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RESEARCH ARTICLE

Genetic diversity associated with agronomic traits using microsatellite markers in Pakistani rice landraces

Zahida Hassan Pervaiz*
Department of Biochemistry
Quaid-i-Azam University
Islamabad, Pakistan
E-mail: zahidahasan82pk@hotmail.com

M. Ashiq Rabbani
Institute of Agri-Biotechnology and Genetic Resources
National Agricultural Research Center
Park Road, Islamabad, Pakistan

Ishtiaq Khaliq
School of Life Sciences
University of Sussex, Brighton, UK

Stephen R. Pearce
School of Life Sciences
University of Sussex, Brighton, UK

Salman A. Malik
Department of Biochemistry
Quaid-i-Azam University
Islamabad, Pakistan

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Genetic diversity underlies the improvement of crops by plant breeding. Land races of rice (*Oryza sativa* L.) can contain some valuable alleles not common in modern germplasm. The aim here was to measure genetic diversity and its effect on agronomic traits among rice land-race genotypes grown in Pakistan. Diversity was measured using thirty-five microsatellite markers and seventy-five genotypes. Among the markers used a total of 142 alleles were detected at 32 polymorphic SSR loci, while three loci were monomorphic in Pakistani rice landraces. The number of alleles identified by each marker ranged from 2 to 13 with a mean of 4.4. Size differences between the smallest and largest alleles varied from 11bp to 71bp. Polymorphism information content ranged from 0.124 to 0.836, with an average of 0.569. At nine microsatellite loci, basmati-type landraces amplified more different alleles than those in the coarse-type. DNA markers RM70 and RM72 divided the rice landraces on the basis of days to flowering. A dendrogram based on total microsatellite polymorphism grouped 75 genotypes into four major clusters at 0.40 similarity coefficient, differentiating tall, late maturing and slender aromatic types from the short, early and bold non-aromatic ones. It inferred that Pakistani landraces have diverse genetic bases and can be utilized in future breeding programs. The DNA markers developed will assist in genotype identification, purity testing and plant variety protection.

Landraces are precious genetic resources, because they contain huge genetic variability which can be used to complement and broaden the gene pool of advanced genotypes (Kobayashi et al. 2006). The extent of genetic diversity in a crop population depends on recombination, mutation, selection and random genetic drift. Mutation and recombination bring new variations to a population, whereas selection and genetic drift remove some alleles, often from agronomically important lines. Basmati is one of the premium aromatic rice varieties in the world and cultivated in foothill of Himalayas spread over Pakistan and India. The main characteristic feature of basmati rice is its typical fragrance and 1 to 1.5-fold elongation of rice during cooking Rice (*Oryza sativa* L.) is one of the significant cereal commodities (Lopez, 2008). Rice fulfills the nutritional requirements of half of the world's population. Pakistan is among world's countries having an abundance

*Corresponding author

of basmati landraces. Unfortunately many have been lost during last three decades after green revolution. A number of traditional varieties and improved cultivars have been released for cultivation in different regions of Pakistan since early nineteen century. Aromatic cultivars and advanced breeding lines have a narrow genetic base as they are all related to selected basmati lines. Further, a very limited number of basmati breeding lines were used repeatedly in breeding programs to advance grain quality. Unfortunately resistance against biotic and abiotic stresses were not priorities. In Pakistan, out of seven basmati varieties currently under cultivation, five have 'Basmati-370' as one of the parents (Rabbani et al. 2008). There is a strong need to not only conserve landrace genotypes but also broaden the gene-pool of aromatic rice for future utilization in breeding of high yielding, superior quality and better-adapted varieties in the country.

Exploring diversity in a landrace collection is very important for identifying new genes and further improvement of the germplasm (Aggarwal et al. 2002; Brondani et al. 2006; Jayamani et al. 2007; and Thomson et al. 2007). In the past many efforts have been made to assess the genetic diversity and relationships among germplasm collections of rice using DNA markers. However, limited genetic analyses of Pakistani rice are available from DNA markers. Microsatellites (also known as simple sequence repeats) are simple; tandemly repeated 5-20 fold; often di- to tetra-nucleotide; sequence motifs; each flanked by unique sequences. They are valuable as genetic markers because: they are co-dominant in nature; show high allelic diversity; are easily and economically assayed by PCR; and their use may be automated. Tens of thousands of potential SSRs have been identified in rice, and over 25,000 have been developed as molecular markers (Temnykh et al. 2000; McCouch et al. 2002; IRGSP 2005). These markers are currently being used to develop high density genetic maps, genotype rice accessions, determine the genetic structure, optimize the assembly of core collections, and for marker-assisted breeding (McCouch et al. 2002; Yu et al. 2003; Garris et al. 2005).

In rice, SSR markers have been effectively utilized for many purposes including (i) genome mapping (Temnykh et al. 2000; McCouch et al. 2002); (ii) assessment of the genetic diversity and relatedness among various cultivars including both aromatic and non-aromatic rice (Ravi et al. 2003; Jain et al. 2004; Saini et al. 2004; Siwach et al. 2004; Ghneim Herrera et al. 2008); (iii) identification and purity testing of varieties (Coburn et al. 2002; Nagaraju et al.

