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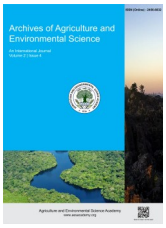

ORIGINAL RESEARCH ARTICLE

Genetic diversity analysis of some Bangladeshi aromatic rice (*Oryza sativa* L.) using simple sequence repeat markers (SSRM)

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ABSTRACT

In order to germplasm management, conservation, parental identification and proper utilization of aromatic rice germplasm of Bangladesh genetic diversity assessment and molecular characterization is necessary. We used ten microsatellite markers across twenty aromatic rice landraces along with four improved varieties to discriminate and characterize among them. The number of alleles per locus ranged from 2 to 8, with an average of 4.30 alleles across 10 loci. A total of 43 polymorphic alleles were detected. The values of Polymorphic information content (PIC) ranged from 0.217 to 0.835 (average 0.495) which indicate high genetic diversity among the studied aromatic rice genotypes. It was concluded by the PIC value of RM5339 that it might be the finest marker for diversity estimation and characterize of these aromatic rice genotypes, followed by RM334, RM414 and RM28502 markers. The UPGMA cluster dendrogram constructed in this study identified seven clusters with a correlation coefficient 0.874. Molecular characterization of aromatic rice landraces of Bangladesh exhibited large variations among the genotypes. Five rice genotypes namely BRRI dhan38, BRRI dhan50, Bashmoti safed, Malaysira, Khas-kani showed highest genetic dissimilarity among the studied rice genotypes. The findings of this study would be useful for background selection in backcross breeding programs for aromatic rice improvement as well as identification of genetically distant and genetically close accessions for maintenance and conservation.

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INTRODUCTION

Rice (*Oryza sativa* L.) is a cereal grain and it is the most widely consumed staple food for a large part of the world's human population, especially in Asia. Around the world rice is cultivated in approximately hundred countries covering almost 158 million hectares of cultivated land and its annual production is above 700 million tons (Anonymous, 2018). Rice cultivation is well-suited to countries and regions with low labor costs and high rainfall, as it is labor-intensive to cultivate and requires ample water. As a result, rice occupies about 70% of the total cropped area of about 13.9 million hectares in Bangladesh (Sajib *et al*., 2012).

Aromatic rice is a small sub-group of rice. In several aromatic rice varieties an aroma component 2-acetyle-l-pyrroline (similar to popcorn), has been found as an imperative flavor component (Weber *et al*., 2000). The aroma, flavor and texture of aromatic rice make it high graded in quality and so procure higher price compared to high quality non-aromatic rice in international market. For example, Aromatic rice such as Basmati from Pakistan, Nepal, and India and Sadri from Iran are highly valued for their aroma and quality (Garris *et al*., 2005). Aromatic rice is widely accepted not only in Asia but also in Europe and USA

(Sajib *et al.*, 2012). Though their high importance, improvement of aromatic rice has been relatively slow. Historically, aromatic rice are cultivated in small areas of Bangladesh. According to conventional taxonomy, Bangladeshi (Indian sub-continent) aromatic rice have been identified as indicas (Khush *et al.*, 2000). Most of the aromatic landraces are low yielding and medium fine grain with strong aroma. After introduction of high yielding rice varieties, the cultivation of land races reduced drastically. As a result, a many aromatic rice as well as other land races have already been lost and many are at the verge of extinction (Singh *et al.*, 2000). But these native rice varieties traditionally cultivated by farmers may contain a substantial genetic diversity which can be a source of germplasm for genetic enhancements of cultivated rice varieties (Choudhury *et al.*, 2013).

