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Fingerprinting and genetic diversity evaluation of rice cultivars using Inter Simple Sequence Repeat marker

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Abstract
Rice as one of the most important agricultural crops has a putative potential for ensuring food security and addressing poverty in the world. In the present study, in order to provide basic information to improve rice through breeding programs, Inter Simple Sequence Repeat marker (ISSR) was used For DNA fingerprinting and finding genetic relationships among 32 different cultivars. In this study, 12 out of 17 used primers amplified 184 distinct and reproducible fragments with high value of polymorphism (88%). also, for fingerprinting the cultivars 29 loci were used that generated high polymorphic bands among the cultivars. Results indicated that similarity index varied between 39% and 88.4 %, furthermore, PIC value with an average of 23% ranged between 0.1 (for primer #3) to 0.34 (for primer #2). Clustering based on Jaccard coefficient similarity index and UPGMA algorithm divided the cultivars into 6 main sub-clusters in cut-off point of 64% similarity index. The two Italian rice cultivars 'Ribe' and 'Roma' were the closest cultivars in addition, 'Vialone nano' and 'Anbarbu' showed the highest dissimilarity. In total, high genetic divergence was observed among the cultivars also, poly (GA)-containing 3-anchored primers amplified the highest number of bands. According to similarity and cluster analysis, it could be inferred that crosses involving Anbarbu cultivar are the most promising ones to improve rice through breeding programs. In fact, results of this study would be promising as a genetic marker for the identification of rice cultivars and an important source of knowledge for subsequent rice researches.
Keywords: Dendrogram, Genetic Similarities, Genome analysis, PCR based Marker, Polymorphism.

Introduction

Rice (*Oryza sativa* L.) as the world's most important crop for more than 2 billion people belongs to Gramineae or grass family and subfamily of *Orazoidea* (1). The people get 60-70% of their daily energy requirements from this cereal grain as a source of complex carbohydrates, nutrients, vitamins and minerals (7, 16). Assessment of rice cultivars to analyze their genetic relationships not only provides basic information to improve productivity and traits related to agronomic parameters but it is also important to variety

registration systems or uniformity and stability testing (10, 12).

DNA fingerprinting and assessment of genetic relationships have become an important tool for varietal identification in plant breeding programs (15, 22). There are several systems including: morphological, chemical, and biochemical markers for evaluation of similarity among genotypes. Nevertheless, these characterizing systems have some limitations, such as being time-consuming, limited to a few characteristics and influenced by factors like temperature, humidity, light or the age of plants which can modify classification based on these systems (24). In contrast, DNA-based marker systems are highly heritable and obtainable in high numbers. They also show enough polymorphism and by simultaneous elimination of environmental impacts, provide a reliable and powerful tool for assessing differences among closely related organisms with high accuracy (20). Several DNA-based marker systems are available for genetic fingerprinting of plants. RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and SSRs (simple sequence repeats or microsatellites) are generally PCR (polymerase chain reaction)-based DNA marker systems (18) but, ISSR as a DNA-based marker is a powerful, fast and low cost method that requires small amounts of DNA as the template. In addition, because of using longer primers with high annealing temperature, it provides reproducible and reliable results. This PCR-based DNA marker does not requires any prior sequence information for implementation and uses a single primer targeting microsatellite motifs generating abundant polymorphisms (13). ISSR has been used successfully to assess variation in a vast range of plants, such as blueberry (5), lingo berry (4), *Auricularia polytricha* (25), *Dioscorea opposite Thunb* (26), *Leonurus cardiaca* (11) and *Oryza Sativa* (9) which was described as a powerful technique to assess genetic diversity and similarities between and within species.

The objective of this study was evaluation of ISSR marker ability to assess genetic diversity among rice cultivars in order to fingerprint and estimate genetic relationships among the cultivars. Actually, the results can generate basic information that could be useful for rice breeding programs, such as cultivar identification, parental choice for developing heterotic hybrids or germplasm preservation.

