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# Genetic diversity analysis of recombinant inbred lines of rice (*Oryza sativa* L.) using microsatellite markers

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## Abstract

Estimation of genetic diversity is an important factor in germplasm conservation and characterization. In rice breeding programs, genetic diversity information on specific regions of genome can be very useful for the application of marker assisted selection (MAS) and for gene mapping. A total of 152 rice lines were considered for breeding programs using microsatellites (SSR) technique. The total number of polymorphic alleles was 206 with an average of 2 alleles per SSR locus. The PIC value for the SSR loci ranged from 0.479 to 0.5 with an average of 0.498. The highest PIC value was observed for primers RM60, RM6832, RM3838 and RM592. RM1, RM237, RM154 and RM84 had the lowest PIC values in a decreasing order. Nei's gene diversity ranged from 0.479 to 0.5 with an average of 0.498. Using Shannon's diversity index, a mean genetic diversity of 0.691 was obtained. The lowest diversity was found for RM1, RM237, RM246, RM154 and RM279 in an ascending order and 27 SSR markers had the highest value (0.693). Cluster analysis using the UPGMA method based on Jaccard's similarity coefficient classified all lines into three clusters. Association analysis by general linear model (GLM) method revealed that 62 SSR markers showed a significant association with 10 studied morphological traits. Twenty eight markers were associated with more than one trait. These may be further investigated in rice breeding programs to be introduced as informative and useful markers. Results showed the potential of SSR markers to identify rice lines at the DNA level. The information will help the selection of lines to serve for efficient rice breeding programs.

***Key words:*** Association analysis, Cluster analysis, Genetic variation, Rice, SSR.

## INTRODUCTION

Rice has an important role as the main crop, supporting more than three billion people and consisting of 50% to 80% of their daily calorie intake (Khush, 2005). RILs can be used as powerful tools for genetic mapping. An RIL (recombinant inbred lines) is a new inbred line with a mosaic genome of the parental genomes formed by crossing two inbred lines followed by repeated sibling mating or selfing. Each RIL is an inbred line and can be multiplied permanently. The advantages of RILs for genetic mapping consists of: phenotyping multiple individuals from each line to decrease number of individuals, measurements and environmental variability; need to genotype each line only once; multiple invasive phenotypes can be created on the same set of genomes; and higher mapping resolution can be earned because in RILs the breakpoints are denser than those that happen in the meiosis (Broman, 2005).

Knowledge on the patterns of genetic variation is important for effective management of germplasms. It predicts potential genetic gains and helps in monitoring germplasm (Hassan *et al.,* 2012). In breeding programs, genetic diversity information of specific regions of rice genome can be very useful for the application of marker assisted selection (MAS) and for gene mapping

(Lapitan *et al.,* 2007). Genetic information among and within closely related varieties can be obtained using the genetic diversity information.

Simple sequence repeat, or microsatellites (SSR) markers, are very effective for a spectrum of breeding and genetic applications because of their co-dominant inheritance, reproducibility, good genome coverage and multi-allelic nature. SSR markers have been highly used as genetic markers and are plentifully dispersed within the genome (Powell and Machray, 1996; Silva *et al*., 2013). SSR markers have been used to recognize the genetic diversity of both cultivated and wild species in rice (Brondani *et al*., 2005; Jeung *et al*., 2005; Neeraja *et al*., 2005; Brondani *et al*., 2006; Rahman *et al*., 2010; Hassan *et al*., 2012; Kumar *et al*., 2012; Allahgholipour *et al*., 2014; Shahriar *et al*., 2014; Nachimuthu *et al*., 2015). These studies indicated that SSR markers are good and impressive in finding discriminating among genotypes and genetic polymorphisms. Rahman *et al*. (2010) evaluated the genetic diversity among 28 local rice varieties using 7 SSR markers. They reported that the effective number of alleles ranged from 4.083 to 11.042 with an average of 8.117, the PIC value ranged from 0.755 to 0.909 with an average of 0.864 and Nei's gene diversity ranged from 0.755 to 0.909 with an average of 0.864. The mean Shannon's diversity index was 2.206 and ranged from 1.53 to 2.511. The UPGMA dendrogram based on Nei's genetic distance placed the varieties into two major clusters. Hassan *et al*. (2012) evaluated the genetic diversity among 59 rice genotypes using 8 SSR markers. They reported that the allele number per/locus ranged from 9 to 27, with an average of 14.25. The mean PIC value was 0.857 and ranged from 0.767 to 0.857. The mean genetic diversity over all SSR loci was 0.870 and ranged from 0.792 to 0.948. Genetic distance between the variety pair ranged from 0.33 to 1.0. The UPGMA dendrogram based on Nei's genetic distance placed the varieties into three clusters. Kumar *et al*. (2012) evaluated the genetic diversity among 64 rice genotypes using 20 SSR markers. They reported that the PIC value for the SSR loci ranged from 0.36 to 0.98 with an average of 0.64. Cluster analysis using the UPGMA method based on Jaccard's similarity coefficient revealed that all genotypes were classified into eight major distinct clusters at the genetic similarity level of 0.40- 0.96. Allahgholipour *et al*. (2014) evaluated the genetic diversity among 94 rice genotypes using 52 SSR markers. They reported that the PIC value ranged from 0.423 to 0.892 with an average of 0.71 and gene diversity ranged from 0.48 to 0.89 with an average of 0.73. The mean Shannon's diversity index was 1.641 and ranged from 0.708 to 2.353. Cluster analysis using the complete linkage method based on Jaccard's similarity coefficient revealed that all genotypes were classified into nine clusters at the genetic similarity level of 0.01- 0.75.

