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Assessment of genetic diversity among selected aromatic rice genotypes using SSR markers

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Abstract

Molecular characterization of genotypes is an important part for the selection of appropriate parents. Twenty four (nineteen aromatic and five non-aromatic) rice genotypes were evaluated for 40 genetic loci at molecular level to identify the desirable genotypes for future breeding program. A total of 159 alleles were found in the present study of which RM 6959 showed the highest number of alleles (8) and RM 5499 showed the lowest number of alleles (2), with an average of 3.98 alleles across the 40 loci. The frequency of the most common allele at each locus ranged from 33% (RM 19, RM 215, RM 452 and RM 1155) to 92% (RM 10). The range of polymorphic information content (PIC) values was from 0.1504 to 0.7584 with an average of 0.532. The highest PIC value (0.7548) was obtained for RM 242 followed by RM 215 and RM 6959 (0.7394). PIC value revealed that RM 242 was the best marker for revealing the genetic constitutions of 24 tested rice genotypes.

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Introduction

Grain quality in rice plays an important role in consumer acceptability. Juliano and Duff (1991) concluded that grain quality is second after yield as the major breeding objective for crop improvement. The quality in rice is considered based on milling quality, grain size, shape, appearance, aroma and other cooking characteristics (Dela *et al.*, 2000). Aroma quality of scented rice is a major character which increases the value of rice in international market (Nayak *et al.*, 2002). Most of the scented rice varieties in Bangladesh are of traditional type, photoperiod sensitive and cultivated during the Aman season. Majority of these indigenous aromatic rice cultivars are low yielding but its higher price and low cost of cultivation generate higher profit margins compared to other varieties.

Like other parts of the world, Bangladesh has already lost a large number of aromatic rice genotypes and many at the verge of extinction (Singh *et al.*, 2000). Rapid adoption of modern varieties is a serious threat for the existence of fine quality rice genotypes for their low yield. The Himalayan foothill including parts of Bangladesh is considered to be the secondary center of diversity of the genus *Oryza* (Morishima, 1984) but information about the characterization or genetic diversity of aromatic rice is very limited. Systematic study and characterization of such germplasm is not only important for utilizing the appropriate attribute based donors, but also essential in the present era for protecting the unique rice. Thus, there is a need to collect, exploit and evaluate the untapped germplasm (Parikh *et al.*, 2012).

For the study of genetic diversity, the plant scientists have used generally morphological, physiological as well as chemical features of plant. The number of scorable morphological characters is varying as compared to the biological active genes. Moreover in most cases, plant genomes have large amount repetitive DNA which are not expressed and do not contribute to the physiological or morphological appearance of plants. Due to stage specific expression of characters and influence of environment, morphological diversity estimates are less reliable. Moreover, at times there may be little morphological diversity among cultivars with related pedigrees. Most of the rice varieties have been developed traditionally by selection, hybridization and back crossing with locally adapted high-yielding lines. The conventional methods of plant selection for aroma are not easy because of the low narrow sense heritability of aroma.

So, there is always a need to study polymorphism at DNA level, which can be an indicative of genetic diversity. Molecular characterization of the genotypes gives precise information about the extent of genetic diversity which helps in the development of an appropriate breeding program (Sajib *et al.*, 2012). Ahn *et al.*(1992) reported a DNA marker closely linked to *fgr* (fragrant) gene for aroma on rice chromosome 8. Rice is also a model crop for the study of genetics and genome organization due to its diploid genetics, relatively small genome size (430 Mb) (Causse*et al.*, 1994; Kurata *et al.*, 1994) and significant level of genetic polymorphism (McCouch*et al.*, 1998; Tanksley, 1983, Wang *et al.*, 1992).Several types of molecular markers *viz.*, RFLP (Botstein *et al.*, 1980), RAPD (Williams *et al.*, 1990), AFLP (Vos *et al.*, 1995), SSR microsatellites (Levinson and Gutman, 1987) and SNP (Vieux *et al.*,2002) have been developed. PCR based markers such as microsatellites are co-dominant, hyper variable, abundant and well distributed throughout the rice genome (Temnykh *et al.*, 2001). Microsatellites have shown great promise in genetic diversity, genome mapping, gene tagging and marker assisted selection (MAS) because they are technically simple, time saving, highly informative and require small amount of DNA. Abundance of microsatellite markers is now available through the published high density linkage map (McCouch *et al.*, 2002) or public database. Therefore, the study was undertaken to assess genetic diversity at molecular level in Bangladeshi local aromatic rice genotypes and to select suitable diverse parents for future breeding program.

Materials and methods

Plant materials

Twenty four genotypes of rice landraces were used for microsatellite DNA markers analysis. The list of the twenty four rice genotypes including their ecotype, BRRI accession number and place of collection is given in Table 1. The whole experiment was conducted at the Marker Assisted Selection (MAS) Laboratory, Plant Breeding Division, Bangladesh Rice Research Institute, Gazipur, Bangladesh. Five grams of seed from each genotype was sown in the earthen pot and young leaves of twenty one days old seedlings were collected for DNA extraction through modified Miniscale method (Zheng*et al.*, 1995).

Table 1. List of 24 test rice genotypes with their different characters.

SL. No.	BRRI Access No.	Variety Name	Ecotype	Aroma	Place of collection
1	4867	Chinigura	T. Aman	Aromatic	Naogaon
2	7413	Basmati India	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
3	7082	Kataribhog	T. Aman	Aromatic	Dinajpur
4	5347	Sakkorkhora	T. Aman	Aromatic	Barguna
5	4497	Basmati Porder	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
6	4904	Basmati 370	T. Aman	Aromatic	Pakistan
7	4496	Basmati Naret	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
8	4495	Basmati IRGC 27782	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
9	4500	Basmati 1	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
10	4501	Basmati 107	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
11	4502	Basmati 134	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
12	4503	Basmati 372	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
13	-	Kamarang	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
14	5950	JamaiAduri	T. Aman	Aromatic	GRSD*, BRRI, Gazipur
15	416	Jhingasail	T. Aman	Non-aromatic	Rajshahi
16	4109	Khutichikon (3)	T. Aman	Aromatic	Comilla
17	4108	Khutichikon (2)	T. Aman	Non-aromatic	Comilla
18	247	Kalijira	T. Aman	Aromatic	Khulna
19	315	Binnaphul	T. Aman	Aromatic	Gaibandha
20	-	Rong-er-gura	T. Aman	Non-aromatic	Bhola
21	245	Chinisagor	T. Aman	Aromatic	Mymensingh
22	-	Rasmala	T. Aman	Non-aromatic	Sherpur
23	7063	SugandhiDhan (2)	T. Aman	Non-aromatic	Nawabganj
24	4490	Basmati T_3	T. Aman	Aromatic	GRSD*, BRRI, Gazipur

GRSD*: Genetic Resources and Seed Division.