Materials and methods

Preparation of plant materials and genomic DNA isolation

The seeds of 32 rice cultivars were obtained from the Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) (Table 1) and cultivated in hydroponic medium in controlled condition; the nutrients for rice hydroponic medium have been described previously by Yoshida *et al*., (23). Genomic DNA was extracted from approximately 0.1 g leaf materials powdered by mortar and pestle using Dellaporta *et al*,. (6) method with some

modifications, for quantification and qualification of the isolated DNA were used spectrophotometer and 0.7% agarose gel electrophoresis.

Table 1. The cultivar names of rice used in this study.

Number	Genotype	Number	Genotype	Number	Genotype	Number	Genotype
1	Hasan Sarai 1	**9**	Onda	**17**	Rashti Sard	**25**	Mosa Tarom
2	Ahlami Tarom	**10**	Ribe	**18**	Sadri	**26**	Mir Tarom
3	Hasan Sarai 2	**11**	Dular	**19**	Baldo	**27**	IR 58
4	CH2-1	**12**	Roma	**20**	Amol 1	**28**	Abji Boji
5	Lemonimo	**13**	Jasmin 85	**21**	Bajar	**29**	Anbar Bou
6	Hasani	**14**	Zir Band P	**22**	Vialone nano	**30**	Domsiah
7	Manjing	**15**	Amol 2	**23**	Ringo	**31**	Amol 3
8	Fujiminori	**16**	Salari	**24**	Cripto	**32**	Sepidroud

ISSR amplification

In the present study, seventeen ISSR primers (Alpha DNA, Canada) were analyzed for detecting polymorphism among the cultivars. Finally, the PCR reactions were performed in a volume of 20 µl reaction mixture containing 40 ng of template DNA, 0.6 mM of each dNTPs, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08 Nonidet P40), 2 mM $MgCl_2$, 1 U of *Taq* DNA polymerase enzyme and 0.6 µM of each ISSR primers. To reduce background amplification, 2 % (v/v) formamide was added to the reaction mixture. Amplification reactions were carried out using a AB Applied Biosystems thermal cycler (Bio-Rad, USA) with an initial denaturation step of 5 min at 95˚C, followed by 35 cycles at 94° C for 40s, 55° C for 1 min, 72° C for 2 min and a final extension step at 72° C for 7 min. Two independent PCRs were carried out for each primer, and the products were fractionated on separate gels. A reaction containing all PCR components except DNA (negative control) was used in each experiment to test DNA contamination of the reagents. PCR amplified fragments were separated on 2.5% agarose gels by electrophoresis at 90 V in 0.5x TBE (Tris/Boric Acid/EDTA) buffer. Gels were stained with ethidium bromide and imaged in Doc-Print VX5 (VIBER LOURMAT) gel documentation system.

Data analysis

ISSR reproducible fragments were scored as present (1) or absent (0). Since ISSR is a dominant marker, the presence of a band was interpreted as either a heterozygote or dominant homozygote and the absence of a band was interpreted as recessive homozygote. The data matrix structure was assembled by binomial (0/1) data and was used as input data for further analysis applying NTSYS version 2.02 software program. To test whether clusters in the dendrogram agrees with the information from similarity matrix, cophenetic matrices were created from the dendrogram and were compared with the similarity matrix. Similarity of ISSR data was computed using Jaccard's similarity index and cluster diagrams generated by UPGMA algorithm and the resulting clusters were expressed as dendrograms.

Results

In the present study, polymorphic information content (PIC) varied ranging from 0.1 (primer #3) to 0.34 with an average of 0.23. The primer ISSR #2 showed the highest value of polymorphism that referred to the efficiency of the primer to detect polymorphism within a population depending on the number of alleles and frequency distribution. Among 17 ISSR primers used, 12 primers that produced distinct and reproducible fragments were selected for fingerprinting and diversity analysis (Table 2).

Table 2. List of 12 ISSR primer sequences used for analysis of the rice cultivars with annealing temperature, size variation, number of bands, polymorphism and PIC value.

