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Morpho-molecular diversity study of rice cultivars in Bangladesh

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Abstract: Rice is one of the frontline cereals in the world and the major cultivated crop in Bangladesh. A total of eleven simple sequence repeats (SSRs) and thirteen sequence-tagged site (STS) markers were used to characterize twenty-four rice cultivars in Bangladesh. Twenty-four markers generated 60 alleles with 2.5 alleles per locus. The average polymorphism information content (PIC) value was 0.40, while the mean value of heterozygosity, gene diversity, and major allele frequency were recorded as 0.10, 0.48 and 0.62, respectively. However, the SSR markers showed more specificity and a higher discrimination power than the STS markers. The cluster analysis displayed four major clusters with a genetic similarity coefficient value of 0.73. The morphological analyses of the grain identified that Binadhan-20 and BRRI dhan34 had the longest and the shortest seed size, respectively, with a variable correlation between the seed length, width and length/width ratio. The phenol reaction test distinguished seven cultivars as *japonica* and seventeen cultivars as *indica* or an intermediate type. All these results regarding the phenotypic data and marker information will be useful for parental selection in modern rice breeding programmes.

Keywords: genetic diversity; phenol reaction test; rice cultivars; SSR marker; STS marker

Rice (*Oryza sativa* L.) is the most diversified crop which adapted to a wide range of geographical, ecological and climatic regions (Yadav et al. 2013), and feeds more than half of the world's population (Sasaki & Burr 2000). The global rice production is 755.49 million tonnes; Bangladesh ranks fourth in terms of area and production (FAO 2019). Bangladesh has a warm and humid climate, sufficient low-lying land, three rice growing seasons (Aus, Aman and Boro), which are suitable for rice cultivation throughout the year. For being a staple food, its production is considered as the key factor for food security in Bangladesh. About 132 000 accessions are housed at the International Rice Gene Bank in the Philippines (IRRI 2019), consisting of two major subspecies *japonica* and *indica* (Oka 1958) that are differentiated on the basis of morphological and genetic characteristics (Caicedo et al. 2007). The *indica* type is widely

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grown in tropical and subtropical climates like in Bangladesh, whereas *japonica* is typically grown in the regions with cooler climates and accounts for more than 75% of the global rice trade (Geist 2006).

The seed size, shape and length/width (L/W) ratio are important features for grain quality assessment (Rita & Sarawgi 2008) that vary from one geographical region to another and among cultivated rice varieties (Azeez & Shafi 1966). A previous study used the grain size as an important characteristic to distinguish the *japonica* and *indica* rice types (Fitzgerald et al. 2009). *Indica* cultivars possess long to short, slender, somewhat flat grains, and their spikelets are awnless, whereas the grain characteristics of *japonica* cultivars are short, roundish, with awnless to long-awned in their spikelets and do not shatter easily (Geist 2006). Differentiation between *indica* and *japonica* rice varieties has traditionally been performed on the morphological characteristics, such as plant height, plant type, and type of grains in combination with some physiological and biochemical features (Oka 1962; Wang & Li 1997).

However, the traditional method using morphological and physiological traits can greatly be influenced by the environmental conditions. With the advent of molecular marker breeding programmes, the characterisation of rice through marker assisted selection is now more accurate and less affected by the environment (Panaud et al. 1996; McCouch et al. 2001). Molecular markers, including microsatellite or simple sequence repeats, are widely distributed across the genome, have high allelic diversity, and are efficient for identification of genes and quantitative trait loci (QTL) (McCouch et al. 2001).

Few studies regarding the characterisation of Bangladeshi Aus rice, and some landraces have been reported thus far using SSR markers (Islam et al. 2017, 2018). A majority of studies are either only limited to the molecular marker or to the biochemical or morphological markers for the characterisation. Considering all of the above aspects, the present study was conducted, which combined molecular markers, a phenol analysis and grain morphological traits together to promise a better understanding of the genetic diversity in Bangladeshi rice cultivars.

