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Research Article

Investigation of population structure and molecular genetic diversity under coastal agro-ecosystem in rice (*Oryza sativa* L.)

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Abstract
Molecular markers are useful tools for evaluating genetic diversity and determining cultivar identity. Thirty nine simple sequence repeat (SSR) markers were selected in order to evaluate the genetic diversity in 88 genotypes. A total of 115 alleles were detected in 39 SSR polymorphic markers. The number of alleles per marker ranged from 1 to 5, with an average of 3 alleles per locus. An average polymorphism information content value of 0.5615 was recorded. It ranged from zero (RM431, RM379 and RM262) to 0.6476 (RM324), indicating significant genetic diversity among and within the rice accessions of the present study. The average observed heterozygosity was 0.1136, while the average expected genetic diversity was 0.6105. A dendrogram constructed using the Unweighted Pair Group Method with Arithmetic means (UPGMA), grouped the 88 rice genotypes into three well differentiated major clusters. The structured program without prior information provided support for the existence of three genetically distinct clusters (K=3).

Key words: Cluster analysis, SSR marker, *Oryza sativa,* Rice breeding, Variability.

INTRODUCTION

Rice (*Oryza sativa* L.) is an annual grass (Gramineae). It is originated in Asia. It is a staple food for peoples of South and South East Asia (Chang, 1976). Rice is cultivated from sea level to around 3000 m altitude and from the 30°S to 49°N. Temperature and day lengths are therefore quite diverse in the growing areas. The wide geographical distribution of rice has resulted in the development of a great diversity of varietal types.

Globally rice is grown in and around 162.06 million ha, occupying one tenth of arable land, with an annual production of 497.7 million tonnes of rough rice (http://www.statista.com, 2019). The average productivity of 3.07 t /ha of rice provides a per capita consumption of 100 to 240 kg/ year across the globe. Over 90 per cent of the world's rice is produced and consumed in Asia and more than half of it comes from India and China, exploiting maximum arable land. More than two billion people obtain their 80 per cent calorie from rice and its derived products from 90 per cent of the world's rice area (FAO, 1999). Rice based production systems and their associated post harvest operations employ nearly a billion people in rural areas in developing countries (IRC, 2003).

In India, rice area accounts for 22.8 per cent of total cropped area, 35.6 per cent of the area under food crop and 43.9 per cent of the area under cereals. Rice production accounts for 31 per cent of food grain production and 45 per cent of cereal production, providing 20 to 25 per cent of agricultural income. The rice production has increased from 20 million tonnes in 1950-51 to 118.87 million tonnes in 2019, with a productivity increase from

0.6 t/ ha to 2.71 t/ ha, during the same period. There has been a substantial increase in area from 30 million ha to 44 million ha from 1950 to 2019 (http://www.statista.com, 2019). This has been due to crop substitution and increased profitability by growing semi-dwarf high yielding varieties under irrigated conditions. At present, the population of India is 136.64 crores, which is increasing by 1.36 crores per year (macrotrends.net, 2019-20). To meet the demand of the growing population, five million tonnes of additional food is required, out of which, two million shares is of rice. Hence, rice demand of 143 million tonnes by 2030 has to be met by increased rice productivity per unit time and area in the future. To meet the increasing demand, rice production must be increased in spite of less land, less water, less pesticides in a sustainable way. Among the various options for increasing rice production, earliness breeding has to be proved to be one of the best strategies and it has tremendous scope in Indian rice agriculture. Grain quality has become an important issue affecting the domestic consumption and possibly international trade of rice. Around 25 million tonnes of rice with a value of 9,940 million US dollars is under world trade (FAO, 1998). India is a country, which is slowly emerging as a self sufficient country in food grain production. In this situation, the country will enter to export the surpluses in the international market. Market quality firstly depends on the physical appearance of the grain like grain length, grain length/breadth ratio. Development of early maturing, rice genotypes with good quality and higher yield needs intensive research in genetics and plant breeding.

