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ASSESSMENT OF GENETIC VARIABILITY IN RICE (*ORYZA SATIVA* L.) GERMPLASM FROM PAKISTAN USING RAPD MARKERS

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Abstract

Information on genetic diversity and relationships among rice genotypes from Pakistan is currently very limited. Molecular marker analysis can truly be beneficial in analyzing the diversity of rice germplasm providing useful information to broaden the genetic base of modern rice cultivars. The objective of this study was to evaluate the genetic polymorphism of 75 rice accessions and improved cultivars using random amplified polymorphic DNA (RAPD) technique. Twenty-eight decamer-primers generated a total of 145 RAPD fragments, of which 116 (80%) were polymorphic. The number of amplification products produced by each primer varied from 3 to 9 with an average of 5.2 alleles primer^{-1}. The size of amplified fragments ranged from 250 to 4000bp. A dendrogram was generated from minimal variance algorithm using Ward method. All the 75 genotypes were grouped into two main groups corresponding to aromatic and non-aromatic types of *indica* rice. Clustering of accessions did not show any significant pattern of association between the RAPD fingerprints and collection sites. This type of analysis grouping different rice accessions in relation to fragrance, a major rice quality determinant, and varietal group is extremely useful to develop a core collection and gene bank management. Further more, the information revealed by the RAPDs regarding genetic variation is helpful to the plant breeder in selecting diverse parents and for future orientation of rice breeding program.

Introduction

Rice (*Oryza sativa* L.) being most important food crop for more than 2 billion people in Asia, provides 27% of dietary energy and 20% of overall dietary protein (Bashir *et al*., 2007). It is cultivated under diverse environmental conditions in Pakistan, occupies 10% of total area in country and accounts for 5.9% of value added in agriculture and 1.3% in GDP (Anon., 2009). Green revolution eradicated most of traditional landraces in almost all of the rice growing countries of the world but Pakistan is among one of the few countries who retained their wealth of traditional rice varieties. In late nineties, Pakistan has released a number of improved cultivars by crossing traditional varieties and advanced breeding lines on the basis of narrow genetic makeup (Rabbani *et al*., 2010). This is all a great contribution to grain quality and development of resistance against biotic and abiotic stresses. More than 2500 accessions collected from different areas of Pakistan are preserved in gene-bank at Institute of Agri-Biotechnology & Genetic Resources, National Agricultural Research Centre, Islamabad, Pakistan. Rice landraces possess several undesirable agronomic traits including tall plant stature, long crop duration, sensitivity to photoperiod, and poor response to fertilizer application resulting

in too low yield (Singh *et al*., 2000). Though, development of evolved Basmati varieties has improved the rice grain quality parameters to a great extent along with increased yield, but it also has contributed to the reduced genetic variability in the modern day cultivars. Several semi-dwarf, high yielding rice cultivars developed fall short of features of traditional varieties like resistance/or tolerance to biotic and abiotic stresses.

Morphological and biochemical markers are not much consistent for diversity analysis because of the impression of environmental factors. For reliable and efficient analysis of genetic variation in the germplasm and to elucidate the intra- and inter-specific relationships, the use of molecular markers is prerequisite (Rabbani *et al*., 2010). Application of molecular markers enhances the efficiency of traditional plant breeding by indirect selection through molecular markers linked to both simple and quantitative traits of interest as these are not influenced by the environment and can be scored at all stages of plant growth (Gupta *et al*., 1999). They can also be used for germplasm characterization, genetic diagnostics, characterization of transformants, study of genome organization, phylogenetic analysis etc (Rafalski & Tingey, 1993).

Random amplified polymorphic DNA (RAPD), due to its simplicity, inexpensive nature and non-requirement of prior information of genetic sequence (Williams *et al*., 1990) is being extensively used for estimating genetic polymorphism. RAPDs have been used to construct genetic linkage maps (Reiter *et al*., 1992), estimate genetic diversity (Chalmers *et al*., 1992; Raghunathachari *et al*., 2000; Porreca *et al*., 2001) and for phylogenetic relationships among species (Landry *et al*., 1994) in a large number of plant species including rice (Neeraja *et al*., 2002; Saker *et al*., 2005; Rabbani *et al*., 2008). A large number of rice landrace genotypes are preserved in gene-bank at National Agricultural Research Centre, Islamabad, Pakistan, but this germplasm has not been properly explored for genetic diversity and other useful information. This study evaluates the pattern of genetic variability and relatedness among landrace genotypes of Pakistan using selective RAPD markers. Data will be definitely useful for sake of maintenance and differentiation of various landraces which are preseved. It would be helpful for plant breeders to select readily the diverse parents which will add new germplasm base for future rice breeding programmes.

