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Genetic fingerprinting for the protection of local rice (*Oryza sativa* L.) cultivars of Bangladesh

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ABSTRACT: Genetic fingerprinting of 110 rice cultivars of Bangladesh was completed with five polymorphic microsatellite DNA markers such as RM153, RM251, RM333, RM335 and RM475. The amplified DNA fragments are known as alleles from Polymerase Chain Reaction (PCR) reactions were separated on 2% agarose gel electrophoresis system, subsequently visualized by high performance ultraviolet transilluminator. In all, 99 distinctive alleles averaging 19.80 alleles/locus from the entire utilized microsatellite loci were counted. Several diversity indexes such as Polymorphism Information Content (PIC), heterozygosity, and cluster analysis were computed in this quantitative investigation. Superior genetic differentiation and inferior gene flow values among the cultivars were revealed from the recorded genetic diversity study of PIC, Effective allele, Shannon index (*I*), Hardy-Weinberg equilibrium (HWE), Nei's gene diversity (*h*), along with genetic differentiation-F_{is} and gene flow-N_m analysis. A total, 5995 varietal pairs were achieved all the way through alternative combinations of 110 rice cultivars where their Nei's genetic distance (*D*) was ranged from zero to 2.832. Nei's genetic-base an Unweight Pair Group Method of Arithmetic Means (UPGMA) diagram was assembled which eventually separated all the cultivars from each other according to their genetic distance and similarity. Thus, the finding of this study will expose such strategies to distinct all the wild relatives, cultivars and commercial varieties of rice or any other crop species having various genetic levels to facilitate further improvement and protection in future.

KEYWORDS: Genetic fingerprinting, *Oryza sativa* L., Plant variety protection, Bangladesh

INTRODUCTION

Rice (*Oryza sativa* L.) having diploid chromosome number 2n=2x=24 belongs to the significant grass family *Gramineae* and subfamily *Oryzoidae* is considered one of the most cultivated annual cereals because almost 1/2 of the world's human population consume rice every day as their predominant staple food [1]. It is occupied in the central position other than rest of the agricultural component to contribute Bangladesh's national economy [2]. It is believed that Asian farmers have been maintaining the selection and domestication process of a wide range of rice cultivars since ancient [3]. The Gene bank of Bangladesh Rice Research Institute has accumulated near about 8,500 rice germplasm from different ecological and indigenous sources of Bangladesh of which 8,044 genotypes have been registered so far [4]. Great variations in rice genotypes have been noticed in Asia, more particularly in China as well as Indian subcontinent regarding morphological, biochemical and molecular aspects [5],[6]. Zhao [7] has reported on the subject of the origin of rice which has been accomplished close to the northeastern part of India (Assam) and southwestern part of China (Yunan), both are truly recommended to the subtropical upland of Himalayas [8]. *Oryza rufipogon* and *Oryza nivara* are the two parts of *Javanica* rice which are assumed feasible immediate progenitors of *Oryza sativa*

[9].Interestingly, the Assam center (nearby of Bangladesh) is also considered for the center of origin of such *Javanica* rice.

Day by day the number of rice genotypes become raise which ultimately harder the flexibility to differentiate of rice cultivars on the premise of morphological and biochemical attributes [4]. Therefore several molecular markers such as SSR (Simple Sequence Repeat), RAPD (Random Amplified Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism), and AFLP (Amplified Fragment Length Polymorphism) etc. are widely utilized to be identified of particular cultivars [10], or quality seed of hybrid varieties [11] and for documentation of the released varieties in seed grain trade as well [12]. Thus DNA fingerprinting data is additionally one of the example which is being globally practiced for the legal evidence of DUS (Distinctness, Uniformity, and Stability) [13]–[20].

PCR-based assays, co-dominant inheritance pattern, and elevated multi allelic variation/polymorphism are the major dynamic factors which steer the microsatellites/SSRs as the precious genetic markers of choice for the breeders [21]–[23]. The motif of the hyper variable microsatellites/SSRs are fairly located and abundantly well distributed all over the rice genome [24]–[28]. Hence, microsatellites/SSRs are also becoming efficient tools for the breeders as well as geneticists to incorporate genetic maps of rice having enormous wealth of diverge genetic variation [29]–[32]. Still, more than 50,000 microsatellites/SSRs have been designed in between *Indica* and *Japonica* rice accessions which are being contributed to construct genetic map for characterization and documentation of rice [30],[33]–[36]. Based on the above scientific reports, such characterization and documentation process have been recently utilized in several varieties/landraces/cultivars/wild types of *Oryza sativa*, *Triticum aestivum*, *Zea mayes*, *Saccharum officinarum*, *Brassica napus*, *Glycine max*, *Solanum tuberosum*, *Corchorus capsularis*, and other crop species of Bangladesh [13]–[19],[37]. However, in this study, the genetic fingerprinting techniques were utilized through five SSR markers for the protection of 110 local rice genotypes grown in Bangladesh. Moreover, the genetic fingerprinting techniques of this research can be further exploited for the protection and establishment of Intellectual Property Rights (IPR) of other crop species of Bangladesh.

MATERIALS AND METHODS

Raising of rice seedlings and extraction of DNA

Genetic Resource and Seed (GSD) division of Bangladesh Rice Research Institute (BRRI) was provider of 108 cultivar's seeds and the seeds of rest two cultivars named ACI-1 and Alok-932024 were supplied by ACI Pvt. Ltd of Bangladesh. Genetic fingerprinting lab of department of Genetics and Plant Breeding (GPB), Bangladesh Agricultural University (BAU), Bangladesh was being concerned to grow these seedlings as well as this experiment to be conducted. Genomic DNA of each cultivar was extracted from 2-5 inner succulent shoots of two-weeks-old fresh seedlings germinated in sterile petridishes. In that case, Rahman *et al*., [13]–[19],[37],[38] illustrated modified CTAB (cetyl trimethyl ammonium bromide) DNA extraction method supported by Aljanabi and Martinez [39] was followed to isolate genomic DNA.

