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Comparisons on Genetic Diversity among the Isonuclear-Alloplasmic Male Sterile Lines and Their Maintainer Lines in Rice

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Abstract: Four sets of rice isonuclear-alloplasmic lines including 16 male sterile lines and their maintainer lines were analyzed by using 91 pairs of SSR primers to study the genetic diversity of nuclear genome and their relative relationships. A total of 169 alleles were detected in the 16 lines, with a frequency of polymorphic loci of 53.85% and an average number of alleles per locus of 1.8, and the average gene diversity was 0.228. Four sets of the isonuclear-alloplasmic male sterile lines shared 146 identical alleles, corresponding to 86.39% of the total alleles; meanwhile, there are 23 different alleles among the tested materials, being 13.61% of the total alleles. On average, 78.70% identical alleles and 21.30% different alleles of the total alleles were detected between the isonuclear-alloplasmic male sterile lines and their maintainer lines. There were 53.85% identical alleles and 46.15% different alleles of the total alleles among the homozygous allonucleus male sterile lines. The fingerprints were established for some male sterile lines and maintainer lines. All the materials tested were divided into three groups at the 0.2 genetic distance based on the cluster analysis. Eight lines of Huanong A and Huayu A (including Huanong B and Huayu B) were in the first group, four lines of Kezhen A (including Kezhen B) in the second group, and four lines of Zhenshan 97A (including Zhenshan 97B) in the third group. For the isonuclear-alloplasmic male sterile lines, the similarity coefficient between Y (Yegong) type and WA (wild abortive) type or between CW (Raoping wild rice) and WA type reached 87-98%.

Key words: hybrid rice; isonuclear-alloplasmic male sterile line; maintainer line; genetic diversity

The key to the exploitation of heterosis for the three-line hybrid rice is to breed outstanding parents, especially the male sterile lines. In China, the hybrid rice combinations with WA (wild abortive) cytoplasm were always the mainly cultivated varieties in rice production. According to the statistical data, the cultivated area for the combinations with WA cytoplasm accounted for 69.0%, 64.7% and 57.8% of the total rice cultivated area in 1996, 1997 and 1998, respectively [1]. The trends of cytoplasm monophyletism are likely to result in the genetic vulnerability. Therefore, to find and utilize excellent male sterile lines with new cytoplasms from all kinds of rice germplasm is an important task. Cai et al from South China Agricultural University have bred several sets of isonuclear-alloplasmic lines with the same nucleus background and different cytoplasms (including Yegong, wild rice abortive and Raoping wild rice) by several backcrosses. By using these materials, the cytoplasmic effect was investigated [2-3], and a series of Y(Yegong) type male sterile lines with new cytoplasm derived from a traditional indica late rice variety Yegong in South China were bred [4]. Among them, Y Huanong A passed achievement appraisal of Guangdong Province in 1997 and registered for national new plant variety authority in China in 2000. By April in 2006, 19 new hybrid rice combinations have been bred with Y Huanong A and have been registered by national or provincial crop variety approval committees for about 30 times. It is shown that these new male sterile lines have great breeding values.

By now, the male sterility [6-7], agronomic characters [8], cytoplasmic effects [3, 9] and resistances to rice blast and the whitebacked planthopper [10-11] of isonuclear-alloplasmic male sterile rice lines have been studied. However, few reports were found on the differentiation of their nuclear genomes. Simple sequence repeat (SSR) marker is newly developed and

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has been widely used in the construction of linkage maps, gene mapping, genetic relationship analysis and variety identification due to its advantages such as high reliability and reproducibility and rich polymorphisms in varieties. Moreover, SSR marker has its obvious advantage on the identification of hybrid rice and its parents because it belongs to the codominance marker [12]. In this study, four sets of rice isonuclear-alloplasmic male sterile lines (including Huanong A, Huayu A, Kezhen 2A and Zhenshan 97A) and their maintainer lines were selected to analyze their genetic diversity of nuclear genomes and their relative relationships.