Traditionally morphological or physiological traits as well as protein or isozyme markers are used to assess genetic diversity in plants. But they are greatly biased by environment, need long time for assessment and show low polymorphism between the genotypes (Chakravarthi and Naravaneni, 2006). In contrast, modern biotechnology provide us molecular markers which are independent of environmental factors, show high polymorphism between the genotypes, allow easy and quick analysis of loci distributed among the plant genome (Chakravarthi and Naravaneni, 2006). As a result, molecular markers have become distinct, reliable and efficient tool for characterization, conservation, management of germplasm. Among the PCR based DNA markers, microsatellites or SSRs (simple sequence repeats) are highly preferred for gene tagging and gene mapping efforts as they have high level of polymorphism content and versatility. They are tandemly repeats of simple sequence which may be a short motif of di, tri, or tetra-nucleotides (Li *et al.*, 2004). SSR markers are also preferred in genetic diversity analysis, molecular map construction and genetic mapping, construction of fingerprinting, genetic purity test, analysis of rice lines diversity test etc. due to their reproducibility and amenability for automation, quickness, simplicity, rice polymorphism stability, accuracy etc. (McCouch *et al.*, 2002; Ma *et al.*, 2011; Roy *et al.*, 2015). Genetically distant and the morphologically close accessions could also be identified by SSR markers (Sajib *et al.*, 2012). In the present study, twenty aromatic landraces of Bangladesh along with four improved aromatic varieties were analyzed for genetic variation using SSR markers. The special objective was to find out genetic diversity and relationship of aromatic landraces, to assist in base broadening of the germplasm for future aromatic rice breeding programs.

MATERIALS AND METHODS

Collection of genetic materials

Experimental material comprised of 20 aromatic rice landraces and 4 improved varieties. List of genotypes with their type, origin, source of collection, kernel size and shape and aroma type are given in Table 1. These rice genotypes were collected from Bangladesh Institute of Nuclear Agriculture, BINA, Maymensingh and Bangladesh Rice Research Institute, BRRI, Gazipur.

Methods for SSR genotyping

DNA was extracted from the leaf tissues of 21 days old seedlings (a single seedling per genotype), based on a modified acetyl trimethyl ammonium bromide (CTAB) method described by (Stein *et al.*, 2001). Twelve SSR markers, one from each chromosome were selected. Among them the primers that showed polymorphic band were selected and primers that showed monomorphic band were excluded. Finally, 10 microsatellite primers were selected for final PCR amplification. Detailed information of the primers we used can be found in web database (http://www.genetics.org). Information about primer sequences and allele sizes is shown in Table 2. Polymerase chain reactions (PCRs) were performed in a thermo cycler (G-STROM, GSI, England). The volume of PCR solution was 10µl, containing 3µl of diluted template DNA, 1.5 µl of 10X× PCR buffer (Mg^{2+} free), 0.2µl of *Taq* DNA polymerase, 0.25µl 10mM of deoxynucleotide triphosphates (dNTPs), 1.8µl of Mg^{2+}, and 0.5µl of each forward and reverse primers and 2.25µl of double distilled H_2O. The following PCR profile used an initial denaturation step for 5 minute at 94°C (hot start and stand separation). After that 35 cycles of denaturation at 94°C for 1 minute, 35 cycles of anneling at 55°C for 1 minute, 35 cycles of primer elongation at 72°C for 2 minute and then final elongation at 72°C for 5 minute. Amplified products were stored at -20°C. The amplified fragments were separated on 8% (w/v) native polyacrylamide gels. The electrophoreses were performed at 70v for 2 h in 1× TBE [Tris-borateethylenediaminetetraacetic acid (EDTA)] buffer, and the gels were stained with ethidium bromide for 25-30 min, kept in dark, and then visualized using an Alpha-Image gel documentation unit linked to a PC

Data analysis

The most intensely amplified fragments were determined by comparing the migration distance of amplified fragments relative to the molecular weight of known size marker, 100 base pair (100bp) DNA-ladder, using Alpha-Ease FC 5.0 software (Alpha Innotech, USA). The band profiles for each SSR primer pair were scored for distinct and reproducible bands as present (1) or absent (0). Jaccard's similarity coefficient values were selected, pair wise genetic distance was calculated and dendrogram (Nei, 1973) based on similarity coefficient values were generate using unweighted pair-group method with arithmetic mean (UPGMA) by using the online dendrogram construction utility Dendro UPGMA (http://genomes.urv.es/UPGMA) (Garcia -Vallvé *et al.*, 1999). The polymorphic information content (PIC) value was calculated using the following formula (Anderson *et al.*, 1993):

$$PIC = 1 - \sum_{i=1}^{k} p_i^2$$

Where, '*k*' is the total number of alleles (bands) detected for one SSR locus and '*p*' is the proportion of the cultivars or genotypes containing the allele (band) in all the samples analyzed.