Primer	Sequence (5' to 3')	Annealing temperature	Fragment size length	Total no. of bands	Polymorphic bands	Polymorphism (%)	PIC
ISSR #2	(GA) 9C	57° C	220-2300	34	30	88	0.34
ISSR #3	(GA) 9T	55° C	420-1150	17	15	88	0.10
ISSR #5	(GT) 8C	51° C	400-1200	17	14	82	0.14
ISSR #8	(CT) 8G	51° C	600-1200	11	11	100	0.10
ISSR #9	(AG) 8C	52° C	500-1620	16	14	87	0.22
ISSR #13	(TC) 8C	51° C	700-1250	7	7	100	0.31
ISSR #15	(AC) 8G	51° C	500-1150	12	10	83	0.31
ISSR #16	(TG) 8A	49° C	680-1920	15	13	86	0.22
ISSR #17	(AC) 8C	51° C	500-1500	17	15	88	0.29
ISSR #18	(ATC) 6T	49° C	550-1150	11	10	90	0.25
ISSR #19	(ATC) 6C	50° C	800-1750	14	12	85	0.23
ISSR #21	(ATA) 6T	42° C	600-1500	13	11	84	0.30

Within the selected primers, there were nine di-nucleotide repeat primers and three tri-nucleotide repeat primers with 3˙ anchors and the other primers produced faint or no distinct bands. Totally, using the 12 selected ISSR primers, 184 fragments with the size ranging between 400-2300 bp were amplified that 162 loci of which were polymorphic and no band was detected in negative controls. Primers ISSR #8 and #13 showed the highest polymorphism value (100%) and the lowest one was produced by ISSR #5.

Fingerprinting analysis

DNA fingerprinting with the aim of cultivar or varietal identification, has become an important tool for genetic identification, germplasm management and registration systems (21). In the present study, 29 polymorphic loci were amplified by more informative primers. The obtained banding patterns were

reproducible for each primer and genotype that were used for fingerprinting the cultivars. The selected primers can be used for genetic distance estimation and identification of the cultivars that are difficult to characterize because of their similar morphological properties (Figure 1). Two-dimensional PCA plot analysis showed that the first, second and third components represented 15%, 13% and 12% of variation respectively, and the first 10 coordinates explained 78% of the total variation that represented appropriate sampling of the primers and scattering over different parts of the genome.

