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ANALYSIS OF GENETIC DIVERSITY OF 102 RICE VARIETIES USING MICROSATELLITE MARKER

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Abstract

Sixty-nine SSR markers were used to analyze the genetic diversity of 102 germplasm resources adapted to different climatic regions of Heilongjiang province. The results indicated that 50 out of 69 have polymorphism. One hundred eighty nine alleles were detected. The average number of alleles per locus was 3.78 with a range from 2 to 9. Genetic diversity index ranged from 0.019 to 0.761, with the mean of 0.355. Polymorphism information content ranged from 0.019 to 0.641, with the mean of 0.318. The expected heterozygosities ranged from 0.055 to 1.492 with an average value of 0.595. UPGMA cluster analysis showed that the 102 rice materials tested could be divided into four groups at a genetic similarity coefficient of 0.698.The 102 rice germplasm resources from different region showed lower genetic diversity. Rice breeders need to introduce innovation of germplasm in the conventional rice breeding program for further increase of rice yield and stress tolerance in Heilongjiang province.

Introduction

Rice is the most important food crop in the world. It originated from subtropical swamps, and evolved into a variety of subspecies such as Asian cultivated rice (*Oryza sativa* L. subsp. *Japonica* Kato and *Oryza sativa* L. subsp. *Indica* Kato), African cultivated rice, and wild rice in millions of years of evolution. The rice varieties planted in northern China are primarily *Oryza sativa* L. subsp. *Japonica* Kato, and their planting area in Heilongjiang Province has reached 413 million ha in 2016. It has made substantial contributions to ensuring the safety of food rations in China. However, limited by the narrow genetic base of breeding resources, rice breeding has not yet made any significant breakthrough in recent years. Using modern molecular biology techniques to study the genetic diversity of *japonica* rice resources that adapted to the ecology and climate of Heilongjiang is essential for the genetic improvement of rice.

In recent years, a lot of scholars have used simple sequence repeat (SSR) markers to analyze the genetic structure of rice varieties and resources of Heilongjiang. Zhang *et al.* (2016) analyzed the genetic diversity of 73 rice varieties confirmed in Heilongjiang in recent years with 24 SSR markers for rice DNA fingerprinting and 38 other SSR markers, dividing these 73 varieties into six clusters. Sun *et al.* (2011) used 42 pairs of SSR primers to analyze the genetic diversity of 80 ricevarieties in Heilongjiang, dividing them into six clusters with a genetic similarity coefficient of 0.5. Yang *et al.* (2008) used 52 pairs of SSR primers to analyze the genetic diversity of rice in Heilongjiang, including 43 bred cultivars obtained after 1990, five major varieties before 1990, andsixvarieties imported from Japan, divided them into three clusters, and demonstrated that most

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of the varieties have close phylogenetic relationships. In this study, 102 rice varieties from the Germplasm Resources Garden in the Rice Research Laboratory of the Institute of Crop Cultivation and Farming, Heilongjiang Academy of Agricultural Sciences were used as study materials, and 50 pairs of SSR markers covering the entire genome of rice uniformly were selected to conduct analysis on the genetic diversity and population structure of rice, aiming to provide a reference for the rice breeding of our group as well as the other breeders.

