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Genetic diversity analysis and DNA fingerprinting of the main japonica rice varieties in Heilongjiang Province

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RESEARCH ARTICLE

Abstract

In order to apply the national agricultural standard of China (NY/T 1433-2007) and its recommended 12 primers, the simple sequence repeat technique and capillary electrophoresis were firstly applied to analyse the genetic diversity and construct DNA fingerprints of 50 japonica rice cultivars in Heilongjiang Province (including the ChaHaYang, JianSanJiang, WuChang, XiangShui and FangZheng regions). A total of 40 alleles were detected in the 50 varieties using the 12 primer pairs. The number of alleles detected by each primer pair ranged from 1-11, with an average of 3.3. According to the unweighted pair group method with arithmetic mean cluster analysis, the similarity coefficients of the varieties ranged from 0.850-0.982, the polymorphism information content values ranged from 0.093-0.493, and the polymorphic frequencies ranged from 0-0.667. The 50 varieties were divided into eight categories with a similarity coefficient of 3.4. DNA fingerprints were constructed for the 50 rice cultivars. According to the above results, the 12 primers recommended in the national standard were proved to be efficient and applicable for the main japonica rice cultivars in Heilongjiang Province, in which those varieties had narrow genetic backgrounds and low genetic diversity. The genetic background can be expanded by introducing new genetic resources.

Keywords: japonica rice, genetic diversity, SSR marker, fingerprints

1. Introduction

The soil in Heilongjiang Province contains a high organic matter content. In this region, the temperature difference between day and night is large, and the daily period with sunshine is long. Heilongjiang Province is the coldest rice cultivation area in the world and the main planting area for japonica rice in China. The rice farming area and production of Heilongjiang Province are increasing annually. Heilongjiang Province is the most important production base for commodity rice in China, with a farming area of more than 4 million ha and production that reached 22 million tons in 2015 (Meyer and Purugganan, 2013; Turner *et al.*, 2008). Rice from the WuChang and FangZheng regions in Heilongjiang Province represents well-known brands and protected products with geographical indications in China. However, rice variety identification, seed purity and authenticity identification, variety rights protection, and rice production and processing safety occur at significantly lower levels in these rice farming areas compared to developed areas, resulting in serious market problems, such as fake and shoddy commodities (Song and Meng, 2016; Zhang *et al.*, 2016). Therefore, identification and origin protection for the main japonica rice varieties in Heilongjiang Province will greatly enhance the authenticity of the brand, promote the healthy operation of the rice market, and provide rice products with high quality and nutrition.

DNA fingerprinting provides an efficient and accurate method for the differential identification of rice varieties. The construction of DNA fingerprints is important for rice breeding, identification, monitoring of regional species varieties, and seed management (Iudith *et al.*, 2013; Song *et*

al., 2016; Wu *et al.*, 2015). DNA fingerprints of rice varieties have been constructed in the United States, Japan, and South Korea. The Sichuan, Liaoning, Zhejiang, and Anhui provinces in China have also completed DNA fingerprint construction for their provincial rice varieties, which have been promoted and tested for years (Cheng *et al.*, 2011; Kobayashi *et al.*, 2006; Luo *et al.*, 2015). Therefore, obtaining a deep understanding of the genetic diversity of the main rice cultivars in Heilongjiang Province has great significance for the exploitation of new germplasm resources and will make good use of the excellent parents.