Materials and Methods

Plant material: Seventy-five landraces including three commercial varieties (Super-basmati, JP5 and IR6) as check used in present study were collected from 22 districts of Punjab, four districts of Sindh and three districts of NWFP covering almost all four ecological zones of rice growing areas in Pakistan and are preserved in gene-bank at Institute of Agri-Biotechnology & Genetic Resources, National Agricultural Research Center, Islamabad (Table 1).

RAPD analysis: Genomic DNA was isolated from rice seeds using method of Kang *et al*., (1998) with minor modifications. Thirty-five decamer oligonucleotides belonging to Operon series (OP) were used for analyzing polymorphisms. After an initial screening, 28 primers were selected on the basis of polymorphism and scorable banding patterns. A modified method of Williams *et al*., (1990) was used to perform PCR amplification. Amplification reactions mix of 20µl contained 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP), 0.2µM of 10-mer primer, 1 unit *Taq* DNA polymerase (Fermantas) and 20ng of template genomic DNA. Amplification was programmed to a 1 cycle of 5 minutes at 94^{o}C for initial strand

separation. This was followed by 45 cycles of 1 minute at 94°C for denaturation, 1 minute at 36°C for annealing and 2 minutes at 72°C for primer extension. Finally, 1 cycle of 7 minutes at 72°C was used for final extension. Amplified products were resolved on a 1.5% agarose gel in 1xTBE (10mM Tris-Borate, 1mM EDTA) buffer containing 0.5µg ml^{-1} of Ethidium bromide along with 1kb standard DNA ladder (Fermantas). After electrophoresis, the gels were documented using UV illuminator system.

Data analysis: Presence or absence of amplified products was scored as '1' or '0', respectively. Only major bands, which amplified consistently, were scored. Size of the amplified products was measured by comparing known size of 1kb plus molecular weight marker. Pair-wise comparisons of the accessions based on the presence or absence of unique and shared amplification products were used to generate similarity coefficients. 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). A dendrogram was constructed using minimal variance algorithms method (Ward, 1963) to assess genetic variability and relationships among the landraces.

Table 1. List of local landraces and improved cultivars of Pakistani rice used in the present study.

Zone	Province	District	Total	Accession number / cultivar
I	Punjab (22 districts)	Sheikhupura	08	6514, 6515, 6516, 6595, 6597, 6599, 6655, 6740
		Gujranwala	05	6505, 6549, 6563, 6605, 6611
		Gujrat	03	6507, 6683, 6684
		Sargodha	03	6570, 6588, 6711
		Pindibhatiyan	03	6527, 6530, 6620
		Leyyah	01	6558
		Narowal	02	6693, 6694
		Sialkot	06	6603, 6613, 6621, 6663, 6664, 6670
		Hafizabad	01	6509
		Muzaffargarh	02	6705, 6706
		D. G. Khan	04	6537, 6717, 6719, 6642
		Rajanpur	02	6590, 6593
		Khanewal	02	6724, 6729
		Faisalabad	06	6622, 6626, 6627, 6633, 6578, 6582
		Lahore	02	6654, 6698
		Pasrur	02	6658, 6577
		Multan	03	6758, 6751, 6755
		Lodhran	02	6560, 6731,6722
		Okara	01	6676
		Jhang	01	6734
		Sahiwal	01	6756
		Kasur	01	6745
III and IV	Sindh (4 districts)	Sukkur	01	6760
		Badin	01	6779
		Larkana	01	6766
		Sangarh	01	6771
II	NWFP (3 districts)	Swat	03	6564, 6638, 6623
		Dir	02	6519, 6520
		Malakand	01	6574
Check varieties		RRI, Kala Shah Kaku		Super-basmati
		RRI, Dokri, Sindh		IR6
		ARI, Mingora, Swat		JP5

