure, significant genetic differentiations were observed in 13 loci (RM21, RM128, RM147, RM169, RM190, RM221, RM231, RM251, RM253, RM317, RM341, RM418, and RM478) for indica varieties and 11 loci (RM101, RM135, RM152, RM159, RM169, RM190, RM251, RM253, RM311, RM418, and RM478) for japonica ones between the two periods. It was found some alleles had been lost in current major rice varieties as comparing with those in 1950s. Therefore, it should be necessary to exploit more alien elite genetic resources for extension of genetic background in current rice breeding program.
Key word: rice (*Oryza sativa*); major varieties; simple sequence repeats; genetic diversity; analysis of molecular variance

In general, crop breeding will lead to the increase of food yield and other products to meet various demands of the people, meanwhile will lead to the decline of crop biological diversity resulting in genetic vulnerability [1-7] to adverse environment. This phenomenon has been revealed by the comparative studies on the genetic diversity changes in DNA level in wheat in Europe [8-9], oat in Canada [10], and maize in France [11]. However, some scientists did not agree with this idea, thinking that due to the progress of breeding techniques and the increasing germplasm exchange in modern crop breeding, the genetic background of the current varieties was enlarged, which has been indicated by those studies with the molecular biological methods on the modern varieties of the spring wheat [12], hard grain wheat [13], winter wheat in England [14], wheat in Argentina [15] and barley in Europe [16]. Fu et al [10] conferred that these different results may be caused by differences of sample size (number) and marker types used.

Rice is the first food crop feeding about 60% population in China. The Chinese modern rice breeding started since 1919. With about 80 years of breeding work, more than 5000 modern varieties have been released to farmers. But systematic studies were deficient on the comparison of DNA diversity for these varieties. Zhuang et al [18] reported that the genetic background of the rice varieties in current years were rather narrow, which was revealed by the result with 50 RFLP markers among 25 indica varieties and their parentages. Yang et al [19] considered that the genetic diversity of modern rice varieties were not lower than that of landraces by SSR analysis on 238 varieties. Qi et al [20] suggested that the genetic diversity of rice varieties was declined

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since 1950s, but increased greatly in 1990s.

In the present study, the genetic diversity of 151 rice varieties (78 in 1950s and 73 in the recent ten years of 1995-2004) was compared by using 40 simple sequence repeats (SSR) markers for assessing the genetic diversity of the varieties planted in the two periods. It would provide useful reference for better exploitation of elite germplasm and parent selection in rice breeding program.

MATERIALS AND METHODS

Rice materials

One hundred and fifty-one major varieties with large planted area were selected for the genetic study (Table 1). To eliminate the possible deflection resulting from sample size (number), 78 and 73 varieties planted in 1950s and the recent ten years (1995-2004) respectively were selected. Among all the tested materials there were 118 varieties, each of which had an annual planted area of more than 67 000 ha during the above mentioned periods, respectively. Moreover, these 151 varieties could be grouped into two subspecies: 79 indica and 72 japonica. All materials tested came from the rice germplasm bank of China National Rice Research Institute (CNRRI).

DNA extraction and SSR analysis

The upper parts (1-2 cm) of fresh young leaves were cut and then ground in liquid nitrogen. DNA was extracted following the modified CTAB procedure as described by Zheng et al [21]. Forty pairs of SSR primers (2-4 pairs each chromosome) were selected for SSR analysis (Table 2).

PCR reaction was carried out in 10 μL reaction system containing 2.0 μL of 10×buffer, 1.0 μL of 2 mmol/L dNTPs，1.0 μL of 25 mmol/L $MgCl_2$, 0.6 μL each of 10 μmol/L forward and reverse primers，0.1 μL *Taq* polymerase (5 U/μL) and 20 ng template DNA. DNA amplification was performed in a PTC-100 96v Thermocycler (MJ Research Inc.). The PCR reaction was conducted with following steps: pre-denature at 94℃ for 1 min, followed by 35 cycles of 94℃ for 5 min, 55℃ for 1 min and 72℃ for 2 min, and final extension at 72℃ for 5 min. The amplified products were electrophoresed on 6% non-denaturing polyacrylamide gel, and detected using silver staining [22].

