SSR ANALYSIS OF CHROMOSOMES 3 AND 7 OF RICE (*ORYZA STAIVA* L.) ASSOCIATED WITH GRAIN LENGTH

KASHIF ASLAM[*] AND MUHAMMAD ARIF

National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan
*Corresponding author's e-mail:kashif.nibge@gmail.com

Abstract

Grain size and weight determine crop yield in cereals, whereas in rice grain size and shape is major attribute to identify market value and also used for classifying rice genotypes. Rice germplasm collection and knowledge of genetic diversity are required for variety improvement. Molecular markers provide powerful tool for identification of genetic variation and mapping of gene/ QTLs. There are a lot of gene/QTLs were identified by different groups on chromosome 3 and 7 controlling grain length. Clustering based on grain length divided the 48 accessions into two major clusters with some contradiction. Genetic relationships among the 48 rice accessions were determined based on allelic diversity using Power Marker tree, structure analyses and PCA using 51 SSR markers located on chromosome 3 and chromosome 7. Two-dimensional PCA scaling and power marker tree analysis showed high-level of differentiation between Basmati and indica rice accessions and divide these rice accessions in two distinct clusters.

Introduction

Grain size and weight contribute for crop yield in cereals, whereas in rice, grain size and shape are major criteria to assess market value and to classify rice genotypes. Grain size with its dimensions for length and width has become a target trait for rice breeding in recent years (Xing & Zhang, 2010). Preferences for grain size and grain shape varies widely between countries; some like long and cylindrical grains (USA and Europe) and others go for short and round grains including China, Japan, and Korea (Bai *et al.,* 2010). Rice varieties show huge amount of variation in grain size (Juliano &Villareal, 1993).

Cultivated rice evolved from minimum two independent domestication events, resulting in the indica and japonica subspecies (Cheng *et al.,* 2003). There are three known subspecies under *Oryza sativa* L. i.e. indica, japonica and javanica subspecies indica originated from India and South China region; japonica subspecies from Japan, Korea, and North China; and javanica from Java and Indonesia. Genetic diversity is an acute section of biodiversity as being a possible source of significant traits variation (Karp *et al.,* 1997; Hughes *et al.,* 2008). The study conducted by (Garris *et al.,* 2005) on rice germplasm indicated five distinct groups in *Oryza sativa* L. named as indica, tropical japonica, temperate japonica, aromatic and aus using SSR markers. Mostly basmati cultivation varieties were developed by Basmati-370 as one of the parents (Rabbani *et al.*, 2010).

Many individual quantitative trait loci (QTLs) studies for grain size have been carried out. These individual studies reported hundreds of QTLs, out of which very few were reported by dozens of studies with different genetic background (Lin *et at*., 1995; Tan *et al.,* 2000; Thomson *et al.,* 2003: Li *et al.,* 2004a, 2004b; Lei *et al.,* 2006; Bai *et al.,* 2010; Shao *et al.,* 2010). Out of many independent studies for identification of QTLs for grain length in rice, located on chromosome 3 and chromosome 7 has been reported number of times in different genetic background. Tsunematsu *et al.,* (1996) mapped two QTLs for grain length on chromosomes 3 and 7, by using F_7 population derived from a cross between Asominari and IR64. Redona

& Mackill (1998) also found seven QTLs for grain length and grain shape were mostly controlled by loci on chromosomes 3 and chromosomes 7. Tan *et al.,* (2000) identified the QTLs for appearance characteristics of rice, and suggested that grain length and grain width were individually controlled by one or two major QTLs and minor QTLs. Numbers of grain length genes was reported by Wan *et al.,* (2006) on chromosome 2, 3, 5, 7 & 9 with varying phenotypic variation 5.8 to 35.6 in three different environments using SSR and EST markers. Dong & Zheng (2002) studied steamed-rice shape and detected three QTLs for length on chromosomes 2, 3 and 10.

The outcome of several studies resulted in identification of QTL for grain length or grain size on chromosome 3 by using different genetic background with biparental populations originating from indica/ indica and indica/japonica crosses (Li *et al.,* 1997; Yu *et al.,* 1997; Xiao *et al.,* 1996; Redonˇa & Mackill 1998; Kubo *et al.,* 2001; Xing *et al.*, 2002; Moncada *et al.,* 2001). Bai *et al.,* (2010) reported QTL for grain length on chromosome7. Shao *et al.,* (2010) also reported QTL on chromosome 7 placed about 13.2 cM away from the QTL qGL7. The genetic separation between these two loci implies that they are distinct from each other. Xu *et al.,* (2002) also identified a QTL associated with variation for grain length (13.9% phenotypic variance) near to the location of qGL7-2. A QTL was found associated with grain length between the interval of SSR markers RM505 and RM248 on chromosome 7 by Zheng *et al.,* (2007). The main aim of this study was to evaluate the correlation between phenotypic diversity analysis and genetic phylogenetic analysis of chromosomes 3 and 7 based on grain length variations.

Materials and Methods

Plant material: In this investigation 48 rice accessions comprising of 10 basmati, 25 non-basmati and 13 IRRI genotypes were used including one land race Basmati 370 which was released in 1933 in Indo-Pak sub-continent. The selection of the genotypes was based on (a) their popularity within Pakistan, India and Philippines (b) diversity for some phenotypic character like grain length, biotic and abiotic resistant etc. as reported in the literature

and these accessions were used for the introgression of biotic and abiotic stress resistant gens in the elite breeding lines. The name of the genotypes and their respective grain length are shown in Table 1.

Phenotypic analysis for grain length: The seeds from 10 plants of each accession, grown at NIBGE, were collected. The grains were submitted to Grain Quality and Nutrition Center (GQNC) International Rice Research Institute (IRRI) for the analysis of dehusked grain length analysis. Unbroken seeds were selected and scanned using a HP scanner. Grain length was measured using image analysis software GIMP 2.8 software (http://www.gimp.org/). Ten seeds of each entry were used for the analysis with three replications and average was taken for further analysis.