Shahriar *et al*. (2014) evaluated the genetic diversity among 30 advanced rice breeding lines ($F_9$ generation) and along with 4 check varieties using three SSR markers. They reported that a total of 29 alleles were detected among the rice genotypes with an average of 9.67 alleles per locus. The PIC value ranged from 0.47 to 0.88 with an average of 0.71 and gene diversity ranged from 0.48 to 0.89 with an average of 0.73. Using UPGMA method 34 genotypes were grouped into four major clusters at the genetic similarity level of 0.3 -0.40. Nachimuthu *et al*. (2015) evaluated the genetic diversity in 192 rice lines using 61 SSR markers. They reported that the number of alleles per locus ranged from 2 to 7 with an average of 3 alleles per locus. The PIC value ranged from 0.146 to 0.756 with an average of 0.468. The expected heterozygosity or gene diversity (He) ranged from 0.16 to 0.75 with an average of 0.52.

Anandan *et al*. (2016) evaluated 96 rice lines using 39 polymorphic SSR markers that presented a total of 128 alleles. They showed that the PIC value ranged from 0.09 to 0.37 with an average of 0.24 and gene diversity ranged from 0.04 to 0.50 with an average of 0.30. Heterozygosity ranged from 0.04 to 0.97 with an average of 0.42. 16 and 10 SSRs had significant association with early seedling vigor trait in rice using general linear model (GLM) and mixed linear model (MLM) methods, respectively. Tabkhkar *et al*. (2018) evaluated the genetic diversity among 83 rice genotypes using 34 SSR markers. They showed that the PIC value ranged from 0.29 to 0.86 with an average of 0.71 and gene diversity ranged from 0.54 to 2.27 with an average of 1.60. The mean Shannon's diversity index was 0.74 which ranged from 0.35 to 0.86. Cluster analysis using the UPGMA method based on the simple matching coefficient revealed that all genotypes were classified into ten clusters. Association analysis using multiple regression analysis showed that 11 and 16 SSR markers had a significant correlation with the plant paddy weight under drought stress and non stress conditions, respectively. Their results showed that four markers (RM231, RM279, RM166, and RM231) showed a significant association with the plant paddy weight under both conditions.

The purpose of the present study was to evaluate the genetic diversity of rice lines using SSR markers.

## MATERIALS AND METHODS

### Plant material and extraction of DNA

The rice material used in genetic evaluations involved 152 families consisting of 150 recombinant inbred lines (RILs) with parents. An $F_8$ generation developed from a cross between two rice cultivars, Sepidroud (an improved rice variety from IRRI, a high yielding and dwarf plant that is resistant to lodging and blast, as the male parent) and Gharib (a native rice variety that is cultivated mainly in Guilan province, it is susceptible to lodging and has a low yield and was used as the female parent). A detailed description of primer sequences is available at http://www.gramene.org/microsat/ssr.txt.

A total of 103 polymorphic markers were used to detect the genotype of 152 plants. Parental surveys were used to recognize polymorphism between the parents using SSR markers. Fresh leaves were used for DNA extraction according to the modified protocol of Saghai-Maroof *et al*. (1984) and the quality of the extracted DNA was checked by electrophoresis on a 1% agarose gel.

The polymerase chain reaction (PCR) was carried out in a total volume of 10 µl per reaction containing 2 µl of template DNA (5 ng / µl), 1 µl 10×PCR buffer, 0.6 µl of forward and reverse primers (5 µM stock concentration), 0.6 µl dNTPs, (2 mM), 0.48 µl of MgCl2 (50 mM) 0.14 µl Taq polymerase (5 U/µl) and 4.58 µL of sterile nano-pure $H_2O$. The PCR amplification reaction was performed in a thermal cycler (Applied Biosystems, Germany) at an initial denaturation temperature of 94 °C for 5 min, then 35 cycles of 94 °C for 30 s, 55 °C for 30 s (primer annealing occurred with most of the primers while some were adjusted), 72 °C for 2 min and final extension at 72 °C for 5 min and then stored at 4 °C. The PCR products were separated by electrophoresis in 3% agarose in 0.5×tris-borate EDTA (TBE) buffer. The determined PCR bands were detected by the safe stain.

### Genetic diversity

Only clearly and reproducible amplified bands were scored for the structure of a data matrix. Amplification products of 152 lines were manually scored using the binary coding system, '0' for the absence of band and '1' for the presence of the band. The observed number of alleles (Na), effective number of alleles (Ne) (Kimura and Crow, 1964), Nei's gene diversity (Nei, 1973), Shannon diversity index (Lewontin, 1972) were calculated using the POPGENE software ver. 1.32 (Yeh *et al*., 1997). The polymorphism information content (PIC) was calculated for each marker using equation 1, according to the method of Anderson *et al*. (1993).

$$(1) \qquad PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Where, $P_{ij}$ is the frequency of the jth allele for the ith marker, and is summed over n alleles. PIC values can range from 0 to 1. At a PIC of 1, the marker would have a limitless number of alleles. At a PIC of 0, the marker has only one allele. The PIC value explains diversity within accessions (intra-populational diversity) and determines the degree of polymorphism in each locus, a PIC value of less than 0.25 showing low polymorphism, a value between 0.25 and 0.5 average polymorphism and a value higher than 0.5 shows a very polymorphic locus (Botstein *et al*., 1980). Nei's amount of the average gene diversity per locus $H_S$ (Nei, 1973) is defined using equation 2:

$$(2) \qquad Hs = \frac{1}{k}\sum_{s=1}^{k} H_{Ss} = \frac{1}{k}\sum_{s=1}^{k}\left[1 - q_s^2 - \left(1 - q_s\right)^2\right]$$

Where $q_s$ is the frequency of appearance one of the two alleles at the sth diallelic locus and k is the total number of loci (differentiating factors), $H_{Ss} = 1 - q_s^2 - (1 - q_s)^2$.

