African Journal of Biotechnology Vol. 9(7), pp. 979-985, 15 February, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1461
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Semi-random PCR markers for DNA fingerprinting of rice hybrids and theirs corresponding parents

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Accepted 28 December, 2009

Molecular markers technology provides novel tools for DNA fingerprinting of rice hybrids to assess hybrid seed purity. Semi- random PCR primers targeting intron-exon splice junctions (ISJ) were used to analyze the rice genome with the aim of evaluating potential of these markers for identification and classification of rice hybrids. A total of 21 primers were tested for screening eight hybrid combination and their corresponding parents. Among 185 amplification products, 42.7% have polymorphic bands. The average number of bands per primer and genotype were 15.41 and 11.56, respectively. The genetic similarity between lines ranged from 0.47 to 0.95 with an average similarity index of 0.75. Cluster analysis based on Dice's similarity coefficient using UPGMA procedure grouped the cultivar into 5 clusters. A set of 5 semi- random primers characterized all the genotypes from each other, which can be used for identification of these hybrid and their parental lines. IT/10-1 and IT31/15 multilocus primers were the most-informative loci for DNA profiling and differentiation. Because of its rapidity and reliability of semi- random primers, it seems to be a suitable method for DNA typing and discrimination of rice hybrids.

Key words: Molecular marker, semi-random PCR, fingerprinting, rice hybrid.

INTRODUCTION

Rice is one of the most important crops in the world and is consumed by more than half the world population. About 20% of the total calorie supply worldwide comes from rice and especially in Asia; more than 2 billion people derive 60 - 70% of their daily energy requirement from rice (Matsumoto et al., 2006). As demands for feeding of rising world population grow, hybrid rice production is inevitable. In these regard, hybrid rice production program has began in other countries, following its success in china (Nandakumr et al., 2004).

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Abbreviations: ISJ, Intron-exon splice junctions; **PCR,** polymerase chain reaction; **CMS,** cytoplasmic male sterility; **DUS,** distinctness, uniformity and stability.

Since rice is strictly a self-pollinated crop, hybrid seed production must be based on male sterility systems. Currently, the most popular male-sterility system in rice is the cytoplasmic male sterility (CMS), that involving 3 lines of male sterile (A line), their cognate iso-nuclear maintainer (B line) and a restorer (R line).

The identification of rice cultivars and lines and determination of their genetic relations 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 (Ichii et al., 2003; Kwon et al., 2005; Kumar, 1999). So, clear-cut identification of elite crop varieties and hybrids is essential for protection and prevention of unauthorized commercial use (Nandakumr et al., 2004). Conventional characterization of hybrids based on specific morphological and agronomic data is time-consuming, restricted to a few characteristics, influenced by environmental condition. In contrast, DNA-based markers are highly heritable,

Table 1. Different rice hybrids and their corresponding parents were used in this study. The numbers at the parenthesis are serial number of 16 rice genotypes.

Genotype	Type of lines	Origin
IR58025A/ IR42686R (15)	Hybrid	Dasht-e-Naz, Sari, Iran
Neda-A/ IR62037-93-1-3-1-IR (11)	Hybrid	Rice Research Institute, Amol, Iran
Neda-A/ IR69726-54-3-IR (6)	Hybrid	Rice Research Institute, Amol, Iran
Neda-A/ IR28 (4)	Hybrid	Rice Research Institute, Amol, Iran
Neda-A/ SA4 (12)	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A/ IR62037-93-1-3-1-IR (9)	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A/ IR69726-54-3-IR (8)	Hybrid	Rice Research Institute, Amol, Iran
Nemat-A/ IR28 (2)	Hybrid	Rice Research Institute, Amol, Iran
IR42686R (16)	Restorer	Rice Research Institute, Amol, Iran
IR62037-93-1-3-1-IR (10)	Restorer	IRRI, Manila, The Philippines
IR69726-54-3-IR (7)	Restorer	IRRI, Manila, The Philippines
IR28 (3)	Restorer	IRRI, Manila, The Philippines
SA4 (13)	Restorer	IRRI, Manila, The Philippines
Neda-A (5)	CMS	Agricultural University, Sari, Iran
Nemat-A (1)	CMS	Agricultural University, Sari, Iran
IR58025A (14)	CMS	IRRI, Manila, The Philippines

available in high numbers, and exhibit enough polymerphism, hence they can be used to discriminate closely related genotypes of a plant (Kumar, 1999; Yashitola et al., 2002; Wang et al., 2005). For this reasons, DNA fingerprinting for cultivar or varietal identification has become an important tool for genetic identification in plant breeding and germplasm management (McGregor et al., 2000).