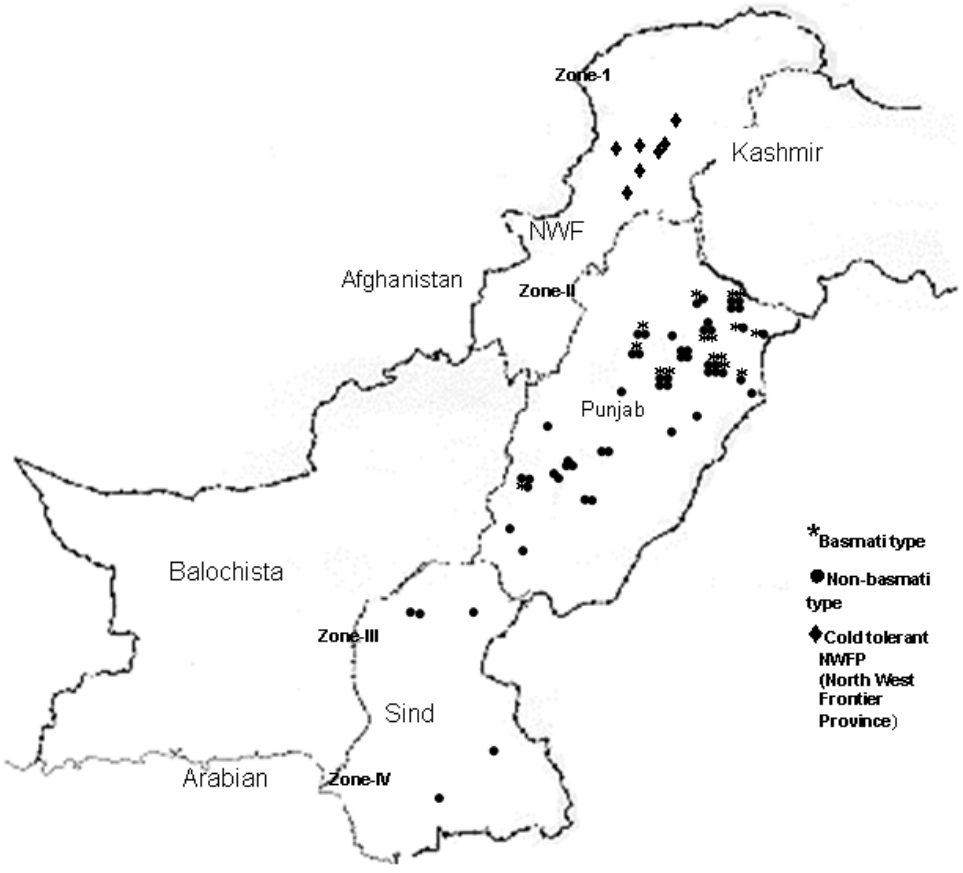

Figure 1. Map showing collection of landraces from four rice growing ecological zones of Pakistan.

2002; Singh et al. 2004; Joshi and Behera 2006) and (iv) determination of the genetic relationship between several sub-species (Ni et al. 2002). In most of these studies, basmati types clustered into a separate group distinct from that of *indica* and *japonica* rice varieties (Aggarwal et al. 2002; Nagaraju et al. 2002; Jain et al. 2004). Therefore, basmati germplasm may have a long, independent and complex pattern of evolution that distinguishes it from other groups within *Oryza sativa* (Jain et al. 2004).

The present study has been conducted to evaluate the pattern and extent of genetic variability and relatedness among some rice landraces of Pakistan based on important agronomic traits using SSR markers. DNA marker analysis will help the identification and differentiation of landraces with different genetic make-up. The information will enable maximized selection of diverse parents and assist in broadening the germplasm base of future rice breeding programs.

MATERIALS AND METHODS

Plant material

Seventy two landrace genotypes and three commercial check varieties, Super Basmati (Indica type, aromatic), IR6 (indica type, non-aromatic) and JP5 (Japonica type, non-aromatic) of rice were used in this study. The research material represented the landraces of local rice collected from four ecological zones of Pakistan (Figure 1). A detailed description of the materials used in present investigation is shown in Table 1 and Table 2.

Microsatellite marker analysis

Total genomic DNA was extracted from 2-3 seed samples from five individuals of each landrace using Kang et al. (1998) with minor modification (mature dry seeds were ground to fine powder rather than whole seed. Purity and concentration of DNA was monitored spectrophotometrically at a wavelength of 260 and 280 nm using NanoDrop ND-1000 Spectrophotometer (Wilmington, USA). All DNA samples were diluted to a working concentration of 20 ng/µl with TE before use. An equal amount of genomic DNA from 5 individuals of each landrace was mixed to make a bulk sample for microsatellite PCR analysis.

