ISJ based markers DNA fingerprinting and diversity study in rice (*Oryza sativa.* L.) populations collected from different growth regions of China

ALY KANSAYE[1,2*], OUSMANE NIANGALY[2], AMADOU APHO BAH[2], CHENG RUI[1], LV SHUZUO[1], DU XIANGHONG[1], XIE SONGFENG[1], SONG WEINING[1, 3, 4]

[1]College of Agronomy Northwest A&F University, Yangling Shaanxi 712100, China

[2]College of Agronomy, IPR / IFRA, Katibougou Bp: 06, Koulikoro, Mali.

[3]Yangling Branch of China Wheat Improvement Center, Northwest A&F University, Yangling, Shaanxi 712100, China

[4]Shaanxi Key Lab of Molecular Biology for Agriculture, Northwest A&F University, Yangling, Shaanxi, 712100, China

Corresponding author*: kansayeali2014@gmail.com

ABSTRACT: Molecular markers technology provides novel tools for DNA fingerprinting of crops. The genetic diversity and DNA fingerprinting of 24 varieties of two different populations of rice (*Oryza sativa* L.) where investigated using ISJ primers. A total of 9 ISJ primers were tested for screening 24 varieties of two different populations. The objective of the present work was to study genetic diversity within and between populations and within varieties. The results revealed a total of 1607 reproducible amplifications products and 1463 (91.03%) polymorphic bands for all primers and all genotypes. The number of polymorphic amplified products ranged from 0 for primer E4 to 237 for primer R2. The number of bands per primer ranged from 24 for primer E4 to 261 for primer R2, and number of bands per genotypes varied from 1 to 10. The primers R2, R4 and E4-1 were the most informative locus for DNA profiling and differentiation. The coefficient of similarities based on ISJ data among varieties ranged from 0.42 to 0.90 with an average similarity index of 0.67. The lowest genetic similarity was observed between You Mi and Xiang Zhao Xian No 24; and the highest genetic similarity was observed between genotypes R207 and R507. Cluster analysis based on Dice's similarity coefficient using UPGMA procedure grouped the varieties into three clusters: Cluster I comprised nine varieties all from Hunan. Cluster II possessed five varieties four of them are from Hunan as well as varieties of cluster I, but the last one yangling Inc.'s locality is unknown. Cluster III possessed most varieties (ten) from Beijing and Hunan.

Keywords: ISJ, DNA fingerprinting, rice, Yangling, China

I. INTRODUCTION

Rice, (Oryza sativa) (2n=24) belonging to the family Gramineae and subfamily Oryzoidea is the staple food for one third of the world's population. After wheat, rice is the second largest source of calories in the human diet and provides approximately 20% of the total calories consumed worldwide. It is a particularly important food source in Asia and provide more than two billion people with 60-70% of their daily energy requirements (1) ;(2). Rice occupies almost one-fifth of the total land area covered under cereals. It is grown under diverse cultural conditions and over wide geographical range. The world's rice production has doubled during last 25 years, largely due to the use of improved technology such as high yielding varieties and better crop management practices (3) Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programs and use of new biotechnological tools. There is wide genetic variability and diversity available in rice among and between landraces leaving a wide scope for future crop improvement.

Moreover, rice is also an ideal model plant for the study of grass genetics and genome organization due to its diploid genetics, relatively small genome size 430 Mb (4), significant level of genetic polymorphism (5), large amount of well-conserved genetically diverse material (100.000 accessions of rice germplasm worldwide) and the availability of widely collected, compatible wild species.

Characterization and quantification of genetic diversity has long been a major goal in evolutionary biology. Information on the genetic diversity within and among closely related crop varieties is essential for a rational use of genetic resources. The analysis of genetic variation both within and among elite breeding materials is of fundamental interest to plant breeders. It contributes to monitoring germplasm and can also be used to predict potential genetic gains or losses.

The identification of rice varieties and

determination the relationships between varieties are very important for plant improvement program, variety registration system, distinctness, uniformity and stability (DUS) testing and the protection of plant variety and breeders' rights (6). So, clear-cut identification of elite crop varieties is essential for protection and prevention of unauthorized commercial use (7).

