Use of Bulk Segregant Analysis for Varietal Identification and Genetic Diversity Estimation in Pakistani Basmati and Non-Basmati Rice Cultivars through Molecular Markers

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Abstract

Identification of alleles for a particular variety through Bulk Segregant Analysis (BSA) is a valuable technique that probes differences specifically in distinguishing traits. Our study was an attempt to develop variety specific marker which differentiate aromatic rice cultivars from non-aromatic. Bulk Segregant Analysis (BSA) approach with RAPD and ISSR assay was undertaken to trace the variety specific alleles among 2 group of popular Pakistani Basmati and non-Basmati rice genotypes to check adulteration by rice exporters. Out of 160 RAPDs and 30 ISSR used in BSA, 29 RAPD and 18 ISSR revealed consistent polymorphisms and were used to assort the advanced disparities amongst 10 varieties. Overall 262 of 359 random amplified alleles and 116 of 151 inter simple sequence alleles were found polymorphic. The number of alleles generated from RAPD and ISSR marker ranged from 5 to 19 and 3-18 respectively. Pair-wise genetic similarity among the varieties with jaccard coefficients was 0.85 for RAPDs and 0.79 for ISSRs. UPGMA dendrogram based on cluster analysis of individual and combine genetic similarity co-efficient resolved 10 rice cultivars into 2 major groups, Basmati and Non-basmati. Moreover, a total of 3 variety specific alleles of 650, 800, 1075 bp were amplified by random primers OPN-05 and OPE-09. While ISSR generated five (UBC-814, UBC-811, UBC-808, UBC-808a and UBC-82) rare alleles for specific genotypes with band sizes of 1500, 1400, 250, 950 and 720 bp respectively. Direct sequencing of rare allelic amplified bands in Sequence Characterized Amplified Regions (SCARs) and sequence tagged sites (STS) might behave useful for recreating phylogenic trees with aim of preserving the integrity of Basmati rice.

Keywords: Bulk segregant analysis; Basmati rice (*Oryza sativa* L.); Genetic diversity; RAPD; ISSR; Varietal identification

Introduction

In recent years, emergence of Asian aromatic rice as a profitable business and globalization of world trade has necessitated formulation of legal tools for rice cultivar protection, certification and registration. The characterization of rice germplasm is obligatory not only for the identification of various cultivars but also to find out genetic relationships among them. Pakistan is globally recognized for cultivating and exporting long-grain aromatic basmati rice and its land is legendary for the production of extremely delicious rice with special characters i.e., aroma, high paste and extra-long grain [1].

In rice exporting countries, adulteration of rice varieties is awfully common. Several techniques (paddy rice visual examination, chemical method and DNA based analysis) have been employed over the last few decades, targeted specifically to the elimination of this problem. As an important export item, proper inspection and sophistication in preserving rice seed quality is imperative which is possible through development of variety specific markers. In addition to it, being evolved from extremely selected lines basmati breeding lines have narrow genetic base contributing only to the grain quality, aroma and high yield [2]. Characterization of the genetic resources is therefore, instantly needed because the strength of the genetic diversity is a requisite for world food security and endurance of human civilization.

DNA markers are valuable tools to resolve the genetic structure of a rice collection and to interpret the evolutionary associations among different groups [3]. Several techniques have been employed for diversity assessment but Bulk Segregant Analysis (BSA) along with RAPD and ISSR markers have proven to be useful in genetic

fingerprinting, genotype identification, tagging the important genes for aroma, cooked kernel elongation, amylose content etc. and gene mapping [4]. BSA approach has been utilized to increase narrower genetic variation since this approach engrosses screening of differences between 2 pooled DNA samples [5]. The combined use of RAPD [6] and ISSR markers [7] allows for a high level of genomic coverage as RAPD markers are potentially associated with functionally important loci [8] and ISSR markers amplify hypervariable non-coding regions [9]. RAPD and ISSR are multi locus profiling techniques able to distinguish genotypes below the species level, such as cultivars and clones, and have been used in numerous diversity studies [10]. Moreover, the advantage of random pairing of primers lies in increasing the variability of polymorphic fragments and also in reducing the cost of designing primers. RAPD and ISSR primers have been successfully used for cultivar analysis in a number of plant species including rice [11]. Present research work was conducted to assess genetic diversity among different Basmati and Non-Basmati varieties via BSA and to identify markers linked to specific commercial varieties via BSA approach. The

information obtained will be useful for adulteration testing as well as variety improvement breeding programs by minimizing the risk of genetic erosion.