Data analysis

All SSR data were obtained according to SSR reference provided by http://www.gramene.org, with one pair of SSR primer referring for one locus and one band for one allele. The number of alleles (*Na*) and

Table 1. Rice varieties used in this study.

Period	Subspecies	Variety
1950s	indica	Mafangxian, Nantehao, Lucaihao, Liantangzao, Guangchang 13, Aijiaonante, Guangchang'ai, Qingxiaojinzao, Liantangzao 4, Zaoxian 503, Nante 16, Bolizhan, Xiazhibai, Leihuozhan, Aizizhan, Hongjiaozhan, Hongjiaozao, Baimifen, Shenglixian (Jiangsu), Shenglixian (Anhui), Wanlixian, Dee-geo-woo-gen, Zhongnong 4, Nanjing 1, Taichung Native 1, Zhongshan 1, Zhechang 9, Zhuyin 2, Zhechang 3, Zhongshanhong, Baotaihong, Huanan 15, Baotai'ai, Shishiqian, Jiaopanzhong, Huanghezi, Hongmidongzhan, Dahuangzhan, Chishubaotaihong, Baimidongzhan, Xiaohongdao, Fanzi
	japonica	Aomori 5, Ginbozu, Ishikari-shiroge, Aikoku, Gong 17, Hejiang 1, Songliao 4, Huangmangdao, Yanzhihong, Baidaozi, Youmangzaojing, Baipidadao, Taichung 65, Chianan 8, Kwangfu 1, Guihuaqiu, Nongken 57, Nongken 58, Tainan 1, Xiaozhanjiangmi, Huangkezaonianri, Feilaifeng, Laolaiqing (Zhejiang), Laolaiqing (Jiangsu), 412, Sishangyu, Sudao 1, Wuzuinuo, 10509, Lizhihong, Laohudao, Taihuqing, Hongkenuo, 261, Hongxujing, Zhumaocu
The recent ten years (1995-2004)	indica	Zhe 733, Xiangzaoxian 11, Zhong 86-44, Xiangzaoxian 13, Xiangzaoxian 14, Jiayu 293, Zhouyou 903, Zhongsi 2, Zhefu 218, Xiangzaoxian 19, Xiangzaoxian 17, Ganzaoxian 37, Zhongyouzao 81, Ganzaoxian 40, Mancang 515, Quannong 3, Zhe 9248, Xiangzaoxian 24, Jiazao 935, Jiayu 948, Zhefu 910, Jiahezaozhan, Xiangzaoxian 31, Zhongjian 100, Shaojia 1, Xian 128, Qishanzhan, Jingxian 89, Qihuangzhan, Xianxiaozhan, Texianzhan 13, Yangdao 6, Yuexiangzhan, Texianzhan 25, Xiangwanxian 3, Xiangwanxian 9, Xiangwanxian 11
	japonica	Dongnong 416, Yujing 6, Liaojing 454, Longjing 8, Jiudao 20, Nongda 7, Liaojing 294, Kenjiandao 7, Ningjing 16, Tong 35, Kendao 8, Wuyoudao 1, Tongyu 124, Kuiku 131, Fujihikari, Kendao 10, Longjing 13, Wuyujing 3, Zaofeng 9, Wuyujing 5, Zhendao 88, Wuyunjing 7, 93-25, Wuyunjing 8, Lianjing 3, Wuxiangjing 14, Ning 67, Xiushui 664, Xiushui 122, Taihujing 2, Bing 91-17, Xiushui 63, Bing 97-59, Bing 96-42, Bing 98-110, Jiahua 1

the Nei's gene diversity index (*He*) [23] were evaluated using POPGENE v 1.31[24], and significance test was conducted for evaluating the changes of *Na* and *He* during the two periods (1950s and the recent ten years) by applying 'Wilcoxon Matched Pairs Test'. The *F*-statistics (*Fst*) were used to analyze the genetic differentiation of the varieties and their tested loci in the two periods based on the allelic discrepancy at each locus by the procedure of AMOVA（Analysis of Molecular Variance）in ARLEQUIN ver 3.0.