Pearson correlation coefficients were used to evaluate the relationships between the genetic diversity parameters and the number of alleles, using SPSS 19. The Jaccard's similarity coefficient was used for cluster analysis of the rice lines using the UPGMA (the Unweighed Pair-Group Method with Arithmetic Mean) method to conclude phylogeny and genetic relationships using SPSS 19. Using the NTSYS-pc ver 2.1 (Rohlf, 2002), the highest value of similarity coefficient was calculated. To determine if the percentage of original cases are classified correctly for cluster analysis outputs and predicted group membership, Fisher's linear discriminant analysis (Fisher, 1936) using SPSS 19 was applied. Stepwise regression was used to identify the informative SSR markers using SPSS 19.

## RESULTS

### Genetic diversity

Estimate of genetic diversity is an important factor in germplasm conservation and characterization. In this research, 103 polymorphic SSR markers well divided on 12 chromosomes were used to distinguish the genetic diversity among 152 rice lines. All studied lines were pure and indicated one band for all studied markers. The observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity (Nei, 1973), Shannon

diversity index (I) for each SSR locus and PIC values are shown in Table 1. The observed number of alleles (Na) in all rice lines was two. The effective number of alleles (Ne) ranged from 1.921 to 2, with an average of 1.992 (Table 1).

Using Nei's gene diversity, a mean genetic diversity of 0.498 was obtained from the analysis, the lowest diversity was found in RM1 (0.479) and some SSR markers such as, RM237 (0.488), RM246 (0.488), RM154 (0.488), RM279 (0.489) and RM84 (0.490) were located in subsequent ranking. Sixteen SSR markers had the highest value (0.5) including RM3340, RM60, RM3838, RM234, RM248, RM420, RM445, RM278, RM3428, RM5474, RM224, RM511, RM463 and RM2197.

Using Shannon's diversity index, overall genetic diversity of 0.691 was obtained, indicating a level of genetic variation among these lines. The lowest diversity was found in RM1 (0.6724) and some SSR markers such as, RM237 (0.681), RM246 (0.686), RM154 (0.681) and RM279 (0.682) were located in subsequent ranking. Twenty seven SSR markers had the highest value (0.693).

**Table 1.** SSR markers used in this study, observed and effective number of alleles and polymorphic information content for all primers.

| Marker | Chromosome number | Repeat motif | Observed number of alleles | Effective number of alleles | Nei's genetic diversity | Shannon's information index | PIC |
|---|---|---|---|---|---|---|---|
| RM5423 | 1 | (TC)16 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM84 | 1 | (TCT)10 | 2 | 1.960 | 0.490 | 0.683 | 0.370 |
| RM1 | 1 | (GA)26 | 2 | 1.921 | 0.479 | 0.672 | 0.364 |
| RM1287 | 1 | (AG)17 | 2 | 1.964 | 0.491 | 0.684 | 0.370 |
| RM237 | 1 | (CT)18 | 2 | 1.954 | 0.488 | 0.681 | 0.369 |
| RM246 | 1 | (CT)20 | 2 | 1.954 | 0.488 | 0.681 | 0.372 |
| RM1268 | 1 | (AG)16 | 2 | 1.974 | 0.494 | 0.687 | 0.372 |
| RM5800 | 1 | (AGG)8 | 2 | 1.987 | 0.497 | 0.690 | 0.373 |
| RM3340 | 2 | (CT)15 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM154 | 2 | (GA)21 | 2 | 1.955 | 0.488 | 0.682 | 0.369 |
| RM279 | 2 | (GA)16 | 2 | 1.958 | 0.489 | 0.682 | 0.375 |
| RM6911 | 2 | (TTA)15 | 2 | 1.985 | 0.496 | 0.689 | 0.373 |
| RM424 | 2 | (CAT)9 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM7624 | 2 | (TTAA)6 | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM3355 | 2 | (CT)15 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM599 | 2 | (GCG)8 | 2 | 1.993 | 0.498 | 0.691 | 0.374 |
| RM60 | 3 | (AATT)5AATCT(AATT) | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM489 | 3 | (ATA)8 | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM282 | 3 | (GA)15 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM3867 | 3 | (GA)30 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM6832 | 3 | (TCT)8 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM5626 | 3 | (AAG)11 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM135 | 3 | (CGG)10 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM416 | 3 | (GA)9 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM7389 | 3 | (GATA)7 | 2 | 1.987 | 0.497 | 0.690 | 0.373 |
| RM8213 | 4 | (TC)10 | 2 | 1.978 | 0.494 | 0.687 | 0.372 |
| RM1359 | 4 | (AG)25 | 2 | 1.969 | 0.492 | 0.685 | 0.371 |
| RM273 | 4 | (GA)11 | 2 | 1.993 | 0.498 | 0.691 | 0.374 |
| RM252 | 4 | (CT)19 | 2 | 1.989 | 0.497 | 0.691 | 0.374 |
| RM317 | 4 | (GC)4(GT)18 | 2 | 1.972 | 0.493 | 0.686 | 0.371 |
| RM255 | 4 | (AGG)5(AG)2-(GA)16 | 2 | 1.989 | 0.497 | 0.690 | 0.374 |
| RM6320 | 5 | (CTT)13 | 2 | 1.994 | 0.499 | 0.692 | 0.374 |
| RM13 | 5 | (GA)6-(GA)16 | 2 | 1.995 | 0.499 | 0.692 | 0.374 |
| RM3838 | 5 | (GA)22 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM440 | 5 | (CTT)22 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM459 | 5 | (CATC)6 | 2 | 1.998 | 0.500 | 0.693 | 0.375 |
| RM305 | 5 | (GT)4+degener | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM3800 | 5 | (GA)19 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM421 | 5 | (AGAT)6 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |

