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ABSTRACT

Assessment of genetic diversity and molecular characterization among elite rice varieties of Bangladesh is very important for germplasm management, varietal identification and DNA fingerprinting. Thirty-four microsatellite markers were studied across 21 types of rice to characterize and discriminate among different varieties. The number of alleles per locus ranged from 2 to 11, with an average of 4.18 alleles across 34 loci. A total of 57 rare alleles were detected at 24 loci, where-as 42 unique alleles were detected at 20 loci. The results revealed that 14 rice varieties produced unique alleles, which could be used for identification, molecular characterization and DNA fingerprinting of these varieties. Polymorphic information content (PIC) values ranged from 0.157 to 0.838, with an average of 0.488, which revealed that much variation was present among the studied varieties. The PIC values revealed that RM401 might be the best marker for identification and diversity estimation of rice varieties, followed by RM566, RM3428, RM463, and RM8094 markers. The UPGMA cluster dendrogram created in this study identified five clusters with a similarity coefficient of 0.50. The SSR polymorphism and diversity could likely be attributed to pedigree. In this study, eight SSR markers (RM10713, RM279, RM424, RM6266, RM1155, RM289, RM20224 and RM5371) were identified that produced specific alleles only in the aromatic rice varieties and were useful for varietal identification and DNA fingerprinting of these aromatic rice varieties. The findings of this study sould be useful for varietal identification and could help in background selection in backcross breeding programs.

KEYWORDS: *Genetic Diversity, Molecular Characterization, Rice, SSR Marker, Breeding.*

INTRODUCTION

Rice (*Oryza sativa* L.) is an important cereal crop consumed exclusively by humans, as it is a staple food for about 50% of the global population (Garris et al. 2005; Ramkumar et al. 2010). The use of DNA markers has been suggested for precise and reliable characterization and discrimination of rice genotypes (Karkousis et al. 2003). For genetic variability assessment, DNA markers are extensively used, because these are not affected by environmental factors. Microsatellites (SSRs) are the marker of choice because of their advantages over other markers. These markers are polymorphic, abundant in eukaryotic organisms and well distributed throughout the genome (Tautz 1989; Morgante and Olivieri 1993). The SSRs are most suitable for rice because of their reproducibility, multiallelic nature, hypervariablility, codominant inheritance, relative abundance, and genome-wide coverage (Powell et al. 1996). In addition, SSRs often have flanking regions that are highly conserved in related species, which allows the use of the same primer pairs in related genomes (Cipriani et al. 1999; Sosinski et al. 2000). The SSR markers are particularly suitable for evaluating genetic diversity and relationships among plant species, populations, or individuals (Kostova et al. 2006; Tu et al. 2007), studying rice germplasm for either conservation or utilization (Sharma et al. 2007); marker-assisted selection breeding (Perez-Sackett et al. 2011; Rani and Adilakshmi 2011); cultivar identification; hybrid purity analysis and gene mapping studies (Weising et al. 1997; Altaf-Khan et al. 2006; Rajendrakumar et al. 2009; Sarao et al. 2010). Selection of parents based on genetic divergence using SSR markers has been successfully utilized in multiple crop species (Xu et al. 2002; Karkousis et al. 2003).

Information on the genetic diversity within and among closely related crop varieties is essential for a rational use of genetic resources and of fundamental interest to plant breeders. It contributes to monitoring germplasm and can also be used to predict potential genetic gains

(Chakravarthi and Naravaneni 2006). Information regarding genetic variability at molecular level could be used to help, identify and develop genetically unique germplasm that complements existing cultivars. Diversity of modern Aman rice varieties of Bangladesh has great importance for food security. So, it is very important to assess the genetic diversity among these varieties. Ours is the first study to evaluate genetic variation among modern Aman rice varieties at DNA level using SSR markers. The objectives of this research were to (i) assess the genetic variation and diversity of 21 elite Aman rice genotypes, (ii) determine the genetic relationship among these genotypes for breeding purposes, and (iii) characterize these rice genotypes.

