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Full Length Research Paper

Genetic diversity analysis of stress tolerant rice (*Oryza sativa* L.)

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Fourteen rice genotypes, composed of six salt tolerant, three submergence tolerant, two drought tolerant genotypes along with three high yielding genotypes, released from Bangladesh Rice Research Institute (BRRI) were used for genetic diversity analysis using 40 simple sequence repeats (SSR) markers. All of the used SSR markers were found polymorphic among the 14 rice genotypes. The amplicon size ranged from 75 bp (RM436) to 330 bp (RM26360). A total of 168 alleles were detected, the number of alleles per locus ranged from 2 (RM252, S03120) to 6 (RM570, S12055, S11033) with an average of 4.2 alleles per locus. Polymorphic information content (PIC) value varied from 0.21 (RM252) to 0.76 (S07024) with an average of 0.57. From genetic distance co-efficient, the highest and lowest genetic distant varieties were found for BRRI dhan28 vs. BRRI dhan43 (0.82%) and BRRI dhan40 vs. BRRI dhan44 (0.37%) respectively. Unweighted pair group method with arithmetic mean (UPGMA)-cluster analysis divided the rice genotypes into four distinct clusters. The information obtained from this study would be useful for planning the breeding program to develop stress tolerant rice variety with high yielding ability and fine grain quality.

Key words: Genetic diversity, simple sequence repeats (SSR) markers, stress tolerant, rice.

INTRODUCTION

Rice, *Oryza sativa* L. (2n = 24) is the staple food for one third of the world's population, growing in over 1.5 billion ha of land (Chakravarthi and Naravaneni, 2006; Jena and Mackill, 2008). In Bangladesh, rice is the first and staple food; accounts for 94% of food grain production. However, rice production of Bangladesh is affected by different abiotic stresses (for example, salinity, submergence, drought etc.) which in combined contribute half of the total loss by both biotic and abiotic stresses (BARC, 2009; Alam et al., 1998). At the same time, population of Bangladesh is increasing steadily with a growth rate of 1.36% that reduces the agricultural land gradually, which ultimately affect the food security in Bangladesh (BARC, 2009). To meet the increasing demand of rice, there is an urgent need to develop rice varieties for saline, submergence and drought prone areas with higher yield potential and with greater yield stability.

Genetic diversity is a pre-requisite for any crop improvement program as it helps in the development of superior recombinants. It is a source of variation, which is a raw material for any improvement work. Accurate assessment of the levels and patterns of genetic diversity can be invaluable in crop breeding. Genetic diversity analysis is used for estimating and establishing of genetic relationship in germplasm collection, identifying diverse parental combinations to create segregating progenies with maximum genetic variability for further selection and introgressing desirable genes from diverse germplasm

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Table 1. Plant materials used in SSR marker analysis.

Variety	Source/origin	Release	Parentage	Feature
BR 23	BRRI	1988	DA29/BR4	MST
BRRI dhan28	BRRI	1994	BR6/ Purbachi	Long slender grain
BRRI dhan40	BRRI	2003	IR4595-4-1-15/BR10	MST
BRRI dhan41	BRRI	2004	BR23/BR1185-2-B-16-1	MST
BRRI dhan42	BRRI	2004	BR14/IR25588-73-1	MUDT
BRRI dhan43	BRRI	2005	BR24/BR21	MUDT
BRRI dhan44	BRRI	2007	BR10/BRRI dhan31	MTST
BRRI dhan47	BRRI	2008	IR51511-B-B-34-B/TCCP266-2-49-B-B-3	HST
BRRI dhan49	BRRI	2008	BR4962-12-4-1/IR33380-7-2-1-3	Long slender grain
BRRI dhan50	BRRI	2010	BR30/IR67684B	Basmati type grain
BRRI dhan51	BRRI	2010	Sarna/IR49830-7-1-2-3	FFST
BRRI dhan52	BRRI	2010	BR11/IR40931-33-1-3-2	FFST
BRRI dhan53	BRRI	2010	BR10//BR23/BR847-76-1-1	MST
BRRI dhan54	BRRI	2010	BR1185-2B-16-1/BR548-128-1-1-3	MST