SSR markers

Sixty SSR markers with clear amplifications were selected for genetic diversity analysis of twenty four rice genotypes.

Genotyping protocol

Miniscale DNA extraction procedure

400 µl of DNA extraction buffer was added to the well of grinding plate where around 3 cm of rice leaf was previously placed. The rice leaf was crushed by glass rod so that the crushed liquid (=leaf juice) turned into greenish colour.

Then another 400 µl DNA extraction buffer was added to the well of grinding plate. About 800 µl Leaf juice was transferred into a 1.5 ml microfuse tube (which was previously labeled by permanent marker pen) by 1 ml pipette.500 µl chloroform was added to the leaf juice. Tubes were shaken in an orbit shaker at 250 rpm for 30 minutes. The microfuse tubes were placed horizontally to the basement of the shaker. The tubes were fitted tightly by adhesive tape in between two tube rakes. Tubes were shaken so that the colour of the liquid turned into milky white. The tubes were centrifuged at a speed of 13,000 rpm for 10 minutes.

Table 2. Different types of reagents for preparing 8% gel.

Reagents	Final conc.	8% gel
Sterile nanopure H_2O		41.35 ml
10X TBE buffer	5X	6.0 ml
40% Acrylamide	8%	12 ml
10% APS	0.1%	600 µl
TEMED(Tetramethylethylenediamine)	1 µl/ml	50 µl
Total		60.0 ml

Table 3. Number of alleles, allele size range, highest frequency allele and polymorphism information content (PIC) found in 24 rice genotypes for 40 microsatellite markers.

Marker	Chr.No	Position (cM)	Motif*	Allele No	Size range (bp)	Highest frequency allele		PIC Value
						Size (bp)	Freq. (%)	
RM 5	1	94.9	(GA)14	3	93-102	93	62	0.4443
RM 283	1	31.4	(GA)18	4	149-165	149	79	0.3379
RM 6	2	154.7	(AG)16	4	158-171	169	50	0.5236
RM 452	2	58.4	(GTC)9	4.	191-212	198	33	0.6874
RM 5427	2	-	(TC)16	4	127-155	127	50	0.6050
RM 322	2	49.8	(CAT)7	3	102-105	105	54	0.5020
RM 489	3	29.2	(ATA)8	5	187-275	246	50	0.5962
RM 545	3	35.3	(GA)30	4	202-230	202	58	0.5524
RM 6959	3	65.4	(TTC)9	8	107-303	107	41	0.7394
RM 307	4	0	(AT)14(GT)21	5	117-202	121	41	0. 6747
RM 537	4	8.5	(CCG)9	3	226-237	226	54	0.5331
RM 551	4	8.5	(AG)18	3	172-178	178	62	0.4080
RM 1155	4	58.9	(AG)13	4	147-169	147	33	0.6519
RM 413	5	26.7	(AG)11	5	67-108	67	58	0.5529
RM 421	5	111.2	(AGAT)6	4	126-171	144	62	0.5060
RM 454	6	99.3	(GCT)8	3	120-130	120	75	0.3414
RM 510	6	20.8	(GA)15	3	116-128	116	91	0.1504
RM 3514	6	91.9	-	4	147-154	154	41	0.6057
RM 125	7	24.8	(GCT)8	4	121-134	121	41	0.6218
RM 455	7	65.7	(TTCT)5	3	259-165	265	87	0.2124
RM 478	7	93.8	(AG)12	4	146-171	171	45	0.5949
RM 5499	7	50.45	(TC)25	2	227	227	83	0.2392
RM 25	8	52.2	(GA)18	4	120-140	123	45	0.6189
RM 223	8	80.5	(CT)25	5	143-158	150	41	0.6647
RM 331	8	60.9	[(CTT)4GTT]2(CTT)11	4	145-175	175	58	0.5233
RM 6471	8	54.3	(GCC)9	4	74-81	81	50	0.5994
RM 215	9	99.4	(CT)16	5	149-154	153	33	0.7394
RM 242	9	73.3	(CT)26	5	194-223	207	25	0.7584
RM 316	9	1.8	(GT)8(TG)9(TTTG)4(TG)4	4	192-209	197	54	0.4770
RM 222	10	11.3	(CT)18	4	176-211	211	50	0.5593
RM 271	10	59.4	(GA)15	3	197-223	197	58	0.4200
RM 467	10	46.8	(TC)21	3	219-230	219	62	0.4683
RM 25425	10	-	(CT)33	4	111-121	111	45	0.5972
RM 144	11	123.2	(ATT)11	3	97-99	97	75	0.3706
RM 287	11	68.6	(GA)21	5	215-238	215	33	0.6443
RM 536	11	55.1	(CT)16	4	138-144	144	45	0.3856
RM 19	12	20.9	(ATC)10	5	112-156	117	50	0.7308
RM 20 A	12	0	(ATT)14	4	113-148	113	45	0.5836
RM 277	12	57.2	(GA)11	3	122-127	127	50	0.4768
RM 27400	12	-	(GA)11	4	234-245	240	45	0.6135

Three layers were created after centrifuging which were light coloured top layer containing DNA, middle layer containing cell digests and debris, greenish bottom layer containing chlorophyll. 500 µl liquid of upper interface was transferred to another microfuse tube by 1 ml pipette. The microfuse tubes were previously labeled. Care was taken so that the middle layer was not disturbed.900 µl ice-cooled (from -20°C) 100% ethanol was added to the 500 µl liquid. Care was taken so that DNA samples were not spilt from one tube to another while closing the cap of the microfuse tubes.

Table 4. Distribution of 24 rice genotypes into six clusters.

Cluster	Number of genotypes	Genotypes
I	9	Chinigura, JamaiAduri, Jhingasail, Khutichikon (2), Binnaphul, Khutichilon (3), Chinisagor, Basmati India and Kataribhog
II	1	Rasmala
III	7	Basmati 107, Basmati 134, Basmati Porder, Basmati 370, Basmati Naret, Basmati IRGC 27782, Basmati 1, Basmati 372
IV	1	SugandhiDhan (2)
V	1	Basmati T_3
VI	4	Kamarang, Sakkorkhora, Kalijira and Rong-er-gura

Table 5. Similarity matrix of rice genotypes analyzed using Nei's original measures of genetic identity.

