Genetic variation and association analysis of some important traits related to grain in rice (*Oryza sativa* L.) germplasm

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ABSTRACT: The identification of genomic loci involved in control of quantitative traits receives growing attention in plant molecular breeding. The present study was carried out to evaluate the genetic variability among 48 rice genotypes and determine the genomic regions associated with ten grain related important traits. A total number of 63 alleles were detected by 18 selected SSR markers from different chromosomes with an average of 3.5 alleles per marker. A model-based Bayesian approach subdivided 48 evaluated rice genotypes into three major subgroups with the consideration of the highest value of ΔK. The mean r^2 value for all loci pairs on the same chromosome was 0.053. A total of 38 significant marker-trait associations were identified ($P< 0.05$) that explaining more than 32% of the total variation. RM315, RM3428, RM289, RM16, RM574 and RM156 markers had highest R^2 and most association with assayed traits, respectively. The findings of this study revealed association of grain properties in rice with some SSR markers that could serve as target genomic regions for further research such as MAS, fine mapping and candidate gene discovery in rice breeding programs.

KEYWORDS: Association analysis, Linkage disequilibrium, Rice, SSR.

INTRODUCTION

Molecular plant breeding has revolutionized conventional breeding techniques in all areas within the last 20 years. Identification of tightly linked markers is necessary for the successful exploiting of DNA markers technology in different breeding programs (1). Various DNA marker systems (such as RFLP, RAPD and AFLP) with different characterizations have closely tracked developments in molecular biology (14). SSR markers, due to having relatively most of characteristics of an ideal molecular marker such as high polymorphism, random genome distribution and co-dominant Mendelian inheritance, are one of the most reliable markers for different genetic studies (7). Association mapping (AM) or LD-based mapping as a reliable genomic approach and complementary to traditional linkage-based mapping have greatly accelerated the identification of chromosome regions associated with important complex traits in crops (11, 16). This approach with exploiting of the historical recombination events that have occurred in natural populations during times, reducing the cost and time for

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analysis (5, 19, 20). Moreover, QTLs and molecular markers detected in the inbred lines and elite germplasm can be used directly in breeding programs. This approach has been used in several plant species, including maize (18), barley (17), hexaploid wheat (10), sorghum (13) and lettuce (15). Considering rice as a model species with relatively small genome size (~430 Mb), and also with high level of genetic variation due to recombination events with cultivation antecedent about 7,000 years (22, 26), therefore association mapping is a feasible and potentially very useful technique for rice germplasm. This study attempts to establish associations of SSR markers with some of assayed grain related important traits of rice. Outcome of this study could be helpful in identification of genes responsible for these traits, which can further be utilized in marker-assisted selection for the development of superior varieties in different rice breeding programs.

MATERIALS AND METHODS

Plant materials

Forty-eight rice varieties including 24 landraces, 15 Iranian breeding lines, and 9 introduced lines were used in this study (Table 1).The varieties originated from different regions of Iran and other parts of the world, which were kindly provided by the Rice Research Institute of Iran (RRII), Rasht, Iran and International Rice Research Institute (IRRI), and have been widely used as parents in rice breeding programs during past decades.

Field trials and phenotyping

For field studies, each rice variety was sown in the research field of Rice Research Institute of Iran (RRII) (Rasht) using a randomized block design. For all varieties, 30-days-old seedlings were transplanted into the field at a spacing of 15cm between plants within each row and 30 cm between the rows. The middle five plants in the central row of each plot were collected and bulked in order to examine the agronomic traits. Ten grain related traits including grain length and width (mm), lemma length and width (mm), palea length and width (mm), awn length (mm), hectoliter weight (test weight) (gr/cm^3), percentage of grain protein (%) and percentage of grain fissure (%) were evaluated. All studied traits were obtained from three replicates and for each trait, the means of the replicates were used in data analysis.

