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DNA fingerprint and diversity in aromatic rice by *Gn1* gene linked SSR markers

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Abstract

Number of grains per panicle is a limiting factor for the yield of aromatic rice. Toovercome this limitation, itmight behelpful to screenaromatic rice using markers linked to grain number.In this study, an elementary DNA fingerprinting database of the nine aromatic rice cultivars was built using four SSR primer pairs linked to *Gn1* gene responsible for grain number in rice. For genotyping of aromatic rice cultivars, DNA was extracted from leaf samples using IRRI standard protocol. Allele scoring was done by using a computer based program Alpha Ease FC 4.0 and data were analyzed by Power Marker version 3.25 software. Nei's genetic distance value and similarity werecomputed. From the analysis, it was found that a total of 21 alleles were detected with an average number of 5.25 alleles per locus having PIC values ranging from 0.3402 (RM5493) to 0.7883 (RM3452) and the average value 0.6629. The highest gene diversity (0.8148) was observed in loci RM3452,and the lowest gene diversity (0.3404) was observed in loci RM5493.From the study, it can be stated that all of the aromatic rice germplasm have bands of the gene that influence the grain but they showed genetic variability.Information obtained from genotyping of varieties helped to analyze the genetic diversity within and among closely related crop varieties which has the potential for crop improvement and to meet the diverse goals like producing cultivars with increased yield of aromatic rice.

Key words: DNA fingerprint, aromatic rice, *Gn1* gene, SSR marker

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Introduction

Rice (*Oryza sativa L.*) is one of the most important cereal crops in the world. It is the second most important agricultural crop in the world (FAO, 2012).Rice as a food and an ingredient has been a subject of a lot of interest over the past few decades. There has been a growing interest in a type of rice called aromatic rice. Three things have led to this interest: globalization, health concerns and culinary changes. The result has been the widespread development of new types of aromatic rice along with increased production in more common types of this rice(Jaiswal*et al*., 2002). Of course, it doesn't just smell good; there are subtleties in the taste of the food as well. Aromatic rice is also preferred by some consumers despite their price and yield. Due to high consumer's demand reportedly, Bangladesh imports around 50 thousand tons of fine aromatic rice eachyearfromneighboring countries. Lack of high yielding, biotic stressresistant and abiotic stress tolerant variety made cultivation of aromatic rice limited gradually. Hence theimprovement of the existing genotypes in respect of yield and aroma is of paramount importance (Hossain *et al*., 2009). Conventional breeding method merely is not sufficient

and therefore, newtechnologies like gene transfer and marker assisted breeding can improve aromatic rice productivity to meet the growing demand.The presence of genetic variability for economic traits is a key factor for improving aromatic rice productivity.

Aromatic rice is popular in Asia, and cultivation of aromatic rice has been gaining popularity in Bangladesh over the recent years, because of its huge demand both for internal consumption and export (Dasand Baqui, 2000). Despite the generally favorable agro-climatic conditions, area of aromatic rice is less than 2% of the national rice acreage of Bangladesh (Kaul*et al*., 1982). Aroma is an important trait that is generally associated with high-quality grains and is developed where temperature is cooler during maturity. The biochemical basis of aroma was identified as 2-acetyl-1-pyrroline (Buttery*et al*., 1983; Tanchotikul and Hsieh, 1991).

Rice varieties in Bangladesh 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 large effect of the environment and the low narrow sense heritability of aroma. This hinders the development of an accurate, rapid and reliable screening technique. Recent progress and technical advances in DNA marker technology permit the rapid and accurate identification of individuals that contain genes of interest (Jones*et al*., 1997). Researches have been done for screening the aromatic rice germplasm by SSR marker, and no works till date have been conducted on the screening of aromatic rice with SSR marker that are linked to grain number.

Gn1 is a gene for cytokinin oxidase/dehydrogenase (OsCKX2), an enzyme that degrades the phytohormonecytokinin. Reduced expression of OsCKX2 causes cytokinin accumulation in inflorescence meristems and increases the number of reproductive organs, resulting in enhanced grain yield. In general, the productivity of aromatic rice is low. However, the presence of *Gn1* gene in aromatic rice would increase the grain number and thus the yield of aromatic rice.The success of genotyping of aromatic rice by *Gn1* gene will ensure more productive aromatic rice varieties. Therefore, the present work was attempted to assess the extent of variability among the aromatic rice varieties using SSR markers linked to *Gn1*gene.