Thirty five primer pairs covering all twelve chromosomes were selected for the genetic diversity analysis on the basis of published rice microsatellite framework map. Three primers (RM5, RM210 and RM229) exhibited monomorphic fragments and were therefore excluded from further analysis. The original source, repeat motifs, primer sequences and chromosomal positions for these markers can be found in the rice genome database (http://www.gramene.org). Microsatellite primer pairs were obtained from Hokkaido Science System (Sapporo, Hokkaido, Japan).

SSR analysis was performed following the protocol of Ravi et al. (2003) with minor modifications. PCR amplification reactions were carried out in a total volume of 20µl containing; 10 mM Tris HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200 µM each of deoxynucleotide triphosphate (dNTP); 0.2 µM of each forward and reverse primer; 1 unit Taq DNA polymerase (Fermentas Life Sciences); and 20 ng of template DNA. The PCR amplifications were carried out using a MyGene Series Peltier Thermal Cycler (UniEquip GmbH, Munich, Germany). Thermal cycler was programmed to 1 cycle of 5 min at 94°C as an initial hot start and strand separation step. This was followed by 35 cycles of 1 min at 94°C for denaturation, 1 min for annealing temperature depending on the marker used (55°C-65°C) and 2 min at 72°C for primer elongation. Finally, 1 cycle of 7 min at 72°C was used for final extension. Amplified products were stored at -20°C until further use. The reproducibility of the amplification products was checked twice for each primer.

Electrophoresis of amplified products

After amplification, a 15 µl aliquot of the amplified SSR samples was combined with 3 µl of a loading buffer (0.4% (w/v) bromo-phenol blue, 0.4% (w/v) xylene cyanole and 5 ml of glycerol) and was analyzed directly on 3% (w/v) Gene Choice High Resolution agarose (CLP, USA) gels in 1xTBE buffer (10mM Tris-Borate, 1mM EDTA) containing 0.5 µg per ml of ethidium bromide. A 25 bp DNA ladder (Biolabs, New England, UK) was used as a size marker to compare the molecular weights of amplified products. After electrophoresis, the gels were documented using an UVI Doc Gel Documentation System (UVITEC, Cambridge, UK).

Allele scoring and data analysis

Ethidium bromide staining of agarose gels generally showed several bands. The size of the most intensively amplified band for each microsatellite marker was determined based on its electrophoretic mobility relative to molecular weight markers (increments of 25 bp). Amplified products from SSR analysis were scored qualitatively for presence and absence of each marker allele-genotype combination. Each SSR band amplified by a given primer was treated as a unit character. Data was entered into a binary matrix as discrete variables, 1 for presence and 0 for absence of the character. The most informative primers were selected based on the extent of polymorphism. The polymorphic information content (PIC) value of a marker was calculated according to Anderson et al. (1993). Mean allele numbers, PIC values, and genetic similarities were calculated on the basis of different rice landraces, chromosomes and microsatellite classes. Pair-wise comparisons of the genotypes based on the proportion of unique and shared amplification products (alleles) were used to measure the genetic similarity by Dice coefficients using PAST program (Hammer et al. 2001). Genetic similarities (F) between all pair of the landraces were

Table 1. Accession number and local name of landraces.

Accession No.	Local name	Accession No.	Local name	Accession No.	Local name
6627	Bungua-147	6549	Basmati-372	6599	Sufaida-20
6722	SathraSufaid-331	6516	SonFine-43	6603	BagarSugdasi-34
6560	Basmati-410	6530	PalampuriBasmati-137	6605	Sonoattar-45
6626	Jhona-145	6771	15	6611	DaggarSufaid-94
6558	KamohBasmati-392	6560	Basmati-410	6613	DhanKasarwala-102-4
Super	Check-1	6574	Begmi-119A	6622	Dhan-133
JP5	Check-2	6623	Begmi-135	6597	RB-3
6593	Rohru-414	6670	MundearaDhan-90S	6745	Jhona-101
6620	Jhona-129	6756	400	6633	Tiri-219
6677	BasmatiSurkh-161	6519	Begmi-51A	6655	MunjiSuffaid-23A
6638	268-A	6505	KalaBunda-50	6676	WhiteMunji-160
6621	DhanKasarwala-131	6507	Hansraj-54	6683	Jhona-178
6642	BamlaSuffaid-320	6693	213-B	6684	180-CoarseVar
6654	BaggiMunji-22	6564	Begmi	6694	Sufaida-222B
6658	CoarseWhite-40	6527	Chamber-128	6698	Tiri-236
6751	336-Tiri	6509	Hansraj-62	6705	274-A
6664	MunjiSufaid-165	IR6	Check variety	6706	Sathri-275
6663	76S-CoarseRice	6711	LalDhan-304	6717	Sathra-318
6779	20-A	6758	Tiri-424-2	6719	Kanhra-8-327
6520	Begmi-51S	6570	LalDhan-304	6724	DhanDesi-336
6740	Baggi-423	6578	Rohdu-150	6729	349-CoarseWhite
6537	Basmati-242	6582	EB-204	6731	353
6563	BasmatiKamon	6588	Dhan-300	6734	368
6766	8	6590	Kanhgra-319	6755	399
6515	Basmati-1-1A	6595	N-35	6760	1-A

calculated according to Nei and Li (1979). A dendrogram was constructed using pair-group method to get genetic relationships among landraces.The reliability of the dendogram was tested by bootstrap analyses with 10,000 replications to assess branch support. Some workers consider that the confidence limits obtained in bootstrap must be over 95% in order to consider the grouping of taxa (a group of genetically similar organisms that are classified together as e.g. species, genus, or family) at a branch to be statistically significant (Felsenstein, 1985). Others use a lower limit (above 50% or at least 50%) as indicating statistical support for the topology at a node (Highton,

