GENETIC DIVERSITY STUDY OF INDIGENOUS RICE ACCESSIONS FROM NORTHERN MOUNTAINOUS AREAS OF PAKISTAN USING MICROSATellite/SSR MARKERS

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Abstract

A total of 96 indigenous rice accessions from Northern areas of Pakistan were used as research material, while IR6, JP5, Nipponbare, and Super-basmati were included as check for comparison. Total genomic DNA was isolated from dehusked dry seeds and young seedlings of all the lines using standard protocols utilizing 36 SSR primers distributed over 12 chromosomes of rice. The major objective of the present study was to estimate the genetic diversity within and among the subpopulation of indigenous rice accessions and secondly to examine the extent of population structure of indigenous rice germplasm from northern Pakistan. A total of 127 different scorable and reproducible alleles were scored. The total number of alleles per loci amplified by each SSR primer ranged from 2-7 with an average of 3.5 alleles per locus. The PIC values ranged widely among loci from 0.39 (RM240 on chromosome 2) to 0.80 (RM119 on chromosome 4) with an average of 0.638 per locus. UPGMA (unweighted pair group method) analysis clustered rice accessions into eight major groups, I to VIII effectively differentiating most of the japonica and other short and long non-aromatic accessions. Microsatellites showed sufficient variation to distinguish between indica and japonica type. A number of SSR markers obtained could be used to generate indica and japonica specific markers, and to differentiate rice grown at high altitudes of Northern Pakistan into two different groups.

Key words: Rice (Oryza sativa L.), SSR markers, Genetic diversity.

Introduction

Oryza sativa is the oldest domesticated crop cultivated throughout the world, while O. glaberrima is restricted to the African continent (Ishikawa et al., 2017; Samaranayake et al., 2018). In the molecular biology studies of Graminaceae, O. sativa is a model plant because of its smaller genome, diploid nature and high level of genetic polymorphism (Samaranayake et al., 2018). Rice is the major staple food of around 3 billion (about 60%) people in the world providing 60–70% daily calories, 20% protein and 2% fats (Fiaz et al., 2019). Asian countries are the main rice producers (Zarei et al., 2018). Pakistan is one of the major exporters of Basmati rice to different countries in the world especially the European Union (Allam et al., 2015). Rice is important as a source of income and employment for common people. The slogan “Rice is life” is most suitable in Pakistan as rice production plays a vital role in our national nutrition security (Younas et al., 2016). Japonica type rice is grown in Khyber Pakhtunkhwa (KP) and Azad Jammu and Kashmir. Short and medium grain type rice commonly called “Begami” is grown in the Swat region. This type of rice has a high global demand especially in Japan, Korea and Russia. The yield and cultivated area under rice tend to decrease in KP since the last decade, which is an alarming condition for poor rice consumers of the country (Bibi, 2017). Genetic improvement of crops as well as increase in yield under different climatic conditions is credited as one of the key approaches for hunger alleviation and improved food production in a fast-growing world population (Roy et al., 2017).

Pakistan lost several rice varieties during the alarming situation of global biodiversity loss. Conservation, as well as an exploration of landrace genotypes with a high yield, could play an important role in improving rice productivity in Pakistan (Khan & Tahir, 2018). Conventionally used agromorphological and biochemical traits do not adequately discriminate variability, and there is a need for more precision. The use of molecular markers especially SSR markers for assessment of genetic diversity in rice is receiving a lot of attention (Wang et al., 2018). Molecular markers are efficient, reliable, and direct method for determining genetic diversity and providing a system for management of plant genetic resources as well as linking phenotypic and genotypic variations in many species (Khush & Brar, 2017). The locus specific markers such as SSRs are widely applied in genetic analysis of many crops (Rawal et al., 2018). SSR markers have been widely used for genetic diversity analysis and cultivar identification because of their abundance, reproducibility, reliability, multi-allelic nature, co-dominance inheritance and requirement of a small amount of DNA for testing (Wang et al., 2017). Up till now, more than 2500 SSR primer pairs have been developed for rice genome (Gupta, 2016). In the present study, 45 SSR markers were used to measure genetic diversity among 96 rice accessions from northern Pakistan.

Material and Methods

A total of 45 SSR markers covering the whole genome of rice were used to characterize and evaluate genetic variation among 92 rice accessions from Northern areas of Pakistan and four rice checks i.e. Super Basmati,
The tubes were incubated for 10 min at 54°C for 60 minutes. Seeds were finely ground using mortar and pestle followed by addition of 500 μL of 2% CTAB solution (100 mM Tris-HCl (pH 8.0), 20 mM EDTA; pH 8.0), 1.4 M NaCl, 1% PVP “polyvinylpyrrolidone” (w/v 40,000, 2% CTAB). DNA was gently separated using chloroform: isoamyl alcohol ratio of 24:1. The sample was centrifuged, and the supernatant was transferred into a new eppendorf tubes. Then, Isopropanol equal to 75% of the total volume was added. The tubes were incubated for 10 minutes at 25°C to precipitate DNA. Then, DNA pellet was washed with 70% ethanol (500 μL). The material was again centrifuged at 10,000 rpm for 5 minutes at room temperature and then washed with 70% ethanol was poured off. The DNA pellet was dried and re-suspended in 100 μL of TE buffer. DNA was removed by addition of 1 μL of RNase (10 mg/mL). DNA concentration was checked using Nano Drop ND-1000 Spectrophotometer and adjusted to 20 ng/μl as working concentration for PCR analysis.