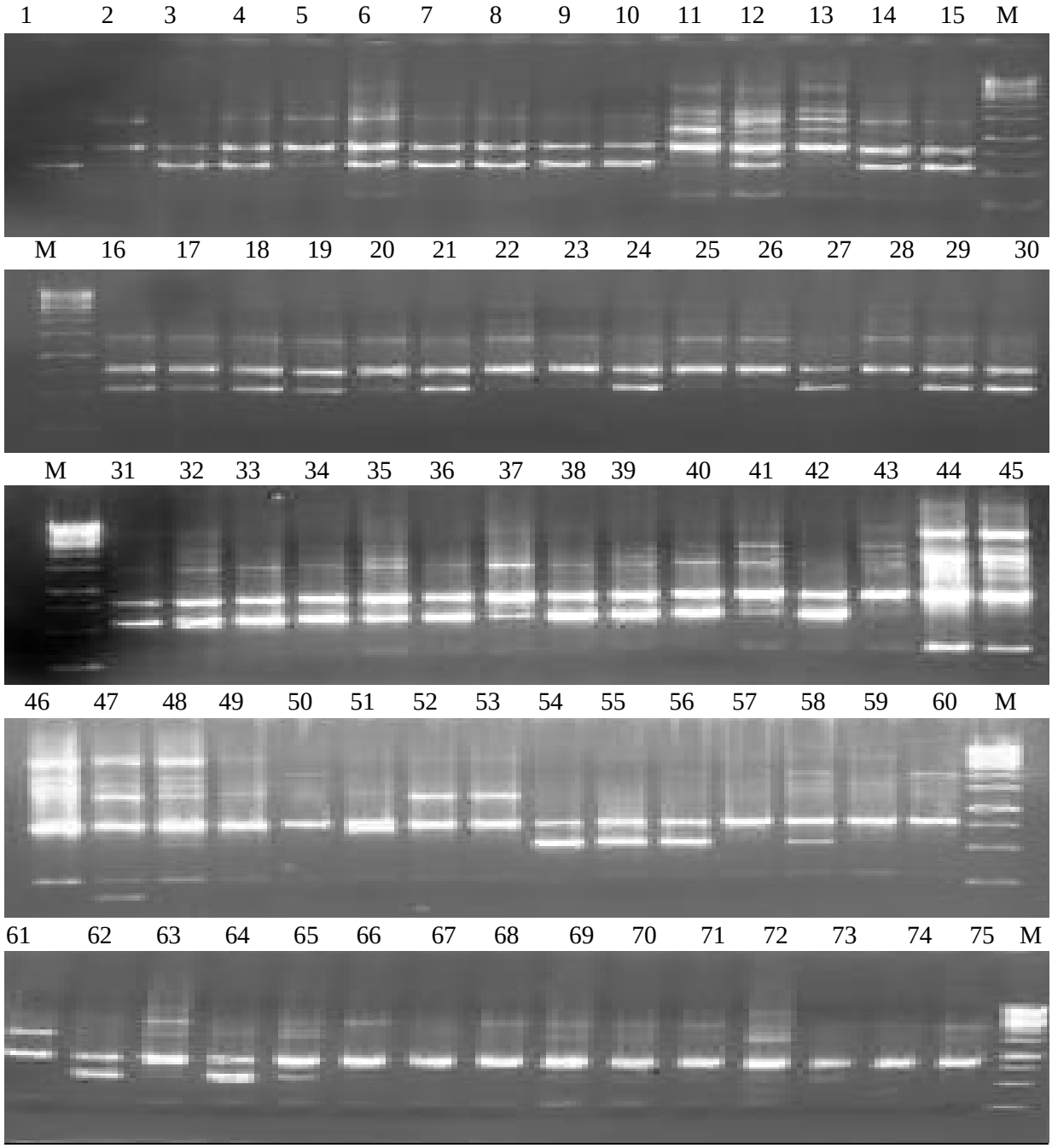

Fig. 1. RAPD banding profiles of 75 Pakistani rice landraces generated by primer OPC-01.