MATERIALS AND METHODS

Plant materials

The rice materials used in the experiment are shown in Table 1, including four sets of the isonuclear -alloplasmic male sterile lines such as Huanong A, Huayu A, Kezhen 2A and Zhenshan 97A, which were bred by using traditional rice variety Yegong, wild rice abortive and Raoping wild rice as cytoplasmic donors, and their four maintainer lines. The male sterile lines and their maintainer lines in the same set shared the common nuclear genome background and different cytoplasmic backgrounds.

DNA extraction

DNA was extracted by the simple SDS extraction method [13] with minor modifications.

PCR reaction

The reaction was conducted in 20 μL containing 3 μL primers, 2 μL 10×PCR buffer (involving 15 mmol/L $MgCl_2$), 0.3 μL dNTPs (10 mmol/L), 50-100 ng template DNA, 1 U *Taq* enzyme. The reaction program was pre-denatured at 94℃ for 5 min, 35 cycles with 1 min at 94℃, 1 min at 55℃ and 1 min at 72℃, finally extended for 5 min at 72℃. Then the amplified products were electrophoresed on the 6% denatured polyacrylamide gel.

Statistical analysis

By comparing with the marker standard bands, the main bands of each SSR marker were read from down to up on the gel. The main bands at different positions were treated as different alleles of a same marker, and '1' was assigned when having an allele while '0' was assigned when the position was empty. The genetic distance was calculated by Treecon software (Version 1.3b) [14] with SSR marker data. The formula of genetic distance (GD) is: $GD=1-GS$, where GS means genetic similarity and its formula is $GS=2N_{ij}/(N_i+N_j)$, N_{ij} means the number of common bands between two genotypes while N_i and N_j mean the number of different bands between two genotypes. Cluster analysis was made by UPGMA method. The genetic diversity index such as polymorphic loci percentage was calculated according to Sun et al [15].

Table 1. Sterile lines and maintainer lines used in the experiment.

Name of sterile or maintainer line	Symbol of line	Cytoplasm type	Generation
Huanong A	N3A	Yegong	BC_{18}
Huanong A	N4A	Wild rice abortive	BC_{18}
Huanong A	N5A	Raoping wild rice	BC_{18}
Huanong B	N3-5B		
Huayu A	N6A	Yegong	BC_{17}
Huayu A	N7A	Wild rice abortive	BC_{17}
Huayu A	N8A	Raoping wild rice	BC_{17}
Huayu B	N6-8B		
Kezhen 2A	N9A	Yegong	BC_{19}
Kezhen 2A	N10A	Wild rice abortive	BC_{33}
Kezhen 2A	N11A	Raoping wild rice	BC_{19}
Kezhen 2B	N9-11B		
Zhenshan 97A	N12A	Yegong	BC_9
Zhenshan 97A	N13A	Wild rice abortive	BC_{32}
Zhenshan 97A	N14A	Raoping wild rice	BC_{30}
Zhenshan 97B	N12-16B		

RESULTS

Genetic diversity of SSR markers

Ninety-one pairs of SSR primers were randomly selected from 12 chromosomes of rice (Table 2) to detect genetic diversity of the 16 accessions. A total of 169 alleles were observed in the tested materials, and the percentage of polymorphic loci was 53.85%. On average, number of alleles per locus, number of alleles per polymorphic locus, and effective number of alleles

Table 2. The primers, alleles and polymorphism index content (PIC) in the experiment.