Conventional characterization of varieties based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environmental conditions. In contrast, DNA-based makers are highly heritable, available in high numbers, and exhibit enough polymorphism; hence they can be used to discriminate closely related genotypes of a plant (8); (9). For this reasons, DNA fingerprinting for cultivars or varietals identification has become an important tool for genetic identification in plant breeding and germplasm management (10).

The abundance of information on DNA sequences of plant genomes permits to design sequence-related primers for PCR amplification (11) The use of semi-random primers targeting the intron-exon splice junction (ISJ), proposed by Weining and Langridge (12) and developed by Rafalski et al. (13) and proved to be very useful for fingerprinting (14). This system is universal for plant because the sequences of primers were based on the consensus sequences of ISJ (7 and 9 bases in length) of plant and necessary for effective splicing (13).

Semi-random markers have been successfully used for cultivar analysis in number of plant species, rye (11), maize (15), wheat and triticale (16), potato (17) and common bean (18). Weining & Langridge (12) identified and mapped polymorphism in cereals using conserved, semi-random and random primers. With this approach, inter- and intra-specific polymorphism could be detected by different combinations of primers.

In the present study, we applied PCR with intron-exon splice junction (ISJ) primers (12) to collections of rice (*Oryza sativa* L.) germplasms to analyze the DNA polymorphism and the relationships between the cultivated varieties in China. The varieties have been collected from three different regions of China.

The main aim of this study is to assess genetic diversity and DNA variability between populations and within varieties.

II. MATERIAL AND METHODS

2.1 Plant material and genomic DNA isolation

Twenty-four rice varieties were used in this study see the list of these varieties on the Table 1. Genomic DNA was isolated from the young and fresh leaves of ten plants of several varieties for testing DNA variation within varieties, whereas the leaves of individual plant were used for all materials for testing genetic diversity between varieties. DNA was extracted according to Weining & Langridge (12) protocol modified by Weining & Henry (19).

DS Extraction buffer: 2 % Sodium Lauroyl sarcosine, 0.1 M Tris-HCl, 10 mM EDTA, pH 8.0.

2.2 Procedure of DNA isolation

Fresh leaves of twenty-one days old were used. Leaf tissues was cut into small pieces, and then grounded to a powder in 2ml tubes in liquid nitrogen, with the glass pestles under liquid nitrogen. The powder was then mixed with 0.6 mL of DS buffer and subsequently add 0.6 mL phenol/chloroform/isoamylalcohol (25:24:1). The whole mixture was shaken well for 30 seconds and leave it on ice for 20 min. During 20 min on ice the mixture was shaken three times with 5 min interval between each other. After 20 min on ice the mixture was centrifuged at 10000 rpm for 10 min in bench centrifuge. The aqueous phase was recovered and transferred to fresh tubes, 0.6 ml of chloroform was added to the obtained solution with subsequently shaken well before centrifugation for 10 min. The upper phase was collected and 0.5 ml of isopropanol and 50ul of 3 MNaAc were added in the tube. Then the tube was inverted gently for few times to precipitate the DNA and centrifuged at 10000 rpm for 5 min. After discarding the supernatant, the pellet was washed two times with 70 % ethanol. Pellet was air dried at room temperature for two hours and then was added 100ul of double distilled H_2O. We checked DNA quality with 1% agarose gel electrophoresis.

2.3 ISJ primers analysis of DNA

Nine ISJ primers were used in this study (see Table 2). PCR amplification was carried out in a total volume of 20μL reaction mixture containing 1.0μL of template DNA, 2.0μL of 10×buffer, 1.6μL dNTPs (2.5mmol /L), 1μL $MgCl_2$ (25mmol/L), 1μmol/L primer, 0.2μL of Taq polymerase (Takara) and 12.6μL of double-distilled H_2O. The amplification reaction was performed according the following cycling program: initial denaturation for 5 min at 94°C, followed by 9 thermal cycles of 1 min at 94°C, 108 s at 48°C, 2 min at 72°C, 20 thermal cycles of 1 min at 94°C, 90 s at 55°C, 2 min at 72°C, and a final extension at 72°C for 10 min.