Genotypes	1	10	11	12	13	14	15	16	17	18	19	2	20	21	22	23	24	3	4	5	6	7	8	9
1	0.000																							
10	0.700	0.000																						
11	0.750	0.475	0.000																					
12	0.725	0.525	0.425	0.000																				
13	0.800	0.500	0.425	0.475	0.000																			
14	0.575	0.575	0.825	0.650	0.700	0.000																		
15	0.600	0.550	0.775	0.750	0.600	0.325	0.000																	
16	0.475	0.575	0.650	0.650	0.550	0.400	0.425	0.000																
17	0.500	0.650	0.800	0.750	0.700	0.500	0.450	0.400	0.000															
18	0.750	0.625	0.700	0.725	0.475	0.650	0.650	0.700	0.700	0.000														
19	0.575	0.500	0.825	0.750	0.650	0.425	0.325	0.400	0.325	0.550	0.000													
2	0.600	0.675	0.750	0.800	0.800	0.525	0.575	0.525	0.575	0.725	0.550	0.000												
20	0.725	0.650	0.625	0.750	0.525	0.650	0.650	0.650	0.650	0.550	0.650	0.700	0.000											
21	0.500	0.700	0.750	0.750	0.700	0.525	0.525	0.325	0.400	0.650	0.425	0.525	0.750	0.000										
22	0.625	0.625	0.800	0.675	0.750	0.525	0.625	0.525	0.625	0.650	0.525	0.525	0.800	0.425	0.000									
23	0.700	0.600	0.675	0.525	0.700	0.625	0.650	0.800	0.700	0.700	0.700	0.700	0.625	0.700	0.675	0.000								

24	0.750	0.650	0.500	0.550	0.575	0.725	0.700	0.675	0.725	0.700	0.700	0.700	0.550	0.700	0.725	0.600	0.000						
3	0.550	0.600	0.700	0.775	0.700	0.475	0.375	0.450	0.450	0.600	0.400	0.350	0.750	0.450	0.550	0.800	0.750	0.000					
4	0.725	0.500	0.625	0.625	0.425	0.625	0.600	0.575	0.725	0.575	0.525	0.550	0.550	0.725	0.700	0.800	0.600	0.525	0.000				
5	0.850	0.425	0.300	0.475	0.500	0.675	0.750	0.625	0.825	0.700	0.750	0.600	0.725	0.700	0.600	0.600	0.550	0.675	0.475	0.000			
6	0.725	0.375	0.500	0.525	0.600	0.575	0.700	0.700	0.700	0.600	0.600	0.600	0.725	0.700	0.625	0.600	0.600	0.700	0.525	0.350	0.000		
7	0.625	0.425	0.425	0.450	0.650	0.500	0.675	0.600	0.650	0.725	0.700	0.650	0.750	0.675	0.650	0.600	0.650	0.650	0.625	0.375	0.300	0.000	
8	0.750	0.450	0.375	0.425	0.600	0.600	0.700	0.675	0.750	0.725	0.725	0.650	0.800	0.725	0.625	0.700	0.650	0.600	0.625	0.300	0.300	0.200	0.000
9	0.775	0.450	0.500	0.425	0.625	0.525	0.625	0.675	0.725	0.700	0.650	0.700	0.825	0.725	0.700	0.575	0.725	0.650	0.600	0.450	0.350	0.275	0.000

1 = Chinigura, 2 = Basmati India, 3 = Kataribhog, 4 = Sakkorkhora, 5 = Basmati Porder, 6 = Basmati 370, 7 = Basmati Naret, 8 = Basmati IRGC 27782, 9 = Basmati 1, 10 = Basmati 107, 11 = Basmati 134, 12 = Basmati 372, 13 = Kamarang, 14 = JamaiAduri, 15 = Jhingasail, 16 = Khutichikon (3), 17 = Khutichikon (2), 18 = Kalijira, 19 = Binnaphul, 20 = Rong-er-gura, 21 = Chinisagor, 22 = Rasmala, 23 = SugandhiDhan (2) and 24 = Basmati T3.

Ethanol was gently mixed with the liquid containing DNA.The tubes were kept in -20°C freezer for overnight to hasten the coagulation of DNA.The tubes were then centrifuged in the next day at 13,000 rpm for 10 minutes. DNA was precipitated at the bottom of the tubes. It was important to have formation of precipitates at the bottom rather than at the side of the tubes.The upper liquid was decanted by pouring out the liquid gently into a beaker. Care was taken so that the precipitate was not displaced or poured out into the beaker. The pellet and the surface of microfuse tubes were washed by 70% ethanol.

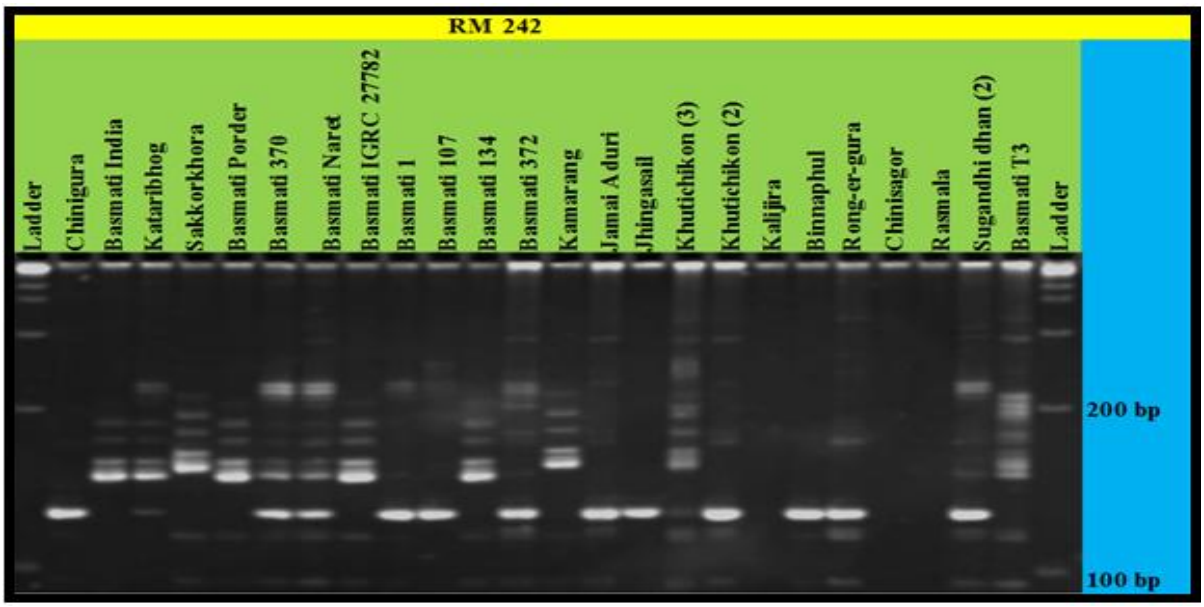

Fig. 1.DNA profile of the 24 rice germplasm with the SSR marker RM 242.