**Table 1 (Continue).** SSR markers used in this study, observed and effective number of alleles and polymorphic information content for all primers.

| Marker | Chromosome number | Repeat motif | Observed number of alleles | Effective number of alleles | Nei's genetic diversity | Shannon's information index | PIC |
|---|---|---|---|---|---|---|---|
| RM480 | 5 | (AC)30 | 2 | 1.991 | 0.498 | 0.691 | 0.374 |
| RM592 | 5 | (ATT)20 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM314 | 6 | (GT)8(CG)3(GT)5 | 2 | 1.974 | 0.494 | 0.687 | 0.372 |
| RM217 | 6 | (CT)20 | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM276 | 6 | (AG)8A3(GA)33 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM402 | 6 | (ATA)7 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM549 | 6 | (CCG)9 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM6836 | 6 | (TCT)14 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM3827 | 6 | (GA)21 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM7434 | 6 | (GTAT)10 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM7579 | 6 | (TCTG)6 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM5371 | 6 | (TC)13 | 2 | 1.991 | 0.498 | 0.691 | 0.374 |
| RM30 | 6 | (AG)9A(GA)12 | 2 | 1.991 | 0.498 | 0.691 | 0.374 |
| RM5211 | 7 | (TA)40 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM5711 | 7 | (AAT)24 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM1243 | 7 | (AG)15 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM8263 | 7 | (TC)13 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM5481 | 7 | (TC)21 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM70 | 7 | (ATT)33 | 2 | 1.989 | 0.497 | 0.691 | 0.374 |
| RM234 | 7 | (CT)25 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM248 | 7 | (CT)25 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM3555 | 7 | (GA)12 | 2 | 1.994 | 0.499 | 0.692 | 0.374 |
| RM420 | 7 | (AAAT)7 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM445 | 7 | (AG)12 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM1235 | 8 | (AG)15 | 2 | 1.998 | 0.500 | 0.693 | 0.375 |
| RM3572 | 8 | (GA)12 | 2 | 1.991 | 0.498 | 0.691 | 0.373 |
| RM42 | 8 | (AG)6-(AG)2T(GA)5 | 2 | 1.985 | 0.496 | 0.689 | 0.373 |
| RM331 | 8 | [(CTT)4GTT]2(CTT)11 | 2 | 1.982 | 0.496 | 0.689 | 0.373 |
| RM404 | 8 | (GA)33 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM6845 | 8 | (TGA)8 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM5485 | 8 | (TC)22 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM219 | 9 | (CT)17 | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM7038 | 9 | (AACA)7 | 2 | 1.994 | 0.499 | 0.692 | 0.374 |
| RM566 | 9 | (AG)15 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM6839 | 9 | (TCT)17 | 2 | 1.996 | 0.499 | 0.692 | 0.374 |
| RM434 | 9 | (TC)12 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM257 | 9 | (CT)24 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM288 | 9 | (GA)7G6(GA)7 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM278 | 9 | (GA)17 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM160 | 9 | (GAA)23 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM201 | 9 | (CT)17 | 2 | 1.982 | 0.496 | 0.689 | 0.373 |
| RM215 | 9 | (CT)16 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM8201 | 10 | (CT)13 | 2 | 1.993 | 0.498 | 0.691 | 0.374 |
| RM5620 | 10 | (AAG)10 | 2 | 1.998 | 0.500 | 0.693 | 0.375 |
| RM311 | 10 | (GT)3(GTAT)8(GT)5 | 2 | 1.994 | 0.499 | 0.692 | 0.374 |
| RM2863 | 10 | (AT)37 | 2 | 1.998 | 0.500 | 0.693 | 0.375 |
| RM333 | 10 | (TAT)19(CTT)19 | 2 | 1.985 | 0.496 | 0.689 | 0.373 |
| RM1812 | 11 | (AT)16 | 2 | 1.987 | 0.497 | 0.690 | 0.373 |
| RM202 | 11 | (CT)30 | 2 | 1.978 | 0.494 | 0.687 | 0.372 |
| RM3428 | 11 | (CT)18 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM5824 | 11 | (ATA)12 | 2 | 1.991 | 0.498 | 0.691 | 0.374 |
| RM286 | 11 | (GA)16 | 2 | 1.981 | 0.495 | 0.688 | 0.372 |
| RM5474 | 11 | (TC)21 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM224 | 11 | (AAG)8(AG)13 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |

**Table 1 (Continue).** SSR markers used in this study, observed and effective number of alleles and polymorphic information content for all primers.

| Marker | Chromosome number | Repeat motif | Observed number of alleles | Effective number of alleles | Nei's genetic diversity | Shannon's information index | PIC |
|---|---|---|---|---|---|---|---|
| RM1337 | 12 | (AG)21 | 2 | 1.997 | 0.499 | 0.692 | 0.375 |
| RM519 | 12 | (AAG)8 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM3331 | 12 | (CT)15 | 2 | 1.987 | 0.497 | 0.690 | 0.373 |
| RM511 | 12 | (GAC)7 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM28166 | 12 | (CT)12 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM463 | 12 | (TTAT)5 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM206 | 12 | (CT)21 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM2935 | 12 | (AT)39 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| RM2197 | 12 | (AT)23 | 2 | 2.000 | 0.500 | 0.693 | 0.375 |
| RM212 | 12 | (CT)24 | 2 | 1.999 | 0.500 | 0.693 | 0.375 |
| MEAN | | | 2 | 1.992 | 0.498 | 0.691 | 0.374 |
| St. Dev | | | 2 | 0.013 | 0.003 | 0.003 | 0.002 |