Primer	Chromosome	No. of alleles	PIC	Primer	Chromosome	No. of alleles	PIC	Primer	Chromosome	No. of alleles	PIC
RM1	1	3	0.59	RM127	4	1	0.00	RM256	8	1	0.00
RM129	1	1	0.00	RM252	4	2	0.12	RM126	8	1	0.00
RM483	1	2	0.49	RM509	5	1	0.00	RM464	9	2	0.34
RM499	1	1	0.00	RM538	5	1	0.00	RM410	9	2	0.50
RM243	1	3	0.50	RM31	5	1	0.00	RM189	9	3	0.59
RM9	1	4	0.53	RM169	5	2	0.06	RM205	9	1	0.00
RM23	1	1	0.00	RM13	5	1	0.00	RM242	9	2	0.48
RM27	2	2	0.50	RM541	6	1	0.00	RM566	9	2	0.26
RM138	2	1	0.00	RM412	6	1	0.00	RM160	9	2	0.47
RM423	2	2	0.48	RM435	6	1	0.00	RM484	10	2	0.47
RM526	2	2	0.08	RM454	6	1	0.00	RM474	10	2	0.39
RM207	2	3	0.52	RM469	6	1	0.00	RM228	10	3	0.45
RM240	2	2	0.12	RM510	6	2	0.49	RM239	10	1	0.00
RM250	2	1	0.00	RM170	6	4	0.50	RM311	10	4	0.59
RM263	2	2	0.26	RM253	6	3	0.53	RM333	10	4	0.71
RM411	3	1	0.00	RM204	6	1	0.00	RM441	11	1	0.00
RM517	3	1	0.00	RM455	7	2	0.50	RM457	11	1	0.00
RM520	3	1	0.00	RM436	7	1	0.00	RM20	11	1	0.00
RM523	3	1	0.00	RM429	7	2	0.30	RM167	11	1	0.00
RM218	3	3	0.63	RM264	7	2	0.26	RM202	11	4	0.53
RM16	3	3	0.49	RM234	7	1	0.00	RM206	11	3	0.52
RM55	3	3	0.45	RM82	7	1	0.00	RM209	11	3	0.43
RM135	3	2	0.30	RM3	7	2	0.17	RM254	11	3	0.46
RM130	3	1	0.00	RM481	7	4	0.58	RM453	12	2	0.49
RM119	4	1	0.00	RM214	7	2	0.06	RM463	12	1	0.00
RM127	4	1	0.00	RM407	8	2	0.38	RM415	12	3	0.62
RM417	4	1	0.00	RM458	8	1	0.00	RM17	12	2	0.50
RM470	4	1	0.00	RM506	8	1	0.00	RM235	12	3	0.53
RM241	4	1	0.00	RM515	8	3	0.55	RM247	12	1	0.00
RM261	4	3	0.31	RM210	8	3	0.65				
RM249	4	1	0.00	RM223	8	2	0.06				

were 1.8, 2.49 and 1.47, respectively, and the gene diversity was 0.228. This indicated that the four sets of isonuclear-alloplasmic rice lines had lower genetic diversity. Among the 91 pairs of primers, the primer RM333 showed the highest polymorphism index content (PIC) at 0.71. The PIC of 21 pairs of primers reached 0.5 above in all, suggesting that these primers could be used to distinguish the tested materials. While 42 pairs of primers in all had only an allele in the 16 lines individually, so their PICs were zero.

SSR fingerprints of the four sets of isonuclear-alloplasmic lines

Some characteristic bands were suitable to identify the tested materials and regarded as fingerprints. As shown in Table 3, the characteristic bands of N13A were found on RM16, RM223,

Table 3. SSR fingerprints of the tested lines.

Rice material	Characteristic SSR bands
N4A	The 3rd band of RM481
N6A	The 1st band of RM526
N7A	The 1st band of RM207
N12A	The 1st bands of RM55 and RM16
N13A	The 1st bands of RM16, RM223 and RM214, and the 2nd band of RM228
N12-16B	The 3rd band of RM235 and the 1st band of RM9
N9A	The 1st band of RM206
N11A	The 1st band of RM253
N9-11B	The 2nd band of RM209
Huanong A and Huang B	The 4th band of RM333
Zhenshan 97A and Zhenshan 97B	The 1st band of RM474
Kezhen 2A and Kezhen 2B	The 2nd and 4th band of RM311, and the 1st band of RM407

RM214 and RM228. The characteristic bands of N12A were the first bands of RM55 and RM16. The third band of RM235 and the first band of RM9 were the fingerprint of N12-16B. There was one characteristic band for N4A, N6A, N7A, N9A, N11A, and N9-11B, respectively. Moreover, for Huanong A (i.e. N3A, N4A and N5A) and Huanong B (i.e. N3-5B) series, the fourth band of RM333 was their characteristic band. For Zhenshan 97A (i.e. N12A, N13A and N14A) and Zhenshan 97B (i.e. N12-16B) series, the first band of RM474 was their fingerprint, and the second band of RM311 and the first band of RM407 were the characteristic bands for Kezhen 2A (i.e. N9A, N10A and N11A) and Kezhen 2B (i.e. N9-11B).