Materials and Methods

Nine aromatic rice genotypes,ChiniSagor, Kalizira, BR-34, Khirsha, LR-189, LR-53, LR-42, UkunMadhuand a Japanese line STG were used in this study. In order to extract genomic DNA for SSR analysis, young, vigorously growing fresh leaf samples were collected from 25 days old seedling of each of the 9 aromatic rice genotypes. STG, a Japanese rice line,leaves were taken from Nagoya University, Japan by the first author (courtesy, Prof. Motoyuki Ashikari).

Genomic DNA extraction: DNA was extracted from the leaves of each genotype using the modified IRRI standard protocol. The simplified mini scale procedure for DNA isolation for PCR analysis was developed at IRRI. The leaf samples were cut into pieces and the sample was ground. Before and after grinding, 600 and 200µl extraction buffer was added, respectively. Then 500 µl chloroform was added and mixed well The samples were shaken at 600 rpm for 20/25 minutes and were centrifuged at 14000rpm for 5 minutes. The supernatant(500 - 600µl) was transferred in eppendorf tube and 20 µl RNase was added. Also, 50µl PCI was added and centrifuged for 10 minutes at 14000 rpm. The supernatant was transferred into another tube. Then 900µl 100% ethanol/isopropanol was added and gently shaken. The samples were kept in freeze -20^{o}C overnight and the samples were taken out from freeze, vortexed and centrifuged 10 minutes at 14000 rpm. Then ethanol was removed and allowed the pellets for air drying 1 hour or more. 1X TE buffer 50µl was added for re-suspension. The samples were then kept in 4^{o}C overnight for chelating reaction. Then the samples were vortexed and stored at -20^{o}C freeze. A concentration of about 50 ng / µl was maintained in the

DNA samples.DNA confirmation was done by using 0.8% agarose gel electrophoresis. Electrophoresis was carried out at 80V for 90 minutes.

Four primers were selected on the basis of their association with the trait (Table 1). The PCR cocktail including DNA had total volume of 10 µl/reaction based on rice protocol, was placed in the PCR tubes and run in the DNA thermal cycler.DNA amplification was performed in an oil-free thermal cycler. The reaction mix was preheated at 94°C for 5 minutes followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and elongation or extension at 72°C for 2 minutes. After the last cycle, a final step for 7 minutes at 72°C to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4°C.

Table 1. Details of SSR primers used in the present study

Primer Name	Primer Sequence		Expected PCR product (bp)	Annealing Temp. (°C)	Motif	Choromosome	SSR start	SSR end
RM10326	F	AGCCGGGCATACAGTCTTTCTC	277	53	AT	1	5368282	5368307
	R	TCTGGCGTGTTGGCACTAGC						
RM3425	F	CAGCAGCAAGAACCCTAGAAATCC	128	50	AG	1	5116190	5116225
	R	CTCGTGATCAACCGACAAAGC						
RM3452	F	TGGACTTGGTCTCTCCAAACTC	190	53	TC	8	24772332	24772369
	R	CAGTATGTTGGTGGGTCAAGC						
RM5493	F	GCGGTAACAAACCAACCAACC	200	50	GA	8	26140988	26141035
	R	AAAGCAGGACACAGTCACACAGG						

About 2 µl of each PCR product was loaded in each well. Before loading, each PCR product was mixed with 2 µl of 2X gel loading dye. DNA marker (25bp DNA ladder) was used for size determination. The electrodes were connected to the power supply and run for about 3-3.5 hours at 80 volts.After completion of electrophoresis, the gel was soaked in ethidium bromide (10mg/ ml) solution for 15-20 min.After staining, the gel was taken out carefully from the staining tray and placed on high performance ultraviolet light box (UV-trans-illuminator) of gel documentation system for checking the DNA bands. The DNA bands were observed and saved.