The abundance of information on DNA sequences of plant genomes permits to design sequence-related primers for PCR amplification (Rafalski et al., 2002). The use of semi-random primers targeting the intron- exon splice junction (ISJ), proposed by Weining and Langridge (1991) and developed by Rafalski et al. (1997) and proved to be very useful for fingerprinting (Przetakiewicz et al., 2002). This system is universal for plants 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 (Rafalski et al., 1997). The additional bases were added at random to extend the length of the primers. The increased length of the majority of primers and elevated annealing temperature all amplification steps resulted in good reproducibility of PCR profiles. The results of amplification of DNA of several monocotyledonous and dicotyledonous plants confirm the versatility of this method (Gawel et al., 2002).

Semi-random markers have been successfully used for cultivar analysis in number of plant species viz. rye (Rafalski et al., 2002), maize (Rafalski et al., 2001), wheat and triticale (Gawel et al., 2002), *Solanum tuberosum* L. (Przetakiewicz et al., 2002), potato (Przetakiewicz et al., 2007), and common bean (Marotti et al., 2007). With concerns about reproducibility of universally semi-specific markers, respect to RAPD markers, semi-random DNA fingerprinting has been used in this study. The main aim of the present study was to check, whether semi-specific markers fingerprinting may be a useful tool for identifycation and differentiation of rice hybrids and their parental lines.

MATERIALS AND METHODS

Plant materials and genomic DNA isolation

Sixteen rice genotypes, including three cytoplasmic male-sterile (CMS) lines, five restorer lines and their eight hybrid combinations were used in this study (Table 1). Genomic DNA was isolated from young leaves of 10 plants of each parental line while the leaves of individual plant were used for hybrids. DNA was extracted according to Dellaporta et al. (1983) procedure with minor modification. The quantity and quality of DNA was assessed with 0.7% agarose gel electrophoresis using diluted uncut lambda phage DNA as size standard.

Semi-random primers analysis

Twenty-one Semi-random primers (Primm, Italy) were tested and twelve polymorphic primers were selected (Table 2). PCR was carried out in 12.5 µl reaction mixture containing 40 ng of template DNA, 200 µM of each dNTPs, 1×*Taq* buffer PCR (10 mM Tric-HCl, 50 mM KCl (pH 8.8), 0.08 Nonidet P40), 2.6 mM of $MgCl_2$, 1.25 U of *Taq* polymerase and 1 µM each of the Semi-random primers using a Techne Genius thermal cycler (Techne Ltd., U.K.). The amplification reaction was performed according to Przetakiewicz et al. (2002) with the following details: 7 cycles at 94°C for 40 s, T_m + 2 for 1 min, 72°C for 2 min and followed by 33 cycles at 94°C for 40 s, T_m + 6 for 1 min, and 72°C for 2 min. Final extension was at 72°C for 10 min. PCR amplified products were separated by electrophoresis in 1.5% agarose gels at 50 V in 0.5 × TBE buffer. Gels stained with ethidium bromide, were imaged in Biometra (UV-solo model) gel documentation system. Each reaction was repeated

Table 2. Data of selected primers were used in semi-random amplification. Temperature melting: T_m; Nucleotide number: NN; Total number of bands: TNB; Number of polymorphic bands: NPB and Percent of polymorphic bands: PPB.