The PCR-amplified products were separated by electrophoresis in 2 % agarose gels with 1×TAE buffer. Gels stained with ethidium bromide, were imaged in Biometra (UV-solo model) gel documentation system. Each reaction was repeated twice and only reproducible bands were considered for analysis.

Table 1. List of rice varieties.

No	Name	Origin	Site
1	Luo Gu b	South China	Hunan (South)
2	Xiang Wan Shan No17	South China（Hunan）	Hunan (South)
3	Xiang Luo	South China（Hunan）	Hunan (South)
4	R227	South China（Hunan）	Hunan (South)
5	Luo Gu a	South China（Hunan）	Hunan (South)
6	Di Fang Zhong a	South China（Hunan）	Hunan (South)
7	R 207	South China（Hunan）	Hunan (South)
8	R 018	South China（Hunan）	Hunan (South)
9	Bing Zhao Luo No1	South China（Hunan）	Hunan (South)
10	You Mi	South China（Hunan）	Hunan (South)
11	R 493	South China（Hunan）	Hunan (South)
12	Tian Long No1	South China（Hunan）	Hunan (South)
13	R 507	South China（Hunan）	Hunan (South)
14	Yangling inc	Unknown	
15	98-11	North China	
16	Huang Hua Zhan	South China （Hunan）	Hua rong
17	Jin Zhao No47	South China （Hunan）	(JinHua) Zhejiang Yue Yang Xian
18	Xiang Wan Xian No12	South China （Hunan）	Hua rong
19	Zao Chun Nuo	South China （Hunan）	Yue Yang Shi Nong Ke Suo
20	Xiang zao xian 31	South China （Hunan）	Lin Xiang
21	Jia Zhao No21	South China （Hunan）	Yue Yang Shi Nong Ke Suo
22	Zhe Fu No7	South China （Hunan）	Hua rong
23	Xiang zao xian 45	South China （Hunan）	Yang Jiang
24	Xiang zao xian 24	South China （Hunan）	-

Table 2. List of 9 ISJ Primers.

ISJ Primers	Sequences
E4	5’-GGAATTCCACCTGCA-3’
E2	5’-GGAATTCCACGTCCA-3’
R1	5’-TCGTGGCTGACTTACCTG-3’
R2	5’-TGCTGGTTTGCAGGT-3’
R3	5’-TGCTGTGTGTGGACG-3’
R4	5’-TCGTGGCTGACTTACCTG-3’
R5	5’-TCGTGGCTGACGTCCATT-3’
R1-1	5’-TCGTGGCTGACTTCACTG-3’
E4-1	5’-GAATTCCAGCCTGCA-3’

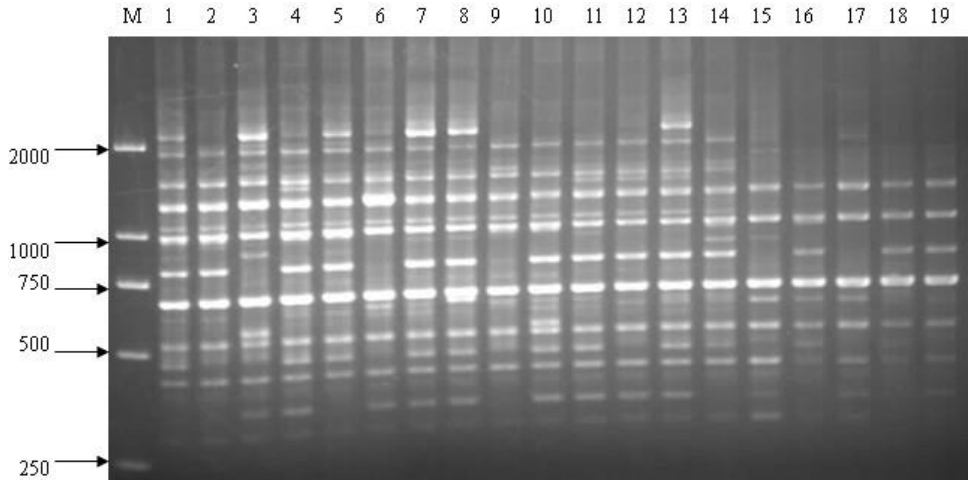

Figure 1 ISJ agarose gel profile of 19 rice genotypes using primer R2.