RESULTS AND DISCUSSION

Overall allelic diversity

Ten published SSR primer pairs were selected from different chromosome. These 10 primer pair generated 43 unambiguous bands with an average of 4.3 bands per primer pair. Each 43 amplified band was polymorphic. The number of polymorphic alleles per locus amplified by each primer pair ranged from 2 (primer pair 7) to 8 (primer pair 3) with an average of 4.3 alleles per locus. The SSR markers were highly informative and polymorphic as evident from its polymorphic information content or PIC value. The PIC value of each primer pair ranged from 0.217 to 0.835 with an average of 0.495 (Table 3). The level of polymorphism determined by the PIC value (mean= 0.48) is consistent with the reported PIC value in previous works (Wong *et al*., 2009; Hossain *et al*., 2012; Sajib *et al*., 2012). The highest PIC value 0.835 was obtained for primer RM5639 (Figure 1). Other primers, such as RM334 (0.655), RM414 (0.580), RM28502 (0.569) also showed high PIC value. This result raveled that marker RM5639 would be the best in screening this 24 rice genotype followed by RM334, RM414 and RM28502. However, primer RM28502 showed band pattern (Figure 2) which was very much similar to the UPGMA dendrogram constructed with the 24 aromatic rice genotypes. The PIC value found in this study indicated high genetic diversity among the studied aromatic rice germplasm. So, despite their cultivation in marginal areas, aromatic rice landraces of Bangladesh showed high genetic diversity. Similar high genetic diversity among Bangladeshi aromatic rice genotypes ware observed by Sajib *et al*. (2012) in their genetic diversity analysis of 12 aromatic rice genotypes cultivated in Bangladesh, using SSR markers. These findings of current study were also supported by the findings of Islam *et al*. (2016) who used 19 quantitative traits to assess the variability of 113 aromatic rice and fine rice genotypes cultivated in Bangladesh. The diverse genetic nature of these aromatic rice landraces possibly be an echo of the prevailing diverse agro-ecological features of these region.

UPGMA cluster of 24 aromatic rice genotypes based on SSR marker analysis

An UPGMA based dendrogram was constructed from the binary data obtained from the SSR marker based DNA profiles of the sample analyzed (Figure 3). The genotypes that were genetically similar clustered together in the dendrogram. Using correlation coefficient (CP = 0.874) we constructed the UPGMA dendrogram of the 24 aromatic rice genotypes. We observe seven major clusters. Cluster I contained only one genotype which is BRRI dhan38. Cluster II also contained one genotype, Maloti. Cluster III consisted of 9 genotypes namely, Malaysira, Katarivogue, Dhanchicon, BRRI dhan34, Sadagura, Chinisail, BR- 5, Gobindovogue and Khas-kani. Cluster IV consisted of 5 genotypes namely, Fulkori, Begunbitchi, Radunipagol, Khasamukpura and Kalojira. Cluster V consisted of two genotypes namely, Oukunmodu and Khas. Cluster VI consisted of 4 genotypes namely, Basmoti Indian, Black, Dudsail and Dubsail. Cluster VII consisted of two genotypes namely, Basmoti safed, BRRI