Table 1. Name and origin of studied rice varieties

No	Variety	Origin
1	Musa Tarom	
2	Hassani	
3	Dom Sorkh	
4	Gharibsiah Reyhani	
5	Ali Kazemi	
6	Sange Tarom	
7	Tarom Mohali	
8	Sadri	
9	Binam	
10	Sangjo	
11	Hashemi	
12	Shahpasand	
13	Dom Syah	Iran
14	Dom Sefid	
15	Salari	
16	Gharib	
17	Zireh	
18	Reyhani	
19	Ghashangeh	
20	Abjibuji	
21	Gardeh	
22	Tarom Amiri	
23	Anbarbu	
24	Mohammadi Chaparsar	
25	Dorfak	
26	Bejar	
27	Nemat	
28	Neda	
29	Dasht	
30	Amol 1	
31	Amol 2	
32	Amol 3	Iran (IL)
33	Khazar	
34	Gil 1	
35	Gil 3	
36	Line 213	
37	Sepidrud	
38	Line 507	
39	Line 4	
40	IR28	
41	IR30	
42	IR36	
43	IR50	
44	IR58	Philippines
45	IR60	
46	IR60	
47	Tetep	
48	Fujiminuri	

IL: Improved Line

SSR genotyping

In total, 18 SSR primer pairs located on different chromosomes were selected for population structure analysis (Table2) (http://www.gramene.org/). Amplification was carried out in an Astec Bio PCR System in a total volume of 10 µl, containing 50 ng of gDNA, 1X PCR buffer, 2.4 mM $MgCl_2$, 0.2 mM of each dNTP, 1U of *Taq* DNA polymerase (Cinnagen Co., Iran), and 0.4 µM primer. The PCR profile composed of an initial denaturation at 94 ℃ for 3 min followed by 10 cycles of 94 ℃ for 30s (denaturing), 65 ℃ for 30s (annealing), and 72 ℃ for 1 min (extension), with 26 subsequent cycles of 94 ℃ for 30s (denaturing), 55 ℃ for 30s (annealing), and 72 ℃ for 1 min with 26 subsequent cycles of 94 ℃ for 30s 55 ℃ for 30s (annealing), and 72 ℃ for 1 min (extension) and a final extension of 72 ℃ for 5 min. The PCR products were detected using the optimized silver staining method in denaturing polyacrylamide gels (6).

Data analysis

Summary statistics for the allele diversity of 18 SSR markers in 48 varieties were calculated using the PowerMarker V3.25 program (9), these included allele number, gene diversity (D_I), and polymorphism

Table 2. Characteristics of used SSR markers.

Primer	Motif	Primer sequence	Chromosome No.	Allele number	Gene diversity	PIC
RM6080	$(CCT)_9$	cagaggaagcaaggagatcg ccatcgggagaaagagag	3	3	0.51	0.46
RM315	$(AT)_4(GT)_{10}$	gaggtacttcctccgtttcac agtcagctcactgtgcagtg	1	5	0.42	0.39
RM288	$(GA)_7G_6(GA)_7$	ccggtcagttcaagctctg acgtacggacgtgacgac	9	4	0.45	0.41
RM289	$G_{11}(GA)_{16}$	ttccatggcacacaagcc ctgtgcacgaacttccaaag	5	4	0.37	0.34
RM201	$(CT)_{17}$	ctcgtttattacctacagtacc ctacctcctttctagaccgata	9	4	0.61	0.54
RM261	$C_9(CT)_8$	ctacttctccccttgtgtcg tgtaccatcgccaaatctcc	4	4	0.47	0.42
RM112	$(GAA)_5$	gggaggagaggcaagcggagag atgacttgatcccgagaacg	2	5	0.34	0.33
RM252	$(CT)_{19}$	ttcgctgacgtgataggttg atgacttgatcccgagaacg	4	2	0.43	0.36
RM16	$(TCG)_5(GA)_{16}$	cgctagggcagcatctaaa aacacagcaggtacgcgc	3	3	0.49	0.44
RM190	$(CT)_{11}$	ctttgtctatctcaagacac ttgcagatgttcttcctgatg	6	4	0.47	0.43
RM6283	$(CTG)_8$	tggagactgagctgatgcc tcaggtggtcggttccttac	3	3	0.36	0.35
RM156	$(CGG)_8$	gccgcaccctcactccctcctc tcttgccggagcgcttgaggtg	3	4	0.54	0.49
RM256	$(CT)_{21}$	gacagggagtgattgaaggc gttgatttcgccaagggc	8	3	0.41	0.37
RM593	$(CT)_{15}(CA)_{10}$	tcccgtatgtaacgtgcca gacaagagaacatcgctagg	5	3	0.36	0.31
RM282	$(GA)_{15}$	ctgtgtcgaaaggctgcac cagtcctgtgttgcagcaag	3	2	0.48	0.42
RM3428	$(CT)_{18}$	attcatgcttcctttcagtg gattactggtttgccatttg	11	3	0.44	0.39
RM574	$(GA)_{11}$	ggcgaattctttgcacttgg acggtttggtagggtgtcac	5	4	0.39	0.38
RM277	(GA)11	cggtcaaatcatcacctgac caaggcttgcaagggaag	12	3	0.34	0.32