MST= Moderate salt tolerant; HST= highly salt tolerant; MUDT = moderate upland drought tolerant; MTST= moderate tidal submergence tolerant; FFST= flush flood submergence tolerant.

into the available genetic base (Smith, 1984; Cox et al., 1986; Barrett and Kidwell, 1998; Thompson et al., 1998; Islam et al., 2012). Genetic diversity analysis guides breeders for rapid progress of breeding program Molecular markers are promising and effective tools for measuring genetic diversity in germplasm collection and elucidating their evolutionary relationships. Several molecular markers for example, restriction fragment length polymorphisms (RFLP), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR) and (single-nucleotide polymorphisms) SNPs are now available to assess the variability and diversity at molecular level. Due to co-dominance, abundance, highly reproducibility and polymorphism, SSRs are an excellent molecular marker for various genetic analyses, including genetic mapping, germplasm surveys, and determination of the genetic structure and diversity patterns and for marker-assisted breeding (Panaud et al., 1996; Temnykh et al., 2000; Garris et al., 2005; Islam et al., 2008, 2011, 2012; Yasmin et al., 2012). SSR markers are efficient in detecting genetic polymorphisms and discriminating among genotypes from germplasms of various sources, even they can detect finer level of variation among closely related breeding lines within a same variety (Lapitan et al., 2007). For rice, 18,828 SSR markers throughout the whole rice genome are now available (IRGSP, 2005).

In this study, we estimate the genetic diversity within 14 modern rice genotypes including 11 stress tolerant and three high quality grain varieties, and determine the genetic relationship among these genotypes using SSR markers. This analysis will help to understand genetic relationship among different groups of stress tolerant genotypes and between stress tolerant and high quality grain genotypes and this information will help to formulate future breeding strategies for developing new multiple stress tolerant and quality rice varieties.

MATERIALS AND METHODS

Plant material and rice deoxyribonucleic acid (DNA) extraction

In this study, 14 rice genotypes comprised of 11 stress tolerant; including six salt tolerant, three submergence tolerant and two drought tolerant genotypes along with three different high quality grain varieties that are stress sensitive were studied (Table 1). The seeds of 14 rice varieties were obtained from plant breeding division of Bangladesh Rice Research Institute (BRRI), Gazipur. Total genomic DNA was isolated using the Miniscale method as described by Zheng et al. (1995) and Islam et al. (2012) from 15-day-old seedling leaves. After DNA extraction, DNA was stored at –20°C for further use.

Simple sequence repeat (SSR) assay

40 simple sequence repeat (SSR) markers distributed on all the 12 chromosomes of rice were used for genetic diversity analysis. These SSR markers were chosen based on their physical position on the 12 chromosomes of rice genome according to the 'Gramene' database (http//:www.gramene.org).

PCR was performed in 96-well plates containing a total 10 µL volume; 2 µL of DNA template, 1.5 µL 10X PCR buffer (containing 200 mM Tris-HCl pH 8.3, 500 m M KCl, 15 mM $MgCl_2$), 1 µL of 1 mM dNTPs, 0.5 µL of 5 µM forward and reverse primers, 0.3 µL of commercial Taq polymerase enzyme and 4.2 µL nano-pure water. PCR was run in G-Storm thermal cycler and with the following cycle profile; initial denaturation for 5 min at 94°C and then 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C and 7 min at 72°C for the final product extension.

PCR amplified products were separated by 8% polyacrylamide

gel electrophoresis at 100 V for 2 to 2.5 h in 0.5X TBE buffer. Gels were stained in ethidium bromide solution and visualized under UV light using the gel documentation system (Alpha Imager, USA).