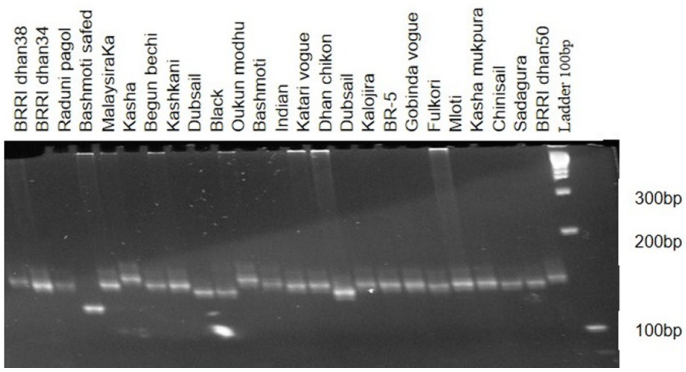

Figure 1. *Amplification profile of primer RM5639 from 24 aromatic rice genotypes in 1.0% agarose gel strained with ethidium bromide. Two micro liter of PCR product was used in each sample.*

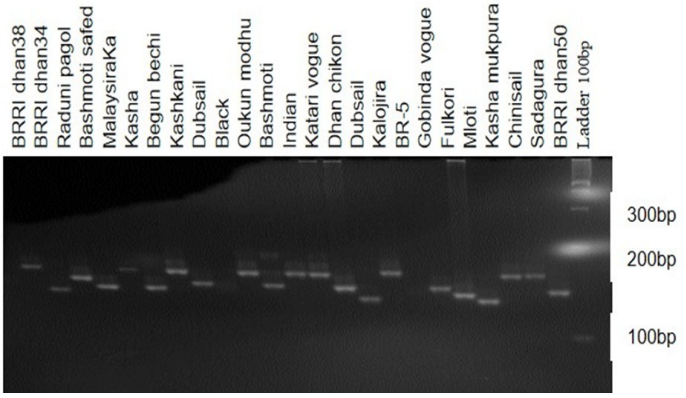

Figure 2. *Amplification profile of primer RM5639 from 24 aromatic rice genotypes in 1.0% agarose gel strained with ethidium bromide. Two micro liter of PCR product was used in each sample.*

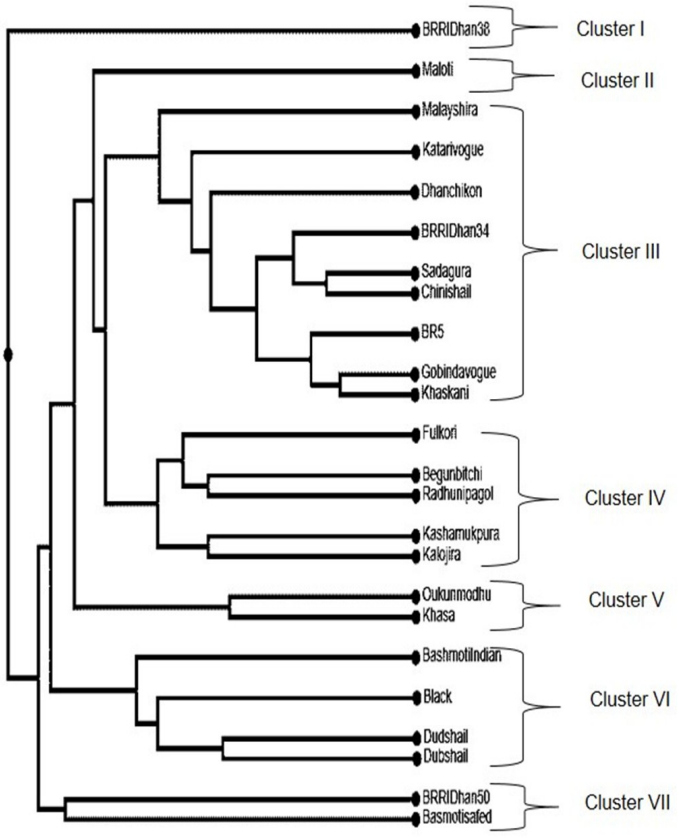

Figure 3. *UPGMA cluster dendrogram in Newick Format showing genetic relationships among 24 genotypes based on 10 SSR markers. Cophenetic Correlation Coefficient (CP) value is 0.874.*

Table 1. List of genotypes with their type, origin, source of collection, kernel size and shape and aroma type.