Materials and Methods

The experiment was carried out at the Rice Innovation Base of Heilongjiang Academy of Agricultural Sciences. The Base is located at 126°49' east longitude and 45°50' north latitude, with a mid-temperate continental monsoon climate. The crops have sufficient sunshine during the growing season, rain and heat together over the same period, and large temperature difference between day and night in the grain-filling period. There is a frost-free period of 140 days, and an annual rainfall of around 500 mm here. Regarding the soil basic fertility, the organic matter content is 33.1 g/kg, the nitrogen from alkaline hydrolysis is 122.6 mg/kg, the available phosphorus is 68.2 mg/kg, and the available potassium is 139.1 mg/kg. The test materials (Table 2) were 102 rice varieties from the Germplasm Resources Garden in the Rice Research Laboratory of the Institute of Crop Cultivation and Farming, Heilongjiang Academy of Agricultural Sciences. All the test varieties were able to be matured normally in Harbin, Heilongjiang. On April 15, 2017, the seedlings were cultivated by the dry-raising seedling and thin-planting method, with a seed amount of 450 g/m^2. The seedlings were transplanted on May 15, 2017. The fertilizer application rates of pure nitrogen, phosphate (P_2O_5) and potassium (K_2O) were all 140 kg/hm^2. The nitrogen fertilizer was used half as the base fertilizer and half as the green fertilizer, whereas all the phosphorus and potassium fertilizers were applied as the base fertilizers. The experiment adopted a completely randomized design, with six rows for each variety and three replications, a row spacing of 29.7×13.2 cm, and the other managements same as in the field production.

At the seedling stage, the young leaves were collected, and the total DNA of rice was extracted with the modified cetyltrimethylammonium bromide (CTAB) method (Wang 2014). At the peak tillering stage of 2017, two leaves from each labeled plant of all rice varieties were collected and placed in an ice box, kept in a –80ºC freezer for later total DNA extraction.

A total of 69 pairs of SSR primers distributed on 12 rice chromosomes were synthesized by Beijing Genomics Institute. Among them, there were 50 primer pairs demonstrating polymorphism regarding different rice varieties. Therefore, these 50 pairs of polymorphic primers were used to analyze the genetic diversity of all tested varieties, with details shown in Table 3.

The amplification protocol was as follows, pre-denaturation at 94ºC for 5 min, denaturation at 92°C for 30 sec, annealing at 92°C for 30 sec, extension at 72ºC for 1 min, for a total of 35 cycles, and followed by extension at 72 ºC for 7 min, and stored at 15ºC. The reaction system as following:

Table 1. The reaction system.

DNA (50 ng/μl)	1 μl
10× Buffer	1 μl
dNTP (2.5 mM)	0.8 μl
Forward primer (10 μM)	0.2 μl
Reverse primer (10 μM)	0.2 μl
Easy Taq	0.05 μl
ddH_2O	6.75μl
Reaction system	10 μl

The preparation of glass plates and polyacrylamide gel, electrophoresis and gel staining were conducted as described in the reference.

One locus was detected for each pair of SSR primers, and each polymorphic strip was treated as one allele. The value of "0" or "1" was used to represent the absence or presence of allelic variation at the corresponding locus of the tested variety. Therefore, with "1" corresponding to the presence of a strip and "0" corresponding to the absence of a strip, the electrophoresis result was transformed into a (0, 1) matrix. The statistics and analysis of the number of alleles (Na), the genetic diversity index (He), and the polymorphism information content (PIC) were conducted as referenced in the literature (Liu and Muse 2005). According to the unweighted pair group method with arithmetic mean (UPGMA), the SHAN module provided by NTSYSpc ver. 2.1 was used to perform the cluster analysis and draw the tree diagram. Further, the statistical software Structure ver. 2.2 was used to analyze the population genetic structure, and the data were submitted to the Structure Harvest website for classification. Then POPgen ver. 1.32 was used to calculate the values of Shannon-Weiner diversity index (Hs), genetic identity (I) and Neis genetic distance (D) within the clusters resulted from Structure ver. 2.2, and the cluster diagram based on genetic distance was plotted as well. In addition, POPGene ver. 1.32 was used to calculate the values of I and D between clusters.