Comparison on difference in the nuclear genome

As shown in Table 4, four sets of rice isonuclear-alloplasmic male sterile lines shared 146 alleles, accounting for 86.39% of the total alleles, and presented 23 different alleles, being 13.61% of the total alleles. This indicated that obvious effects could be obtained by backcrossing. Among them, the minimum difference of nuclear genome was shown in Huanong A and Huayu A series, and the identical alleles in the same sets accounted for 91.12% of the total alleles. Kezhen 2A series took the second place, with 88.17% identical alleles of the total alleles. The maximum difference of nuclear genome was in Zhenshan 97A series, with the different allele percentage of 24.85%, which might be attributed to the fewer backcross generations for N12A of Zhenshan 97A (only backcrossed to BC_9), making it had much more differences with the other two male sterile lines (N13A, backcrossed to BC_{32}; N14A, backcrossed to BC_{30}). On average, 133 identical alleles (78.70% of the total alleles) and 36 different alleles (21.30% of the total alleles) were detected between the isonuclear-alloplasmic male sterile lines and their maintainer lines. Though they were both isonuclear-alloplasmic lines, the different alleles between the maintainer lines and their isonuclear-alloplasmic male sterile lines were 7.69 percent points more than that within the isonuclear-alloplasmic male sterile lines. Among the isoplasmic-allonuclear male sterile lines, 91 identical alleles were found on average, accounting for 53.85% of the total alleles, while 78 different alleles were noted, with 46.15% of the total alleles. The results showed that the numbers of identical alleles were the highest among the

Table 4. Comparison of nuclear genome for the four sets of isonuclear-alloplasmic rice.

Material	Number of identical alleles	Percentage (%)	Number of different alleles	Percentage (%)
Among the isonuclear-alloplasmic sterile lines [a]				
Huanong A	154	91.12	15	8.88
Huayu A	154	91.12	15	8.88
Kezhen 2A	149	88.17	20	11.83
Zhenshan 97A	127	75.15	42	24.85
Average	146	86.39	23	13.61
Between the isonuclear-alloplasmic sterile lines and their maintainer lines				
Huanong A and Huanong B	133	78.70	36	21.30
Huayu A and Huayu B	143	84.62	26	15.38
Kezhen 2A and Kezhen 2B	141	83.43	28	16.57
Zhenshan 97A and Zhenshan 97B	116	68.64	53	31.36
Average	133	78.70	36	21.30
Among the isoplasmic-allonuclear sterile lines [b]				
Y type of cytoplasm sterile lines	90	53.25	79	46.75
WA type of cytoplasm sterile lines	90	53.25	79	46.75
CW type of cytoplasm sterile lines	93	55.03	76	44.97
Average	91	53.85	78	46.15

[a] Huanong A includes N3A, N4A and N5A; Huayu A includes N6A, N7A and N8A; Kezhen 2A includes N9A, N10A and N11A; Zhenshan 97A includes N12A, N13A and N14A. Each set of sterile lines shares the same nuclear genome and different cytoplasms.

[b] Y type of cytoplasm sterile lines with Yegong cytoplasm includes N3A, N6A, N9A and N12A; WA type of cytoplasm sterile lines with wild rice abortive cytoplasm includes N4A, N7A, N10A and N13A; CW type of cytoplasm sterile lines with Raoping wild rice cytoplasm includes N5A, N8A, and N11A and N14A.

isonuclear-alloplasmic male sterile lines while their differences were the least, the second between the isonuclear-alloplasmic male sterile lines and their maintainer lines, and the lowest among the isoplasmic-allonuclear male sterile lines while they had the largest differences.