Simple sequence repeat (SSR) molecular markers have been widely used for the construction of DNA fingerprints, genetic diversity analyses, and the identification of the purity and authenticity of cultivars due to advantages including a simple and rapid operation, good reproducibility, abundance, and high polymorphism detection rate (Latif *et al.*, 2011; Liu and Zhang, 2010; Wang *et al.*, 2014). Sarao *et al.* (2010) used 75 SSR marker primer pairs to genetically analyse 14 rice cultivars cultivated in India. Akagi *et al.* (1997) analysed the genetic diversity of 59 Japanese japonica rice varieties using 20 SSR markers. In China, Xiao *et al.* (2006) used 24 pairs of SSR marker primers to construct DNA fingerprints and perform cultivar identification of the 42 main rice varieties in Sichuan Province. Li *et al.* (2014) used the 12 pairs of rice SSR primers recommended by the Ministry of Agriculture to construct DNA fingerprints of 46 conventional japonica rice cultivars bred in the main breeding institutes in Zhejiang Province since 2008 and analysed their genetic relationships. Zhang *et al.* (2013), Wang *et al.* (2014), and Liu *et al.* (2015) analysed the inheritance of the rice cultivars in Heilongjiang Province based on SSR markers, but the range of the selected cultivars was small, and the main cultivars were not fully covered. Furthermore, the polymerase chain reaction (PCR) products in those studies were mostly analysed using gel electrophoresis, which has an accuracy and stability of band counting that is inferior compared to capillary electrophoresis and thus affects the accuracy of the results.

In this study, the 12 preferred SSR primers recommended by NY/T 1433-2007 were firstly applied for a genetic diversity analysis and DNA fingerprint construction of 50 japonica rice cultivars in Heilongjiang Province (Ministry of Agriculture of the People's Republic of China, 2007). This national standard could be technical application and present useful direction for both here and other provinces. Therefore, the SSR marker-based PCR products from the NY/T 1433-2007 recommendation were tested by capillary electrophoresis, which improved the accuracy of the results and provided technical support for the identification and conservation of japonica rice varieties in Heilongjiang Province.

2. Materials and methods

Selection of test materials and simple sequence repeat primers

A total of 50 japonica rice cultivars were collected from five farming regions in Heilongjiang Province, including 11 cultivars from ChaHaYang, 12 cultivars from JianSanJiang, 4 cultivars from WuChang, 12 cultivars from FangZheng, and 11 cultivars from XiangShui. The distributions of the cultivars and regions are shown in Figure 1. The SSR primers are shown in Table 1 (Ministry of Agriculture of the People's Republic of China, 2007).

Genomic DNA extraction and PCR amplification

Fifty rice seeds were germinated to the 3-leaf stage in an incubator with a constant temperature of 30 °C and constant humidity. After the tips of the leaves were collected, DNA was extracted and purified with the modified cetyltrimethylammonium bromide method; the obtained DNA samples were stored at -20 °C for later use (Doyle and Doyle, 1987).

Fluorescent primers were synthesised based on the pre-experiment results. The 5' end of the primer was labelled with FAM/HEX/Tamra. The PCR amplification reaction solution contained 0.6 µl of the forward and reverse primers (µM), 2 µl of 5×PCR buffer, 1 µl of Enhancer, 0.8 mmol/l of dNTPs (10 mM), 0.12 µl of Taq DNA polymerase, 1 ng of the DNA template, and the appropriate amount of ddH_2O to obtain a total volume of 10 µl.

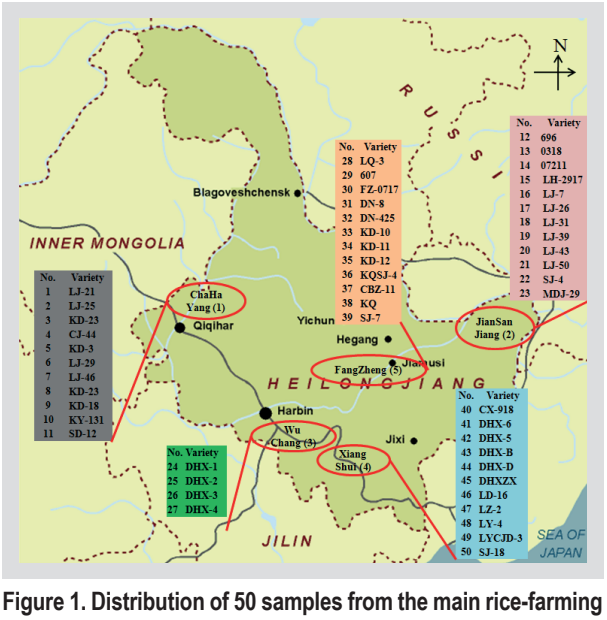

Figure 1. Distribution of 50 samples from the main rice-farming areas of Heilongjiang Province, China. 1 = ChaHaYang; 2 = JianSanJiang; 3 = WuChang; 4 = XiangShui; 5 = FangZheng.

