RESEARCH ARTICLE

Effectiveness of molecular markers for improving grain quality in Iranian rice

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ABSTRACT: Grain and cooking qualities in rice are measured by several physical and chemical traits, each of them controlled by several genes. Molecular markers have become fast and reliable tools for screening genotypes for grain quality. However, As different populations may carry different genes, the efficacy of previously developed markers in new populations should be tested. In order to assess the effectiveness of molecular markers in predicting grain quality in Iranian rice genotypes, a total of 38 genotypes from three different backgrounds were fingerprinted by 9 grain quality specific molecular markers and 10 laboratory traits. A total of 31 alleles were detected with an average of 3.1 alleles per locus and the polymorphic information content values ranged from 0.245 to 0.74. Cluster analysis based on molecular markers divided the rice genotypes into three major clusters and effectively differentiated between various genotypes. However, the dendrogram based on the common set of qualitative traits didn't succeed in discriminating between original groups. There were significant associations between molecular markers and quality traits except for milling factor. However, these associations weren't necessarily specific to their supposed traits. Therefore, association of markers developed in other rice populations, specially those developed in Japonioca populations should be tested prior to application in Iranian rice marker assisted breeding programs.

KEYWORDS: Rice, Grain Quality, Molecular Markers, Polymorphic Information Content, Marker Assisted Selection.

INTRODUCTION

Rice is the second most important cereal after wheat, and more than half of the world's population depends on rice as the main food. Rice breeding in Iran aims to develop cultivars with high yield that retain good grain quality. Grain quality is the main factor in acceptance of rice by consumers and it determines the economic value of rice varieties. However, most of high yielding cultivars, despite having grains with physical resemblance to local cultivars, have inferior cooking quality. Grain quality should be considered as a combination of milling quality, grain size, grain shape, appearance, fragrance and cooking properties [1]. Amylose content (AC), Gelatinization Temperature (GT) and Gel Consistency (GC) are suggested to be directly related to cooking and eating quality [2]. Based on the result of Singh et al. [3], cultivars with intermediate AC, GT, softer GC and linear grain elongation after cooking are generally preferred among rice consumers.

Although quality traits can be measured according to standard laboratory methods, but these methods are time and labor consuming and are measured only after harvest. Molecular markers that are closely linked to grain quality genes are specially useful for improving the rice grain quality as they can select for desired plants at early stages and can save time and labor . Zhou et al. [4] employed a combination of SSR, RFLP and AFLP molecular markers in their study, for simultaneously improving four quality traits in the elite rice variety Zhenshan 97. Development of such markers needs a vast knowledge on genomic regions harboring grain quality genes.

Studies have indicated that AC is controlled by a single major locus along with modifications by some minor genes [1]. The Granule-Bound Starch Synthase (GBSS) in rice is encoded by the Waxy (*Wx)* gene that controls the synthesis of amylase [5]. *Wx* region has also a large effect on GC [6].

GT in rice is mainly determined by an alkali decadent locus on chromosome 6, encoding the Starch Synthase IIa (*SSIIa*) gene [7]. Fragrance is controlled by a single recessive gene (*fgr*) closely linked to the FRLP clone RG28 on chromosome 8 [8] and some QTLs in other parts of rice genome [9].

Appearance of rice, which includes grain size and shape, translucency and chalkiness [1] is a crucial factor in affecting consumer attitudes. Grain shape is usually evaluated by grain length, grain breadth and grain length-breadth ratio, which correlate positively with grain weight [10]. Quantitative trait locus analysis indicated that *GS3* gene has a great effect on grain length and weight variation [11]. Tan et al. [12] showed that chalkiness is a quantitative trait controlled by polygenes and is highly influenced by environmental factors. According to Kunze [13], milling quality of rice grain, which is basically determined by the head rice yield (HRY), can be reduced in presence of fissures in rice kernel.