MATERIALS AND METHODS

Plant Materials and Markers

Twenty-one elite and high-yielding rice varieties were studied (Table 1). Seeds were germinated in germination chambers and, after three days, germinated seedlings were sown in pots. The pots were then kept in a net-house. Thirty-four SSR markers distributed across 12 chromosomes were used for diversity analysis (Table 2).

SSR Marker Genotyping

DNA was collected from young leaves of 28-day-old seedlings following a modified miniscale protocol (Lang 2002). Polymerase chain reaction was performed in a solution of a 10 µl containing 1.5 µl 10x PCR buffer, 1.0 µl dNTPs (0.25 µl each of the dNTPs), 1.0 µl of each of primer (including forward and reverse primer), 0.5 µl taq DNA polymerase, 1.0 µl template DNA and a suitable amount of double distilled water. Amplification was carried out using a G-storm PCR machine (Gene Technologies Ltd., England). The initial denaturation was carried out for 5 min at 94 ^{0}C. Subsequently, 35 cycles of PCR were performed, and each

cycle was completed by denaturation (1 min at 94 ^{0}C), annealing (1 min at appropriate temperature) and extension (2 min at 72 ^{0}C). A final extension was performed for 7 min at 72 ^{0}C. Bromophenol blue (2 µl 5x) was added to each well of the PCR plate, and 3.5 µl of PCR amplification products was loaded in each well of the gel using fine-tipped 10 µl pipettes. A DNA ladder (3.0 µl) was also loaded between two sets of wells loaded with the PCR product. The gel was then run for 45 min to 1.15 hours (depending upon the allele size) at 100 mA current. SYBR-safe staining solution was used for staining the gels. The gels were stained for 30-35 min in the dark and were documented using a UVPRO Alpha Innotech gel documentation unit.

Data analysis

Molecular weight of each amplified allele was measured in base pairs using the Alpha-Ease FC 5.0 software. The allele frequency data from Power Marker version 3.25 (Liu and Muse 2005) was used to export the data in binary format (allele presence="1" and allele absence = "0") for analysis with Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) version 2.2 (Rohlf 2002). Polymorphic information content (PIC) values were calculated with the following formula (Anderson et al. 1993):

$$\text{PICi} = 1 - \sum_{j=1}^{n} (Pij)^2$$

where n is the number of marker alleles for marker i and P_{ij} is the frequency of the jth allele for marker i.

Summary statistics, including the number of alleles per locus, major allele frequency, gene diversity and PIC values were determined using PowerMarker version 3.25 (Liu and Muse 2005). For the unrooted phylogenetic tree, genetic distance was calculated using the 'Nei distance' (Nei 1973), followed by phylogeny reconstruction using neighbor-joining, as

implemented in PowerMarker, and the tree was viewed via Treeview (Page 1996). A similarity matrix was calculated with the Simqual subprogram using the Dice coefficient, followed by cluster analysis with the SAHN subprogram using the UPGMA clustering method as implemented in NTSYS-pc, and this was used to construct a dendrogram that showed relationships among the genotypes. The similarity matrix was also used for principal coordinate analysis (PCoA) with the DCenter, Eigen, Output, and MXPlot subprograms in the computer program NTSYS-pc.

RESULTS

Number of alleles

A total of 142 alleles were detected at 34 SSR markers among 21 rice genotypes. The number of alleles per locus ranged from 2 (RM10713, RM10927, RM6266, RM5371, RM436, RM256 and RM590) to 11 (RM401) with an average of 4.18 alleles across 34 loci, with 2 alleles for 7 markers, 3 alleles for 8 markers, 4 alleles for 6 markers, 5 alleles for 5 markers, 6 alleles for 5 markers, 7 alleles for two markers and 11 alleles for one marker. The overall size of the amplified products ranged from 80 bp (RM436) to 453 bp (RM401). The number of alleles, frequent alleles, rare alleles and unique alleles, the range of allele size, the highest frequency allele and the PIC values of different rice varieties for 34 SSR markers are shown in Table 2. The differences in molecular size between the smallest and the largest allele for a given SSR locus varied from 3 bp (RM436) to 263 bp (RM401).