**Table 2.** Correlations (Pearson's coefficient) between the genetic diversity parameters and the number of alleles on data derived from the analysis of 103 SSRs on the 152 lines.

| Genetic diversity parameters | Effective number of alleles | Nei's genetic diversity | Shannon's information index | PIC |
|---|---|---|---|---|
| Effective number of alleles | 1 | 1** | 1** | 0.941** |
| Nei's genetic diversity | | 1 | 1** | 0.941** |
| Shannon's information index | | | 1 | 0.942** |
| PIC | | | | 1 |

**: Correlation is significant at the 0.01 probability level.

The PIC values indicate allele frequency and diversity among the lines, also changed from one locus to another. The higher PIC values may be the effect of diverse lines and the lower PIC value may be the effect of closely related lines. The PIC value for the SSR loci ranged from 0.479 to 0.5 with an average of 0.498 that showed average polymorphism (Table 1). The highest PIC value (0.5) was observed for primers RM60, RM6832 (on chromosome 3), RM3838 and RM 592 (on chromosome 5). RM1 (on chromosome 1) had the lowest PIC value (0.479) and some SSR markers such as, RM237 (0.488), RM154 (0.488) and RM84 (0.489) had low PIC values and were located in the next ranking. Positive and significant correlations were found between the effective number of alleles (Ne), Nei's gene diversity, Shannon diversity index and PIC (Table 2).

**Cluster analysis**

Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped 152 lines into three main clusters at the similarity coefficient of 0.54 (the highest value rather than other similarity coefficients) and included 58, 47 and 47 lines, respectively (Figure 1). Results of the discriminant analysis showed that the three cluster groups were confirmed at high levels of the correct percent (100%) (data not shown). Each cluster differentiates the lines from the others. Sepidroud and Gharib (parental lines) were clustered in a separate cluster as they have far genetic backgrounds from each other. Sepidroud along with 57 $F_8$ pure lines (1, 2, 3, 5, 9, 15, 16, 19, 29, 35, 39, 44, 45, 47, 48, 57, 62, 72, 73, 77, 78, 79, 80, 82, 89, 95, 98, 100, 108, 110, 114, 116, 118, 122, 124, 125, 130, 131, 134, 135, 137, 138, 139, 140, 141, 146, 152, 153, 155, 159, 160, 166, 175, 177, 178, 179, and 184) were located in one cluster and Gharib along with 46 $F_8$ pure lines (7, 17, 21, 22, 23, 24, 26, 32, 33, 34, 38, 40, 42, 49, 52, 54, 55, 56, 59, 60, 61, 63, 65, 69, 75, 76, 84, 85, 87, 90, 105, 106, 121, 123, 133, 143, 144, 145, 147, 151, 157, 162, 170, 172, 173, and 176) were located in another cluster. Fourty seven $F_8$ pure lines (6, 8, 10, 12, 14, 20, 25, 27, 28, 37, 41, 46, 50, 53, 64, 67, 68, 70, 74, 81, 88, 91, 92, 93, 94, 96, 99, 102, 103, 104, 107, 111, 117, 119, 120, 126, 127, 128, 132, 142, 148, 156, 163, 167, 181, 185, and 186) were located in other separate cluster.

**Association analysis**

Stepwise regression was performed to estimate significant regressions for some morphological traits

(as the dependent variables) on 103 SSR marker alleles (as the independent variables) (Table 3). The regression analysis showed significant regressions for days to 50% flowering (DF) on 11 SSR marker alleles. These associated markers, namely RM154, RM237, RM3838, RM8201, RM1287, RM463, RM279, RM3331, RM6832, RM6839, and RM6845 accounted for 82.9% of the total variation in DF. For days to 85% maturity (DM), associated alleles on 10 SSR markers were found including RM154, RM3838, RM8201, RM311, RM279, RM1287, RM202, RM6839, RM1359, and RM3428 accounted for 84.9% of the total variation in DM. For tiller number per plant (NTP), associated alleles on 5 SSR markers were found including RM273, RM421, RM160, RM549, and RM3800 accounted for 25.4% of the total variation in NTP. For panicle number per plant (NPP), associated alleles on 9 SSR markers were found including RM273, RM421, RM549, RM5371, RM511, RM489, RM3355, RM305, and RM566 accounted for 73.4% of the total variation in NPP.