Materials and Methods

Plant material and DNA extraction

10 *Indica* type Pakistani rice genotypes from two groups i) Basmati (Super Basmati, Basmati-198, Basmati-2000, Basmati-385, Basmati-Pak and Shaheen Basmati) and ii) Non-basmati (Pk-386, Supri, Supra and Super Fine) were selected and grown in field of Agriculture Biotechnology Division, National Institute of Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan (Table 1). Total genomic DNA of each genotype was extracted from fresh leaves by CTAB method [12] whereas, quality and quantity of DNA was checked on 1.2% agarose gel and by spectrophotometer, respectively. The extracted DNA was diluted to a final concentration of 50 ng μL^{-1} with 1 X TE buffer and stored at -20°C.

Bulk Seggregant RAPD and ISSR analysis

Two bulked DNA samples are generated to discriminate Basmati from Non-Basmati varieties. The bulks are screened for differences using RAPD assay. A total of 160 RAPD primers from Operon Technologies Inc, Alabama, USA series C, D, E, R and Gene Link Series A, B, M, N were used. RAPD amplification reaction were carried out in a 20 μL reaction containing 40 ng genomic DNA, 3 μL of primer, 100 μM dATP, dCTP, dGTP and dTTP, 1 unit of Taq polymerase (fermentas), $1 \times$ Taq polymerase buffer and 2.5 mM $MgCl_2$. DNA amplification reaction was performed for RAPD-PCR as: Initial denaturation of 5 min at 95°C, 40 cycles of 94°C for 1 min (denaturation), 37°C for 1 min (annealing), 72°C for 2 min and final extension at 72°C for 10 minutes according to Williams et al. [6]. Same samples were analyzed by 30 ISSR markers of which 18 showed good amplification and were used for analysis. Amplification was performed in a 25 μL reaction mixture containing 40 ng of genomic DNA, 0.3 μM each primer, $1 \times$ Taq DNA polymerase reaction buffer, 1.5 U of Taq DNA polymerase and 0.2 mM each dNTPs [13]. Reproducibility of amplified RAPD and ISSR markers were tested for their ability to prime PCR amplification of three selected cultivars with independent amplifications from three independent DNA isolations per cultivar. For the choice of RAPD/ISSR primers, unambiguous and qualitative (present or absent) fragments that gave repeatable patterns two or three times with the same cultivar were considered. Primers that amplified consistently reproducible polymorphisms were selected and used to analyze all the 10 cultivars. The amplified products of both RAPD and ISSR were resolved on 1.2% agarose gels with ethidium bromide staining, photographed by UV light and scored for the presence and absence of DNA fragments. The PCR product was scored by means of polymorphic banding pattern.

Data analysis

The amplified products were scored for the presence (1) or absence (0) of bands of various sizes across the genotypes to generate a binary matrix. To quantify the genetic shift, a similarity matrix was generated from the binary data of RAPD and ISSR individually and combinely using Jaccard coefficients in program of XLSt at 2014. Cluster analysis, done with the UPGMA constructed dendrogram based on similarity co-efficient was used to assess pattern of genetic diversity among rice genotypes. After determining that each string of 1 and 0 scores was a unique array (each individual had a unique string of traits), all possible pair wise genetic distance values were calculated by Jaccard indexes [14]. Polymorphic information content (PIC) values were calculated for each RAPD and ISSR primer according to the formula: $PIC=1-\Sigma(P_{ij})^2$, where P_{ij} is the frequency of the ith pattern revealed by the jth primer summed across all patterns revealed by the primers [15]. Principal Coordinate analysis (PCoA) was performed using GenAlEx version 6.501.

Results

Bulk segregant analysis and cultivar identification

Among the tested markers, 55 primers were found to be polymorphic in bulk segregant RAPD analysis. Out of these markers, 29 showed lucid and consistent banding patterns for amplification of each cultivar were ultimately chosen for examining the genetic diversity and discrimination among the cultivars used (Table 2). While 18 polymorphic ISSR markers exhibited several bands that were shared among the basmati and other fine cultivars, whereas a few bands were obtained from some primers that revealed variety specific bands. Amplification profile revealed by two of the random polymorphic markers (OPN-05 and OPE-09) shows a characteristic variety specific band (Figure 1).

Number of alleles and percentage polymorphism

The level of polymorphism among these rice cultivars was estimated by calculating allele number and polymorphism for each of the evaluated 29 RAPDs and 18 ISSR. A total of 262 polymorphic alleles were detected using these markers. The index of polymorphism, which was estimated as the proportion of variable random loci in the total number of loci studied, was 70.4% (average over all loci), varying from 25-94.4% depending on primers used (Table 2). The number of alleles per locus generated by each RAPD marker varied from 5 to 19 with an average of 12.4 alleles per locus. Maximum numbers of 17 polymorphic alleles were obtained with the marker GML-18, while the minimum 2 alleles/marker was amplified with OPC-04 and OPD-04, with an average of 9 polymorphic bands.

