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Establishment of DNA Fingerprinting of Liaojing Series of Japonica Rice

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Abstract: In this study, Liaoning Japonica rice were chosen as materials to construct its DNA fingerprints by SSR markers .The results showed that the optimal SSR-PCR reaction system in rice included composition as 1.5mmol/L Mg2+,0.25mmol/L dNTP,1.5 U TaqDNA polymerase, 0.4 mmol/L primer. Amplification protocol was predenaturing at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 1minute, annealing at 50, 55 or 60°C for 1 minute, extension at 72°C for 1 minutes and 8 minute final extension at 72°C. In the 22 selected higher genetic polymorphism primer pairs, RM19, RM224, RM311, RM278, RM210, RM336, RM225, RM267, RM335, RM232, RM226 and RM1195 were able to amplify stable, clear and easily identified bands. Establishing a unique DNA fingerprint of the tested rice varieties by their fingerprints can distinguish each of the tested materials, such as genetic mapping construction, genetic diversity, variety identification, seed purity testing and accessorial breeding. This study provided the basis for protection of new varieties and seeds identification of authenticity and purity.

Key words: Japonica rice · Fingerprint map · Agronomic SSR · Identify

INTRODUCTION

Simple sequence repeat (SSR) is an effective tool for genetic variation identification of germplasms [1-2]. SSR markers have some merits such as quickness, simplicity, rich polymorphism and high stability, thus being widely applied in molecular map construction and gene mapping [3-4], construction of fingerprints [5], genetic purity test [6], analysis of germplasm diversity [7-9] and utilization of heterosis [10-11], especially in identification of the species with closer genetic relationships [12-14]. Using SSR molecular markers, Qi *et al* [15] studied the trends in genetic diversity of Chinese rice varieties developed in the last 50 years and found that the genetic diversities analyzed by the two methods were highly consistent. Wang *et al* [16] showed that the combination of pedigree analysis, SSR molecular marker technology and cluster analysis could further improve the research techniques of genetic diversity of species.

China has begun implementation of new varieties protection and it is necessary to establish DNA fingerprint database. In the northeast old industrial leading industries, Liaoning Agricultural development is one of the priorities of this project [17]. But there are few reports about constructions of DNA fingerprints and database of Japonica. In this study, a series of Liaoning Japonica rice are used to map their fingerprints by applying DNA molecular marker techniques, which will provide molecular basis for the protection of new varieties and the identification of seed purity and authenticity.

MATERIALS AND METHODS

Liaoning Japonica rice was provided by Rice Research Institute of Liaoning Agricultural Sciences Academy.

DNA Isolation: The total DNA of all the rice materials is extracted by using simple method [18].

The Detection of DNA Concentration: Agarose gel electrophoresis and sub-photometric method were used for monitoring the concentration and purity of DNA respectively.

Amplification of Microsatellite and Design of Ssr Primers: Public Rice genome physical map was used to find the 41 pairs of SSR primers (Table 1-2) distributed in 12 rice chromosomes. Polymorphic marker selecting tests were made by applying these primers.

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Table 1.1: A list of rice varieties used in experiment

Number	Varieties	Number	Varieties
1	Liaoning 326	6	Liaoning 294
2	Liaoning 5	7	Liaoning 6
3	Liaoning 180	8	Liaoxing 9
4	Liaoxing 10	9	Liaoning 287
5	Liaoning 371	10	Liaoning 454

Table 1-2: Basic information of SSR Markers

Marker	Chromosome	Marker	Chromosome	Marker	Chromosome	Marker	Chromosome
RM151	1	RM218	3	RM238	6	RM269	10
RM1195	1	RM5414	4	RM125	7	RM224	11
RM297	1	RM273	4	RM336	7	RM209	11
RM266	2	RM335	4	RM18	7	RM101	12
RM263	2	RM26	5	RM320	7	RM19	12
RM154	2	RM267	5	RM210	8	RM17	12
RM48	2	RM274	5	RM223	8		
RM530	2	RM334	5	RM278	9		
RM232	3	RM225	6	RM219	9		
RM85	3	RM133	6	RM311	10		
RM55	3	RM204	6	RM258	10		

Table 1-3: SSR-PCR recipe

Component	Concentration	Aliquot (ul)
10×Buffer	1	2.5ul
25mM $MgCl_2$	1.5mM	1.5ul
5uM Primer	0.2uM	1ul
5uM Primer	0.2uM	1ul
10mM dNTP	0.2mM	0.5ul
5U/ul Taq Enzyme	1.25U	0.25ul
25ng/ul DNA	50ng	2ul
ddH_2O		16.25ul
Total		25ul

The public Rice genome physical map is available by logging http://www.gramene.org/mirosat/ssr.html. These primers were synthesized by Shanghai Bio-Engineering Co. Ltd.