Cluster analysis of the four sets of isonuclear-alloplasmic rice

Fig. 1 showed the cluster analysis results of the four sets of isonuclear-alloplasmic male sterile lines and their maintainer lines according to the data from 91 pairs of primers. All the materials tested could be divided into three groups at the 0.2 of the genetic distance. The first group consisted of eight lines including the male sterile lines and their maintainer lines of Huanong A and Huayu A series, suggesting these two sets had the closest relative relationship. The second group included four isonuclear-alloplasmic lines of Kezhen 2A series (N9A, N10A, N11A and N9-11B). The third group had four isonuclear-alloplasmic lines of Zhenshan 97 series (N12A, N13A, N14A and N12-16B). The isonuclear-alloplasmic lines in the same set were firstly clustered except N3-5B, showing these materials had the close genetic relationship each other and the near nuclear background.

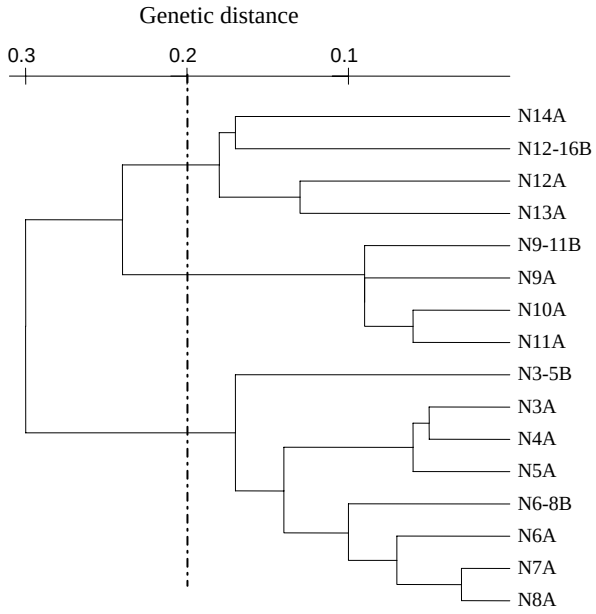

Fig. 1. Cluster analysis of the four sets of rice isonuclear-alloplasmic lines.

Relationship between the new cytoplasmic male sterile lines and WA male sterile lines

The genetic distances between the new cytoplasmic male sterile lines (i.e. Y type with Yegong cytoplasm and CW type with Raoping wild rice cytoplasm) and the WA type with wild abortive cytoplasm were listed in Table 5. It revealed that the genetic distances between the isonuclear-alloplasmic male sterile lines of Y type and WA type, or that between CW type and WA type were 0.02-0.13. For example, the genetic distance between N3A and N4A was 0.04, and the genetic distance between N7A and N8A was 0.02. In other words, the similarity coefficient between these two isonuclear-alloplasmic male sterile lines is 87-98%. It will be very helpful to further study the cytoplasmic effects of these male sterile lines. For those with different genome backgrounds and different cytoplasmic backgrounds, Huanong A with Y type cytoplasm or CW type cytoplasm was close to Huayu A with WA type cytoplasm, for example, the genetic distance between N3A and N7A was 0.12. Similarly, Kezhen 2A with Y type cytoplasm or CW type cytoplasm was close to Zhenshan 97A with WA type cytoplasm, for example, the distance of N9A and N13A was 0.16. However, Huanong A and Huayu A with Y type or CW type cytoplasm showed further relationship with Kezhen 2A and Zhenshan 97A with WA type cytoplasm, with

Table 5. Genetic distances between Y type or CW type and WA type male sterile lines.

WA type male sterile line	Y type male sterile line				CW type male sterile line			
	N3A	N6A	N9A	N12A	N5A	N8A	N11A	N14A
N4A	0.04	0.11	0.25	0.26	0.05	0.11	0.26	0.22
N7A	0.12	0.05	0.23	0.24	0.11	0.02	0.23	0.20
N10A	0.25	0.22	0.07	0.13	0.24	0.22	0.05	0.20
N13A	0.24	0.21	0.16	0.09	0.25	0.21	0.19	0.13

the genetic distance at 0.20-0.26.

