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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 (*Oryzae 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 *Graminae*, *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 espicially 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 agro-morphological 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, Nihonbare, IR6, JP5. The coat was removed from the seed and 2-3 grains containing the storage tissue were placed in 1.5 ml eppendorf tubes. 400 µl of extraction buffer 0.5% SDS, 200 mM NaCl, 25 mM EDTA, and 200 mM Tris-HCl (pH 8.0) containing Proteinases K (50 µg) were added to the tubes. Each sample was incubated at 37°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), 1.4M 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 new eppendorf tubes. Then Isopropanol equal to ⅔ of the total volume was added. The tubes were incubated for 10 minutes at 25°C to precipitate DNA, followed by centrifugation at 12,500 rpm for 12 minutes and removal of the supernatant. 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 70% ethanol was poured off. The DNA pellet was dried and re-suspended in 100 µl of TE buffer. RNA 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 20ng/µl as working concentration for PCR analysis.

For microsatellite analysis, PCR reaction was carried out in 20µl PCR tube containing 1x PCR buffer, $MgCl_2$, 0.2mM of each dNTPs, 0.4µM each of forward and reverse primer, 20-50ng genomic DNA and 0.5-unit Taq polymerase. And then the following thermal cycler profile was used: a denaturing step at 94ºC (5 min) followed by 30 cycles each of denaturation at 94ºC for 30 seconds, 54°C for 40 seconds (annealing) and 72ºC for 2 min (primer elongation). A final extension step at 72ºC 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 the 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 (sequentials, 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 northern areas germplasm. In most of the cases, *Japonica* cultivars, 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 ınformation 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 yield^{-1}. 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. Additionaly 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 45 accessions with maximum mean grain breadth and thickness. Cluster VII had nine accessions including a cultivated long grain variety (Super Basmati). This cluster showed maximum mean culm diameter and grain L/B ratio, while for all other traits medium mean values were recorded. Cluster VIII represented only two accessions which are early in days to maturity, while all other character's medium mean value was scored for accessions of cluster VIII. Accessions '7629' and '7654' showed maximum genetic diversity.

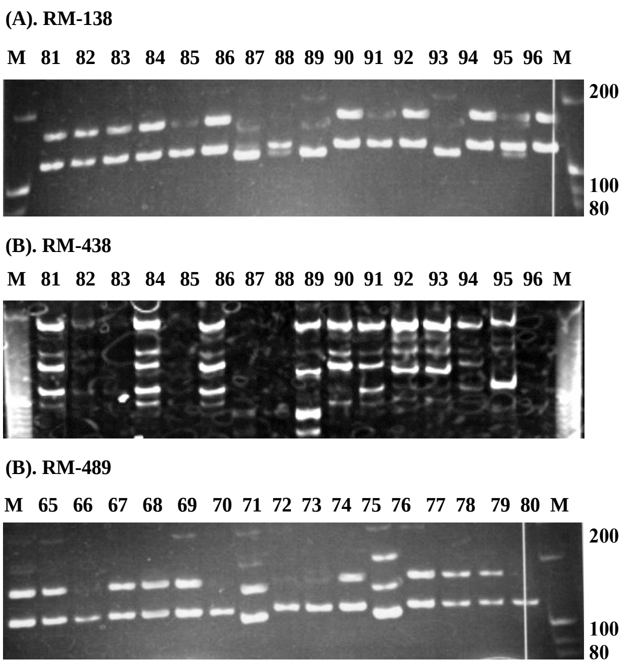

Fig. 1(A-C). SSR banding pattern of rice accessions generated by different primer pairs.

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.

Table 1. PIC values and number of alleles for SSR markers in rice.