Quantification and optimization of DNA concentration

UV-absorption spectrophotometer (Spectronic® Genesis™) was utilized at 260nm absorption to measure the purity and concentration of DNA which was subsequently estimated and converted into 25ng/µl with TE buffer, and finally stored at 4ºC before to amplify with SSR primers.

Selection of polymorphic SSR markers for rice genotypes

A total, 50 SSRs comprising on all the twelve chromosomes of rice were obtained. A set of seventeen SSR primers described previously [38] were selected from them by surveying allelic polymorphism data from the available rice genome database (http://www.gramene.org) as illustrated by Rahman *et al.,* [13]–[19],[37]. At first, three to five of those primers were tested through ten randomly selected genotypes setting with the recommended PCR thermal profile [13]–[19]. The expected ranges (base pairs length) PCR product was then validated based on the most excellent response to amplify the target genomic region of the template DNA. In such a way five SSR primers *viz*., RM153, RM251, RM333, RM335 and RM475 which depict 5, 3, 10, 4, and 2 of rice chromosome [36] were preferred by visualizing clear and predictable amplified alleles, and finally employed for SSR analysis in this study (Figure 1). The selected primers were subsequently run with all 110 cultivars at once which displayed clear and repeatable polymorphic bands.

Polymerase chain reaction (PCR) amplification profile for SSRs

Eppendorf™ oil-free thermal cycler gradient was utilized in this PCR reaction. Approximate 15 µl reaction volume consists of 50ng sample DNA (2.0 µl), 10x PCR Buffer (3.0 µl), 1 µM of each forward and reverse SSR primer (1.0 µl), 0.25 mM dNTPs (1.5 µl), 1 unit ampli Taq DNA polymerase (0.5 µl), and nuclease free double distilled deionized water (6.0 µl) were utilized to perform PCR reaction. PCR settings were carried out by the conditions described by Panaud *et al.*, [34],[36] with minor modifications suggested by previous research [13]–[19],[37] as follows: 95°C for 5 min (an initial denaturation) followed by entire 35 cycles, 95°C for 40 sec (denaturation), 55°C for 30 sec (annealing) and 72°C for 1 min (elongation/extension), then a final elongation/extension cycle at 72°C for 7 min. Amplified PCR reactions were then stored at –20°C for further utilization.

Electrophoresis and inspection of banding patterns

Top vision submarine horizontal electrophoresis system (BIORAD™ Sequencing Cell) was utilized to electrophoresis the PCR reactions (Figure 1). Prior to electrophoresis, each 07 µL amplified PCR aliquot and 3 µL of loading buffer [38] were mixed gently. This mixture was then loaded on 2% agarose gel, and placed into the submarine horizontal gel chamber with 1x TBE running buffer (Trizma base, boric acid and EDTA; pH 8.0). A five microlitre (5 µL) 100 bp standard DNA (Gene ruler, Fermentas®) ladder was added in both left and right side of the gel to compare the molecular weight of the amplified PCR products of each cultivar. PCR mixtures (10 μl) were subjected to electrophoresis at 100V and 50W for 2 hrs 40 mins. The electrophoresis sample was then kept as photographic image by the camera polaroid gel documentation system (UVP, BioDoc-It™ System).

Genotyping of alleles and data analysis

DNA FRAG v3.03 computer software [39] was utilized to measure the most profoundly amplified alleles per loci (Figure 1) by using 100bp recognizing size standard DNA ladder [40]. The individual unambiguous DNA fragments were referred as alleles of the respective SSR markers. The allele frequency data (DNA fragment) was exported as diploid datasheet arrangement (AA, AB, CC and so on) on POPGENE v1.31 computer program [42], and therefore, utilized for the several statistical analysis including "observed number of alleles-N*a* [42]", "effective number of alleles-N*e*" [43], "allelic diversity index (PIC=1-$\sum X_i^2$, where X_i indicates the frequency of the i^{th} allele), Shannon's Information index-*I* [44]", "Hardy-Weinberg equilibrium (H*e* and H*o* of Levene [45] and Gene flow-N_m)", "Nei's gene diversity index (*h*) [46]", "chi-square & probability index", and "Wright's fixation index-F_{is} [47]". POPGENE v1.31 software was also applied to estimate genetic distance and similarity among the genotypes. Finally, an UPGMA (Unweighted Pair Group Method of Arithmetic Means) phylogenetic tree (Figure 2) was assembled by means of Nei's [48] genetic distance (*D*) which was visualized via Treeview computer software [49]. The generated cluster on UPGMA diagram (Figure 2) was then used to explain the relationships among the cultivars in this study.

RESULTS

Allele frequency and allelic diversity index (PIC)

Using 5 polymorphic loci in 110 cultivars of rice, a complete of 99 alleles were found in this quantitative investigation where RM335 revealed the foremost observed alleles (25) followed by RM333 (21), RM251 (20), RM475 (19) and RM153 (14) as shown in Table 1. The Highest effective number (N*e*) of alleles (19.852) was also found in RM335 (Table 2). The diversity index of alleles or Polymorphism Information Content (PIC=1-$\sum Xi^2$) values usually reflect particular allele diversity into a species [50]. The average PIC value was 0.864 with the number ranging from 0.879 (RM153) to 0.949 (RM335) (Table 1).

Genetic variation statistics

Aspect of allele frequency of each cultivars and total cultivars were taken into consideration to calculate the comprehensive Shannon's Information Index (*I*) which is fluctuated from 2.284 (RM153) to 3.080 (RM335). Fixation index (F_{is}) (a measure of genetic differentiation) was recorded from 0.687 to 0.958 having 0.838 average values (Table 2). Highest gene flow (N_m) was estimated through RM251 microsatellite loci (0.046) while RM153 showed the lowest gene flow (0.005) in this study (Table 2).