Allele scoring and data analysis

The size (in nucleotide base pair) of the amplified band for each SSR marker was determined based on its migration with comparison to a known molecular weight marker (1Kb DNA Ladder) with the help of Alpha Ease FC 5.0 software. The program power marker version 3.25 (Liu and Muse, 2005) was used for analyzing SSR markers alleles including number of alleles per locus, major allele frequency, gene diversity, polymorphism information content (PIC) values, etc. Polymorphic information content (PIC) values were calculated with the following formula (Anderson et al., 1993):

$$PIC_i = 1 - \sum_{j=1}^{n} P^2_{ij}$$

Where, n is the number of marker alleles for marker *i* and P_{ij} is the frequency of the *j* th allele for marker *i.*

The CS Chord (1967) distant coefficient and a dendrogram representing the genetic relationships between genotypes, based on the un-weighted pair-group method with arithmetic averages (UPGMA) were constructed using the same program.

RESULTS

Overall SSR diversity

Every SSR marker used in this study generated polymorphic band among 14 rice genotypes. The largest band size was produced by RM26360 (330 bp) and the smallest by RM436 (75 bp). The total 168 alleles were detected and ranged from 2 (RM252, S03120) to 6 (RM570, S12055, S11033) with an average of 4.2 alleles per locus among 14 rice genotypes. Polymorphism information content (PIC) value ranged from 0.21 to 0.76, with an average of 0.57. The highest PIC value (0.76) was found for the marker S07024 followed by RM570 (0.74), S11033, S02026 (0.73), RM471, RM400 (0.72), and the lowest for RM252 (0.21).

The gene diversity value ranged from 0.2449 to 0.7901 with an average value of 0.6231. S07024 was responsible for the highest level of gene diversity (0.7901) and the lowest level of gene diversity (0.2449) was found for RM252 (Table 2). Examples of SSR alleles as resolved with PCR assay for RM71 and RM471 are depicted in Figure 2.

Genetic distance-based

The highest genetic distance (82.73%) was found between BRRI dhan28 and BRRI dhan43. BRRI dhan28 had more than 70% genetic distance coefficient with BRRI dhan47, BRRI dhan49, BRRI dhan51, BRRI dhan52 and BRRI dhan53. BR23 was also more than 70% genetic distance coefficient with BRRI dhan42, BRRI dhan47, BRRI dhan50, BRRI dhan51, BRRI dhan52 and BRRI dhan53. BRRI dhan43 and BRRI dhan54, BRRI dhan43 and BRRI dhan49 and BRRI dhan44 and BRRI dhan53 were found distant by high coefficient values of 0.74, 0.73 and 0.71, respectively. BRRI dhan40 vs. BRRI dhan44 had the lowest genetic distance (37%) (Table 3).

Unweighted pair group method with arithmetic mean (UPGMA) dendrogram

14 genotypes were differentiated into four distinct clusters (Cluster-1, 2, 3 and 4). Cluster-1 consisted of two genotypes, BRRI dhan23 and BRRI dhan28. Cluster-2 was composed of only one genotype, BRRI dhan43. Cluster-3 was divided into two sub-cluster, sub-cluster-3A and sub-cluster-3B. Two salt tolerant genotypes, BRRI dhan47 and BRRI dhan53 were grouped in sub-cluster-3A. Sub-cluster-3B consisted of two submergence tolerant genotypes (BRRI dhan51 and BRRI dhan52) and two high yielding genotypes (BRRI dhan49 and BRRI dhan50). Cluster-4 has also two sub-clusters, sub-cluster-4A and sub-cluster-4B. BRRI dhan42 alone made sub-cluster-4A. Sub-cluster-4B clustered three salt tolerant genotypes (BRRI dhan40, BRRI dhan41 and BRRI dhan54) along with one submergence tolerant genotype (BRRI dhan44) (Figure 1).

DISCUSSION

A set of 40 SSR markers covering all the 12 chromosomes of rice were used for this study based on published rice microsatellite map. Chromosomal position, repeat motif and primer sequences were found from rice genome database (http:// www.gramene.org).