The SSR-PCR Amplification and Optimization in the Reaction System of Genomic DNA: The reaction conditions of PCR is pre-denaturing at 94°C for 5 min, 35 cycle of denaturing at 94°C for 1 min, annealing at the primer's temperature 50°C, 55°C or 60°C for 1 min and extending at 72°C for 1 min, extending at 72°C for 8 min finally. Reaction mixture volume is 25 ul (Table 1-3).

The four major components affecting PCR reactions include $MgCl_2$, Primer, dNTP and Taq enzyme. We use different combinations of gradients from which the best combination is selected.

Detection of Amplified Products: SSR-PCR reaction system is optimized with 3% agarose gel electrophoresis and SSR markers are analysed with 6% denaturing polyacrylamide gel electrophoresis.

Mapping of Japonica Dna Fingerprint

Screening of Ssr Primers with Dna Fingerprinting: olymorphism is analysed with composited 41 pairs of SSR primers and optimized SSR-PCR system to determine the appropriate SSR markers applied in fingerprint mapping.

Mapping of DNA Fingerprint: Map the series of Liaojing varieties DNA fingerprinting with screened primers.

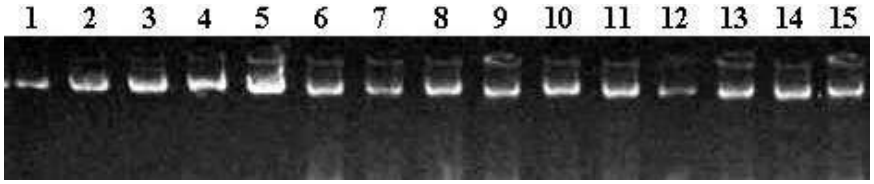

Fig. 1-1: The total DNA isolates checked by 0.8% Agarose gel.
1-5 The λDNA is 25ng, 50ng, 100ng, 150ng and 200ng; 6-15 is Liaojing 326. Liaojing 5. Liaojing 180. Liaoxing 10. Liaojing 371. Liaojing 294. Liaoxing 9. Liaojing 287. Liaojing 454.

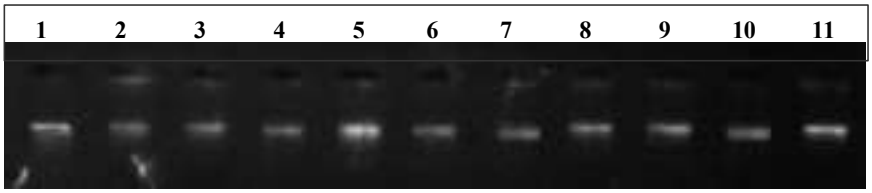

Fig. 1-2: The diluted DNA isolates checked by 0.8% Agarose gel.
1 50ng λDNA 2-11 is diluted DNA of Liaojing 326. Liaojing 5. Liaojing 180. Liaoxing 10. Liaojing 371. Liaojing 294. Liaojing 6. Liaoxing 9. Liaojing 287. Liaojing 454.

RESULTS AND DISCUSSION

DNA Isolation and Detection: The concentration of extracted DNA samples was between 200~400ng/ul. Clear and tidy bands showed by 0.8% agarose gel electrophoresis (Figure 1-1). Compared with ëDNA, obtained DNA molecular weight were larger and fulfilled the requirements of SSR-PCR amplification. The concentration of extracted genomic DNA was diluted around 25-50ng for PCR amplification (Figure 1-2).

Optimization of Ssr Reaction System : The concentration gradients are set according to concentration ranges of various ingredients used in conventional SSR. The PCR amplification results are assessed based on the number of gel electrophoresis bands and their brightness and stability to determine the optimum system.