number, gene diversity (D_l), and polymorphism information content (PIC). Gene diversity, referred to as expected heterozygosity, is defined as the probability of two randomly chosen alleles from a different population. Where f is the inbreeding coefficient and estimated from the data using the method of moments. Polymorphism information content (PIC_l) is estimated following the formula proposed by Botstein *et al.* (1980):

$$D_l = (1 - \sum_{u=1}^{k} p_{lu}^2) / (1 - \frac{1+f}{n})$$

$$PIC_l = 1 - \sum_{u=1}^{k} p_{lu}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^{k} 2 p_{lu}^2 p_{lv}^2$$

Population structure analysis was carried out through a Bayesian-based model using Structure 2.3.4 software (12) for assayed 48 rice varieties using a burn-in of 100,000, run length of 100,000, and a model allowing for admixture and correlated allele frequencies. At least five runs of Structure were done by setting the number of subgroups (K) from 1 to 10, and an average likelihood value L (K), was calculated for each K across all runs. The model choice criterion to detect the most probable value of K was ΔK, an *ad hoc* quantity related to the second-order change of the log probability of data with respect to the number of clusters inferred by Structure (3, 24). Inferred ancestry estimates of individuals (Q-matrix) were derived for the selected subpopulation (12). The calculation of kinship coefficients (K-matrix), r^2, and D' values and also cluster analysis based on neighbor joining method and MLM-based association analysis (http://www.maizegenetics.net).

RESULTS

Phenotypic variation

The summary statistics for ten studied traits of the 48 rice varieties are shown in Table 3. There was a reasonable level of variation in the majority of the measured quantitative traits in this rice panel, indicating that genetic gain by means of selection is likely. For example, for grain length (GL), grain width (GW) and lemma length (LL), the maximum values were approximately two times larger than the minimum values in the studied varieties.

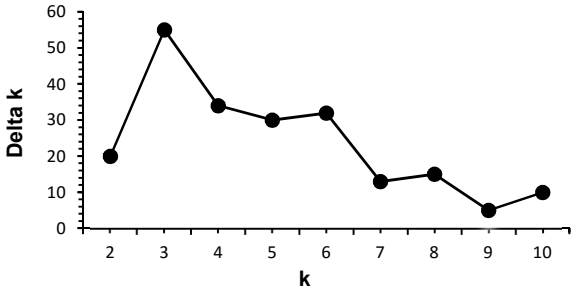

Figure 1. Bilateral charts to determine the optimal number of K identified by Structure program

These relatively large levels of phenotypic variability were measured for all traits, indicating that this collection of varieties were appropriate to be used in association studies of agronomic traits. Also the phenotypic value for each studied trait was normally distributed, indicating that the traits are controlled by quantitative genes (25).

Allelic variation and evaluation of population structure

A total of 18 SSR primer pairs selected from different chromosomes were used to evaluate the genetic diversity of the studied genotypes. These primers were generated a total of 63 bands ranging from 2 (RM252) to 5 (RM315), with an average of 3.5 bands per primer (Table 2). The average genetic diversity over all SSR loci was 0.44 that shows the probability of two alleles varies between two individuals (1), with a mean polymorphism information content (PIC) value of 0.397 (Table 2) As a prerequisite, population structure matrix (Q) varies between two individuals (1),

Table 3. Summary statistics for grain related assayed traits.

Traits	Min	Max	Range	Mean	SD
GL (mm)	4.90	8.48	3.58	6.88	0.81
GW (mm)	1.74	3.03	1.29	2.18	0.30
LL (mm)	7.07	13.81	6.74	9.03	0.96
LW (mm)	1.81	2.70	0.89	2.18	0.18
PL (mm)	6.73	11.54	4.81	8.78	0.97
PW (mm)	1.05	1.68	0.63	1.28	0.11
AL (mm)	0.00	5.71	5.71	2.74	0.42
HW (gr/cm^3)	3.51	4.70	1.19	4.12	0.23
PP (%)	0.70	3.30	2.6	1.86	0.74
PGF (%)	0.00	100	100	27.43	19.24