Primer	5´- 3´Sequence	T_m	NN	TNB	NPB	PPB
IT/10-1	ACGTCCAGAC	32	10	25	21	84
IT/10-2	ACGTCCAGGT	32	10	15	9	60
IT/10-3	ACGTCCAGCA	32	10	9	2	22
IT/10-4	ACGTCCACCA	32	10	15	4	26.6
IT/10-5	ACGTCCACAG	32	10	8	3	37.5
IT/10-6	ACGTCCATCC	32	10	20	8	40
IT31/15	GAAGCCGCAGGTAAG	48	15	22	13	63.63
IT33/15	GATGCCCCAGGTAAG	48	15	8	1	12.5
IT 34/15	GCGGCATCAGGTAAG	48	15	13	3	23.07
IT35/15	CGAAGCCCAGGTAAG	48	15	17	9	52.94
MEN-1	TGCGCGATCG	34	10	14	4	28.57
MEN-2	GTCCATGCCA	32	10	16	2	12.5

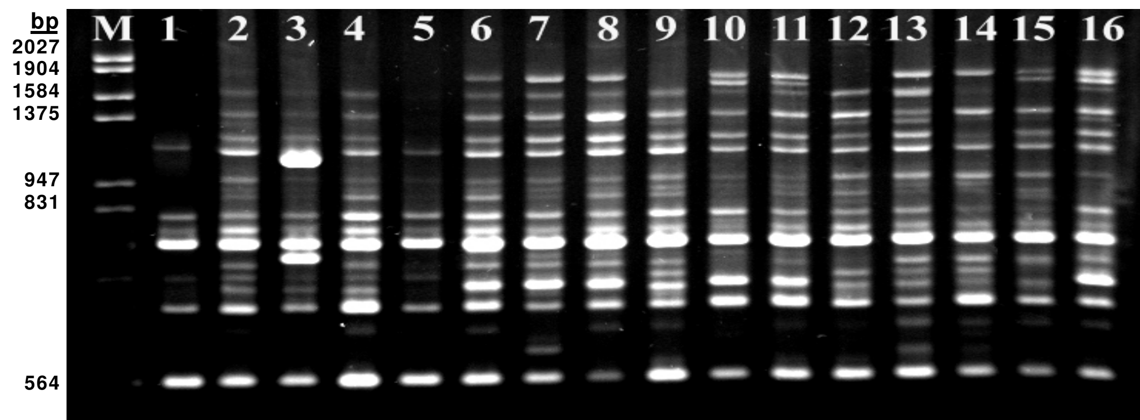

Figure 1. Semi-random agarose gel profile of 16 rice genotypes using primer IT (10-1). Lane numbers represent serial number of rice genotypes listed in Table 1; M = molecular marker ladder.

twice and only reproducible bands were considered for analysis.

Data analysis

Semi-random reproducible fragments were scored as present or absent (1, 0). The ISJ matrices were then analyzed using (NTSYS) version 2.02 (Rohlf, 1998). 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.

RESULTS

Fingerprinting analysis

Twenty-one oligomer primers (Primm, Italy) from different series (IT and ET type) were tested and IT type polymorphic primers were selected (Table 2). The size of amplified fragments ranged from 200 to 1800 bp. A total of 185 reproducible amplifications products were observed and 79 polymorphic bands were scored. The number of polymorphic amplified products ranged from 1 for primers IT33/15 to 21 for primer IT/10-1 (Table 2). The average number of bands per primer and genotypes were 15.41 and 11.56, respectively. IT/10-1 and IT31/15 markers were the most-informative locus for DNA profiling and differentiation. An example of an ISJ pattern produced using the IT/10-1 primer is presented in Figure 1. The rice CMS lines and restorer lines could be uniquely identified by semi-random multilocus amplified profile at five informative loci: IT/10-1, IT/10-2, IT/10-3, IT/10-6 and IT31/15. Figure 2 shows the schematic presentation of amplification pattern in these loci.