S. No.	SSR primer	No. of alleles	Frequency	PIC value
1.	OSR13	4	0.348	0.652
2.	RM25	3	0.411	0.589
3.	RM29	3	0.341	0.659
4.	RM104	4	0.254	0.746
5.	RM105	3	0.378	0.622
6.	RM118	3	0.399	0.601
7.	RM119	7	0.193	0.807
8.	RM124	3	0.403	0.597
9.	RM125	3	0.484	0.516
10.	RM138	3	0.429	0.571
11.	RM144	3	0.348	0.652
12.	RM125	3	0.341	0.659
13.	RM240	2	0.609	0.391
14.	RM249	4	0.264	0.736
15.	RM250	4	0.292	0.708
16.	RM271	2	0.538	0.462
17.	RM277	4	0.577	0.423
18.	RM287	3	0.335	0.665
19.	RM315	3	0.419	0.581
20.	RM321	3	0.433	0.567
21.	RM331	3	0.335	0.665
22.	RM334	4	0.280	0.720
23.	RM433	3	0.423	0.577
24.	RM438	6	0.198	0.802
25.	RM457	5	0.227	0.773
26.	RM474	5	0.247	0.753
27.	RM484	4	0.267	0.733
28.	RM486	3	0.367	0.633
29.	RM487	3	0.514	0.486
30.	RM489	4	0.279	0.721
31.	RM494	3	0.341	0.659
32.	RM504	3	0.395	0.605
33.	RM510	5	0.303	0.697
34.	RM545	5	0.285	0.715
35.	RM552	2	0.506	0.494
36.	RM587	4	0.276	0.724
	Average	3.583	0.362	0.638

Discussion

Genetic markers are useful tools to provide a comparatively unbiased estimate of genetic similarity in crops (Crofts *et al.*, 2018). The study of the genetic relationship between the germplasm from Northern areas is very limited. Only one report on morphological studies of Northern areas germplasm was available (Katsuta *et al.*, 1996). This study was the first report for the characterization of the molecular diversity of Northern Pakistan rice germplasm. The number of fragments recorded in this study corresponded well with the earlier report of Vu *et al.*, (2016) who observed the genetic dissimilarity using 30 SSR markers in Vietnamese lowland rice varieties. Shah *et al.*, (2013) also had similar findings. These results were also comparable to 2-8 numbers of alleles per marker with a mean number of three alleles; using 51 SSR markers that are distributed along the chromosome three and seven; using improved cultivars from Pakistan as reported by Aslam & Arif (2014).