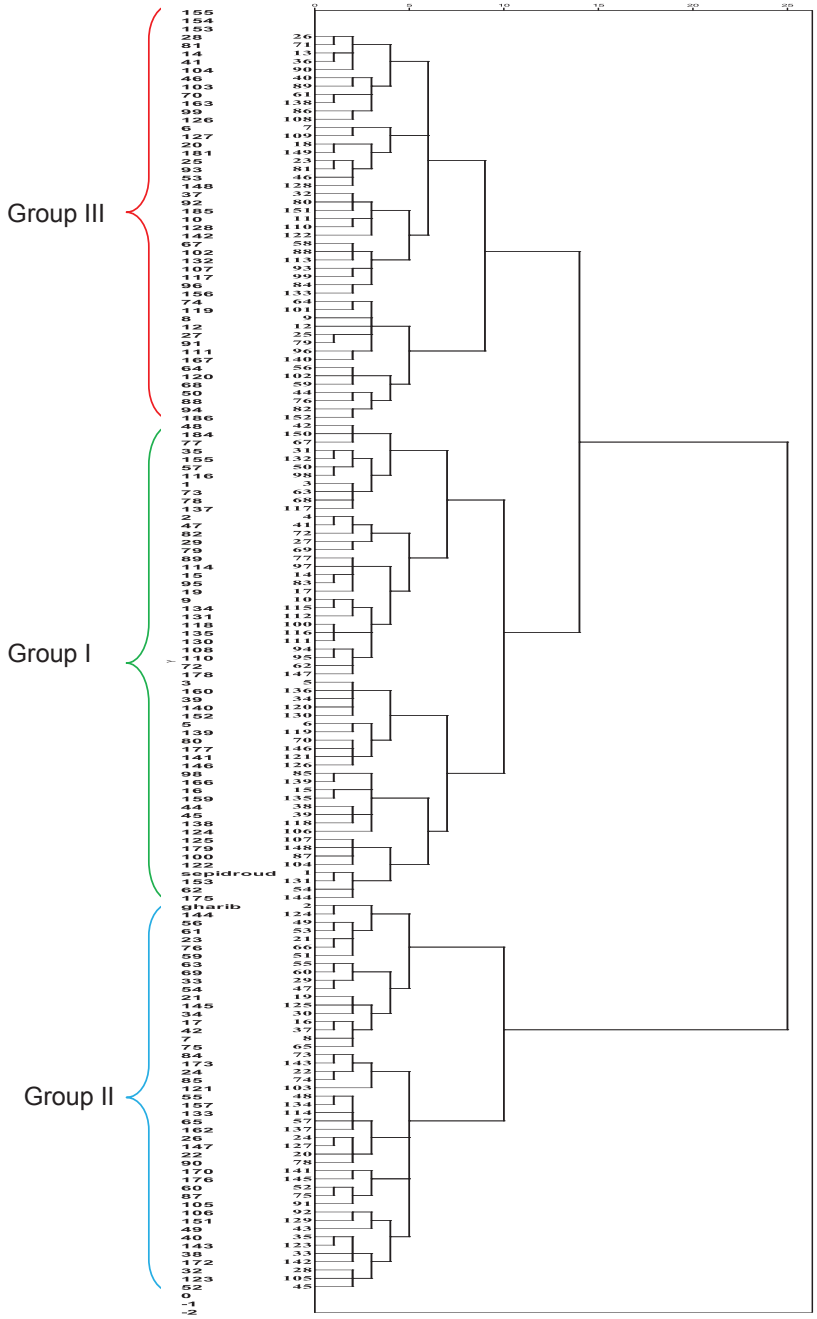


**Figure 1.** Dendrogram of cluster analysis from the Jaccard's similarity coefficient and UPGMA method to group 152 rice lines based on 103 SSR markers data (3 distinct groups).

**Table 3.** Informative SSR markers related to some morphological traits identified by stepwise regression analysis.

| Trait | Marker | Beta | $R^2$ | Sig |
|---|---|---|---|---|
| Days to 50% flowering | RM154 | -0.442** | 0.072 | 0.000 |
| | RM237 | -0.119* | 0.052 | 0.050 |
| | RM3838 | -0.200** | 0.099 | 0.000 |
| | RM8201 | -0.119* | 0.034 | 0.032 |
| | RM1287 | -0.190** | 0.057 | 0.001 |
| | RM463 | 0.117* | 0.075 | 0.015 |
| | RM279 | -0.127** | 0.090 | 0.010 |
| | RM3331 | -0.118* | 0.100 | 0.013 |
| | RM6832 | 0.110* | 0.108 | 0.017 |
| | RM6839 | -0.114* | 0.117 | 0.032 |
| | RM6845 | -0.092* | 0.025 | 0.050 |
| Days to 85% maturity | RM154 | -0.389** | 0.074 | 0.000 |
| | RM3838 | -0.202** | 0.055 | 0.000 |
| | RM8201 | -0.159** | 0.101 | 0.004 |
| | RM311 | -0.134** | 0.141 | 0.005 |
| | RM279 | -0.112* | 0.064 | 0.020 |
| | RM1287 | -0.172** | 0.083 | 0.001 |
| | RM202 | -0.091* | 0.098 | 0.050 |
| | RM6839 | -0.128* | 0.107 | 0.014 |
| | RM1359 | -0.122* | 0.115 | 0.016 |
| | RM3428 | -0.093* | 0.011 | 0.046 |
| Tiller number per plant | RM273 | -0.360** | 0.022 | 0.000 |
| | RM421 | -0.274** | 0.020 | 0.000 |
| | RM160 | -0.225** | 0.054 | 0.000 |
| | RM549 | -0.157** | 0.072 | 0.007 |
| | RM3800 | -0.119* | 0.086 | 0.030 |
| Panicle number per plant | RM273 | -0.296** | 0.094 | 0.000 |
| | RM421 | -0.305** | 0.091 | 0.000 |
| | RM549 | -0.185** | 0.117 | 0.000 |
| | RM5371 | -0.255** | 0.131 | 0.001 |
| | RM511 | -0.117* | 0.043 | 0.019 |
| | RM489 | 0.106* | 0.051 | 0.028 |
| | RM3355 | 0.135** | 0.060 | 0.006 |
| | RM305 | -0.120* | 0.069 | 0.015 |
| | RM566 | 0.106* | 0.078 | 0.026 |
| Plant height | RM3555 | 0.194** | 0.060 | 0.002 |
| | RM276 | 0.139* | 0.071 | 0.018 |
| | RM217 | 0.136* | 0.098 | 0.028 |
| | RM13 | 0.094* | 0.023 | 0.050 |
| | RM445 | 0.182** | 0.040 | 0.000 |
| | RM5211 | 0.100* | 0.058 | 0.022 |
| | RM248 | 0.166** | 0.069 | 0.006 |
| | RM519 | -0.104** | 0.078 | 0.006 |
| | RM8213 | 0.130** | 0.085 | 0.003 |
| | RM6832 | -0.098** | 0.092 | 0.009 |
| | RM7624 | 0.133** | 0.079 | 0.003 |
| | RM237 | 0.097* | 0.006 | 0.012 |
| | RM6836 | -0.078* | 0.012 | 0.036 |
| | RM440 | -0.089* | 0.017 | 0.022 |
| | RM70 | 0.095* | 0.023 | 0.016 |
| | RM434 | -0.081* | 0.028 | 0.047 |