Similarity and cluster analysis

The coefficient of similarities based on semi-random data among genotypes ranged from 0.47 to 0.95 with an average

Loci (bp) \ Genotypes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IT/10-1(1900)							■	■		■	■		■	■	■	■
IT/10-1(1800)										■	■				■	■
IT/10-1(1300)	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■
IT/10-1(1250)		■														
IT/10-1(650)		■														
IT/10-1(400)	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■
IT/10-2(830)	■		■	■	■	■			■		■	■	■			
IT/10-3(800)								■		■	■		■	■		
IT/10-6 (870)	■	■		■	■	■		■	■			■				
IT31/15 (1200)	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■
IT31/15 (1100)	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■
IT31/15 (940)		■														
IT31/15 (700)						■		■		■						
IT31/15 (650)	■	■	■	■	■	■		■	■		■	■	■	■	■	■
IT31/15 (560)			■													
IT31/15 (300)	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■
IT31/15 (250)				■												
IT33/15 (830)	■	■	■	■	■	■		■	■		■	■		■	■	■
IT34/15 (500)		■												■	■	
IT35/15 (1200)	■	■	■	■	■	■	■	■	■		■	■	■	■	■	

Figure 2. Schematic representation of semi-random profiling of eight rice hybrids and their parental lines. The numbers at the top table are serial number of 16 genotypes listed in Table 1. Shaded block indicate the presence of allelic bands by the unique respective semi-random loci.

similarity index of 0.75. The lowest genetic similarity was observed between local male sterile line Neda-A and restorer line IR62037-93-1-3-1-IR from IRRI and the highest one belongs to Neda-A/ IR28 and Nemat-A/ IR62037-93-1-3-1-IR hybrids from local breeding line. The lowest genetic similarity between CMS lines and restorer lines were observed between local CMS line Nede-A (from Sari University of Agricultural sciences) and IR62037-93-1-3-1-IR line from IRRI and the highest one belongs to IR58025A and SA4 lines (from IRRI) (Table 3).

Cluster analysis based on Dice's similarity coefficient using UPGMA procedure grouped the cultivars and lines into five clusters, each containing 12.5, 62.5, 6.25, 12.5 and 6.25% of genotypes (Figure 3). Cluster I comprised two local cytoplasmic male sterile, Nada-A and Nemat-A lines (two sister lines that developed by IR58025A). Cluster II possessed most hybrid lines: Nemat-A / IR28, Neda-A/ IR28, Nemat-A/ IR62037-93-1-3-1-IR, Neda-A/ SA4, Neda-A/ IR69726-54-3-IR, Nemat-A/ IR69726-54-3-IR, Neda-A/ IR62037-93-1-3-1-IR (breeding line from Iran); 2 restorer lines: IR28, SA4 and one CMS line: IR58025A (from IRRI). Cluster III included restorer line IR69726-54-3-IR (from IRRI). IR58025A/ IR42686R (breeding line), IR42686R and IR62037-93-1-3-1-IR (from IRRI) were allocated in cluster IV and V, respectively.

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. The main advantage of semi-specific primer is that it avoids targeting the heterochromatic regions of the plant genome (Przetakiewicz et al., 2002). This is of particular importance in cereals, where repeated sequence regions are very abundant (Rafalski et al., 2002). Out of 21 semi-random primers (IT & ET type) used in this study, only the molecular data that belong to intron-targeting primers were satisfactory results; that chosen for molecular

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

Serial number of genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	1															
2	0.84	1														
3	0.77	0.82	1													
4	0.85	0.92	0.81	1												
5	0.93	0.72	0.64	0.77	1											
6	0.89	0.86	0.78	0.9	0.72	1										
7	0.75	0.71	0.7	0.72	0.6	0.82	1									
8	0.8	0.8	0.76	0.8	0.63	0.9	0.75	1								
9	0.88	0.92	0.81	0.95	0.79	0.88	0.74	0.79	1							
10	0.52	0.5	0.54	0.56	0.47	0.62	0.65	0.61	0.56	1						
11	0.72	0.79	0.75	0.8	0.59	0.85	0.75	0.83	0.76	0.7	1					
12	0.85	0.88	0.75	0.87	0.77	0.82	0.68	0.73	0.9	0.53	0.73	1				
13	0.81	0.87	0.8	0.84	0.66	0.84	0.76	0.8	0.85	0.59	0.81	0.81	1			
14	0.79	0.84	0.75	0.8	0.7	0.82	0.77	0.78	0.81	0.59	0.81	0.78	0.87	1		
15	0.67	0.74	0.64	0.7	0.62	0.75	0.63	0.71	0.72	0.5	0.72	0.75	0.71	0.81	1	
16	0.65	0.74	0.66	0.73	0.57	0.76	0.66	0.72	0.75	0.58	0.73	0.74	0.77	0.76	0.77	1