Group	Local Name	Remarks
Basmati	Super Basmati	Strong aroma, extra-long grain, excellent cooking quality but very late maturing
	Basmati-198	Moderately aromatic, long grain but short stature, late maturing
	Basmati-2000	Strongly aromatic, extra-long grain, excellent cooking quality High yielding, local variety
	Basmati-385	Aromatic, long grain, excellent cooking quality after Super Basmati, short stature, early maturing, High yielding
	Basmati-Pak (Kernal Basmati)	Moderately aromatic, extra-long grain, excellent cooking quality, local variety, late maturing, tall, High yielding,
	Shaheen Basmati	Moderately aromatic, extra-long grain, excellent cooking quality, local variety
Non-Basmati	PK-386	High yielding, **Non-aromatic**, long grain, early maturing variety, poor cooking quality
	Supri	High yielding, **Non-aromatic**, long grain, early maturing variety
	Supra	High yielding, **Non-aromatic**, long grain, early maturing variety
	Super fine	High yielding, **Non-aromatic**, long grain, early maturing variety

Table 1: List of Rice varieties used for RAPD and ISSR analysis.

S No	Primers	Sequence (5'-3')	Number of loci		Polymorphism (%)	Maj. Allele freq.	Var. Spec band	PIC
			Total	Polymorphic				
RAPD primers								
1	GLN-05	ACTGAACGCC	14	12	85.7	0.2	1	0.8680
2	GLN-09	TGCCGGCTTG	9	6	66.6	0.4	2	0.7300
3	GLN-11	TCGCCGCAAA	14	10	71.4	0.2		0.8442
4	GLN-12	CACAGACACC	13	10	76.9	0.3		0.7978
5	GLN-14	TCGTGCGGGT	15	13	86.6	0.3		0.8232
6	GLN-15	CAGCGACTGT	10	5	50	0.8		0.3142
7	GLN-16	AAGCGACCTG	11	6	54.5	0.2		0.8196
8	GLN-19	GTCCGTACTG	18	13	72.2	0.3		0.8232
9	GLN-20	GGTGCTCCGT	11	7	63.6	0.3		0.7978
10	GLA-04	AATCGGGCTG	10	5	50	0.4		0.7014
11	GLA-17	GACCGCTTGT	14	11	78.5	0.1		0.8910
12	GLB-01	GTTTCGCTCC	7	6	85.7	0.4		0.6756
13	GLB-08	GTCCACACGG	12	10	83.3	0.2		0.8680
14	OPC-04	CCGCATCTAC	8	2	25	0.4		0.5632
15	OPD-04	TCTGGTGAGG	5	2	40	0.6		0.5700
16	OPE-03	CCAGATGCAC	16	9	56.2	0.2		0.8442
17	OPE-04	GTGACATGCC	13	11	84.6	0.3		0.7724
18	OPE-06	AAGACCCCTC	7	6	85.7	0.2		0.8660
19	OPE-09	CTTCACCCGA	7	3	42.8	0.6		0.5200
20	GLM-02	ACAACGCCTC	13	11	84.6	0.3		0.8232
21	GLM-03	GGGGGATGAG	14	10	71.4	0.3		0.7716
22	GLM-04	GGCGGTTGTC	16	12	75	0.2		0.8680
23	GLM-09	GTCTTGCGGA	15	14	93.3	0.1		0.8910
24	GLM-11	GTCCACTGTG	15	13	86.6	0.2		0.8680
25	GLM-13	GGTGGTCAAG	12	9	75	0.2		0.7754
26	GLM-15	GACCTACCAC	14	7	50	0.3		0.7210
27	GLM-16	GTAACCAGCC	9	6	66.6	0.3		0.7042
28	GLM-18	CACCATCCGT	18	17	94.4	0.2		0.8823
29	GLM-20	AGGTCTTGGG	19	16	84.2	0.2		0.6562
	Total		**359**	**262**		**8.7**	**3**	**22.05**
	Average		**12.4**	**9**	**70.4**	**0.3**	**1.5**	**0.760**
ISSR Primers								
1	UBC-807	AGAGAGAGAGAGAGAGT	11	6	54.5	0.4		0.7300
2	UBC-808	AGAGAGAGAGAGAGAGC	18	11	61.1	0.1	1	0.8680
3	UBC-810	AGAGAGAGAGAGAGAGT	13	10	76.9	0.2		0.8910
4	UBC-811	GAGAGAGAGAGAGAGAC	6	4	66.6	0.2	1	0.7942
5	UBC-814	CTCTCTCTCTCTCTCTA	6	5	83.3	0.5	2	0.6730
6	UBC-816	CACACACACACACACAT	6	5	83.3	0.6		0.6980
7	UBC-820	GTGTGTGTGTGTGTGTC	11	10	90.9	0.1	1	0.8910
8	UBC-821	GTGTGTGTGTGTGTGTC	9	8	88.8	0.4	1	0.7578
9	UBC-824	TCTCTCTCTCTCTCTCG	7	6	85.7	0.2		0.8442
10	UBC-826	ACACACACACACACACC	14	12	85.7	0.3		0.8232
11	UBC-827	ACACACACACACACACCG	7	5	71.4	0.3		0.7978
12	UBC-828	TGTGTGTGTGTGTGTGA	5	4	80.0	0.4		0.7300
13	UBC-829	TGTGTGTGTGTGTGTGC	6	5	83.3	0.3		0.7716
14	UBC-834a	AGAGAGAGAGAGAGAGCT	5	3	60.0	0.8		0.2688
15	UBC-834b	AGAGAGAGAGAGAGAGTT	3	1	33.3	0.5		0.4918
16	UBC-836a	AGAGAGAGAGAGAGAGCA	7	4	57.1	0.3		0.7716
17	UBC-836b	AGAGAGAGAGAGAGAGTA	11	10	90.9	0.2		0.8442
18	UBC-840	GAGAGAGAGAGAGAGATT	9	8	88.8	0.2		0.8680
	Total		**151**	**116**		**6**	**6**	**13.5**
	Average		**8.882**	**6.82**	**76.99**	**0.33**	**1.2**	**0.75**