The tubes were then centrifuged at 13,000 rpm for 5 minutes. Again the upper liquid was decanted by pouring out the liquid gently into a beaker.Themicrofuse tubes were soak-dried by placing the tubes on the tissue paper upside down for a while.

The pellet was dried under fume hood for half an hour to allow alcohol to be evaporated.100 µl of 1X TE buffer was added into DNA pellet.

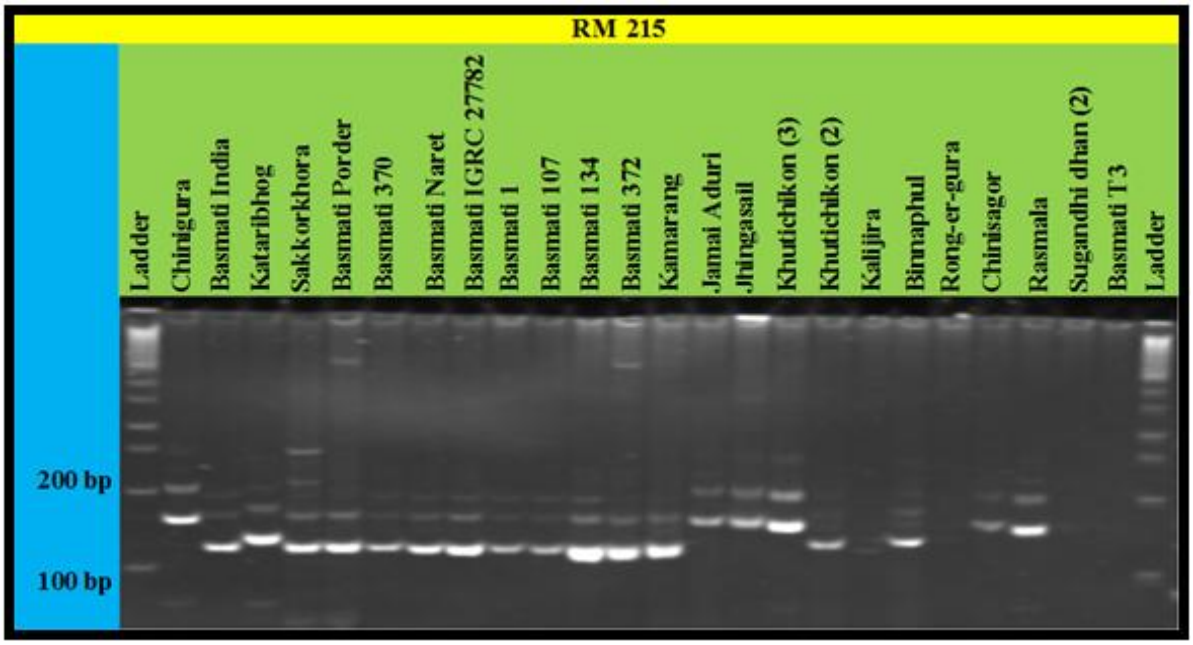

Fig. 2.DNA profile of the 24 rice germplasm with the SSR marker RM 215.

The tubes containing DNA were placed in a 4°C refrigerator overnight to complete chelating reaction of Na2EDTA. DNA was not used at once for PCR reaction. DNA remaining in the pellet mixes up automatically in TE buffer, that's why the pellets were not disturbed.

Polymerase chain reaction (PCR)

PCR was performed in 10 µl reactions containing around 25 ng of DNA template (3 µl DNA with 20X dilution factor), 1 µl 10X reaction buffer (containing 200 mM Tris-HCl pH 8.3, 500 mM KCl), 1.35 µl 25 mM $MgCl_2$, 0.2 µl of 10 mM dNTP, 0.5 µl each of 10 µM forward and reverse primers and 0.2 µl of Taq DNA polymerase (5 U/µl) using GStorm thermal cycler (Chen *et al.,* 1997; Neeraja *et al.,* 2007). Twelve-channel pipette was used for transferring DNA from dilution plate to PCR plate. Ten micro liter of mineral oil was added in each well to prevent evaporation and the PCR plate was wrapped with adhesive film.

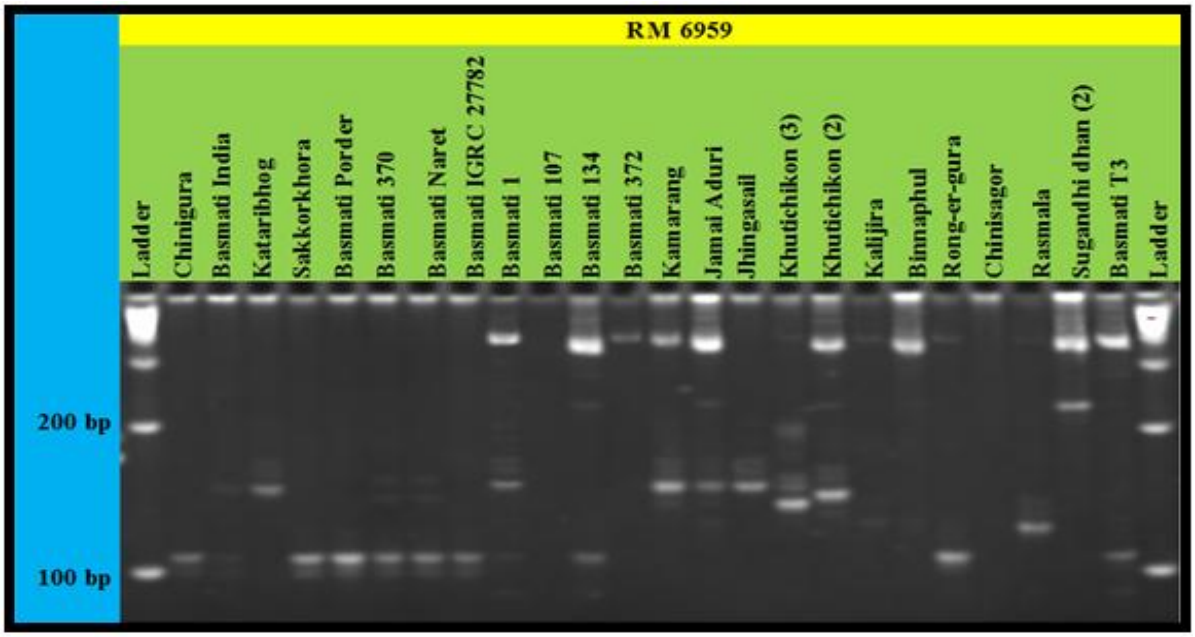

Fig. 3.DNA profile of the 24 rice germplasm with the SSR marker RM 6959.