Effect of Mg^{2+} Concentration on Ssr Amplification Products: Mg^{2+} was used as the activator of Taq enzyme and its concentration directly affect the specificity and efficiency of PCR amplification. If the concentration of Mg^{2+} is too low, Taq enzyme activity decrease significantly. While too high, it may cause non-specific amplification products. Eight concentration gradient of Mg^{2+} is set in this experiment. That is 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mmol / L. Addition to the concentration of 0.5mmol / L, others all have amplified products (Figure 1-3). The results show the bands are vaguer when Mg^{2+} concentration is 1.0mmol / L and relatively clear when 1.5 ~ 2.5mmol / L and non-specific bands are produced when 3.0 ~ 4.0mmol / L .Consequently, 1.5mmol / L is selected as the optimal concentration.

The Effect of *dNTPs Concentration on SSR Amplification* Products: dNTP is the substrate of PCR reaction. If dNTP concentration is too low, it may result in low productions. While too high it will cause error incorporation. Seven dNTP concentration gradients are set in this experiment and all have amplified products (Figure 1-4). The results show the amplified products are lighter and vaguer when dNTP concentration is 0.10 ~ 0.20mmol / L and brighter when 0.40mmol / L and the amplification effect is better when 0.25 ~ 0.35mmol / L. Therefore, in the view of economic point, 0.25mmol / L of dNTP concentration is appropriate.

The Effect of Primer Concentration on SSR Amplification Products: Primer concentration is an important factor of PCR reaction system. If primer concentration is too low, it may result in low amplification productions and uneasy detective DNA bands. While too high it will cause non-specific amplification products and the formation of primer dimmer. As can be seen from Figure 1-5, the concentration of primers is a greater effect on SSR reaction. The results show the PCR products are low when primer concentration is 0.2mmol / L and non-specific amplification is generated when 0.6 ~ 1.0mmol /L and with the increase of primers concentration the non-specific products increase. The effect of amplification is better when primer concentration is 0.4mmol / L.

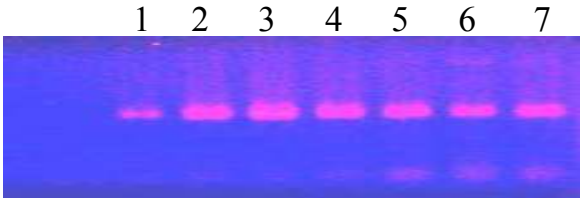

Fig. 1-3: The effect of Mg2+ concentration on SSR profiles.
1-8 Mg2+ concentration is 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0mM

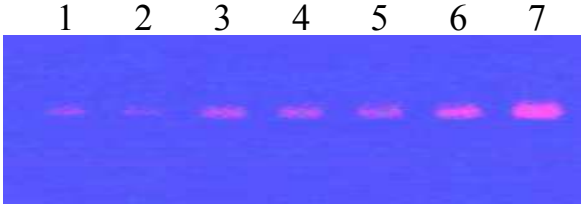

Fig. 1-4: The effect of dNTPs concentration on SSR profiles.
1-7 The dNTPs concentration is 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 and 0.40mM

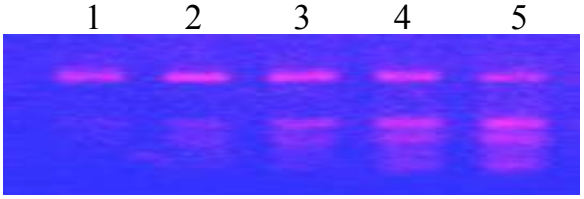

Fig. 1-5: The effect of primer concentration on SSR profiles.
1-5The primer concentration is 0.2, 0.4, 0.6, 0.8 and 1.0mM

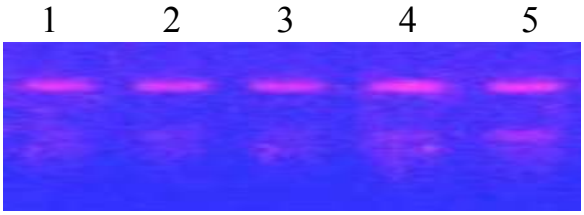

Fig. 1-6: The effect of Taq DNA polymerase concentration on SSR profiles.
1-5 The Taq DNA polymerase concentration is 0.5, 1.0, 1.5, 2.0 and 2.5U

The Effect of Taq DNA Polymerase Concentration on SSR Amplification Products: Taq enzyme concentration is an essential factor for PCR products. Amplification effects of different Taq DNA polymerase manufacturers and batches are vastly different. If Taq enzyme concentration is too low, it can not be amplified. While too high, non-specific amplification products may generate. The results show that the amplification effect is better and the bands are clearer when Taq DNA polymerase concentration is 1.0U and 1.5U. For economy considerations, the optimal Taq DNA polymerase concentration is 1.25U (Figure 1-6).