Genetically diverse parents are likely to combine well, which will in turn result in the evolution of high yielding heterotic hybrids and/or superior segregants, as inferred in rice (Banumathy *et al.,* 2010) and in Okra (Akotkar *et al.,* 2010). Mahalanobis' D^2 statistic is generally used to measure the genetic distance between the genotypes, classically. However, it depends upon morphological traits, which are supposed to be influenced by environments. Many different types of molecular markers have been used for diversity analysis (Melchinger, 1999). Among PCR-based markers, SSR markers have proved to be an effective tool for measuring genetic diversity. SSR markers are well distributed in the genome and exhibit more polymorphism. They are also co-dominant markers that are widely used for following a targeted gene (Morgante and Olivieri, 1993). SSR markers are very appropriate to measure the genetic diversity in rice (Powell *et al.,* 1996; Ghneim Herrera *et al.,* 2008; Choudhary *et al.,* 2013 and Li *et al.,* 2014). However, a study that was performed to comprehensively investigate genetic diversity and characterization of different duration has not been found. The present study was undertaken to assess the genetic diversity and characterization of 88 rice genotypes differing in flowering time using SSR markers.

MATERIALS AND METHODS

DNA from 88 rice genotypes (**Table 1**) was isolated by following the modified CTAB method (Murray and Thompson, 1980). Thirty nine molecular markers (**Table 2**) of the gene for the first flowering time were amplified with the isolated 88 DNA samples. The isolated DNA samples were amplified using a T100 thermal cycler (BIO-RAD, USA) with a total of 100 µl of 50 ng DNA, 1 µl of Tris-HCL 10 mM (pH 8.3), 0.5 µl of 1.5 mM $MgCl_2$, 0.1 µl of 0.5 unit of Taq polymerase (New England Biolabs, U.K), 1 µl of 50 µM dNTP mixture and 0.2 µl each of forward and revere primers (5 pico molar).

Conditions for carrying out DNA amplification were as follows: initial denaturation at 94°C for 5 min, followed by 35 repeated cycles of denaturation at 94°C for 45s, annealing for 45s (temperature specific to primer) and extension for the 60s at 72°C followed by a final extension for 8 min. at 72°C. Bromophenol blue was added to the samples and the molecular weight of the amplified DNA was estimated in 3.5% agarose gel with 50-bp ladder (New England Biolabs, UK) as standard in 1X Tris-Boric acid-EDTA (TBE) buffer. The resolved PCR bands were documented using the molecular Imager Gel Doc XR system (Bio-Rad).

Only clear and intense bands were recorded. The molecular size of the amplified fragments was determined by image lab software (Bio-Rad) using a 50 bp DNA ladder as standard. Amplification of DNA samples with primers was recorded as '1' for the amplified and 'O' for the unamplified regions according to the molecular size of the marker. A data matrix with 'O' or '1' against the molecular size was prepared for further analysis. Marker genetic diversity parameters *viz., the* number of alleles, allele frequency, genetic diversity index and heterozygosity were calculated by power marker software.

Polymorphism information content (PIC) for every polymorphic marker was computed using the formula

$$PIC = 1 - \Sigma Pi2 - \Sigma\ \Sigma Pi2\ Pj2.$$

Where,'i' is the sum of alleles detected 'Pi' is the frequency of the i^{th} allele and j=i+1. A neighbour-joining tree with bootstrap value (1000) was constructed using the unweighted pair group method with arithmetic average (UPGMA) algorithm with the help of DARwin version 6.0.

RESULTS AND DISCUSSION

Eighty- eight rice genotypes of different eco-geographic were genotyped using 39 SSR markers, which produced a total of 115 alleles (**Table 3**). Among these 115 alleles, 1% were considered as rare (showed an allele frequency of <1%). The number of alleles per loci varied from 1 to 5 with an average of 3 alleles per locus. The highest number of alleles (5) were detected