For microsatellite analysis, PCR reaction was carried out in 20 μL PCR tube containing 1X PCR buffer, MgCl₂, 0.2 mM of each dNTPs, 0.4 μM each of forward and reverse primer, 20-50ng genomic DNA and 0.5-unit Taq polymerase. The following thermal cycler profile was used: a denaturing step at 94°C (5 min) followed by 30 cycles each of denaturation at 94°C for 30 seconds, 54°C for 40 seconds (annealing) and 72°C for 2 min (primer elongation). A final extension step at 72°C for 7 min was performed. Amplified DNA products were examined by electrophoresis in 12% Poly Acrylamide Gel (PAGE). The gels were stained and visualized.

**Allele scoring and data analysis:** SSR banding profile generated by each set of SSR primers was compiled into a binary data matrix. Coding system (0) for absence and (1) for presence of bands was used to construct the binary data matrix. Each band amplified by a given SSR primer was treated as a unit character. Only clear and unambiguous bands amplified consistently were recorded. The molecular size of the amplified bands was scored based on the known size of DNA bands of a 20 bp DNA ladder. Genetic similarities between pairs of accessions were derived by the simple matching coefficient and by the similarity index. Estimation of genetic similarities (F) was calculated between all pairs of the rice accessions according to Nei and Li (1979). UPGMA analysis (unweighted pair groups method) employing SAHN clustering (sequential, agglomerative hierarchic and non-overlapping) based on the genetic distance matrix was applied for construction of dendrogram through NTSYS-pc (version 2.2) software package. The term polymorphism information content (PIC) denotes the significance of a marker. In the current study, the PIC value of SSR marker was calculated using Power Marker version 3.25.

**Structure analysis:** STRUCTURE V2.3.1 Software was used for historical lineages that showed clusters of similar accessions. Due to the distribution of accessions not showing a clear cutoff point and to detect the numbers of subpopulations, an ad hoc measure DK was applied. For the membership of each accession, an admixture model was run from the value of K = 1 to 15. To subdivide the germplasm into different subgroups run with the maximum likelihood was used.

**Results**

A total of 45 (SSR) markers covering the whole genome of rice were used to characterize and evaluate genetic similarity among 92 rice accessions and four rice checks i.e. Super Basmati, Nihonbare, IR6, JP5. Out of these 4 markers (RM60, RM122, RM 237 and RM178) were monomorphic, five could not amplify some of the rice accessions, while thirty-six were polymorphic. Amplification profile as revealed by some of the polymorphic markers (RM-138, RM-438, RM-489) across Northern areas germplasm is depicted in (Fig. 1A-C). A significant level of variation was observed among accessions of the Japonica type varieties, Nihonbare and JP5, had unique and shared common bands with other rice accessions. A total of 127 different reproducible and scorable alleles were recorded. The number of bands per locus amplified by each SSR primer ranged from 2-7 with an average of 3.5 alleles per locus (Table 1). The difference in size between the largest and smallest allele at a given SSR locus ranged from 110 bp (RM315) to 300 bp (RM484, RM474, RM48). A maximum number of alleles per SSR primer was determined to be seven for RM119, while the minimum number of two alleles per SSR primer was amplified by RM240, RM271 and RM552. Accessions “7654” and “7660” produced the highest number of alleles (84). It was followed by accession “7657” and “7209” scoring 82 and 79 alleles, respectively, while accessions “7611”, “7214” and “7604” gave the lowest number of alleles (i.e. 14, 25 and 31, respectively). The number of alleles varied from 2 to 7 with most cultivars having 3 alleles per SSR locus.

**Polymorphism information content (PIC):** The level of polymorphism among the 96 accessions was assessed by scoring PIC values for each of the 36 SSR markers. The PIC values ranged from 0.39 (RM240 on chromosome 2) to 0.80 (RM119 on chromosome 4) with an average (0.638) per locus among each locus (Table 1).