Genotypic analysis

SSR genotyping: SSR primers for nuclear DNA analysis were selected from the Gramene data base (http://www.gramene.org/) for chromosome 3 and 7 as presented in Fig. 1. Forty one and thirty SSR markers mapped on chromosome 3 & 7 respectively were surveyed. Thirty SSR markers randomly distributed on remaining chromosomes were also surveyed.

DNA extraction: For DNA isolation leaves were collected from 21 days old seedlings sown in nursery. Murray & Thompson (1980) protocol was followed with some modifications. Rice leaves were ground by pestle and mortar using liquid nitrogen. Ground tissues were transferred in 2ml centrifuge tubes and 800µl of 65°C warm 2x CTAB buffer with 2% mercaptoethanol was added. After mixing, the samples were incubated at 65°C in oven for 30 minutes with intermittent shaking after every 10 minutes. Then 800µl chloroform-isoamly (24:1)

was added and samples were placed on shaker for 15 minutes to allow precipitation of proteins. In the next step, samples were centrifuged at 12000 rpm for 10 minutes by using Beckman coulter centrifuge (Model: microfuge® 18). Aqueous part of the samples was transferred to 2ml deep well plate and chilled isopropanol (600µl) was added. Samples were stored at -20^0C for overnight for DNA precipitation and centrifuged at 12000 rpm for 10 minutes. After air drying the pellets were washed with 70% ethanol and dried in oven at 37°C for 30 minutes. DNA was dissolved in 100µl TE buffer. The DNA of each sample were run on 1% agarose gel for an hour and stained with Sybr® safe to visualize the DNA under UV light.

Polymerase chain reaction (PCR): To screen the primers on diverse population, PCR was performed with total volume of 15µl reaction mixture having final concentration of 1X PCR buffer, 2.5mM $MgCl_2$, 1mM dNTPs, 0.2 µM each of the forward and reverse primers and 1U of homemade Taq polymerase (IRRI). An Allerga 25R centrifuge machine (Beckman Coulter, Brea, CA, SA) was used to mix the components by centrifuging for 1 minute. PCR was performed in G-Strom (Surrey, UK) thermal cycler using PCR profile as 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing 55-60°C for 45 sec and cyclic extension 72˚C for 45 sec, final extension at 72°C for 10 minutes. The amplified products were run on 8% PAGE and after staining with Sybr® safe the gels were viewed under UV light. Gel images taken by Alpha Imager® were placed in the Microsoft Office Excel sheet with accession IDs. The bands on the gel were scored according to the presence from down to up as A, B, C....N bands for each of the accessions.

Table 1. List of rice genotypes used in this study along with their grain length (mm).

| S. #	Varieties	Grain length (mm)	S.N#	Varieties	Grain length (mm)
1.	IRBL12-M	5.4	25.	TKM 6	5.3
2.	IRBLz5-CA	5.0	26.	DR-92	7.2
3.	Supri	7.1	27.	Sufaid 86	7.1
4.	PSBRC80	7.0	28.	PK386	7.2
5.	IRBLt-K59	5.1	29.	Supra	8.3
6.	IRBL9-W	6.1	30.	Apo	5.9
7.	IRBLi-F5	4.8	31.	Basmati 198	7.1
8.	IRBLz-Fu	5.3	32.	Basmati 2000	6.9
9.	NIAB-IR-9	7.4	33.	Basmati 370	6.7
10.	IR 74371 -54-1-1	6.3	34.	IR 78875-131-B-1-1	6.9
11.	Vandana	5.8	35.	Basmati 385	6.7
12.	IR 71525-19-1-1	6.3	36.	Nona Bokra	5.7
13.	IRBB 57	6.6	37.	Pokkali	5.6
14.	Super fine	7.6	38.	IR-6	7.1
15.	IR55419-04	6.3	39.	DR-58	7.1
16.	IR 74371-3-1-1	6.7	40.	IR-64	7.0
17.	Pusa basmati 1121	8.8	41.	IR-24	5.0
18.	IR 74371-46-1-1	6.5	42.	Azucena	6.4
19.	Basmati Pak	7.6	43.	Super Basmati	7.4
20.	IR 78875-131-B-1-4	6.4	44.	IRBL11-Zh	7.6
21.	Shaheen basmati	7.1	45.	IRBL19-A	7.4
22.	DR-82	6.2	46.	KSK Line 99512	7.2
23.	KSK-133	6.7	47.	BAS-515	7.5
24.	KS-282	7.3	48.	KSK Line 99417	7.4
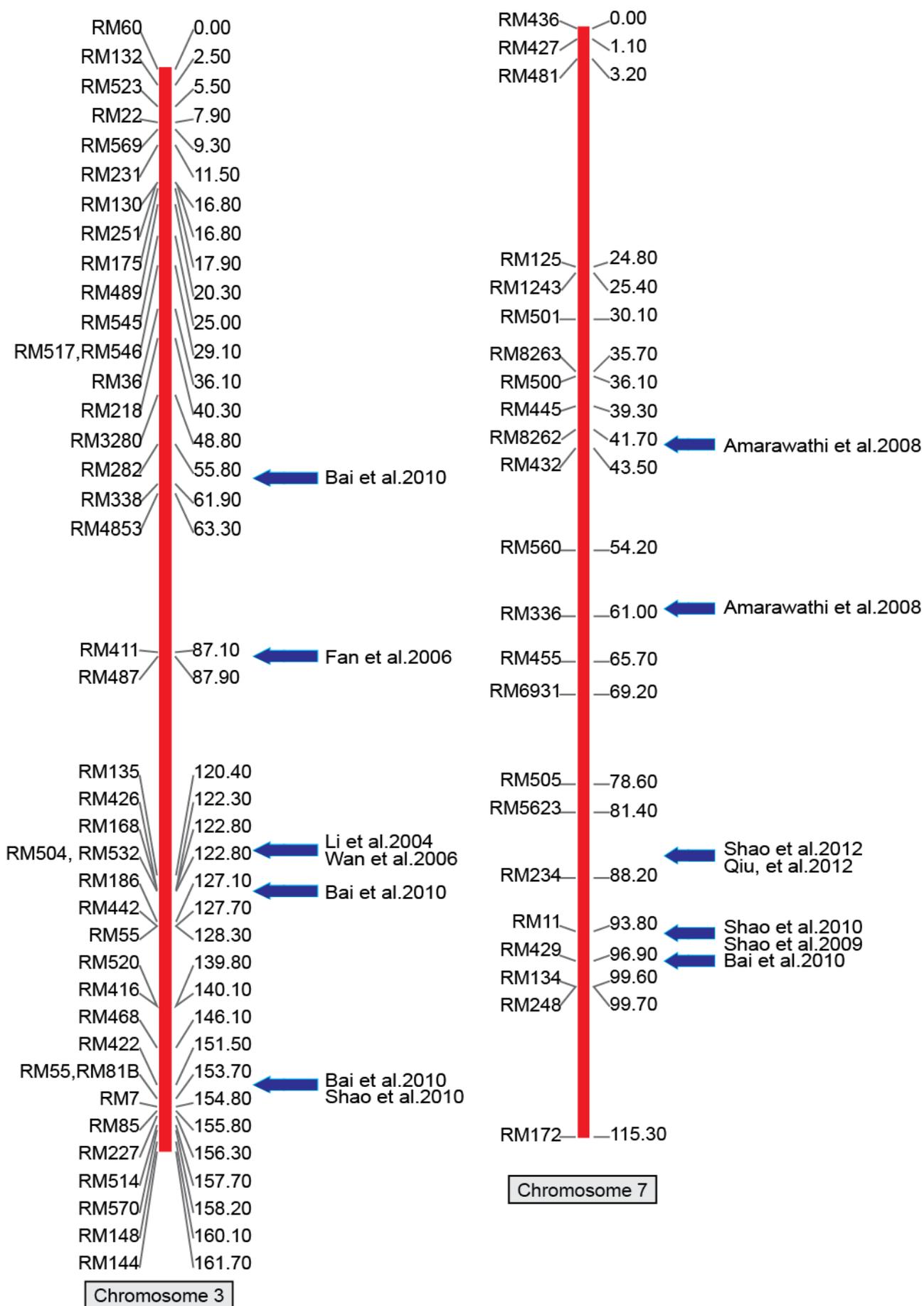
Fig. 1. Position of SSR markers along with position surveyed on chromosome 3 and chromosome 7.