1993). In our study we used the lower limits to assess grouping of taxa to be statistically significant because we observed that as the number of test sample increases the confidence interval decreases.

RESULTS

Thirty-five microsatellite or SSR markers (Table 3) covering all 12 chromosomes were utilized to characterize and assess genetic diversity among seventy-five diverse rice landraces from Pakistan. Only three loci (RM5, RM210 and RM229) were observed monomorphic in Pakistani landrace genotypes. A considerable level of variability was observed among different landraces for remaining thirty two microsatellite loci. In most of the cases, basmati check and other aromatic cultivars exhibited similar banding patterns. The microsatellites exhibited several bands that were shared among the check Super Basmati and some landraces, whereas a few bands were shared among IR6 and other landraces Pakistani rice. Nine accessions (6626, 6756, 6530, 6519, 6549, 6514, 6698, 6654, and 6751) displayed unique bands in comparison with all other genotypes with different microsatellite markers. Interestingly, many loci revealed characteristic alleles in some landraces which were not produced in any of the check varieties used.

Each of the primer pairs differed significantly in their ability to determine variability among the landraces (Table 3). Some primers generated several markers, while others generated only few. A total of 145 alleles were detected across 75 rice cultivars/landraces using 32 SSR markers (Table 3). Of these, 142 (98%) were found to be polymorphic. The maximum number of polymorphic alleles (*i.e.* 13 bands) was obtained with the marker RM70, while the minimum number of polymorphic bands (2 alleles) was amplified with the markers RM10, RM13, etc. The average number of polymorphic alleles per marker was 4.4. The overall size of the amplified product varied from 75 bp (RM1) to 349 bp (RM182). The size difference between the smallest and the largest allele at a given SSR locus varied from 12 (RM122) to 71 bp (RM302).

A total of 32 (22%) rare alleles were observed among 12 of the 32 SSR loci, with an average of 2.7 rare alleles per locus. The maximum number of rare alleles were observed at RM163 locus (6 alleles), followed by RM70 and RM252 loci (5 alleles each). In general, markers detecting a greater number of alleles per locus detected more rare alleles.