In this study, 14 rice genotypes were fingerprinted with 40 SSR markers and all markers were polymorphic. The total 168 alleles were detected. The number of alleles ranged from 2 to 6 per locus, with an average of 4.2 alleles per locus. The average number of allele of this study was comparable to the result of Pervaiz et al. (2009) using different Asian rice germplasm and observed an average of 4.5 alleles per locus (range of 2 to 13). Similarly, the average number of alleles per locus of this study was lower than that reported earlier by Islam et al. (2008), who reported an average of 5 alleles per locus using different rice germplasm of Bangladesh. These inconsistencies might be due to the genotypes used and selection of SSR markers. Markers that have the ability to detect high number of discernable alleles are the suitable marker for molecular characterization and genetic diversity analysis (Islam et al., 2008). In this

Table 2. Summary results of 40 SSR markers across 14 genotypes.

Marker	Chromosome number	Position (cM)	Amplicon size (bp)	Allele number	Gene diversity	PIC value
RM71	2	49.8	125-148	5	0.6735	0.6319
RM122	5	3	244-261	4	0.6942	0.6368
RM125	7	41.7	86-115	5	0.6667	0.6221
RM222	10	5.5	217-230	5	0.6735	0.6319
RM252	4	99	215-219	2	0.2449	0.2149
RM279	2	13.4	130-178	4	0.6122	0.5407
RM322	2	33.6-36.3	104-109	3	0.5417	0.4598
RM324	2	51.1	221-239	4	0.7347	0.6847
RM327	2	51.9	205-217	4	0.5816	0.5199
RM400	6	110.6	253-301	5	0.7551	0.7161
RM436	4	11	75-78	4	0.6122	0.5407
RM471	2	44.0-48.3	44.0-48.3	5	0.7551	0.7161
RM555	4	19.0-20.9	167-175	4	0.6942	0.6368
RM559	3	129.6	167-182	3	0.4388	0.3862
RM570	5	158.2	179-211	6	0.7755	0.7406
RM1024	1	14.1	288-300	4	0.5306	0.4832
RM1287	1	58.1	147-171	3	0.6020	0.5229
RM6208	8	42.9	132-146	5	0.7245	0.6853
RM10793	1	66.5	137-193	5	0.5799	0.5475
RM10927	1	73.1-73.4	145-152	4	0.4592	0.4275
RM18459	5	60.7	240-255	5	0.7041	0.6574
RM18877	5	98.3-101.0	167-192	3	0.5612	0.4650
RM23668	9	0.0-0.5	102-113	4	0.6122	0.5407
RM24712	9	83.2	185-198	3	0.6531	0.5798
RM26360	11	35.6-45.3	311-330	4	0.7347	0.6847
S01038	1	38.8	239-247	3	0.6122	0.5298
S 01160	1	160.4	173-197	5	0.6735	0.6319
S02026	2	26.9	185-194	5	0.7653	0.7261
S02054	2	54.6	158-172	4	0.6939	0.6414
S03120	3	120.4	248-251	2	0.4082	0.3249
S04065	4	65	236-241	3	0.5694	0.4768
S05009	5	9	184-221	5	0.6746	0.6231
S07011	7	11	202-214	5	0.7347	0.6894
S07024	7	24.8	226-242	5	0.7901	0.7560
S08055	8	55.4	196-219	5	0.6735	0.6169
S09073	9	73.6	244-251	5	0.6224	0.5874
S11033	11	33.4-34.8	184-220	6	0.7692	0.7346
S12055	12	55.9	153-176	6	0.7101	0.6743
AP3206(F)	−	−	140-170	3	0.2551	0.2401
Pec4	−	−	193-213	3	0.3571	0.3254
Mean				4.2	0.6231	0.5713

"−"= Not found.

study, the highest number of alleles (6) were found for RM570, S12055, S11033 followed by RM71, RM125, RM222, RM400, RM471, RM6208, RM110793, RM18459, S01160, S02026, S05009, S07011, S07024, S08055 and S09077 (5). This result suggests that these markers could be used as effective tools to assess the genetic diversity of rice germplasm of various sources. Only two markers; RM252 and S03120 produced lowest number of alleles (2).