Table 1. List of genotypes used in the study

S. No.	Name	Duration	S. No.	Name	Duration
1	Jayanthidhan	Long	45	CR dhan 101	Medium
2	CR 1014	Long	46	CR dhaan 202	Medium
3	Reeta	Long	47	CR dhan 310	Medium
4	CR dhan 601	Long	48	Khitish	Medium
5	Jalamani	Long	49	Pyari	Medium
6	Lunishru	Long	50	Naveen	Medium
7	CR dhan 408	Long	51	IC-206447	Medium
8	CR dhan 307	Long	52	IC-125757	Medium
9	Varshadhan	Long	53	IC-0514489	Medium
10	Tapaswini	Long	54	IC-135318	Medium
11	Hanswari	Long	55	IC-124436	Medium
12	Improve lalat	Long	56	IC-0627835	Medium
13	CR dhan 500	Long	57	IC-114312	Medium
14	Swarna Sub 1	Long	58	IC-0627836	Medium
15	IC-ARC-7220	Long	59	IC-0623213	Medium
16	IC-ARC-7078	Long	60	IC-114188	Medium
17	IC-ARC-11547	Long	61	IC-0517840	Medium
18	IC-299694	Long	62	IC-214312	Medium
19	IC-ARC-7408	Long	63	IC-135191	Medium
20	IC-ARC- 13300	Long	64	IC-114971	Medium
21	IC-379792	Long	65	ADT 38	Medium
22	IC- 215370	Long	66	ADT 39	Medium
23	IC- 377869	Long	67	CO 50	Medium
24	IC-ARC- 1119	Long	68	Vandana	Early
25	IC-379136	Long	69	IC-0098989	Early
26	IC-611162	Long	70	IC-0124198	Early
27	IC-386231	Long	71	IC-0203398	Early
28	IC-ARC-11203	Long	72	IC-0135769	Early
29	IC-300981	Long	73	IC-0123756	Early
30	IC-67725	Long	74	IC-0207960	Early
31	IC-264987	Long	75	IC-0135529	Early
32	IC-518987	Long	76	IC-0134873	Early
33	IC-ARC-7432	Long	77	IC-0209056	Early
34	IC-ARC-10595	Long	78	IC-0207992	Early
35	ADT 44	Long	79	IC-0135063	Early
36	ADT 50	Long	80	IC-0207955	Early
37	CR 1009 Sub 1	Long	81	ADT-36	Early
38	ADT 52	Long	82	ADT-37	Early
39	Annanda	Medium	83	ADT-42	Early
40	Satyabhama	Medium	84	ADT-43	Early
41	Phalguni	Medium	85	ADT-45	Early
42	CR dhan 203	Medium	86	ADT-48	Early
43	CR dhan 305	Medium	87	ASD-16	Early
44	Sahabhagidhan	Medium	88	PTB-15	Early