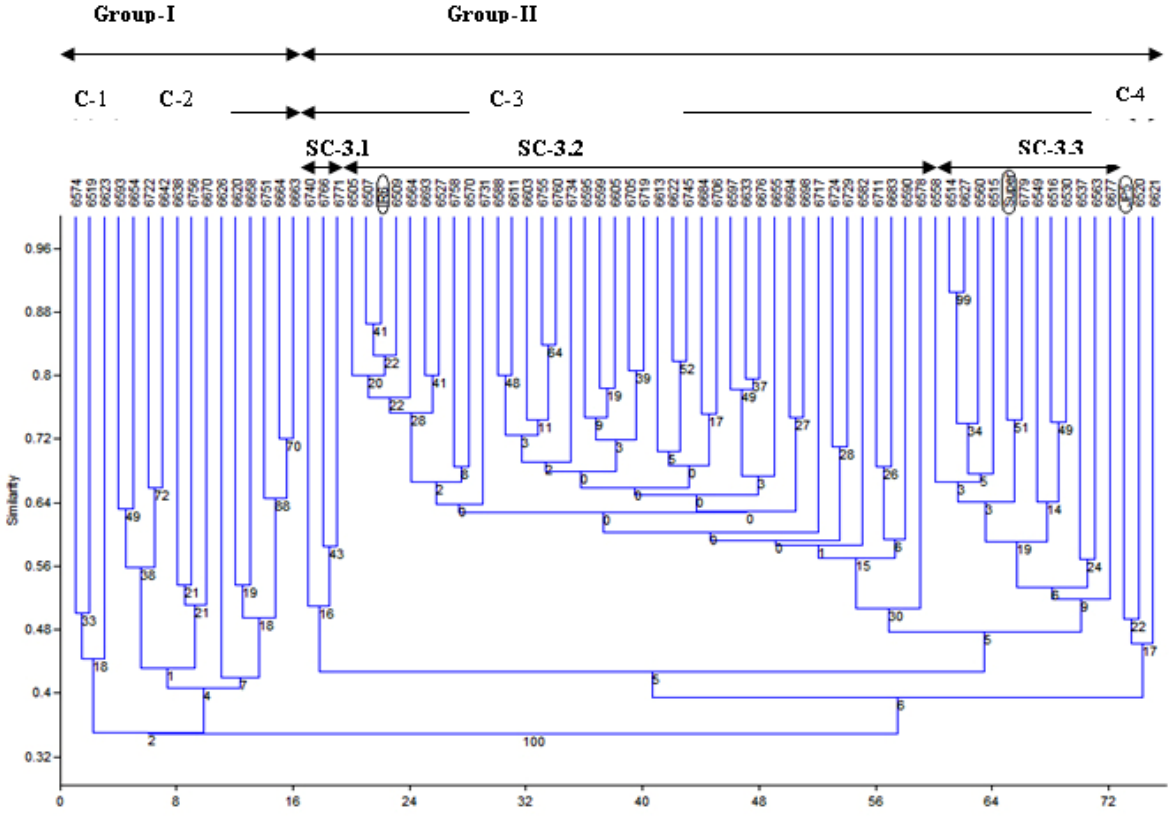

Figure 2. UPGMA cluster analysis showing the diversity and relationship among 75 landraces of rice based on 145 alleles generated by 32 SSR markers. Super-basmati, IR6 and JP5 indicated in circles.Group I (C = Cluster, SC= Sub Cluster)) C1: cluster 1 (cold tolerant, short grained genotypes), C2: cluster 2 (early maturing, tall height, medium seed length, zone II), Group II C3: cluster 3 (Three Sub clusters(SC): SC1: Early maturing Zone III, SC2: Non-Basmati type, SC3: Basmati type) C4: Cluster 4 (Japonica type landraces from northern areas).

Twenty-one (60%) of the rice landraces had rare SSR alleles. The maximum number of rare alleles were present in two landraces, 6626 and 6740 (3 rare alleles each). Two rare alleles were found in each of '6722', '6558, '6621', '6654', '6652', '6530', and '6756', while 12 landraces had single rare allele at individual locus. A landrace was assigned null allele for an SSR locus whenever an amplification product could not be detected for a particular genotype-marker combination. In the set of 75 genotypes, 2 loci showed null alleles. Null alleles were observed in 'Super-basmati' at the RM241 locus and, in '6574'and '6623' at the RM60 locus.

The level of polymorphism among the 75 genotypes was evaluated by calculating PIC values for each of the 32 SSR loci. The PIC values varied widely among loci and ranged from 0.124 (RM10) to 0.836 (RM163) with an average of 0.569 per locus (Table 3). PIC values showed a significant positive linear correlation with number of alleles at SSR locus ($r = 0.73$; $P < 0.01$, analysis of variance). '6558' and '6717' gave the maximum numbers of alleles (43). It was followed by '6677' and '6515' producing 40 alleles each, while cultivars 'JP5', '6771', '6574' and '6623' gave the least number of alleles (*i.e.* 34 alleles each) followed by 6626 and 6745 gave 35 alleles each.

A similarity matrix based on the proportion of shared SSR fragments was used to establish the level of relatedness between the various landraces surveyed. Pair-wise estimates of similarity ranged from 0.14 to 0.90 and the average similarity among all 75 landraces was 0.52 (data not shown). Two genotypes 6627 and 6514 were the closest related genotypes with the highest similarity index of 90%. This was followed by 86% similarity between two pairs of genotypes 6507 and check variety IR-6. The lowest similarity (13%) was observed between genotypes 6626 and 6745. As expected, genetic similarities between the IR-6 and landraces were comparatively high. Among the basmati type landraces, similarity coefficients ranged from 0.22 to 0.90, whereas non-basmati types showed similarity coefficients of 0.19 to 0.72. Although comparison of basmati type landraces indicated that these were closely related. Of the landraces analyzed, 6626 had the greatest dissimilarity with all the other tested genotypes. The similarity coefficients of 6626 with all the other landraces ranged from 0.13 to 0.49.

A UPGMA cluster diagram grouped the 75 Pakistani landraces into two major groups at 100% confidence interval, effectively differentiating the slender aromatic rice cultivars from the short bold and long bold non-aromatic landraces at similarity coefficient value of 0.34 (Figure 2). Group-I consisted 16 genotypes which are further subdivided into two clusters, cluster-I containing three cold tolerant short grained genotypes, 6574 (Malakand), 6519 (Dir) and 6623 (Swat) while cluster-II consist of 13 landraces (Early maturing, tall heighted and medium seed length) from different regions of Punjab province (Zone-II) except one 6638 from NWFP (Zone-I). Group-II consisted of two major clusters, one grouping two short grained landraces with JP5 a japonica type check variety, while other consisting of total 56 landraces further divided into three sub clusters, one combining IR6, a non-basmati check variety with 39 landraces and 12 basmati type long grained and tall plants together with Super- basmati check variety, third sub cluster grouping non basmati type, two landraces from Zone-III, 6771 (Sangarh) and 6766 (Larhkana) and one from (6740) Sheikhupura. Cluster analysis placed most of the traditional tall, long-grained cultivars together showing a high level of genetic relatedness. However, the clusters formed by the basmati type were distinct from those of the non-aromatic group. Three *japonica* genotypes were clustered together in group II, and closer to the aromatic cultivars than to the non-aromatic *indica* group. Dendograms showed that the genotypes that are derivatives of genetically similar types clustered together. Landraces in the same subgroup usually shared a high proportion of ancestry and/or agronomic characteristics such as height, maturity, quality traits, etc. The landraces originating from various regions of Pakistan form well defined, distinct, groups in the cluster analysis, indicating association between the SSR patterns and the geographic origin of genotypes used.