Frequent, rare and unique alleles

Very frequent alleles were considered to be those occurring in more than 10% of the varieties in the collection, whereas those occurring between 2% and 10% of the varieties in the collection were classified as rare alleles (Alvarez et al. 2007). In this study, 85 frequent

alleles were identified at 34 microsatellite loci, with an average of 2.50 alleles per locus. The frequency of the most common allele at each locus ranged from 23.81% (RM463) to 90.48% (RM256 and RM436). On an average, 59.42% of the rice varieties shared a common major allele at any given locus. A total of 57 rare alleles were identified at 24 loci among 34 microsatellite loci, with an average of 1.68 alleles per locus (Table 2). Of these, 10 were present in a variety named BR25. Eighteen rice genotypes displayed one or more of such rare alleles. Most of these rare alleles were present in four of the rice varieties: Rajasail (7 alleles), BR5 (6 alleles), BRRI dhan41 (6 alleles) and BRRI dhan46 (6 alleles). Higher numbers of rare alleles were observed at the RM401 locus (8 alleles), followed by the RM3412, RM8094 and RM286 loci (4 alleles each).

A total of 42 unique alleles were detected at 20 microsatellite loci (Table 2). Fourteen of the rice varieties had unique alleles for at least one microsatellite locus. Notably, eight SSR markers, RM10713 (chromosome 01, 123 bp), RM279 (chromosome 02, 159 bp), RM424 (chromosome 02, 230 bp), RM6266 (chromosome 03, 183 bp), RM1155 (chromosome 04, 135 bp), RM289 (chromosome 05, 92 bp), RM20224 (chromosome 06, 169 bp) and RM5371 (chromosome 06, 102 bp), amplified specific alleles only in four aromatic rice varieties (BR5, BRRI dhan34, BRRI dhan37 and BRRI dhan38). Of these four aromatic rice varieties, BR5 had unique alleles at the RM3412 (206 bp), RM8094 (178 bp), RM10696B (292 bp), RM489 (309 bp), RM401 (226 bp) and RM242 (225 bp) loci. BRRI dhan34 had unique alleles at the RM3412 (157 bp), RM8094 (225 bp), RM38 (116 bp) and RM286 (232 bp) loci. BRRI dhan37 and BRRI dhan38 had similar rare alleles at the RM8094 (189 bp) and RM10696B (310 bp) loci.

Polymorphism in SSR markers

All of the 34 SSR markers used in this study generated polymorphic bands among the rice

varieties, and no band was found to be monomorphic. A similar result was found by Giarrocco et al. (2007) and Chakravarthi and Naravaneni (2006). The PIC values for the SSR loci ranged from 0.157 (RM436 and RM256) to 0.838 (RM401), with an average of 0.488. The highest PIC value (0.838) was obtained for RM401, followed by RM566 (0.754), RM3428 (0.748), RM463 (0.745) and RM8094 (0.730) (Table 2). The lowest PIC value (0.157) was obtained for RM436 and RM256.

Genetic distance-based analysis

An unrooted neighbor-joining tree (Fig. 1) showed the genetic relationships among the rice varieties. Interestingly, four aromatic fine rice varieties (BR5, BRRI dhan34, BRRI dhan37 and BRRI dhan38) were far from the other varieties and clustered in the same group. A moderate salt-tolerant variety Rajasail, which is grown mainly in the coastal saline areas through direct seeding, was close to the tallest variety BR25 (Fig. 1), a fine rice variety whose grain color is very attractive (reddish brown). BR3 and BRRI dhan33 were very similar in plant stature and grain characters, and might be considered in the same group. Other varieties were close to each other.