Based on the vast information on rice genome, either specific markers have been designed for different quality traits, or SSR markers have been reported with close linkage to such genes. For example, in a recent study, 110 SSR markers have been used effectively to map QTLs associated with 7 important quality traits, namely grain length, grain breadth, grain length-breadth ratio, cooked kernel elongation ratio, amylose content, alkali spreading value (ASV) and aroma [14]. Although molecular markers are robust and promising in populations where they have been developed, their efficiency should be evaluated in target populations. It is because usually there

are several different genes that are responsible for a single quality trait, and not all of them are present in a single population. For example, Bradbury et al. [15] reported that *BAD2* gene is polymorphic between aromatic and non-aromatic genotypes. However, there are several cases of aromatic rice that don't carry the *BAD2* gene [16]. Therefore, the effectiveness of molecular markers should be confirmed in breeding populations. The main objectives of this study were to evaluate the possibility of predicting the grain quality of Iranian rice based on previously established molecular markers, and to compare the efficiency of molecular markers with the traditional set of laboratory-measured quality traits in determining the quality of rice genotypes.

MATERIALS AND METHODS

Plant material

Seeds of 38 rice cultivars including 14 Iranian local cultivars, 11 improved lines, 12 promising varieties and one IRRI improved line (Table 1) were provided by the Rice Research Institute of Iran (RRII).

Table 1. List of rice genotypes used in this study

	Genotypes	Category*		Genotypes	Category*
1	Anbarboo	LV	20	Fajr	HYC
2	Domsiah	LV	21	Kadous	HYC
3	Rashti	LV	22	Dasht	HYC
4	Sang tarom	LV	23	Nemat	HYC
5	Tarom mahalli	LV	24	Neda	HYC
6	Hasansaraie	LV	25	Khazar	HYC
7	Salari	LV	26	NA3	AL
8	Ghashenge	LV	27	NA6	AL
9	Moosatarom	LV	28	NA9	AL
10	Deilamani	LV	29	NA11	AL
11	Sadri	LV	30	NA12	AL
12	Mohamadi chaparsar	LV	31	NA15	AL
13	Zirebandpey	LV	32	NA17	AL
14	Rashti Sard	LV	33	ST401	AL
15	Keshvari	HYC	34	ST601	AL
16	Kuhsar	HYC	35	ST1-503	AL
17	Shafagh	HYC	36	ST2-306	AL
18	Sahel	HYC	37	ST4-210	AL
19	Shiroudi	HYC	38	IR64	HYC

*LV: Local variety, HYC: High yielding cultivar, AL: Advanced line

Table 2. List of molecular markers sequences used in this study

Primer	type	sequence	Chromosome	Related Traits	Reference
NF1-NR2		F- CGAGGCGCAGCACAACAG R- GGCCGTGCAGATCTTAACCAT			
	STS		6	GT	46
F22-R21		F- CAAGGAGAGCTGGAGGGGGC R- ACATGCCGCGCACCTGGAAA			
RM190	SSR	F- CTTTGTCTATCTCAAGACAC R- TTGCAGATGTTCTTCCTGATG	6	AC- GC	47
RM42	SSR	F- ATCCTACCGCTGACCATGAG R- TTTGGTCTACGTGGCGTACA	8	Aroma	41
RM587	SSR	F- ACGCGAACAAATTAACAGCC R- CTTTGCTACCAGTAGATCCAGC	6	GC	48
RM204	SSR	F- GTGACTGACTTGGTCATAGGG R- GCTAGCCATGCTCTCGTACC	6	AC	49
RM234	SSR	F- ACAGTATCCAAGGCCCTGG R- CACGTGAGACAAAGACGGAG	7	Chalky grain	50
RM1339	SSR	F- ATCAAAGCATGTAAACCAGC R- CGTAAGATCTCCCTACCACC	1	FK	51
RM21938	SSR	F- CCAAATTGCTTCCTCGGATATAGG R- CGGATTTAGGGAGTTCGTGTTCG	7	Chalky grain	50
8G-16	Indel	F- GACGGTCAATGTTGCTCAG R- GTCACGCATTAAATCCAAG	8	Chalky grain	52