DISCUSSION

One main cause of eradication of plant genetic resources has been the adaptation of narrowly based advanced varieties for intensive cultivation practices. Variation in landraces is helpful for broadening the crop gene pool (Frankel and Soulé, 1981). Diversity exploration among plant landraces made necessary by the failure of the green revolution to be sustained. Little was known about the relationship between Pakistani agronomic crop cultivars in general and rice landraces in particular on the basis of SSR analysis. Here, 35 microsatellite markers were used to assess the genetic diversity of 75 genotypes of rice including three check varieties Super-basmati (Indica type, aromatic), IR6 (Indica type, Non-aromatic) and JP5 (Japonica type, non-aromatic). Some agronomic features of selected rice landraces *e.g.* plant height, days to maturity and seed length along with their local names and location are included in the Table 1 and Table 2. The results indicated significant genetic variation among the rice landraces of Pakistan. Microsatellite assays identified a number of alleles that were shared among the Super-basmati and some landraces. A close relationship between 'Super-basmati' and a group of twelve landrace genotypes (16%) was observed. That phenomenon could be a favour towards strong discrimination power of some of the DNA markers. However, the similarity coefficient of these landraces ranged from 0.47 to 0.75 compared with Super-basmati rice. These landraces also shared many morphological and agronomic traits with Super-basmati (Indica type) that strengthen the supposition of close relationship between them. 'Check variety 'IR6' (Indica type and non-aromatic) shared limited number of fragments with 54% of landraces (shown in group II of dendrogram),

Table 2. Classification of landraces on the basis of Agronomic traits and geographical location.

Agronomic traits and locations	Levels	Accessions
*Days to Maturity	Very Early	6514, JP5, 6638, 6621, 6560, 6756, 6758, 6570, 6655, 6731, 6734, 6755
	Early	6627,6722, 6626, 6593, 6620, 6642, 6654, 6658, 6751, 6664, 6663, 6779, 6740, 6766, 6771, 6574, 6623, 6670, 6693, 6711, 6578, 6582, 6586, 6590, 6595, 6599, 6603, 6605, 6611, 6613, 6622, 6597, 6745, 6633, 6676, 6684, 6694, 6698, 6717,6729
	Medium	6719, 6724, 6705, 6760, 6507, 6564, 6509, IR6
	Late	6558, Super- Basmati, 6677, 6520, 6537, 6563, 6515, 6549, 6516, 6530, 6519, 6505, 6527, 6683, 6706
*Plant Height	Tall	6627,6722, 6593, 6620, 6642, 6654, 6658, 6751, 6664, 6663, 6779, 6740, 6766, 6771, 6574, 6623, 6670, 6693, 6711, 6578, 6582, 6586, 6590, 6595, 6599, 6603, 6605, 6611, 6613, 6622, 6597, 6745, 6633, 6676, 6684, 6694, 6698, 6717,6729, 6514, JP5, 6638, 6560, 6756, 6758, 6570, 6655, 6731, 6734, 6755, 6719, 6724, 6705, 6760, 6507, 6509, 6558, 6677, 6520, 6537, 6563, 6515, 6549, 6516, 6530, 6519, 6505, 6527, 6683, 6706
	Intermediate	Super-Basmati, JP5, 6621, 6564
	Dwarf	6626, IR6
*Seed Length	Short	6626, JP5, 6593, 6638, 6621, 6520, 6516, 6574, 6623, 6519, 6564, 6711, 6758, 6578, 6582, 6588, 6595, 6597, 6745, 6676, 6694, 6705, 6719, 6724, 6731, 6734, 6760
	Medium	IR6, 6514, 6560, 6756, 6758, 6570, 6655, 6731, 6755, 6627,6722, 6620, 6654, 6658, 6751, 6664, 6663, 6779, 6740, 6766, 6771, 6574, 6670, 6693, 6711, 6586, 6590, 6599, 6603, 6605, 6611, 6613, 6622, 6633, 6684, 6698, 6717,6729, 6705, 6507, 6564, 6509, 6558, 6677, 6520, 6537, 6563, 6515, 6549, 6516, 6530, 6519, 6527, 6683, 6706
	Long	Super-basmati, 6642, 6756, 6505
Geographical distribution	Zone-I	6564, 6638, 6623, 6519, 6520, 6574
	Zone-II	6514, 6515, 6516, 6595, 6597, 6599, 6655, 6740, 6505, 6549, 6563, 6605, 6611, 6507, 6683, 6684, 6570, 6588, 6711, 6527, 6530, 6620, 6558, 6693, 6694, 6603, 6613, 6621, 6663, 6664, 6670, 6509, 6705, 6706, 6537, 6717, 6719, 6642, 6590, 6593, 6724, 6729, 6622, 6626, 6627, 6633, 6578, 6582, 6654, 6698, 6658, 6577, 6758, 6751, 6755, 6560, 6731, 6676, 6734, 6756, 6745
	Zone-III	6766, 6771
	Zone-IV	6779, 6760

