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Studies on genetic diversity in rice (*Oryza sativa* L.) using SSR markers

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Abstract
Rice is the most important staple food grain of all cereal crops and feeds more than half of the world's population. Assessment of genetic diversity is of utmost important in rice breeding from the perspective of selection, conservation and proper utilization. The present study was undertaken with an objective to assess the genetic diversity among 28 rice cultivars by use of 22 SSR markers. A total of 41 alleles were detected across 28 rice cultivars used. PIC values varied widely among SSR loci tested and it ranged from 0.753 to 0.067, with a mean value of 0.376. The 28 rice cultivars were grouped into two clusters *i.e.,* cluster I and II with dissimilarity coefficient 0.42. Cluster I was sub divided into two minor sub-groups IA and IB having 6 and 11 genotypes respectively. IB was further subdivided into minor groups. In similar way, the second main cluster *i.e.* Cluster II was also sub divided into two minor sub-groups that is IIA and IIB having 3 and 8 genotypes respectively. IIB further divided into minor groups. This indicated presence of considerable diversity in the genotypes studied. The most diverse cultivars were HUR4-3 and GSRIR1DQ125-L2-D2.

Keywords: Rice, dendrogram, molecular diversity, SSR markers

Introduction
Rice is the most important cereal crop in the world. India shared 24.52% in global rice production. For more than 70% of Asian population, rice is grain of life. Rice has deeply embedded itself in Indian culture. Worldwide rice is grown on an area of 163 million hectares with a production of 751.9 million tonnes and with an average productivity being 4.52 tonnes per hectare (FAO Rice Market Monitor 2017) [6], out of which 90 per cent of world's rice is produced and consumed in Asia. To feed the ever growing population, the targeted rice production of the world, China and India for the year 2030 is envisioned as 771.02, 168.90 and 130.02 million tonnes respectively (United State Department of Agriculture 2014) [29]. To get success in accomplishing the target, the increase in rice productivity is the only option left, since the other alternatives like cultivable land, water and other natural resources are either stagnant or declining (Singh *et al.* 2013) [18]. Characterization and quantification of genetic diversity has long been a major goal in evolutionary biology. Information on the genetic diversity within and among closely related crop varieties are essential for a rational use of genetic resources. The analysis of genetic variation both within and among elite breeding materials is of fundamental interest to the plant breeders. It contributes to the monitoring of germplasm and can also be used to predict potential genetic gain.

Rice is chief staple food crop for which complete genome sequence is available. It is the best model plant for study of grass genetics and genome organization due to its relatively small genome size of 430 Mb (Causse *et al.* 1994) [2]. The various features of rice like availability of saturated molecular markers, smallest genome, precise genome sequence, diploid, self pollinating crop make it extensively studied among cereals and model crop for genetic studies.

Molecular Marker based Genetic Diversity Analysis (MMGDA) also has potential for evaluating changes in genetic diversity over time and space (Duvick 1984) [5]. Numerous molecular markers are being used for fingerprinting and diversity analysis among crop plants such as Restriction Fragment Length Polymorphism (RFLP) (Sun *et al.* 2001) [25], Random Amplified Polymorphic DNA (RAPD) (Williams *et al.* 1990) [31], microsatellites or Simple Sequence Repeats (SSRs) (Gao *et al.* 2005) [7] and Amplified Fragment Length Polymorphism (AFLP) (Aggarwal *et al.* 1999) [1]. Simple sequence repeat (SSR) is a vital tool for genetic variation identification of germplasm (Powell *et al.* 1996) [15] and hence, being extensively applied in genetic diversity analysis, analysis of germplasm diversity (Jin *et al.* 2010) [8]. They are also known as microsatellites or short tandem repeats (STRs). It is a type of variable number tandem repeats (VNTR).

Microsatellite markers have been ideal for identification and purity checking of rice varieties. Microsatellites are PCR-based markers, they are abundant, co-dominant, highly reproducible and interspersed throughout the genome (Panaud *et al.* 1996, Temnykh *et al*. 2000) [14, 3]. In particular, they are able to detect high levels of allelic diversity (McCouch *et al.* 1997) [11]. These markers can detect a significantly higher degree of polymorphism in rice (Ni *et al*. 2002, Okoshi *et al*. 2004) [12, 13] which becomes ideal for studies on genetic diversity and intensive genetic mapping (Cho *et al*. 2000). In the light of above points, the present investigation was undertaken to study genetic diversity among 28 rice cultivars so as to identify varying genotypes which would aid in rice breeding programmes.