Data analysis: Phenotypic diversity analysis of grain length of 48 germplasm by Gower-paired method was performedwith the help of PAST (PAlaeontological Statistics) (Hammer *et al.,* 2004). Power Marker version 3.25 (Liu & Muse, 2005) (http// www.powermarker.net) was run for phylogenetic analysis and TreeView software was used dendrogram view. For the phylogenetic tree, genetic distance was calculated using the "Nei (1987)" distance matrix followed by phylogeny reconstruction using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. Nei (1987) define the genetic distance as "the genetic distance is the extent of gene differences between populations or species that is measured by some numerical quantity." STRUCTURE software (Pritchard *et al.,* 2000; Falush *et al.,* 2003) was used to conclude population structure using a burning length of 20,000 and run length of 30,000 for K level ranging from 2 to 10. Structure analysis is based on Fst value which estimates the amount of genetic differentiation between population and in subdivision of population. STRUCTURE has a main advantage of providing information about the subgroups and then identifying individuals to their subgroups (Zhang *et al.*, 2011).Matrix plot PCA was performed using NTSYS pc 2.1.

Results

Grain length variation: Phenotypic analysis of 48 rice accessions, using image analysis software, showed statistically significant difference in grain length. Grain length variation was ranged from 4.8mm to 8.8mm. The average grain length was 6.69mm was found among these accessions. Maximum grain length was found in Indian cultivar Pusa Basmati 1121 (8.8mm) showed in Fig. 2 followed by Supra (8.3mm). The minimum grain length was found in non-basmati rice accession IRBLi-F5. However, Basmati rice accessions showed a higher average grain length (7.31mm) in comparison to non-basmati rice accessions (6.52mm).

Phenotypic Diversity: Cluster analysis based on grain length, clearly differentiated the accessions into two major groups. Only one accession from India Pusa Basmati-1121 did not fall into any sub cluster. Group-1 consists of ten accessions with grain length below 6 mm. However, this group can be further subdivided into two sub groups. Group-1a includes Pokkali, IRBLi-K59, IRBL-z5-CA and IRBLz-Fu, while group-1b comprises of four accessions (Vandana, Nona Bokra, TKM6 and IR24) as shown in Fig. 3. The range of grain length variation in group-1a is 4.8 to 5.5mm and group-1b is 5.0 to 5.8mm. Thirty seven accessions were included in group-2. Group-2 was further subdivided into two sub clusters. Group-2a comprises of 21 rice accessions having grain length range from 6.01mm to 7.00mm. However, group-2b comprises of 16 accessions having grain length more than 7.01mm. All Basmati varieties except Basmati 2000 and Basmati 198 were members of group-2b along with other non-basmati long grain accessions Supra, Super Fine NIAB IR-9. The only contradiction in group-2b was supra having grain length of 8.3mm.

SSR marker analysis: Genetic associations among 48 accessions were analyzed, based on phenotypic variation of grain length with the help of 51 SSR markers distributed along the chromosome three and seven. Out of 51 SSR markers, five primers were found monomorphic across all accessions. A total of 154 alleles were amplified ranging from 2-8 numbers of alleles per marker. The mean number of allele was 3.24. Maximum number of alleles (8) were amplified by marker RM 481 marker located on chromosome7 followed by marker RM282 (06) on chromosome 3. The gene diversity ranged from 0.040 to 0.792 with an average of 0.46. The PIC value across markers ranged from 0.040 to 0.762 with an average of 0.402. Maximum PIC on chromosome 7 was 0.762 at marker RM481 followed by marker RM2823 (0.742) as shown in Figs. 4 and 5).