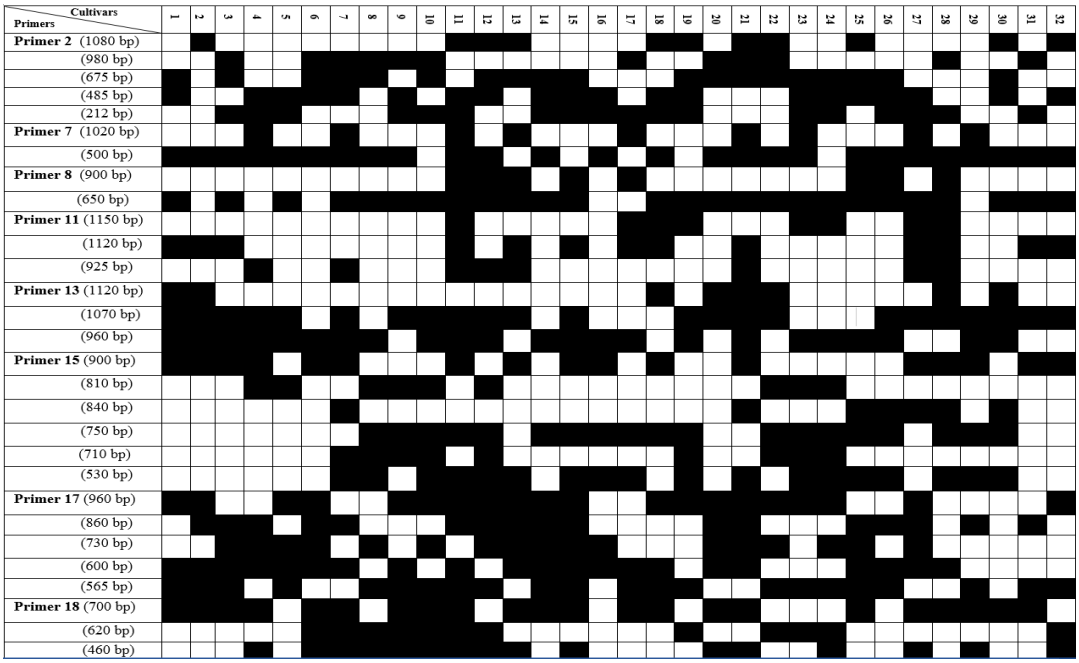

Figure 1. The fingerprinting profile of 32 rice varieties obtained by ISSR markers. The black blocks indicate presence of band and white blocks indicate absence of band.

Genetic relationships

Knowledge about genetic constitute differences of genotypes is highly important in breeding programs. The importance is because of the need to identify parents with high genetic distances to produce progenies with higher heterosis (2). In this study, generated similarity matrix by ISSR markers exhibited a range of similarity indexes between 39%- 88.4% (Table 3). The two Italian rice cultivars 'Ribe' and 'Roma' were the closest genotypes with the highest similarity index (88.4 %) that were followed by two Iranian rice cultivars 'Hasan Sarai 1' and 'Hasan Sarai 2' with 87% similarity index. The high level of similarity could be due to having common ancestors and or selecting similar traits during breeding programs in Italian or Iranian rice cultivars. The highest distance (61%) was between 'Vialone nano' and 'Anbarbu' followed by distance

between 'Lemonino' and 'Anbarbu' cultivars with 58.7% dissimilarity index.