Highlights

Number of alleles generated per locus by each marker ranged from 2-6 with an average of 2.733. UPGMA based cluster analysis revealed considerable diversity among all studied genotypes. A dendrogram based on Jaccard's dissimilarity coefficient identified HUR4-3 and GSRIR1DQ125-L2-D2 as most diverse genotypes.

Materials and Methods

The present study was carried out in 2017 in Molecular biology lab (Niche Area of Excellence) of Department of Genetics and Plant Breeding, Institute of Agricultural sciences, Banaras Hindu University, Varanasi (U.P). The experimental material for this investigation comprised of 28 rice genotypes as given in Table 1. Total number of twenty two SSR primers were used in present study. The details of SSR primers used are presented in Table 2.

Table 1: List of rice genotypes used in the study

S. No.	Genotypes	Source of Seeds	S. No.	Genotypes	Source of Seeds
1.	GSRIR1-DQ125-L2-D2	IRRI, Philippines	7.	GSRIR1-DQ62-D6-D1	IRRI, Philippines
2.	GSRIR1-DQ125-R4-Y1	IRRI, Philippines	8.	GSRIR1-DQ129-Y4-L1	IRRI, Philippines
3.	GSRIR1-DQ142-Y1-Y1	IRRI, Philippines	9.	GSRIR1-DQ139-R1-L2	IRRI, Philippines
4.	GSRIR1-DQ187-Y3-D1	IRRI, Philippines	10.	GSRIR1-DQ150-R5-Y1	IRRI, Philippines
5.	GSRIR1-DQ121-LI9-L1	IRRI, Philippines	11.	GSRIR1-DQ138-LI1-Y1	IRRI, Philippines
6.	GSRIR1-DQ62-D7-D1	IRRI, Philippines	12.	GSRIR1-DQ122-D2-D1	IRRI, Philippines
13.	GSRIR1-DQ112-Y1-D2	IRRI, Philippines	21.	URG-24	BHU, Varanasi
14.	GSRIR1-DQ136-LI5-Y1	IRRI, Philippines	22.	URG-30	BHU, Varanasi
15.	URG-1	BHU, Varanasi	23.	HUR4-3	BHU, Varanasi
16.	URG-3	BHU, Varanasi	24.	HUBR5-1	BHU, Varanasi
17.	URG-5	BHU, Varanasi	25.	HUR5-2	BHU, Varanasi
18.	URG-8	BHU, Varanasi	26.	HUBR10-9	BHU, Varanasi
19.	URG-19	BHU, Varanasi	27.	HUR-105	BHU, Varanasi
20.	URG-22	BHU, Varanasi	28.	HUR-3022	BHU, Varanasi

Table 2: Details of the microsatellite primers used in present studies

Microsatellite locus	Location/ Chromosome	Forward/ Reverse	Sequence 5'----------> 3'	Temp. (0C)
RM60	3	Forward	AGTCCCATGTTCCACTTCCG	53
		Reverse	ATGGCTACTGCCTGTACTAC	
RM22	3	Forward	GGTTTGGGAGCCCATAATCT	53
		Reverse	CTGGGCTTCTTTCACTCGTC	
RM1	1	Forward	GCGAAAACACAATGCAAAA	55
		Reverse	GCGTTGGTTGGACCTGAC	
RM19	12	Forward	CAAAAACAGAGCAGATGAC	55
		Reverse	CTCAAGATGGACGCCAAGA	
RM108	9	Forward	TCTCTTGCGCGCACACTGGCAC	61
		Reverse	CGTGCACCACCACCACCACCAC	
RM161	5	Forward	TGCAGATGAGAAGCGGCGCCTC	61
		Reverse	TGTGTCATCAGACGGCGCTCCG	
RM162	6	Forward	GCCAGCAAAACCAGGGATCCGG	61
		Reverse	CAAGGTCTTGTGCGGCTTGCGG	
RM178	5	Forward	TCGCGTGAAAGATAAGCGGCGC	69
		Reverse	GATCACCGTTCCCTCCGCCTGC	
RM223	8	Forward	GAGTGAGCTTGGGCTGAAAC	54
		Reverse	GAAGGCAAGTCTTGGCACTG	
RM237	1	Forward	CAAATCCCGACTGCTGTCC	55
		Reverse	TGGGAAGAGAGCACTACAGC	
RM259	1	Forward	TGGAGTTTGAGAGGAGGG	55
		Reverse	CTTGTTGCATGGTGCCATGT	
RM283	1	Forward	GTCTACATGTACCCTTGTTGGG	61
		Reverse	CGGCATGAGAGTCTGTGATG	
RM408	8	Forward	CAACGAGCTAACTTCCGTCC	55
		Reverse	ACTGCTACTTGGGTAGCTGACC	
RM495	1	Forward	AATCCAAGGTGCAGAGATGG	55
		Reverse	CAACGATGACGAACACAACC	
RM452	2	Forward	CTGATCGAGAGCGTTAAGGG	61
		Reverse	GGGATCAAACCACGTTTCTG	