Results and Discussion

Among the 69 pairs of primers tested in this study, 50 demonstrated polymorphisms, accounting for 72.5% of the total number of primer pairs. A total number of 189 allelic variations were detected in the 102 tested varieties with these 50 polymorphic primer pairs, giving an average of 3.78 alleles detected per primer pair, and the range of allelic variations detected by a single primer pair was 2 to 6. The primers with two allelic variations detected were RM495, RM237, RM55, RM293, RM471, RM510, RM454, and RM433; those with three allelic variations detected were RM265, RM452, RM208, RM338, RM514, RM251, PSM326, RM190, RM455, RM118, RM316 and RM171; those with four allelic variations detected were RM1, RM5, RM327, RM341, RM335, RM161, RM125, RM408, RM25, RM447, RM105, RM484, RM536, RM287, RM332, and RM19; those with five allelic variations detected were RM246, RM231, RM162, RM11 RM223, RM284, PSM338, and RM311; and those with six allelic variations detected were PSM132, RM215, RM219, PSM406, and RM247. The He value ranged from 0.019 to 0.761, with an average of 0.355; the PIC ranged from 0.019 to 0.641, with an average of 0.318; and the Shannon-Weiner index ranged from 0.055 to 1.492, with an average of 0.595. The Na, He and PIC values of five pairs of primers, PSM132, RM215, RM219, PSM406 and RM223, exclusively ranked top five among all primer pairs, indicating that these five pairs of primers were the most abundant polymorphic markers for the 102 tested varieties. In addition, the Na, He and PIC values of the other six pairs of primers, RM247, RM246, RM231, RM162, RM284, and PSM338, ranked high as well, indicating abundant polymorphic of these primers regarding the 102 tested varieties.

According to the genetic similarity coefficient matrix, UPGMA was used to make the genetic correlation plot of the 102 tested varieties (Fig. 1). It can be seen from Fig. 1 that with a genetic similarity coefficient of 0.698, the 102 tested varieties could be divided into 4 clusters, among which, Kenheidao, Yangeng 19 and Tonggeng 299 were separately branched, indicating that these three varieties were genetically distant from the other tested varieties.

Based on the genetic similarity coefficient matrix analysis it was evedent that both Longjing 30 and Liaoyan 16 inherit genes from Luyu 132. Both Mudanjiang 27 and Longdao 10 are descendants of Yueguang, while Yueguang continues the bloodline of Luyu 132. Both Longdao 10 and Aizhixu are descendants of Xu. The cultivars Longdao 5 and Yueguang from the Germplasm