Table 1. Fingerprinting Alleles and polymorphism information content (PIC) of five SSR loci across 110 rice cultivars

Allele	RM153 (Chromosome 5)	RM251 (Chromosome 3)	RM333 (Chromosome 10)	RM335 (Chromosome 4)	RM475 (Chromosome 2)	Mean
Allele A	0.005	0.023	0.018	0.014	0.005	
Allele B	0.023	0.055	0.018	0.041	0.014	
Allele C	0.032	0.005	0.018	0.018	0.014	
Allele D	0.032	0.041	0.023	0.018	0.005	
Allele E	0.141	0.041	0.018	0.055	0.014	
Allele F	0.200	0.086	0.009	0.055	0.023	
Allele G	0.118	0.077	0.014	0.009	0.050	
Allele H	0.123	0.091	0.005	0.041	0.041	
Allele I	0.118	0.086	0.009	0.027	0.105	
Allele J	0.100	0.018	0.036	0.055	0.123	
Allele K	0.064	0.096	0.082	0.068	0.064	
Allele L	0.018	0.059	0.064	0.041	0.055	
Allele M	0.018	0.123	0.073	0.055	0.136	
Allele N	0.009	0.077	0.191	0.082	0.141	
Allele O		0.005	0.191	0.050	0.109	
Allele P		0.036	0.055	0.064	0.055	
Allele Q		0.014	0.096	0.041	0.018	
Allele R		0.036	0.041	0.073	0.009	
Allele S		0.027	0.014	0.032	0.023	
Allele T		0.005	0.018	0.018		
Allele U			0.009	0.055		
Allele V				0.041		
Allele W				0.023		
Allele X				0.018		
Allele Y				0.009		
Diversity Index (PIC=1-$\sum Xi^2$)	0.879	0.926	0.893	0.949	0.907	0.864
Observed number of alleles	14	20	21	25	19	19.80
Effective number of alleles	8.3190	13.5879	9.3617	19.8523	10.8036	12.38
% of Effectiveness	29.167	41.047	46.377	44.169	33.277	38.81
Sequence of primers (5′-3′)	F: gcctcgagcatcatcatcag R: atcaacctgcacttgcctgg	F: gaatggcaatggcgctag R: atgcggttcaagattcgatc	F: gtacgactacgagtgtcaccaa R: gtcttcgcgatcactcgc	F: gttcegtgttcegtgccecc R: gaacagagaacagagccacc	F: cctcacgatttttcctccaac R: acggtgggattagactgtgc	
Motif	$(GAA)_9$	$(CT)_{29}$	$(TAT)_{19}(CTT)_{19}$	$(CTT)_{20}$	$(TATC)_8$	

Heterozygosity statistics

Across 110 rice varieties, RM251 (0.290) yielded the very best average heterozygosity (H_O) in current study followed by RM475 (0.172), RM335 (0.154), RM333 (0.090) and RM153 (0.036) (Table 2). Highest heterozygosity can be explained as a result of length and distance of RM251 marker on the genetic map relative to centromere [14].

Varietal identifications

Comparative SSR profiles and DNA molecular weight (band position) against five SSR primers (Figure 1), all the varieties were distinguished from each other with a minimum of single and/or arrangement of five primers.

Table 2. Summary statistics of the utilized genetic diversity parameters in the study

Loci	Polymo-rphism	Size range (bp)	N*a**	N*e**	*I**	Ho*	He*	*h**	Chi-Sq.	Prob.	Level of Significance	*F_{is}	F_{st}	N_m*
RM153	100%	163–244	14.00	08.319	2.284	0.036	0.883	0.879	10.113	0.810	NS	0.958	0.979	0.005
RM251	100%	108–210	20.00	13.589	2.736	0.290	0.930	0.926	49.314	0.001	**	0.687	0.843	0.046
RM333	100%	169–355	21.00	09.361	2.557	0.090	0.897	0.893	60.523	0.001	**	0.898	0.949	0.013
RM335	100%	69–203	25.00	19.853	3.080	0.154	0.954	0.949	51.925	0.000	**	0.838	0.918	0.022
RM475	100%	151–263	19.00	10.803	2.568	0.172	0.911	0.907	77.421	0.000	***	0.810	0.904	0.026
Mean	100%	132-255	19.80	12.384	2.645	0.149	0.915	0.911				0.838	0.918	0.022

*N*a* = Number of observed alleles, *N*e* = Number of effective alleles =$1/(\sum X_i^2)$, Where X_i is the frequency of the i^{th} allele [43], **I* = Shannon's Information index by Lewontin [44], *Unbiased Expected Heterozygosity (H*e*) was computed using Levene [45], **h* = Nei's (1973) gene diversity index [46], NS=not significant, * P<0.05, ** P<0.01, *** P<0.001, *F_{is} = Wright's fixation index (F_{is}) as a measure of heterozygote deficiency or excess (H*e* - Ho) / H*e*) [47], and *N_m = Gene flow estimated from F_{st} = 0.25(1 - F_{st})/F_{st}

Table 3. Distinction of 110 rice cultivars all the way through SSR band positions