**Cluster analysis:** The genetic similarity matrix among the rice accessions was assessed by a UPGMA cluster analysis. A UPGMA cluster analysis grouped rice accessions into eight clusters (I-VIII) effectively, differentiating most of the Japonica and other short and long non-aromatic accessions (Fig. 2). Cluster I consisted of one long grain, non-aromatic indica type IR6 variety and other 15 accessions from Northern Pakistan (Table 2). Members of cluster I had minimum mean flag leaf width and seed yield11. Cluster II comprised of two Japonica type varieties, Nihonbare and JP5, grouped with 5 accessions. Cluster II had medium
mean leaf width, leaf length, flag leaf length and ligule length while having the minimum mean number of culms. Additionally members of cluster II were recorded to have medium mean culm length and diameter, while having minimum mean number of sterile culms plant \(^{-1}\) and panicle length. Cluster III comprised of 13 accessions. Cluster III was recorded to have maximum mean flag leaf length; while having the maximum mean number of culms and the minimum mean number of sterile culms plant \(^{-1}\) and maximum mean panicles \(^{-1}\) (Table 3). Cluster IV had three accessions which showed maximum mean leaf length, ligule length, plant height, seed yield plant \(^{-1}\), thousand grain weight, and grain length. Cluster V had only one accession. Cluster VI had 8.3% (8) of accessions used in structure analysis, A, B, C, D, E, F, G and H represented 8.3% (8), 9.4% (9), 17.7% (7), 8.3% (8), 12.5% (12), 30.2% (29) and 8.3% (8) of accessions used in structure analysis, respectively. Thus, the most structured population was G, followed by F, B, A, E, H, D and C, having shown similar results to the UPGMA tree (Fig. 2) by sorting rice accessions into 8 major clusters.

**Population structure:** Population structure with admixture model was run with iterations using all 96 accessions and 36 polymorphic SSR primers. All accessions were grouped into eight sub-groups assigned the letters (A-H) respectively (Fig. 3). The sub-population A, B, C, D, E, F, G and H represented 8.3% (8), 9.4% (9), 5.2% (5), 17.7% (7), 8.3% (8), 12.5% (12), 30.2% (29) and 8.3% (8) of accessions used in structure analysis, respectively. Thus, the most structured population was G, followed by F, B, A, E, H, D and C, having shown similar results to the UPGMA tree (Fig. 2) by sorting rice accessions into 8 major clusters.
Fig. 2. Dendrogram showing the relationship among 96 *O. sativa* accessions based on SSR banding pattern.
The number of polymorphic bands observed in the present research was higher than the average number of alleles (2.4) recorded by Rabiei et al., (2015) using wild rice accessions from Behar. Joachim (2015) studied rice varieties from India that revealed a mean 2.33 alleles per locus. Shah et al., (2013) compared non-basmati and basmati rice varieties from Pakistan with a mean of 2.75 alleles per locus. The PIC values scored in the present research were like previous assessed microsatellite markers in rice. Panigrahi (2016) reported the PIC values (0.000 to 0.794) with a mean of 0.606. The PIC value in this research was higher than the earlier observations by Roy et al., (2017) and Zhang et al., (2014) who reported an average PIC value of 0.402 and 0.4831, respectively. Present study PIC values are less than that previously recorded by Molla et al., (2015) and Aslam & Arif (2014) who reported PIC values of 0.710 and 0.811, respectively, they used more diverse rice material, amplified relatively higher number of alleles as reported earlier in rice. Joachim (2015) used 24 SSR markers in black glutinous rice accessions and recorded an average of 0.50 dissimilarity coefficient.

Kaur et al., (2015) observed the lower genetic similarity in aromatic rice germplasm than the currently reported similarity value in germplasm. He observed 0.119 genetic similarity in aromatic rice germplasm than 0.40 in the germplasm used or use of different markers. The main reason for higher similarity observed in these research studies might be due to the presence of low intra-specific variability in the germplasm used or use of same ancestors and selection of similar traits as compared to rice genotypes used in the current study. Recombination among the genotypes with a high level of genotypic diversity would be an effective breeding technique.
SSR markers grouping pattern revealed partial geographical homogeneity within clusters, e.g. Cluster I had genotypes from District Mansehra (ten accessions), 25 genotypes in Cluster VI were collected from Chitral and 8 accessions from Swat fall in Cluster III. However, a few genotypes from other districts were also grouped in these clusters. Ishikawa et al., (2017) and Joachim (2015) also reported the same association between genetic and geographic diversities.

Conclusions and Recommendations

Ninety-six accessions were selected through morphological and biochemical characterization for the determination of allelic variability through microsatellite analysis using 45 SSR markers. An average of 3.5 alleles per locus was observed. PIC value with an average of 0.638 per locus was observed. From the results it can be concluded that SSR markers are effective in detecting polymorphism in the accessions studied. A considerable variation was noticed in cluster analysis; different groups were formed based on SSR marker results. It is suggested that representative accessions from different groups should be chosen for core collection and for a breeding program aimed at varietal improvement.

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References


Gupta, L. 2016. Phenotyping and genetic analysis of jnp lines in rice for yield and quality traits. JNKVV.


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