Polymorphic information content (PIC) value ranged from 0.21 to 0.76 with an average of 0.57. The PIC values observed in this analysis were consisted with the previous report involved in microsatellite marker analysis in rice: Islam et al. (2008) reported a PIC value of 0.55

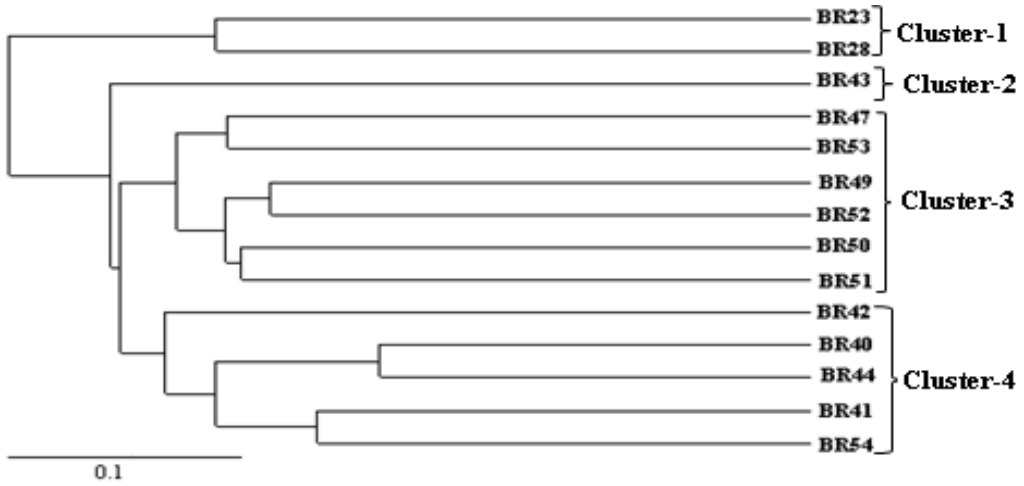

Figure 1. Cluster analysis of the 14 rice genotypes using distant coefficient.

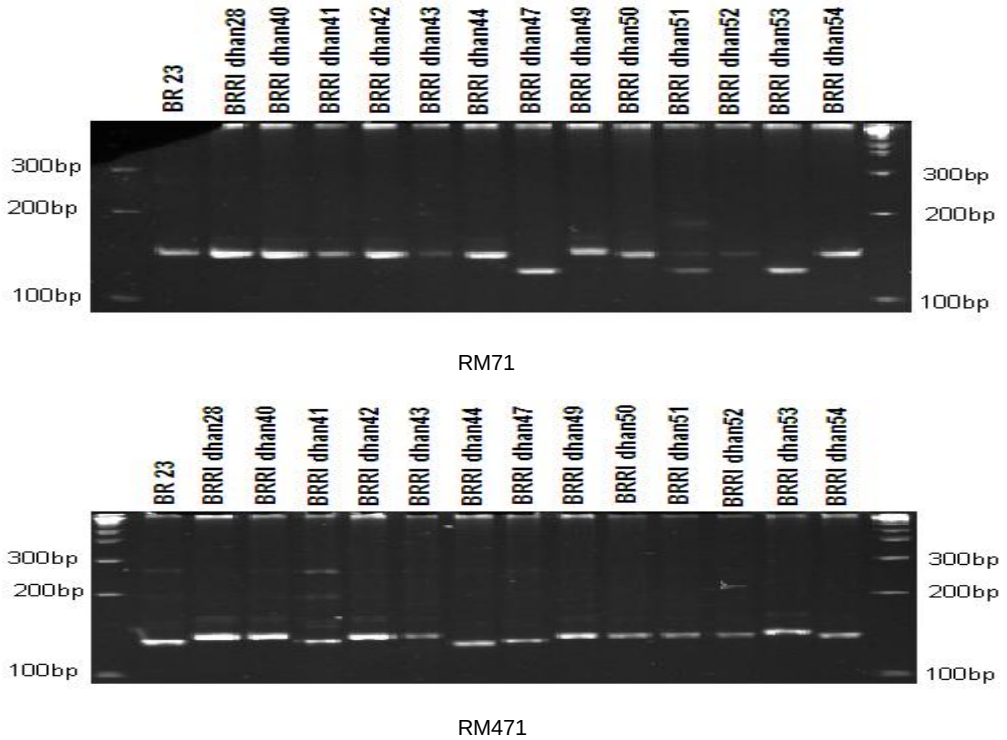

Figure 2. Banding pattern of the 14 rice genotypes generated by primer RM71and RM471.

