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RESEARCH PAPER

SSR based molecular diversity study in local rice (*Oryza sativa* L.) genotypes of Bangladesh

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ABSTRACT

Genetic diversity is one of the most important factors considered in plant breeding and molecular approaches are well accepted and precise to determine the diversity. This study was conducted to determine the genetic diversity of 28 local rice varieties using Simple Sequence Repeat (SSR) markers. A set of five SSR markers namely RM55, RM259, RM413, RM474 and RM481 were used to study the diversity at molecular level. All of the microsatellite loci were amplified by the polymerase chain reaction (PCR) and found to be 100% polymorphic. Across 28 varieties, 62 alleles were identified. The highest no. (15) of was identified by RM474. On the other hands RM55, RM259 and RM481 identified the lowest 11 alleles and RM473 observed 14 alleles. Observed heterozygosity for RM55, RM259, RM413, RM474 and RM481 were 0.071, 0.285, 0.107, 0.017 and 0.178, respectively. Genetic differentiation values were found in the range of 0.045 to 0.954 with an average of 0.912. Variation also observed in allele frequency and diversity index where diversity index ranged from 0.776789 to 0.917101. Over all Nei's genetic distance value (D) ranged from nil to 2.890 among 178 varietals pairs resulting as a means of permutation combination of 28 rice varieties. The UPGMA dendrogram based on Nei's genetic distance separated all the varieties among with each other. 28 varieties were identified with at least one and/or combination of 5 primers. The approach of this study will be useful for the development of desirable varieties by selection of parents as well as to protect the varieties through DNA fingerprinting.

Key words: Molecular diversity, SSR, *Oryza sativa*, Bangladesh

Introduction

Rice (*Oryza sativa* L.) is the staple food for nearly half of the world's population. This crop is widely distributed around the world, in as broad range as 50° N to 40° S and from the sea level to altitudes of more than 2500m. Rice is grown in Bangladesh under diverse ecosystem of irrigated, rain fed and deep water conditions in three distinct seasons namely aus, aman and boro (Rashid, 1994).

Large variations in respect of morphological, biochemical and DNA traits exist among rice varieties leaving a wide scope for future improvement (Chakravarthi and Naravaneni, 2006). Both breeders and farmers in many cases find the variations in their varieties and fields tend to take the advantages of appropriate selection technique in order to maintain the purity or even to screen for a new type. As the number of rice cultivars increases, the ability to distinguish them on the basis of morphological, molecular and biochemical traits becomes more difficult mostly due to genotype-environment interaction.

Molecular markers have been successfully applied in variety and cultivar identification (Mailer *et al*. 1994), or controls of seed purity of hybrid varieties (Marshall *et al.* 1994). A powerful technique for DNA fingerprinting is successful PCR amplification of tandem repeat sequences, which have long been known to be polymorphic and widespread in plant genomes referred to

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as Simple Sequence Repeats (SSR).

In plants, it has been demonstrated that SSRs are highly informative, locus specific markers in many species (Akkaya *et al*. 1992; Legarcrantz and Anderson 1993; Wu and Tanksley 1993). SSRs are increasingly useful for integrating the genetic, physical, and sequence-based maps of rice, and they simultaneously provide breeders and geneticists with an efficient tool to link phenotypic and genotypic variation.

In Bangladesh, 20 crop species including rice, wheat, maize, barley, rapeseed, soybean, potato and other crops were tasted with SSR by Rahman *et al*. (2006 and 2007) to know the phenotypic and genotypic variation with a view of documentation and characterization.

Materials and Methods

Isolation of genomic DNA

Bulked DNA was isolated from 2-5 juvenile culms of 15-day-old seedlings of each of 28 rice varieties/cultivars following the protocol described by Aljanabi and Martinez (1997) and also used by Rahman *et al*. (2007) with some modifications. Excluding usage of CTAB, the modified protocol included digestion with homogenization buffer (Solution: Tris-50 mM, EDTA-25 mM, NaCI-300 mM, 1% SDS and deionized water) at 65℃ for 30 min, extraction with phenol: chloroform: isoamyl alcohol (25:24:1), precipitation with ice-cold and extra pure isopropyl alcohol and purification with absolute ethanol (Plus sodium acetate, 3M) and 70% ethanol chronologically. Finally, DNA sample of each rice variety dissolving in 30-40μl of TE buffer within 1.5 ml eppendorf tube was preserved separately at -20℃. Presence of genomic DNA was confirmed on 1% agarose gel qualitatively.