Similarity matrix: The genetic distance of 0.0637 to 0.6520 was observed with an average of 0.2027 among the accessions. The highest genetic distance of 0.6520 was observed between IRBLi-F5 and DR-82 while the highest similarity was observed between Bas-515 and Super basmati because Super basmati was used in the cross of Bas-515 for maintain the basmati traits. On an average, the highest similarity was observed in IR 74371-54-1-1. On other hand, the highest average genetic distance was found for super fine. Among the basmati accessions, maximum genetic distance (0.5490) was observed between Pusa Basmati 1121 and Basmati 385. Pusa Basmati 1121 was derived from P614-1-2 and P614-2-4-3 crossed in India Agricultural Research Institute (IARA) New Delhi having extra-long grain length with good cooking quality.

Phylogenetic analysis based on genotype: Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Cluster analysis with the help of Power Marker based on Nie (1987) clearly divided rice accessions into two groups based on SSR marker analysis (Fig. 6). Group-I comprised of twenty three accessions while twenty five accessions were included in group-II. Group-I was further subdivided into five sub clusters. These five subclusters were comprised of nineteen accessions while four accessions were found with admixed ancestry. Sub cluster -1 consisted of two accessions NIAB-IR-9 and Supra from Pakistan. Four rice accession, constituted the second sub cluster, were from IRRI. Sub cluster three was the biggest cluster of group-I comprised of six accessions. Out of six, four accessions are from Pakistan (DR-58, IR-6, DR-92 and PK-386), one from Philippines (IR78857-131-B-1-4) and one from India (Pokkali). Sub cluster-IV consisted of two accessions while sub cluster five consisted of four accessions. Group-II was further subdivided into four sub clusters. Only one accession from India i.e., Pusa Basmati-1121 did not fall into any sub cluster. Sub cluster-I include seven accessions. The elite basmati rice of Pakistan Super Basmati was the member of this group. Ten varieties were gathered in sub cluster-II. Out of ten rice accessions, seven accessions were from Philippines followed by two rice accessions from Pakistan and one variety from India. Sub group-III includes four basmati accessions from Pakistan including land race Basmati 370. Sub group-IV encircle three accessions was the smallest sub cluster of group-II.

IR24 (Indica)   **Pusa Basmati 1121**   **Super basmati**
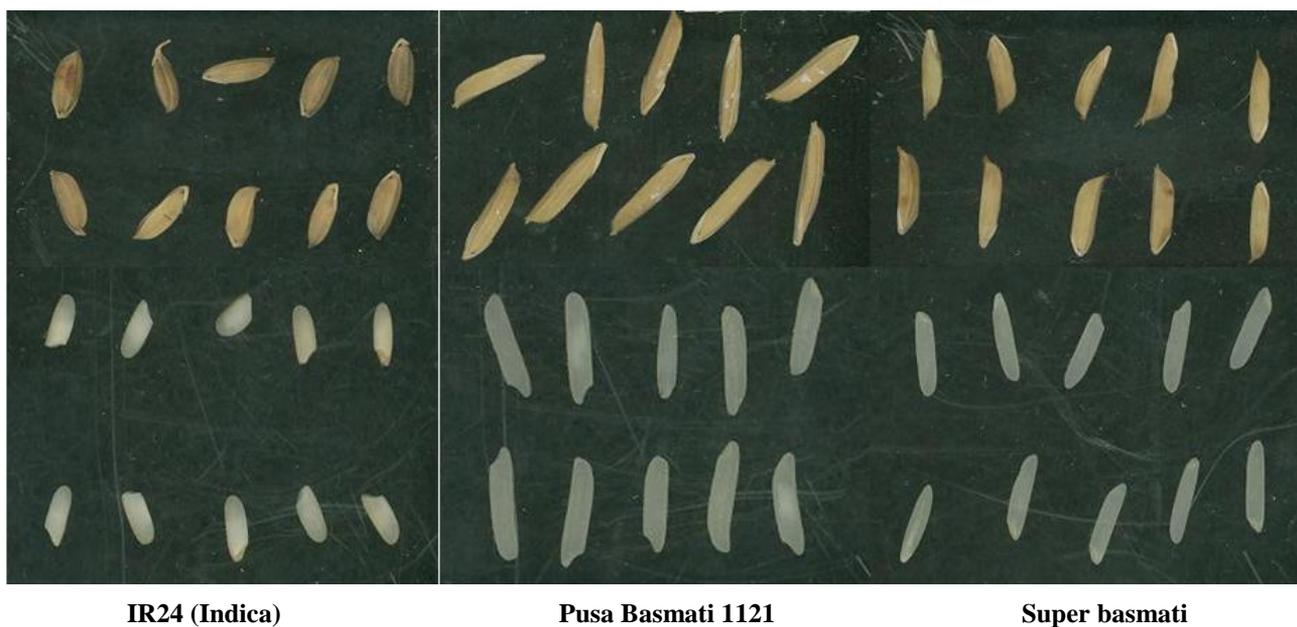
Fig. 2. Grain length of 3 different rice accessions from Philippines (IR24), India (Pusa Basmati 1121 and Pakistan (Super Basmati).
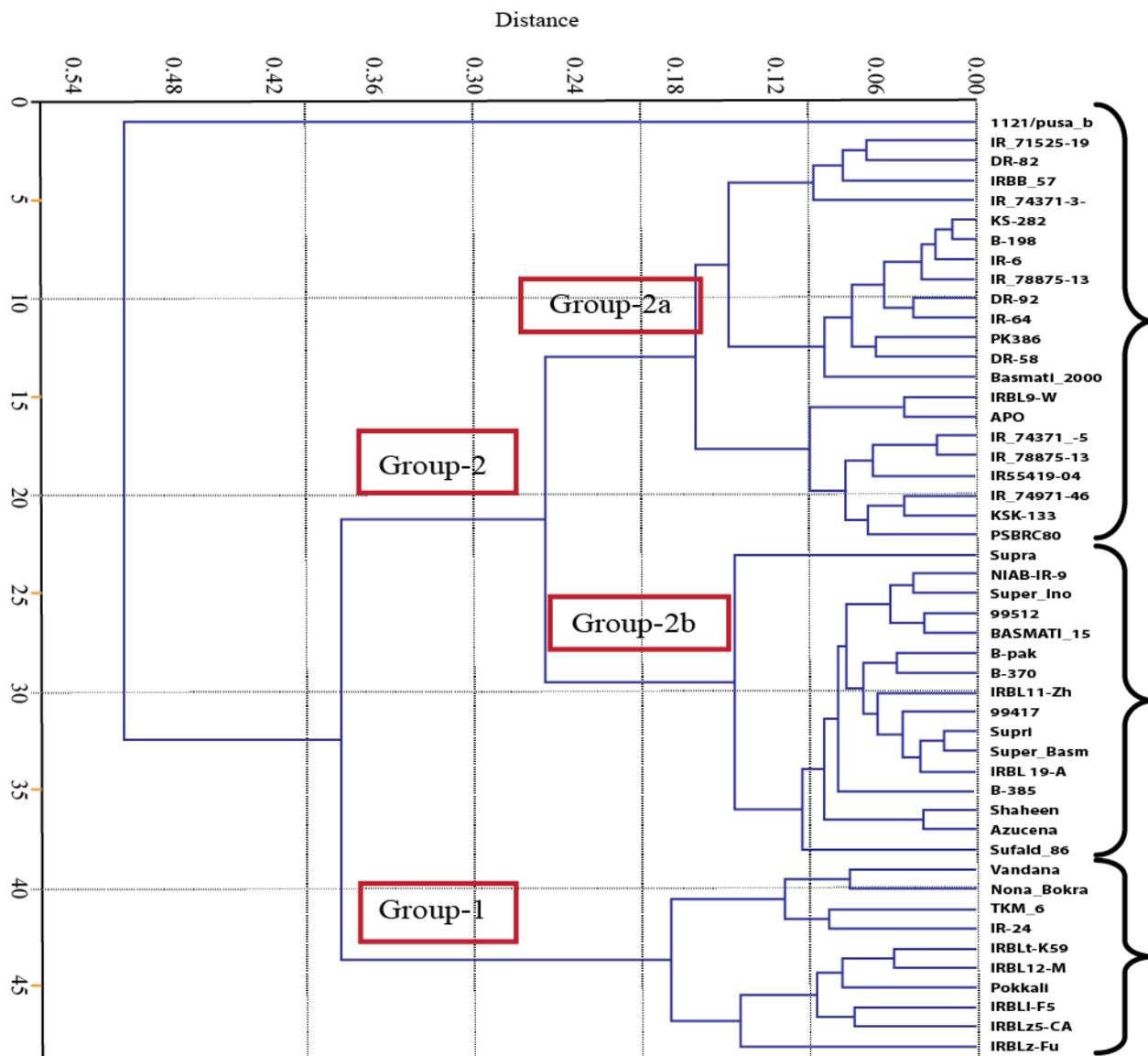