Laboratory quality traits

The total of 38 genotypes were planted at the RRII field in Mazandaran, Amol under a randomized complete block arrangement with two replications in 2017. After harvesting, seed samples from each plot were used to evaluate the quality traits of grain and cooking, namely AC, GT, GC, fragrance, Elongation Rate (ER), Length After cooking (LA), Length Before cooking (LB), Head Rice Yield (HRY), Fissured Kernels (FK), Milling Factor (MF) and percentage of hulls. The method of Juliano [17] was used for measuring AC. To determine GT, the ASV was used as described by Little et al. [18]. GC was measured by the method of Cagampang et al. [19]. The fragrance of cultivars was determined according to the Dela Cruz and Khush method [1]. LB and LA of raw and cooked rice were measured on 10 random grains and ER was calculated using the formula given by Juliano and Perez [20]. MF was measured according to He et al. [21]. HRY was calculated as the ratio of rough rice mass that remained as head rice. The percentage of hulls of rough rice was calculated according to Dela Cruz and Khush's [1] formula:

$$Hull(\%) = \frac{\text{weight of hulls}}{\text{weight of rough rice}} \times 100$$

The percentage of FK was estimated using the Velupillai and Pandey's method [22].

DNA extraction and PCR amplification

For DNA extraction and quality analysis, 5 seeds of each genotype were sown in a small pot. Leaf samples were taken from 20-days young seedlings and kept at -80°C. Genomic DNA was extracted from young leaves by the Plant Genomic DNA Miniprep Kit (CAT.NO:MBK0011). Purity and concentration of extracted DNA was monitored by electrophoresis in 1% agarose gel and also by spectrophotometer. SSR Polymerase Chain Reaction (PCR) was performed on a thermal cycler (Bio-Rad, USA) and ampliqon was used to prepare the reaction mixture, which included all of the required factors for PCR reactions. PCR reactions were carried out with the total reaction volume of 25 µL containing 12.5 µl of ampliqon, 5.5 µl of distilled water, 5 µl of template DNA and 1 µl of each primer. The temperature cycles were programmed as follows: an initial denaturation phase at 94°C for 4 min, followed by 45 cycles at 94°C for 45 s, annealing at 62 - 72°C (different for each primer) for 45 s, 45 s at 72°C for primer extension and then 4 min at 72°C for the final extension. The amplified products were separated by horizontal electrophoresis in 1.5% agarose gel. The gels were stained with ethidium bromide for visualization on a UV transilluminator.

Molecular markers

Seven polymorphic Simple Sequence Repeats (SSRs), one Indel and one sets of STS marker related to grain and cooking quality parameters were used to genotype the population (Table 2).

Table 3. Means of quality traits in local varieties, high yielding genotypes and advanced lines

Type		GT	GC (mm)	AC	ER	LA (mm)	LB (mm)	HRY	FK	Hull
Local	min	3.05	56.5	16.00	1.695	9.565	5.62	58.45	7.80	21.0
	max	3.8	97.5	20.85	1.955	13.630	7.05	68.55	20.75	27.4
Improved	min	3	38.5	19.30	1.535	11.565	6.81	65.55	6.85	19.8
	max	7	85.0	25.65	1.760	13.910	8.59	71.40	19.10	24.9
New lines	min	4	39.0	16.40	1.665	11.640	6.99	62.35	7.40	20.6
	max	6.8	96.5	24.30	1.850	15.005	8.76	69.00	22.85	24.3
Overall	min	3	38.5	16.00	1.535	9.565	5.62	58.45	6.85	19.8
	max	7	97.5	25.65	1.955	15.005	8.76	71.40	22.85	27.4

Table 4. Phenotypic correlation between appearance and quality traits

Quality traits	GT	GC	AC	ER	LA	LB	FK	Hull	HRY
GT	1								
GC	0.451**	1							
AC	-0.689**	-0.472**	1						
ER	-0.181	-0.336*	0.356*	1					
LA	0.455**	-0.152	0.330*	0.053	1				
LB	0.497**	-0.311	-0.468**	-0.414**	0.886**	1			
FK	0.266	0.040	0.021	0.343*	0.236	0.055	1		
Hull	-0.144	0.110	-0.195	0.245	-0.156	-0.237	0.165	1	
HRY	0.027	-0.111	0.220	-0.307	-0.008	0.122	-0.292	-0.831**	1