Quantification and optimization of DNA concentration

The amount of genomic DNA was quantified at 260nm spectrophotometrically (Spectronic® Genesis™). Using the absorbance reading obtained for DNA sample of each rice variety, the original DNA concentrations were determined and adjusted to 25 ng/μl.

Identification and selection of microsatellite/SSR primers

A set of seventeen microsatellite primer pairs (RM5, RM55, RM105, RM151, RM153, RM170, RM206, RM264, RM266, RM278, RM287, RM307, RM333, RM334, RM335 RM475 and RM481) distributed in the rice genome were identified from the available data-based search (http://www.gramene.org/) for rice SSR markers as described by Akagi *et al*. (1996), Panaud *et al*. (1996), Temnykh *et al*. (2000 and 2001) and McCouch *et al*. (2002). From those identified primers, at first 3 to 5 primers were tested against 5 randomly selected varieties with a recommended PCR thermal profile. Based on better responsiveness in amplifying the target genomic region of template DNA, the expected PCR product sizes in base pairs was then going to check. The selected primers were then screened against 28 varieties at a time.

In this way five primer pairs viz. RM55, RM259, RM413, RM474 and RM481 representing chromosome numbers 3, 1, 5, 10 and 7 of rice genome (Temnykh *et al*. 2001) with clear and expected amplified product sizes were selected and used for microsatellite analysis in the present study.

Polymerase chain reaction (PCR)

Polymerase chain reaction were done in a volume of 10 µl containing 10x PCR Buffer, 0.25 mM each of the dNTPs. 1 µM of each of primer, 1 unit ampliTaq DNA polymerase, 50 ng template DNA and a suitable amount of sterile deionized water. Amplification was carried out in a oil free thermal cycler (Thermal cycler gradient, Eppendorf) with the following thermal profile: initial denaturation step at 94℃ for 3 min. followed by 35 cycles at 95℃ for 30 sec., 55℃ for 30 sec. and 72℃ for 1 min. and a final cycle at 72℃ for 7 min. PCR was confirmed by electrophoresis on 2% agarose gel.

Electrophoretic separation and visualization of PCR products

PCR was confirmed by electrophoresis on 2% agarose gel. Prior to electrophoresis each PCR-products was prepared with loading dye (0.25% xylene cyanol, 0.25% bromophenol blue, 30% glycerol and 1 mM EDTA). Loading dye was used for monitoring the loading and the progress of electrophoresis and to increase the weight of the sample so that it stayed in the well of the gel. A molecular weight marker DNA (100 bp) was loaded on either side of the gel. The gel was carefully placed in the electrophoresis gel chamber (Blue Marine Serva) keeping the gel horizontal and submerged in 1× TBE buffer (running buffer) and final level of buffer was ~5mm above the gel. Electrophoresis was performed at 100 V for 1 hour and 30 minutes using the power supply (EPS 301, Amersham, Pharmacia Biotech). The DNA migrate from negative to positive electrode (black to red). The electrophoresis was stopped after the bromophenol blue dye had reached three-fourths of the gel length. PCR-products were electrophoresed on a 2% agarose gel. Electrophoresis was done using the SequiGen GT Sequencing Cell (BIO-RAD Laboratories, Hercules, CA) electrophoresis system. Before running the gel, the power supply which connects with gel chamber was set for 2hrs and 40 minutes, 100V and 50W. For documentation, the gel was taken out from the electrophoresis chamber and placed on the high performance ultraviolet transilluminator (UVP, BioDoc-It™ System) at the wave-length of 302 nm to observe the quality of the genomic DNA.Then the gel was examined and was photographed by using a Gel Cam Polaroid camera and finally saved in computer.