Considering the above factors, the optimal rice SSR amplification conditions are that Mg^{2+} concentration is 1.5mmol / L, dNTP concentration is 0.25mmol / L, Taq polymerase concentration is 1.25U and primer concentration is 0.4mmol / L.

In the SSR-PCR reactions, each SSR primer has its own optimal annealing temperature and the annealing temperatures of two single primers in some SSR primers are largely different. We have found through land-PCR amplified that better amplification results can be achieved in the same PCR reaction procedure with primers of different annealing temperatures. In this reaction system, the reaction procedures are pre-denaturing at 94°C for 5min, 35 cycles of denaturing at 94°C for 1min, annealing at the primer' s temperature 65~54°C for 1min and extending at 72°C for 2 min, then extension at 72°C for 8 min finally. Among procedures, each annealing temperature minus 1°C in each cycle. With the optimized amplification system and process, the specificity of amplified bands is notably enhanced.

Detection of PCR Amplified Products: In this study, we detect the PCR amplification products with 3% agarose gel electrophoresis and find the distinguished length of DNA fragment is 300bp which is worse than the products of polyacrylamide gel electrophoresis (Figure1-7) 6% variability and 8% non-denaturing polyacrylamide gel electrophoresis were compared in this experiment. The results showed that 8% non-denaturing gel generate more mixed bands while 6% denaturing gel cause wide bands which can be regulated by adjusting the voltage. In general, the detection effect is better with 6% denaturing gel.

Selecting of Polymorphism SSR Primer: In this study, 41 pairs of SSR primers distributed in 12 rice chromosomes are selected within the experimental materials. The results showed that products amplified with 22 pairs of primers out of 41 exhibit polymorphism (Figure 1-8, Table 1-4). The polymorphism performance rate is 53.7%, indicating a high polymorphism of SSR markers. Based on a pair of primers and a large number of alleles in each chromosome, SSR primers used to map DNA fingerprinting are selected from the above 22 pairs of primers.

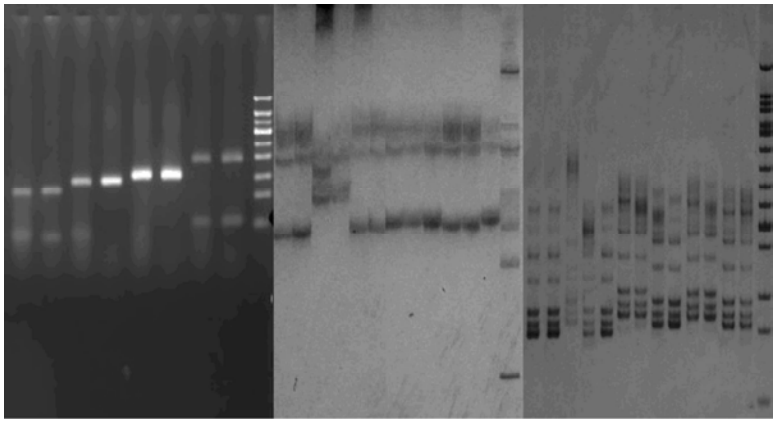

Fig. 1-7: SSR profiles detected by three different methods

A: by 3% agarose gel electrophoresis; B: by 8% non-denaturing acrylamide gel electrophoresis; C: by 6% denaturing acrylamide gel electrophoresis.

