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DNA fingerprinting of rice (*Oryza sativa* L.) cultivars using microsatellite markers

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Abstract

Microsatellite combines several features of an ultimate molecular marker and they are used increasingly in various plant genetic studies and applications. In this work, we report on the utilization of a small set of three previously developed rice microsatellite markers for the identification and discrimination of 17 HYVs and 17 local rice cultivars including two wild rice cultivars. All analyzed microsatellite markers were found to be polymorphic with an average number of 6.33 alleles per locus. These three markers were able to identify 15 local rice cultivars and 11 HYVs. A total of three variety specific alleles, RM-11/147, RM-151/289 and RM-153/178 were identified for BR-11, Badshabhog and BR-19 cultivars respectively. DNA fingerprints of rice cultivars by means of microsatellites provided meaningful data, which can be extended by additional microsatellite markers. The data obtained can be used for the protection of plant genetic resources.

Key words: Rice; microsatellite; markers; variety identification

Introduction

Rice (*Oryza sativa* L.) (2n=24) belonging to the family *Graminae* and subfamily *Orazoidea* is the staple food for one third world's population and occupies almost one-fifth to the total land area covered under cereals. It is grown under diverse cultural conditions and over wide geographical range. Most of the world's rice is cultivated and consumed in Asia, which constitutes more than half of the global population. Approximately 11% of the world's arable land is planted annually to rice, and it ranks next to wheat. Further scope of crop improvement depends on the conserved use of genetic variability and diversity in plant breeding programme and use of new biotechnological tools. There is wide genetic variability available in rice among and between landraces leaving a wide scope for future crop improvement.

Plant scientists have long been used morphological, pigmentation, quality or other characteristics to classify and distinguish plant genotypes within a species. With the advent of the plant variety protection act, the developer of a cultivar could "protect" it from commercial exploitation by others. While not having the same exclusivity as a patent, plant variety protection, nonetheless, offers the owner of a cultivar some legal protection for the exclusive sale of a protected cultivar. In the case of Rice (*Oryza sativa* L.), and most other cases leaf sheath color, inflorescence color, awn presence and other conventional morphological traits, together with stress resistance, have been, and continue to being used to distinguish the uniqueness of a new cultivar. As more cultivar receives protection, and thereby increases the size of the PVPO database, it becomes

more difficult to distinguish new cultivars from those in the database. Moreover, since new cultivars normally arise from hybridizations between members of an elite group of genetically similar parents, the amount of genetic variability among newly developed cultivars is likely to become even smaller. This will further complicate the task of unambiguously identifying new cultivars by the use of conventional characteristics alone. It is thus apparent that the use of molecular genetic markers would provide one solution to the problem of providing unique DNA profiles for the protection of new rice cultivars. Microsatellites are tandemly repeated nucleotide units of 1 to 6 base pairs and alleles usually differ in the number of repeated units. These are generally co-dominant and highly polymorphic DNA based markers. Genetically mapped microsatellite markers cover the whole rice genome with at least one microsatellite every 16 to 20 CM (Chen et al., 1997). This genome coverage enables microsatellites to be used to anchor randomly generated PCR markers such as AFLPs to known regions of the rice genome providing an economical means of producing genetic maps (McCouch et al., 1997). Rice microsatellites also have a demonstrated utility for gene-tagging and marker-assisted selection (Chen et al., 1997 and McCouch et al., 1997) and are polymorphic between (Akagi et al., 1996 and Panaud et al., 1996) and within rice varieties (Olufowote et al., 1997). Because of the high level of polymorphism, and therefore the greater informativeness of microsatellite markers, it seemed likely that these markers would be particularly useful for developing unique DNA profiles of rice genotypes. Such profiles would be especially valuable to unambiguously distinguish rice cultivars in order to obtain plant variety protection. The objective of the work reported here was to distinguish 34 diverse rice genotypes of Bangladesh at a small number of loci using microsatellite DNA markers.

Materials and methods

Plant materials

Thirty four rice samples from Bangladesh, including 17 commercial varieties, 15 landraces and 2 wild rice accessions (Table 1) were used for microsatellite analysis. Seeds were collected from Genetic Resources Centre (GRC), Bangladesh Rice Research Institute (BRRI). Seeds were germinated at aseptic condition and grown in glass house.

Genomic DNA isolation

Genomic DNA was isolated from the rice seedlings following protocol described by Aljanabi et al. (1997) with modification. Fresh leaf samples of 22-days-old seedling were used as the source of genomic DNA. Leaf tissues were cut into small pieces, homogenized and digested with extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 300 mM NaCl and 1% SDS, pH 8.0). After incubation for 20 minutes at 65^0C with intermittent swirling, the mixture was emulsified with an equal volume of phenol: chloroform: isoamyl alcohol (25 : 24 : 1, v/v/v). DNA was precipitated using two volume of absolute alcohol in presence of 0.3M sodium acetate and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air dried and resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH=8.0). DNA quality was checked by electrophoresis in a minigel and quantification was accomplished using a spectrophotometer (Spectronic® Genesis™, Spectronic Instruments Inc., USA).