Genotypes	Type	Origin	Source of collection	Kernel size and shape	Aroma type
BRRI dhan38	Improved variety	Bangladesh	BRRI	Medium, slender	Scented
BRRI dhan34	Improved variety	Bangladesh	BRRI	Short, medium	Scented
Radunipagol	Land races	Bangladesh	BINA	Short, medium	Scented
Basmoti safed	Land races	Bangladesh	BINA	Medium, slender	Lightly scented
Malaysira	Land races	Bangladesh	BINA	Short, bold	Lightly scented
Khasa	Land races	Bangladesh	BINA	Short, medium	Scented
Begunbechi	Land races	Bangladesh	BINA	Short, bold	Lightly scented
Khaskani	Land races	Bangladesh	BINA	Short, medium	Scented
Dubsail	Land races	Bangladesh	BINA	Short, bold	Scented
Black	Land races	Bangladesh	BINA	Short, bold	Scented
Oukun modhu	Land races	Bangladesh	BINA	Short, medium	Scented
Basmoti Indian	Land races	India	BINA	Long, slender	Lightly scented
Katarivogue	Land races	Bangladesh	BINA	Short, medium	Scented
Dhanchikon	Land races	Bangladesh	BINA	Short, medium	Lightly scented
Dudsail	Land races	Bangladesh	BINA	Short, medium	Lightly scented
Kalojira	Land races	Bangladesh	BINA	Short, medium	Scented
BR-5	Improved variety	Bangladesh	BRRI	Short, bold	Scented
Gobindavogue	Land races	Bangladesh	BINA	Short, medium	Lightly scented
Fulkori	Land races	Bangladesh	BINA	Short, bold	Lightly scented
Maloti	Land races	Bangladesh	BINA	Short, medium	Lightly scented
Khasa mukpura	Land races	Bangladesh	BINA	Short, medium	Scented
Chinisail	Land races	Bangladesh	BINA	Short, medium	Lightly scented
Shadagura	Land races	Bangladesh	BINA	Short, medium	Lightly scented
BRRI dhan50	Improved variety	Bangladesh	BRRI	Long, slender	Lightly scented

Table 2. Information of microsatellite markers used for molecular characterization.

Primer name	Chromosome	Primer sequences	Locus	Expected length (bp)
RM526	2	CCCAAGCAATACGTCCCTAG ACCTGGTCATGACAAGGAGG	$(TAAT)_5$	121
RM5639	3	GGAAGAACAGAGTTGCTCGG GTGCCATTTATTTCCGTCCC	$(AAG)_{13}$	123
RM334	5	GTTCAGTGTTCAGTGCCACC GACTTTGATCTTTGGTGGACG	$(CTT)_{20}$	182
RM314	6	CTAGCAGGAACTCCTTTCAGG AACATTCCACACACACACGC	$(GT)_8(CG)_3(GT)_5$	118
RM234	7	ACAGTATCCAAGGCCCTGG CACGTGAGACAAAGACGGAG	$(CT)_{25}$	156
RM407	8	GATTGAGGAGACGAGCCATC CTTTTTCAGATCTGCGCTCC	$(AG)_{13}$	172
RM242	9	GGCCAACGTGTGTATGTCTC TATATGCCAAGACGGATGGG	$(CT)_{26}$	225
RM228	10	CTGGCCATTAGTCCTTGG GCTTGCGGCTCTGCTTAC	$(CA)_6(GA)_{36}$	154
RM224	11	ATCGATCGATCTTCACGAGG TGCTATAAAAGGCATTCGGG	$(AAG)_8(AG)_{13}$	157
RM28502	12	CGAGCAGATCTGATGTCGTCTTCC CTTTGCTTTGCATGCCTCACG	$(GA)_{26}$	155

Table 3. Allele number, number of polymorphic alleles, number of band patterns and PIC values of the SSR markers.