Lane numbers represent serial number of rice genotypes listed in table1;

M=molecular marker ladder.

2.4. Data analysis

Semi-random reproducible bands were scored as present or absent (1, 0). The ISJ matrices were then analyzed using (NTSYS) version 2.02. Similarity for semi-random data was computed using the Dice's similarity index and similarity estimates were analyzed by the UPGMA algorithm. The resulting clusters were expressed as dendrogram.

IV. RESULTS

4.1 Fingerprinting analysis

Nine primers from different series (R and E) were used. The size of amplified fragments ranged from 250 to 2000bp (see Figure 1). A total of 1607 reproducible amplifications products were observed and 1463 polymorphic bands were scored for all primers and all genotypes (91.03% of polymorphism). The number of polymorphic amplified products ranged from 0 for primer E4 to 237 for primer R2. The number of bands per primer ranged from 24 for primer E4 to 261 for primer R2, and number of bands per genotypes varied from 1 to 10. The primers R2, R4 and E4-1 were the most informative locus for DNA profiling and differentiation. An example of an ISJ pattern produced using the R2 primer is presented in Figure1.

4.2 Similarity and cluster analyses

The coefficient of similarities based on semi-random data among varieties ranged from 0.42 to 0.90 with an average similarity index of 0.67. The lowest genetic similarity was observed between You Mi and Xiang Zhao Xian No24; and the highest genetic similarity was observed between genotypes R207 and R507 (see Table 3).

Cluster analysis based on Dice's similarity coefficient using UPGMA procedure grouped the varieties into three clusters (see Figure 2). Cluster I comprised nine varieties, Cluster II possessed five varieties and Cluster III possessed ten varieties.