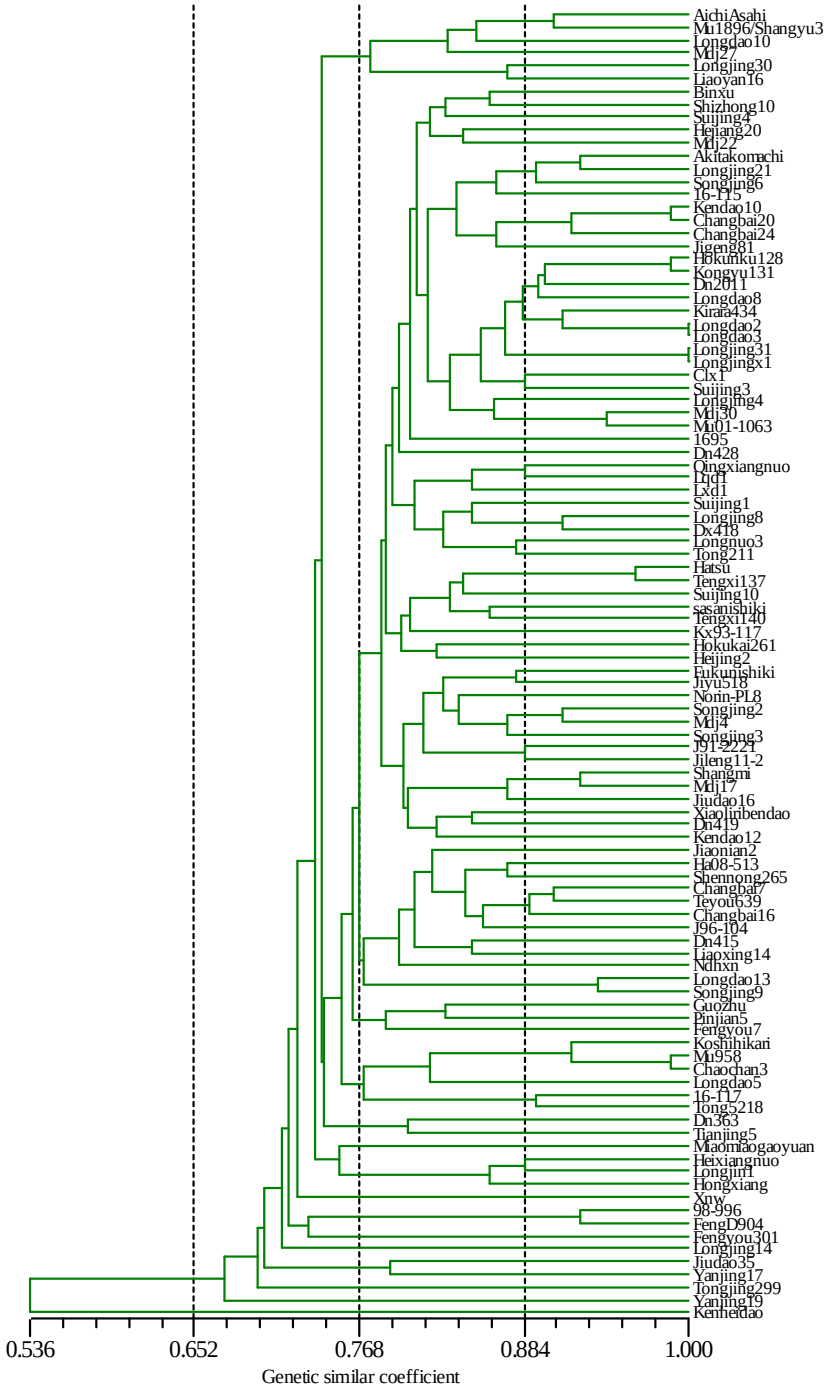

Fig 1. The dendrogram of 102 materials.

Resources Garden bred by our lab are descendants of Luyu 132, Nonglin 6, and Nonglin 8. While the cultivars Chaochan 3 and Mu 958 were bred, respectively by the Rice Research Institute of Jilin Academy of Agricultural Sciences and the Mudanjiang Branch of Heilongjiang Academy of Agricultural Sciences, both of which may use Japanese varieties for rice variety improvement. The cultivar 16-117 and Tong 5218 were regional trial varieties of Jilin Academy of Agricultural Sciences, and may also be descendants of Japanese varieties, hence they were clustered together. Both Yangeng 19 and Yangeng 17 are descendants of Matsumae rice.

A parent of Jiudao 35, Jiuyin 1 might also be the parent of Yangeng 17, Qushou 1. Therefore, Yangeng 17 is the closest to Jiudao 35. While 98-996, Feng D904 and Fengyou 301 are all Jilin varieties. Longjing 14 is a special variety. The aromatic rice Xiangnianwang, Hongxiang, Longjin 1 and Heixiangyu are closely related to each other. Among them, Xiangnianwang has the most complex genetic background and the most heterozygous loci. Tianjing 5 is a type of *Japonica* upland rice. One parent of Longdao 13 is Songgeng 10, which was bred from Liaogeng 5 and Hejiang 20. While one parent of Songgeng 9 was also a hybrid of Liaogeng 5 and Hejiang 20. Therefore, Longdao 13 and Songgeng 9 are closely related to each other. Changbai 7, Teyou 639, Changbai 16, and Ji 96-104 were all bred cultivars of Jilin Academy of Agricultural Sciences. Jiudao 19, Shangmi, Mudanjiang 17, Dongnong 419 and Kendao 12 all inherited genes from Qiuguang.