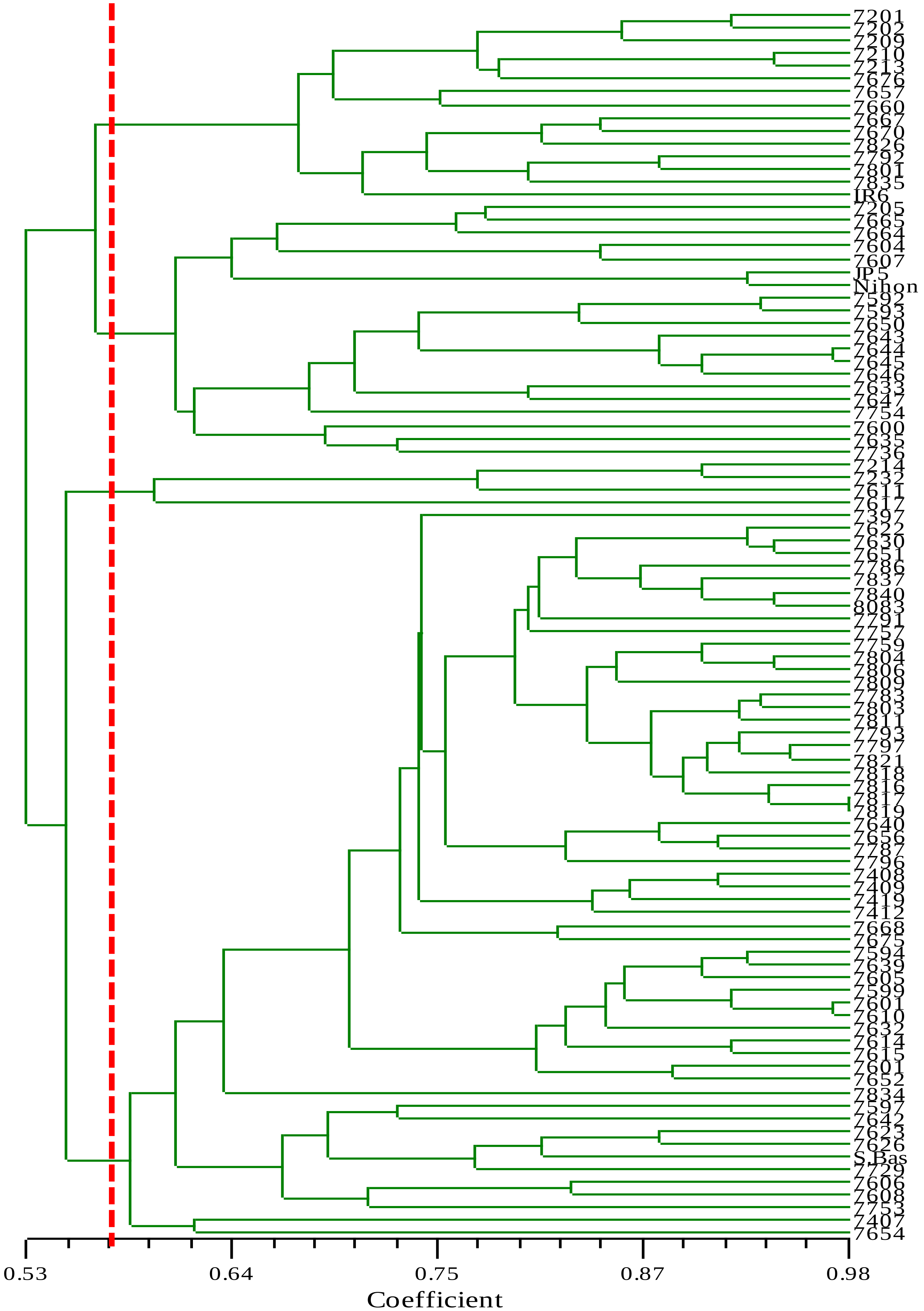

Fig. 2. Dendrogram showing the relationship among 96 *O. sativa* accessions based on SSR banding pattern.

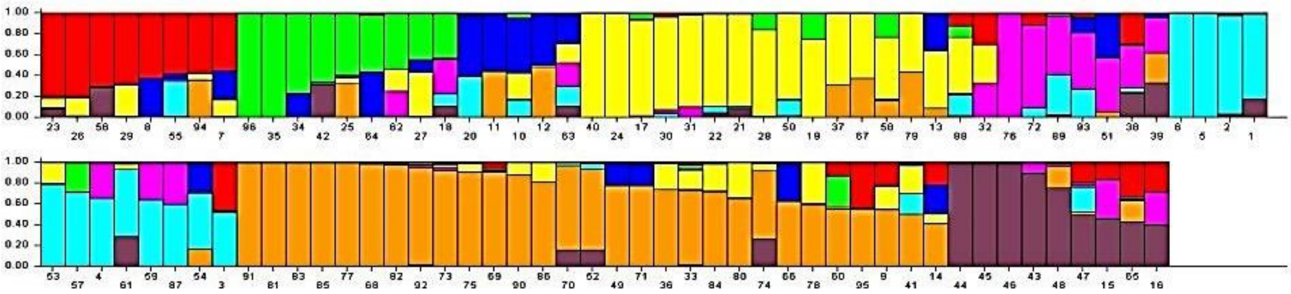

Fig. 3. Population assignment for each accession at K=8 based on STRUCTURE analysis.

Table 2. Accessions in eight clusters based on 36 SSR markers in rice germplasm.

Clusters	Frequency	Accessions
Cluster I	16	7201, 7202, 7209, 7210, 7213, 7676, 7659, 7660, 7667, 7670, 7826, 7792, 7801, 7802, 7835, IR6
Cluster II	7	7205, 7665, 7664, 7604, 7607, JP5, Nihonbare
Cluster III	13	7592, 7593, 7650, 7643, 7644, 7645, 7646, 7633, 7647, 7754, 7600, 7635, 7736
Cluster IV	3	7214, 7232, 7611
Cluster V	1	7617
Cluster VI	45	7397, 7622, 7630, 7651, 7786, 7837, 7840, 8083, 7791, 7757, 7759, 7804, 7806, 7809, 7783, 7803, 7811, 7793, 7797, 7821, 7818, 7816, 7817, 7819, 7640, 7656 7787, 7796, 7408, 7409, 7412, 7668, 7675, 7594, 7639, 7605, 7599, 7601, 7610, 7632, 7614, 7615, 7601, 7652, 7834
Cluster VII	9	7597, 7642, 7623, 7626, Super Basmati, 7729, 7606, 7608, 7753
Cluster VIII	2	7407, 7654

Table 3. Mean±standard deviation for some important quantitative traits of clusters based on SSR data of rice germplasm.