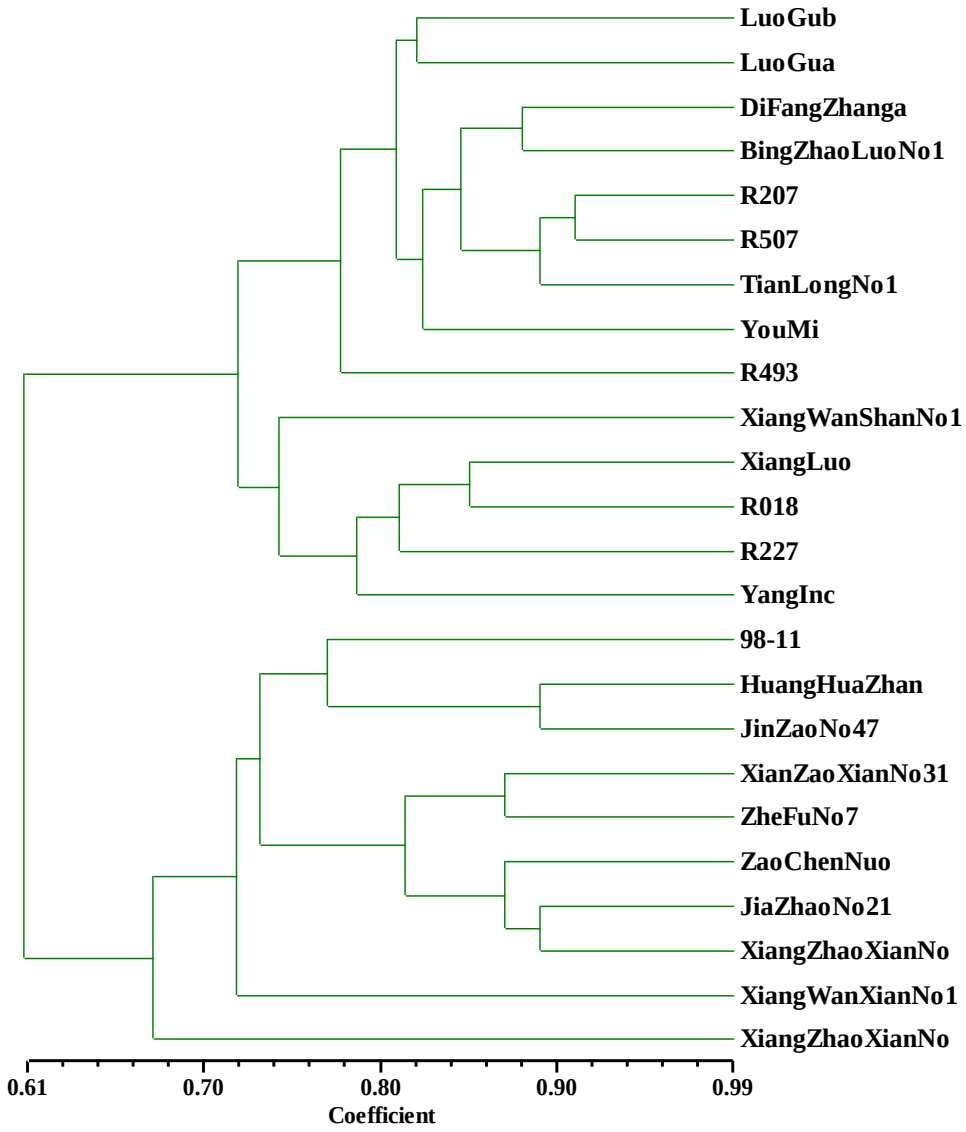

Figure 2. Dendrogram of rice varieties based on Dice's similarity index by ISJ markers.

Table 3. Dice's similarity coefficient matrix for rice genotypes based on ISJ amplification data

	x1	x2	x3	x4	x5	x6	x7	x8	x9	x10	x11	x12	x13	x14	x15	x16	x17	x18	x19	x20	x21	x22	x23	x24
x1	1.00																							
x2	0.75	1.00																						
x3	0.69	0.75	1.00																					
x4	0.68	0.74	0.80	1.00																				
x5	0.81	0.64	0.62	0.79	1.00																			
x6	0.82	0.71	0.71	0.74	0.83	1.00																		
x7	0.80	0.66	0.76	0.75	0.79	0.89	1.00																	
x8	0.80	0.75	0.84	0.81	0.72	0.80	0.89	1.00																
x9	0.83	0.72	0.70	0.73	0.80	0.87	0.84	0.81	1.00															
x10	0.80	0.60	0.65	0.66	0.76	0.82	0.83	0.75	0.80	1.00														
x11	0.72	0.60	0.66	0.69	0.71	0.78	0.82	0.72	0.73	0.76	1.00													
x12	0.80	0.67	0.73	0.76	0.81	0.88	0.87	0.80	0.81	0.82	0.83	1.00												
x13	0.80	0.64	0.72	0.73	0.79	0.83	0.90	0.80	0.77	0.81	0.84	0.89	1.00											
x14	0.73	0.73	0.80	0.74	0.66	0.73	0.74	0.80	0.76	0.65	0.66	0.77	0.78	1.00										