Table 1. Distribution of the 12 recommended preferred simple sequence repeat markers in the rice genome and the primer sequences (NY/T 1433-2007).

Marker	Chromosome	Physical location	Forward primer (5'→3')	Forward T_m/°C	Reverse primer (5'→3')	Reverse T_m/°C
RM297	1	32093949	TCTTTGGAGGCGAGCTGAG	59.7	CGAAGGGTACATCTGCTTAG	57.8
RM71	2	8761504	CTAGAGGCGAAAACGAGATG	57.8	GGGTGGGCGAGGTAATAATG	59.8
RM85	3	36287160	CCAAAGATGAAACCTGGATTG	56.1	GCACAAGGTGAGCAGTCC	59.1
RM5414	4	2021760	ACCATGGTTCAAGAGTGAAA	53.7	ACAGCTCAACCTGTTGAGTG	57.8
RM274	5	26661876	CCTCGCTTATGAGAGCTTCG	59.8	CTTCTCCATCACTCCCATGG	59.8
RM190	6	1764638	CTTTGTCTATCTCAAGACAC	53.7	TTGCAGATGTTCTTCCTGATG	56.7
RM336	7	21818658	CTTACAGAGAAACGGCATCG	57.8	GCTGGTTTGTTTCAGGTTCG	57.8
RM72	8	6757363	CCGGCGATAAAACAATGAG	55.4	GCATCGGTCCTAACTAAGGG	59.8
RM219	9	7887585	CGTCGGATGATGTAAAGCCT	57.8	CATATCGGCATTCGCCTG	57.3
RM311	10	9347372	TGGTAGTATAGGTACTAAACAT	52.6	TCCTATACACATACAAACATAC	52.6
RM209	11	17767906	ATATGAGTTGCTGTCGTGCG	57.8	CAACTTGCATCCTCCCCTCC	61.9
RM19	12	2432080	CAAAAACAGAGCAGATGAC	53.2	CTCAAGATGGACGCCAAGA	57.6

The PCR reaction program was as follows: pre-denaturation at 94 °C for 2 min; forty cycles of denaturation at 94 °C for 30 s, annealing for 30 s (the annealing temperatures for the different primers are shown in Table 1), and extension at 72 °C for 30 s; a final extension at 72 °C for 5 min for a total of 40 cycles and storage at 10 °C.

Capillary electrophoresis

The experiment was performed with a 3730XL sequencer (Applied Biosystems, Foster City, CA, USA). The original data obtained from the sequencer were analysed using the fragment analysis software (Plant) in Genemarker (SoftGenetics, State College, PA, USA). The fragment size was obtained by comparing the position of the internal standard of each lane with the peak position of each sample (Cheng *et al.*, 2011).

Data and statistical analysis

The genetic distance, genetic similarity, polymorphism frequency (PF), number of alleles, effective number of alleles (Kimura and Crow, 1964), polymorphism information content (PIC), Nei's (1973) gene diversity, and Shannon's diversity index (Francis *et al.*, 2000) were calculated using POPGENE version 1.32 software (University of Alberta, Edmonton, Canada). The principal component analysis was performed using OriginPro version 9 (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

Simple sequence repeat marker polymorphisms

The polymorphism information for the 12 primer pairs is shown in Table 2. A total of 40 alleles were detected in the capillary electrophoresis assay, with an average of 3.3 alleles amplified by each primer pair showing good polymorphisms. Marker RM72 showed 11 alleles, which was the highest number of alleles detected in all rice varieties, whereas only one allele was detected using

Table 2. Polymorphism information for the 12 pairs of primers.[1]