Table 2: RAPD and ISSR primers used to detect polymorphism, No. of polymorphic bands among different cultivars per primer.

Eighteen ISSR primers showing reproducible and polymorphic patterns were chosen for cultivar identification and generated a total of 151 bands, with an average of 8.8 bands per primer. The size ranged from 250 to 3000 bp. Of the 151 bands produced, 116 (76.9%) were polymorphic. The number of polymorphic bands detected with each primer ranged from 3 (UBC834b) to 18 (primer UBC808) with an average of 6.82 per locus (Table 2).

Rare alleles

Alleles with a frequency less than 5%, were recognized as rare alleles. Generally, markers identifying a greater number of alleles per locus manifest more rare alleles. In our study, a total of 9 rare alleles were observed, 3 from 262 random polymorphic loci and 6 from ISSR loci. The cultivar Pk-386 with primer OPN-05 displayed a unique band of about 650 bp in comparison with all other cultivars. Interestingly, another primer OPE-09 revealed characteristic fragment of 1075 bp in Super Fine and 800 bp in each Supra and Pak-386 which were not produced in any of the other cultivars used (Figure 1). Among ISSR markers, five markers were cultivar specific and able to distinguish them clearly.

Similarity matrix and cluster analysis

A similarity matrix based on the proportion of shared alleles between RAPD and ISSR was used to establish the level of relatedness between the cultivars surveyed (Table 3). Pair-wise genetic similarity coefficients by RAPD markers among these varieties varied from 0.527 to 0.849 with an average similarity of 0.67 (Table 3 lower diagonal). The genetic relationship among the rice cultivars was assessed by a cluster analysis of the similarity matrix. Dendrogram based on RAPD marker revealed that at about 0.61 similarity coefficients, the 10 rice genotypes grouped into two major clusters, which effectively differentiated the basmati from non-basmati cultivars (Figure 2). Landraces with aroma trait (Basmati-Shaheen, Basmati-385, Basmati-Pak, Basmati-2000, B-198 and Super Basmati) showed limited variability and clustered together in group-I at similarity co-efficients of 0.68 (68%). Group II included Non-Basmati, non-aromatic quality cultivars i.e., Supri, Super fine, Supra and Pak-386, which were clustered together at similarity coefficient of 69.6% (0.696). Maximum percentage of genetic variation was present within the groups i.e., 72.9% followed by 27% diffrences among cluster. This Variance among and within the populations are highly significant.

The ISSR genetic similarity values based on Jaccard's coefficient ranged from 0.4 between 'Supri' and 'SB' to 0.89 between 'B.000 and B. Pak (Table 3 upper diagonal). A dendrogram that was constructed according to ISSR data of ten rice cultivars allowed us to divide them into two main clusters (Figure 3). First cluster included all Basmati varieties i.e., Basmati-Shaheen, Basmati-385, Basmati-Pak,

Basmati-2000, B-198 and second included all non-Basmati varieties i.e., Super fine, Supra and Pak-386 except Supri. The greatest similarity (78%). was observed between species B. Pak and B.2000 (Figure 3). A dendrogram constructed from combine data generated by RAPD and ISSR markers also clearly discriminate Basmati varieties from Non-Basmati group (Figure 4).