Sl. No	Variety Name	Distinguishing Primer(s)	Band positions due to specific primers (bp)				
			RM153	RM251	RM333	RM335	RM475
1	Lal Amon	RM153 + RM251	191	108	194	106	178
2	Lau Jan	RM153 + RM333	184	108	171	88	173
3	Nara Aswina	RM153 + RM333	195	149	182	84	185
4	Buna Dhan	RM251	191	149, 104	207	120	178
5	Begun Bechi	RM333	195	140	242	88	200, 167
6	Bhasha Manik	RM251 + RM333	195	145	212	84	205
7	Kochu Dhola	RM335	184	126	228	69	173
8	Sunga Wala	RM153 + RM251	184	113	296	79	161
9	Konek Chul	RM333 + RM335	195	133	220	79	173
10	Horinkhur Panati	RM335 + RM475	195	133	207	74	190
11	Ganjia	RM251/RM335/RM475	191	176, 108	207	120, 74	173, 151
12	Dudhsar	RM335	195	140	319	125, 74	173
13	Mathia	RM153 + RM251 + RM333	191	126	207	88	167
14	Khirma Pat	RM335	206	176, 113	200	125, 84	178
15	Mukut Sail	RM335	200	149	207	130, 79	178
16	Ban Kolom	RM153 + RM251 + RM333	200	149	235	84	151
17	Safa Har (3)	RM153 + RM251	222	126	220	95	173
18	Kal Nania	RM251 + RM333	200	113	207	95	185
19	Shil Pan	RM251/ RM475	200	154, 145	256	88	210, 173
20	Jabar Sail	RM153 + RM251	200	133	188	84	167
21	Moisha Mida	RM251 + RM333	200	113	169	100	185
22	Paglakushyari	RM251	200	120	200	130	178
23	Pan Kaich	RM153 + RM251	195	140	200	154	205, 173
24	Lal Patjat	RM333	200	140	207, 176	142	178
25	Chandda Gotok	RM251	200	162, 108	188	175, 142	178
26	Moisha Mira	RM335	195	133	207	180, 120	205
27	Choia Mora	RM335	191	140	228	120, 100	185
28	Modhu Maloti	RM335	191	140	319	142, 106	190
29	Kali Gochya	RM335	177	190	188	175, 135	173
30	Ludi Gochya	RM335	163	184	200	161, 125	167
31	Kala Gura	RM153 + RM251	171	168	207	106	190
32	Monura	RM153 + RM335	171	204	228	135	178
33	Moisa Mira	RM251 + RM333	177	210	207	135	178
34	Chand Moni (3)	RM335 + RM475	171	133	200	115	173
35	Kamoni Sail	RM251	177	200, 176	200	142	205
36	Juna	RM333	191	184	263	106	178
37	Kurki	RM251 + RM333	177	210	188	106	178
38	Arai Raj	RM251 + RM333	177	184, 168	355	175, 142	185
39	Kala Gora	RM251 + RM333	177	204	220	125	200
40	Mulai	RM333 + RM335	177	204	188	115	200
41	Dharga Sail	RM251	163	210, 184	212	106	195
42	Bondyl	RM335/RM475	177	168	220, 188	125	240
43	Chakkol	RM153 + RM335	177	204	302, 200	115	200
44	Rajamun	RM251	171	176, 133	228	161	210
45	Kanchon Mogi	RM251/RM333/RM475	184	184, 162	235, 188	125	237, 200
46	Hiruyal	RM475	184	184, 168	200	161	247, 200
47	Chapa Mali	RM153 + RM335	171	204	228	106	205
48	Deppol	RM153 + RM251	191	204	194	100	205
49	Pushon	RM333/RM475	177	162	302, 207	120	231
50	Kali Cochr	RM333 + RM335	200	145	220	142	200
51	Ghori Amon	RM475	177	176, 113	235	148	240, 200
52	Muirol	RM153 + RM251	191	190	207	148	195
53	Muar Sail	RM475	184	168	207	115	247
54	Jhaw Lota	RM153 + RM251	171	140	212	100	195
55	Bhoban	RM475	191	176	200	88	263, 210
56	Ful Badam	RM153 + RM251	206	190	228	88	195
57	Galong	RM333 + RM335	200	149	212	130	185
58	Hasna Chikon	RM251 + RM333	206	158	182	125	185
59	Thakor	RM153 + RM251	195	158	220	200	167
60	Tembur	RM153 + RM251 + RM333	200	145	207	135	178
61	Tulsi Mala	RM153	222, 206	162	188	142	151
62	Giring	RM251	200	190, 149	355	180	200, 167
63	Nag Pechi	RM251 + RM333	206	176	228	142	185
64	Bhua Dhan	RM475	230	140	200	200	200, 178
65	Sandik Sail	RM251 + RM333	206	158	200	135	185
66	Jhoria Sail	RM251	206	176, 140	200	195	205, 185
67	Halde Medi	RM333 + RM335	206	176	212	142	185
68	Maitya Cheng	RM334	206	176	207	168	173
69	Kala Bail	RM333/RM335	222	158	302	200, 125	190
70	Murki Balam	RM251/RM475	206	184, 133	302, 200	200, 130	215, 190
71	Gulchamlaish	RM251 + RM333	206	158	296	195	195
72	Raj Kumari	RM251	171	176, 145	336	200, 130	190
73	Hati Banda	RM333 + RM334	206	162	235, 188	161	215
74	Kui Sail (2)	RM153	244, 177	162	182	130	200
75	Chakkol (Muta) Raozan	RM153 + RM251	214	176	207	190	195
76	Muijuri	RM153 + RM333	200	162	228	175	178
77	Munsi Sail	RM251 + RM333	195	168, 126	188	175	200
78	Thakur Dhan	RM251 + RM333	206	133	194	175	224
79	Moina Sail	RM251 + RM333	200	162	194	154	215
80	Butu Balam (2)	RM335	191	168, 126	220, 182	115	200
81	Kali Jira (2)	RM153 + RM251	184	168, 133	200	148	205
82	Jhual Kata	RM335	200	162	182	154, 110	200
83	Raj Bhog	RM251 + RM335	200	176	200	110	205
84	Lal Modonga	RM251	222	162, 145	171	110	205
85	Surma Sail	RM475	195	154	212	180	210, 178
86	Madhu Mala	RM251	200	158, 131	176	125	190
87	Dumai Sail	RM251/ RM335	200	140, 120	200	203, 180	178
88	Chadlash	RM251	214	145, 133	207	130	167
89	Jola Bhangh	RM153 + RM475	184	140	200	180	173
90	Mutonga (2)	RM251 + RM333	184	140	235	203	205
91	Jula Gudi	RM153	206	149	212	175	157
92	Gutok	RM153/RM251	230, 191	154, 149	207	130	161
93	Pani Torong	RM251	214	162, 149	336	161	185
94	Randar	RM153 + RM251 + RM333	200	149	200	190	173
95	Bhor Gelam	RM251	184	168, 149	207	180	205, 173
96	Neel Huri	RM333/RM335	206	149	248, 200	200, 175	185
97	Jol Kumari	RM153 + RM251	191	162	207	125	205, 185
98	Ful Kari	RM153 + RM251	184	158	212	180	185
99	Biropa	RM153 + RM251 + RM333	191	145	200	125	185
100	Asham Baba	RM153 + RM251 + RM333	206	158	207	120	200
101	Dhul Abiz	RM153 + RM333	200	158	194	120	200
102	Porangi	RM251	200	168, 145	220	110	190
103	Tulo Sail	RM153/RM251	214, 177	168, 158	188	115	215
104	Khoni Dhan	RM251/RM475	184	158, 140	200	110	231, 190
105	Choro	RM153 + RM475	195	168, 133	200	106	210
106	Sundar Sail	RM153 + RM251	168	190	194	115	224
107	Bokra	RM251	184	168, 140	228	148	215
108	Nagra	RM251/ RM333	168	168, 154	256, 200	148	210
109	ACI 1	RM153 + RM251	230	140	188	148	215
110	Aalok 932024	RM335/ RM475	195	149	207	154, 106	224, 190