Scoring and analysis of microsatellite data

Microsatellite DNA profiles of all rice varieties against five primers are shown in Fig. 1. Photograph from Gel Cam Polaroid camera was printed on 20cm X 25 cm graph paper. The bands representing particular alleles at the microsatellite loci were scored manually and designated the bands as A, B, C, etc. from the top to the bottom of the gel by four experienced scientists individually. The genotypes of different individuals were hypothetically scored as AA, BB, CC, etc. for homozygous

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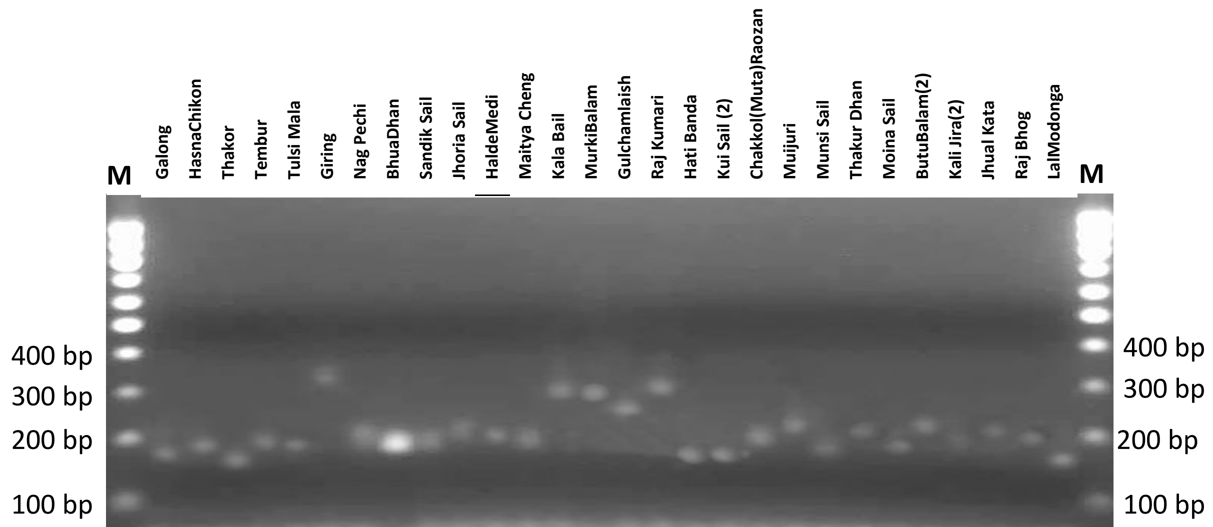

Fig.1: Microsatellite profiles of 28 local rice varieties at locus **RM 55**; using 100bp DNA ladder

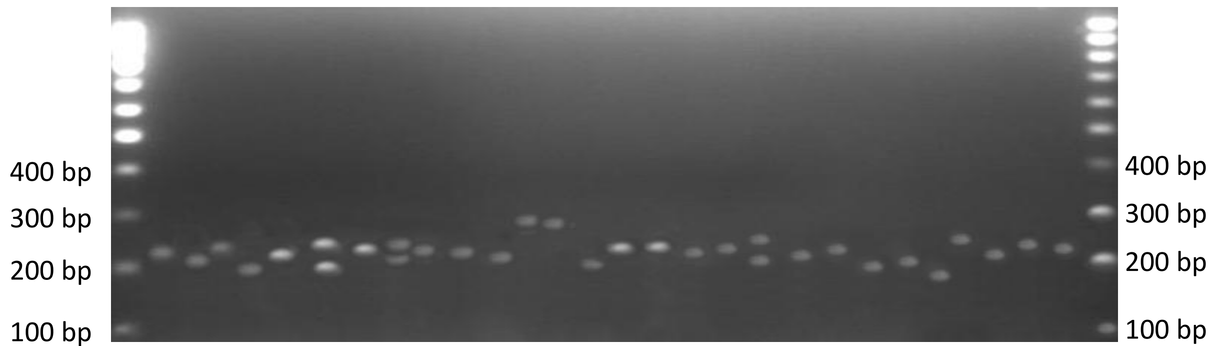

Fig.2: Microsatellite profiles of 28 local rice varieties at locus **RM 259**; using 100bp DNA ladder