RESULTS

SSR diversity

Among 40 pairs of SSR primers used, 39 pairs (97.5%) showed polymorphisms in 151 rice varieties planted in 1950s and the recent ten years. Only RM479 displayed single locus with an allele of 253 bp (Table 2). Thus, 39 polymorphic SSR primers were used for comparison analysis of genetic diversity in

Table 2. Chromosome location, number of rare alleles, number of alleles, and Nei's gene diversity index (*He*) at 40 SSR loci in 151 Chinese major rice varieties.

Locus	Location	Number of rare alleles			Number of alleles (*Na*)			Nei's gene diversity index (*He*)		
		indica	japonica	Total	indica	japonica	Total	indica	japonica	Total
RM128	1	4	3	4	6	4	7	0.571	0.081	0.655
RM265	1	0	1	0	3	4	4	0.532	0.598	0.715
RM174	2	1	1	0	3	3	3	0.366	0.224	0.309
RM211	2	2	2	2	5	3	5	0.365	0.081	0.569
RM221	2	0	1	0	2	2	3	0.292	0.080	0.580
RM341	2	6	3	4	9	6	9	0.575	0.553	0.757
RM135	3	1	2	2	2	5	6	0.049	0.674	0.659
RM231	3	0	1	0	6	4	6	0.701	0.600	0.785
RM251	3	2	3	4	5	5	7	0.544	0.448	0.712
RM293	3	0	1	0	2	3	3	0.162	0.340	0.573
RM142	4	1	1	0	2	2	2	0.025	0.054	0.498
RM261	4	1	3	1	5	5	5	0.664	0.253	0.711
RM317	4	1	1	3	5	2	6	0.603	0.027	0.666
RM335	4	1	4	5	8	8	10	0.683	0.545	0.793
RM159	5	2	2	2	5	6	7	0.631	0.698	0.795
RM161	5	0	2	1	2	4	5	0.240	0.348	0.632
RM169	5	2	2	2	8	4	8	0.807	0.462	0.790
RM178	5	1	1	1	3	2	3	0.184	0.027	0.506
RM162	6	0	2	1	1	6	6	0.000	0.738	0.660
RM190	6	4	1	3	7	4	7	0.498	0.608	0.721
RM253	6	3	2	3	8	5	8	0.712	0.594	0.763
RM125	7	0	0	1	1	2	3	0.000	0.153	0.534
RM418	7	1	1	2	8	6	9	0.848	0.786	0.869
RM427	7	0	1	0	2	2	2	0.119	0.054	0.500
RM478	7	0	1	0	4	3	4	0.497	0.156	0.624
RM152	8	1	2	3	4	4	6	0.672	0.513	0.641
RM230	8	2	1	3	6	5	7	0.670	0.683	0.694
RM215	9	1	1	2	4	5	5	0.553	0.561	0.717
RM285	9	3	0	2	5	4	6	0.189	0.721	0.593
RM288	9	0	2	2	2	3	4	0.096	0.081	0.530
RM147	10	0	1	0	2	2	2	0.292	0.054	0.494
RM222	10	1	0	2	5	5	6	0.648	0.683	0.696
RM269	10	0	2	0	5	3	5	0.778	0.107	0.641
RM311	10	1	1	1	4	3	6	0.633	0.488	0.776
RM21	11	3	4	3	9	8	11	0.844	0.747	0.855
RM332	11	1	0	1	5	3	5	0.651	0.596	0.699
RM479	11	0	0	0	1	1	1	0.000	0.000	0.000
RM101	12	1	3	1	5	7	7	0.517	0.755	0.712
RM277	12	1	1	1	3	2	3	0.186	0.054	0.537
RM463	12	0	0	0	2	2	2	0.450	0.176	0.349

the present study.