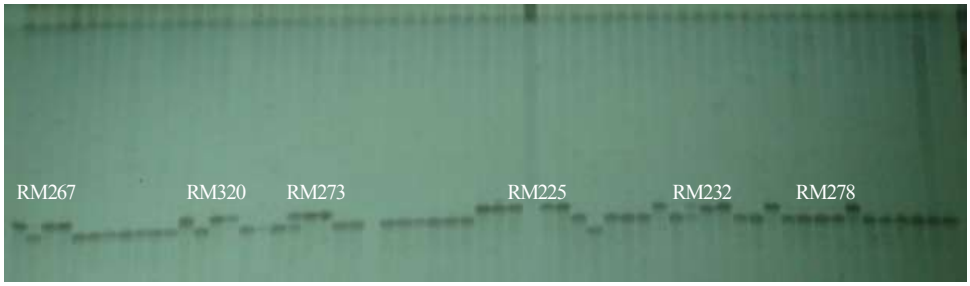

Fig. 1-8: Selection of polymorphism SSR primer

1-60 is result of different primer of Liaojing 326. Liaojing 5. Liaojing 180. Liaoxing 10. Liaojing 371. Liaojing 294. Liaojing 6. Liaoxing 9. Liaojing 287. Liaojing 454.

Table 1-4: Results of SSR polymorphism of the rice variety

	Rice variety											Rice variety											Rice variety									
Primer	1	2	3	4	5	6	7	8	9	10	Primer	1	2	3	4	5	6	7	8	9	10	Primer	1	2	3	4	5	6	7	8	9	10
RM278	0	0	0	1	0	0	0	0	0	0	RM210	0	0	1	0	0	0	0	0	0	0	RM1195	1	0	0	0	0	1	0	1	0	0
	1	1	1	0	1	1	1	1	1	1		0	0	0	1	0	0	0	0	0	0		0	0	1	1	1	0	1	0	0	0
RM225	1	1	1	9	1	1	0	0	0	0		1	0	1	1	0	1	1	1	0	0		0	1	0	0	0	0	0	0	1	1
	0	0	0	0	0	0	1	0	1	1		0	1	0	0	1	0	0	0	0	1	RM273	1	1	0	9	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	0	0	RM335	0	0	0	1	0	0	0	0	0	0		1	0	1	1	1	1	1	1	1	1
RM133	1	1	1	1	1	1	1	1	1	1		0	0	0	0	0	0	0	1	0	0	RM232	0	1	0	1	1	1	0	0	1	0
RM224	1	0	0	1	0	0	1	0	0	0		1	1	1	0	1	1	1	0	1	1		1	0	1	1	0	0	1	1	0	1
	0	0	1	0	0	1	0	1	0	0	RM320	1	0	1	1	0	0	0	0	1	1	RM209	0	0	0	0	0	0	0	0	0	0
	0	1	0	0	1	0	0	0	1	1		0	1	0	0	1	1	1	1	1	0		1	1	1	1	1	1	1	1	1	1
RM223	0	1	0	0	0	0	0	0	0	0	RM264	1	0	0	0	0	1	0	1	1	0	RM530	1	1	0	1	0	1	1	1	1	1
	1	0	1	1	1	1	1	1	1	1		1	1	1	1	1	0	1	0	0	1		0	0	1	0	1	0	0	0	0	0
RM5414	1	1	1	0	1	1	1	0	1	1	RM266	1	1	1	0	0	0	0	0	0	0	RM297	0	0	0	1	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	0	0		1	0	1	0	0	0	0	0	0	0		0	9	0	0	0	0	1	0	0	0
	0	0	0	1	0	0	0	0	0	0		0	0	0	0	1	0	1	1	0	1		1	1	1	0	1	1	0	1	1	0
RM19	1	1	1	1	1	1	1	1	1	1		0	0	0	1	0	0	0	0	0	0		0	0	0	0	0	0	0	0	0	1
	1	0	0	1	1	1	1	1	0	0	RM311	0	0	1	1	0	0	1	0	0	0	RM336	0	0	1	0	0	0	1	0	0	0
RM18	1	1	1	0	1	1	1	0	1	1		1	0	0	0	0	1	0	1	0	0		0	1	0	0	1	0	1	0	0	0
	0	0	0	1	0	0	0	0	0	0		0	1	0	0	1	1	0	0	1	1		1	0	0	1	0	0	0	0	0	0
	0	0	0	0	0	0	0	1	0	0	RM469	1	1	1	1	1	1	1	1	1	1	RM267	1	0	1	1	0	0	0	0	0	0
RM238	1	1	1	1	1	1	1	1	1	1																						
	0	1	0	0	0	0	0	0	0	0																						