using 21 rice genotypes, Joshi and Behrea (2006) reported 0.54 using 38 rice genotypes, and Pervaiz et al. (2009) reported 0.60 using 35 rice genotypes. PIC value of this study was higher than the average PIC value of 0.33 reported by Singh et al. (2004) using aromatic rice germplasm but lower than that earlier reported by Xu et al. (2004). Brondani et al. (2006) and Giarrocco et al. (2005) observed an average PIC value of 0.66, 0.73 and 0.69 for the world collection, traditional Brazilian rice varieties and Argentine rice germplasm, respectively. Findings of these high PIC value might be due to inclusion of more diverse set of rice germplasm. The PIC value of a marker reflects marker allele diversity and frequency among the cultivars. Higher the PIC value of a marker indicates higher probability of detecting the number of allele among cultivars. S07024 had the highest

Table 3. Genetic distance indices between pairs of rice genotypes.

Genotype	BR23	BR28	BR40	BR41	BR42	BR43	BR44	BR47	BR49	BR50	BR51	BR52	BR53
BR28	0.5110												
BR40	0.6695	0.6327											
BR41	0.4380	0.6252	0.4867										
BR42	0.7345	0.6327	0.4739	0.5752									
BR43	0.7057	0.8273	0.5840	0.6252	0.5752								
BR44	0.6464	0.6160	0.3694	0.4867	0.5540	0.5110							
BR47	0.7387	0.7787	0.6695	0.6327	0.5686	0.5353	0.5771						
BR49	0.6871	0.7057	0.6160	0.6327	0.5686	0.7253	0.5310	0.4975					
BR50	0.7002	0.6688	0.6252	0.5561	0.6502	0.5826	0.5110	0.5502	0.5002				
BR51	0.7002	0.7717	0.6752	0.5916	0.6945	0.5402	0.6752	0.5002	0.5402	0.4887			
BR52	0.7878	0.7596	0.5064	0.6389	0.6680	0.5064	0.5064	0.5908	0.4647	0.4937	0.4783		
BR53	0.7753	0.7002	0.6252	0.6431	0.5752	0.6945	0.7057	0.5002	0.5002	0.6174	0.4887	0.6099	
BR54	0.6431	0.5659	0.5916	0.4237	0.6174	0.7414	0.4752	0.6174	0.5145	0.4237	0.4766	0.5808	0.5502

PIC value (0.76), followed by RM570 (0.74), S11033, S02026 (0.73), RM471, RM400 (0.72), S07011, RM6208 (0.69), RM324, RM26360 (0.68), S12055 (0.67), and RM18459 (0.66). Therefore, SSR marker, S07024 was found to be superior for the analysis of genetic diversity in future. This result describes the usefulness of these markers for characterizing rice genotypes.