Results

RAPD fingerprints were generated to evaluate genetic variation among 75 rice accessions and check cultivars (Table 1) using 28 random decamer oligonucleotide primers generating informative and easily scorable RAPD profiles. Figure 1 shows the amplification profiles generated with primer OPC-01 across 72 landrace genotypes and 3 commercial check varieties. Most of the landraces possessed similar banding patterns as in case of monomorphic alleles. A check variety 'JP5' shared least number of bands with rest of the accessions showing it's more distant behavior with other rice landraces. Instead it shared more common alleles with aromatic basmati types as compared to non-aromatic coarse genotypes. Landraces 6638 (Swat), 6621 (Sialkot) and 6626 (Faisalabad) displayed unique banding profile as compared with all other genotypes. A number of primers produced characteristic fragments among these three landraces, not amplified in

any other, while 'IR6' and 'Super-basmati" shared their most of the fragments with other landraces. Some landraces with same local name and from same location but with different accession numbers e.g., Lal Dhan-304 (Sargodha) (6570 and 6711) possessed different banding profiles.

Among 35 decamer oligonucleotide primers, 28 were selected on the basis of their varying ability to resolve variability among landraces. A total of 145 fragments were amplified across 75 genotypes with 80% (116) polymorphism (Table 2). The number of amplified fragments generated by each primer varied from 3 (OPK-11 and OPK-12) to 9 (OPA-09 and OPK-02) with an average of 5.2 fragments. The size of the amplified products ranged from 250 (OPK-12) to 4000bp (OPB-13). Landraces 6588 (Sargodha), 6582 (Faisalabad), 6578 (Faisalabad) and 6595 (Sheikhupura) revealed novel alleles of 1450bp, 600bp, 570bp and 550bp, respectively with primer OPA-09, OPA-16 and OPA-17.

Similarity matrix based on RAPD profiles was used to reveal the extent of variability among landraces and their genetic relationship with three check varieties. Landrace 6622 (Faisalabad) showed close association with 6655 (Sheikhupura) and 6711(Sargodha) (0.92). Accession 6570 (Sargodha) showed least similarity coefficient of 0.71 value with 6603 (Sialkot). Pairwise estimates of similarity coefficients ranged from 0.71 to 0.92. Similarity coefficients ranged from 0.77 to 0.88 for Super-basmati and IR6 with all other landraces. Almost 55 and 26% landraces showed similarity coefficients more than 0.80 with IR6 and Super-basmati, respectively. Only 19% landrace genotypes possessed more than 80% similarity in banding profile with JP5 and similarity coefficients ranged from 0.73 to 0.88 (data not shown).

Table 2. Primers used for generating RAPDs in 75 landraces and cultivars of rice from Pakistan.

Sr. No.	Primer's name	Sequence (5'-3')	Amplified bands	Polymorphic bands	Polymorphism (%)	Size range (bp)
1	OPA-02	TGCCGAGCTG	5	4	80	400-1350
2	OPA-03	AGTCAGCCAC	4	2	50	300-1500
3	OPA-04	AATCGGGCTG	6	4	66.6	500-1600
4	OPA-09	GGGTAACGCC	9	7	77.7	450-1450
5	OPA-10	GTGATCGCAG	6	3	50	400-1000
6	OPA-16	AGCCAGCGAA	4	3	74	500-1200
7	OPA-17	GACCGCTTGT	4	4	100	550-2800
8	OPA-18	AGGTGACCGT	4	2	50	400-1600
9	OPB-08	GTCCACACGG	7	6	85.7	350-2500
10	OPB-12	CCTTGACGCA	4	4	100	700-2000
11	OPB-13	TTCCCCCGCT	6	4	66.6	500-4000
12	OPB-18	CCACAGCAGT	5	4	80	600-3100
13	OPC-01	TTCGAGCCAG	5	4	80	300-2700
14	OPC-07	GTCCCGACGA	4	4	100	250-1800
15	OPC-10	TGTCTGGGTG	6	5	83.3	400-1900
16	OPC-12	TGTCATCCCC	7	7	100	270-1500
17	OPC-15	GACGGATCAG	4	3	75	250-1200
18	OPF-01	ACGGATCCTG	8	8	100	300-1700
19	OPF-14	TGCTGCAGGT	4	4	100	300-2200
20	OPJ-02	CCCGTTGGGA	4	2	50	250-1200
21	OPJ-08	CATACCGTGG	4	4	100	300-900
22	OPK-01	CATTCGAGCC	3	2	66.6	350-1500
23	OPK-02	GTCTCCGCAA	9	9	100	300-3000
24	OPK-06	CACCTTTCCC	6	3	50	300-2800
25	OPK-10	GTGCAACGTG	3	2	66.6	600-1300
26	OPK-11	AATGCCCCAG	3	3	100	400-700
27	OPK-12	TGGCCCTCAC	3	2	66.6	250-700
28	OPK-17	CCCAGCTGTG	8	7	87.5	300-1800
Total	**28**		**145**	**116**	**80**	**250-3100**