MATERIAL AND METHODS

Rice cultivars. A total of 24 rice cultivars comprising four different types (landraces, HYV, aromatic and *indica* type Japonica) were used in this study (Table 1). All the cultivars were grown under natural field conditions in the experimental field of the Bangladesh Institute of Nuclear Agriculture (BINA), Mymensingh, Bangladesh in Kharif-1, in 2018 and the phenotypic evaluation, molecular analyses were executed in 2019.

Identification and selection of molecular markers. After checking the initial performance, a total of 24 molecular markers consisted of eleven simple sequence repeat (SSR) and thirteen sequence-tagged site (STS) markers were finally selected as presented in Table 2. The SSR markers were derived from the Gramene database (https://archive.gramene.org/markers/) for rice (McCouch et al. 2002) and the STS markers used the information from Chin et al. (2007).

Measurement of length, width and weight of rice grain and observation of awn. The phenotypic data were recorded from grains of each cultivar. The length and width of the rice grains were measured by digital slide callipers (Mitutoyo, JAPAN) and the mean value of each trait was calculated from ten randomly selected grains, where the 1 000-grain weight was measured. The presence or absence of awns was scored visually.

Phenol reaction test. A 2% aqueous phenol solution was used to differentiate the rice cultivars into *japonica* and *indica* types. At first, twenty grains of each variety were soaked in distilled water for 24 h and then ten out of twenty grains were soaked in 4 ml of the 2% phenol solution for 48 h. The remaining ten seeds were used as an untreated control which were soaked in 2 ml of distilled water for the same period. Upon treatment, the grain colour change was compared with the untreated grains from the same sample to assess the colour change. The cultivars were designated as an *indica* type when showing darkening after exposure to the phenol solution, while the *japonica* type showed no change in grain colour (Reflinur et al. 2018).

Isolation of genomic DNA, PCR amplification and allele scoring. Genomic DNA was extracted from the fresh young leaves of twenty-day-old seedlings using the sodium dodecyl-sulfate (SDS) method according to Rahman et al. (2007). Polymerase chain reaction (PCR) amplification was carried in a 10 μL reaction and performed as described by Akter et al. (2014). The amplified PCR products were checked in 8% polyacrylamide gel (in 1× TBE) using a 100 bp ladder (Thermo Scientific, USA) followed by visualisation in a gel documentation system (AlphaImager

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HP Imaging System). Allele scores for both the SSR and STS markers were tabulated by observing a clear unique band pattern from the PCR product and coded using a digital number.

Data analysis. The molecular data gathered from all the primers used in the present study were prepared as a binary matrix, with one (1) and zero (0) representing the presence of an allele at each locus on 24 rice cultivars. A phylogenetic dendrogram was generated based on Jaccard's similarity coefficient with the unweighted pair group method and arithmetic average (UPGMA) using NTSYS-pc Ver. 2.1 (Rohlf 2002). The statistical summary, including values of the polymorphic information content (PIC), heterozygosity, major allele frequency and allele number was calculated for the total population and markers using PowerMarker (Ver. 3.25) with a bootstrap analysis of 1000 permutations (Felsenstein 1985; Liu & Muse 2005).

The collected phenotypic data were analysed to examine differences in the phenotypic values between the rice cultivars using a one-way analysis of variance (ANOVA) or Student's *t*-tests. Duncan's multiple range test was conducted to make further comparisons if the results of the analyses were significant ($P < 0.05$). The statistical analyses were performed using the SAS software (Ver. 9.1.3) and, for the principal component analysis, the statistical tool R (Ver. 3.5.1, package "factoextra") was applied.

RESULTS AND DISCUSSION

Phenotypic evaluation. Grain characteristics are key agronomic traits that determine the rice yield.