Name of Primer	Number of alleles	Number of polymorphic alleles	Number of Band patterns	Polymorphic Information Content (PIC)
RM526	6	6	7	0.349
RM5639	8	8	8	0.835
RM334	5	5	5	0.655
RM314	4	4	5	0.580
RM234	2	2	2	0.498
RM407	3	3	3	0.217
RM242	4	4	4	0.4896
RM228	3	3	5	0.319
RM224	3	3	5	0.439
RM28502	5	5	5	0.569
Total	43	43	-	-
Average	4.3	4.3	4.9	0.495

Table 4. Pairwise genetic distance among 24 aromatic rice genotypes obtained from microsatellite marker analysis.

	G1	G 2	G 3	G 4	G 5	G 6	G 7	G 8	G 9	G 10	G 11	G 12	G 13	G 14	G 15	G 16	G 17	G 18	G 19	G 20	G 21	G 22	G 23	G 24
G1	0	0.89	0.75	0.95	0.92	0.81	0.75	0.90	0.77	0.81	0.89	0.73	0.77	0.89	0.83	0.77	0.90	0.90	0.89	0.71	0.88	0.88	0.88	0.89
G2		0	0.63	0.72	0.50	0.69	0.43	0.36	0.72	0.83	0.71	0.83	0.47	0.53	0.72	0.65	0.40	0.23	0.71	0.67	0.69	0.25	0.25	0.78
G3			0	0.65	0.65	0.77	0.43	0.72	0.47	0.69	0.78	0.69	0.65	0.78	0.65	0.56	0.67	0.65	0.53	0.67	0.60	0.69	0.69	0.63
G4				0	0.67	0.78	0.65	0.80	0.74	0.84	0.79	0.84	0.80	0.85	0.80	0.74	0.75	0.74	0.56	0.81	0.71	0.78	0.78	0.72
G5					0	0.82	0.50	0.53	0.78	0.87	0.77	0.87	0.60	0.65	0.78	0.67	0.39	0.44	0.65	0.68	0.63	0.56	0.56	0.88
G6						0	0.69	0.71	0.71	0.82	0.39	0.75	0.78	0.77	0.71	0.78	0.72	0.71	0.83	0.72	0.75	0.57	0.57	0.69
G7							0	0.56	0.56	0.69	0.71	0.69	0.47	0.63	0.65	0.47	0.50	0.47	0.43	0.59	0.60	0.50	0.50	0.78
G8								0	0.80	0.77	0.47	0.90	0.50	0.23	0.80	0.67	0.21	0.15	0.79	0.61	0.78	0.31	0.31	0.85
G9									0	0.53	0.72	0.53	0.74	0.85	0.40	0.74	0.75	0.74	0.72	0.75	0.78	0.78	0.78	0.72
G10										0	0.69	0.57	0.71	0.69	0.53	0.63	0.79	0.78	0.69	0.79	0.75	0.89	0.89	0.89
G11											0	0.77	0.79	0.53	0.72	0.72	0.59	0.56	0.84	0.67	0.83	0.69	0.60	0.78
G12												0	0.71	0.83	0.63	0.63	0.85	0.84	0.69	0.79	0.75	0.89	0.82	0.83
G13													0	0.47	0.74	0.50	0.44	0.50	0.56	0.75	0.63	0.41	0.41	0.91
G14														0	0.72	0.56	0.40	0.36	0.71	0.67	0.69	0.50	0.50	0.90
G15															0	0.72	0.81	0.74	0.65	0.81	0.71	0.78	0.78	0.72
G16																0	0.61	0.59	0.47	0.61	0.43	0.71	0.63	0.91
G17																	0	0.21	0.74	0.56	0.65	0.36	0.36	0.86
G18																		0	0.72	0.61	0.71	0.31	0.31	0.79
G19																			0	0.80	0.50	0.77	0.77	0.78
G20																				0	0.72	0.72	0.65	0.80
G21																					0	0.67	0.75	0.83
G22																						0	0.18	0.77
G23																							0	0.83
G24																								0