Table 3. Similarity index values among the rice cultivars using Jaccard coefficient method.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1	1																															
2	0.86	1																														
3	0.88	0.84	1																													
4	0.74	0.68	0.79	1																												
5	0.81	0.74	0.75	0.7	1																											
6	0.76	0.72	0.78	0.77	0.69	1																										
7	0.71	0.65	0.73	0.77	0.63	0.77	1																									
8	0.64	0.59	0.67	0.62	0.67	0.65	0.68	1																								
9	0.76	0.7	0.73	0.68	0.76	0.72	0.74	0.79	1																							
10	0.73	0.65	0.71	0.7	0.76	0.67	0.7	0.81	0.85	1																						
11	0.68	0.65	0.68	0.68	0.63	0.69	0.76	0.62	0.71	0.69	1																					
12	0.7	0.65	0.68	0.68	0.75	0.67	0.71	0.78	0.8	0.88	0.73	1																				
13	0.68	0.64	0.7	0.74	0.59	0.71	0.78	0.59	0.67	0.66	0.77	0.66	1																			
14	0.81	0.75	0.84	0.73	0.74	0.77	0.7	0.73	0.79	0.76	0.67	0.74	0.69	1																		
15	0.76	0.68	0.78	0.75	0.67	0.7	0.74	0.7	0.72	0.76	0.73	0.73	0.8	0.79	1																	
16	0.68	0.66	0.72	0.72	0.68	0.64	0.71	0.58	0.62	0.62	0.64	0.62	0.72	0.67	0.76	1																
17	0.61	0.62	0.59	0.52	0.62	0.57	0.52	0.51	0.61	0.54	0.49	0.53	0.49	0.68	0.52	0.58	1															
18	0.71	0.64	0.66	0.55	0.59	0.64	0.58	0.56	0.65	0.58	0.6	0.57	0.57	0.72	0.61	0.52	0.61	1														
19	0.7	0.62	0.64	0.59	0.73	0.62	0.67	0.72	0.76	0.82	0.71	0.82	0.6	0.69	0.67	0.57	0.51	0.59	1													
20	0.76	0.7	0.76	0.75	0.69	0.82	0.77	0.64	0.77	0.7	0.7	0.7	0.78	0.83	0.75	0.64	0.58	0.65	0.65	1												
21	0.77	0.71	0.77	0.81	0.66	0.75	0.87	0.65	0.71	0.71	0.76	0.7	0.86	0.76	0.82	0.75	0.52	0.6	0.62	0.83	1											
22	0.59	0.54	0.59	0.57	0.64	0.56	0.55	0.68	0.72	0.74	0.53	0.71	0.5	0.65	0.59	0.51	0.52	0.62	0.69	0.59	0.55	1										
23	0.68	0.62	0.65	0.67	0.7	0.62	0.67	0.76	0.78	0.82	0.66	0.79	0.6	0.73	0.69	0.59	0.57	0.59	0.82	0.65	0.66	0.81	1									
24	0.66	0.59	0.64	0.66	0.71	0.63	0.65	0.79	0.78	0.87	0.65	0.84	0.6	0.74	0.74	0.6	0.52	0.56	0.85	0.65	0.67	0.74	0.86	1								
25	0.67	0.61	0.72	0.6	0.61	0.64	0.68	0.66	0.66	0.64	0.64	0.66	0.65	0.76	0.73	0.58	0.54	0.7	0.59	0.68	0.7	0.66	0.61	0.6	1							
26	0.76	0.72	0.76	0.64	0.77	0.68	0.72	0.68	0.72	0.7	0.7	0.74	0.67	0.8	0.77	0.69	0.65	0.65	0.69	0.73	0.73	0.59	0.69	0.65	0.78	1						
27	0.72	0.69	0.77	0.78	0.64	0.73	0.73	0.61	0.69	0.69	0.72	0.67	0.81	0.73	0.73	0.68	0.56	0.6	0.6	0.76	0.83	0.57	0.66	0.65	0.65	0.67	1					
28	0.69	0.63	0.67	0.56	0.56	0.59	0.64	0.53	0.64	0.59	0.61	0.6	0.6	0.66	0.64	0.49	0.49	0.58	0.56	0.66	0.67	0.52	0.56	0.54	0.66	0.7	0.63	1				
29	0.51	0.52	0.54	0.53	0.41	0.52	0.6	0.51	0.49	0.51	0.56	0.48	0.56	0.51	0.51	0.46	0.43	0.46	0.46	0.51	0.57	0.39	0.48	0.44	0.49	0.51	0.53	0.54	1			
30	0.72	0.64	0.68	0.61	0.61	0.59	0.67	0.62	0.64	0.66	0.6	0.66	0.63	0.69	0.67	0.56	0.49	0.54	0.64	0.67	0.68	0.53	0.63	0.6	0.67	0.74	0.62	0.67	0.65	1		
31	0.62	0.58	0.62	0.57	0.53	0.54	0.57	0.52	0.57	0.55	0.51	0.51	0.58	0.59	0.57	0.55	0.52	0.57	0.54	0.59	0.57	0.53	0.58	0.51	0.52	0.54	0.57	0.47	0.56	0.7	1	
32	0.64	0.6	0.66	0.59	0.59	0.56	0.61	0.56	0.59	0.54	0.53	0.53	0.64	0.63	0.61	0.66	0.56	0.52	0.51	0.61	0.64	0.47	0.57	0.53	0.54	0.65	0.67	0.51	0.58	0.71	0.75	1

Cluster analysis

Evaluation similarity coefficients or dendrogram helps to select superior individuals to obtain hybrids with greater segregation and heterotic effects during recombination (3). In this study, clustering based on Jaccard's coefficient similarity index and UPGMA algorithm divided the cultivars into 6 main sub-clusters. The cultivars were grouped based on the cut-off point of 64% similarity equivalent to the mean genetic similarity of all the cultivars Figure 2. Cophenetic correlation created from comparing similarity and output matrix of the dendrogram was 0.91, that indicates the used similarity coefficient and cluster analysis method were reliable for grouping the rice cultivars. Majority of cultivars were placed in cluster I that consisted of five sub-groups with a range of similarity between 73%- 0.79%. This range of similarity suggests the existence of relatively less divergence in group I, that may be because of the origin of some of the cultivars from closely related ancestors, For example, 'Mosa Tarom' with 'Mir Tarom' and 'Hasan Sari1' with 'Hasan Sari2' are actually identical varieties that were separated from each other during cultivation in different regions.