*Days to maturity (≤ 100 days = Very early, 101-115 days = Early, 116-130 days = Medium, 131-145 days = Late, ≥ 145 days = Very late).
*Plant height (≤ 110 cm = Semi dwarf, 111-130 cm = Intermediate, ≥130 cm = Tall).
*Seed length (8 mm = Short grain, 8-10 mm = Medium grain, 10 mm = Long grain).

suggesting a close association. Similarity coefficients of 'IR6' with landraces of this group ranged from 0.25 to 0.86.

The number of alleles detected by microsatellite markers varied from 2 to 13 with an average of 4.5 alleles per locus and were similar to those reported in basmati and non-basmati rice varieties (Siwach et al. 2004; Neeraja et al. 2005; Ghneim Herrera et al. 2008). The 2.0-5.5 alleles per SSR locus for various classes of microsatellites were similar to those reported by Cho et al. (2000) using a

Table 3. Details of SSR markers used, indicating their location on rice chromosomes, number of alleles detected, allele size range and polymorphism information content (PIC).

Marker	Chromosome	SSR motifs	Total	Polymorphic	Size range	Difference	PIC
RM1	1	(GA)26	5	5	75 - 135	60	0.678
RM10	2	(GA)15	2	2	142 - 159	17	0.124
RM13	5	(GA)16	2	2	130 - 150	20	0.336
RM16	3	(GA)15	3	3	165 - 187	22	0.256
RM17	12	(GA)21	3	3	160 - 185	15	0.395
RM19	4	(ATC)10	2	2	192 - 252	60	0.191
RM44	8	(GA)16	4	4	100 - 125	25	0.373
RM55	5	(GA)17	2	2	216 - 247	31	0.468
RM60	3	(AATT)5AATCT(AATT)	2	2	162 - 176	14	0.394
RM70	7	(ATT)33	13	13	128 - 167	39	0.800
RM72	8	(TAT)5C(ATT)15	6	6	151 - 200	49	0.726
RM110	2	(GA)15	5	5	138 - 159	21	0.731
RM122	5	(GA)11	5	5	229 - 240	11	0.757
RM163	5	(GGAGA)4(GA)11C(GA)20	9	9	130 - 175	45	**0.836**
RM170	6	(CCT)7	4	4	99 - 119	20	0.654
RM182	7	(AT)16	8	8	328 - 349	21	0.833
RM201	9	(CT)17	4	4	144 - 159	15	0.540
RM202	11	(GA)30	5	5	161 - 190	29	0.735
RM222	10	(CT)18	3	3	199 - 225	26	0.545
RM223	8	(GA)25	4	4	140 - 170	30	0.623
RM224	11	(GA)13	4	4	118 - 157	39	0.662
RM234	7	(GA)25	3	3	133 - 163	30	0.633
RM241	4	(CT)31	5	5	104 - 149	45	0.831
RM242	9	(CT)26	3	3	197 - 255	58	0.444
RM252	4	(GA)19	9	9	194 - 262	68	0.738
RM253	6	(GA)25	4	3	117 - 146	29	0.434
RM257	9	(CT)24	3	3	132 - 147	15	0.653
RM263	2	(CT)34	5	5	162 - 199	37	0.374
RM302	1	(GT)30(AT)8	6	6	120 - 191	71	0.623
RM310	8	(GT)19	5	5	87 - 123	36	0.766
RM333	10	**(TAT)19(CTT)19**	4	4	166 - 196	30	0.629
RM348	4	(CAG)7	3	3	131 - 143	12	0.422
Total			**145**	**142**			
Average			**4.53**	**4.44**			**0.569**

different set of rice germplasm. However, the average numbers of alleles detected here were significantly higher than those reported in Indian aromatic rice varieties by some researchers (Nagaraju et al. 2002; Singh et al. 2004; Joshi and Behera 2006). This could be due to inclusion of several landraces of diverse origin, in this study. In

contrast, the average numbers of alleles noticed in present study were lower than those reported previously (Ni et al. 2002; Jain et al. 2004; Xu et al. 2004; Lu et al. 2005; Brondani et al. 2006; Jayamani et al. 2007; Thomson et al. 2007). Those reports had an average of 6.8, 7.8, 11.9, 6.6, 14.6, 7.7 and 13.0 alleles per locus. They used rice subspecies, Indian quality rice germplasm; US rice genetic resources, traditional varieties of Brazilian rice, a diverse collection of Portuguese rice and an Indonesian rice germplasm, respectively. The inconsistency among reports might be due to the genotypes used and selection of microsatellite primers with scorable alleles.