Table 2. List of SSR markers used in the study

S. No.	Marker	Chr. No.	Forward Primer	Reverse Primer	Motif	Number of Repeats	Annealing Temperature
1	RM200	5	CGCTAGGGAATTTGGATTGA	CGATGAGCAGGTATCGATGAGAAG	GA	16	55
2	RM207	2	ATCCTAGTGGATAAGGCACAGACTGG	CCCTTGCTCTTCCACCTCATCC	AG	29	55
3	RM214	7	GAACATGCTTTCAACCATCAGG	GATCCTCTCAGTTCAGTGCAAGC	AG	32	55
4	RM431	1	GCTTGCTTGTATCTGCATTGGTAGG	GGGATGATCCACTCTCTGTTTGG	AG	16	55
5	RM231	3	CCAGATTATTTCCTGAGGTC	CACTTGCATAGTTCTGCATTG	CT	16	55
6	RM261	4	CTACTTCTCCCCTTGTGTCG	TGTACCATCGCCAAATCTCC	C (CT)	9(8)	55
7	RM282	3	CTGTGTCGAAAGGCTGCAC	CAGTCCTGTGTTGCAGCAAG	GA	15	55
8	RM302	1	TGCAGGTAGAAACTTGAAGC	AGTGGATGTTAGGTGTAACAGG	AT	13	55
9	RM319	1	ATCAAGGTACCTAGACCACCAC	TCCTGGTGCAGCTATGTCTG	GT	10	55
10	RM569	3	CTGCGTCAGATTTCTCCTCTTCG	ACATTCTCGCTTGCTCCTCTCG	AG	16	55
11	RM11	7	ATCGGTGCTTGGCTGGATAGC	CCACCTTCTTCTCCTCCTCTTCC	AG	15	55
12	RM223	8	GAGTGAGCTTGGGCTGAAAC	GAAGGCAAGTCTTGGCACTG	CT	25	55
13	RM250	2	GTTCAAACCAAGCTGATCACAAGC	GGCGTCAGAGTCAGAGATGAAGG	AG	27	55
14	RM21	11	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	GA	18	55
15	RM118	7	CCAATCGGAGCCACCGGAGAGC	CACATCCTCCAGCGACGCCGAG	GA	8	55
16	RM153	5	GCCTCGAGCATCATCATCAG	ATCAACCTGCACTTGCCTGG	GAA	9	55
17	RM218	3	TCAAACCAAGGTCCTTCAACTGC	TTTCTTCCACCGTCCATGTATCC	AG	24	55
18	RM221	2	ACATGTCAGCATGCCACATC	TGCAAGAATCTGACCCGG	(TC) T, C, (TC) (CT)	(4) 3, 3, (2)	55
19	RM258	10	CTCCCTGGCCTTTAAAGCTGTCG	GACGAACAGCAGCAGAAGAGAAGC	AG	11	55
20	RM259	1	GAAGTGCTCCCTAAACTTGTTGC	TTATGGAGGATGGATTCGAAGG	AG	22	55
21	RM262	2	CATTCCGTCTCGGCTCAACT	CAGAGCAAGGTGGCTTGC	CT	16	55
22	RM286	11	CTGGCCTCTAGCTACAACCTTGC	AAACTCTCGCTGGATTCGATAGG	AG	21	55
23	RM314	6	CTAGCAGGAACTCCTTTCAGG	AACATTCCACACACACACGC	GT, CG, GT	8, 3, 5	55
24	RM315	1	AAGCTCATTTGAGGGTATCG	AGGGCAAGAGAAACTATTGG	AC	15	55
25	RM404	8	GGAGCAGCTAAGGCAGATAAGAGG	GCCTTCATGCTTCAGAAGACAGC	AG	29	55
26	RM487	3	TTTCTCGAACGCAGGAGAAC	GCTAGGAACATCAACCCGAG	AC	10	55
27	RM521	2	ATGACCCAATTTCTGACTCTAGCC	CATGGGTGGTGCTGTAGATGG	AG	14	55
28	RM555	2	TTGACATGCGAAATGGAGATGG	TTGGATCAGCCAAAGGAGACC	AG	11	55
29	RM9	1	GGCCCTCATCACCTTCGTAGC	CGTCCTCCCTCTCCCTATCTCC	AG	14	55
30	RM227	3	ACCTTTCGTCATAAAGACGAG	GATTGGAGAGAAAAGAAGCC	CT	10	55
31	RM224	11	ATCGATCGATCTTCACGAGG	TGCTATAAAAGGCATTCGGG	AAG, A	8, 13	55
32	RM206	11	ATCGATCCGTATGGGTTCTAGC	GTCCATGTAGCCAATCTTATGTGG	AG	33	55
33	RM168	3	TGTCGTCGAGGATTTGGAGATCG	GAATCAATCCACGGCACAGTCC	AC	10	55
34	RM81	3	GAGTGCTTGTGCAAGATCCA	CTTCTTCACTCATGCAGTTC	TCT	10	55
35	RM237	1	CAAATCCCGACTGCTGTCC	TGGGAAGAGAGCACTACAGC	CT	18	55
36	RM324	2	GATTCCACGTCAGGATCTTCTGG	GCTCACCAGTTGAGATTGAAAGG	ATC	9	55
37	RM263	2	AATCTATGGACCTGGGAGGAACC	TGACGAGAGTGCTACGTTTGAGC	AG	14	55
38	RM163	5	CGCCTTTATGAGGAGGAGATGG	AAACTCTTCGACACGCCTTGC	AG	15	55
39	RM248	7	AGAGAGCAAGTTTGAAGCGAAGC	ACCAAGAGGGTAGCCTAGCATGG	AG	15	55