Table 1. Phenotypic variation of the grains in the different rice cultivars

No.	Cultivar name	Cultivar type	Length (mm)	Width (mm)	Length/ width ratio	1 000 grain weight (g)
1	Kalo mota	Bangladeshi landrace	8.45^{e}	3.12^{bc}	2.71^{g}	21. 11^{f}
2	Kacha mota	Bangladeshi landrace	7.94^{fg}	3.58^{a}	2.22^{j}	26.65^{b}
3	BRRI dhan77	HYV, BRRI	8.38^{e}	3.19^{bc}	2.62^{hi}	26.69^{b}
4	BRRI dhan76	HYV, BRRI	7.97^{fg}	3.17^{bc}	2.52^{hi}	23.61^{de}
5	BR5	HYV, BRRI	6.09^{i}	2.28^{g}	2.68^{hi}	10.13^{j}
6	Katarivog	Bangladeshi landrace	7.73^{g}	2.05^{h}	3.77^{bcd}	11.47^{i}
7	Dud kalam	Bangladeshi landrace	7.26^{h}	3.07^{cd}	2.36^{ij}	24.10^{d}
8	Moulota	Bangladeshi landrace	8.80^{de}	2.92^{d}	3.02^{fg}	24.62^{c}
9	Sada mota	Bangladeshi landrace	8.61^{e}	3.43^{ab}	2.51^{hij}	23.53^{cd}
10	Lal mota	Bangladeshi landrace	7.57^{g}	3.22^{bc}	2.35^{ij}	26.35^{b}
11	Benapol	Bangladeshi landrace	9.39^{bc}	2.31^{f}	4.07^{bc}	24.11^{cd}
12	Kachra	Bangladeshi landrace	8.16^{e}	3.18^{bc}	2.56^{h}	30.67^{a}
13	Jothui	Bangladeshi landrace	8.31^{f}	3.12^{bc}	2.66^{h}	23.33^{de}
14	Kamina Saru	Bangladeshi landrace	7.47^{gh}	2.53^{fg}	2.99^{fg}	16.57^{g}
15	Baila Amon	Bangladeshi landrace	8.32^{ef}	2.96^{de}	2.81^{fgh}	23.46^{de}
16	Gahinda	Bangladeshi landrace	7.84^{g}	2.07^{h}	3.79^{bcd}	13.42^{h}
17	Saubail	Bangladeshi landrace	8.33^{e}	2.43^{f}	3.42^{e}	13.37^{h}
18	Bashful	Bangladeshi landrace	7.46^{gh}	2.84^{de}	2.62^{h}	16.60^{g}
19	Begun Bichi	Bangladeshi landrace	5.83^{i}	2.24^{g}	2.60^{h}	11.13^{i}
20	Tulsi Mala	Bangladeshi landrace	9.57^{b}	2.40^{f}	3.99^{bcd}	25.68^{bc}
21	Binadhan-17	HYV, BINA	9.39^{b}	2.25^{g}	4.17^{b}	21.28^{f}
22	Binadhan-20	HYV, BINA	10.37^{a}	2.26^{g}	4.59^{a}	25.82^{bc}
23	BRRI dhan34	HYV, BRRI	6.25^{c}	1.97^{h}	3.17^{ef}	10.16^{j}
24	Japonica-1	HYV, Korean	9.218^{bcd}	2.66^{ef}	3.47^{e}	24.41^{cd}
LSD value			0.45	0.18	0.20	0.95

In a column, similar letters do not differ significantly whereas a dissimilar letter differs significantly as per Duncan's multiple range test; $P < 0.05$ as value of the probability level

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All the measured phenotypic traits amongst the studied cultivars were significantly different (Table 1). The range of the mean performance of the seed length, seed width and length/width ratio of the different cultivars varied from 5.83 to 10.37 mm, 1.97 to 3.58 mm and 2.22 to 4.59 mm, respectively. Binadhan-20 produced the longest seed and Begun Bichi displayed the shortest grain length. Moreover, the highest grain width was observed in Kacha mota and the lowest grain width was found in BRRI dhan34 (Table 1). The results are favourably supported by previous reports (Yoon et al. 2006; Srivastava & Jaiswal 2013).