After initial denaturation for 5 min at 94°C, each cycle comprises 45 sec denaturation at 94°C, 45 sec annealing at 55°C, and 1:30 min extension at 72°C with a final extension for 7 min at 72°C at the end of 35 cycles. The PCR product was preserved at 10°C temperature in the thermal cycler in case of necessity.

Polyacrylamide gel electrophoresis (PAGE)

Assembly of the glass plate

The glass plates were washed properly using laboratory detergent (bleaching powder based) and rinsed with water. Glass plates were also washed by 0.5 M NaOH solution. There should not have any stickiness of previously used gel on the surface of glass. Glass plates were air dried or lint-free tissue papers were used to dry up the plates quickly. The chosen inner surfaces of the plate was sprayed with 100% ethanol and wiped with lint-free tissue. The short plate (round-bottom) was hold and the rubber gasket was attached starting from one side of the plate. The notches on the gasket were aligned on the corners. The circular portion of the gasket was exposed to the inner side of the plate. The short plate was laid on the table with the inner side up. The spacers were put along the inside edges of the gasket. The other plate was put on top of the short plate. The clamps were set on both sides of the plates and the plate assembly was laid flat on the table. The surface of the table was even.

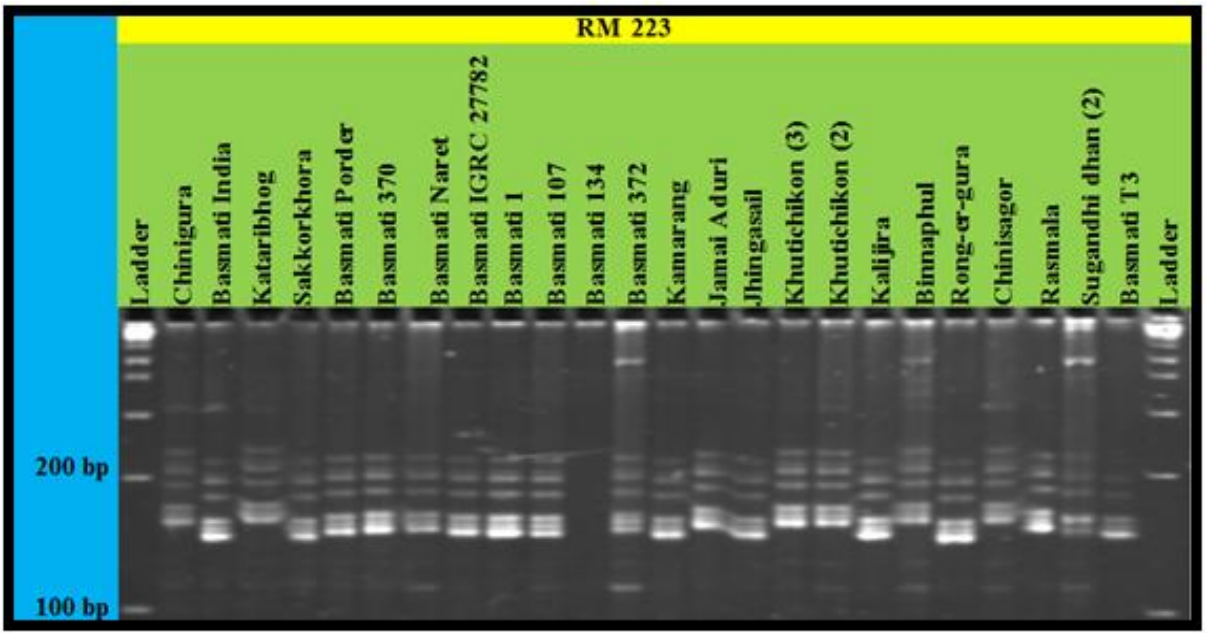

Fig. 4.DNA profile of the 24 rice germplasm with the SSR marker RM 223.

Preparation of gel

The gel solution was prepared in a beaker with a magnetic stirring bar. The reagents were added as given below in Table 2.The concentration of gels used for PAGE was 8%. After adding TEMED, the solution was stirred using magnetic stirrer for few seconds on a stirrer machine at a speed to mix the chemicals properly. The speed of stirring was maintained such so that mixing was achieved but no chemicals were spilt away. Immediately after stirring, the gel solution was poured into glass plate assembly. Gel solution was poured smoothly and continuously avoiding air bubbles starting from one corner until it reached top portion of the short plate.

The comb was inserted in the gel gently. The comb was inserted almost fully in case of comb with short teeth, whereas the comb with long teeth was inserted partially (half to three-fourth). The gel was allowed to polymerize for 20 minutes.

Setting glass plate in vertical rig

After the gel was polymerized, the gasket was removed starting from one corner of the plate assembly. Around 500 ml of 0.5X TBE buffer was added in the base of the tank. The plate assembly was attached with clamps in one side of the tank such that the short plate was facing the inner side.

The same was done on the other side. It was made sure that there were no air bubbles at the bottom to avoid short circuit. Around 300 ml of 0.5X TBE buffer was again added on top of the tank and the comb was removed gently. Twoμl of 10X loading dye was added to the each well containing 10 μl PCR product and the plates were centrifuged at a speed of 3600 rpm for 1 min in a high speed refrigerated centrifuge machine to mix the loading dye with PCR product. Around 2.5 μl of the mixer was loaded in the wells of PAGE gel with the help of 2-2.5 μl pipette. DNA size marker like 1 Kb^+ DNA ladder was loaded for size determination. The cover of the tank was put and the electrodes were connected to the power supply and the gel was run for about 2.0-2.5 hours at 100 volts. It was noted that running time depended on the size of PCR fragments.