Microsatellite markers and PCR amplification

Three previously developed rice microsatellite primer pairs (RM 11, RM 151 and RM 153) were used in the analysis (Table 2). Polymerase Chain Reactions were done in a volume of 10 µl containing 50 ng template DNA, 1 µl 10X PCR buffer containing 15mM $MgCl_2$, 0.25 mM each of the dNTPs, 0.25 µM of each primer, 1 U ampli *Taq* DNA polymerase (GENEI Pvt. Ltd. Bangalore, India) and a suitable amount of sterilize deionized water. Amplification were carried out in a oil free thermal cycler (Thermal cycler gradient, Eppendorf, Germany) with the following program: Initial denaturation at 94^oC for 3 min followed by 35 cycles at 94 ^{o}C for 30 sec, 55 ^{o}C for 30 sec, and 72 ^{o}C for 1 min and a final cycle at 72 ^{o}C for 7 min. PCR products were checked in 2% agarose gel.

Determination of microsatellite allele lengths

PCR products were separated on 6% denatured polyacrylamide gel containing 19:1 Acrylamide : Bis acrylamide and 7M urea. Electrophoresis was carried out on Sequi Gen GT eletrophoresis cell (Bio-Rad laboratories, USA). Gels were stained with silver nitrate using the Promega Silver Stquence™ protocol (Gustavo et al., 1994).

Data analysis

The size (in nucleotides) of the most intensely amplified band for each microsatellite marker was determined based on its migration relative to the molecular weight (mw) size markers, 100bp DNA ladder (GENEI Pvt. Ltd. Bangalore, India) using the software DNA frag Ver. 3.03 (Nash, 1991).

Table 1. Rice Cultivars used in the present study

A. High Yielding Varieties			
Sl No	**Name**	**Ecotype**	**Pedigree**
1	Binadhan-4	Aman	BR 4 X Iratom 38 (Mutation)
2	Binadhan-5	Boro	Dular x Iratom 24 (Mutation)
3	Binadhan-6	Boro	Dular x Iratom 24 (Mutation)
4	Binasail	Aman	Nizersail (Mutation)
5	Iratom-24	Aus	IR 8 (Mutant)
6	BR-11	Aman	IR 20 X IR 5-47-2
7	BR-14	Aus	IR 5 (D) X BR-3
8	BR-19	Boro	IR 2180-2 X IR 2178-1
9	BR-21	Aus	C 22 X IIT 1444
10	BR-26	Aus	IR 18348-36-3-3 X IR 25863-61-3-2X IR 58
11	BRRI dhan-27	Aus	KN 1 B-361-1-8-6-9X C 168
12	BRRI dhan-28	Boro	IR-28 X Purbachi
13	BRRI dhan-29	Boro	BG 92 X BR 51-46-5
14	BRRI dhan-30	Aman	IR 2058-78-1-3-2-3 X BR 4
15	BRRI dhan-36	Boro	IR 64 X IR 35293-125-3-2-3
16	BRRI dhan-38	Aman	Bashmoti D x BR-5
17	BRRI dhan-40	Aman	IR 4595-4-1-15 X BR 10
B. Local Cultivars			
Sl No	**Name**	**Ecotype**	**Accession No.**
18	Badshabhog	Aman	4355
19	Dular	Aus	0022
20	Hashikalmi	Aus	3575
21	Hizaldigha	Deep water Aman	1263
22	Kataktara	Aus	2059
23	Kataribhog	Aman	4362
24	Khaiyaboro	Boro	0936
25	Maliabhanger	Deep water Aman	0046
26	Nizersail	Aman	0741
27	Nonasail	Aman	0599
28	Pajam	Aman	0646
29	Pakhbiroi	Boro	5702
30	Pashusail	Boro	0054
31	Razasail	Aman	0784
32	Tepiboro	Boro	0930
33	Wild rice-2	Deep water Aman	4338
34	Wild rice-7	Deep water Aman	4316

The polymorphism information content (PIC) was calculated according to Nei's statistics (Nei, 1973): PIC=1-$\sum(p_i^2)$, where p_i is the frequency of the ith allele detected in the germplasm. Allele frequency was determined using software POPGENE (version 1.31) (Yeh et al., 1999)

Results

All thirty four rice cultivars were successfully amplified with the three microsatellite primer pairs (RM 11, RM 151 and RM 153). Based on previous results (Sefc et al., 2000) primer pairs will be referred to as loci and DNA bands as alleles. All three microsatellite markers were found to be polymorphic, revealing a total of 18 alleles with an average number of 6.33 alleles per locus in the thirty four rice cultivars examined (Table 3). At the RM-151 locus, a total of 9 different alleles were identified among the 34 rice genotypes ranging in size from 178 bp to 347 bp. Likewise, 5 alleles (size ranging from 143 bp-180 bp) and 4 alleles (size ranging from 181 bp-223 bp) were detected at the locus RM-11 and RM-153 respectively (Table 3). Banding patterns generated by primer pairs RM 151 and RM 153 in 15 rice cultivars are shown in Fig 1.