	Cluster I	Cluster II	Cluster III	Cluster IV	Cluster VI	Cluster VII	Cluster VII
FLL	34.6 ± 7.5	42.4 ± 7.5	53.0 ± 9.3	42.0 ± 4.3	39.4 ± 10.5	47.9 ±10.5	42.0 ±1.8
FLW	1.3 ± 0.3	1.4 ± 0.3	1.4 ± 0.1	1.8 ± 0.1	1.3 ± 0.2	1.6 ± 0.2	1.4 ± 0.1
LL	45.4 ± 5.7	51.1 ± 11.5	50.9 ± 5.3	61.3 ± 11.4	46.7 ± 10.4	55.4 ± 6.9	45.5 ± 8.1
LW	1.2 ± 0.3	1.2 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	1.1 ± 0.2	1.3 ± 0.2	1.4 ± 0.1
LgL	1.6 ± 0.4	1.7 ± 0.5	1.7 ± 0.3	1.9 ± 0.4	1.4 ±0.3	1.5 ± 0.2	1.4 ± 0.0
NoC	21.0 ± 7.3	18.8 ± 4.9	30.6 ± 7.0	25.9 ± 3.5	20.4 ± 6.3	20.2 ± 4.8	21.0 ± 4.0
CL	106.2 ± 19.9	106.9 ±17.1	112.1 ± 8.6	115.9 ± 16.5	102.8 ±15.3	114.7 ± 15.2	99.9 ± 15.7
CD	2.6 ± 0.6	2.6 ± 0.5	2.7 ± 0.3	3.2 ± 0.2	2.5 ± 0.4	2.9 ± 0.4	2.7 ± 0.0
SC/P	5.8 ± 7.0	3.6 ± 1.8	4.7 ± 3.5	7.5 ± 3.6	3.8 ± 3.1	4.6 ± 2.8	8.1 ± 1.5
P/P	15.3 ± 4.1	15.2 ± 3.7	25.9 ± 9.1	18.4 ± 2.0	16.6 ± 5.7	15.6 ± 4.6	12.9 ± 2.5
PL	26.9 ± 3.6	26.8 ± 3.6	27.3 ± 3.1	32.8 ± 1.1	27.3 ± 2.9	30.1 ± 5.6	27.3 ± 3.3
PH	133.1 ± 21.8	133.6 ± 19.0	139.4 ± 10.0	148.7 ± 16.3	130.1 ± 16.2	144.9 ± 18.7	127.2 ± 19
DM	83.2 ± 2.5	85.5 ± 6.0	86.7 ± 7.9	82.7 ± 0.5	83.5 ± 3.9	90.4 ± 16.8	82.0 ± 1.0
SY/P	64.1 ± 14.6	67.0 ± 6.1	85.4 ± 26.7	90.4 ± 12.8	73.3 ± 19.2	70.3 ± 22.1	72.0 ± 6.9
TSW	29.0 ± 1.6	28.6 ± 3.4	24.2 ± 2.8	29.0 ± 1.5	28.0 ± 3.7	25.3 ± 2.2	28.4 ± 0.3
PGL	6.1 ± 0.5	6.1 ± 0.8	6.1 ± 0.7	6.7 ± 0.4	5.8 ± 0.4	6.1 ± 0.9	5.7 ± 0.1
PGB	2.7 ± 0.2	2.6 ± 0.3	2.5 ± 0.2	2.6 ± 0.3	2.9 ± 0.3	2.5 ± 0.4	2.6 ± 0.1
PGT	2.0 ± 0.1	1.9 ± 0.1	1.8 ±0.1	1.9 ± 0.0	2.1 ± 0.2	1.9 ±0.1	1.9 ± 0.1
GL/B	2.3 ± 0.3	2.4 ± 0.5	2.4 ±0.3	2.6 ± 0.5	2.0 ± 0.3	2.5 ± 0.8	2.2 ± 0.1

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. Ramchander *et al.*, (2018) 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 previously 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 using three SSR markers. Pair-wise similarity coefficient varied from 0.10 to 0.99 with an average of 0.54 (54%) in Pakistani rice cultivars of basmati and non-basmati types (Aslam and Arif, 2014). The main reason for a higher level of genetic 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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