Table 3. Genetic diversity parameters and PIC values of the 39 markers

S. No.	Marker	Allele Number	Major Allele Frequency	Genetic Diversity	Heterozygosity	PIC
1	RM200	3	0.8011	0.3337	0.1591	0.3023
2	RM207	3	0.8011	0.3299	0.0341	0.2935
3	RM214	4	0.9148	0.1590	0.0114	0.1521
4	RM431	1	1.0000	0.0000	0.0000	0.0000
5	RM231	3	0.6932	0.4321	0.0227	0.3480
6	RM261	2	0.9091	0.1653	0.0455	0.1516
7	RM282	2	0.9886	0.0225	0.0000	0.0222
8	RM302	3	0.5227	0.6126	0.0455	0.5438
9	RM319	1	1.0000	0.0000	0.0000	0.0000
10	RM569	4	0.9375	0.1195	0.0114	0.1167
11	RM11	3	0.7841	0.3603	0.0227	0.3294
12	RM223	3	0.8239	0.3049	0.0341	0.2827
13	RM250	3	0.7614	0.3840	0.0000	0.3418
14	RM21	4	0.4716	0.6562	0.0568	0.5968
15	RM118	3	0.9659	0.0664	0.0000	0.0652
16	RM153	3	0.6932	0.4693	0.0000	0.4200
17	RM218	3	0.5511	0.5897	0.0114	0.5204
18	RM221	1	1.0000	0.0000	0.0000	0.0000
19	RM258	3	0.7330	0.4026	0.0114	0.3378
20	RM259	3	0.9545	0.0878	0.0000	0.0859
21	RM262	1	1.0000	0.0000	0.0000	0.0000
22	RM286	4	0.8636	0.2459	0.0568	0.2337
23	RM314	3	0.4773	0.5850	0.0000	0.4960
24	RM315	2	0.9545	0.0868	0.0000	0.0830
25	RM404	3	0.8693	0.2350	0.0114	0.2208
26	RM487	3	0.9489	0.0982	0.0341	0.0956
27	RM521	3	0.8807	0.2136	0.1250	0.1970
28	RM555	3	0.6648	0.4773	0.0795	0.4055
29	RM9	3	0.5284	0.5188	0.7614	0.4058
30	RM227	2	0.9602	0.0764	0.0795	0.0735
31	RM224	4	0.6818	0.4949	0.0455	0.4567
32	RM206	5	0.5227	0.6020	0.1705	0.5318
33	RM168	3	0.6250	0.4848	0.0000	0.3873
34	RM81	3	0.8523	0.2621	0.0455	0.2454
35	RM237	2	0.9659	0.0659	0.0000	0.0637
36	RM324	5	0.3580	0.7033	0.0795	0.6476
37	RM263	3	0.8864	0.2069	0.0227	0.1951
38	RM163	4	0.5625	0.6105	0.1136	0.5615
39	RM248	5	0.3239	0.7492	0.0682	0.7055
	Mean	2.9744	0.7752	0.3131	0.0554	0.2799

for the loci RM 206, RM 324 and RM 248 and the lowest was detected for a group of markers *viz.,* RM431, RM319, RM221 and RM262. A similar number of alleles (2 to 5) for SSR markers were reported in 141 basmati rice accessions of North Western Himalaya (Salgotra *et al.,* 2005). The PIC value represents the relative informativeness of each marker and in the present study, the average PIC value was found to be 0.5615. The PIC values ranged from zero for RM431, RM319 and RM 262 to 0.6476 for RM324. Singh *et al.* (2016) reported a mean PIC value of 0.29, in different sets of rice varieties which were in agreement with the present study. Heterozygosity was found to be very low which may be due to the autogamous nature of rice. Expected heterozygosity or gene diversity (He) computed according to Nei (1973) varied from 0.00 to 0.7614 (RM9) with an average of 0.0554 (**Table 3**). The low level of heterozygosity was reported in other studies on rice (Choudhury *et al.,* 2014) and Nachimuthu *et al.,* 2015) and this could be attributed itself pollination behaviour. The genetic diversity ranged from 0.000 to 0.7033 (RM324) with an average of 0.6105. Gene diversity obtained in the present study was quite low (0.52).