RM55	3	Forward	CCGTCGCCGTAGTAGAGAAG	55
		Reverse	TCCCGGTTATTTTAAGGCG	
RM212	1	Forward	CCACTTTCAGCTACTACCAG	51.5
		Reverse	CACCCATTTGTCTCTCATTATG	
RM455	7	Forward	AACAACCCACCACCTGTCTC	57
		Reverse	AGAAGGAAAAGGGCTCGATC	
RM5	1	Forward	TGCAACTTCTAGCTGCTCGA	57
		Reverse	GCATCCGATCTTGATGGG	
RM431	1	Forward	TCCTGCGAACTGAAGAGTTG	55
		Reverse	AGAGCAAAACCCTGGTTCAC	
RM154	2	Forward	ACCCTCTCCGCCTCGCCTCCTC	61
		Reverse	CTCCTCCTCCTGCGACCGCTCC	
RM312	1	Forward	GTATGCATATTTGATAAGAG	55
		Reverse	AAGTCACCGAGTTTACCTTC	

DNA isolation from plant material

Collection of young leaves from 21-15 days old seedling was done, leaves were stored at -20˚ C. DNA was extracted following CTAB extraction method according to Doyle and Doyle, 1987 [4] with few modifications.

DNA quality estimation

Quantification of genomic DNA was done spectro photometrically at both 260 and 280 nm wavelengths. Ratio of OD of 260/280 as 1.8 indicate purity of DNA while ratio less than 1.8 indicate contamination of phenol or proteins, higher value indicate presence of RNA.

PCR analysis

22 SSR markers were used for genetic diversity analysis. PCR amplification was carried out following the standard procedures. All components remain constant for all primer pairs except annealing temperature which changes. The reactions are carried out in PCR tubes in Thermo-cycler.

Scoring of Bands

Amplified PCR products were separated on 2.5% of agarose gel with ethidium bromide. TAE buffer was used as running buffer. In order to standardize band size ladder of 100 bp was used. All this were subjected to electrophoresis on 60-80 volts for 2 hours. The amplified products were visualized and photographed under UV light source in a gel documentation system. Band position of given SSR marker for each genotype was scored from the respective gel images. SSR profile giving constant amplification for all the genotype was included in this study. Scoring was simplified by using pattern as '1' for the presence and '0' for the absence of a band to generate the 0 and 1 matrix. Such binary data matrix was then utilized to generate genetic similarity data among the 28 lines of rice genotypes.

Genetic Similarity analysis

NTSYS-pc version 2.11W (Rohlf 1997) [21] was used to analyse binary data matrix generated by polymorphic SSR markers. To calculate the Jaccard's dissimilarity coefficient SIMQUAL programme was used.

UPGMA based cluster analysis

The dissimilarity matrix was used as an input for analysis of clusters. UPGMA-based clustering was done using Sequential Agglomerative Hierarchial Non-Overlapping (SAHN) module and utilizing Jaccard's Co-efficient of NTSYS-pc for dendrogram construction. In Unweighted pair-group method with arithmetic averages (UPGMA), clusters were joined based on the average distance between all members in one group.