Rice grains are classified into three groups, namely long, medium, and short grains according to the Standard Evaluation System (IRRI 1998). Here, Binadhan-20 possessed the highest L/W ratio and the lowest ratio was recorded Kacha mota (Table 1). The results indicated that the length/width ratios of the measured cultivars were recorded as long and medium sized grains indicating the existence of sufficient variation among the genotypes for certain traits. A high L/W ratio value observed in Binadhan-20 as a high yielding variety (HYV) in comparison to that in Kacha mota as Bangladeshi landrace that are probably related to differential the genetic background, geographical distribution and ecological habits. It is caused by the fact that the variation in the grain length to width ratio observed in the present study are not in accordance with Bangladeshi rice haplotypes. On the contrary, Zhang et al. (2021) reported that the haplotypes with the largest frequency in the cultivars predominantly consisted of *indica* subspecies, which had much slenderer and longer grains than those in the landraces. Considering the presence or absence of awns, we found only two cultivars

Table 2. Allele frequency, gene diversity and heterozygosity of 24 markers

No.	Marker	Marker type	Chr	MAF	No. of allele	H_e	H_o	PIC
1	RM167	SSR	11	0.71	3	0.45	0.00	0.41
2	RM337	SSR	08	0.69	4	0.49	0.04	0.45
3	RM407	SSR	08	0.53	2	0.50	0.19	0.37
4	RM434	SSR	09	0.5	2	0.50	0.08	0.38
5	RM1287	SSR	01	0.75	2	0.38	0.05	0.30
6	RM3825	SSR	01	0.52	3	0.59	0.00	0.51
7	RM5461	SSR	01	0.33	4	0.73	0.00	0.68
8	RM5639	SSR	03	0.43	4	0.66	0.05	0.59
9	RM5749	SSR	04	0.43	4	0.68	0.09	0.63
10	RM5806	SSR	10	0.43	3	0.63	0.09	0.55
11	RM10115	SSR	01	0.85	2	0.26	0.13	0.22
12	S1054	STS	01	0.80	2	0.31	0.04	0.27
13	S1140	STS	01	0.85	2	0.25	0.21	0.22
14	S3048	STS	03	0.53	3	0.59	0.21	0.51
15	S4097	STS	04	0.67	2	0.44	0.17	0.35
16	S5080	STS	05	0.52	2	0.50	0.09	0.37
17	S7114	STS	07	0.83	2	0.28	0.17	0.24
18	S8020	STS	08	0.78	2	0.34	0.00	0.28
19	S9000	STS	09	0.67	2	0.44	0.08	0.35
20	S9075	STS	09	0.59	2	0.48	0.13	0.37
21	S10072	STS	10	0.59	2	0.48	0.09	0.37
22	S11117	STS	11	0.58	2	0.49	0.05	0.37
23	S12011B	STS	12	0.58	2	0.49	0.17	0.37
24	S12091	STS	12	0.63	2	0.47	0.25	0.36
Mean		–	–	0.62	2.5	0.48	0.10	0.40

SSR – simple sequence repeat; STS – sequence tagged site; Chr – chromosome No.; MAF – major allele frequency; H_e – gene diversity; H_o – observed heterozygosity; PIC – polymorphism information content

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Table 3. Correlation among the length, width, length/width ratio and weight of the grains (N = 24)

		Length	Width	Ratio	Weight
Length	Pearson correlation	1	0.09	0.59**	0.64**
	significance (2-tailed)		0.65	0.00	0.00
Width	Pearson correlation	0.09	1	–0.74**	0.69**
	significance (2-tailed)	0.65		0.00	0.00
Ratio	Pearson correlation	0.59**	–0.74**	1	–0.12
	significance (2-tailed)	0.00	0.00		0.59
Weight	Pearson correlation	0.64**	0.69**	–0.12	1
	significance (2-tailed)	0.00	0.00	0.59	