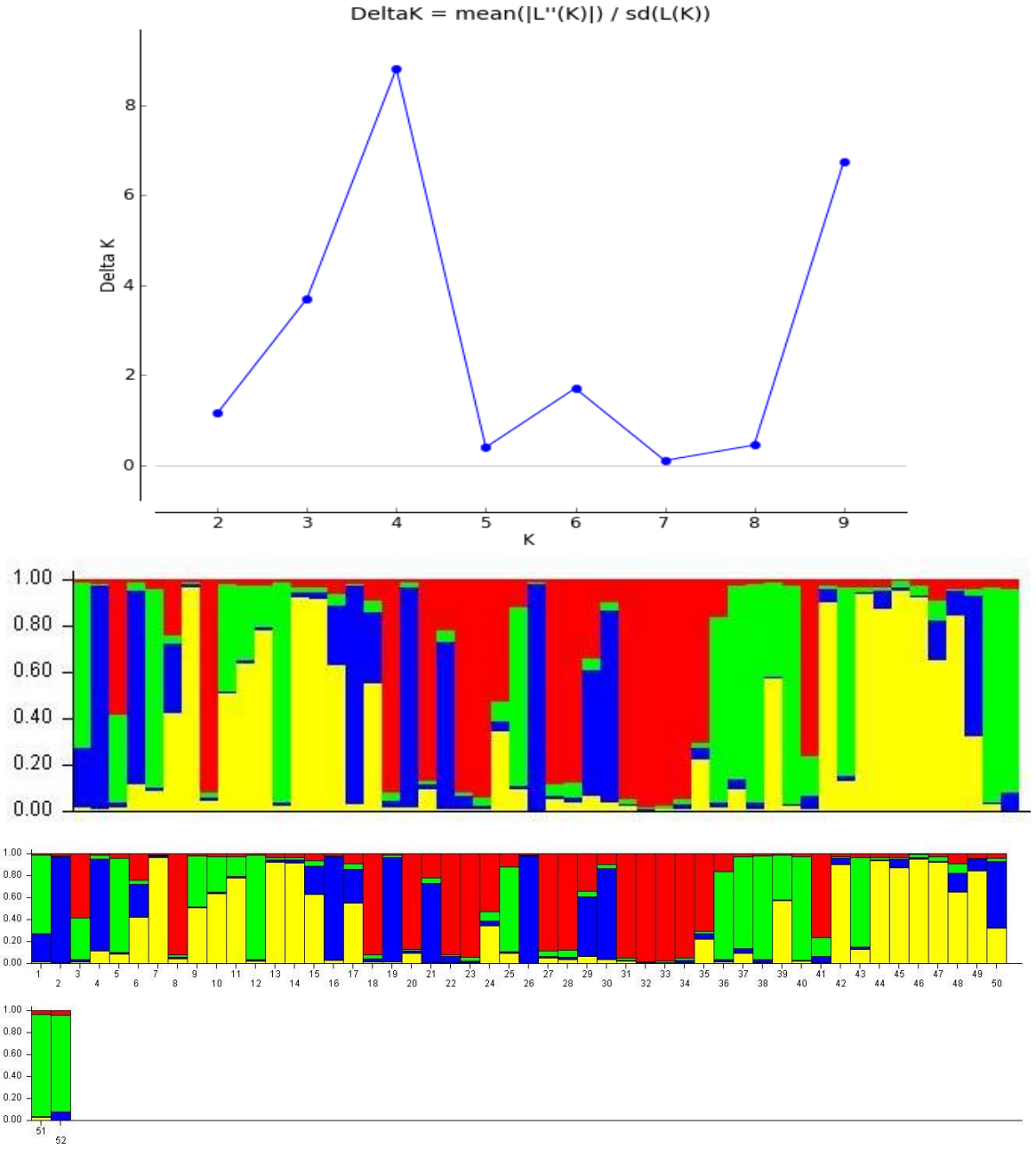

Fig. 1. Population structure

The population structure of the 88 germplasm line was analysed by Bayesian based approach. The log likelihood revealed by structure showed the optimum value as 3 (K=3). Similarly, the maximum of adhoc measures AK was found to be KM (**Fig. 1**) which indicated that the entire population can be grouped into three subgroups. Based on the membership fractions, the accessions with the probability of ≥ 80% were assigned to corresponding subgroups with others categorized as admixture. Earlier studies on population structure have reported two to eight sub population using different rice collections (Roy *et al.,* 2015 and Upadhyay *et al.* 2012).

Clustering analysis based on unweighted pair group method with arithmetic mean (UPGMA) method using DARwin separated the accessions into three main groups (**Fig. 2**), which showed similar results as structure analysis. This grouping was further supported by earlier studies of Upadhyay *et al.* (2012) and Das *et al.* (2013). Group II in the UPGMA tree consists of maximum accessions. In the UPGMA tree, the accessions within group I, II and III clustered into smaller subgroups based on their origin and types. Hence, the clustering analysis by two classification methods re veals a high level of similarity in the clustering of the genotypes. All these points to the accuracy and usefulness of the SSR markers in tracing the phylogency or pedigree of a germplasm or breeding materials. These observations, corresponds to the previous observations of other rice germplasm studies (Bonny *et al.,* 2015 and Masuduzzaman *et al.,* 2016). Similarly, a clustering pattern has also been reported by Pachauri *et al.* (2013) based on allelic and morphological data along with the location in rice varieties using SSR markers. Accessions that are found clustered together are assumed to have high genetic similarity, while those that are found far away from each other are considered to be divergent.