SSR	Na	PF value	PIC value	Ne	h	I
RM297	5	0.411	0.093	1.107	0.093	0.188
RM71	2	0.035	0.093	1.107	0.093	0.188
RM85	1	0.040	0.163	1.317	0.203	0.330
RM5414	4	0.481	0.365	1.041	0.039	0.098
RM274	1	0.000	0.269	1.368	0.269	0.440
RM190	2	0.240	0.154	1.159	0.120	0.203
RM336	4	0.360	0.144	1.484	0.285	0.435
RM72	11	0.521	0.493	1.000	0.000	0.000
RM219	5	0.667	0.205	1.569	0.302	0.442
RM311	2	0.315	0.467	1.498	0.250	0.346
RM209	1	0.000	0.245	1.658	0.371	0.549
RM19	2	0.035	0.493	1.779	0.422	0.608

[1] SSR = simple sequence repeat; Na = number of alleles; PF = polymorphism frequency; PIC = polymorphism information content; Ne = effective number of alleles; h = gene diversity; I = Shannon's diversity index.

RM85, RM209, and RM274 as the markers, which was the smallest number of alleles. Five alleles were detected using markers RM297 and RM219, four alleles were detected using markers RM5414 and RM336, and two alleles were detected using markers RM71, RM190, RM311, and RM19. These findings are similar to the results of Luo *et al.* (2015) in the study of DNA fingerprint construction for the conventional japonica rice cultivars in the Taihu Lake area. In Luo *et al.*'s study, 99 alleles were detected in 19 japonica rice varieties using 24 primer pairs, with the primers RM297, RM5414, RM190, RM311, RM72, RM71, and RM19 showing the highest polymorphisms among the varieties. While RM297, RM85, RM190, RM219 markers indicated a balanced polymorphisms in 46 rice cultivars in Zhengjiang Province using the recommended national 12 SSR primers (Li *et al.*, 2014).

Therefore, it is applicable to use the primers recommended from the national standard in China to identify the rice polymorphisms. The results could be a reasonable test for the 50 rice varieties and show potential application in other regions of China. 354 single primers were ever summarised among the total 988 SSR primers from 3,388 rice varieties in 17 provinces of China and compared with the recommended ones of the national standard (Zhijiang Li *et al.*, unpublished data). 96% anastomoses were matched with those primers in national standard recommendation. Therefore, the above results firstly indicated valuable and applicable authority in Heilongjiang rice varieties.

The PF values of the markers varied from 0-0.667, with an average of 0.259. The polymorphisms identified using the markers differed. The PF values of RM72 and RM219 were higher than 0.500, while those RM71 and RM209 were higher than 0.500 in rice cultivars of Zhengjiang Province (Li *et al.*, 2014). The PF values of markers RM274 and RM209 were 0, showing no allele polymorphism in the 50 rice cultivars. The results obtained with markers RM274 and RM209 were consistent with the results obtained for the conventional japonica rice varieties by Ying *et al.* (2007). The PIC values of the SSR markers ranged from 0.093-0.493, with an average of 0.265. While those values in the range of 0.249-0.834, with an average of 0.728 in 48 rice cultivars of Guizhou Province and 0.038-0.729, with an average of 0.456 in 50 ones of Hunan Province were higher than those in Heilongjiang Province under the same recommended SSR primers (Wang *et al.*, 2012; Xu *et al.*, 2017).

The RM72 and RM19 chromosomes had the highest PIC value (0.493), followed by the RM311 chromosome (0.467), whereas the RM297 and RM71 chromosomes had the lowest PIC value (0.093). The SSR marker polymorphisms were not abundant in the 50 cultivars. This result reflected the relatively low genetic diversity of the rice varieties in Heilongjiang Province, which was similar to the results obtained by Liu *et al.* (2015) based on polymorphism analysis of 125 samples from Heilongjiang Province.

The effective allele number (Ne) ranged from 1.000 (RM72)-1.779 (RM19), with an average of 1.341. Nei's genetic diversity ranged from 0-0.442, with an average of 0.204. The Shannon diversity index ranged from 0-0.608, with an average of 0.319.