**Correlation is significant at a 0.01 level (2-tailed)

having awns, namely Kachra and Tulsi Mala, while the others were observed as being awnless (Figure 1) indicating that rice cultivars analysed in the present study are closely related to each other and probably belong to the same varietal group. The grain weight is positively associated with the grain size, which is determined by the grain length, grain width, and grain thickness (Yang et al. 2014). In the present study, the highest 1 000-seed weight was recorded in Kachra and the lowest was noted in BR5 (Table 1). All the measured traits among the cultivars were significantly different and showed a strong correlation among the traits. For most of the correlations, the direction (+ and –) and degree of correlation was similar with previous studies (Yoon et al. 2006). In agreement with previous studies, the seed length showed positive correlations with the length/width ratio (r = 0.59**) and 1 000-grain weight (r = 0.64**) and the width also showed a positive correlation with the 1 000-grain weight (r = 0.69**). Negative correlations existed between the seed width and length/width ratio (r = –0.74**) (Table 3).

All the principal components were positively associated with the grain length, grain width, L/W ratio and thousand grain weight. PC1 was strongly associated with the grain width and thousand grain weight. The cultivars having high values in PC1 had a greater reduction in those traits (Table 4) and all the cultivars were widely scattered across the different quarters (Figure 2). The grain length and L/W ratio are mostly the contributors of PC2 which account for 44.62% of the total variation. Besides, PC3 accounted for only 2.76% of the variation; hence, it will not be used as a good component. A similar type of performance and pattern for the phenotypic variables were reported by Maji & Shaibu (2012).

Phenol reaction test. The phenol reaction test can distinguish the cultivar as either the *indica* type or *japonica* type or an intermediate one (Gross et

Figure 1. Presence of awns in Kachra (A) and Tulsi Mala (B); and absence in the Kalomota (C), and Lalmota (D) rice cultivars

White bars represent a 5 mm scale

Table 4. Correlation matrix of three principal components (PC), Eigen values, the relative and cumulative proportion of the total variance on all the traits of the evaluated rice genotypes

Trait	PC1	PC2	PC3
Grain length (mm)	0.06	0.91	0.28
Grain width (mm)	0.95	0.03	0.02
L/W ratio	0.39	0.60	0.00
Thousand grain weight (g)	0.69	0.24	0.06
SD	1.45	1.34	0.33
Proportion of variance (%)	52.49	44.62	2.76
Cumulative proportion (%)	–	97.11	99.87
Eigen value observed	2.10	1.78	0.11

L/W ratio – length/width ratio; SD – standard deviation

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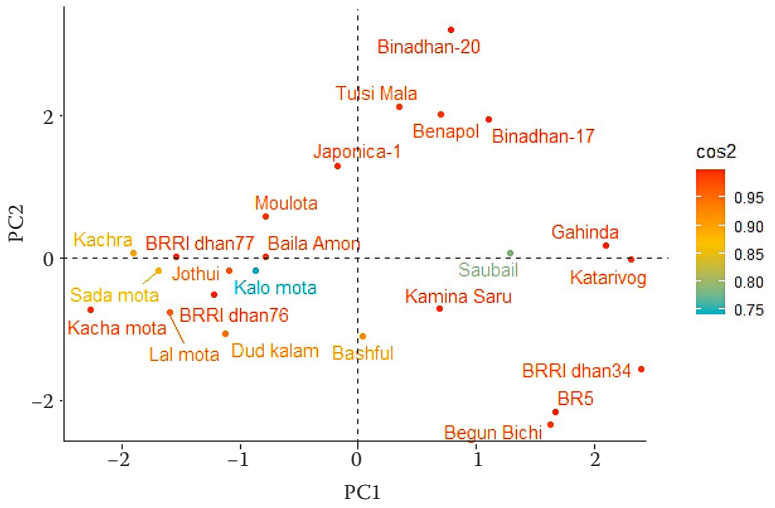