Principal Coordinate Analysis (PCoA)

In Principal Coordinate Analysis (PCoA) the first three coordinates explained 64.2% of the total variation, 27.4% explained by the first coordinate with 22.1% and 14.7% explained by the second and third coordinate respectively. These coordinates (I and II) included all Basmati genotypes except Shaheen Basmati and B.198 while coordinates (III and IV) grouped all Non-Basmati varieties (Figures 5I and 6).

Discussion

Basmati rice from India and Pakistan known as special aromatic rice is circumscribed commercially for gene transformation, and hence, has been maintained with little variability across populations. Due to high value of Basmati rice in foreign exchange, its adulteration is a common issue. Moreover, Varietal homozygosity and lack of inter-population variation among Basmati varieties reinforce the determination of its genetic structure and identification of variety specific marker. Bulk segregant analysis (BSA) is a fast and inexpensive approach that avoids the necessity to genotype population on individual basis. The technique also allows probes candidate genes for a trait to be tested for differences in allele frequency between lines selected to differ specifically in that
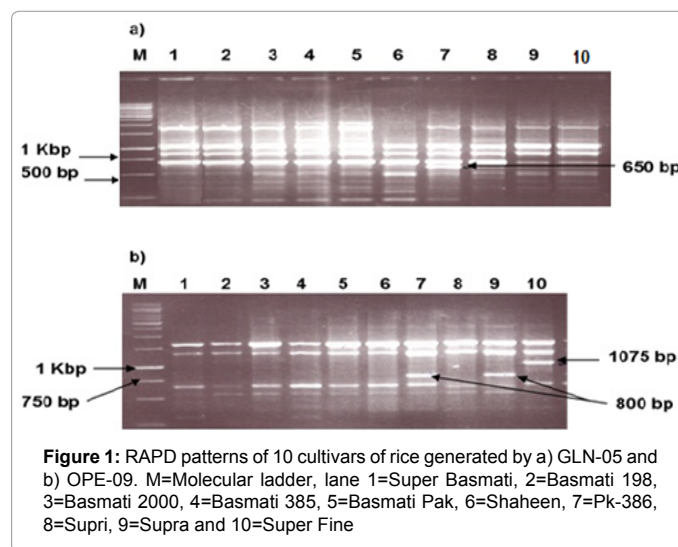

Figure 1: RAPD patterns of 10 cultivars of rice generated by a) GLN-05 and b) OPE-09. M=Molecular ladder, lane 1=Super Basmati, 2=Basmati 198, 3=Basmati 2000, 4=Basmati 385, 5=Basmati Pak, 6=Shaheen, 7=Pk-386, 8=Supri, 9=Supra and 10=Super Fine

	SB	B- 198	B-2000	B- 385	B-Pak	B-Shaheen	Pk-386	Supri	Supra	Super Fine
SB	****	0.638	0.752	0.722	0.821	0.613	0.584	0.403	0.561	0.588
B-198	0.669	****	0.604	0.604	0.600	0.532	0.514	0.417	0.546	0.482
B-2000	0.688	0.822	****	0.719	0.788	0.623	0.581	0.457	0.530	0.585
B-385	0.678	0.738	0.810	****	0.772	0.681	0.637	0.470	0.571	0.572
B-Pak	0.710	0.740	0.799	0.774	****	0.633	0.661	0.435	0.569	0.634
B-Shaheen	0.683	0.743	0.777	0.849	0.807	****	0.517	0.443	0.504	0.536
B-386	0.573	0.623	0.642	0.593	0.653	0.668	****	0.504	0.666	0.628
Supri	0.547	0.620	0.631	0.591	0.613	0.618	0.739	****	0.490	0.583
Supra	0.572	0.673	0.659	0.640	0.654	0.621	0.678	0.716	****	0.605
Super Fine	0.527	0.570	0.597	0.593	0.608	0.629	0.676	0.765	0.695	****