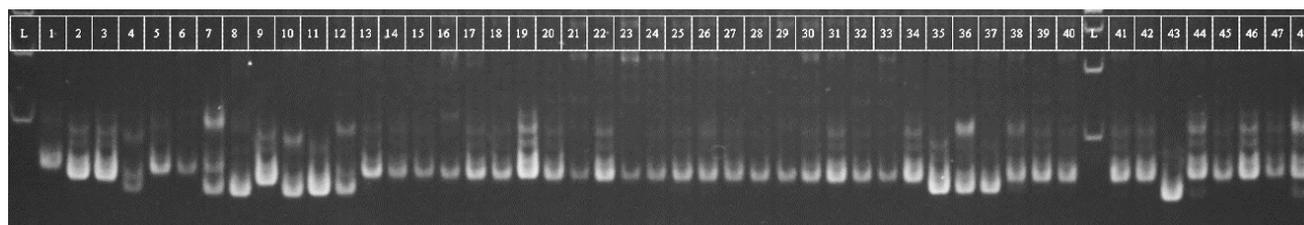Fig. 3. Phylogenetic Analysis of 48 rice germplasm by Gower-paired method on the basis of grain length.

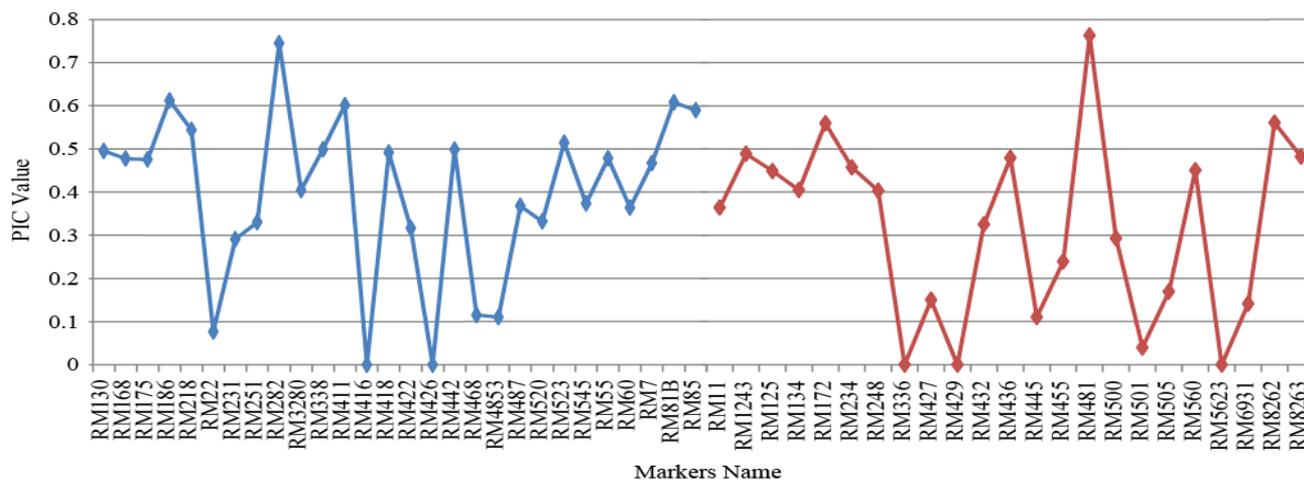Fig. 4. RM338 (183bp) showing the genetic diversity between 48 germplasm on 8 % PAGE.