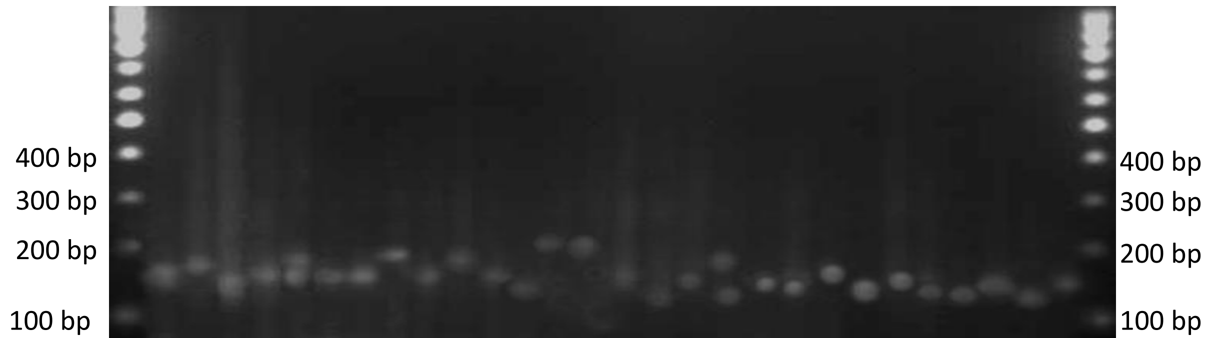

Fig.3: Microsatellite profiles of 28 local rice varieties at locus **RM 413**; using 100bp DNA ladder

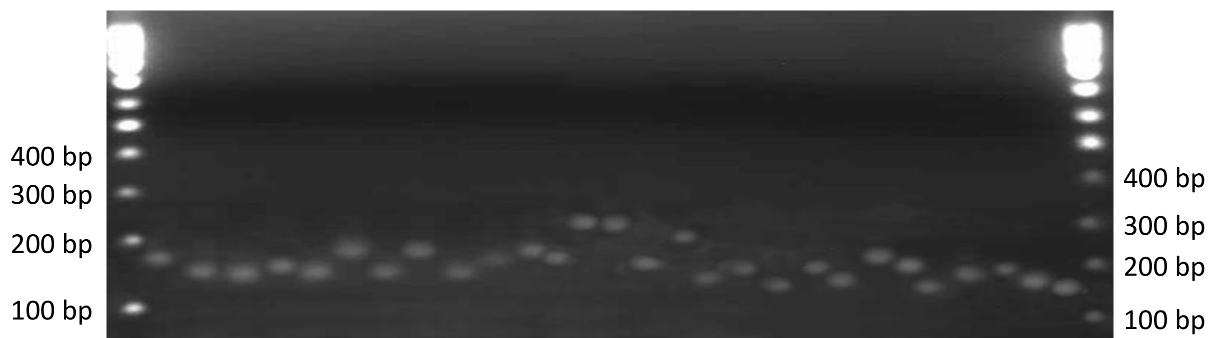

Fig.4: Microsatellite profiles of 28 local rice varieties at locus **RM 474**; using 100bp DNA ladder

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or as AB, AC, BC etc. for heterozygous. A single genotypic data matrix was constructed for all loci. Polymorphism Information Content (PIC) was computed by deducting sum of square values for all the frequencies of different alleles produced by a single marker locus from one using the formula: PIC= 1- ΣXi^2, where, Xi is the frequency of the i-th allele of a particular locus. PIC provides an estimate of the discriminatory power of a marker by taking into account, not only the number of alleles that are expressed, but also the relative frequencies of those alleles. PIC values range from 0 (monomorphic) to 1 (very high discriminative, with many alleles in equal frequencies). The software DNA FRAG version 3.03 was used to estimate allelic length (Nash, 1991). Expected (He) and observed heterozygosity (Ho) were also calculated as per Nei (1972) formula and with the help of POPGENE (version 1.31) (Yeh *et al.* 1999) computer package program. Estimation of Nei's genetic distance values (D) (Nei, 1972) and construction of UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram was constructed using the software POPGENE (Version 1.31) (Yeh *et al.* 1999).