Figure 2. Principal component (PC) analysis of the 24 rice cultivars based on the morphological traits

al. 2009; Tiwari et al. 2013). Based on the colour change, the phenol reaction test distinguished the cultivars BR5, Kachra, BRRI dhan34, Saubail, Katarivog, Tulsimala, Begunbichi as an aromatic rice and, thus, closer to the *japonica* type. On the contrary, BRRI dhan76, Gahinda, Binadhan-17, Japonica-1 (Milyang23, a Tongil-type rice derived from an *indica* and *japonica* cross with a genetic background resembling *indica*) showed an intermediate colour change and the rest of the cultivars identified as *indica* are basically Aus (Figure 3) indicating that most of the cultivars were developed and cultivated under similar environments for specific breeding purposes.

Molecular marker analysis. A total of 60 alleles with a mean of 2.5 alleles per marker have been detected in 24 rice varieties using 24 markers (Table 2). The allele number per locus ranged from 2 to 4. The major allele frequency ranged from 0.33–0.83 and the gene diversity ranged from 0.25–0.73. The banding patterns of the 24 rice cultivars at the representative loci, viz. S4097, S5080, S8020 and RM5806, are shown in Figure 4.

The mean PIC value for all the markers was 0.40 with a range of 0.22 to 0.68. According to the definition of informative level (Botstein et al. 1980), six (25%) markers were highly informative (PIC > 0.5), fifteen (62.5%) were reasonably informative (0.5 > PIC > 0.25), and three (12.5%) were only slightly informative (PIC < 0.25). Here, the mean of the observed heterozygosity of all the markers were 0.10 with a range from 0 to 0.25 which was slightly higher than the average heterozygosity of 0.07 reported by Babu et al. (2014) and 0.02 reported by Pathaichindachote et al. (2019). In addition, the mean value for the gene

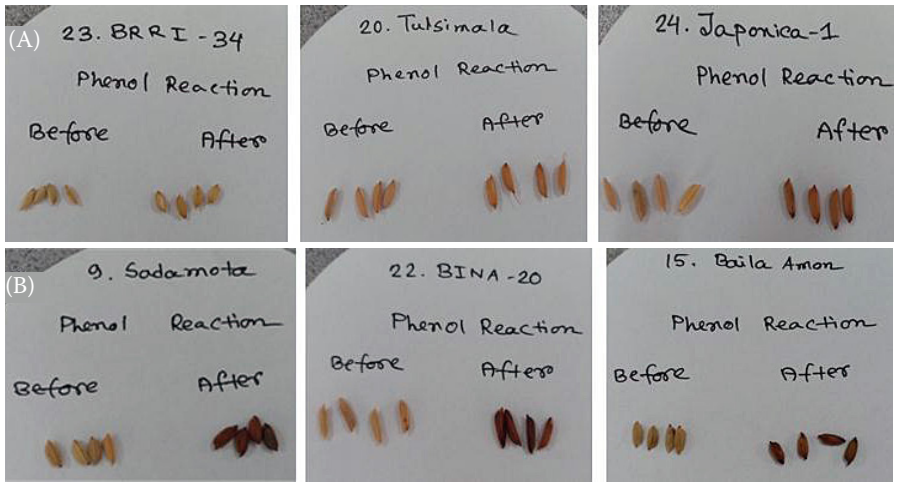

Figure 3. The phenol reaction rice hulls of different rice cultivars: upper panel with no colour change (A) and lower panel showing grain darkening (B)