GL: Grain length, GW: Grain width, LL: Lemma length, LW: Lemma width, PL: Palea length, PW: Palea width, AL: Awn length, HW: Hectoliter weight, PP: Percentage of grain protein, PGF: Percentage of grain fissure

with a mean polymorphism information content (PIC) value of 0.397 (Table 2) As a prerequisite, population structure matrix (Q) should be considered to avoid false positive associations (21). The analysis of population genetic structure showed a significant population structure in these rice varieties. Since the distribution of L (K) did not show a distinct mode for the true number of K, to overcome difficulties in determining the real value of K, another measurement (ΔK) has been used (3). In our study, the collection of 48 assayed rice genotypes was partitioned into three subgroups (K=3) with the consideration of the highest value of ΔK (Figure 1). Cluster analysis based on neighbor-joining algorithm, subdivided all of the 48 genotypes into three major Subgroups (Figure 2). Therefore, we used the respective Q matrix output of the three subgroups runs for association analysis based on population structure.

LD values determination and Association analysis

In order to evaluate LD extent, the squared correlations (r^2) of allele frequency and D' indices were calculated by analysis of a total of 63 loci pairs on the same chromosome obtained from the 18 selected SSR markers using the program TASSEL 3.0. The r^2 and D' values for used primer pairs ranged from 0.001 to 0.331and 0.009 to 0.74, with the average value of 0.053 and 0.241, respectively. Meanwhile, for five primer pairs, r^2 was more than 0.1, possibly due to convergent selection pressure between distant markers (24). Marker trait associations (P< 0.05) for all measured traits

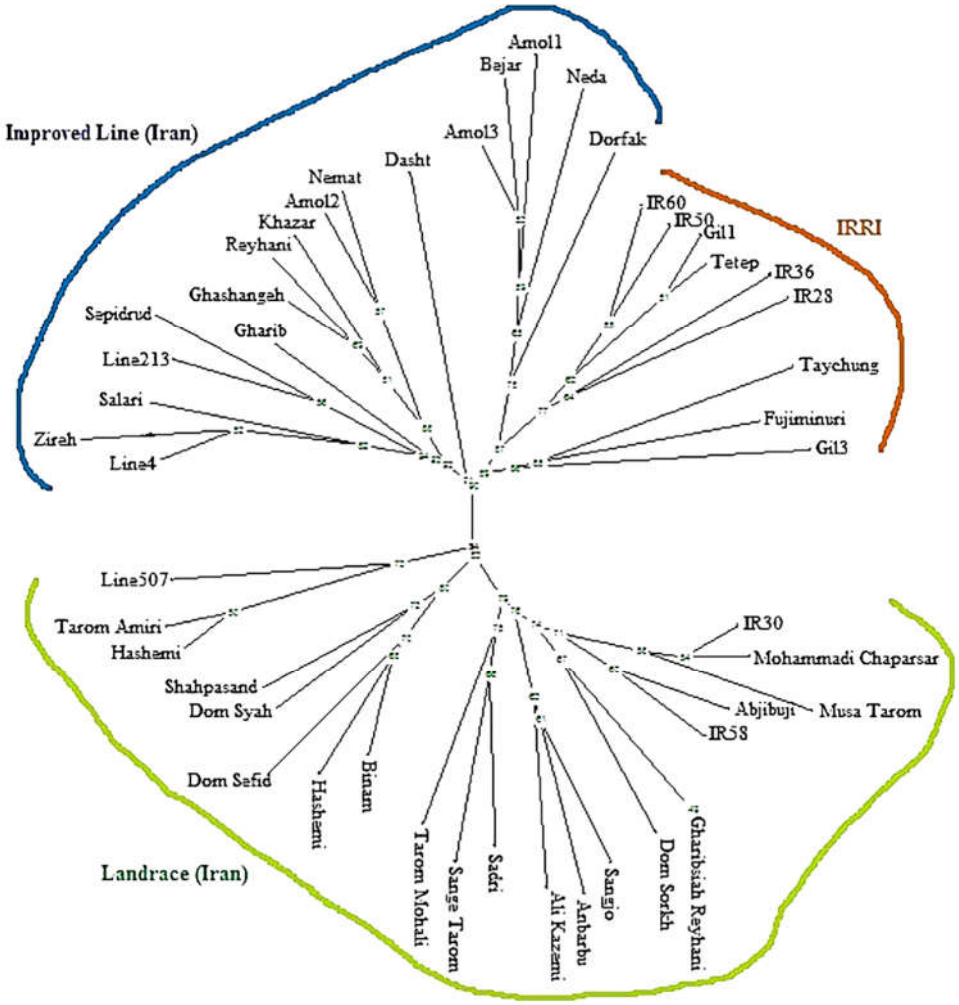

Figure 2. Classification of 48 rice varieties based on neighbor-joining algorithm.