As shown in Table 2, a total of 213 alleles were identified in 151 rice varieties by 39 pairs of SSR primers, with variations of 95-320 bp. The number of alleles among tested loci varied drastically from 2 to 11 with an average of 5.5 per locus. Of the 213 alleles, 62 were of rare alleles with low gene frequency < 5%, occupying 29.1% of the total alleles. Average Nei's gene diversity index (*He*) was 0.649, varying from 0.309 (RM174) to 0.869 (RM418). Considering gene diversity index being the function of the allele number and frequency [24], therefore, those SSR markers with higher gene diversity index, like RM418, showed higher tested efficiency.

The two subspecies indica and japonica showed a distinguished difference in SSR diversity. There were 173 alleles identified from 79 indica varieties, ranging from 1 to 9 per locus with an average of 4.4. Among them, 48 were low frequency (< 5%) alleles, and the average gene diversity index (*He*) was 0.458 with the range of 0-0.848. For 72 japonica varieties, 156 alleles were identified, being 9.8% less than that of the indica ones. Among them, 60 were rare alleles, and the average allele number (*Na*) was 4.0 with the range of 2-8 and the Nei's gene diversity index (*He*) was 0.395 ranging from 0.027 to 0.786, which was 13.8% lower than that of indica subspecies.

By the analysis on allele frequency distributions in indica and japonica varieties, it was found that there were 18 SSR markers showing specific characteristics responsible to subspecies (RM128, RM221, RM341, RM135, RM251, RM293, RM142, RM317, RM335, RM161, RM178, RM162, RM125, RM427, RM288, RM147, RM311 and RM277). These markers could be used for identifying indica and japonica rices.

Genetic diversity in different periods

Higher genetic diversity was noted in varieties planted both in 1950s and the recent ten years, with rich variation in each SSR locus (Table 3). The *Na* and *He* were 5.2 and 0.646 in 78 varieties in 1950s, respectively, slightly higher than those of 73 varieties in the recent ten years (*Na* and *He* were 4.8 and 0.630, respectively). Wilcoxon Matched Pairs Test showed that the *Na* changed at significant level (*Na*: $z = 2.259$, $P = 0.024$) whereas the *He* did not (*He*: $z = 1.828$, $P = 0.068$). The trends of *Na* and *He* changes within indica and japonica subspecies were similar to entire sample set, e.g. the *Na* and *He* of the varieties in 1950s were higher than those in recent ten years. The *Na* change was significant (for indica, $z = 2.677$, $P = 0.007$; for japonica, $z = 3.441$, $P = 0.001$) whereas *He* change was not (for indica, $z = 1.471$, $P = 0.141$; for japonica, $z = 1.932$, $P = 0.053$). These differences of *Na* and *He* changes between the two periods might be related to rare alleles.

Genetic differentiation in the periods

AMOVA analysis showed that most variations of SSR were within periods (Table 4). The genetic variation was only 1.9% between the periods, but it was significant at 5% level. The genetic differentiations between the two periods both in indica and japonica varieties were at very significant level ($P<0.001$), being 5.0% and 8.2% of variation for indica and japonica respectively, both higher than that of entire sample set. On the other hand, the genetic differentiations in various loci in indica and japonica were different. Of 39 SSR loci in indica varieties, the alleles of 13 SSR loci (33.3%) (RM21, RM128, RM147, RM169, RM190, RM221, RM231, RM251,

Table 3. Comparison of mean number of alleles per locus (*Na*) and average Nei's gene diversity index (*He*) between the two periods.