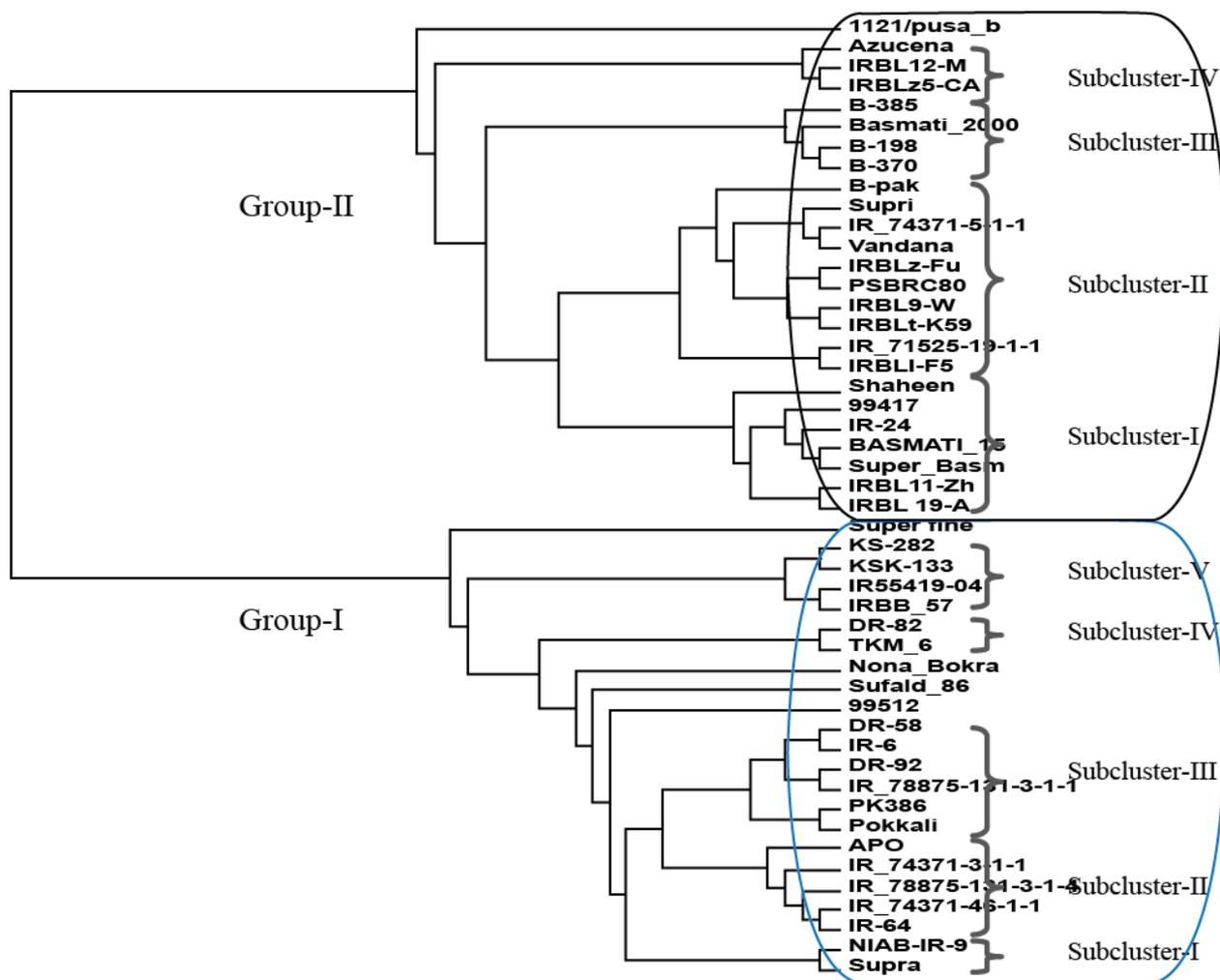Fig. 5. PIC value of SSR marker surveyed on Chromosome 3 (blue) and chromosome 7(red).