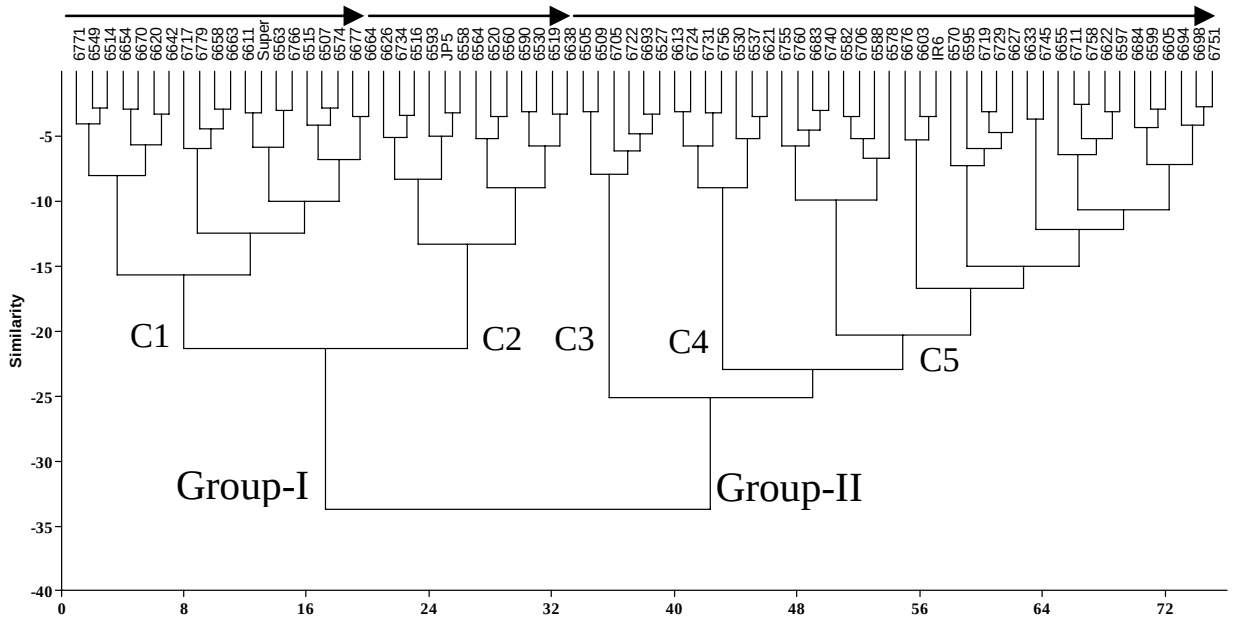

Fig. 2. UPGMA cluster analysis showing the diversity among rice landraces of Pakistan based on 145 RAPD fragments generated by 28 random primers.

A dandrogram produced by clustering using Ward's minimal variance algorithm method, grouped all the landraces into two main groups. One major group constituted aromatic type landraces along-with Super-basmati and JP5, while other group contained around 54% of all the landraces and check variety IR6. Further these clusters were differentiated into 5 sub-clusters. Subcluster-1 consisted of 19 genotypes (26.4%) including Super-basmati, an aromatic check variety. Subcluster-2 constituted 13 landraces (18%) including JP5, while subcluster-3 and 4 grouped six and seven landraces from southern Punjab, respectively. Thirty-eight percent of the landraces from different zones of Pakistan comprised subcluster-5 along-with IR6, a coarse check variety.