(Table 3).

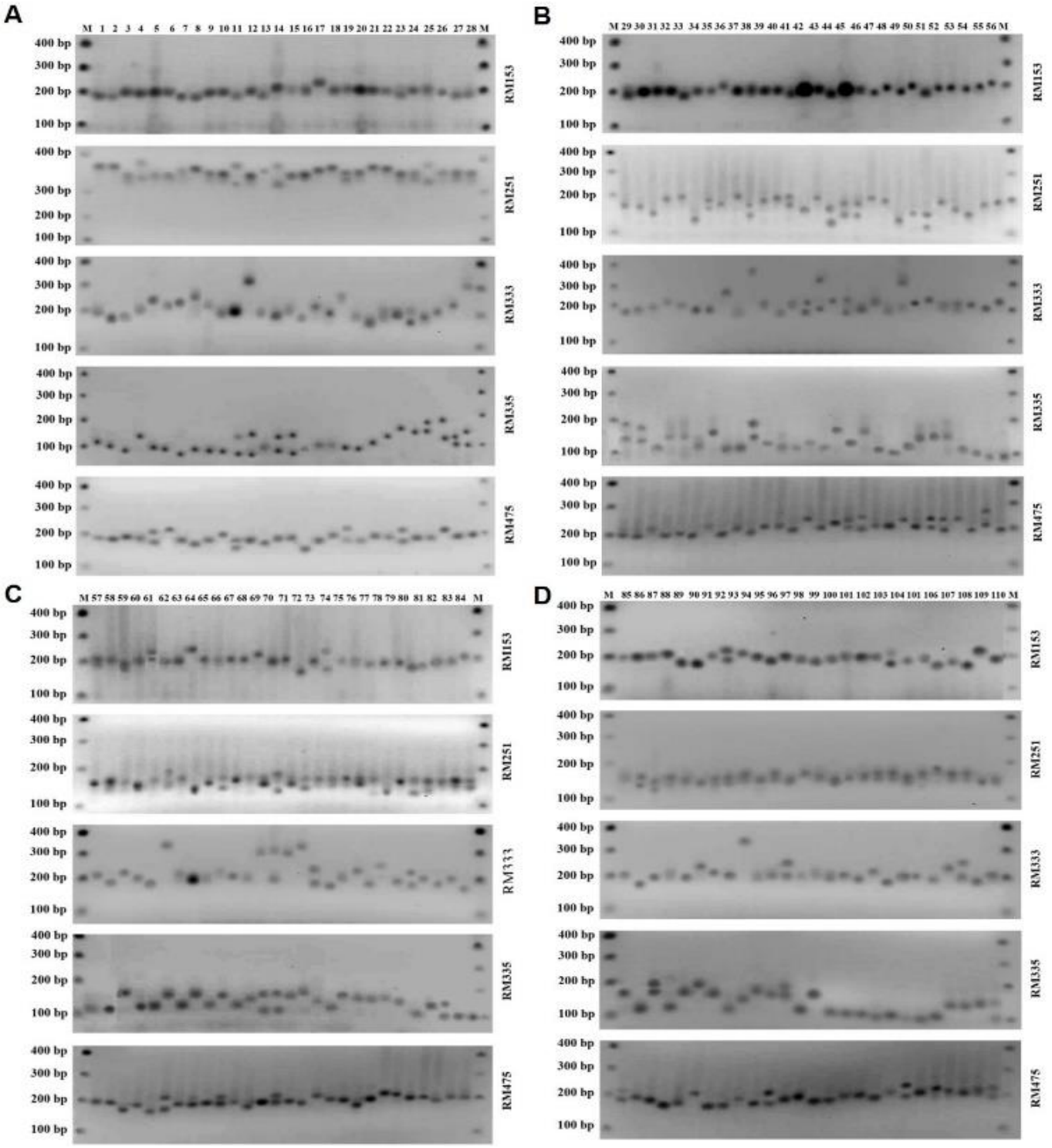

Figure 1. A) Representative gel pictures of amplified alleles from five SSR markers RM153, RM251, RM333, RM335 and RM475 by using several rice cultivars of Bangladesh (Lane M=100bp Ladder; Lanes 1 to 28 represents rice cultivars (1=Lal Amon, 2=Lau Jan, 3=Nara Aswina, 4=Buna Dhan, 5=Begun Bechi, 6=Bhasha Manik, 7=Kochu Dhola, 8=Sunga Wala, 9=Konek Chul, 10=Horinkhur Panati, 11=Ganjia, 12=Dudhsar, 13=Mathia, 14=Khirma Pat, 15=Mukut Sail, 16=Ban Kolom, 17=Safa Har (3), 18=Kal Nania, 19=Shil Pan, 20=Jabar Sail, 21=Moisha Mida, 22=Paglakushyari, 23=Pan Kaich, 24=Lal Patjat, 25=Chandda Gotok, 26=Moisha Mira, 27=Choia Mora, 28=Modhu Maloti) [38]. B) B. Representative gel pictures of amplified alleles from five SSR markers RM153, RM251, RM333, RM335 and RM475 by using several rice cultivars of Bangladesh (Lane M=100bp Ladder; Lanes 29 to 56 represents rice cultivars (29=Kali Gochya, 30=Ludi Gochya, 31=Kala Gura, 32=Monura, 33=Moisa Mira, 34=Chand Moni (3), 35=Kamoni Sail, 36=Juna, 37=Kurki, 38=Arai Raj, 39=Kala Gora, 40=Mulai, 41=Dharga Sail, 42=Bondyl, 43=Chakkol, 44=Rajamun, 45=Kanchon Mogi, 46=Hiruyal, 47=Chapa Mali, 48=Deppol, 49=Pushon, 50=Kali Cochr, 51=Ghori Amon, 52=Muirol, 53=Muar Sail, 54=Jhaw Lota, 55=Bhoban, 56=Ful Badam), C) Representative gel pictures of amplified alleles from five SSR markers RM153, RM251, RM333, RM335 and RM475 by using several rice cultivars of Bangladesh (Lane M=100bp Ladder; Lanes 57 to 84 represents rice cultivars (57=Galong, 58=Hasna Chikon, 59=Thakor, 60=Tembur, 61=Tulsi Mala, 62=Giring, 63=Nag Pechi, 64=Bhua Dhan, 65=Sandik Sail, 66=Jhoria Sail, 67=Halde Medi, 68=Maitya Cheng, 69=Kala Bail, 70=Murki Balam, 71=Gulchamlaish, 72=Raj Kumari, 73=Hati Banda, 74=Kui Sail (2), 75=Chakkol (Muta) Raozan, 76=Muijuri, 77=Munsi Sail, 78=Thakur Dhan, 79=Moina Sail, 80=Butu Balam(2), 81=Kali Jira (2), 82=Jhual Kata, 83=Raj Bhog, 84=Lal Modonga), and D) Representative gel pictures of amplified alleles from five SSR markers RM153, RM251, RM333, RM335 and RM475 by using several rice cultivars of Bangladesh (Lane M=100bp Ladder; Lanes 85 to 110 represents rice cultivars (85=Surma Sail, 86=Madhu Mala, 87=Dumai Sail, 88=Chadlash, 89=Jola Bhangh, 90=Mutonga (2), 91=Jula Gudi, 92=Gutok, 93=Pani Torong, 94=Randar, 95=Bhor Gelam, 96=Neel Huri, 97=Jol Kumari, 98=Ful Kari, 99=Biropa, 100=Asham Baba, 101=Dhul Abiz, 102=Porangi, 103=Tulo Sail, 104=Khoni Dhan, 105=Choro, 106=Sundar Sail, 107=Bokra, 108=Nagra, 109=ACI 1, 110=Aalok 932024).