Polymorphic information content

PIC value of markers was calculated. The calculation of PIC (Weir, 1996) for the i^{th} marker is PIC = $1 - \Sigma P_{ij}$ $(j=1,2,\ldots..n)$, where P_{ij} is the frequency of the j^{th} pattern for the $\boldsymbol{i}^{th}$ marker and the summation extends over (n) patterns (Peng and Lapitan 2005) [16].

Results and Discussion

The assessment of genetic diversity is an indispensable component in germplasm characterization and conservation. The results derived from analyses of genetic diversity at the DNA level could be used for planning effective breeding programs aimed at broadening the genetic bases of commercially grown varieties. (Wang and Chee 2010) [30]. In the present study, a total of 22 SSR markers were used to assess the extent of genetic diversity across 28 rice cultivars (Fig 3). Out of 22 SSR markers, 15 were found to be polymorphic. A total of 41 alleles were detected across 28 rice cultivars used. The number of alleles generated per locus by each marker ranged from 2 to 6 with an average of 2.733. The results obtained in present study were comparable to the observations made by (Rabey *et al*. 2013 and Shah *et al*. 2013) [20, 22], where average number of alleles detected were 3.83 and 2.75 in genetic diversity of eight rice cultivars and diversity within the aromatic and non-aromatic rice varieties respectively. However, the result observed in present study was higher than the result reported by (Kibria *et al.* 2009) [9] with an average number of alleles 1.78 per locus in order to assess the genetic diversity among aromatic rice genotypes using simple sequence repeat (SSR) and randomly amplified polymorphic DNA (RAPD) markers through marker aided selection (MAS). This was also higher than the report of (Prabakaran *et al.* 2010) [17] for genetic divergence of rice land races where in they reported average alleles of 2.2 per locus. Similarly the present result was lower than the results reported by (Pachauri *et al.* 2013) [18] with an average number of alleles per locus detected as 2.79 in molecular and morphological characterization of Indian farmers rice varieties.