The size of most intensely amplified fragments was determined by comparing the migrated distance of amplified fragments relative to the molecular weight of known size markers, 25bp and 100bp DNA ladder using Alpha-Ease FC 5.0 software (Alpha Innotech, USA). The number of alleles per locus, major allele frequency, gene diversity, PIC and Nei's genetic identity and genetic distance values were calculated using Power Marker version 3.25. All the genotypes were scored for the presence and absence of the SSR bands for further analysis with NTSYS-pc version 2.2. MEGA software was used to construct a UPGMA (Unweighted Pair Group Method with Arithmetic Averages) dendrogram showing the distancebased interrelationship among the genotypes.

Results and Discussion

The evaluation of genetic diversity is very important factor for rice improvement that can be obtained through DNA marker based approaches, which is capable of exhibiting large number of loci for extensive variability. In the present study selected rice varieties were analyzed using a highly repeatable PCR based assay known as Simple Sequence Repeat (SSR) or microsatellites markers. These microsatellite DNA

markers produce higher level of DNA polymorphism with respect to rice genotypes. The variation found at molecular level is briefly discussed here.

Allelic and loci variation within the genotypes: In this study 9 genotypes of rice were analyzed using 4 sets of primers: RM10326, RM3425, RM3452, RM5493. Amplified microsatellite loci were analyzed and the result revealed that all primers showed polymorphism among the rice genotypes (Figure 1-4). The microsatellite loci detected 3to 6 alleles per locus with a mean of 5.25 alleles per locus in the present studyand the alleles were co-dominant suggesting their superiority in detecting DNA polymorphism. Using 4 SSR markers, a total of 21 alleles were detected among the 9 rice genotypes. In respect of primer RM10326, allele size ranged from 261–278bp, whereas primer RM3425, RM3452, RM5493 showed a range 124–178bp, 180-188bp, 190–210bp, respectively (data not shown). The average number of allele per locus was 5.25 (Table 2).