PCoA was used to characterize the sub-groups of the germplasm set. A two dimensional scatter plot involving all 88 genotypes showed that the first three PCA axes accounted for 8.33, 16.45 and 23.65 per cent of the

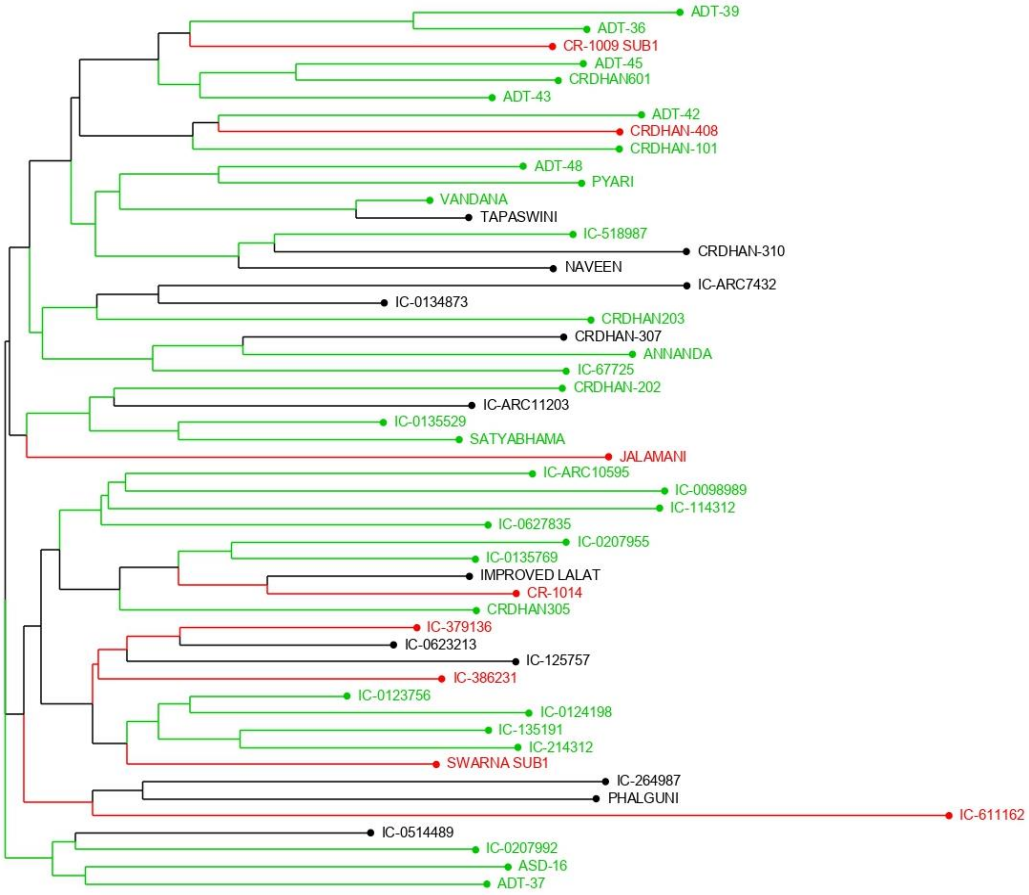

Fig. 2. Dendrogram showing the genetic dissimilarity among the rice genotypes

cumulative variation among populations (**Table 4**). In PCoA, rice varieties were labelled with three different colours which represent the three populations obtained from population structure. The POP 1 and POP 2 showed distinct grouping whereas the individuals of POP 3 were distributed over POP 1 and POP 2 (**Fig. 3**).

The hierarchical distribution of molecular variance by ANOVA and pair wise analysis revealed highly significant genetic differentiation among groups. It revealed that 5% of the total variation was among the population, while 78 per cent was among individuals within groups and within individuals it was 17 per cent (**Table 5**). Calculation of Wright's F statistics at all SSR loci revealed that FIS was 0.819 and FIT was 0.828. Determination of FST for the polymorphic loci across all accessions showed FST as 0.047, which implies high genetic variation. The pair wise FST estimate among subgroups indicated that the two groups are significantly different from each other. Consequently, the differential between the overall groups and their geographical groups had really happened and resulted in high genetic diversity. Variation of similar pattern as observed in a previous study (Mazid *et al.,* 2013). In one study, which involved 41 rice genotypes from three populations, 67 per cent of the total variation was attributed to variation within the genotype, while variation among the three populations represents the high level of genetic differentiation which will further strengthen the divergence of the population. High genetic differentiation is very important within the germplasm for creating a desirable heterotic group in base breeding populations (Alam *et al.,* 2015). Thus, genetic diversity characterization is very important as it provides the basis for a planning conservation strategy, utilization, and establishment of breeding and improvement for rice plants (Li *et al.,* 2011).