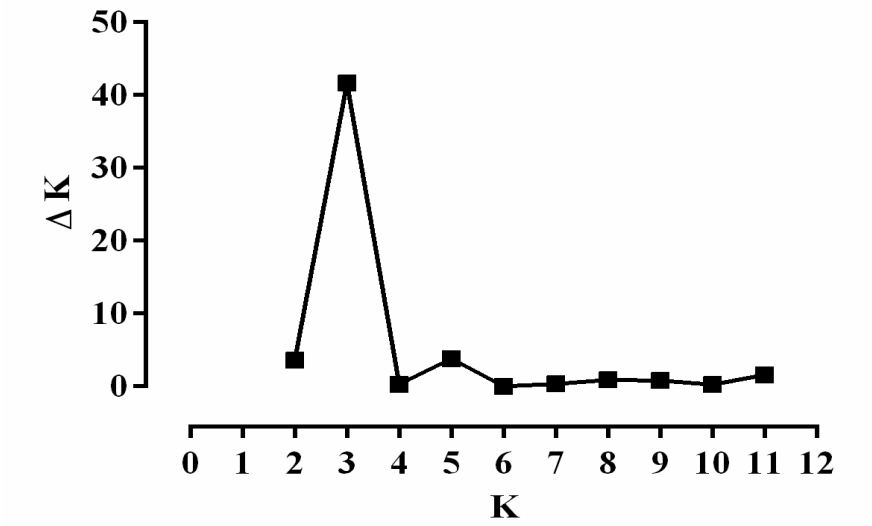

Fig. 2.Graphical relationship between K and ΔK.

Structure ver. 2.2 was used to perform genetic structure analysis on the tested 102 varieties. The value of ΔK reached a maximum value when K was set to 3 (Figs 2 and 3), which meant that the genetic structure of the 102 varieties could be divided into three clusters.

In general, the three clusters shared high genetic identity and small genetic distance. Among them, groups I and II have the smallest genetic identity, with a value of 0.9153, and the largest genetic distance, with a value of 0.0885. Followed by groups I and III, a genetic identity value of 0.9356 and a genetic distance of 0.0665. Where groups II and III have the largest genetic identity value of 0.9447 and the smallest genetic distance of 0.0569 (Tables 2-4).

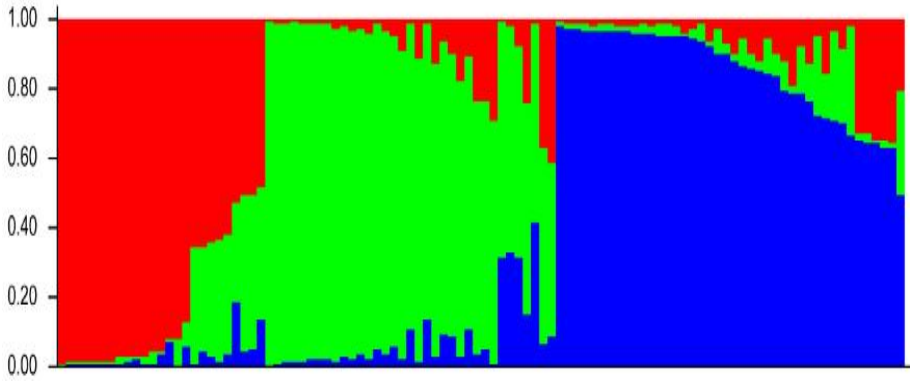

Fig. 3. The genetic structure of 102 rice materials (K=3).