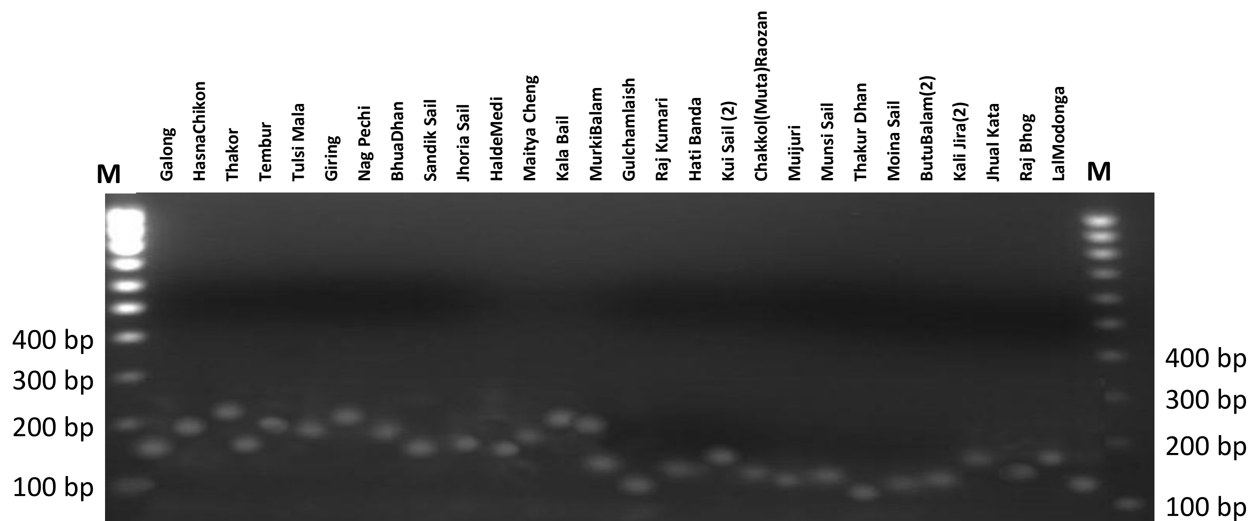

Fig.5. Microsatellite profiles of 28 local rice varieties at locus RM 481; using 100bp DNA ladder

Table 1: Summary of genetic variation and heterozygosity statistics for all loci

Locus	na*	ne*	I*	Fst	Nm*	Obs Hom	Obs. Het	Exp. Hom*	Exp Het*	Nei**	Ave_ Het
RM55	11.00	4.48	1.856	0.954	0.012	0.928	0.071	0.209	0.790	0.776	0.035
RM259	11.00	6.56	2.086	0.831	0.050	0.714	0.285	0.137	0.863	0.847	0.142
RM413	14.00	9.98		0.940	0.015	0.892	0.107	0.083	0.916	0.899	0.053
RM474	15.00	12.06		0.941	0.015	0.892	0.107	0.066	0.933	0.917	0.053
RM481	11.00	7.95	2.461	0.897	0.028	0.821	0.178	0.109	0.890	0.874	0.089
Mean	12.4	8.21	2.592	0.912	0.024	0.849	0.149	0.120	0.878	0.862	0.074
St. Dev	1.74	2.63	0.262	0.045	0.014	0.076	0.076	0.050	0.050	0.049	0.038

*na = Observed number of alleles
* ne = Effective number of alleles
*I = Shannon's Information Index
*Nm = Gene flow estimated from Fst = 0.25(1 - Fst)/Fst.
* Expected homozygosity and heterozygosity were computed using Levene (1949)

Results and Discussion

SSR has become one of the most widely used molecular markers for genetic studies in recent years. Enriching the AFLP or specific adaptor-amplified DNA fragments is a simple and efficient approach for SSR isolation and has been successfully applied to a number of plant genomes.

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Allelic and Loci Variation within the genotypes

All the microsatellite loci (RM55, RM259, RM413, RM474 and RM481) amplified were found to be polymorphic. The microsatellite profiles of loci RM55, RM259, RM413, RM474 and RM481 are shown in Fig 1-5. Using five primers across 28 varieties, 62 allele were identified. The number of alleles ranged from eleven to fifteen per locus. The locus RM474 had the highest number of alleles (15) while the locus RM55, RM259 and RM481 had the lowest number of alleles (11) and RM413 had fourteen allele **(Tab 2).** The effective number of allele was also the highest (19.852) for RM474 (12.06) and the lowest for RM55 (4.48) (Table 2). Yang *et al*. (1994) found up to 25 alleles for 10 microsatellite markers among 238 accessions of *Indica* and *Japonica* cultivars and landraces.