The average Ne and PIC values in the experiments in the present study were 1.341 and 0.265. These values were lower than the values reported in the literature (Zhang *et al.* 2012, 2013), indicating that the northern rice had low genetic diversity. Therefore, the selection of appropriate breeding methods is important to enhance the genetic diversity of the typical japonica rice in Heilongjiang Province.

Genetic similarity coefficient and genetic distance among the five rice farming areas

The statistical analysis of the genetic information obtained for the five rice farming areas is shown in Table 3. The genetic similarity coefficient ranged from 0.850 to 0.982, with an average of 0.928. The genetic similarity was highest between the rice cultivars in JianSanJiang and ChaHaYang (0.982) and lowest between the rice cultivars in WuChang and ChaHaYang (0.850). The overall genetic variation was small, and the genetic similarity between the rice cultivars among the five rice farming areas was high with a narrow

Table 3. Genetic similarity coefficient and genetic distances among the five groups.[1]

ID	ChaHaYang	JianSanJiang	WuChang	FangZheng	XiangShui
ChaHaYang	–	0.982	0.850	0.947	0.941
JianSanJiang	0.018	–	0.855	0.972	0.938
WuChang	0.162	0.157	–	0.877	0.936
FangZheng	0.055	0.029	0.131	–	0.941
XiangShui	0.061	0.064	0.066	0.061	–

[1] The upper triangle shows the genetic similarity coefficients between the corresponding groups, and the lower triangle shows the genetic distances between the corresponding groups.

genetic basis, in which these results were firstly reported in Heilongjiang Province. Zhang *et al.* (2016) used 62 SSR markers to analyse the genetic diversity of 73 conventional rice cultivars validated in Heilongjiang Province in recent years, in which the variance of the genetic similarity coefficient between the cultivars was 0.622-0.966, with an average of 0.759. This finding indicated that the genetic relationships among the 73 varieties were close, which was similar to the findings of the present study.

Differences in the geographical distribution, accumulated temperature, and planting may also lead to an increase in the genetic diversity of the rice (Das *et al.*, 2013). Genetic distance values were in the range of 0.018 to 0.162 (Table 3). And those WuChang indicated the bigger genetic differences with ChaHaYang (0.162) and JianshanJiang (0.157) rice varieties, respectively. Therefore, the Daohuaxiang and Longjing japonica rice series in this study showed significant genetic differences between different planting areas, such as the four DHX varieties in WuChang, the five varieties in XiangShui, and the LJ and KD varieties in all areas. Studies have shown that the early northeast rice varieties are mostly bred from Japan; thus, the genetic distance of the northeast rice varieties is still narrow after hybrid breeding (Wang *et al.*, 2014). Therefore, elite genes should be properly introduced and breeding methods suitable for the local planting conditions should be developed to improve the germplasm of quality japonica rice in Heilongjiang Province. Moreover, genetic modification with genes to improve the rice's nutritional quality and sensory characteristics should be considered to improve the genetic diversity and the food quality of the rice.

Similarly, Li *et al.* (2014) used the 12 preferred SSR primers recommended by the Ministry of Agriculture to analyse the genetic diversity of 46 new japonica rice cultivars in Zhejiang Province. The similarity coefficients between the varieties ranged from 0.735-1.000, with an average of 0.879, indicating that the genetic relationships among the japonica rice varieties in Zhejiang Province were close and that the genetic background was narrow. A DNA fingerprint database of 279 cultivars has been successfully constructed (Cheng, 2009). These successes demonstrated that the construction of specific rice varieties and investigations of their consistency over different years using these primers are feasible. Based on the above findings, the analysis of the genetic relationships of rice varieties in a certain region using the preferred primers recommended by the Ministry of Agriculture is effective, and the identification results firstly demonstrated here are stable for the varieties in Heilongjiang Province.

Cluster analysis among the rice varieties

Using the OriginPro software, we performed a cluster analysis of the 50 japonica rice cultivars by calculating the nearest neighbour based on the Euclidean distance. As shown in Figure 2, the 50 cultivars could be divided into eight categories with a similarity coefficient of 3.4.