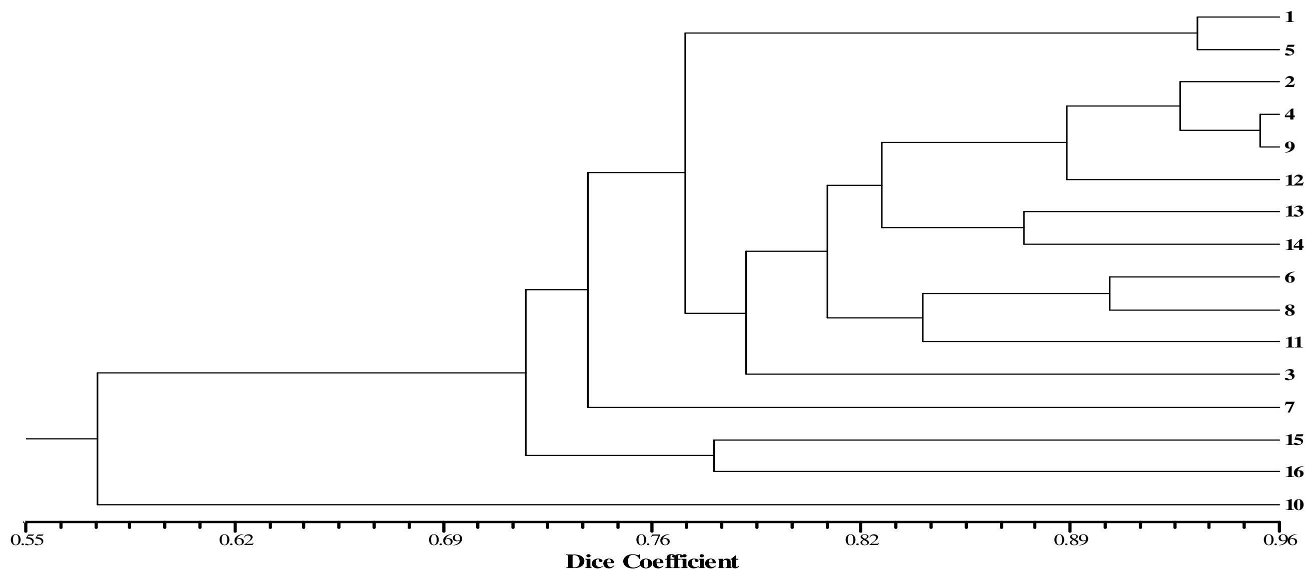

Figure 3. Dendrogram of 16 rice genotypes based on Dice's similarity index by semi-random markers. The numbers at the right of dendrogram are serial number of rice genotypes listed in Table 1.

analyses. The average number of bands per primer and genotype were 15.41 and 11.56, respectively. These estimates were shown higher than the RAPD markers data that were evaluated in the previous study (Hashemi et al., 2009).

The results clearly revealed that IT/10-1, IT/10-2, IT/10-3, IT/10-6 and IT31/15 primers have the highest efficacy for DNA profiling and discrimination of rice hybrids and lines. Gawel et al. (2002) reported that this 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. Molecular marker analysis also indicated that there are small differences among two local CMS lines viz., Neda-A and Nemat-A. This is likely due to the fact that their parental breeding lines used to build up these lines are very close to each other. Neda-A and Nemat-A are sister lines developed by transferring of wild abortive (WA) cytoplasm from IR58025A to them via backcrossing (Nematzadeh et al., 2006). According to similarity co-