Table 4. Associations between SSR markers and ten agronomic traits (as described in Table 2) with P< 0.05.

Trait	SSR marker [a]	Chromosome No.	P-value	R^{2b}
GW	RM289	5	0.001	0.69
GW	RM156	3	0.012	0.48
GW	RM252	4	0.023	0.36
GL	RM574	5	0.002	0.64
GL	RM190	6	0.018	0.39
GL	RM256	8	0.006	0.58
GL	RM593	5	0.032	0.43
LW	RM282	3	0.011	0.41
LW	RM288	9	0.024	0.33
LW	RM156	3	0.004	0.62
LL	RM6283	3	0.017	0.51
LL	RM289	5	0.005	0.63
LL	RM3428	11	0.002	0.72
LL	RM277	12	0.036	0.46
PW	RM190	6	0.041	0.32
PW	RM6283	3	0.001	0.59
PL	RM6283	3	0.008	0.63
PL	RM16	3	0.016	0.54
PL	RM289	5	0.003	0.67
AL	RM16	3	0.009	0.65
AL	RM315	1	0.001	0.74
AL	RM277	12	0.021	0.53
AL	RM593	5	0.035	0.32
AL	RM574	5	0.014	0.49
AL	RM112	2	0.043	0.42
AL	RM201	9	0.013	0.57
PP	RM315	1	0.001	0.71
PP	RM112	2	0.017	0.56
PP	RM289	5	0.007	0.67
PP	RM3428	11	0.025	0.49
PP	RM256	8	0.036	0.29
HW	RM593	5	0.019	0.57
HW	RM16	3	0.002	0.68
HW	RM156	3	0.012	0.46
PGF	RM6283	3	0.015	0.49
PGF	RM593	5	0.004	0.62
PGF	RM6080	3	0.019	0.54
PGF	RM574	5	0.028	0.47

[a] Only SSR markers with a significant marker-trait association are reported (P<0.05). r^{2b} indicates the percentage of total variation explained.

were evaluated in different chromosomes using the program TASSEL 3.0. By carrying out genome scanning, a total of 38 marker-trait associations related to studied traits were identified using 18 different SSR markers and explained more than 32% of the total variation. The 38 marker-trait associations for the 10 assayed traits in studied rice varieties ranged from two to seven associations, with the average value of 3.8 for each trait: Awn length (AL) was associated with seven markers, percentage of grain protein (PP) with five markers, grain length (GL), lemma length (LL) and percentage of grain fissure (PGF) with four markers, grain width (GW), lemma width (LW), palea length (PL) and hectoliter weight (HW) were associated with three markers and palea width (PW) with two markers. The highest coefficients of determination (R^2) were belong to RM315 (R^2=0.74, AL), RM3428 (R^2=0.72, LL), RM315 (R^2=0.71, PP), RM289 (R^2=0.69, GW), RM16 (R^2=0.68, HW), RM289 (R^2=0.67, PL), RM574 (R^2=0.64, GL) and RM156 (R^2=0.62, LW) markers, that showing strong association between these markers and mentioned traits. Also the results showed that some identified loci were associated with two or more traits simultaneously (Table 4). For instance, RM289 on chromosome 5 was associated with four traits (GW, LL, PL, and PP), while RM282, RM201, RM288, and RM6080 were only associated with one trait. Having knowledge of these loci could make a valuable contribution to rice breeding programs.

DISCUSSION

Since rice is completely sequenced, it is well suited to genome-wide association studies. Among the factors influencing accuracy of marker–traits association, the admixture of used population and its level of diversity are very important. Since the ΔK statistics showed a clear distribution of true K value (3), it was used in the present study to an accurate estimation and determination of true number of subgroups, K (Figure 1). The rice varieties were significantly divided into three subgroups based on results from population structure analysis. Association analysis with regard to simultaneous population structure (Q) and kinship (K) matrices, could eliminate biased results, considering the structured population and relatedness characteristics in rice germplasm (12, 21). Therefore to achieve results with high accuracy, a mixed linear model