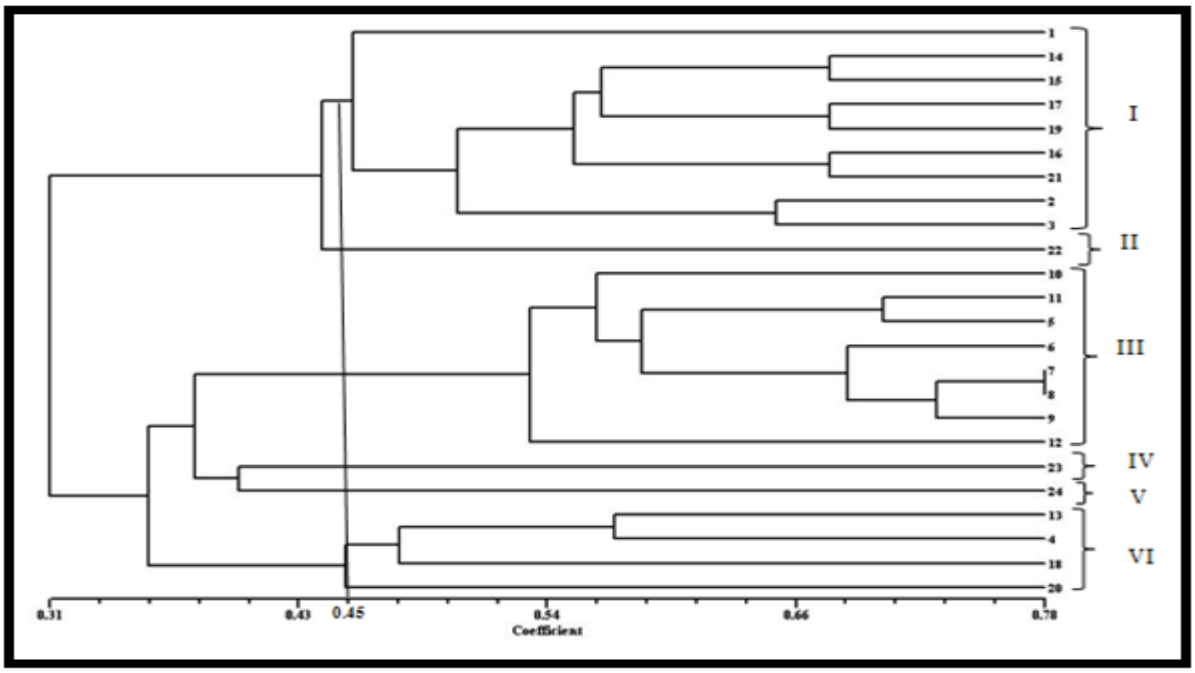

Fig. 5. UPGMA cluster dendrogram showing the genetic relationship among 24 rice genotypes based on the alleles detected by 40 microsatellite markers.

Legend:

1 = Chinigura, 2 = Basmati India, 3 = Kataribhog, 4 = Sakkorkhora, 5 = Basmati Porder, 6 = Basmati 370, 7 = Basmati Naret, 8 = Basmati IRGC 27782, 9 = Basmati 1, 10 = Basmati 107, 11 = Basmati 134, 12 = Basmati 372, 13 = Kamarang, 14 = JamaiAduri, 15 = Jhingasail, 16 = Khutichikon (3), 17 = Khutichikon (2), 18 = Kalijira, 19 = Binnaphul, 20 = Rong-er-gura, 21 = Chinisagor, 22 = Rasmala, 23 = SugandhiDhan (2) and 24 = Basmati T3.

Staining and visualization of the gel

The power supply unit was turned off and the plates were removed from the tank. The glass plates were separated using a knife. The acrylamide gel was removed carefully and transferred in the ethidium bromide staining solution (0.5 mg/ml) for around 25 minutes.

The stained gels were put in the exposure cabinet of the gel documentation system (Bio-Rad Company).

The gel was viewed in the computer monitor by exposing it first to white light.

The necessary adjustments were made by moving the gel inside the exposure box. A gel barrier was used to keep the gel straight. The gel image resolution might be adjusted using the camera setting. The gel was exposed to UV light and the gel image was saved as a Jpeg file.

Scoring and analysis of microsatellite data

Molecular weight for each amplified allele was measured in base pair using Alpha-Ease FC 4.0 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 NTSYS-pc version 2.2 (Rohlf, 2002).The summery statistics including the number of alleles per locus, major allele frequency, gene diversity, polymorphism information content (PIC) values were determined using Power Marker version 3.25 (Liu and Muse, 2005). A similarity matrix was calculated with the Simqualsub program using the DICE coefficient, followed by cluster analysis with the SAHN subprogram using the UPGMA clustering method as implemented in NTSYS-pc was used to construct a dendogram showing relationship among the genotypes. The similarity matrix was also used for principal coordinate analysis (PCoA) with the DCcnter, Eigen, Output and MXPlot subprograms in computer program Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc).