DISCUSSION

The genetic diversity for 16 male sterile lines and their maintainer lines in the study revealed that there were 169 alleles in all. The percentage of polymorphic loci was 53.85%, the average number of alleles per locus was 1.8, and the average gene diversity was 0.228. Previously, it was reported that on average there were 6.8 alleles per SSR primer between cultivated rice and wild rice [16], 2.34 alleles per RFLP probe among cultivated rice [15], and 3.6 alleles per isozyme locus among cultivated rice [17]. The average number of alleles per locus in our study was rather fewer compared with the former reports. Zhu et al [18] found that the average gene diversity within cultivated rice and common wild rice were 0.67 and 0.90, respectively, when comparisons were made for the genetic diversity between Asian cultivated rice and common wild rice with SSR markers. Tang et al [19] analyzed 4408 accessions of Chinese cultivated rice germplasm with 12 isozyme loci and found that the average gene diversity was 0.012-0.547. In our experiment, the results showed that the genetic diversity of the tested materials was very low. It is because the difference of four maintainer lines is rather low on the one hand, for example, Huanong B, Huayu B and Kezhen 2B share one or more common parents; on the other hand, it is also because 16 accessions belong to four sets of isonuclear-alloplasmic lines with the materials in the same set share the similar pedigree, and a majority of materials were backcrossed for 17-33 generations thus the nuclear genome is close.

Generally, the isonuclear-alloplasmic male sterile lines are bred by backcross for many generations. Therefore, a pair of male sterile lines and its maintainer lines only shows fertility difference theoretically and almost no difference for nuclear genome when checked with molecular marker. However, our results revealed that there were averagely 86.39% identical alleles for the same set of isonuclear-alloplasmic male sterile lines, with the highest one was 91.12%. This showed that obvious effects could be achieved by backcross for many generations and artificial selection. However, though the four sets of isonuclear-alloplasmic male sterile lines were backcrossed for many generations, the average different allele percentage was still 13.61%, and the average different allele percentage between isonuclear-alloplasmic male sterile lines and their maintainer lines reached 21.30%. The reasons may be as the following: 1) Being attributed to their different pedigrees. The backcross parents are incompletely identical during breeding these male sterile lines, for example, the backcross parents of Huanong A includes Jianmeizao, 6964, Xianhui and BⅡ44-5, those of Kezhen 2A includes Jianmeizao, Erjiuai 4, Kezhen, and the ones of Zhenshan 97A includes Jianmeizao and Zhenshan 97. Moreover, the backcross generations with the final backcross parents are generally 5-6 generations, and the total backcross generations are not same for each male sterile line [20]. So their nuclear genome backgrounds are complicated. 2) Due to only selection by morphology (especially by the fertility characters) in breeding of these male sterile lines, which might result in different nuclear genomes though having the same morphological characteristics. Moreover, the total rice genome includes a lot of loci, thus it will be very slow for recovered to the background of backcross parents. However, the mechanism is still to be clarified. Backcross will achieve good effects if backcross is selected with molecular marker assistance.

Though many tested materials shared the similar genome background, even were bred by the same central parents, polymorphism could still be detected to a certain extent with SSR marker. This indicated SSR marker could be applied to analyze and identify the pedigree of hybrid rice and their parents. To screen highly polymorphic SSR primers within the parents of hybrid rice for establishing their SSR fingerprints is a good solution to identification for male sterile lines, maintainer lines and its hybrids in rice.

Moreover, our results showed that the four sets of isonuclear alloplasmic male sterile lines and their maintainer lines had close relative relationship and their similarity coefficient reached 70% above. Li et al [21] found that male sterile lines used in rice production with 70% of total cultivated area had rather low genetic variation, and their similarity coefficients were

at 0.81-0.96. The similar results were gotten in our experiments. Therefore, to exploit and utilize the new excellent male sterile lines with new cytoplasm from all kinds of rice germplasm resources, and to pay more attention to breeding male sterile lines with widely genetic diversity, are still the important tasks for three-line hybrid rice breeding.

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