** and * mean correlation is significant at the 1% and 5% levels of probability, respectively

Data analysis

Quality traits

Data collected on grain and cooking quality characteristics were subjected to normality test and analysis of variance (ANOVA), and the traits that didn't show significant differences were removed from subsequent analyses. Phenotypic correlations were calculated by SPSS 18 software (23), and the genetic similarity between varieties was calculated using the Unweighted Pair-Group Method with Arithmetic mean algorithm (UPGMA) by NTSYSpc program [24].

Molecular data

The amplified bands were scored as present (1) or absent (0), generating a binary data matrix comprising '1' and '0' for each marker. The Polymorphic Information Content (PIC) of each SSR marker was calculated using the following formula developed by Ni et al. [25]:

$PIC_i = 1 - \Sigma p_{ij}^2$

Where: P_{ij} is the frequency of the jth allele for marker i.

Molecular data was used to construct dendrogram using the UPGMA method by the NTSYS Spc program [24]. A t-test was run for testing association between the loci and the quality traits.

RESULTS

Variations in laboratory quality traits

The analysis of variance for laboratory quality traits indicated that there were significant differences among the genotypes for all of the traits except for MF (table not presented). So, MF was exempted from further analyses. Ranges of quality traits in local varieties, high yielding cultivars and advanced lines are presented in Table 3. As can be seen in this table, the range of variations for GC, GT and AC in the two latter groups exceeds from desired ranges. This can explain why most of older high yielding cultivars have inferior grain and cooking quality.

Correlation values between pairs of grain quality characters are summarized in Table 4. As the table shows, AC was negatively correlated with GC and GT, and there was a positive correlation between GC and GT. FK was negatively correlated with HRY. ER was positively correlated with AC and FK.

Cluster analysis, using quality traits separated the accessions into 5 main groups (the figure is not shown). Results showed that the genotypes with various origins and quality trait values were clustered together into same group.

Table 5. Number of alleles, allele size range, allele frequency and polymorphism information content (PIC) of 9 markers across 38 cultivars

Marker	No. of alleles	Allelic frequency							PIC Value	Size range (bp)
		1	2	3	4	5	6	7		
RM 204	4	6.66	13.32	36.66	46.66	-	-	-	0.650	190-120
RM42	2	52.95	47.05	-	-	-	-	-	0.499	163-150
RM190	2	71.05	28.95	-	-	-	-	-	0.412	140-110
RM21938	2	14.29	85.71	-	-	-	-	-	0.245	200-138
RM234	7	41.18	5.88	5.88	26.48	5.88	5.88	8.82	0.740	200-120
RM1339	5	20.00	5.73	2.85	11.42	60.00	-	-	0.583	164-100
8G-16	3	12.90	80.64	6.46	-	-	-	-	0.329	266-220
RM587	4	27.27	12.12	27.27	34.34	-	-	-	0.723	220-170
NF1-NR2 F22-R21	2	75.72	24.28	-	-	-	-	-	0.400	800-300

Table 7. Association between marker loci and grain quality traits

	GT	GC	AC	ER	LA	LB	HRY	FK	Hull
RM190	**		**		**	**		*	
RM 42	*	*	**		*	*			
RM 204	**	**	**		*		*	*	
RM 21938	**				**	**		*	
RM 234	**	**	**	*	*	**			**
RM1339	*	**	*					*	*
8G16			*	*				*	
RM 587								*	
NF1NR2	**	*	**		**	**	*	**	

* and ** represent significance at 0.05 and 0.01 level of probability, respectively.