The UPGMA-based dendrogram, which was obtained from the binary data that was deduced from the DNA profiles of the samples analyzed, added a new dimension to the genetic similarity perspective. Five distinct groups were created from the analysis of the pooled SSR marker data at a similarity coefficient of 0.50 (Fig. 2). The cluster analysis showed high genetic variation among the rice cultivars studied, with similarity coefficient value ranging from 0.20 to 0.84.

DISCUSSION

Polymorphism in SSR markers

In this study, a microsatellite fingerprint database was generated for 21 rice genotypes using 34 SSRs. High levels of polymorphism were observed among the different rice genotypes. On a per locus basis, an average of 4.18 alleles is comparable with the average of 2.0-5.5 alleles per locus for various classes of SSRs reported by Cho et al. (2000) and Wong et al. (2009). Furthermore, the level of polymorphism, as assessed by the PIC values, was quite high and varied considerably among SSR loci (range 0.157 to 0.838, average value 0.418). High PIC values can be attributed to the use of more informative markers (Akkaya and Buyukunal-Bal 2004). It was also found that the higher the PIC value of a locus, the higher the number of alleles detected (Wong et al. 2009). The PIC values indicated that RM401 might be the best marker for diversity analysis of rice genotypes, followed by RM566, RM3428, RM463, and RM8094. RM436 and RM256 were likely the least powerful markers. Thus, these microsatellite sequences may be useful tools in future genetic studies of rice germplasm.

Genetic relationship among the rice varieties

The dendrogram obtained using the UPGMA method revealed cultivars that were genetically similar and thus clustered together and explained the relationship among the test rice varieties. In this study, five clusters were obtained with similarity coefficients of 0.50. In general, this genetic diversity is likely to be associated with the substantial variation in aroma producing ability, ecological and climatic conditions, agro-ecosystems, and especially breeding and ancestral history. In this study, the grouping of cultivars based on SSR polymorphisms corresponded well to their known pedigree data. Interestingly, all the aromatic rice varieties clustered in the same group and showed the maximum divergence from the other clusters (80% dissimilarity). These aromatic rice varieties were developed by crossing with Basmati (a local aromatic rice of India) or by improving the local aromatic germplasm (Table 1).

Indeed, aromatic rice varieties constitute a special group of rice strains that are well known for the presence of aroma and/or superfine grain quality (Jain et al. 2004). Among these aromatic rice varieties, BRRI dhan37 and BRRI dhan38 were developed from the same cross (Bashmati (D) × BR5) and showed maximum similarity (83% similarity). In unrooted neighbor-joining tree, this relationship is presented clearly (Fig. 1).

Among the other clusters, Rajasail, a moderately salinity-tolerant variety grown mainly in saline coastal ecosystems (Lisa et al. 2004), which had no relation to the other varieties in the pedigree record, formed a single cluster. BR25, which had a plant type similar to Rajasail and was developed from a cross between two completely divergent parents (IR26 × PAJAM) that had no relation to the other varieties from breeding records, also formed a distinct cluster. BR3 and BRRI dhan33 that have similar plant type, yield and grain characters, placed in the same cluster. The remaining varieties formed a single cluster, where BR4 and BR10, developed by crossing the same parents, formed a sub-cluster. BR11, BR22, BR23 and BRRI dhan30 formed another sub-cluster where all these varieties, except BR22, were related directly to IR20 via their ancestry or indirectly through BR4 (developed from a cross with IR20). BRRI dhan31, BRRI dhan32, BRRI dhan39, BRRI dhan40, BRRI dhan41, BRRI dhan44 and BRRI dhan46 formed a third sub-cluster, and they were indirectly related to the ancestral genotype IR20 through BR10, BR11 or BR4. From this study, it is clear that breeding history had a large effect on the diversity in rice.

Characterization of varieties

The practical approach developed in this study was useful in DNA fingerprinting. Among the 34 SSR markers studied, 20 markers spread across 11 chromosomes were found to be useful in fingerprinting 14 genotypes of the present study. It was found that fingerprinting of BR5 could be done by 6 different markers, followed by Rajasail with 5 markers and BRRI dhan34,

BR25, and BR3 with 4 markers each. Fingerprinting of the remaining cultivars can be done using a minimum of one marker. This fingerprinting makes identification and characterization of genotype very easy and further it will be of great help in background selections in backcross breeding programs.