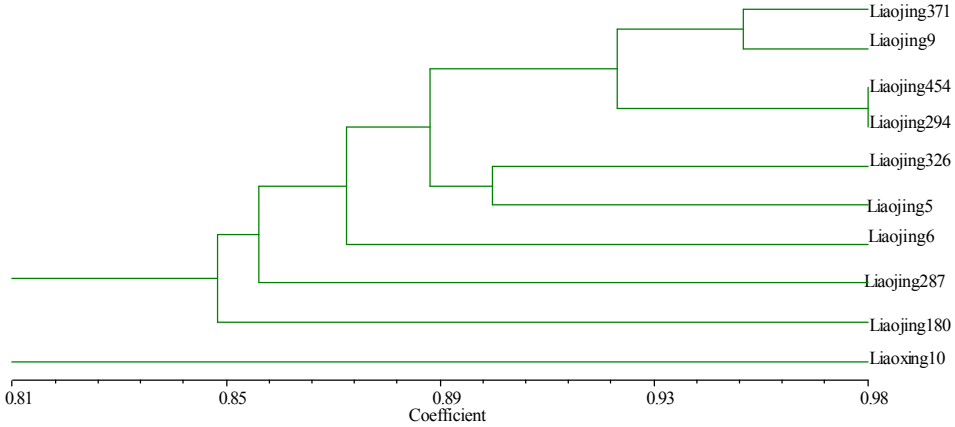

Fig. 1-9: Phylogenetic tree analysis of tested materials

Table 1-5: Basic information of finger-printing SSR Markers

Primer Makers	RM1195	RM226	RM232	RM335	RM267	RM225	RM336	RM210	RM278	RM311	RM224	RM19
Chromosome	1	2	3	4	5	6	7	8	9	10	11	12
The number of allele	4	5	3	6	2	4	4	5	3	3	5	3

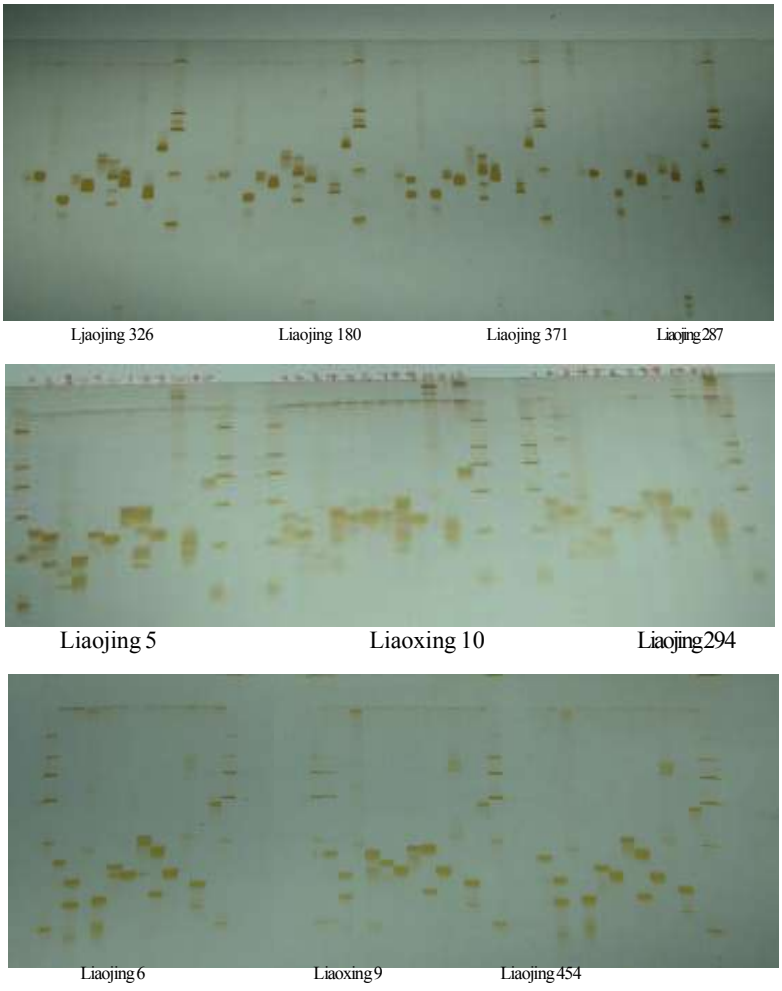

Fig. 1-10: Finger-print of ten rice

Cluster Analysis: According to 01 database, the phylogenetic tree of SSR analysis is obtained through UPGMA cluster analysis (Figure 1-9)

According to the results of phylogenetic tree, ten rice varieties were divided into five groups according to the standard genetic similarity which is 0.89. After dividing groups, the average genetic distance of inter-groups is greater than the distance within group.