x15	0.65	0.73	0.75	0.68	0.60	0.71	0.70	0.73	0.66	0.60	0.60	0.69	0.70	0.77	1.00									
x16	0.65	0.67	0.65	0.66	0.64	0.65	0.62	0.60	0.66	0.61	0.60	0.67	0.66	0.69	0.77	1.00								
x17	0.63	0.63	0.65	0.66	0.66	0.67	0.66	0.63	0.62	0.65	0.62	0.71	0.72	0.71	0.77	0.88	1.00							
x18	0.54	0.65	0.52	0.64	0.60	0.52	0.49	0.52	0.55	0.48	0.47	0.56	0.53	0.58	0.63	0.77	0.71	1.00						
x19	0.64	0.59	0.62	0.65	0.67	0.68	0.67	0.62	0.65	0.66	0.63	0.70	0.71	0.66	0.72	0.80	0.85	0.74	1.00					
x20	0.60	0.56	0.54	0.62	0.68	0.58	0.53	0.50	0.59	0.52	0.55	0.58	0.59	0.60	0.61	0.75	0.73	0.71	0.81	1.00				
x21	0.65	0.61	0.61	0.60	0.64	0.60	0.62	0.60	0.62	0.60	0.60	0.60	0.66	0.67	0.71	0.77	0.75	0.69	0.85	0.82	1.00			
x22	0.54	0.54	0.52	0.60	0.62	0.54	0.51	0.46	0.55	0.46	0.51	0.54	0.55	0.56	0.60	0.75	0.69	0.79	0.76	0.86	0.80	1.00		
x23	0.60	0.56	0.56	0.62	0.66	0.60	0.62	0.54	0.62	0.56	0.60	0.63	0.66	0.61	0.67	0.79	0.79	0.71	0.87	0.82	0.88	0.82	1.00	
x24	0.46	0.54	0.54	0.60	0.51	0.48	0.45	0.46	0.49	0.42	0.60	0.52	0.49	0.54	0.54	0.71	0.63	0.67	0.66	0.77	0.65	0.75	0.67	1.00