Legends: G1= BRRI dhan38, G2= BRRI dhan34, G3= Raduni pagol, G4=Basmoti safed, G5= Malaysira, G6= Khasa, G7= Begun bitchi, G8= Khas-kani, G9= Dubsail, G910= Black, G11= Oukun modu, G12= Basmoti Indian, G13= Katari vogue, G14= Dhan chikon, G15= Dudsail, G16=Kalojira, G17= BR-5, G18= Gobindo vogue, G19= Fulkori, G20= Maloti, G21= Khasa-mukpura, G22= Chini sail, G23= Shada gura, G24= BRRI dhan50.

dhan50. All the genotypes in a particular cluster showed higher distance with the genotypes in another cluster, which indicate the presence of wide diversity amongst genotypes of different cluster (Table 4). This also indicate the homogeneous nature of the rice genotypes present in the same cluster. The results were supported by the findings of Iftekharuddaula *et al.* (2002), Rahman *et al.* (2012) and Islam *et al.* (2017).

Pairwise genetic dissimilarity

A dissimilarity matrix constructed based on Jaccard's coefficient (Table 4.). It was used to determine the level of genetic dissimilarity among the cultivars studied. The pairwise genetic dissimilarity matrix indicated that the highest genetic dissimilarity was found 0.95 between the genotypes BRRI dhan38 and Bashmoti safed. This pair were followed by BRRI dhan38 and Malaysira (0.913), Khas-kani and Basmoti Indian (0.90), BRRI dhan38 and BR-5(0.90), Katarivogue and BRRI dhan50 (0.90), Kalojira and BRRI dhan50 (0.90), Dhan chikon and BRRI dhan50 (0.90) BRRI dhan38 and Khas-kani (0.895), BRRI dhan38 and Gobindo vogue (0.895), Black and BRRI dhan50 (0.895), BRRI dhan38 and Oukunmodu (0.889), Basmoti Indian and Chinisail (0.889), Black and Chinisail (0.889), BRRI dhan38 and BRRI dhan34 (0.889), BRRI dhan38 and Dhanchikon (0.889), BRRI dhan38 and Fulkori (0.889), Black and Sada gura (0.889), BRRI dhan38 and BRRI dhan50 (0.889), BRRI dhan38 and Khasamukpura (0.882), BRRI dhan38 and Chinisail (0.882), BRRI dhan38 and Sada gura (0.882), Malaysira and Basmoti Indian (0.87), Malaysira and BRRI dhan50 (0.875), Malaysira and Black (0.87), Basmoti Indian and BR-5 (0.85), Basmoti safed and Dhancikon (0.85), Khas-kani and BRRI dhan50 (0.85) and so on. The pairwise genetic dissimilarity matrix indicated that the lowest genetic dissimilarity was found 0.15 between the genotypes Kash-kani and Gobindo vogue. This pair were followed by the genotypes Chinisail and Sadagura (0.18), BR-5 and Gobindovogue (0.21), Kash-kani and BR-5 (0.21), Kash-kani and Dhanchikon (0.23), BRRI dhan34 and Chinisail (0.25), BRRI dhan34 and Sadagura (0.25). The pair wise genetic dissimilarity co-efficient indicated high genetic distance among most of the aromatic rice genotypes. Among them five rice genotypes (BRRI dhan38, BRRI dhan50, Bashmoti safed, Malaysira, Khas-kani) which showed highest genetic distance, might be utilized as possible parents for the development of fine grain aromatic rice varieties. High genetic distance between the aromatic rice genotypes cultivated in Bangladesh was also reported by Sajib *et al.* (2012). Similarly, high genetic distance was reported by Islam *et al.* (2017) in their genetic diversity assessment of 53 aromatic rice genotypes cultivated in Bangladesh using 16 quantitative traits.

Conclusion

In conclusion, molecular characterization of Bangladeshi aromatic rice landraces exposed that great variation exists amongst the aromatic rice genotypes. The genetic diversity preserved in these aromatic rice gene pool could be a valuable resource for further improvement and developing new varieties.

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Conflict of interest

The authors declare that they have no conflict of interest regarding the materials used in this manuscript.

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