Genetic differentiation (Fst) values were found in the ranges 0.045 to 0.954 with an average of 0.912 and gene flow (Nm) values ranged from 0.012 to 0.050 with an average of 0.024 (Table 1) indicating comparatively higher level of genetic differentiation and low level of gene flow values in 28 rice (*O. sativa* L.) varieties which are the expression of diversity among the varieties studied. Variation also found in number of alleles, observed and expected heterozygosity. Across 28 rice varieties, RM259 showed the highest average heterozygosity (0.142) while RM55 (0.035) yielded lowest average heterozygosity. Over all Nei's genetic distance value ranged from 0.0492 to 0.917.

Variation also found in allele frequency and diversity index values where diversity index ranged from 0.776789 to 0.917101 (Table 2). Higher diversity index was 0.917101 for RM474. Lower PIC value may be the result of closely related genotypes and higher PIC values might be the result of diverse genotypes.

Table 2: Frequency of alleles and Wright's fixation index at 5 SSR loci across 28 rice *(Oryza sativa* L.) varieties

Allele	RM55 (Chromosome 3)	RM259 (Chromosome 1)	RM413 (Chromosome 5)	RM474 (Chromosome 10)	RM481 (Chromosome 7)
Allele A	0.0179	0.0179	0.0357	0.1429	0.0357
Allele B	0.0357	0.0179	0.0357	0.0714	0.0893
Allele C	0.0893	0.2143	0.0536	0.0357	0.1250
Allele D	0.0357	0.0536	0.0357	0.0357	0.1964
Allele E	0.3750	0.2321	0.0179	0.1071	0.0714
Allele F	0.2500	0.1786	0.0714	0.0357	0.0714
Allele G	0.0714	0.0536	0.0536	0.0357	0.1964
Allele H	0.0357	0.0714	0.0714	0.0536	0.0893
Allele I	0.0357	0.0536	0.1071	0.0357	0.0357
Allele J	0.0179	0.0714	0.1964	0.1071	0.0536
Allele K	0.0357	0.0357	0.0714	0.0714	0.0357
Allele L			0.0893	0.0893	
Allele M			0.1250	0.0536	
Allele N			0.0357	0.0357	
Allele O				0.0893	
Diversity Index (PIC=1-$\sum Xi^2$)	0.776789	0.847577	0.899899	0.917101	0.874388
Observed number of alleles	11	11	14	15	11
Sequence of primers (5´-3´)	f: ccgtcgccgtagtagagaag	f: tggagtttgagaggaggg	f: ggcgattcttggatgaagag	f: aagatgtacgggtggcattc	f: tagctagccgattgaatggc
	r: tcccggttattttaaggcg	r: cttgttgcatggtgccatgt	r: accccaccaaacaagacaac	r: tatgagctggtgagcaatgg	r: ctccacctcctatgttgttg
Motif	$(ga)_{17}$	$(ct)_{17}$	$(ag)_{11}$	$(at)_{13}$	$(caa)_{12}$

Wright's fixation index:

The ranges of wrights fixation index which is the measure of heterozygote deficiency or excess for five primers RM55, RM259, RM413, RM474 and RM481 were 0.0179-0.3750; 0.0179-0.2321; 0.0179-0.1964; 0.0357-0.1429 and 0.357-0.1964 respectively (**Table 2**).

Genetic distance:

The highest Nei's genetic distance (2.890) was estimated in Munsi Sail-Kali Jira (2), the lowest genetic distance (0.00) was calculated in 178 combination (eg. Galong - Thakor, Thakor - Tembur and Hasna Chikon – Tembur etc.)