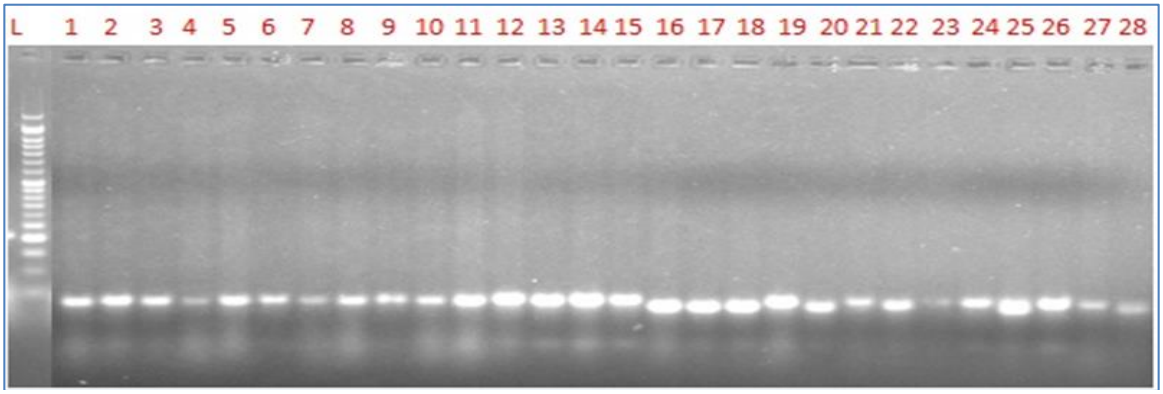

Fig 1: SSR banding profile obtained by marker RM 19

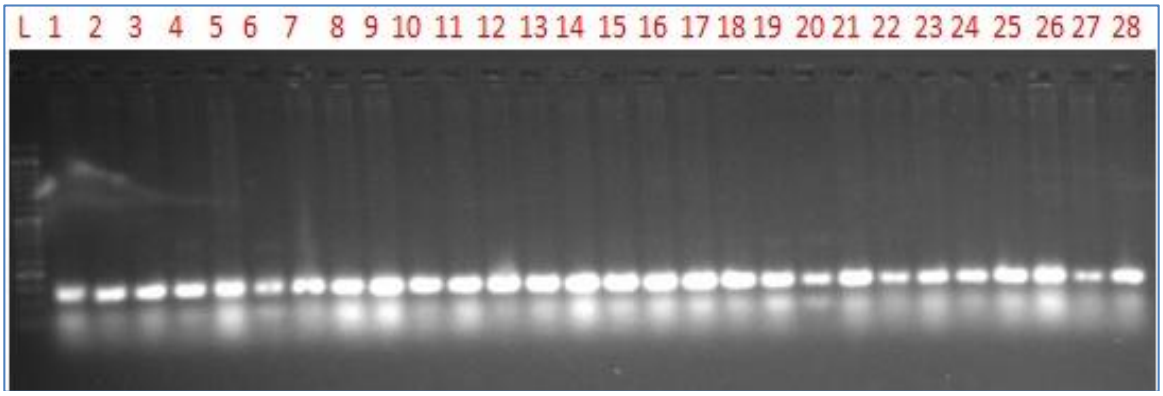

Fig 2: SSR banding profile obtained by marker RM 408

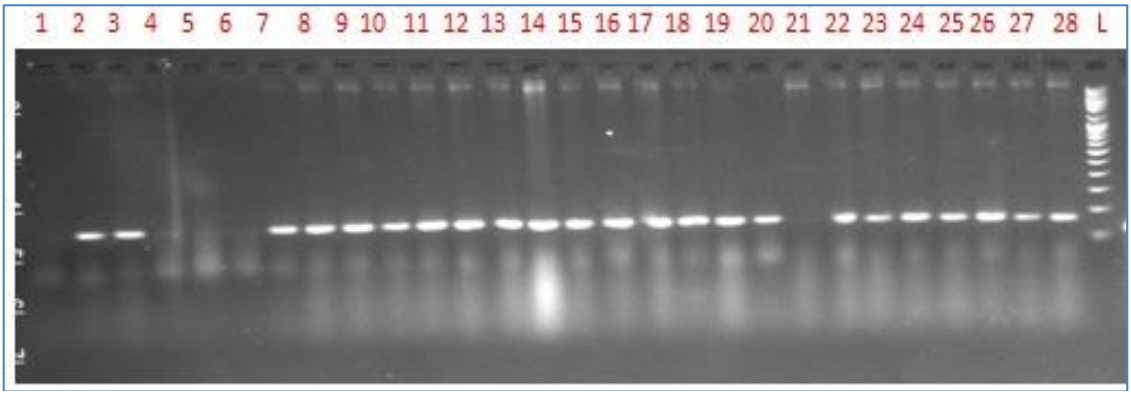

Fig 3: SSR banding profile obtained by marker RM 60
L = 100 bp Ladder1= GSRIR1-DQ125-L2-D2, 2= GSRIR1-DQ125-R4-Y1, 3= GSRIR1-DQ142-Y1-Y1, 4, GSRIR1-DQ187-Y3-D1, 5= GSRIR1-DQ121-LI9-L1, 6 GSRIR1-DQ62-D7-D1, 7= GSRIR1-DQ62-D6-D1, 8= GSRIR1-DQ129-Y4-L1, 9= GSRIR1-DQ139-R1-L2, 10= GSRIR1-DQ150-R5-Y1, 11= GSRIR1-DQ138-LI1-Y1, 12= GSRIR1-DQ122-D2-D1, 13= GSRIR1-DQ112-Y1-D2, 14= GSRIR1-DQ136-L15-Y1, 15= URG-1, 16= URG-3 17=URG-5, 18URG-8, 19=URG-19, 20=URG-22, 21=URG-24, 22=URG-30, 23= HUR4-3, 24=HUBR5-1, 25=HUR5-2, 26=HUBR10-9 27=HUR105, 28=HUR3022

UPGMA based cluster analysis

A dendrogram (Fig 4) based on Jaccard's dissimilarity coefficient was constructed. Cluster Analysis was performed by using UPGMA based on dissimilarity co-efficient values. It resolved 28 rice cultivars lines into one main cluster and two sub clusters *i.e.*, cluster I and II with dissimilarity coefficient 0.42. Cluster I was sub divided into two minor sub-groups IA and IB having 6 and 11 genotypes respectively. IB was further subdivided into minor groups. In similar way, the second main cluster *i.e.* Cluster II was also sub divided into two minor sub-groups that is IIA and IIB having 3 and 8 genotypes respectively. IIB further divided into minor groups. This indicated presence of considerable diversity in the genotypes studied.