The first group Liaojing 326, Liaojing 5, Liaojing 371, Liaojing 294, Liaoxing 9, Liaojing 454

- The second group Liaojing 6
- The third group Liaojing 287
- The fourth group Liaojing 180
- The fifth group Liaoxing 10

Mapping of DNA Fingerprint: Mapping the DNA fingerprint of ten rice varieties applies the twelve pairs of SSR primers which can amplify clear bands and have more alleles (Figure 1-10). The twelve pairs of SSR primers are selected from the polymorphic primers (Table 1-5).

DNA Fingerprint Identification System of Molecular Markers: In this study, a complete SSR molecular markers system is established by SSR molecular maker technology used Liaojing tested materials. This established system can be applied for mapping of DNA fingerprint. The optimal SSR amplification conditions are that Mg^{2+} concentration is 1.5mmol / L, dNTP concentration is 0.25mmol / L, Taq polymerase concentration is 1.25U and primer concentration is 0.4mmol / L.

In this reaction system, the reaction procedures are pre-denaturing at 94 for 5min, 35 cycles of denaturing at 94 for 1min, annealing at the primer §s temperature 50, 55 or 60 for 1min and extending at 72 for 1 min, then extension at 72 for 8 min finally.

Construction of the Standard Dna Fingerprints of Rice: In this study, 12 pairs of primers are selected from the 22 pairs of primers which have strong genetic polymorphism. Stable, clear and easily bands can be amplified by these filtered 12 pairs of primers. They are RM19, RM224, RM311, RM278, RM210, RM336, RM225, RM267 RM335, RM232, RM226 and RM1195. Specific DNA fingerprints of rice are constituted by using the combination of their fingerprints. So we can distinguish them one by one from the tested materials.

DISCUSSION

Phenotype is the interaction of genotype and environment. It is an important and effective method for investigating genetic structure and diversity using phenotypes [19]. Application of SSR markers has been an important way to reveal the genetic diversity of rice materials for its high polymorphism, high abundance, even distribution in the genome and codominance. SSR markers derived from functional genes are effective tools to study the classification of rice germplasms, geographical distribution and pedigree analysis [20-21] .In this study, comprehensive comparisons were conducted on 10 high-quality japonica rice germplasms using SSR markers, pedigree information and mapping the DNA fingerprint, which may provide necessary basis for fully understanding their properties and making full use of them.

The establishment of DNA fingerprint database provides the scientific basis for the rice seeds quality supervision and intellectual property protection. So we should make clear the purpose of constructing DNA fingerprinting database and apply it to test practice for future. At first, material selection should focus on main varieties, the protection of new varieties and authorized varieties. To make sure the varieties have greater practical value, the variety resources should be representative. Secondly, the choosing of SSR markers should have rich polymorphic primers in the selected materials. How to determine the core primer is a key. Core primer amplification haves several characteristics, such as stablely, repeatability and rich polymorphism information. Good varieties of rice are the basis to ensure sustained production. Because of the use of morphological characteristics in identifying hybrid rice germplasm and the purity has certain limitations, thus the use of molecular marker in distinguishing different rice materials has aroused people's great interest. Some Chinese scholars have tried to use SSR molecular markers to establish the DNA fingerprint that can apply to the purity identificate or market rights [22-23], some scholars also establish the particular hybrid rice or the checklist of DNA fingerprint [24-25].

With the use of DNA fingerprint process, the constructed database may reveal its limitations on the emergence of new varieties. So, we need to increase the number of fingerprint database varieties and the amount of core primer to make the database get improved.

Therefore, the construction of 10 japonica varieties DNA fingerprint in this study can be used as a primary database.

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