Germplasm resources are the material basis for rice breeding. Knowledge of the genetic characteristics of a variety of rice varieties is essential for creating more genetic variations, and cultivating rice varieties with higher yield and better quality. Scholars at home and abroad have already conducted extensive studies on rice genetic diversity. Li *et al.* (2011) analyzed the genetic diversity of 18 rice varieties from Northeast China and 13 Japanese rice varieties with 40 SSR markers, and found that the genetic variation was primarily among the varieties, and only small differences between populations. Cheng *et al*. (2014) used 48 pairs of SSR primers to analyze the genetic diversity of 36 rice varieties from various breeding units in Heilongjiang, and gave a He value ranged from 0.050 to 0.662, a PIC value ranged from 0.053 to 0.588, a Shannon-Weiner index value ranged from 0.127 to 1.168, and a genetic coefficient ranged from 0.38 to 1.00. Li *et al*. (2009) investigated the genetic diversity of 107 rice varieties in the three northeastern provinces of China with 53 SSR markers, and demonstrated more newly added alleles than the old disappeared alleles over time, and a narrow genetic base among the rice varieties of the three provinces. Ma *et al.* (2014) studied 63 rice varieties from various countries with 63 pairs of SSR primers, and showed that the genetic diversity among rice varieties was closely related to latitude. The closer the latitude, the closer the genetic relationship of corresponding varieties. Tang *et al.* (2009) used 60 SSR markers to analyze the genetic diversity of 370 aromatic rice varieties from home and abroad, detected a total number of 361 alleles, and demonstrated an average Nei gene diversity index value of 0.663, and a higher genetic diversity of indica rice than japonica rice. Wang *et al.* (2014) used 154 SSR markers to analyze the genetic diversity of 278 rice varieties from Northeast Asia, and gave the order of diversity from high to low as: Heilongjiang landraces, Jilin landraces, Japanese varieties, bred cultivars of Heilongjiang, varieties from Russia and Far Eastern, bred cultivars of Liaoning, bred cultivars of Jilin, South Korean varieties, and Korean varieties. Bajracharya*et al*. (2006) used 39 SSR markers to study the genetic diversity of 147 rice landraces, and revealed a narrow genetic base of the Jumla landrace population. Swain *et al*. (2017) analyzed the genetic diversity of 12 common wild rice, 14 Indian wild rice and 4 rice varieties using 54 pairs of SSR primers,and showed that the Jaccards' similarity coefficient of these varieties reached a value of 0.8249, though the two types of wild rice were grouped into different clusters. Ravi *et al.* (2003) used 499 RAPD markers and 38 SSR markers, respectively to analyze the genetic diversity of 40 cultivated rice varieties and five wild rice varieties, and revealed that SSR markers could distinguish different clusters more accurately. Singh *et al*. (2013) used 36 SSR and SNP markers to analyze the genetic diversity and genetic structure of 375 rice varieties, and showed that the SSR markers worked better regarding genetic diversity analysis.

Table 2. Experiment materials.