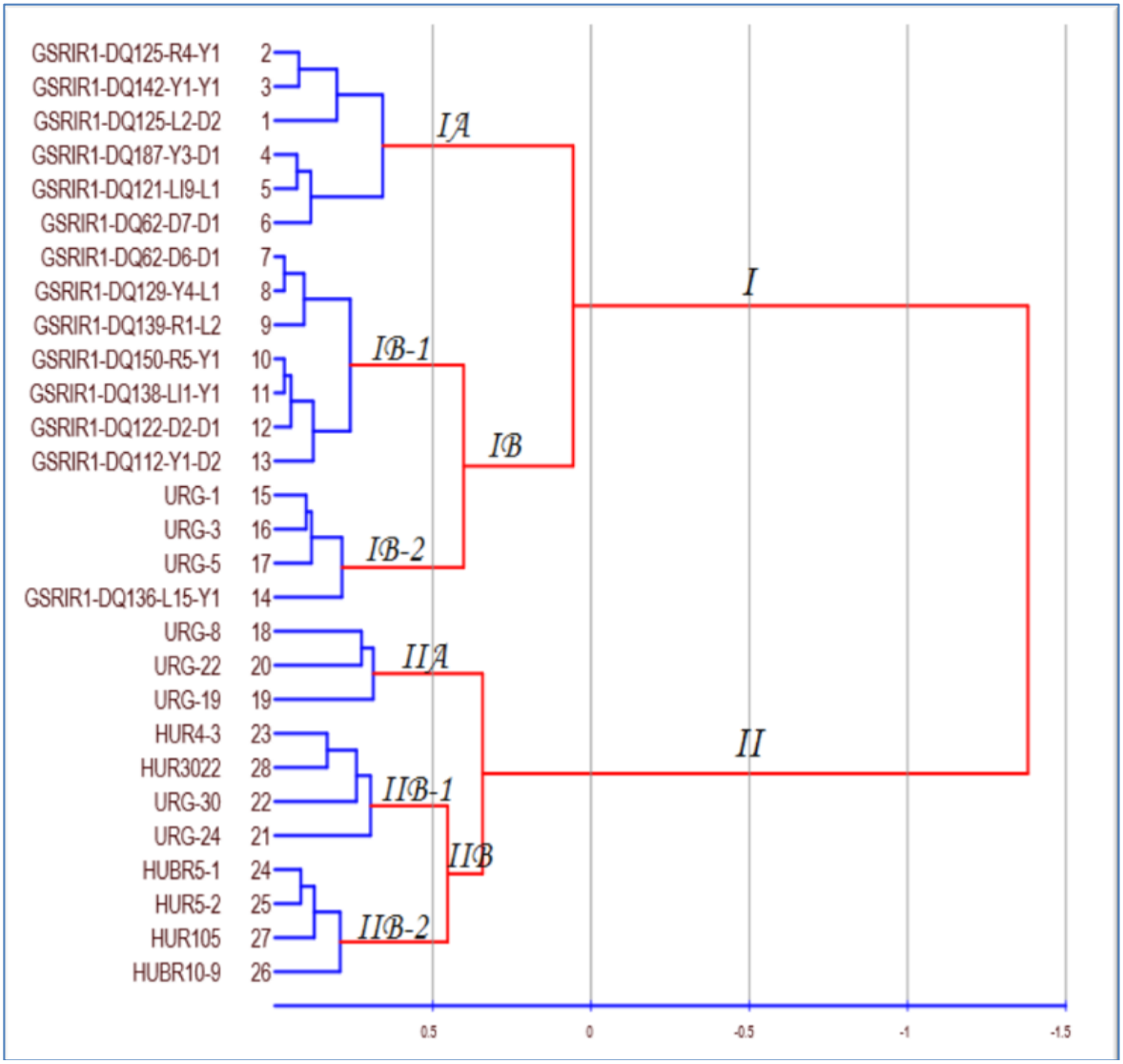

Fig 4: Cluster analysis with UPGMA method in 28 genotypes of rice using SSR fingerprint data and a Jaccard dissimilarity matrix.