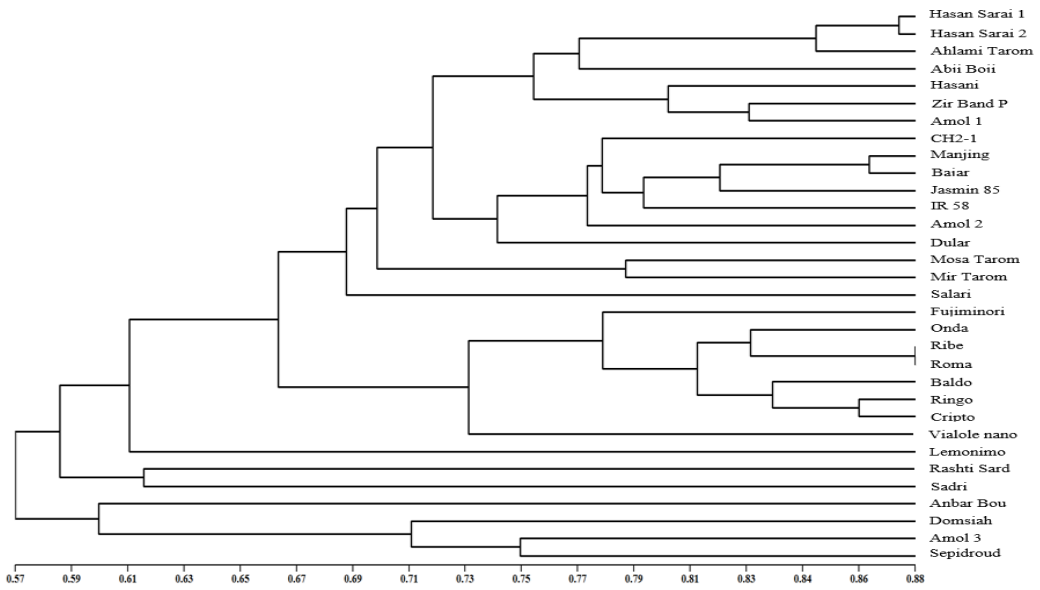

Figure 2. UPGMA dendrogram based on the Jaccard's similarity index calculated from an ISSR analysis of different rice cultivars.

Discussion

This study is on the use of ISSR markers for creating genetic fingerprints and analyzing relationships among domestic and foreign rice cultivars in Iran, which allows breeders to identify cultivars by combined use of the generated bands. According to Ferreira *et al*., (8) heterosis and combining ability of parents depend directly on the genetic diversity between them, and the chance of finding promising combinations is more when more divergent materials are used. The similarity matrix results indicated a considerable level of genetic variation and a wide range of genetic distance among the cultivars, which indicates the importance of studying relationships and genetic distance among the cultivars. In the established fingerprint (Figure 1) the position of bands was different among cultivars, and differed from bands that were unique for some cultivars in comparison with those which were present in approximately 50% of them. It is important to distinguish the identical cultivars that are called by different names in different regions of a country and different cultivars with identical names and or germplasm preservation. It has been approved by Prevost and Wilkinson (19) that generated profiles by ISSR markers are a quick, reliable and highly informative system for DNA fingerprinting. In the present study, the "Anbarbu" cultivar showed the highest genetic distance in comparison with other cultivars, that can be considered the most interesting and promising cultivar for producing progenies with higher heterosise. Based on the similarity coefficients and dendrogram results, it could be inferred that crossing between "Vialone nano" and "Anbarbu" cultivars was the most interesting and promising cross to produce progenies