**Table 3 (Continue).** Informative SSR markers related to some morphological traits identified by stepwise regression analysis.

| Trait | Marker | Beta | $R^2$ | Sig |
|---|---|---|---|---|
| Panicle length | RM234 | -0.341** | 0.016 | 0.000 |
| | RM434 | -0.257** | 0.023 | 0.000 |
| | RM286 | 0.151** | 0.059 | 0.009 |
| | RM6320 | -0.180** | 0.084 | 0.000 |
| | RM2863 | -0.171** | 0.107 | 0.001 |
| | RM1235 | -0.134** | 0.128 | 0.008 |
| | RM257 | -0.165** | 0.145 | 0.006 |
| | RM276 | 0.116* | 0.154 | 0.049 |
| Number of total spikelet per panicle | RM1268 | -0.265** | 0.043 | 0.000 |
| | RM5800 | -0.226** | 0.046 | 0.000 |
| | RM311 | -0.202** | 0.087 | 0.000 |
| | RM219 | -0.245** | 0.055 | 0.000 |
| | RM234 | -0.177** | 0.079 | 0.000 |
| | RM7389 | -0.091* | 0.093 | 0.050 |
| | RM489 | 0.121* | 0.104 | 0.017 |
| | RM549 | -0.122** | 0.115 | 0.008 |
| | RM5481 | 0.110* | 0.026 | 0.015 |
| | RM1 | 0.123* | 0.037 | 0.017 |
| Spikelet fertility | RM463 | -0.288** | 0.056 | 0.000 |
| | RM70 | -0.181** | 0.075 | 0.002 |
| | RM219 | -0.187* | 0.044 | 0.021 |
| | RM305 | 0.230** | 0.195 | 0.000 |
| | RM5800 | 0.232** | 0.031 | 0.000 |
| | RM489 | -0.220** | 0.055 | 0.000 |
| | RM252 | -0.257** | 0.07 | 0.003 |
| | RM257 | 0.166** | 0.083 | 0.003 |
| | RM424 | -0.121* | 0.099 | 0.031 |
| | RM248 | 0.136* | 0.015 | 0.011 |
| | RM3331 | 0.121* | 0.026 | 0.023 |
| | RM135 | 0.112* | 0.037 | 0.038 |
| Thousand grain weight | RM311 | 0.335** | 0.079 | 0.000 |
| | RM282 | -0.134* | 0.046 | 0.027 |
| | RM489 | -0.168** | 0.084 | 0.001 |
| | RM70 | -0.196** | 0.116 | 0.000 |
| | RM1268 | 0.174** | 0.042 | 0.001 |
| | RM1 | -0.174** | 0.066 | 0.003 |
| | RM6839 | 0.134** | 0.082 | 0.005 |
| | RM30 | 0.118* | 0.095 | 0.011 |
| | RM402 | -0.129** | 0.108 | 0.006 |
| | RM3572 | 0.107* | 0.016 | 0.044 |
| Grain yield | RM549 | -0.370** | 0.040 | 0.000 |
| | RM5824 | -0.250* | 0.097 | 0.004 |
| | RM5711 | -0.143* | 0.024 | 0.032 |
| | RM234 | -0.184** | 0.049 | 0.001 |
| | RM3800 | -0.144* | 0.068 | 0.008 |
| | RM1268 | -0.140* | 0.082 | 0.011 |
| | RM42 | 0.134* | 0.096 | 0.018 |
| | RM421 | -0.119* | 0.108 | 0.029 |
| | RM3428 | 0.110* | 0.120 | 0.035 |

For plant height (PH), associated alleles on 16 SSR markers were found including RM3555, RM276, RM217, RM13, RM445, RM5211, RM248, RM519, RM8213, RM6832, RM7624, RM237, RM6836, RM440, RM70, and RM434 accounted for 83.9% of the total variation in PH. For panicle length (PL), associated alleles on 8 SSR markers were found including RM234, RM434, RM286, RM6320, RM2863, RM1235, RM257, and RM276 accounted for 71.6% of the total variation in PL. For number of total spikelet per panicle (NTSP), associated alleles on 10 SSR markers were found including RM1268, RM5800, RM311, RM219, RM234, RM7389, RM489, RM549, RM5481, and RM1 accounted for 68.5% of the total variation in NTSP. For spikelet fertility (SF), associated alleles on 12 SSR markers were found including RM463, RM70, RM219, RM305, RM5800, RM489, RM252, RM257, RM424, RM248, RM3331, and RM135 accounted for 78.6% of the total variation in SF. For thousand grain weight (GW), associated alleles on 10 SSR markers were found including RM311, RM282, RM489, RM70, RM1268, RM1, RM6839, RM30, RM402, and RM3572 accounted for 73.4% of the total variation in GW. For grain yield (GY), associated alleles on 9 SSR markers were found including RM549, RM5824, RM5711, RM234, RM3800, RM1268, RM42, RM421, and RM3428 accounted for 68.4% of the total variation in GY.