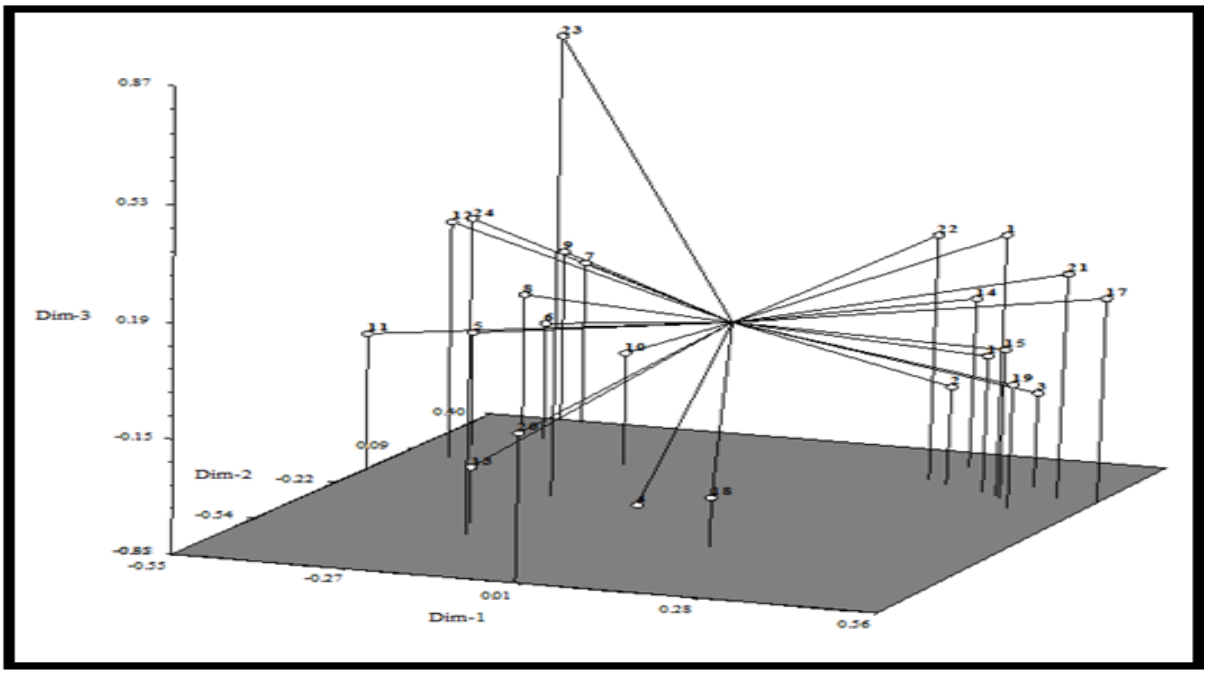

Fig. 6. Two-dimensional view of Principal Coordinate Analysis (PCoA) with 40 microsatellite markers over 24 rice genotypes.

Legend:

1 = Chinigura, 2 = Basmati India, 3 = Kataribhog, 4 = Sakkorkhora, 5 = Basmati Porder, 6 = Basmati 370, 7 = Basmati Naret, 8 = Basmati IRGC 27782, 9 = Basmati 1, 10 = Basmati 107, 11 = Basmati 134, 12 = Basmati 372, 13 = Kamarang, 14 = JamaiAduri, 15 = Jhingasail, 16 = Khutichikon (3), 17 = Khutichikon (2), 18 = Kalijira, 19 = Binnaphul, 20 = Rong-er-gura, 21 = Chinisagor, 22 = Rasmala, 23 = Sugandhi Dhan (2) and 24 = Basmati T3.

Results and discussion

Overall microsatellite diversity

Out of 60 primers forty primers were polymorphic, ten were monomorphic and ten were not amplified. Using 40 primers across 24 rice genotypes, a total of 159 alleles were found in the present study of which RM 6959 showed the highest number of alleles (8) and RM 5499 showed the lowest number of alleles (2), with an average of 3.98 alleles across the 40 loci (Table 3).

The frequency of the most common allele at each locus ranged from 33% (RM 19, RM 215, RM 452 and RM 1155) to 92% (RM 10), which was comparable with that of Thomson *et al.* (2007). On an average, 41% of the 24 aromatic and non-aromatic rice genotypes shared a common major allele at any given locus. This result is comparable with Hossain *et al.*, 2007; Pervaiz *et al.*, 2010; Hossain *et al.*, 2012;Sajib*et al.*, 2012; Meti*et al.*, 2013; Siddique *et al.*, 2014.

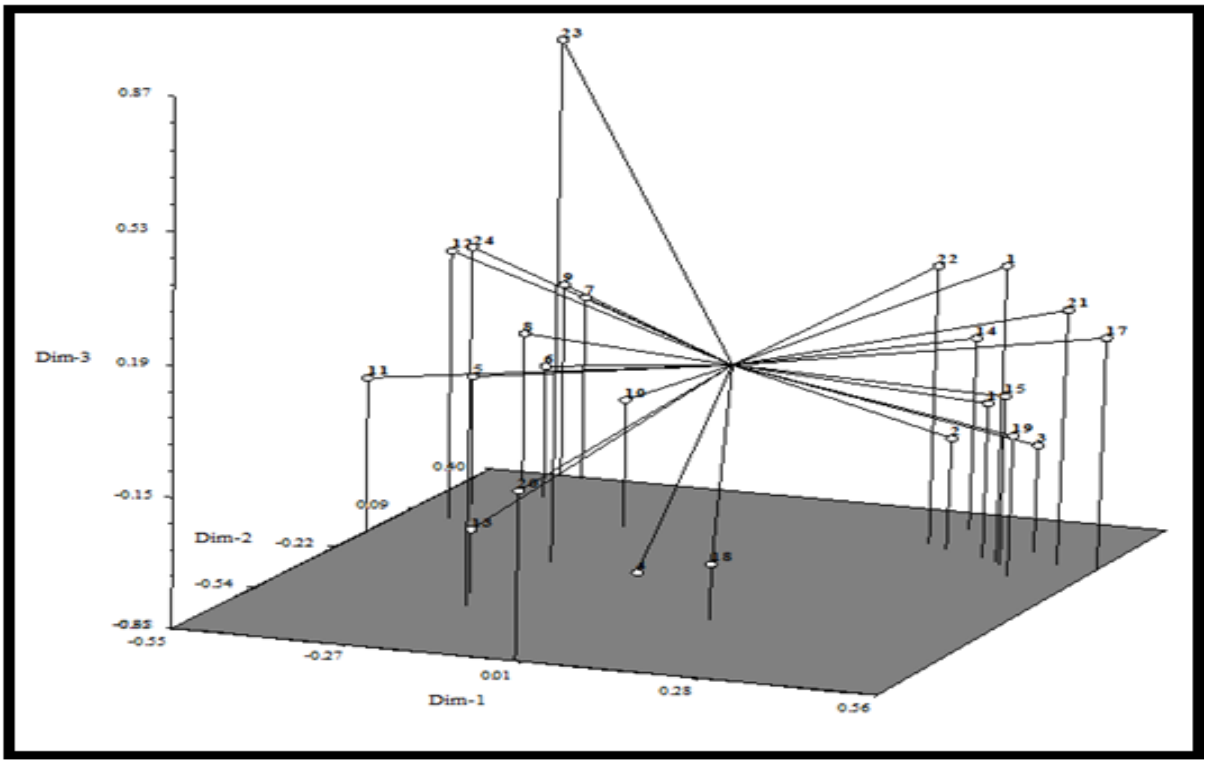

Fig. 7. Three-dimensional view of Principal Coordinate Analysis (PCoA) with 40 microsatellite markers over 24 rice genotypes.