Table 3: Genetic similarity coefficients of 10 rice genotypes based on RAPD (below diagonal) and ISSR (upper diagonal) markers.
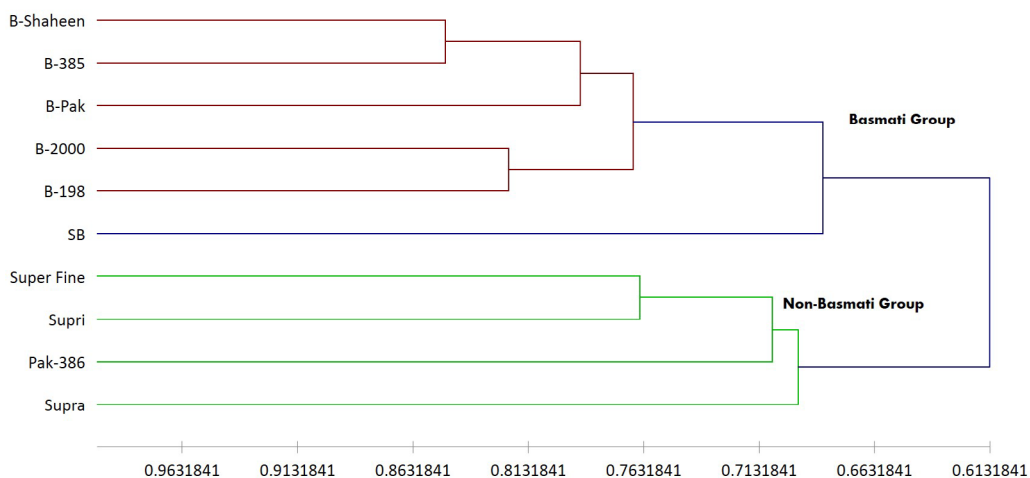
Figure 2: UPGMA dendrogram showing genetic similarity and phylogenetic relationship based on 29 RAPD primers between 10 rice genotypes. Scale on bottom is jaccard coefficients of similarity.
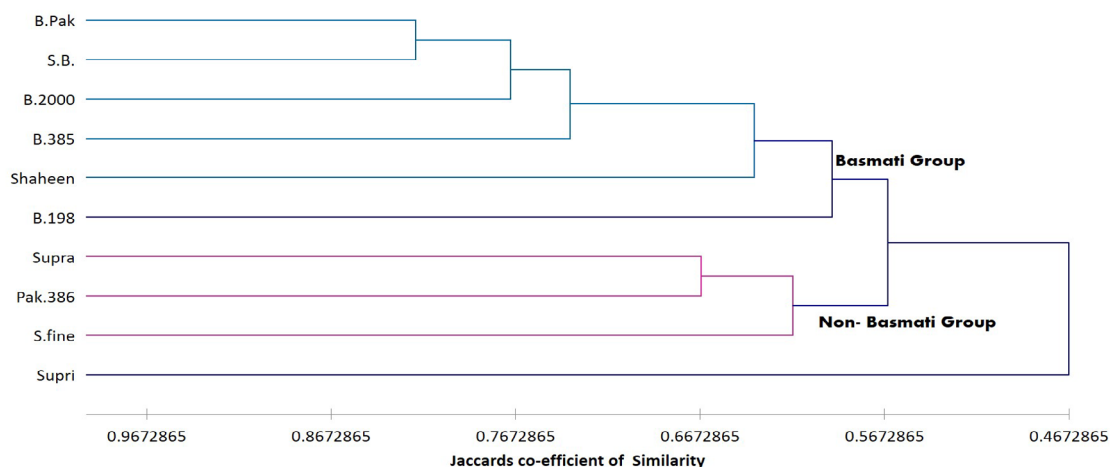

Figure 3: UPGMA dendrogram showing genetic similarity and phylogenetic relationship based on 18 ISSR primers between 10 rice genotypes. Scale on bottom is jaccard coefficients of similarity.

trait. In past few years, several analyses have been performed using RAPD and ISSR-BSA to solve genome wide relationships among different species. RAPDs have successfully been used for cultivar analysis in a number of plant species including walnut [16], wheat [17], lolium [18], and stevia [19] for being technically simpler than other DNA based markers. The advantage in the use of ISSR marker lies in their linkage to SSR loci which are likely to mark gene rich regions [20]. The variety specific markers using 10-mer arbitrary primers have been successfully used in distinguishing the rice varieties of distinctly different origins and with distinctly different characteristics.

In our study, a considerable level of variability was observed among ten different cultivars, though, in most of the cases, basmati and other aromatic cultivars exhibited similar banding patterns in BSA. RAPD markers exhibited several bands that were shared among the basmati and other non-basmati fine cultivars, whereas few primers generated the distinctive bands that were not amplified in other tested genotypes and were considered to be variety specific alleles. Allele number and polymorphism for each of the 29 evaluated RAPDs primer differed

significantly in its ability to determine variability among the cultivars and ranged from 17 to 2 alleles per marker with an average of 12.4 alleles per locus. This observed pattern was reliable with the report based on much larger RAPD markers with same sample size [21]. Identified level of polymorphism with 262 random amplified polymorphic alleles was ranged from 25-94.4% depending on primers used (Table 2). Similar levels of polymorphism have been reported previously among various panels of rice genotypes under different analysis [22]). It has been reported that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system [23]. One of the reasons for this high level of polymorphism could be that the intra-specific variation in rice is extensive [24].