Evaluation of molecular markers

An important criterion for measuring the usefulness of a molecular marker in a plant population is the amount of its polymorphic content. The PIC value is a measure of polymorphism in a marker locus among individuals and represents the relative informativeness of the marker. The 10 SSR and STS molecular markers showed suitable levels of polymorphism, and generated a total of 31 alleles with an average of 3.1 alleles per locus (Table 5). The number of alleles per locus produced by each marker varied from 2 (RM42, RM190, NF1-NR2, F22-R21) to 7 (RM234). The STS marker, as expected, produced only two bands. The PIC values in this study varied from the lowest of 0.245 (RM21938) to the highest of 0.74 (RM234). Also, the RM42 marker showed clear polymorphism between aromatic and non-aromatic cultivars with the total size of amplified products ranged from159 bp for the aromatic genotypes to 165 bp for the non-aromatic genotypes (Table 6).

Table 6. Genotypes with aromatic alleles

NA11	Sahel	Domsiah
NA12	Kadus	Sadri
NA15	Shafagh	Zirebandpey
ST1-503	Koohsar	Rashtisard
Fajr	Hasan Sarayi	Moosatarom
Anbarboo	Sange Tarom	Mohamadi Chaparsar
Salari	Tarommahali	Deilamani
	Neda	

Association between markers and quality traits

Results of t-tests for testing associations between allelic bands and quality traits are summarized in Table 7. Among the markers, RM587 only showed a slight association with percentage of cracked grains. On the other hand, rest of the markers showed associations with several traits, even traits they were not suggested for. For example, RM204, which was recommended for AC, showed highly significant associations with AC, GC and GT along with significant associations with some other traits.

Cluster analysis based on molecular data

The genetic relationship between the rice genotypes was demonstrated in a dendrogram based on informative microsatellite alleles (Figure 1). The 38 rice cultivars were divided into three major clusters at the level of genetic similarity of 0.74. Cluster I included Iranian landraces and four improved high yielding varieties lines viz, Keshvari, Shafagh, Koohsar and Neda. This cluster included mostly high quality local cultivars and some high-yielding genotypes with desirable cooking and eating quality. Cluster II can be divided into 3 sub-clusters IIA, IIB and IIC. Sub-clusters of IIA and IIB had high-yielding genotypes with appropriate quality properties. In the IIC sub-cluster, 3 high-yielding improved cultivars,
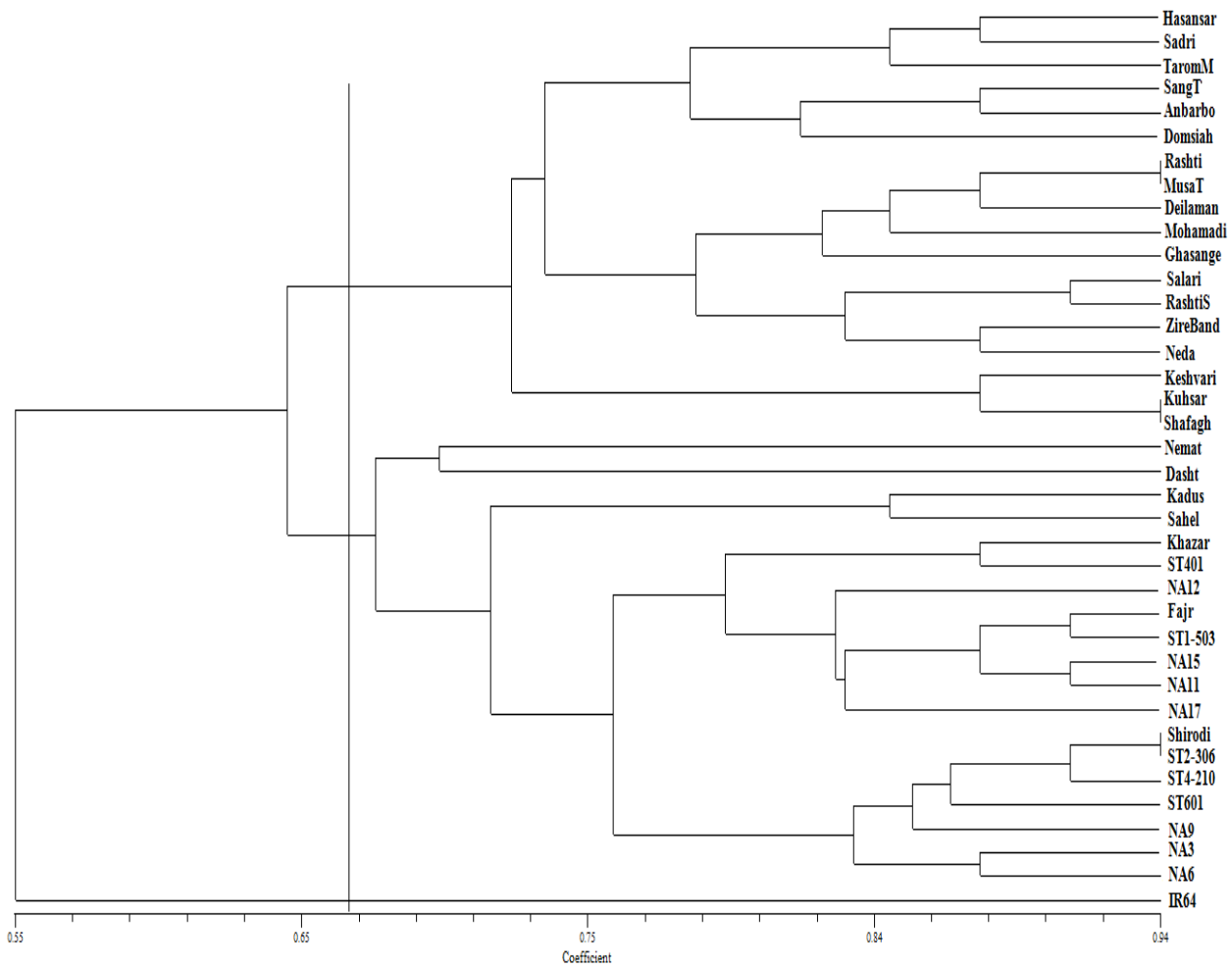
Figure1. Dendrogram of 38 rice cultivars, constructed from UPGMA cluster analysis based on simple matching coefficient by 10 molecular markers.