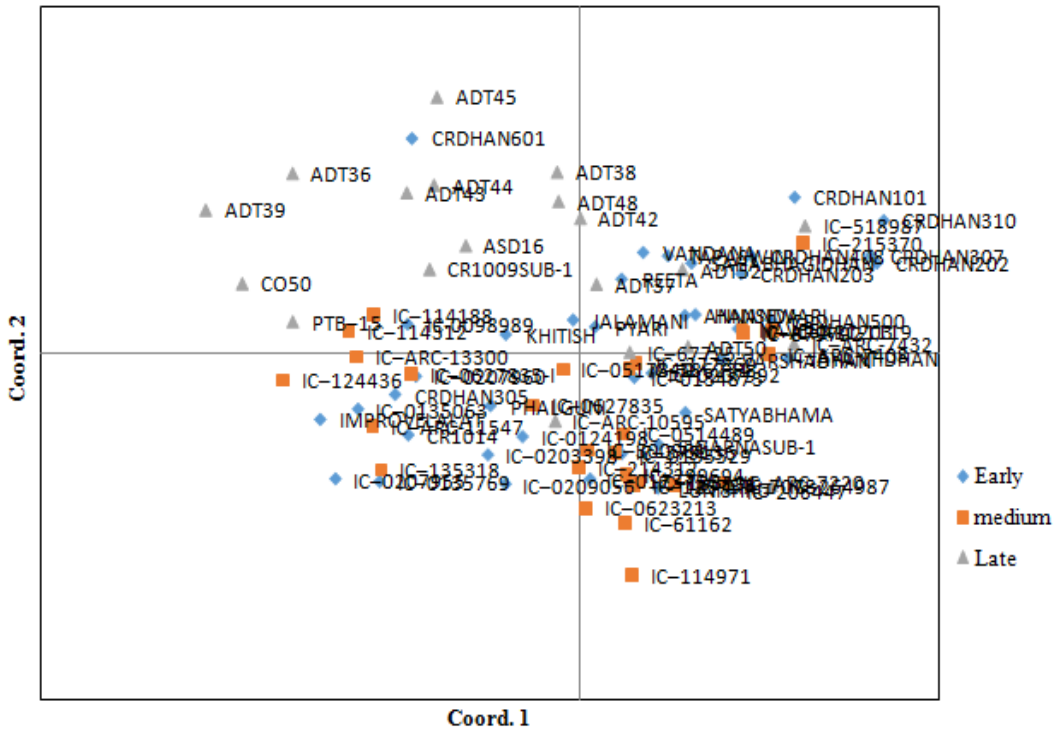

Fig. 3. Principal coordinates (PCoA)

Table 4. Percentage of variation explained by the first 3 axes

Axis	1	2	3
Percentage	8.33	8.13	7.20
Cumulative %	8.33	16.45	23.65

Table 5. Summary of AMOVA

Source	df	SS	MS	Est. Var	%
Among pops	2	55.085	27.543	0.296	5
Among individual	85	924.591	10.878	4.899	78
Within individual	8	95.000	1.080	1.080	17
Total	175	1074.676		6.274	100

F- Statistics	Value	P (Rand >=data)
Fst	0.047	0.001
Fis	0.819	0.001
Fit	0.828	0.001
Fst max	0.694	
F'st	0.068	
Nm	5.055	

Genetic diversity is an important concept in any breeding program. It can be studied using SSR markers for the identification of potential parents in order to achieve heterosis in future rice breeding programs. SSR markers were exploited to provide an unbiased estimate of the diversity pattern in this rice germplasm. The current study found the existence of high levels of diversity among. 88 rice accessions which are good for the introduction of new genes in the existing genotypes. The dendrogram, constructed to identify the genetic similarities among these genotypes showed that accessions from the same region were found to cluster together as well as in different clusters implying that genetic diversity is not related fully to geographical diversity. Clustering patterns on the basis of SSR markers provide amble information that is significantly crucial for the development of genetic manipulation through crop breeding.

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