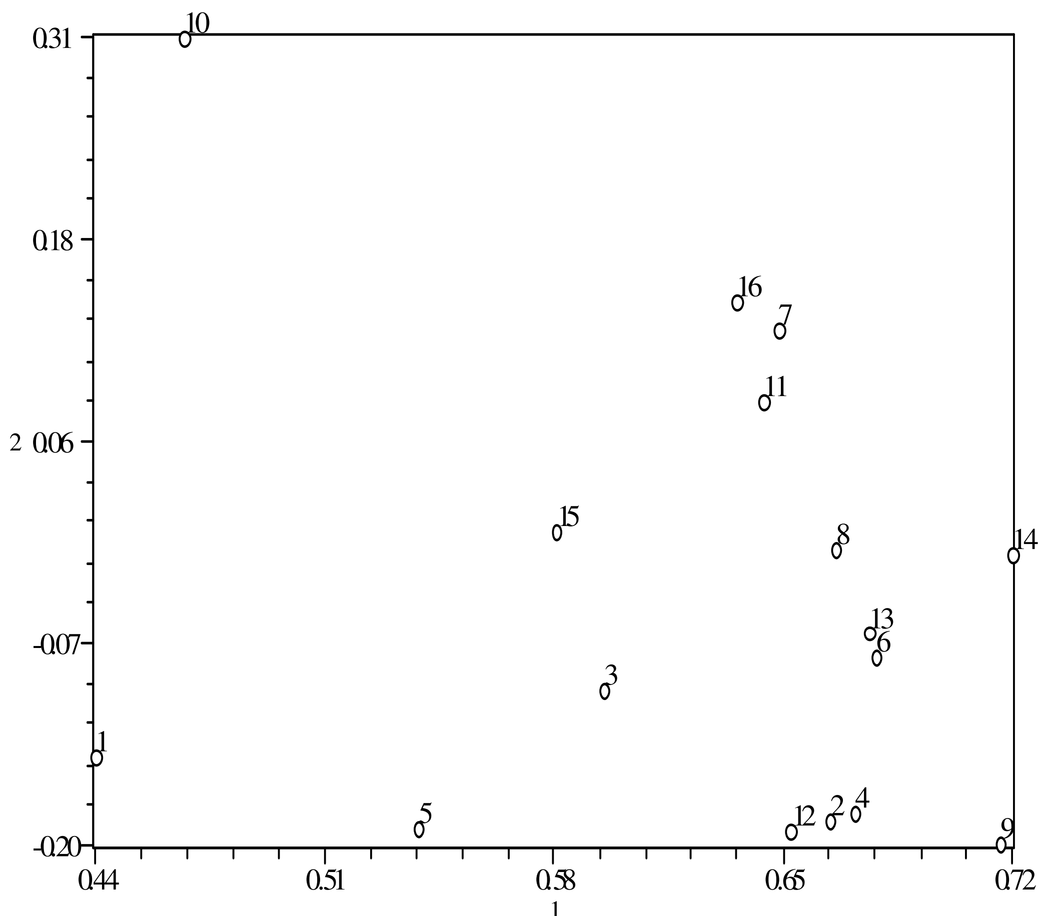

Figure 4. Principle coordinate analysis grouping of 16 rice genotypes based on semi-random data. The numbers in the image are serial number of rice genotypes listed in Table 1.

efficient matrix, four hybrids including IR58025A/IR42686R, Nemat-A/IR62037-93-1-3-1-IR, Nemat-A/IR69726-54-3-IR and Nemat-A/IR28 were more similar to female parent, while the other hybrids were more similar to its male parent. Similarity to female parent may be because of the identity of cytoplasmic genome between hybrid and its corresponding female parent.

A dendrogram was generated from intron-targeting primers' data that grouped the rice variety or lines into five clusters. The results of the clustering are consistent with their known origin with only few exceptions. Principle component analysis was also done to display genetic relationships among the rice genotypes. The first three components derived from this analysis explained 66.78% of the variation. The two-dimensional plot also confirms that cluster analysis results (Figure 4). Our results indicated that, the semi-random markers can identify all DNA of rice hybrids and their parental lines with five specific primers. Neda-A and Nemat-A, two closely relate lines and their resultant hybrids, generated unique profile and could be differentiated to each other. This study utilized intron-targeting markers in rice and indicated that these markers not only could be used for identification and classification of rice genotypes, but also is useful for predicting heterotic groups in breeding programs.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Rice and Citrus Research Institute and Isfahan University of Technology for financial support.

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