In our study, eight microsatellite markers (e.g., RM10713, RM279, RM424, RM6266, RM1155, RM289, RM20224 and RM5371) were identified that produced specific alleles only in four aromatic rice varieties, which could readily distinguish the aromatic varieties from the others. Siwach et al.(2004) identified seven SSR markers (RM1, RM21, RM38, RM170, RM210, RM226 and RM229) that could distinguish Basmati rice varieties from non-Basmati rice varieties. Thus, fingerprinting technology using SSRs is a powerful tool to help in the detection of aromatic and/or premium quality rice varieties, which safeguards the interests of both consumers and producers of aromatic and/or premium quality rice (Bligh et al. 1999; Nagaraju et al. 2002; Siwach et al. 2004). Further, SSRs can be used to prepare a DNA fingerprint database of newly developed rice varieties, which can be used for varietal registration and protection of plant breeders' rights.

This study demonstrated that the tested samples possessed a high level of microsatellite variation. The markers used here were of value for characterizing rice cultivars, DNA fingerprinting of rice varieties and constructing a database for breeding programs, especially in background selections during backcross breeding.

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Table 1: List of rice (*Oryza sativa* L.) varieties used in this study

SL. No.	Name of variety	Year of release	Parents/crosses
01	BR3	1973	IR506-1-133 × Latisail
02	BR4	1975	IR20 × IR5-114-3-1
03	BR5	1976	Progeny selection from Badshahvog
04	BR10	1980	IR20 × IR5-114-3-1
05	BR11	1980	IR20 × IR5-47-2
06	BR22	1988	BR51-46-5 × Nizersail
07	BR23	1988	DA29 × BR4
08	BR25	1992	IR26 × PAJAM
09	BRRI dhan30	1994	IR2058-78-1-3-2-3 × BR4
10	BRRI dhan31	1994	BR11 × ARC10550
11	BRRI dhan32	1994	BR4 × BR2662
12	BRRI dhan33	1997	BG388 × BG367-4
13	BRRI dhan34	1997	Selection (Acc. No 4341)
14	BRRI dhan37	1998	Bashmati (D) × BR5
15	BRRI dhan38	1998	Bashmati (D) × BR5
16	BRRI dhan39	1999	BR1185-2B-56-2-1-1×BR1674-28-3-1-1
17	BRRI dhan40	2003	BR10 × IR4595-4-1-15
18	BRRI dhan41	2003	BR23 × BR1185-2B-16-1
19	BRRI dhan44	2005	BR10 × BRRI dhan31
20	BRRI dhan46	2007	BR11 × ARC14766 × Swarnolota
21	Rajasail	Local variety	Selection

BR = Bangladesh Rice, BRRI = Bangladesh Rice Research Institute

Table 2: Number of alleles, range of allele size, highest frequency allele and polymorphism information content (PIC) values of different rice varieties for 34 SSR markers