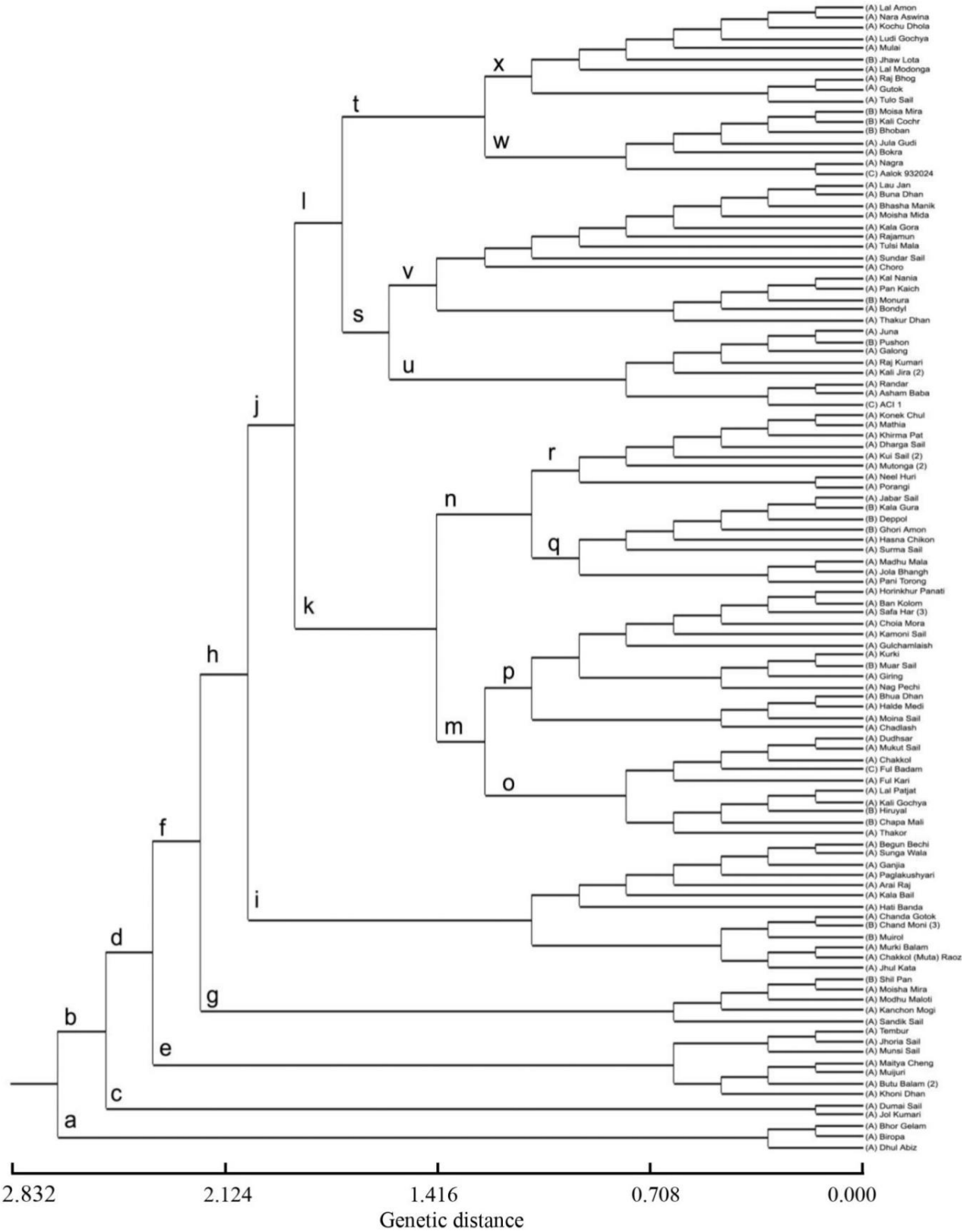

Figure 2. UPGMA phylogenetic tree based on Nei's [63] genetic distance showing the genetic relationship among 110 rice cultivars (Group, A= Transplant Aman, B= Broadcast Aman, C= Boro and Jhum)

Analysis of genetic distance and phylogenetic tree

The summary of Nei's genetic distance (*D*) from 5995 varietal pairs among 110 rice cultivars varied from zero to 2.832. Out of these varietal pairs, 58.87% (3529) showed no genetic distance [11]. Such genetic distance and similarity in this study separated all the 110 cultivars into several clusters ("a" to "x") at once on the UPGMA diagram (Figure 2).

DISCUSSION

All the utilized polymorphic SSR markers recorded a complete of 99 unique alleles (Table 1) which was significantly higher than the total number of alleles reported by the several previous researches [13]–[19],[33]. On an average it yielded 19.80 alleles per primer with an effect of 38.81%. In agreement with earlier works [13]–[19],[33] reported a total of 18 alleles [16],[17] through analyzing with three primers (RM11; RM151 and RM153) and 78 [15] alleles with five primers (RM1; RM151; RM153; RM334 and RM335), respectively, while running on diverse ecotypes of rice genotypes of Bangladesh from the protected rice materials of the BRRI. In those investigations, PIC values were recorded 0.670; 0.707; 0.698 [16],[17] & 0.862; 0.923; 0.831; 0.865 and 0.910, respectively [15]. In another study, a total of 238 rice accessions (*Indica* and *Japonica)* by using entire ten microsatellite markers were investigated by Yang and his associates [51] where they observed maximum 25 exclusive alleles. Genotypes under the low PIC value study represent closely related variants, while superior PIC indicates considerable enormous diversity, which is ideal for the development of new variants as well [52]. The frequency of short tandem repeats of microsatellites as well as their repeat sequences have a command on the quantity of amplified alleles and their resultant PIC values of the experimental genotypes [35],[36],[53],[54]. In addition, Ni *et al.*, revealed from his investigation that more extensive repeats including GA- sequenced repeats acquiesce more quantity of distinctive alleles with superior PIC standards [54]. In contrast, it has been suggested that the motif of $(CTT)_n$ and amplified AT-affluent trinucleotide repeats also exhibits adequate and greater polymorphism of alleles [35]. RM333 primer containing $(CTT)_n$ motif was one of the most instructional SSR marker because it gave 6-7 distinctive alleles and standard PIC range in Temnykh's experiment [36]. In support of RM335 [$(CTT)_{20}$] SSR primers, 25 