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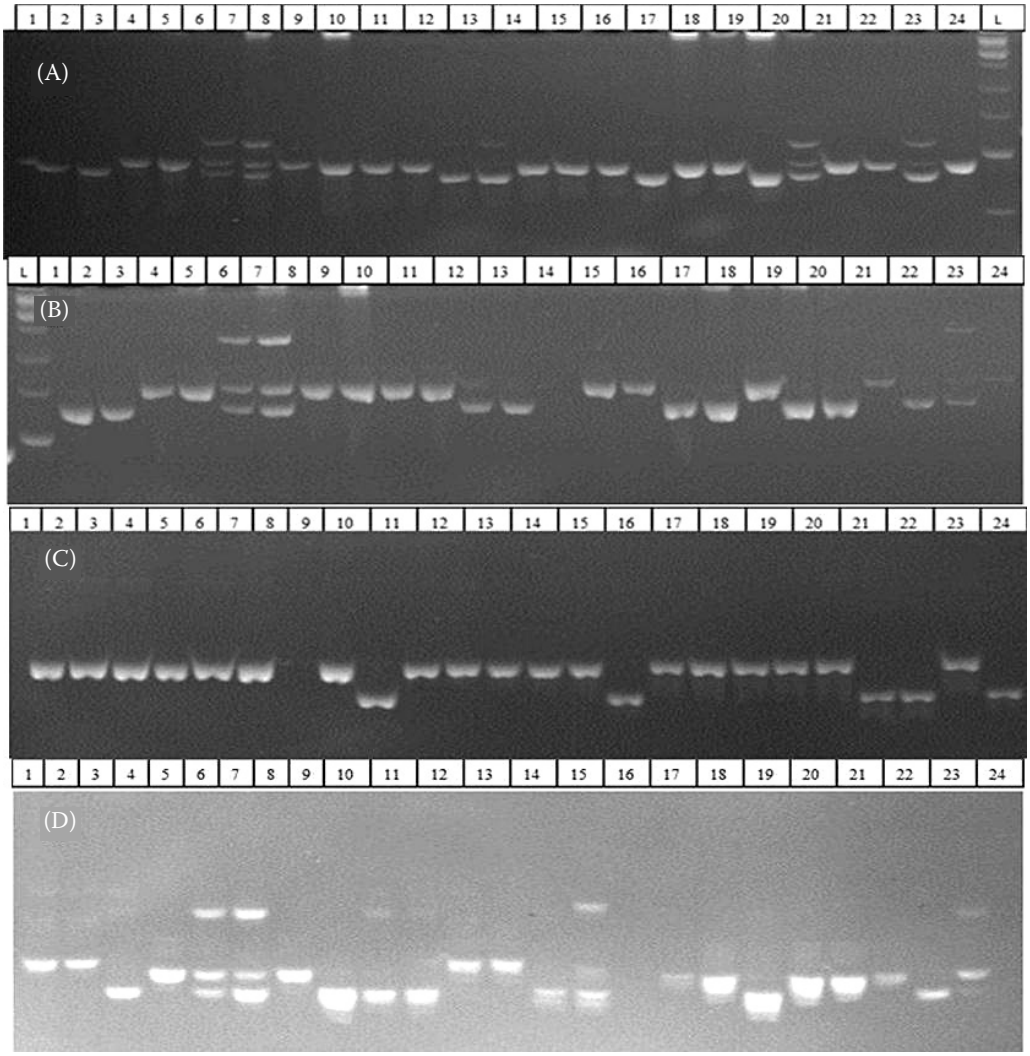

Figure 4. Banding profiles of the 24 rice cultivars (lanes 1–24) with markers: S4097 (A); S5080 (B); S8020 (C); RM5806 (D)
L – 100 bp DNA ladder

diversity was 0.48, which ranged from 0.25 to 0.68. The highest gene diversity (0.68) was found in the RM5749 marker and the lowest was found in the S1140 marker. The highest major allele frequency (0.85) was estimated in RM10115 and S1140, while the lowest (0.33) was found in R5461. Recently, similar results were reported on the polymorphic levels, gene diversity and heterozygosity of molecular markers (Anupam et al. 2017; Li et al. 2017).