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Table 3: Nei's genetic distance among the 28 rice varieties

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	****																											
2	1.609	****																										
3	0.000	1.609	****																									
4	1.609	0.000	0.000	****																								
5	0.000	2.250	0.000	0.000	****																							
6	1.092	0.000	2.191	1.498	0.000	****																						
7	1.609	0.916	0.000	0.000	1.151	0.000	****																					
8	0.000	0.000	1.557	2.250	0.000	2.832	0.000	****																				
9	1.609	0.519	1.609	1.609	2.250	0.000	0.916	1.557	****																			
10	2.191	1.092	0.000	0.000	2.138	0.000	0.805	1.040	0.582	****																		
11	0.916	0.916	0.000	0.000	1.151	0.000	0.223	0.000	0.916	0.805	****																	
12	0.000	1.609	0.000	1.609	2.250	0.000	0.916	0.000	1.609	1.092	0.916	****																
13	0.000	1.151	1.151	0.000	2.197	0.000	0.000	2.197	1.557	0.000	0.000	0.000	****															
14	2.047	1.354	2.047	0.000	1.995	0.000	1.354	1.301	0.949	0.837	1.354	1.354	1.078	****														
15	0.000	0.916	1.609	0.000	2.250	0.000	1.609	0.000	0.916	0.805	1.609	1.609	1.557	1.354	****													
16	0.000	0.000	1.498	2.191	0.000	2.773	2.191	1.222	0.000	2.773	2.191	2.191	1.445	1.530	0.000	****												
17	0.000	1.557	0.000	0.000	0.811	0.000	1.557	0.000	1.557	1.445	1.557	1.557	0.000	0.896	1.557	0.000	****											
18	1.557	1.557	0.000	0.000	1.504	2.138	0.000	2.197	0.000	0.000	0.000	0.000	0.000	1.995	0.000	2.138	1.504	****										
19	0.000	0.000	0.000	1.609	0.000	0.000	1.609	0.000	0.000	2.191	1.609	0.916	0.000	0.000	1.609	2.191	0.000	0.000	****									
20	1.609	0.000	0.000	0.916	1.557	1.498	1.609	2.250	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.557	1.557	0.000	****								
21	0.000	0.000	1.557	0.000	1.504	2.138	0.000	2.197	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.138	2.197	1.504	0.000	1.557	****							
22	0.000	1.609	0.000	0.000	2.250	0.000	1.609	0.000	1.609	1.498	1.609	1.609	0.000	0.949	1.609	0.000	1.557	0.000	0.000	1.609	1.557	****						
23	1.609	0.000	0.000	1.609	1.557	1.498	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.047	0.000	0.000	0.864	1.557	0.000	0.916	0.000	1.609	****					
24	0.000	2.191	2.191	0.000	0.000	2.079	0.000	2.138	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.079	0.000	1.040	0.000	0.000	1.040	0.000	0.000	****				
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	1.504	1.557	1.040	0.000	0.000	0.000	1.589	0.000	0.000	0.000	0.000	0.000	0.000	2.890	2.250	0.000	2.832	****			
26	1.557	1.557	0.000	1.557	1.504	1.040	0.000	2.197	0.000	0.000	0.000	0.000	0.000	0.000	0.000	2.138	1.504	0.406	0.000	0.864	1.504	0.000	0.641	1.040	0.000	****		
27	1.609	0.000	0.000	1.609	0.000	1.498	1.609	1.557	1.609	0.805	1.609	1.609	0.000	2.047	0.000	2.191	0.000	0.000	1.609	1.609	0.000	0.000	1.609	0.000	0.864	1.151	****	
28	0.000	0.000	0.000	2.250	1.504	0.000	0.000	0.000	0.000	2.138	0.000	0.000	1.504	0.000	0.000	2.832	2.197	2.197	0.000	2.250	0.000	0.000	2.250	0.000	1.504	1.504	0.864	****

Name of the genotypes
1. Galong
2. Hasna Chikon
3. Thakor
4. Tembur
5. Tulsi Mala
6. Giring
7. Nag Pechi
8. Bhua Dhan
9. Sandik Sail
10. Jhoria Sail
11. Halde Medi
12. Maitya Cheng
13. Kala Bail
14. Murki Balam
15. Gulchamlaish
16. Raj Kumari
17. Hati Banda
18. Kui Sail (2)
19. Chakkol(Muta)Raozan
20. Muijuri
21. Munsi Sail
22. Thakur Dhan
23. Moina Sail
24. Butu Balam(2)
25. Kali Jira(2)
26. Jhual Kata
27. Raj Bhog
28. Lal Modonga

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Dendrogram

Geographical distance is an important factor that influences the genetic relatedness of populations. UPGMA dendrogram based on Nei's (1972) genetic distance indicated segregation of 28 varieties rice into two main clusters: In cluster: A) Sandik Sail, Raj Kumari and Muijuri are different from cluster B and B) Containing other 25 varieties in one grouped. The clusters were again separated into sub-clusters and sub subclusters subsequently separated into sub cluster and so on as remarking the cluster and subcluster as C, D, E, F and so on.