unique alleles with 0.910 PIC numeral were recorded which were the foremost alleles and the maximum PIC numeral in this investigation. The PIC valuation is considered as the discriminating strength of a promising marker to the genetic diversity study of the breeding materials selection program for the breeders because it regulates the frequency of observed and effective alleles of a particular DNA marker [55]–[57]. However, remarkably elevated PIC (0.879 to 0.949) values in this investigation indicated that the chosen markers have the required properties to be used in this DNA fingerprinting research among the 110 rice cultivars grown in Bangladesh[58]. However, the observation of this study was partially supported by the points of accuracy and usefulness from the above discussions.

Location of particular DNA genetic markers on the precise chromosomes, frequencies and size of alleles through their PIC numeral are given in Table 1. Mutation and chromosomal crossover are the two general events of heredity which are usually occupied at distal proximity from the centromere of the chromosome. They usually effort the formation of abundant alleles and eventually diversity of a specific locus [13]–[16],[18],[19],[37]. 24.7cM (chromosome 5); 79.1 cM (chromosome 3); 110.4cM (chromosome 10); 21.5cM (chromosome 4) and 92.5cM (chromosome 2) are the located positions of RM153; RM251; RM333; RM335 and RM475 primers on rice chromosome [36]. These primers were applied in the current observation of 110 rice cultivars including 2 hybrids and one variety of Jhum cultivation system. Overall gene flow values and genetic diversity were observed in several populations of *Oryza officinalis* and computed 0.316 and 0.442, respectively, by examining of entire 14 microsatellite markers [59]. A wide range of allele frequency along with PIC value, major genetic variation in sense of observed and expected heterozygosity (H*o* and H*e*) were detected in this investigation. The observed and expected heterozygosity (H*o* and H*e*) values were estimated from 0.036 (RM155) to 0.290 (RM 251), and from 0.883 (RM155) to 0.954 (RM335), respectively. Superior expected heterozygosity (H*e*) content than the observed heterozygosity in this study indicated that the selected SSR markers were remarkably abundant informative for the DNA fingerprinting among the rice cultivars [60],[61]. Partial consistent in theses' observations were computed in several Bangladeshi local rice cultivars by a number of previous research groups [4],[17],[38]. Greater genetic variation and a lower gene flow value in 110 rice varieties argued that the most studied varieties in this experiment were landraces [38].