Previously various studies has already been done with RAPD and ISSR markers for variety specific allele mining and genetic diversity estimation identification such as in cardamom [25] and this marker has proved its efficiency. Here ISSR analysis was an efficient tool for diversity analysis, and differentiation of rice landraces on the basis of polymorphism. Basmati landraces except B. 198 (Figure 3 Cluster
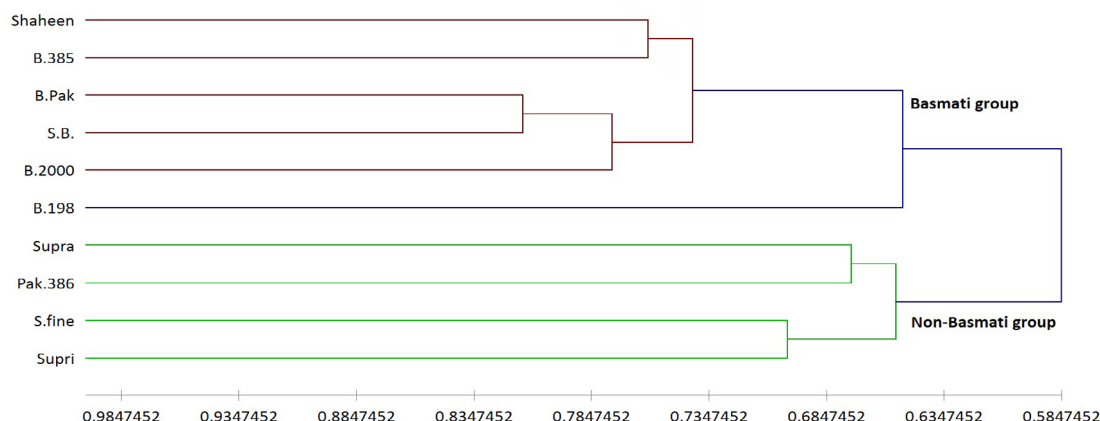
Figure 4: UPGMA dendrogram showing genetic similarity and phylogenetic relationship based on 29 RAPD and 18 ISSR primers between 10 rice genotypes. Scale on bottom is jaccard coefficients of similarity.
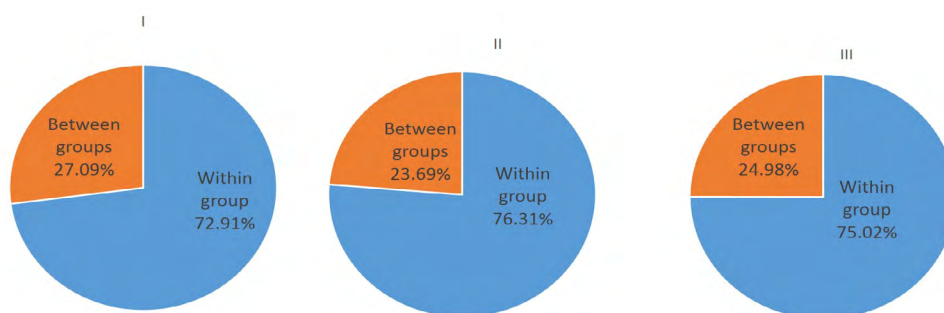

Figure 5: Pie chart showing Percentage of variance for I. RAPD markers II. Both RAPD and ISSR markers III. ISSR markers.
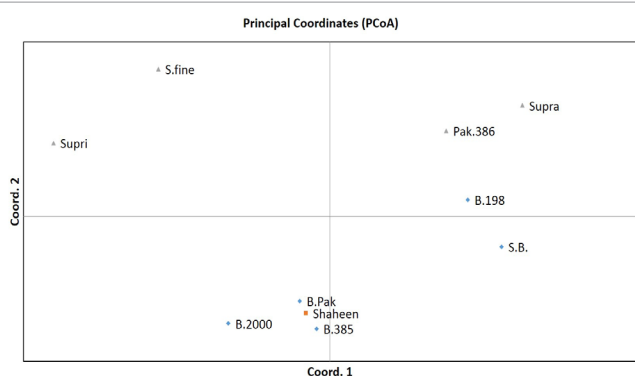

Figure 6: Principal coordinates (PCoA) analysis of the combined data from ISSR and RAPD markers.

I) were grouped together showing similarity at genomic level and difference from other landraces. The reason for B.198 out grouping by ISSR marker might be the diverse genetic background because it meets with Basmati-Pak at o.56. B.198 is also dissimilar with Non-Basmati varieties Supri (7.7) and Pak-386 (7.1%). Thus, these two groups are distinctly diverse (4.85%). The intraspecific polymorphism (75.02%) observed by ISSR markers is higher than interspecific piolymorphism (24.98%) (Figure 5III).