No.	Variety	Source	No.	Variety	Source	No.	Variety	Source
1	Aichi Asahi	Japan	35	Songjing 3	HLJ	69	MU 01-1063	HLJ
2	Qinxu	"	36	Songjing 6	"	70	1695	JL
3	Hatsu	"	37	Songjing 9	"	71	16-115	JL
4	Fukunishiki	"	38	Suijing 3	"	72	16-117	JL
5	Jiaonian 2	"	39	Suijing 4	"	73	98-996	JL
6	Miaomiaogaoyuan	"	40	Suijing10	"	74	Heixiangnuo	JL
7	Qingxiangnuo	"	41	Suijing 1	"	75	Hongxiang	JL
8	Shangmi	"	42	Hejiang 20	"	76	Changbai 7	JL
9	Shizhong 10	"	43	Longjing 4	"	77	Changbai 16	JL
10	Xiaoliribendao	"	44	Llongjing 8	"	78	Changbai 20	JL
11	Koshihikari	"	45	Longjing 14	"	79	Changbai 24	JL
12	Akitakomachi	"	46	Longjing21	"	80	Chaochan 3	JL
13	Sasanishiki	"	47	Longjing 30	"	81	Fengyou 7	JL
14	Guozhu	"	48	Longjing 31	"	82	Feng D904	JL
15	Norin-PL8	"	49	Longnuo 31	"	83	Fengyou 301	JL
16	Hokukai 261	"	50	1 Longjingx 1	"	84	J91-2221	JL
17	Hokuriku 128	"	51	Pinjian 5	"	85	J96-104	JL
18	Kongyu 131	"	52	Kendao 10	"	86	Jigeng 81	JL
19	Kirara 434	"	53	Kendao 12	"	87	Jiyu 518	JL
20	Tengxi 137	"	54	Kenheidao	"	88	Longjin 1	JL
21	Tengxi 140	"	55	Kx 93-117	"	89	Teyou 639	JL
22	Xnw	HLJ	56	Dn 2011	"	90	Tianjing 5	JL
23	CLX 1	"	57	Dn 415	"	91	Jileng 11-2	JL
24	HA 08-513	"	58	Dx 418	"	92	Ndhxn	JL
25	Longdao 2	"	59	Dn 419	"	93	Jiudao 16	JL
26	Longdao 3	"	60	Dn 428	"	94	Jiudao 35	JL
27	Longdao 5	"	61	Dn 363	"	95	Yanjing17	JL
28	Longdao 8	"	62	Deijing 2	"	96	Yanjing 19	JL
29	Longdao 10	"	63	Mdj 4	"	97	Tong 211	JL
30	Longdao 13	"	64	Mdj 17	"	98	Tong 5218	JL
31	Lqd 1	"	65	Mdj 22	"	99	Tongjing 299	JL
32	Lxd 1	"	66	Mdj 27	"	100	Liaoxing 1T4	LN
33	MU 1896/Shangyu 397	"	67	Mdj30	"	101	Liaoyan 16	LN
34	Songjing 2	"	68	Mu 958	"	102	Shennong265	LN

Table 3.The genetic diversity of 50 SSR with polymorphism.

Marker	Link group	Na	He	PIC	Shannon-Weiner
RM495	1	2	0.019	0.019	0.055
RM1	1	4	0.435	0.397	0.760
RM283	1	3	0.545	0.440	0.693
RM5	1	4	0.576	0.511	0.936
RM246	1	5	0.557	0.504	0.983
RM237	1	2	0.038	0.037	0.096
RM265	1	3	0.497	0.406	0.650
RM327	2	4	0.589	0.511	0.956
RM452	2	3	0.161	0.151	0.327
RM208	2	3	0.621	0.547	1.029
RM341	2	4	0.504	0.460	0.712
RM338	3	3	0.094	0.091	0.134
PSM132	3	6	0.687	0.641	1.249
RM55	3	2	0.262	0.228	0.432
RM514	3	3	0.307	0.271	0.453
RM231	3	5	0.555	0.487	0.920
RM251	3	3	0.094	0.091	0.098
RM293	3	2	0.075	0.072	0.164
PSM326	4	3	0.093	0.090	0.165
RM335	4	4	0.469	0.440	0.922
RM471	4	2	0.249	0.218	0.415
RM161	5	4	0.549	0.496	0.910
RM510	6	2	0.313	0.264	0.492
RM454	6	2	0.460	0.354	0.653
RM162	6	5	0.502	0.463	0.933
RM190	6	3	0.505	0.396	0.762
RM125	7	4	0.490	0.388	0.711
RM11	7	5	0.199	0.193	0.384
RM455	7	3	0.094	0.091	0.134
RM118	7	3	0.129	0.124	0.100
RM408	8	4	0.211	0.199	0.393
RM25	8	4	0.130	0.127	0.230
RM223	8	5	0.655	0.590	1.096
RM284	8	5	0.540	0.439	0.846
RM433	8	2	0.038	0.037	0.096
RM447	8	4	0.146	0.142	0.291
RM316	9	3	0.075	0.073	0.056
RM105	9	4	0.164	0.160	0.299
PSM338	9	5	0.574	0.530	1.005
RM215	9	6	0.755	0.719	1.446
RM219	9	6	0.761	0.724	1.492
PSM406	10	6	0.668	0.632	1.252
RM171	10	3	0.284	0.257	0.405
RM484	10	4	0.254	0.230	0.435
RM311	10	5	0.315	0.289	0.514
RM536	11	4	0.502	0.429	0.787
RM287	11	4	0.146	0.142	0.252
RM332	11	4	0.488	0.441	0.794
RM19	12	4	0.048	0.047	0.086
RM247	12	6	0.341	0.329	0.730
Mean		3.78	0.355	0.318	0.595

Table 4.The materials in every genetic structure population.