Khazar, Fajr, Shiroudi and 12 promising genotypes (produced by RRII) were in the same branch. These promising lines had poor to intermediate eating and cooking quality. Group III included only IR64 cultivar which was developed by the International Rice Research Institute (IRRI) and showed intermediate AC, GC and GT and good cooking and eating quality.

DISCUSSION

Genetic variation is the play ground for selection and hybridization programs in rice breeding. The genotypes in this study showed significant variations for the grain quality traits. Lee et al. [26] reported significant differences for physicochemical properties and palatability factors between 69 Korean rice landraces. The same result were observed by Graham-Acquaah et al. [27], who evaluated the agronomic and grain quality traits variations in forty-five rice varieties.

In this research, negative correlations were found between the AC and GT and GC, which were supported by the results of Nakamura et al. [28]. This correlation is partly bcause the Waxy gene region that controls AC has also a large effect on GC [6]. Zhang et al. [29] reported that fissures negatively affected HRY. As a consequence, maintaining HRY during drying process has been a main goal of the rice industry [30]. During milling, chalky kernels tend to break more [31], which leads to decrease in HRY.

Clustering of genotypes based on quality specific SSR markers was able to separate genotypes based on eating and cooking properties and the origin of their development. Unlike the molecular markers, quality traits could not classify the 38 rice cultivars into distinguished quality groups, presumably because they are affected by genetic background, environment, and gene- environment interactions [32].

The number of alleles for some of the SSR markers used in this study was rather low, compared with some of previous studies that have reported higher numbers of alleles in each locus . Talukdar et al. [33] reported an average number of alleles of 3.7 per locus (range 2-6 per locus) in a population structure analysis study of 40 Joha and 14 non-Joha rice genotypes via 143 simple sequence repeat markers. The level of PIC value is comparable to that reported by Nachimuthu et al. [34 (PIC 0.468)] and Talukdar et al. [33 (PIC 0.59)].