In these research materials, a total of 92, 15, 02 and 01 cultivars are recommended for transplant aman (T. Aman), broadcast aman (B. Aman), boro and jhum ecotype by BRRI [14]. In diallel fashion, a total of 5995

varietal pairs were possible among 110 cultivars where 2466 (41.13%) varietal pairs were computed as to be prominent genetic distance (Nei's genetic distance-*D*) with each other [14]. In a previous study, while analyzing 94 varieties of six different ecotypes of rice grown in Bangladesh, a total of 4371 varietal pairs were computed of which 37% appeared nil genetic distance, and merely 1% showed highest genetic distance (2.583) [15],[62]. This closeness may be possible due to the genetic make-up of the locus for which the primers were responsible to distinguish along with low ecotype variation. Among the 5995 varietal pairs, only 0.917% appeared highest (2.832) genetic distance in this research. However, the superior genetic distance (*D*) is often observed while the cultivars or any genotypes were occupied from the landraces or wild relatives, in one side, and the high-yielding varieties (HYVs) on the opposite side in their crossing events [38]. Thus, the variation between highest and lowest genetic distance among the 110 cultivars proved their existence of variability. The resulting such genetic variability of the cultivars can be applied as a parent material in the future variety improvement programs to seek out the most efficient cultivars for further crossing or breeding.

However, Nei's genetic distance (*D*) while analyzed on the UPGMA dendrogram considering 110 cultivars at a time, the dendogram separated the varieties, Dhul Abiz, Biropa and Bhor Gelam (Cluster "a") from other 107 cultivars (Cluster "b"). Cluster "b" subsequently separated into sub-cluster "c" (Dumai Sail and Jol kumari) and sub-cluster "d" containing other 105 rice varieties. Sub-cluster subsequently formed other sub-clusters namely, "e", "f", "g", "h" and so on (Figure 2). The varieties, as for example, Jol Kumari, Sandik sail, Jhul Kata, Thakor, Aalok 932024 and Tulu Sail were found in different sub-clusters "c", "g", "i", "o", "w" and "x", respectively, due to their genetic distance. The major sub-clusters ("u"-"x") were found to cover 39 of the 110 cultivars starting from ACI 1 to Lal Amon, all of which are traditional rice varieties of Bangladesh except ACI 1 and Aalok 932024. UPGMA dendrogram within the groups; Transplant Aman (A), Broadcast Aman (B), Boro and Jhum (C) are given in Figure 2. As two Boro varieties (ACI 1 and Aalok 932024) and one Jhum variety (Ful Badam) were used in this experiment it was not possible to analyze them individually: they were therefore combined for a single analysis. An attempt was made to distinguish the varieties as their ecotype situations. The UPGMA dendrogram was constructed for this purpose (Figure 2). The results showed that the ecotypes have distinct clusters to represent Jhum and BRRI Accessions have formed the unique cluster different from all others as expected. The groups Transplant Aman (T. Aman) and Broadcast Aman (B. Aman) formed two closely linked sub-sub-clusters under one sub-cluster, while showing distinct difference from these sub-groups. Jhum formed a unique cluster and Boro a sub-cluster.

In these 110 cultivars, all the cultivars were distinguished from one another with either through 1st, 2nd, 3rd, 4th, and 5th SSR primers (Table 3) and also through qualitative and quantitative traits of Breeders [18]. Many varieties had similar names, which had created a number of problems related to final characterization. The varieties Moisha Mida (T. Aman), Moisha Mira (T. Aman), Moisa Mira (B. Aman); Kala Gura (B. Aman), Kala Gora (T. Aman) and Thakor (T. Aman), Thakur Dhan (T. Aman) have similar names but when studied by both qualitative and genetic fingerprinting those showed distinct differences [14].

CONCLUSIONS

In this study, the registered local rice cultivars grown in Bangladesh were exploited to distinct each and every rice cultivar based on identification of the DNA band patterns by means of specific primers, generally termed as genetic fingerprinting. All the utilized cultivars in this research were distinguished from one another with either 1st, 2nd, 3rd, 4th, or 5th SSRs. It is important to note that some of the varieties of traditional types had very similar names, which usually gave an understanding of repeats, but it was interestingly found to be distinctly different from one another due to molecular data. An example is the varieties named Moisa Mira, Moisha Mira, Moisha Mida, Thakor, Thakur Dhan, both pairs of which were distinct from one another on molecular traits. Therefore, these were not repeated but individually distinct cultivars or land races of rice collected from different source areas of Bangladesh by BRRI scientists at different times. However, Intellectual Property Rights (IPR) and Plant Variety Protection (PVP) of wild relatives, landraces, cultivars and commercial varieties of Bangladeshi rice will be guided from the outcomes of this research. Such series of works on more rice genetic materials as well as other crop species should be done as a regular study by the genetic resource centers of different institutes in collaboration with universities, where government should give adequate financial and special manpower support with appropriate incentives for those who will lead the program.

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AUTHOR CONTRIBUTIONS

LR, MSEA and MKB were involved in conception and design of the experiments. MSR and MKHS contributed to perform the experiments. LR, UKN, MSR and MKHS contributed to drafting the article. LR and MSR contributed to revising it critically for important intellectual content. MSR made the final approval of the version to be published.

CONFLICTS OF INTEREST

Authors declared that they have no conflict of interest.

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