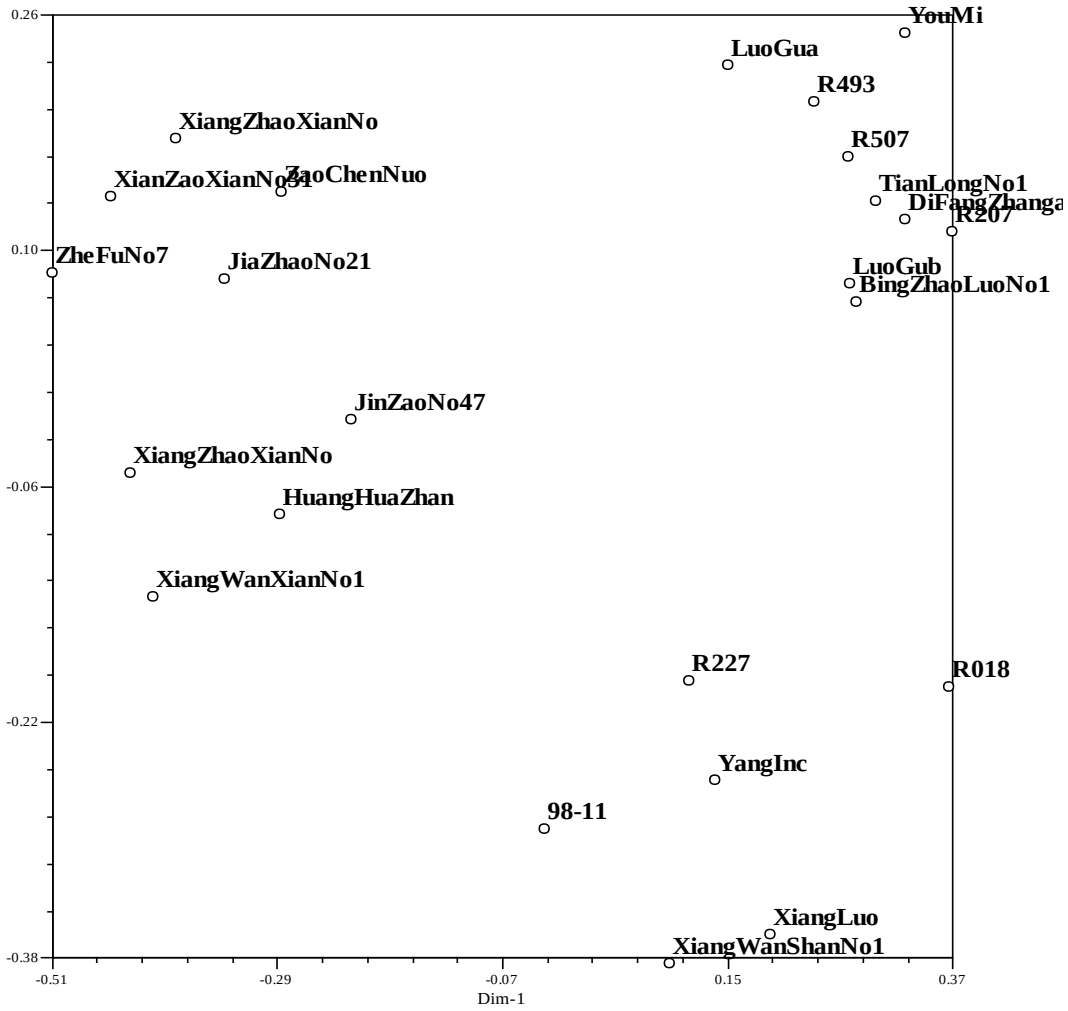

Figure 3. Principle coordinate analysis grouping of 24 genotypes based on ISJ data

V. DISCUSSION

DNA fingerprinting for cultivar or varietal identification has become an important tool for genetic identification in germplasm management and plant registration system. Different molecular marker techniques are accessible today for fingerprinting plant germplasm but information on their relative efficacy in particular crops is not clear (20). The main advantage of semi-random primer is that; it avoids targeting the heterochromatic regions of the plant genome (14). This is of particular importance in cereals, where repeated sequence regions are very abundant (11).

The results of molecular analyses in this study revealed that the number of bands per primer and genotype ranged from 24 to 261 and from 1 to 10. The average number of bands per primer and varieties were 178.55 and 7.43, respectively. These estimates were shown higher than the RAPD markers data that were evaluated in the previous study (21).

The results clearly revealed that eight of nine primers have the highest efficacy for DNA profiling and discrimination of rice varieties. Only primer E4 was the least informative. Gawel et al. (16) reported that DNA fingerprinting system not only was cheap and fast as RAPD, but also unlike the latter, the semi-random primers generated more complex band patterns with a high degree of polymorphism.

A dendrogram was generated from intro-targeting primers' data that grouped the rice varieties into three clusters (see Figure 2). The results of the clustering are consistent their known origin with only few exceptions. Our results indicated that, the semi-random markers can identify all DNA of rice varieties from different origins. This study utilized intron-targeting markers in rice varieties and confirm the hypothesis that, markers not only could be used for identification and classification of rice genotypes, but also is useful for predicting heterotic groups in breeding programs (20). In addition, fingerprinting makes identification and characterization of genotype very easy and further it will be greater help in background selections during backcross breeding

(22).

REFERENCES

[1] Chakravarki B. K. and Naravaneni R., 2006. SSR marker based DNA fingerprinting and diversity study in rice (Oryza sativa L.) Afr J Biotechnol 5(9): 684-688.

[2] FAO, 2008. Food Consumption: Pattern of main food itemsshare in total dietary energy consumption. FAO Statistic Division. Bulletin

[3] Byerlee D 1996. Knowledge. Intensive. Crop Management Technologies: Concepts, Impacts and Prospects in Asian Agriculture. International Rice Research Conference, Bangkok, Thailand, 3-5 June, 1996.

[4] Causse MA, Fulton TM, Cho YG, Ahu SN, Chunwongse J, Wu K, Xiao J, Yu Z, Ronald PC, Harrington SE, Scond G, McCouch SR, Tansksley SD 1994. Satured molecular map of the rice genome based in an interspecific backcross population genetics 138:1251-1274.

[5] McCouch SR, Temnykh S, Lukashova A, Cbum J, Declerck G, Cartinhou S Harrington S, Thomson M, Septiningsi E, Semon M, Moncada P, Jiming L 2001. Microsatellite markers in rice: Abundance, diversity and applications In: Rice Genetics IV. IRRI. Manila, Philippines. Pp 117-135.

[6] Kwon YS, Lee JM, Yi GB, Yi Si, Kim KM, Soh EH, Bae KM, Park, EK, Song IH, Kim BD 2005. Use of SSR markers to complement tests of distinctiveness, uniformity and stability (DUS) of pepper (Capsicum annum L.) varieties. Mol. Cell.19: 428-435.