This analysis indicated the presence of considerable diversity in the germplasm studied. The diverse genotypes are therefore, important in order to select desirable genotypes for utilizing in breeding programmes. On the basis of the dendrogram, the highest similarity was observed between cultivar GSRIR1-DQ129-Y4-L1 and GSRIR1-DQ62-D6-D1 (0.0625), GSRIR1-DQ138-LI1-Y1 and GSRIR1-DQ150-R5-Y1 (0.0625), GSRIR1-DQ122-D2-D1 and GSRIR1-DQ138-LI1-Y1 (0.0625). The most diverse cultivars were HUR4-3 and GSRIR1-DQ125-L2-D2. The results were comparable to Upadhyay *et al.* 2011 [28] who had reported clustering of 29 rice genotypes into major clusters while studying the development of molecular tags for rice lines. Sonkar *et al.* 2016 [23] also conducted molecular diversity analysis of 36 rice cultivars using 4 SSR primers and found similar results in which the germplasm was grouped into four clusters. This analysis indicated the presence of considerable diversity in the germplasm studied. The diverse genotypes are therefore, important in order to select desirable genotypes for utilizing in breeding programmes.

Jaccard's dissimilarity coefficient

The dissimilarity coefficient varies from one to zero, close to one shows high similarity while close to zero shows high dissimilarity. The average of dissimilarity coefficient varies from 1.0 to 0.0625. The total average of dissimilarity coefficient of all 28 genotypes is 0.6413. The dissimilarity coefficient varied from the largest value 1.0 between the cultivar HUR4-3 and GSRIR1-DQ125-L2-D2 followed by dissimilarity coefficient value 0.9583 between the cultivar URG-19 andGSRIR1-DQ125-L2-D2 followed by cultivar value 0.9545 between the cultivar HUR4-3 and GSRIR1-DQ138-LI1-Y1 which shows high dissimilarity between them. The lowest value 0.0625 was found betweenGSRIR1-DQ129-Y4-L1 and GSRIR1-DQ62-D6-D1, GSRIR1-DQ138-LI1-Y1and GSRIR1-DQ150-R5-Y1, GSRIR1-DQ122-D2-D1 and GSRIR1-DQ138-LI1-Y1. Similar result was found by Lapitan *et al.* (2007) [10] and Siva *et al.* (2010) [26].

PIC Value

In the present study, PIC values varied widely among SSR loci tested and it ranged from 0.753 RM (60) to 0.067 RM (161), with a mean value of 0.376 (Table 3.). Among polymorphic SSR markers, RM 60 showed highest PIC value 0.753. Similar result were also obtained by Ravi *et al.* (2003) who evaluated 40 cultivars and 5 wild relatives for polymorphism after amplification with 36 primers. So, PIC value is totally related to polymorphism of markers. High PIC value means high polymorphisms. However, the PIC values reported in this study were lower than those obtained by Sonkar *et al.* 2016 [23] who reported a mean PIC value of 0.92.

Table 3: Allele size (bp) and polymorphism information content (PIC) of SSR primers used in the present study

Marker	PIC
RM 60	0.753
RM 22	0.374
RM 1	0.132
RM 19	0.370
RM 161	0.067
RM 162	0.407
RM 178	0.528
RM 223	0.269
RM 237	0.368
RM 259	0.339
RM 283	0.558
RM 408	0.690
RM 495	0.305
RM 452	0.237
RM 108	0.237

Conclusion

The 28 rice cultivars were grouped into two clusters *i.e.*, cluster I and II with dissimilarity coefficient 0.42. Cluster I was sub divided into two minor sub-groups IA and IB having 6 and 11 genotypes respectively. IB was further subdivided into minor groups. In similar way, the second main cluster *i.e.* Cluster II was also sub divided into two minor sub-groups that is IIA and IIB having 3 and 8 genotypes respectively. This indicates presence of significant diversity among genotypes studied. Based on Jaccard's dissimilarity coefficient HUR4-3 and GSRIR1-DQ125-L2-D2 are most diverse cultivars. The lowest value of it 0.0625 was found between GSRIR1-DQ129-Y4-L1 and GSRIR1-DQ62-D6-D1, GSRIR1-DQ138-LI1-Y1 and GSRIR1-DQ150-R5-Y1, GSRIR1-DQ122-D2-D1 and GSRIR1-DQ138-LI1-Y1 which indicate highest similarity between them. Among polymorphic SSR markers, RM 60 showed highest PIC value 0.753.

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