with higher heterosise because of their greatest genetic distance (60.9 %) and valuable traits for breeding programs such as, long and highly aromatic grains for "Anbarbu" and very tasty grains for "Vialole nano" (Table 3, Figure 2). The result is in accordance with the studies conducted by Manonmani *et al*., (14) in Rice (*Oryza sativa* L) and Orlovskaya *et al*., (17) in spring triticale accessions. They reported the existence of positive correlation between genetic distances derived from PCR based markers and heterosis. Furthermore, the increase in genetic distance value between parental components leads to higher probability of obtaining heterotic hybrids. Using 12 ISSR primers, different numbers of loci were amplified with the range of 7-34 bands, which poly (GA)-containing 3-anchored primers produced the highest number of bands. The result suggests the existence of a high frequency of dinucleotide simple sequence repeats especially poly (GA) motifs in genome of rice, thus primer ISSR #2 was introduced as an efficient primer to detect polymorphism and fingerprinting within rice population because of its high value of polymorphism, PIC, frequency distribution and poly (GA)-containing 3- anchors. Overall, the results indicated that there is a high degree of diversity among the cultivars for genetic relationship evaluation and the ISSR marker is an informative, quick and reproducible approach that generates sufficient polymorphisms for large-scale DNA fingerprinting purposes in rice. The results would be promising as the genetic markers for identification of rice cultivars and gleaned critical data are considered as a source of knowledge for future rice research, such as identification and genotyping of the cultivars, germplasm improvement and parental selection for breeding purposes. For a more detailed review, any given collection of rice can be sampled in different regions and used molecular techniques for characterizing and fingerprinting the varieties.

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انگشت‌نگاری و ارزیابی تنوع ژنتیکی ارقام برنج با استفاده از نشانگر ISSR (Inter Simple Sequence Repeat)

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چکیده

برنج به عنوان یکی از مهمترین محصولات کشاورزی دارای پتانسیلی بالقوه جهت مبارزه با فقر و تامین امنیت غذایی در جهان است. در این مطالعه به منظور بدست آوردن اطلاعات پایه در برنامه‌های اصلاحی برنج از مارکر Inter Simple Sequence Repeat (ISSR) برای انگشت‌نگاری و تعیین روابط خویشاوندی بین ۳۲ رقم برنج استفاده شد. از میان ۱۷ پرایمر استفاده شده ۱۲ پرایمر، ۱۸۴ قطعه‌ی مجزا و تکرارپذیر با پلیمرفیسم ۸۸٪ را تکثیر کردند و از این بین ۲۹ لوکوس که دارای بیشترین میزان پلیمرفیسم بودند جهت انگشت‌نگاری ارقام انتخاب شدند. نتایج بدست آمده نشان داد که میزان شباهت ارقام مابین ۳۹٪ -۸۸.۴٪ و مقدار PIC با میانگین ۲۳٪ مابین ۰.۱ (برای پرایمر #۳) تا ۰.۳۴ (برای پرایمر #۲) متغیر است. خوشه‌بندی براساس ضریب Jaccard و الگوریتم UPGMA ارقام را به شش گروه در نقطه‌ی برش ۶۴٪ تقسیم کرد که دو رقم ایتالیایی Ribe و Roma کمترین فاصله‌ی ژنتیکی و ارقام Vialonenano و Anbar Bou بیشترین فاصله‌ی ژنتیکی را نسبت به یکدیگر نشان دادند. در کل بررسی روابط ژنتیکی با استفاده از مارکر ISSR تنوع بالایی میان ارقام مورد مطالعه نشان داد و پرایمرهای واجد تکرارهای GA بیشترین تعداد باندهای حاصل از تکثیر لوکوس‌ها را به خود اختصاص دادند که بیانگر فراوانی موتیفی بیشتر این تکرارها در ژنوم گیاه برنج است. نتایج بدست آمده از ماتریکس تشابه و آنالیز خوشه‌ای نشان داد که که رقم عنبربو دارای بیشترین فاصله‌ی ژنتیکی در مقایسه با سایر ارقام مورد بررسی است و می‌تواند به عنوان والد در برنامه‌های اصلاحی مورد استفاده قرار گیرد. نتایج بدست آمده از این تحقیق می‌تواند به عنوان مارکری ژنتیکی جهت شناسایی ارقام برنج و منبعی برای تحقیقات آینده با هدف بهبود و اصلاح ارقام برنج باشد.

کلمات کلیدی: دندروگرام، شباهت ژنتیکی، آنالیز ژنوم، مارکر مبتنی بر PCR، چند شکلی.