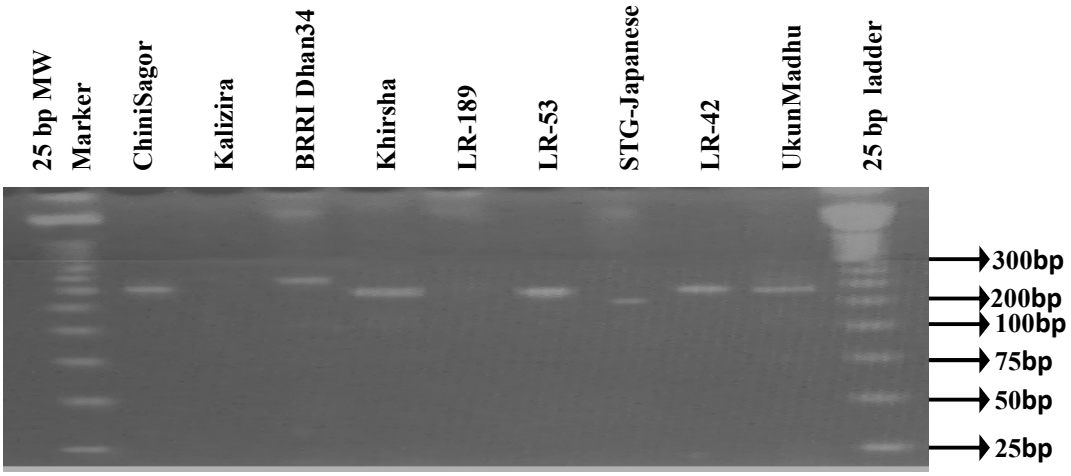

Figure 1. SSR profile of 9 rice genotypes using primer RM10326

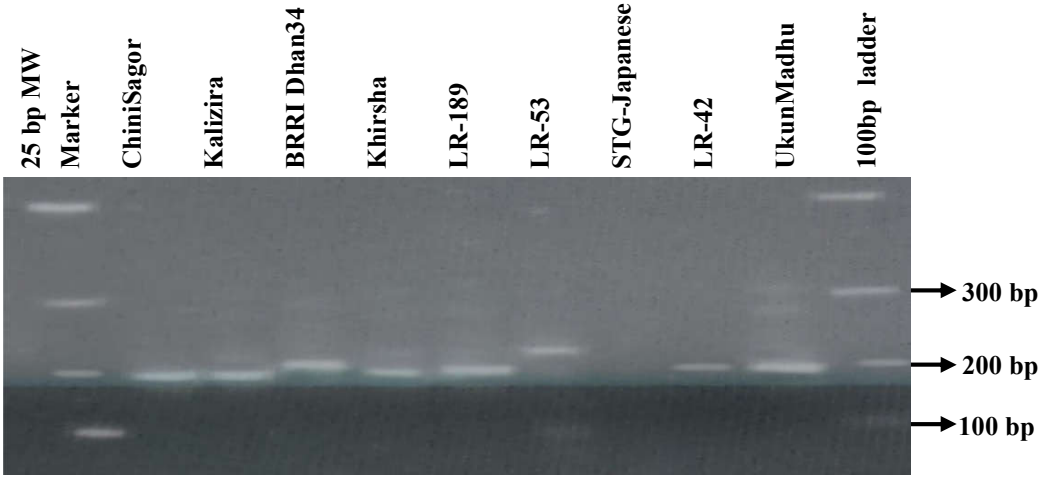

Figure 2. SSR profile of 9 rice genotypes using primer RM3425

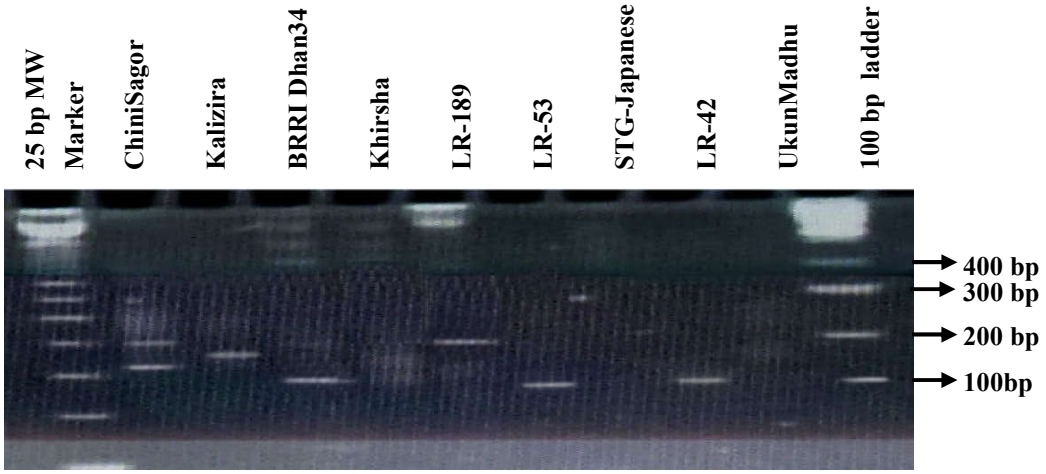

Figure 3. SSR profile of 9 rice genotypes using primer RM3452

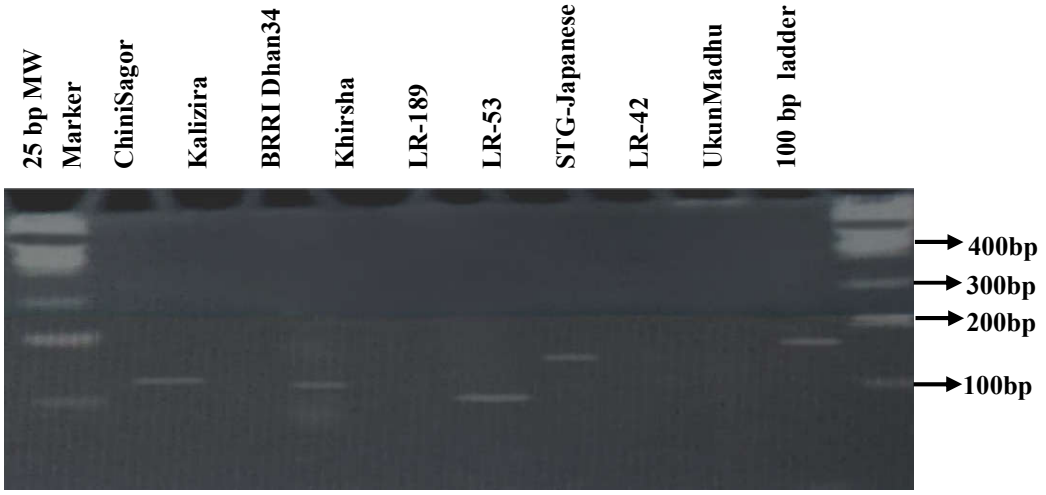

Figure 4. SSR profile of 9 rice genotypes using primer RM5493

An allele observed in less than 5% of the 22 accessions is considered to be rare (Islam, 2012). There were no rare alleles found in this study. Total absence of an allele indicates null allele. In this study the average value of null allele was 0.25. Only the primer RM5493 showed1null allele.

As a measure of the informative nature of microsatellites, the PIC values ranged from 0.7883 (RM3452) to 0.7615 (RM10326, RM3425). RM5493 showed the lowest (0.3402) PIC value and the average value was 0.6629 (data not shown). PIC values also showed a significant, positive correlation with the number of alleles and allele size ranged for microsatellites evaluated in this study. Dhar *et al.* (2012) observed PIC values ranging from a low of 0.7459 (RM152) to a high of 0.8908 (RM10701) and average 0.857. Major allele isdefined as the allele with the highest frequency and also known as most common allele at each locus. The frequency of the most

common allele at each locus ranged from 16.7% (RM167, RM3452, ART5) to 66.7% (RM 585) with a mean frequency of 30% (Table 2). The size of the different major alleles at different loci ranged from 129bp (RM 167) to 203bp (RM585). Dhar *et al*. (2012) observed that the frequency of the most common allele at each locus ranged from 15.38% (RM10701) to 37.51% (RM152) with a mean frequency of 24.15%.