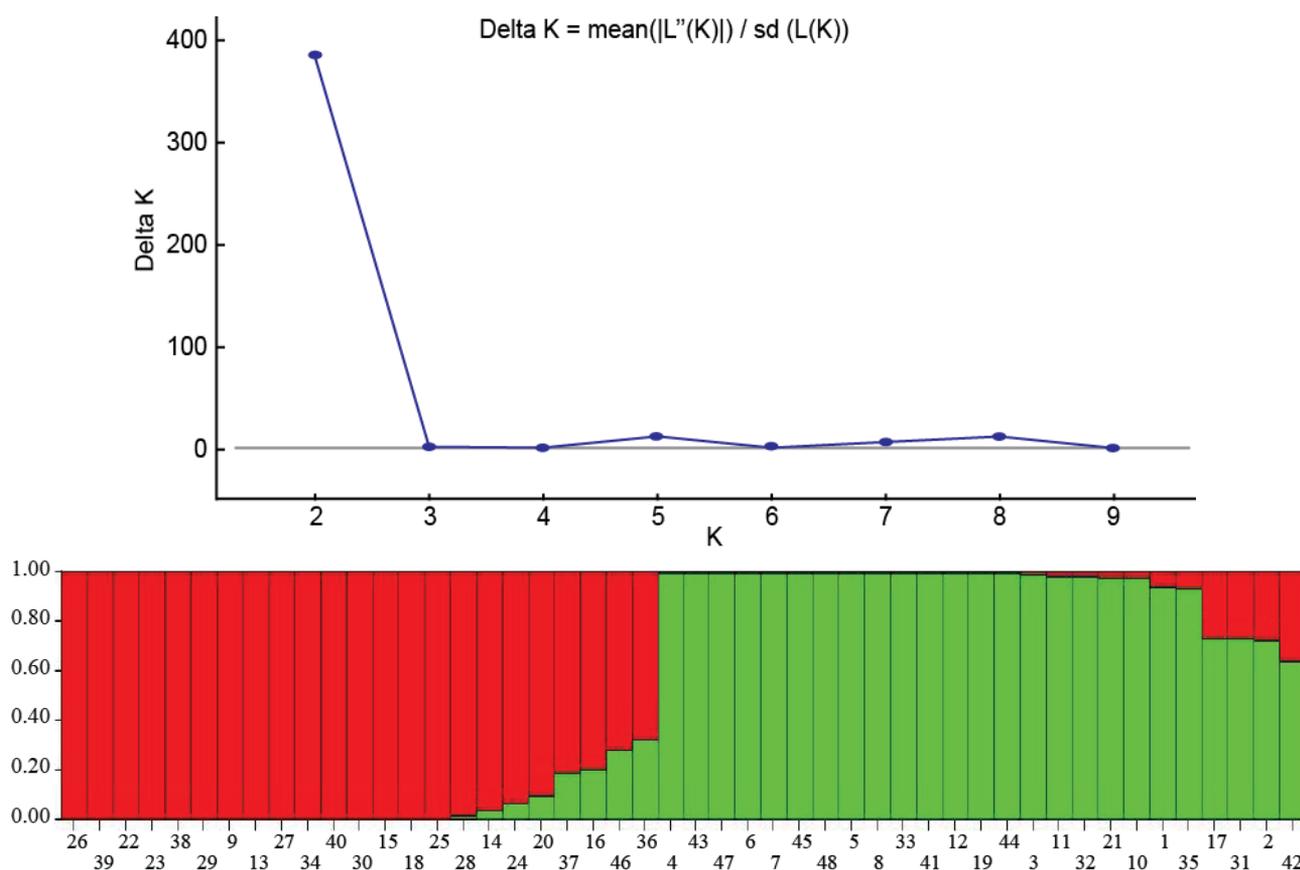Fig. 6. UPGMA tree based on Nei's similarity coefficient of 48 rice accessions.

Fig. 7. Graph showing likely hood of ΔK (a) highest log of likely hood at K=2 Pritchard Structure analysis of 48 genotypes using chromosome 3 & 7 (b) at different K-2.

Population structure: The Pritchard structure analysis of 48 accessions of rice divided these genotypes into 2 major subpopulations using SSR markers on chromosome 3 and 7 as shown in Fig. 7. The online application "structure harvester" determined that subpopulation (K) on the basis of natural log of probability data (LnP(D)) as shown in Fig. 7a. The Fig. 7a showed three peaks out of which 2 peaks has very low LnP(D) so K=2 was selected for the grouping of these rice accession. Group 1 includes mostly indica varieties (15) with 99% purity while Group 2(15 accessions) comprised of mostly Basmati varieties (Basmati 370, Super Basmati, Shaheen Basmati etc.) and some other accessions as shown in Fig. 7. Shaheen Basmati, Basmati 2000, and Basmati 385 showed more than 93% similarities with the traditional basmati (Basmati370). A cross-breed Pusa Basmati 1121 showed 73% similarity with basmati group with the admixture of indica.

Principle coordinate analysis (PCA): A total of 51 SSR markers on 48 rice accessions were used for PCA analysis. Results of two dimensional PCA analyses revealed two major clusters as shown in Fig. 8. Cluster 1 comprises of (IR-64, IR-6 and DR82, DR92, NIAB IR-9, APO) most of accessions were related to indica accessions. However, two lines released from Rice Research Institute (RRI) KSK Pakistan (KS282 and KSK133) found distant from other indica accessions. Cluster 2 can be divided into two sub clusters; sub cluster

2-1 and sub cluster 2-2. sub cluster 2-1 comprises mostly basmati varieties such as Basmati 370 (6), Super Basmati (43), Bas-515(8) and Shaheen Basmati (41). However, all basmati lines showed high genetic diversity on the bases of chromosome 3 and chromosome 7. Basmati Pak (10) and Pusa Basmati 1121 (40) showed more diverse and not falling in sub cluster 2-2. Sub cluster 2-2 includes mostly IRRI accessions such as IRBLt-K59 (30), IRBL9-W (28), IRBLi-F5 (29), IR 71525-19-1-1 (14) and IR 74371 -54-1-1 (15) and two major Indian abiotic stresses resistant varieties such as Pokkali (39) and Vandana (48).