## DISCUSSION

The result of the present research could be beneficial in genetic variation, Marker Assisted Selection (MAS), genome mapping studies and could be applied as the base line information for further research. To identify the polymorphism rate of a marker at a specific locus and genetic studies, markers with PIC values of 0.5 or greater are highly informative (DeWoody *et al*., 1995). The SSR analysis of rice lines in this study showed sufficient polymorphism to fully differentiate intra-population diversity. In the current study, considering the fact that the population containing pure lines in the $F_8$ generation, the mean PIC value observed was 0.498 with a range of 0.479 to 0.5. This result was consistent with the findings of Nachimuthu *et al*. (2015) (the average PIC value of 0.468 with a range of 0.146 to 0.756) while the mean PIC value was lower than the mean PIC value recorded by Rahman *et al*. (2010) (which ranged from 0.755 to 0.909 with an average of 0.864), Hassan *et al*. (2012) (ranged from 0.767 to 0.857 with an average of 0.857), Kumar *et al*. (2012) (ranged from 0.36 to 0.98 with an average of 0.64), Allahgholipour *et al*. (2014) (ranged from 0.423 to 0.892 with an average of 0.71), Shahriar *et al*. (2014) (ranged from 0.47 to 0.88 with an average of 0.71), and Tabkhkar *et al*. (2018) (ranged from 0.29 to 0.86 with an average of 0.71). The average PIC value was also higher than those reported by Anandan *et al*. (2016) (ranged from 0.09 to 0.37 with an average of 0.24).

The highest PIC values were recorded for RM60, RM6832 (on chromosome 3) and RM3838 and RM 592 (on chromosome 5). These markers are more suitable for diversity analysis in studied rice lines. In the current study, Nei's genetic diversity ranged from 0.479 to 0.5 with an average of 0.498 that was approximately similar to mean genetic diversity reported by Nachimuthu *et al*. (2015) and Anandan *et al*. (2016) (ranged from 0.04 to 0.50 with an average of 0.30) while it was lower than results were reported previously by Rahman *et al*. (2010), Hassan *et al*. (2012), Allahgholipour *et al*. (2014), Shahriar *et al*. (2014), and Tabkhkar *et al*. (2018). Shannon's diversity index ranged from 0.672 to 0.693 with an average of 0.691 that was approximately similar to Shannon's diversity index reported by Tabkhkar *et al*. (2018) (ranged from 0.35 to 0.86 with an average of 0.74) and lower than results were reported by Rahman *et al*. (2010) (ranged from 1.53 to 2.511 with an average of 2.206) and Allahgholipour *et al*. (2014) (ranged from 0.708 to 2.353 with an average of 1.641).

In the current study, cluster analysis using the UPGMA method based on Jaccard's similarity coefficient revealed that all of the 152 lines were classified into three clusters. Rahman *et al*. (2010) reported that using the UPGMA dendrogram based on Nei's genetic distance 28 varieties using 7 SSR markers were placed into two major clusters. Hassan *et al*. (2012) reported that using the UPGMA dendrogram based on Nei's genetic distance using 8 SSR markers, 59 rice genotypes were placed into three clusters. Kumar *et al*. (2012) reported that using the UPGMA method based on Jaccard's similarity coefficient 64 rice genotypes were classified into eight major distinct clusters using 20 SSR markers. Allahgholipour *et al*. (2014) reported that by cluster analysis using the complete linkage method based on Jaccard's similarity coefficient 94 rice genotypes were classified into nine clusters using 52 SSR markers. Shahriar *et al*. (2014) reported that based on the UPGMA method 34 genotypes were grouped into four major clusters using 3 SSR markers. Tabkhkar *et al*. (2018) have reported that cluster analysis using the UPGMA method based on the simple matching coefficient revealed that 83 rice genotypes using 34 SSR markers were classified to ten clusters.

In the current study, association analysis 62 SSR markers showed significant associations with 10 morphological traits by general linear model (GLM) method. These informative SSR markers can be useful for improving studied morphological traits in the breeding programs using marker-based methods. Anandan *et al*. (2016) reported that 16 and 10 SSR markers significantly associated with early seedling vigor trait in rice using general linear model (GLM) and mixed linear model (MLM) methods, respectively. Tabkhkar *et al*. (2018) reported that 11 and 16 SSR markers were significantly associated with the plant paddy weight using multiple regression analysis under drought stress and non stress conditions, respectively. Their results showed that four markers (RM231, RM279, RM166, and RM231) showed a significant association with the plant paddy weight under both conditions.

In our study, a total of 28 markers (RM1, RM1287, RM154, RM1268, RM219, RM234, RM237, RM248, RM257, RM273, RM276, RM279, RM305, RM311, RM3331, RM3428, RM3800, RM3838, RM421, RM434, RM463, RM489, RM549, RM5800, RM6832, RM6839, RM70, and RM8201) were associated with more than one morphological trait. Eight markers were associated with more than two morphological traits. RM549 was associated with NTP, NPP, NTSP, and GY. RM489 was associated with NPP, NTSP, SF, and GW. RM1268 was associated with NTSP, GW, and GY. RM234 was associated with PL, NTSP, and GY. RM70 was associated with PH, SF, and GW. RM6839 was associated with DF, DM, and GW. RM311 was associated with DM, NTSP, and GW. RM421 was associated with NTP, NPP, and GY. These markers may be further investigated in rice breeding programs to be introduced as informative and useful markers.

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