Table 2. Allele number, allele size, allele frequency, gene diversity and PIC of 9 rice genotypes using four Gn1 markers

Marker	Allele size (bp)	Allele Number	Major Allele Frequency	Gene Diversity	PIC	Number of Null allele
RM10326	261–278	6.00	0.3333	0.7901	0.7615	0
RM3425	124–178	6.00	0.3333	0.7901	0.7615	0
RM3452	180-188	6.00	0.2222	0.8148	0.7883	0
RM5493	190–210	3.00	0.7778	0.3704	0.3402	1
Mean		5.25	0.4167	0.6914	0.6629	0.25

***Gene diversity*:** According to Nei (1973), the highest gene diversity (0.8148) was observed in loci RM3452 and the lowest gene diversity (0.3704) was observed in loci RM5493 with a mean diversity of 0.6914 (Table 3).

Table 3. Gene diversity among 9 aromatic rice genotypes for 4 Gn1 markers.

Locus	Genotype no	Gene diversity
RM10326	6.0000	0.7901
RM3425	6.0000	0.7901
RM3452	6.0000	0.8148
RM5493	3.0000	0.3704
Mean	5.2500	0.6914

It was observed that marker detecting the lower number of alleles showed lower gene diversity than those which detected higher number of alleles which revealed higher gene diversity. This result is in consistent with previous work done by Heenan *et al.*(2000), who observed that the gene diversity at each SSR locus was significantly correlated with the number of alleles detected, number of repeat motif and with the allele size range. Dhar*et al*. (2012) observed that the highest level of gene diversity value (0.899) was observed in loci RM1070 and the lowest level of gene diversity value (0.774) was observed in loci RM152 with a mean diversity of 0.854.

***Genetic distance based analysis*:** Pair-wise comparison value of (Nei, 1973) genetic distance (D) between varieties was computed from data of 4 primers and ranged from 0.25 to 1.0 (Table 4). The lowest genetic distance (0.25) was observed between kalizira and Khirsha. The highest genetic distance of 1.0 was observed between most of the pairs.