[7] Nandakumr n, Singh A, Sharma K, Mohapara RK, Prabhu TKV, Zaman FU 2004. Molecular fingerprinting of hybrids and assessment of genetic purity of seeds in rice using microsatellite markers. Euphytica, 136:257-264.

[8] Yashitola J, Thirumarugan T, Sundaram RM, Naseerullah M K, Ramesha M S, Sarma N P, and Sonti Ramesh V. 2002. Assessment of purity of rice hybrids using microsatellite and STS markers, Crop Science, 42: 1369-1373.

[9] Wang Y, Xue Y, Li J 2005. Towards molecular breeding and improvement of rice in China. Trends Plant Sci. 10:610-614.

[10] Mc Gregor C E, C A Lambert M M., Greyling J H, Louw and Warnich L., 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tretraploid potato (*Solanum tuberosum* L.) germplasm. Euphytica 113: 135-144.

[11] Rafalski A, Madej L, Wisnieuzka I, and Gawel M 2002. The genetic diversity of components of rye hybrids. CellMol Biol Lett. 7: 471-475

[12] Weining S and Langridge P 1991. Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction, Theor. Appl Genet, 82:209-216.

[13] Rafalski A, Gidzinska M, and WiCniewska I 1997. PCR-based systems for evaluation of relationships among miaze inbreeds. In: Tsaftaris A Sd. Genetics. Biotechnology and breeding of maize and sorghum. Royal Soc.Chem. Cambridge. UK, 106-111.

[14] Przetakiewicz J, Nadolska-Orczyk A, Orczyk W 2002. The use of RAPD and semi-random markers to verify somatic hybrid between diploid lines of Solanum tuberosum L. Cell. Mol. Biol. Lett. 7: 671-676.

[15] Rafalski A., Cheng A, Bhatteramakki D., Morgante M., Dolan M., Register J. C., Smith O. S. and Tingey S.,2001. SNP markers in maize discovery and applications. In: Plant and animal genome, IX. The international conferences on the status of plant and animal genome research, 13-17, January 2001, San Diego, USA, W149.

[16] Gawel M, Wisniewska I, Rafalski A 2002. Semi-specific PCR for the evolution of diversity among cultivars of Wheat and triticale. Cell. Mol. Biol. Lett. 577-582.

[17] Przetakiewicz J, Nadolska-Orczk A, Kuc D, Orczyk W 2007. Tetraploid somatic hybrids of potato (Solanum tuberosum L.) obtained from diploid breeding lines. Cell Mol Biol Lett. 12 : 253-267.

[18] Marotti Ilaria, Alessandra Bonetti, Maurizio Minelli, Pietro Catizone, Giovenni Dinelli 2007. Characterization of some Italian common bean (Phaseolus vulgaris L.) Landraces by RAPD, Semi-random and ISSR Molecular Makers. Genet. Resour. Crop evol. 54: pp 175-188.

[19] Weining Song and Robert J Henry 1995. Molecular analysis of the DNA polymorphism of wild barley (Hordeum spontaneum) germplasm using the polymerase chain reaction. Genetic Resources and Crop evolution 42: 273-281.

[20] Seyyed Hamidreza Hashemi-Petroudi, Seyyed Ali Mohammad Mirmohammadi Maibody, Ghorban Ali Nematzadeh and Ahmad Arzani 2010. Semi-random PCR markers for DNA fingerprinting of rice hybrids and theirs corresponding parents. African Journal of Biotechnology vol.9 (7), pp 979-985.

[21] Hashemi SHR, Mirmohammadi-Maibody SAM, Nematzadeh GA, Arzani A 2009. Identification of rice hybrids using microsatellite and RAPD markers. Afr. J. Biol. 8:2094-2101.

[22] Kalyan Chakravarthi and Rambabu Naravaneni 2006. SSR marker based DNA fingerprinting and diversity study in rice (Oryza sativa L.). African Journal of Biotechnology vol.5 (9), pp 684-688