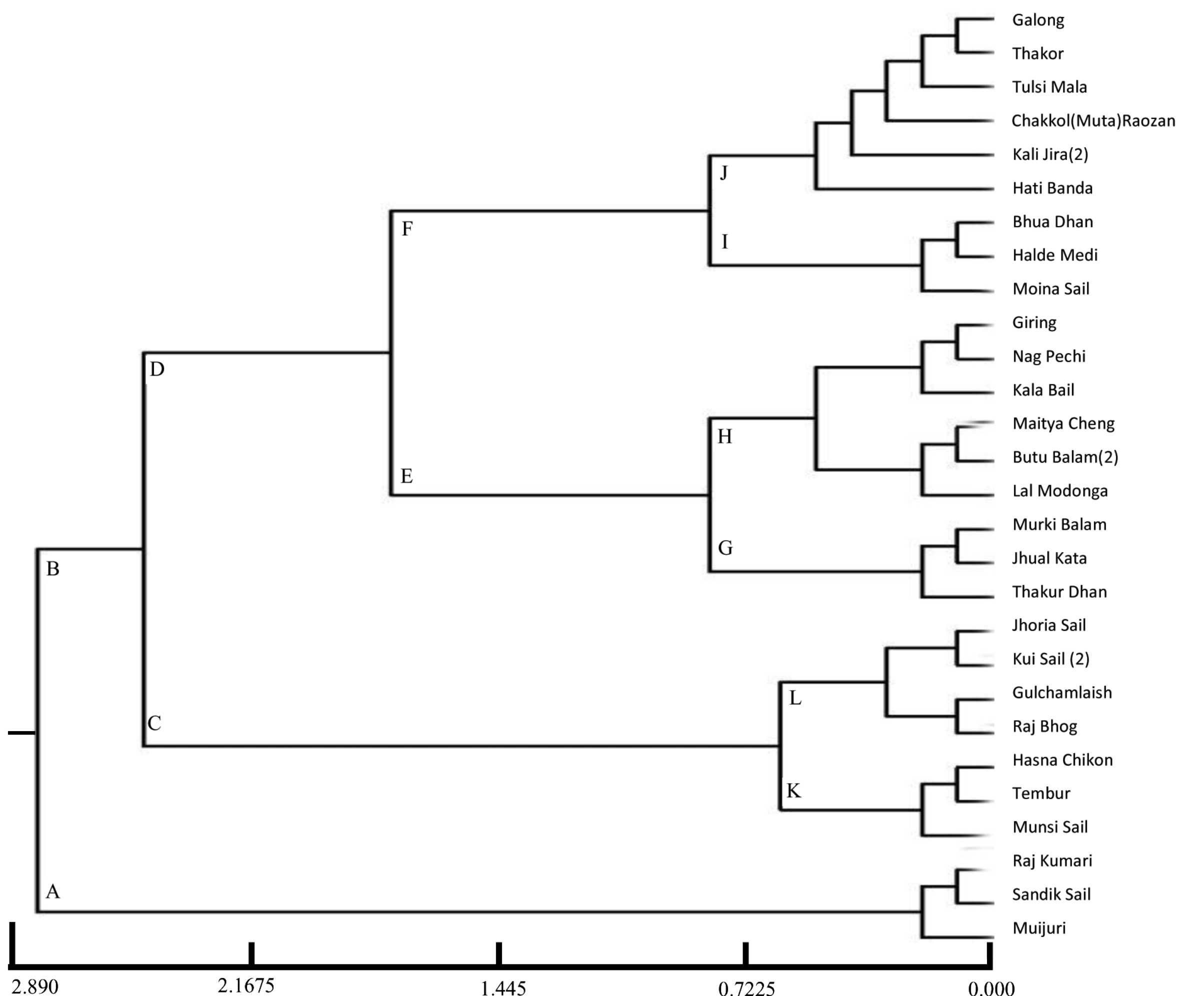

Fig 6: UPGMA dendogram based on Nei's (1972) genetic distance indicated segregation of 28 varieties

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