(MLM) approach considering Q and K matrices (Q+K) was used to less possible spurious associations (24). Association analysis results showed a total of 38 associations between 18 different SSR markers and assayed traits (Table 4). Furthermore, the coefficient of determination (R^2) for majority of the identified associations was more than 32%, indicating that they could be major associations (4). The results of this study showed some of marker-trait associations that had been previously reported (Gramene web site) in associated with QTLs controlling some of assayed traits in foreign rice germplasm. Thus association analysis can be used as an efficient method for validation of identified QTLs in other population. Also the obtained results revealed that association analysis technique can be considerate as an alternative method for conventional linkage mapping. Recently, successful studies were implemented using association analysis. For example, association analysis between amino acid contents and 25 SSR markers were performed in 84 rice landrace accessions by Zhao *et al.* (2009). In another study, in order to identify genomic regions associated with 12 agronomic traits, association analysis was carried out using 274 SSR markers in 150 rice landraces (23). Also Kumar *et al.* (2015) evaluated 220 rice accessions using 6,000 SNPs through association analysis to identify loci related to salinity tolerance in rice. The results of these researches suggest that association analysis could be considerate as a cost and time effective and efficient approach in unveiling genomic regions/candidate genes related to different complex traits in rice. In this study, some of markers were significantly associated with several traits simultaneously (such as association between RM289 marker on chromosome 5 with GW, LL, PL, and PP traits), that may be due to multiple pleiotropic effects and could be used in marker-aided selection to improve breeding programs efficiency. Also, the linkage between RM289 and QTLs of grain shape that was mentioned in Gramene website and results from this study confirmed it, suggesting use of this marker (RM289) for MAS in different rice breeding programs. In addition, it looks that adjacent genomic region of RM289 marker is important in control of grain shape properties. Therefore, due to the availability of complete information of rice sequence, the putative genes controlling traits related to grain shape can be predicted by *in silico* analysis. In conclusion, the grain related traits are effective on the quality and quantity of rice yield, directly or indirectly. Therefore, the identification of genic regions controlling these traits is valuable to promotion of rice breeding programs. The findings of our study, demonstrated that association analysis of complex traits in rice with a relatively small number of markers is an efficient method that could provide basic information for further research such as MAS, fine mapping and candidate gene discovery in terms of grain properties and other important agronomic traits.

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تجزیه ارتباطی و تنوع ژنتیکی برخی از صفات مهم مرتبط با دانه در ژرم‌پلاسم برنج (*Oryza sativa* L.)

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چکیده

شناسایی مکان‌های ژنومی دخیل در کنترل صفات کمّی در اصلاح مولکولی گیاهان روز به روز در کانون توجه بیشتری قرار می‌گیرند. مطالعه حاضر به منظور ارزیابی تنوع ژنتیکی بین ۴۸ ژنوتیپ برنج و تعیین مناطق ژنومی مرتبط با ۱۰ صفت مهم مربوط به دانه انجام گرفت. در کل تعداد ۶۳ آلل با استفاده از ۱۸ نشانگر SSR انتخاب شده از کروموزوم‌های مختلف با میانگین ۳/۵ آلل برای هر نشانگر آشکارسازی شد. روش مبتنی بر مدل بیزین با در نظر گرفتن حداکثر مقدار ΔK، ۴۸ ژنوتیپ مورد ارزیابی را در سه زیر گروه اصلی تقسیم‌بندی کرد. میانگین مقدار r^2 برای کلیه جفت مکان‌های روی کروموزوم‌های مشابه، ۰/۰۵۳ بود. در کل ۳۸ ارتباط نشانگر-صفت معنی‌دار ($P< 0.05$) شناسایی شد که بیشتر از ۳۲ درصد کل تغییرات را توجیه نمودند. نشانگرهای RM315، RM3428، RM289، RM16، RM574 و RM156 به ترتیب دارای بیشترین مقدار R^2 و بنابراین مرتبط‌ترین نشانگرها با صفات مورد ارزیابی بودند. یافته‌های حاصل از این مطالعه ارتباط خصوصیات دانه در برنج را با برخی از نشانگرهای SSR آشکار نمود که می‌توانند به عنوان مکان‌های ژنومی هدف برای تحقیقات بیشتر مانند گزینش به کمک نشانگر، نقشه‌یابی دقیق و کشف ژن‌های کاندید در برنامه‌های اصلاح برنج بکار گرفته شوند.

کلمات کلیدی: تجزیه ارتباطی، عدم تعادل پیوستگی، برنج، SSR