Period	Subset	No. of varieties	Number of alleles per locus (*Na*)			Nei's gene diversity index (*He*)		
			Mean±*SD*	Minimum	Maximum	Mean±*SD*	Minimum	Maximum
1950s	indica	42	4.1±2.2	1	9	0.454±0.281	0	0.830
	japonica	36	3.6±1.4	2	7	0.395±0.245	0.054	0.759
	Total	78	5.2±2.2	2	10	0.646±0.127	0.311	0.849
The recent ten years (1995-2004)	indica	37	3.5±1.7	1	8	0.425±0.237	0	0.824
	japonica	36	3.0±1.4	1	6	0.349±0.277	0	0.753
	Total	73	4.8±2.1	2	11	0.630±0.125	0.267	0.872

Table 4. Analysis of molecular variance (AMOVA) for varieties between the periods of 1950s and the recent ten years (1995-2004).

Sample subset	Source	*df*	Variance component	Percentage of variation (%)	*P*
Entire sample	Between periods	1	0.246	1.9	0.016
	Within periods	149	12.614	98.1	
	Total	150	12.860		
indica	Between periods	1	0.466	5.0	<0.001
	Within periods	77	8.803	95.0	
	Total	78	9.269		
japonica	Between periods	1	0.669	8.2	<0.001
	Within periods	70	7.467	91.8	
	Total	71	8.136		

RM253, RM317, RM341, RM418 and RM478) exhibited significant differences between the two periods. Among them, RM221 had the highest genetic differentiation in the two periods, accounting for 37.8% of the total variation. The number of SSR loci with significant differences between the two periods in japonica varieties was slightly lower than that in indica ones. There were 11 SSR loci (28.2%) with significant differences (RM101, RM135, RM152, RM159, RM169, RM190, RM251, RM253, RM311, RM418 and RM478) between the two periods. RM152 had the highest genetic differentiation, in which 55.8% of the genetic variation was contributed from the difference between the two periods (unpublished data).

DISCUSSION

As comparing with allozyme analysis, it is clear that SSR could assess richer polymorphism of rice germplasm [26]. In the present study, 151 main varieties showed higher SSR diversity. Of 40 SSR loci, 39 revealed genetic diversity with average 5.5 alleles per locus, higher than that of main hybrids [27-28] as well as a part of Yunnan landraces [29] cultivated in China, but 56.3% lower than that of the core collection of Chinese modern varieties [20]. Moreover, the genetic diversity of 72 japonica varieties in the present study was lower than that of 72 japonica varieties from 11 ecotypes of China and abroad detected by Liu et al [30]. This might be attributed to the differences in materials and SSR markers used. Besides, we found about 45% of the SSR markers in the study showing indica-japonica differentiation, similar to the results of the previous studies by other researchers [28, 31-32], suggesting that SSR markers combined with agronomic traits could be used to well identify indica and japonica subspecies.

In the previous study, Qi et al [20] had analyzed the genetic diversity of 257 varieties from 1950s to 1990s in China by using SSR markers and agronomic traits, and considered that the genetic diversity index declined from 1950s to 1980s but increased significantly in 1990s, whereas allele number and phenotype number were lower in 1950s and the highest values were found in 1990s. In our study, indica and japonica varieties showed similar temporal trends, i.e. both Nei's gene diversity index (*He*) and average allele number (*Na*) of the varieties in 1950s were higher than those of the recent ten years. The *He* was not significantly different between the two periods, but the *Na* was significant, suggesting some genes had been lost for rice varieties planted in the recent ten years. Therefore, strengthening the introduction and utilization of alien elite germplasm should be considered as an important issue for rice breeding program. The different results from our and Qi's studies were possibly attributed to the difference in materials used as well as sample size.

In this study, AMOVA analysis indicated that the genetic differentiation was significant although the genetic variance was smaller (1.9%) between the two periods, supporting the results from comparison study on average allele number and Nei diversity index. Meanwhile, AMOVA analysis could provide better way for analyzing temporal change of SSR loci.

Therefore, by combining with AMOVA analysis and other genetic diversity parameters, we could well evaluate genetic diversity changes for various ecotypes of rice germplasms.

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