Discussion

Considerable levels of phenotypic variation were observed among aromatic and non-aromatic rice accessions. Tehrim *et al.,* (2012) also reported high level of polymorphism in different agronomic traits. Genetic relationships among 48 rice accessions was determined on the basis of SSR markers located on chromosome 3 and chromosome 7 based on allelic diversity using Power Marker tree, structure analyses and PCA. Two-dimensional PCA scaling and power marker tree analysis showed high-level of differentiation between Basmati and indica rice accessions and divide these rice accessions in two distinct clusters. There is very little information available on genetic diversity of rice subgroups for specific chromosomes (Jain *et al.,* 2006).
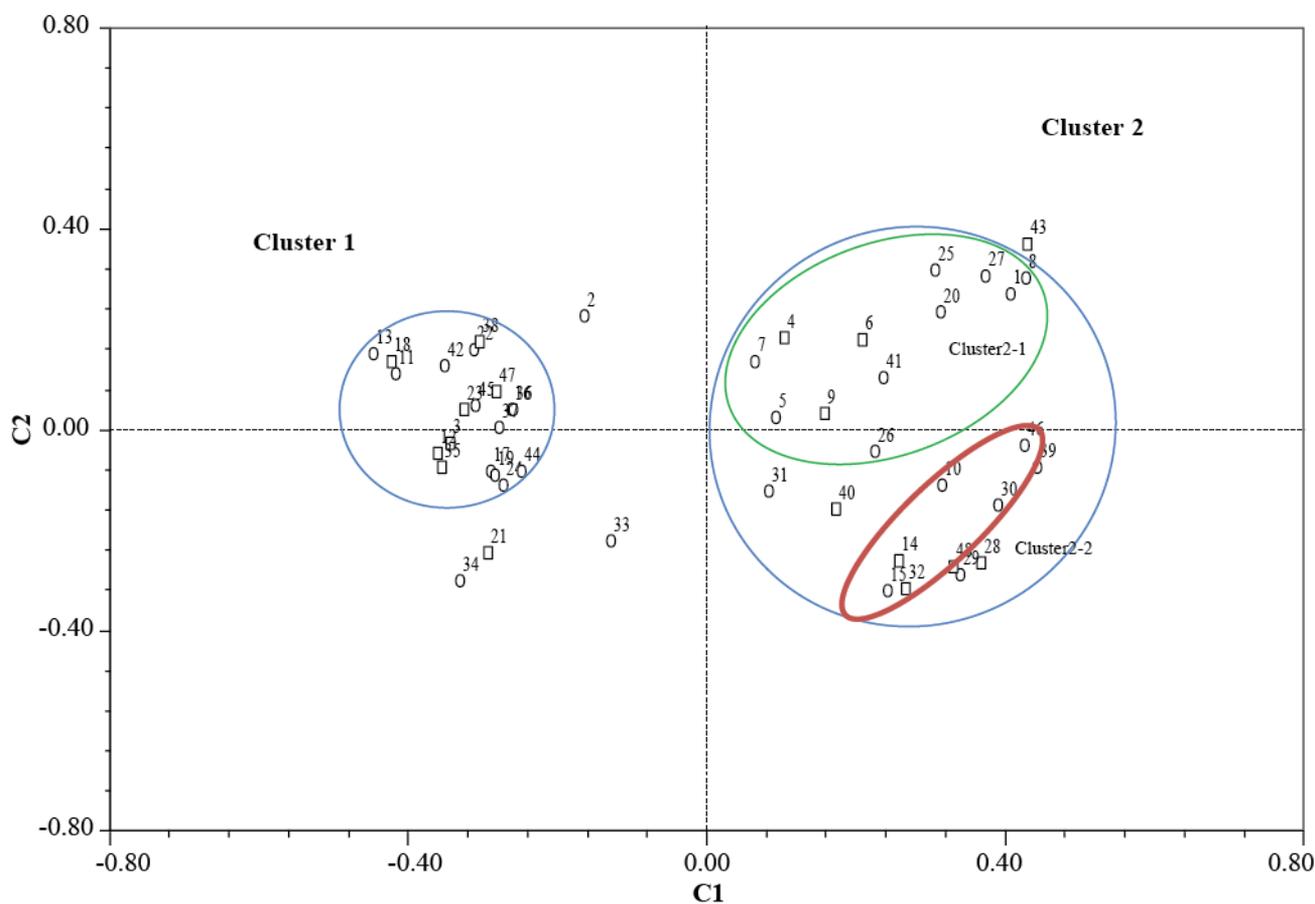
Fig. 8. PCA analysis of 48 rice accessions.

The cross-bred Basmati accessions which were derived from crosses and backcrosses of Basmati and indica rice parents showed different levels of similarity with the Basmati land race (Basmati 370). Among the cross-bred rice varieties, Basmati 198, Basmati 2000 and Basmati 385 showed genetic similarities with Basmati 370, while others Super Basmati and Bas515 were closer to indica on the basis of Power Marker results. Jain *et al.,* (2006) found genetic similarities between Bas370 and Basmati385 using 30 SSR markers covering whole genome, however, these accessions were distant from each other on the base of chromosome 8 analysis. These results indicated higher genetic content from their respective Basmati/indica parents for chromosome 3 and 7. While chromosome 3 and chromosome 7 based SSR dataset showed Basmati Pak and Pusa Basmati 1121 to be quite divergent from tradition variety (Bas-370). However, PCA results showed more genetic dissimilarities between the basmati accessions under studied as compared to non-basmati accessions. The similar patterns of clustering were found in both PCA and Power Marker results in Basmati accessions.

Genetic relationships observed between Basmati, indica and japonica rice accessions using chromosome 3 and chromosome 7 SSR marker data, were similar to the results of whole genome diversity analysis as reported earlier using isoenzyme (Glaszmann, 1987; 1988) and PCR based DNA markers (SSR, AFLP and ISSR) (Garris *et al.,* 2005; Jain *et al.,* 2004; Nagaraju *et al.,*

2002; Saini *et al.*, 2004). Chromosome 3 and chromosome 7 SSR markers datasets based analyses showed that Basmati rice accessions (Group V; Glaszmann 1987, 1988; Garris *et al.,* 2005) are distinct from indica accessions such as IR64, IR6 and japonica (Azucena) rice sub-groups. Both similarity coefficient data and PCA analysis showed that basmati group is closer to japonica group as compared to indica particularly based on Chromosome 3 and chromosome 7. Garris *et al.,* (2005) also reported that basmati group was more near to japonica group based on SSR marker analysis. Phenotypic analysis ad genotypic analysis did not conceded with each other because the grain length is a quantitative trait and affected by numbers of genes/QTLs, which was not covered by using SSR markers only located on chromosome 3 and &7. These observations demonstrate that molecular marker especially SSR technology can be useful to track the genomic regions from different rice parents including those for grain length and can greatly improve the precision and efficiency of rice breeding programs.

Conclusion

Very narrow genetic diversity is reported within basmati accessions of Pakistan but by using trait specific markers we can find some allelic variations in this group.SSR markers are an efficient tool for diversity analysis of inter and intra basmati rice accessions. SSR data generated in this study will allow other researchers to

recognize alleles, coming from the different donor parents and also provide the basis of association mapping studies and help in identification of gene flow in different rice accessions. The overall results derived from analyses of genetic diversity could be used for designing effective breeding programs aimed at broadening the genetic base of commercially grown basmati varieties in Pakistan.

Acknowledgement

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