The polymorphism information content (PIC) values, were quite high and varied (range 0.124 to 0.836, average value 0.569) considerably among SSR loci. The PIC values observed in this study were comparable to those reported in some studies (Jain et al. 2004; Saini et al. 2004; Siwach et al. 2004; Lu et al. 2005; Jayamani et al. 2007; Thomson et al. 2007), but higher than those reported by Singh et al. (2004) and Joshi and Behera (2006). However, this study report lower PIC values compared to those described by Xu et al. (2004) and Brondani et al. (2006), who observed an average PIC value of 0.73 and 0.74 for the world collection and traditional varieties of Brazilian rice, respectively. But this difference might be linked with selection of different markers and more diverse set of varieties.

Lower bootstrap values were observed at some node points in dendrogram in present investigation as compared to Ghneim Herrera et al. (2008). The reason may be the sample size in present investigation we used 75 genotypes and in previous one only eleven genotypes were studied. The cluster analysis based on similarity coefficients places 75 rice genotypes into two major groups at 0.34, while at 0.40 four clusters are formed. Most of the basmati-type landraces fell into the same group along with Super-basmati check variety. Cluster analysis also grouped most of the basmati landraces from different districts of Punjab together indicating that they are genetically similar with each other and have common ancestors. These rice landraces might share basmati parents in their pedigree. A similar study, conducted by Kobayashi et al. (2006) using 18 microsatellite markers, grouped 23 rice landraces into two groups, one small cluster of two indica cultivars, while other of japonica type landraces.

The microsatellite markers used in this study were well distributed amongst the 12 chromosomes, and were located in both coding and non-coding segments of the genome (Cho et al. 2000; Temnykh et al. 2000). Only three markers were monomorphic, while remaining 32 gave polymorphic alleles. RM241 located at chromosome number 4 (106.2-106.2 cm) gave five polymorphic alleles with PIC value of 0.831 in Pakistani landraces while this marker was monomorphic when used previously by Kobayashi et al. 2006 in analyzing genetic diversity of an old Japanese landrace, 'Echizen'. This is a co-localized marker linked to some quantitative traits as well as qualitative traits *e.g.* 1,000 grain weight, awn length, biomass yield, chlorophyll contents and hull color, and flour color (www.gramene.org/db/qtl). This showed that Pakistani landrace germplasm is heterogeneous at the genomic level. Some landraces with similar morphologies in field studies proved to be diverse by DNA marker analyses.

Amplification of microsatellite markers RM70 and RM72 resulted in 13 and 6 polymorphic alleles with size ranges from 128 bp to 167 bp and 151 bp to 200 bp respectively. Separate cluster analysis of these two markers showed groups of late, early and very early maturing accessions (not included in this work). Therefore, earliness, an agronomically important trait, may be linked to these markers. There is no clear evidence for this from the literature except that microsatellite loci RM72 is present as a neighboring marker at chromosome 8 (69-69 cm) with co-localized markers (RM483, RM404, RZ617 and RM44) that were linked to days to flowering in rice (www.gramene.org/db/qtl). Another evidence is that the QTL (Quantitative Trait Locus) for days to flowering/heading (Hd-4 and Hd-5) are located on chromosome 7 and 8 respectively (Yano et al. 1997). Further analysis will be required to prove this hypothesis.

The genetic diversity of rice landraces has been studied using several methods involving their morphological and physiological characters (Oka, 1988), isozymes (Glaszmann, 1987), RFLP markers (McCouch and Tanksley, 1991) or microsatellite markers (Yang et al. 1994; Akagi et al. 1997). However, such studies generally used each rice landrace or cultivar as one single sample and did not focus on the diversity within them. On the other hand, rice landraces are reported to be heterogeneous and to include different genotypes within the population (Fukuoka et al. 2006). Microsatellite markers proved to be a useful tool for clarifying the genetic diversity among landrace genotypes. Ecogeographical adaptation of landraces was also reflected in DNA profiles also at specific loci. Three cold tolerant landraces from Malakand, Swat and Dir (Cluster-1) were grouped together showing similarity at genomic level and difference from other landraces.The reason for this grouping might be the same genetic background with limited out-crossing and farmer choice/priority in these hilly areas.

Here microsatellite analysis was an efficient tool for diversity analysis, and differentiation of rice landraces on the basis of different traits. Overall results show that Pakistani landrace germplasm of rice is not japonica type. The few landraces which grouped with JP5 in the SSR-based analysis of aromatic and quality rice implied a long, independent and complex pattern of evolution for basmati germplasm. The present investigation further indicated that genetically basmati type rice was different from that of coarse/non-aromatic and *japonica* type. In addition, marker-based identification and differentiation of basmati rice may help to maintain the integrity of this high quality product to the benefit of both farmers and consumers. The

microsatellite assay generated cultivar-specific alleles in some of the genotypes screened; these may be used as DNA fingerprints for cultivar identification. This would be of enormous assistance for the establishment and defense of proprietary rights and the determination of cultivar purity.

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