Legend:

1 = Chinigura, 2 = Basmati India, 3 = Kataribhog, 4 = Sakkorkhora, 5 = Basmati Porder, 6 = Basmati 370, 7 = Basmati Naret, 8 = Basmati IRGC 27782, 9 = Basmati 1, 10 = Basmati 107, 11 = Basmati 134, 12 = Basmati 372, 13 = Kamarang, 14 = JamaiAduri, 15 = Jhingasail, 16 = Khutichikon (3), 17 = Khutichikon (2), 18 = Kalijira, 19 = Binnaphul, 20 = Rong-er-gura, 21 = Chinisagor, 22 = Rasmala, 23 = Sugandhi Dhan (2) and 24 = Basmati T3.

The range of polymorphic information content (PIC) values was from 0.1504 to 0.7584 with an average 0.532. Lower PIC value indicates that the genotypes under study are of closely related types, while the higher value of the PIC indicates higher diversity of the materials which is better for development of newer varieties. The PIC values observed in this study, were comparable to previous estimates of microsattelite analysis in rice viz. 0.67-0.88 (Gohain *et al*., 2006) and 0.34-0.88 (Thomson *et al*., 2007), 0.27-0.56 (Roy *et al*., 2015), 0.0-0.83 (Shankar *et al*., 2015) and 0.65-0.91 (Siddique *et al*., 2014).Rahman *et al*. (2006, 2008) working on rice varieties of different ecotypes of Bangladesh from the preserved materials of the BRRI have reported 18 and 78 alleles, respectively while analyzing with 3 primers (RM 11, RM 151 and RM 153), 5 primers (RM 1, RM 151, RM 153, RM 334 and RM 335), respectively.

In those studies PIC values were 0.670, 0.707, 0.698 and 0.862, 0.923, 0.831, 0.865 and 0.910, respectively. The highest PIC value (0.7548) was obtained for RM 242 followed by for RM 215 and RM 6959 (0.7394) (Table 3). PIC value revealed that RM 242 was as the best marker for 24 genotypes. Fig. 1-4showed gel pictures of amplified fragment using SSR primers RM 242, RM 215, RM 6959 and RM 223 for 24 genotypes.

Genetic distance-based analysis

Cluster analysis based on UPGMA (Unweighted Pair-Group Method for Arithmetic Average) with DICE genetic distance, divided twenty four rice genotypes into two major groups (Fig. 5) cluster I and cluster II. Cluster I was divided into two clusters, i.e. cluster IA and cluster IB. Cluster IA had nine genotypes *viz*. Chinigura, JamaiAduri, Jhingasail, Khutichikon (2)

andBinnaphul, Khutichikon (3), Chinisagor, Basmati India and Kataribhog. Cluster IB had only one genotype viz. Rasmala. Cluster II was divided into two clusters, i.e. cluster IIA and cluster IIB. Cluster IIA had ten genotypes viz. Basmati 107, Basmati 134, Basmati Porder, Basmati 370, Basmati Naret, Basmati IRGC 27782, Basmati 1, Basmati 372, Basmati T3 and Sugandhi Dhan (2). Cluster IIB had four genotypes *viz.*Kamarang, Sakkorkhora, Kalijira and Rong-er-gura.

Moreover, when similarity coefficient was considered as 45%, twenty four rice genotypes were classified into six clusters (Table 4). Cluster I had nine genotypes, cluster II had only one genotype, cluster III had seven genotypes, cluster IV and cluster V both had one genotype and cluster VI had four genotypes.
It was also obtained from Cluster analysis based on UPGMA that the pair of genotypes (Basmati Naret and Basmati IRGC 27782) was exactly same (100% similarity). The result indicated that the genotypes *viz.* Basmati Naret, Basmati IRGC 27782 might be same genetic background which could be verified using more markers.

The two dimensional graphical view of Principal Coordinate Analysis (PCoA) showed the spatial distribution of the genotypes along the two principal axes. The genotypes *viz.* Basmati Naret, Basmati 1, Basmati 134, Khutuchikon (2) and Rong-er-gura were found far away from centroid of the cluster and rest of the genotypes were placed more or less around the centroid (Fig. 6). The result indicated that the genotypes were placed far away from the centroid were more genetically diverse while the genotypes were placed near around the centroid possessed more or less similar background. However, centroid may be defined as the vector representing the middle point of the cluster which contained at least one number for each variable. The connecting line between the each genotype and the centroid represented eigen vectors for the respective genotypes.

The three dimensional (3D) graphical view of Principal Coordinate Analysis (PCoA) showed the spatial distribution of the 24 genotypes along the three principal axes.

Principal Coordinate Analysis also conformed to the results from UPGMA cluster analysis. The 3D diagram (Fig. 7) helped to visualize six major clusters where Sugandhi Dhan (2) was far away from the other genotypes. The genotypes Basmati Naret with Basmati 1 and Kataribhog with Binnaphul were closely associated that creates very little or no usefulness in crossing among these four genotypes for generating transgressive segregants through hybridization.

The pair-wise genetic dissimilarity coefficients indicated that the highest genetic distance was recognized between the genotypes Chinigura and Basmati Porder (0.85) followed by 0.83 for the genotypes JamaiAduri and Basmati 134, Binnaphul and Basmati 134, 0.80 for Kamarang and Chinigura, Khutichikon (2) and Basmati 134, Rasmala and Basmati 134, Basmati India and Basmati 372, Kamarang and Basmati India, Khutichikon (3) and SugandhiDhan (2), Rasmala and Rong-er-gura, Basmati IRGC 27782 and Rong-er-gura, Sugandhi Dhan (2) and Kataribhog and Sugandhi Dhan (2) and Sakkorkhora (Table 5). These results were in agreement with the findings of Principal Coordinate Analysis and suggested that these genotypes are diverged. In crop improvement program these genetically diverse genotypes could be chosen as parents for crossing program to create genetic variability and transgressive segregants. On the other hand, lowest genetic distance was obtained between the genotypes Basmati Naret and Basmati IRGC 27782 (0.23) followed by 0.28 for the genotypes Basmati Naret and Basmati 1 and Basmati IRGC 27782 and Basmati India and 0.30 for Basmati Porder and Basmati 134, Basmati Porder and Basmati 370, Basmati Naret and Basmati 370 and Basmati IRGC 27782 and Basmati 370.

Conclusion

In crop improvement program, crossing should not be made between pairs of genotypes having less genetic distance. The less genetic distance between pairs of genotypes may be obtained due to same genetic background.

Hence, microsatellite marker based molecular fingerprinting could serve as a potential basis in the identification of genetically distant accessions as well as in the duplicate sorting of the morphologically close accessions.

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