Marker	Alleles				Allele size (bp)		Highest frequency allele		PIC value
	Total alleles	Frequent alleles	rare alleles	Unique alleles	Range	Difference	Size (bp)	Frequency (%)	
RM05	3	2	1	1	156-175	19	165	80.95	0.292
RM490	4	4	0	0	120-140	20	140	57.14	0.570
RM1287	3	2	1	0	145-162	17	162	65.00	0.442
RM3412	6	2	4	4	157-206	49	187	52.38	0.584
RM8094	7	3	4	3	178-225	47	200	38.10	0.730
RM7075	3	2	1	1	150-209	59	161	80.00	0.303
RM10696B	5	2	3	1	244-310	66	253	52.38	0.604
RM10696	4	2	2	1	221-281	60	232	71.43	0.425
RM10713	2	2	0	0	123-130	7	130	80.95	0.261
RM10720	5	2	3	2	167-194	27	194	66.67	0.491
RM10927	2	2	0	0	122-127	5	122	85.71	0.215
RM279	6	3	3	3	140-199	59	140	47.62	0.654
RM424	3	2	1	0	230-281	51	261	71.43	0.398
RM489	3	2	1	1	248-309	61	248	80.95	0.292
RM6266	2	2	0	0	163-183	20	163	80.95	0.261
RM401	11	3	8	7	190-453	263	453	28.57	0.838
RM1155	4	4	0	0	135-166	31	162	28.57	0.696
RM1024	5	3	2	1	368-387	19	378	33.33	0.683
RM289	3	3	0	0	92-119	27	119	57.14	0.516
RM469	4	3	1	1	199-210	11	199	71.43	0.407
RM20224	3	3	0	0	169-204	35	188	61.90	0.478
RM5371	2	2	0	0	89-102	13	89	80.95	0.261
RM436	2	1	1	0	80-83	3	83	90.48	0.157
RM455	3	3	0	0	168-175	8	172	38.10	0.578
RM38	4	1	3	1	104-116	12	112	76.19	0.375
RM256	2	1	1	0	102-129	27	102	90.48	0.157
RM566	6	4	2	1	129-196	67	163	33.33	0.754
RM242	5	3	2	2	225-271	46	260	33.33	0.625
RM258	5	2	3	3	337-398	61	337	52.38	0.541
RM590	2	2	0	0	135-142	7	142	68.42	0.339
RM3428	6	4	2	1	393-436	43	399	33.33	0.748
RM286	7	3	4	4	181-232	51	187	42.86	0.695
RM17	4	2	2	2	281-307	26	303	64.29	0.483
RM463	6	4	2	2	379-415	36	390	23.81	0.745
Total	**142**	**85**	**57**	**42**					
Average	**4.18**	**2.5**	**1.68**	**1.24**				**59.42**	**0.488**

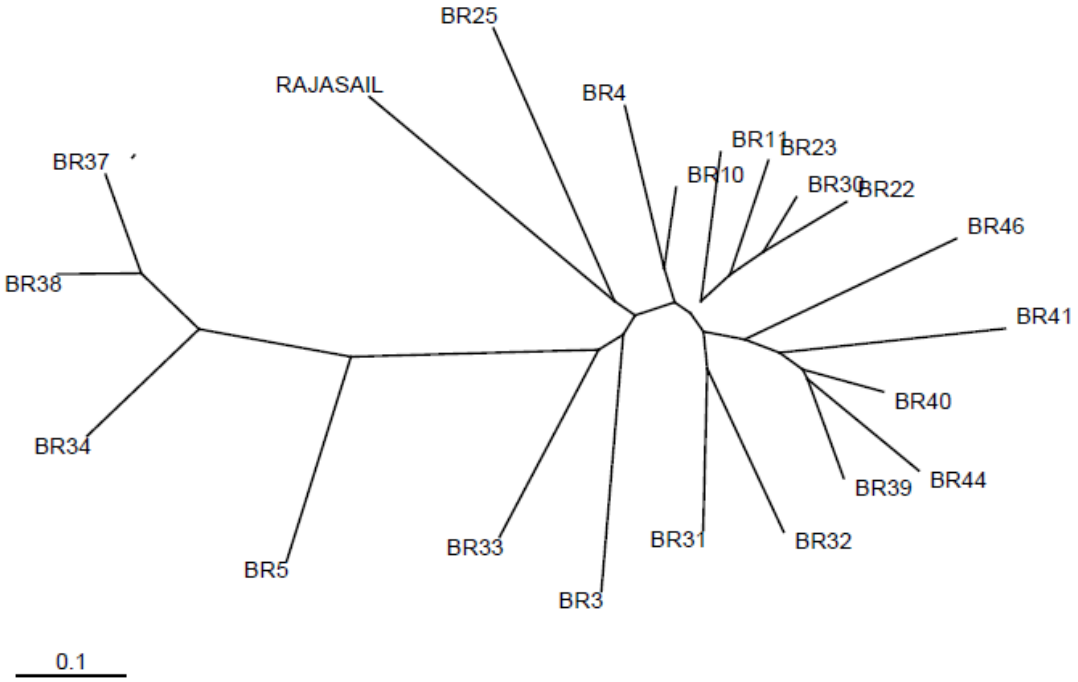

Fig. 1: An unrooted neighbor-joining tree showing the genetic relationships among rice varieties based on the alleles detected by 34 SSR markers.