High PIC values can be attributed to the use of more informative markers [26]. It was also found that the higher the PIC value of a locus, the higher the number of alleles detected [27]. The PIC values indicated by GLA-17 (0.89) and UBC-810 (0.89) might be the best marker for diversity analysis of rice varieties while UBC-834 (0.26) and GLN-15 (0.314) are least polymorphic primers (Table 2). Thus, these loci may be useful tools in future genetic studies of rice germplasm.

An allele that was observed in only one or two of the 10 cultivars was considered rare. Our study identified 3 rare alleles of sizes, 650 bp in variety Pk-386 with primer OPN-05, 1075 bp in Super Fine and 800 bp in each Supra and Pak-386 with OPE-09 (Figure 1). Among ISSR, UBC-814 produces allele of approximately 350 bp in Supri and Basmati. 386. This marker also amplifies a rare allele in Super. Fine of approximately 1500 bp in size. Rare allele for Supri was detected by UBC-820 with band size of 950 bp while in case of B. Pak and S. Basmati, rare allele of sizes 1400 bp and 250 bp was observed in UBC811 and UBC 808 respectively. Moreover, UBC-821 amplifies Super Fine and Basmati. 385 specific allele of size 720 bp that was absent in other varieties. The presence of rare alleles in these cultivars indicates that the materials could be used to estimate the adulteration in basmati varieties and useful for plant breeders and geneticists for future breeding programs as well.

Values of genetic diversity in terms of similarity matrix explained the probability of two randomly chosen alleles originated from different ancestors. In our study, Pair-wise genetic similarity coefficients with RAPD marker varied from 0.527 to 0.849 with an average similarity of 0.67 (Table 3 lower diagonal). This 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. Moreover, these findings are in consistent with our previous findings among 19 rice genotypes

of Pakistan using 40 RAPDs with genetic similarity values ranged from 0.4-0.85 [28]. Another study on 42 elite rice varieties which have been commonly used in Indian breeding programs also reported narrow genetic variability in improved cultivars and similarity values ranged from 0.59 to 0.95 [29].

Cluster analysis based on jaccards similarity co-efficient [14] divide the 10 rice cultivars into two major clusters, which effectively differentiated the basmati (Basmati-Shaheen, Basmati-385, Basmati-Pak, Basmati-2000, B-198 and Super Basmati) and other quality rice cultivars from the long non-aromatic coarse cultivars (Supri, Super fine, Supra and Pak-386). These findings are consistent with the studies conducted by some others reports [30,31]. Supri and Super fine in cluster II were found 76.5% similar while Pk-386 met both of these varieties at 0.708 genetic similarity coefficients. Moreover, landrace supra were found 69.6% similar to these varieties. A higher genetic similarity co-efficient of 68% among Basmati cultivars in cluster I revealed low genetic variability and conserveness of alleles. B-Shaheen and B-385 were very close with similarity co-efficient of 85% while B-2000 showed 82% (0.822) similarity with B-198. Moreover, Super Basmati variety with RAPD markers was found distinct from other aromatic varieties in Basmati group and met them at genetic similarity estimates of 0.685 (68%). Similarly, B.198 with ISSR markers showed outcrossing. The most plausible explanation of this is, it might be due to very specific characteristics present in Super Basmati and B.198 but absent in remaining aromatic cultivars. Dendrogram also revealed that cultivars in the same group usually shared a high proportion of ancestry and/or agronomic characteristics such as height, maturity, quality traits etc. Overall genetic diversity among aromatic varieties was quite low (32%). This was expected because of evidences of close association of morphological characters due to selection for similar characteristics [22] and their origin from similar parent B-370.

The dendrograms generated by RAPD and ISSR markers grouped the cultivars differently. This can be explained by two factors: differences in the number of markers (29 and 18 by RAPD and ISSR, respectively) generated by the two techniques, and the fact that ISSR primers target-specific genome regions, whereas RAPD primers amplify arbitrary regions [32]. For instance, the UPGMA cluster analysis of the cultivars based on combined RAPD and ISSR data illustrated that the dendrogram at a similarity of 0.65, resulted in two clusters, with five Basmati cultivars falling to main cluster I and Non-Basmati to cluster II (Figure 4). In addition, the grouping obtained from PCoA confirmed that obtained from UPGMA dendogram.

Our results demonstrated the ability of BSA along with RAPD and ISSR markers to provide a baseline for variety specific marker development. The adaptation of these identified rare alleles in Sequence Characterized Amplified Regions (SCARs) and sequence tagged sites (STS) will be useful for checking Basmati adulteration and effective in rice breeding programs by preserving the integrity of basmati rice. This will also provide the baseline for future management, planning and research.

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