The set of markers that we used in this study have been developed in rice populations from different countries, and mostly using Japonica rice. Therefore, their usefulness in Iranian rice populations depends on their PIC as well as retaining their association with the traits that they are recommended for. In this study the markers showed a reasonable allelic variability, however their linkage to the target traits couldn't be established. It implies that grain quality in Iranian varieties should be controlled by different set of genes. Therefore, for association studies their usefulness has to be studied more and also specific markers for Iranian rice should be developed. However, these markers could be satisfactorily used for genetic diversity analyses and clustering of genotypes. Babu et al. [35] reported that SSR analysis whould present a higher level of efficiency and would be principally useful in clustering genotypes.

Distance-based clustering analysis classified most of the genotypes according to the geographical region, pedigree and genetic similarity. The results of our study showed that molecular markers have potential for evaluating genetic variation for cooking and eating quality of rice genotypes. Our results revealed that the studied genotypes had different eating and cooking quality properties. Such differences among accessions could be used in various breeding and crop improvement programs. Development of high-yielding varieties accompanied with superior quality of cooking is a priority in rice breeding programs. Conventional breeding based on the time-consuming phenotypic parameters is, expensive and limited by environmental conditions. In order to enhance the efficiency of selection, MAS could help breeders to select for lines with desirable quality genes at early growth stage and early segregating generations. Furthermore, distant genotypes that have been identified in current study, especially IR64 and local genotypes, could be employed by rice breeders in hybridization programs for further improvement of high yielding good quality genotypes.

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کارایی نشانگرهای مولکولی در بهبود کیفیت دانه برنج‌های ایرانی

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چکیده

کیفیت دانه و پخت در برنج توسط چندین صفت فیزیکی و شیمیایی اندازه‌گیری می‌شوند که هر کدام از آنها توسط چند ژن کنترل می‌شوند. نشانگرهای مولکولی به ابزارهای سریع و قابل اعتمادی برای غربال ژنوتیپ‌های برنج برای کیفیت دانه تبدیل شده‌اند. با این وجود، از آنجا که جمعیت‌های مختلف ممکن است ژن‌های مختلفی را با خود حمل کنند، کارایی نشانگرهای مولکولی که پیشتر توسعه داده شده‌اند در جوامع جدید باید مورد بررسی قرار بگیرد. به منظور ارزیابی کارایی نشانگرهای مولکولی در پیش‌بینی کیفیت دانه در ژنوتیپ‌های ایرانی، تنوع ژنتیکی ۳۸ ژنوتیپ برنج ایرانی از سه زمینه ژنتیکی مختلف با استفاده از ۹ نشانگر مولکولی مربوط به کیفیت دانه و ۱۰ صفت کیفی آزمایشگاهی دانه مورد بررسی قرار داده شد. در مجموع ۳۱ آلل با میانگین ۳/۱ آلل به ازای هر جایگاه ژنی تولید شده و مقدار محتوای اطلاعات چند شکل نیز در دامنه ۰/۲۴۵ تا ۰/۷۴ متغیر بود. دسته‌بندی ژنوتیپ‌ها با استفاده از داده‌های نشانگری آن‌ها را به طور موثری به سه گروه اصلی تقسیم کرد و اختلاف بین گروه‌های ژنوتیپی را شناسایی کرد. هرچند، دندروگرام رسم شده بر اساس صفات کیفی معمول آزمایشگاهی نتوانست ارقام بومی را از ارقام پرمحصول و لاین‌های امیدبخش جدا کند. در این آزمایش پیوستگی‌های معنی‌داری بین نشانگرهای مولکولی و صفات کیفی دانه، به‌جز ضریب تبدیل، دیده شد. با این وجود، این پیوستگی‌ها الزاماً به صفاتی که این نشانگرها به آن‌ها منتسب بودند منحصر نبود. بنابراین، پیش از کاربرد نشانگرهایی که در جوامع دیگر، خصوصا جوامع ژاپونیکا، توسعه داده شده‌اند در برنامه‌های اصلاح به کمک نشانگر در جوامع ایرانی، بایستی پیوستگی آن‌ها با صفات کیفی مورد بررسی قرار بگیرد.

کلمات کلیدی: برنج، صفات کیفی، نشانگرهای مولکولی اختصاصی، ضریب اطلاعات چندشکلی، گزینش به کمک نشانگر