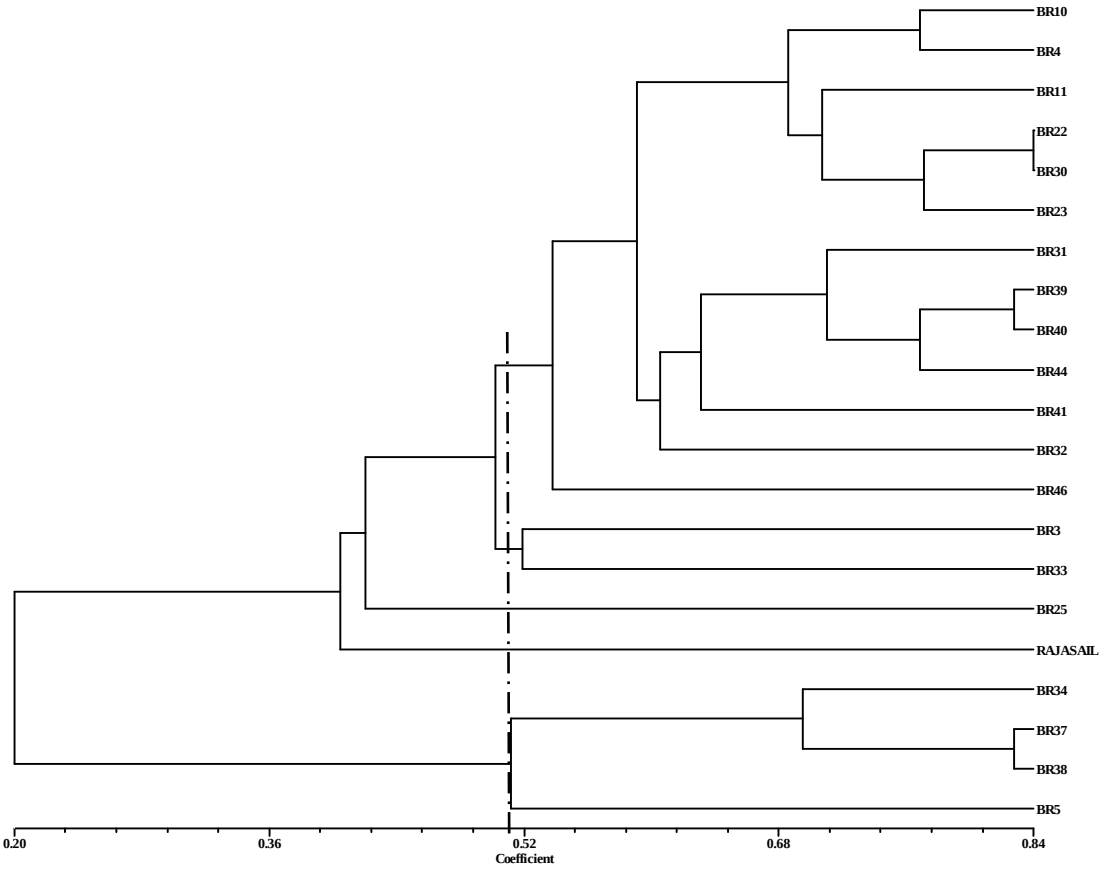

Fig. 2. A UPGMA cluster dendrogram showing the genetic relationships among 21 rice varieties based on the alleles detected by 34 SSR markers.