Discussion

A clear and thorough knowledge of the extent of genetic variation and inter and intra-specific relationship is essential for devising strategies to efficiently utilize and maintain the rice germplasm conservations. In the investigation reported here, RAPD markers were used to examine the relationships among 72 landrace accessions collected from different locations within the country including 3 commercial cultivars. Uses of RAPDs as a tool to study the genetic diversity and relationships among different cultivars have previously been reported (Chalmers *et al*., 1992; Landry *et al*., 1994; Porreca *et al*., 2001; Neeraja *et al*., 2002; Rabbani *et al*., 2008). A considerable level of genetic variability was observed among landraces; cultivars 'Super-basmati' and 'IR6' shared limited number of fragments with majority of the landraces. Also lower numbers of bands were common among landrace genotypes and japonica type check variety (JP5) demonstrating that Pakistani landraces are closer to indica varietal group.

RAPD assay interferes in true diversity analysis by producing alleles from both homozygous and heterozygous conditions leading to reduced diversity estimates. However some modifications in RAPD procedure allow estimation of the genetic parameters (Lynch & Milligan, 1994). Similarity index for accessions varied from 0.72 to 0.91 with an average value of 0.80. Similar levels of diversity have been reported previously among various panels of rice genotypes under different analysis (Davierwala *et al*., 2000; Ren *et al*., 2003). However, relatively higher similarity value (90%) in

comparison with 25 to 77.5% (Raghunathachari *et al*., 2000) could be the result of reduced intra-specific variations in Pakistani rice due to the common ancestors and the selection of primers for few selected traits as compared to Indian scented rice germplasm.

DNA fingerprinting helped in grouping of rice landraces at subspecies level. In cluster analysis, most of the Pakistani landraces fell into a close sub-group corresponding to aromatic rice. Thirty of the 72 landraces, which have similar characteristics for various morphological traits, were grouped into the upper portion of the dendrogram. This may lead to conclusion that majority of Pakistani landraces are Basmati. Recent reports by Kovach *et al*., (2009) demonstrated that basmati-like accessions were nearly identical to the japonica type regardless of their fragrance phenotype, demonstrating a close evolutionary relationship between Basmati varieties and the japonica gene pool.

Ren *et al*., (2003) also reported that RAPD based dendrogram supported the clustering of two distinct groups with exceptions. This diversity analysis also differentiated most of the landraces, which shared same local name but different accession numbers e.g., Jhona-129 (6620), Jhona-145 (6626), Jhona-101 (6745) collected from different geographical locations. Medium grained Jhona-129 (6620) grouped with Super-basmati and Jhona-101 (6745) was grouped with 'IR6', while short grained Jhona-101 (6626) was grouped with 'JP5'. Similarly Rohru-414 (6593) and Rohru-150 (6578) were entirely different in banding pattern. Studies conducted previously using RAPD markers grouped the long-grain basmati cultivars into a single cluster, whereas the other short-grained rice landraces fell into a different group (Rabbani *et al*., 2008). They reported a low level of variability among Pakistani basmati rice varieties using RAPD markers.

It is concluded that the Pakistani rice landraces possess considerable variation in their genetic base. The study showed that most of the Pakistani landraces are morphologically indica type and genetically grouped with 'Super-basmati' as well as 'IR6' check variety. Grouping of 'JP5' and 'Super-basmati' into one major cluster is an indication that basmati rice is closer to japonica type than with indica group. Genetic makeup of a crop species is not affected by environment and only expression profiles can be the targets of environment proved in present study as the landraces collected from different geographical zones grouped together on the basis of RAPD banding profiles. The landraces with wide genetic distance can be used as parents to exploit heterosis in future rice breeding programs. The rare or unique alleles observed can further be employed for marker assisted selection programs. The primers which proved more informative can be converted to sequence tagged sites (STS) and sequence characterized amplified regions (SCAR) for amplification of specific alleles which could be further utilized in rice genome analysis. Also the information gained from clustering behavior of accessions can be useful to design strategies for their management in the gene-bank.

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