***Genetic similarity analysis using unweighted pair group method of arithmetic means (UPGMA)*:** A dendrogram was constructed based on the Nei's (1973) genetic distance calculated by 4 markers among 9 rice genotypes. All 9 rice genotypes could be easily distinguished. UPGMA analysis 9 rice genotypes grouped into 6 clusters (Figure 5). According to the dendrogram and inter-germplasm similarities index it was found that the higher genetic distance indicated the lower inter-germplasm similarity index. On the contrary, the lower genetic distance indicated the higher value of inter germplasm similarity index. Cluster I comprised BRRI dhan34 which is a high

yielding rice variety. The cluster II comprised of LR-189. Cluster III included ChiniSagor, kalizira andKhirsha,while Cluster IV had UkunMadhu. The Japanese line STG was found in a separates cluster number V. The cluster VI comprised of two lines LR-42 and LR-53.

Table 4. Nei's (1973) unbiased measures of genetic distance found of 9 rice genotypes for four SSR markers

	BRRI Dhan34	Chini Sagor	Kalizira	Khirsha	LR-189	LR-42	LR-53	STG	Ukun Madhu
BRRI Dhan34									
ChiniSagor	1.0000								
Kalizira	1.0000	0.5000							
Khirsha	1.0000	0.5000	0.2500						
LR-189	1.0000	1.0000	0.7500	1.0000					
LR-42	1.0000	0.7500	0.7500	0.7500	1.0000				
LR-53	0.7500	0.7500	0.7500	0.7500	1.0000	0.5000			
STG	1.0000	0.7500	0.5000	0.7500	0.7500	0.5000	0.7500		
UkunMadhu	1.0000	0.7500	0.7500	0.7500	1.0000	0.5000	0.7500	0.7500	

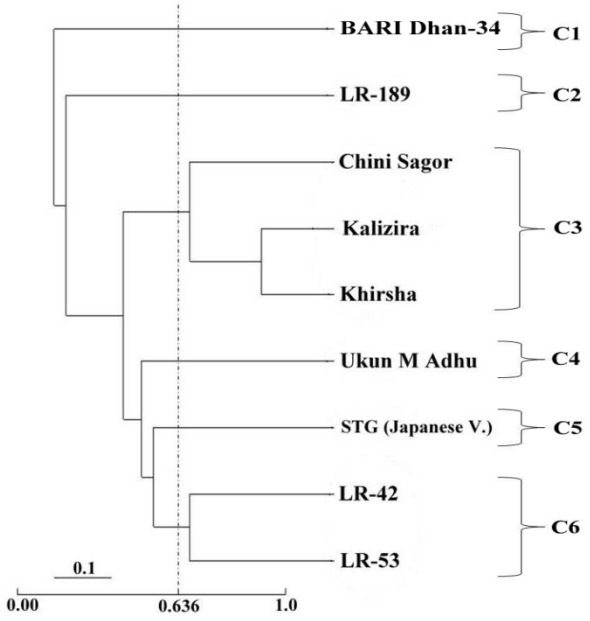

Figure 5. UPGMA dendrogram based on Nei's (1973) genetic distance summarizing the differentiation among 9 rice germplasm according to SSR analysis.

The highest inter-genotype similarity was found in accession Chinisagor, Kalizira, Khirsha and LR-42 and LR-53 also. The lower inter genotype similarity found in BRRI dhan34 and LR-53 means that they are heterozygous in nature. Popi*et al.* (2000) reported that higher similarity could be found of the germplasm that are collected from the same location and they are homozygous in nature.

Success of a crop improvement program depends on the magnitude of genetic variability and the extent to which the desirable characters are heritable (Ravi *et al*., 2003). Hence assessment of genetic diversity becomes important in establishing relationships among different cultivars (Sivaranjani *et al.,* 2010; Kibria *et al*., 2009; Nagaraju *et al*., 2002).

Conclusion

PCR products of all the four primers have showed excellent bands in agarose gel. The DNA based markers offered an opportunity to detect variation

among aromatic rice genotypes that can be utilized to improve aromatic rice. The findings of the study will be very useful for the future screening of aromatic rice germplasm by Gn1 marker. High yielding capacity of aromatic rice is very necessary for our country. So researchers should come forward to take steps for the improvement of aromatic rice variety by breeding for high yield using Gn1 marker.

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