Genetic diversity of different rice cultivars. An unrooted neighbour-joining tree was constructed to observe the genetic relationships among 24 rice cultivars based on the alleles detected by 24 markers. As seen in Figure 5, most of the popular high yielding varieties (HYVs) were grouped in cluster 3 except for BRRI dhan34 and BRRI dhan77 which might have a different background. Besides cluster 1, consisting of three cultivars; Kalomota, Tulsimala and Kacha mota and Cluster 2 consisting of two cultivars; BRRI dhan77 and Gahinda. Cluster 4 had only three cultivars with two sub clusters containing Lalmota, Japonica-1 and BRRI dhan34. Therefore, BRRI dhan34 is an HYV aromatic rice that formed a distinct group and displayed genetic dissimilarity to the other cultivars. The released varieties having same genetic relationship with the local cultivars may be descendent from common or closely related genetic background (ancestors). The analysis of the SSR and STS data by a dendrogram was matched with the previous studies of Lin et al. (2012). Therefore, further studies using more molecular markers are needed to deeply classify the rice genetic groups into indica, Aus, aromatic and japonica.

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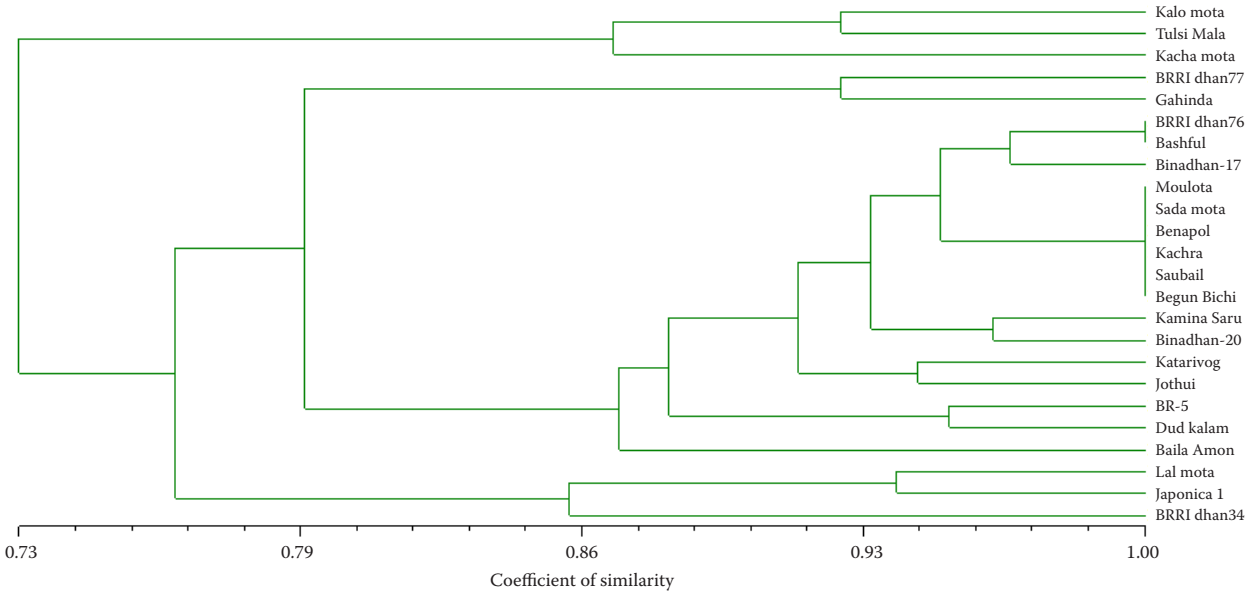

Figure 5. Dendrogram summarising the data on the differentiation between the 24 rice cultivars based on a combination of two different types of molecular markers, simple sequence repeat (SSR) and sequence-tagged site (STS), constructed using an unweighted pair group mean average (UPGMA) cluster analysis using the Jaccard's similarity coefficient

CONCLUSION

This study examined the diversity of 24 cultivars based on the grain morphological traits and 24 molecular markers. The phenol reaction test on the rice cultivars clearly distinguished seven cultivars including small grain aromatic rice as *japonica* and seventeen cultivars as *indica*/*aus* or the intermediate type. The genetic diversity analysis revealed considerable genetic diversity within the studied genotypes and, thus, suggests usefulness for future plant breeding activities to develop superior rice varieties.

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