Genetic distance co-efficient, indicating genetic relationship among the studied germplasm is an efficient tool for breeder to plan a recombination strategy in breeding program for developing potential recombinant. Genetic distance between genotypes ranged from 37 to 83%, which indicated high genetic divergence among the genotypes. The highest genetic distance was found between BRRI dhan28 and BRRI dhan43 (83%). This result is expected because these two varieties originated from different germplasms; BRRI dhan28 is a high yielding genotype for irrigated ecosystem and BRRI dhan43 is a drought tolerant genotypes suitable for upland ecosystem. BRRI dhan40 (salt tolerant genotype) and BRRI dhan44 (tidal submergence tolerant genotype) had the lowest genetic distance (37%). The result is obvious because they have common ancestor 'BR 10' as one of the parents and both of them are suitable for tidal coastal region. From Table 3, it was observed that 60% or higher genetic distance existed between stress tolerant genotypes viz. BRRI dhan40, 41, 42,43, 47, 51, 52, 53 and 54 (for example, salt, submergence and drought tolerant) and high yielding genotypes (BRRI dhan28, 49 and 50). Submergence tolerant genotypes (BRRI dhan51 and 52) have same genetic distance range only with BRRI dhan28. This observation could be utilized for getting stress tolerant rice genotypes with high yielding and fine grain quality through crossing among them. Besides, salt tolerant genotypes, such as BR 23, BRRI dhan40, BRRI dhan41 and BRRI dhan54-all were moderate salt tolerant genotypes and had genetic distance range from 60 to 74% with BRRI dhan47 as well as with submergence and drought tolerant genotypes. This result support the findings of Islam et al. (2008) that high salt tolerant genotypes could be crossed with salt sensitive as well as low level salt tolerant genotypes for enhancing salt tolerant level and for broadening the genetic base for wider adaptability. According to this finding, BRRI dhan47 along with moderate salt tolerant genotypes could be utilized in breeding program for enhancing salt tolerant level. BRRI dhan47 also could be used for crossing submergence tolerant genotypes for developing new genotypes with both submergence and salt tolerant ability.

Cluster analysis presents an overview about genetic diversity and similarity among the studied germplasm. In this analysis, 14 rice genotypes were divided into four distinct clusters (Cluster-1, 2, 3 and 4). Cluster-1 contained one salt tolerant genotype (BR 23) and one high yielding genotype (BRRI dhan28). This alignment indicates that they are genetically different from other genotypes. This may be due to the presence of Bangladeshi local variety (DA 29) and exotic variety (Purbachi, Chinese origin) in the genetic background of BR 23 and BRRI dhan28, respectively. BRRI dhan43, drought tolerant genotype, alone consisted Cluster-2. This is because BRRI dhan43 was derived from a cross, BR 24× BR 21 and these varieties were developed from a common exotic genotype, named C 22 (Philippine upland variety). Therefore, genetic background of this genotype is different from other genotypes. Cluster-3 had two sub-clusters; sub-cluster-3A and sub-cluster-3B. Two salt tolerant genotypes were grouped in sub-cluster-3A and sub-cluster-3B grouped two submergence tolerant genotypes (BRRI dhan51 and BRRI dhan52) and two high yielding genotypes (BRRI dhan49 and BRRI dhan50), although they were different in nature. This may be due to existence of common ancestor in their ancestry. Cluster-4 had also two sub-clusters; sub-cluster-4A and sub-cluster-4B. Sub-cluster-4A composed

of only BRRI dhan42 (drought tolerant genotype). Sub-cluster-4B had two more sub-sub-cluster, each of which has two genotypes. Two salt tolerant genotypes (BRRI dhan41 and BRRI dhan54) were grouped in one sub-sub-cluster due to sharing a common parent 'BR 1185-2B-16-1' in their pedigree and other sub-sub-cluster grouped two genotypes; BRRI dhan40 and 44 were suitable for coastal tidal submergence. They had similarity with respect to genetic base because they were derivative from a common parent (BR10). The dendrogram indicate that genotypes that are derivate from the common ancestor clustered together.

Microsatellite marker analysis could be utilized for genetic diversity analysis and differentiation of different rice germplasm according to their genetic base. The level of diversity exhibited by the markers used in this study, suggest that these markers could be exploited for characterizing both genetically distant and closely related rice genotypes. Since the markers were chosen from all the chromosome of rice, the diversity results of this study are likely to be unbiased. So, these markers could be also utilized for testing purity of the commercial seed lots and for maintenance of seed purity at different levels of seed production. The diversity analysis found for less than 1% of whole rice genome. So, more primers from different chromosome are needed for analyzing even much large germplasm for getting a reproducible data that can be used as ready reference by concerned breeder, variety registration authority and seed production agencies. The information about the genetic diversity of these stress tolerant rice genotypes will be useful to breeder for proper identification and selection of appropriate parents' in future breeding program for developing new stress tolerant rice varieties with high yielding and fine grain quality.

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