Population	Acce.	Material name
PopulationI	25	Aichi Asahi, 128Hokuriku 128, 131 Kongyu 131, Shangyu 434, clx 1, Longdao 2, Longdao 5, Longdao 8, Longdao 10, Mu 1896/shangyu 397, Suijing 10, Suijing 1, Longjing 4, Longjing 30, Longjing 31, Longnuo 31, Longjingx 1, Kenheidao, Dn 2011, Mdj 27,Mdj 30, Mu 01-1063/1695, Tong 211, Liaoyan 16
PopulationII	35	Hatsu, jiaonian 2, sasanishiki, guozhu, Tengxi 137, Tengxi 140, Ha 08-513, Longdao 13, Songjing 6, Songjing 9, Suijing 3, Shennong265, Longjing 8, Pinjian 5, Kx 93-117, Dn 415, Dx 418, Heijing 2, 16-115, 98-996, Hxn, Hx, Changbai16, Fengyou7, Fengd904, Ji96-104, Longjin1hao, Teyou639, Ndhxn, Jiudao35, Yanjing17, Yanjing19, Tongjing299, Liaoxing14
PopulationIII	42	Binxu, Fukunishiki, Miaomiaogaoyuan, Qingxiangnuo, Shangmi, Shizhong 10, Xiaoliribendao, Koshihikari, Akitakomachi, Norin-Pl8, Hokukai 261, Xnw, Longdao 3. Lqd 1, Lxd 1, Songjing 2, Songjing 3, Suijing 4, Hejiang 20、Longjing 4, Longjing21, Kendao 10, Kendao 12, Dn 419, Dn 428, Dn 363, Mdj 4, Mdj 17, Mdj 22, Mu 958/16-117, Changbai 20, Changbai 24, Chaochan 3, Fengyou 301, Ji 91-2221, Jigeng 81, Jiyu 518, Tianjing 5, Jileng 11-2, Jiudao 16, Tong 5218

Table 5.The genetic identity and genetic distance between 3 groups.

	Population I	Population II	Population III
Population I	****	0.9153	0.9356
Population II	0.0885	****	0.9447
Population III	0.0665	0.0569	****

Nei,s genetic identity (above diagonal) and genetic distance (below diagonal).

Nowadays, SSR markers are still effective and economical molecular markers for rice genetic diversity analysis. The 102 rice varieties involved in this study were divided into three clusters. The variety distribution was associated with the regionality that, most of the cultivars from one same breeding unit were grouped in one cluster. The genetic identity values between the three clusters were 0.9153, 0.9356 and 0.9477, respectively, indicating small genetic differences among the clusters. Using the 50 SSR markers to analyze the 102 tested rice varieties gave an average He value of 0.355, an average PIC value of 0.318, and an average Shannon-Weiner index of 0.595, consistent with previous studies. Scholars have conducted numerous studies on the genetic diversity and genetic structure of rice varieties in Northeast China and Japan, and shown a narrow genetic diversity of rice varieties in these regions. Presumably, this is closely related to the ecological environment of involved regions. The germplasm resources of *Japonica* rice are not as rich as *Indica*rice due to high latitudes. And this is the key to limiting the broadening of rice genetic diversity in high latitudes. In the future, the introduction of rice germplasm resources from areas with similar or same latitudes worldwide, hybridization and molecular biology means, may be effective methods and approaches to broaden the genetic diversity of rice in high latitude regions.

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