The rice varieties in the five japonica rice farming areas of Heilongjiang Province could be further distinguished according to the similarity coefficients of the genes. Here, category 1 contains up to 12 varieties, and category 3 has at least three varieties. All four varieties of WuChang were distributed in one category, whereas the five rice varieties in XiangShui and the four rice varieties in FangZheng belonged to categories 4 and 8, respectively (as shown in Table 4). Thus, the genetic information for the japonica rice in Heilongjiang Province was complex, but the Wuchang rice was accurately identified based on the geographical indication.

The results showed that the genetic distance of the rice varieties in Wuchang was greatly different from other areas, although the overall genetic distance was narrow. The SSR breed identification technique can detect some minor differences at the DNA level. However, some varieties with significant differences in morphological characteristics may be identical when identified using fewer primers (i.e. the selected primers are not necessarily guaranteed to identify the cultivar). Similarly, Wang *et al.* (2012), Li *et al.* (2014) and Xu *et al.* (2017) also applied the recommended 12 SSR primers to conclude that those rice cultivars indicated narrow genetic similarity coefficients, which could be the reasons of close affinity and breeding background.

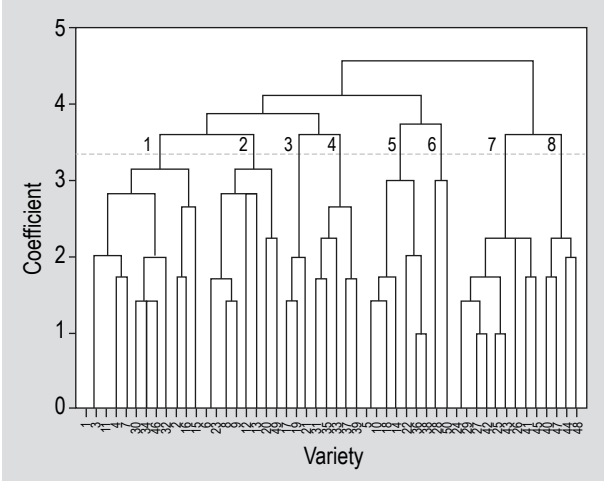

Figure 2. Cluster analysis of the 50 varieties.

Table 4. Accessions numbers of each cluster.

Category	ChaHaYang	JianSanJiang	WuChang	XiangShui	FangZheng	Total
1	6	2		3	1	12
2	3	4			1	8
3		3				3
4				5		5
5	2	3		2		7
6				1	1	2
7			4	1	4	9
8					4	4
Total	11	12	4	12	11	50

Construction of DNA fingerprints

According to the amplification results obtained using the 12 pairs of preferred rice primers and capillary electrophoresis, the presence or absence of polymorphic fragments was recorded in the form of 0/1 to construct the DNA fingerprints of the 50 cultivars.

Cultivars showing different phenotypic traits are not necessarily different in genotype, and the fingerprint differences based on the molecular markers are not necessarily expressed in the phenotypic traits (Cheng *et al.*, 2011). Therefore, differences in DNA fingerprints cannot be used as the only basis for the identification of a variety. Specific identification should be performed in accordance with the requirements of the application using the application purpose as the starting point.

4. Conclusions

To our knowledge, it is very important to establish the national standard to identify the crop in genetic level. In China, the 12 rice primers are recommended to assess the authenticity of japonica rice and useful to protect germplasm resources and the brands in Heilongjiang by SSR technique. Based on the results above, the national standard of NY/T 1433-2007 could be effectively applied for the rice varieties, in which those could extendedly present the improvement of rice germplasm resources and progress in breeding technology not only greatly improve the food quality and planting of rice but also lead to complexity in the genetic diversity of elite and famous varieties. Also the genetic backgrounds of rice varieties in Heilongjiang Province firstly indicated that a narrow characteristic which would be useful in the breeding and planting area. Therefore, those studies present the guarantee for both the cultivation area and production of japonica rice in Heilongjiang Province in China.

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