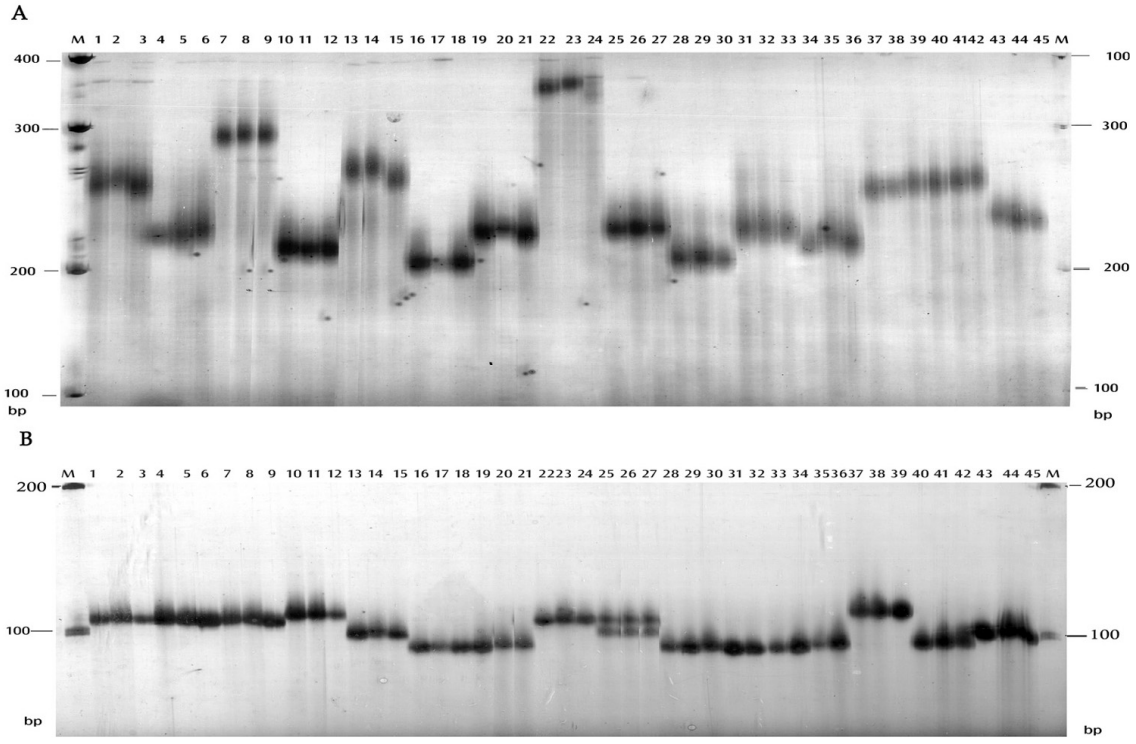

Fig 1. Microsatellite profile of fifteen rice (*Oryza sativa* L.) cultivars at the locus RM 151 (A) and RM 153 (B). M: Molecular wt. marker (100 bp ladder)

Lane	Variety	Lane	Variety	Lane	Variety	Lane	Variety	Lane	Variety
1,2,3:	BRRI dhan38	10,11,12:	Dular	19,20,21:	Kataktara	28,29,30:	Maliabhanger	37,38,39:	Pajam
4,5,6:	BRRI dhan40	13,14,15:	Hashikalmi	22,23,24:	Kataribhog	31,32,33:	Nizersail	40,41,42:	Pakhbiroi
7,8,9:	Badshabhog	16,17,18:	Hizaldhigha	25,26,27:	Khaiyaboro	34,35,36:	Nonasail	43,44,45:	Pashusail

Table 2. Details of the microsatellite markers used for rice genotype identification

Sl.	Locus	Core motif and Number of repeat	Chr.	Forward Primer	Reverse Primer	Ann.T.	Ref
1.	RM 11	$(GA)_{17}$	7	TCT CCT CTT CCC CCG ATC	ATA GCG GGC GAG GCT TAG	55^0C	Panaud et al, 1996
2.	RM 151	$(TA)_{23}$	1	GGC TGC TCA TCA GCT GCA TGC G	TCG GCA GTG GTA GAG TTT GAT CTG C	55^0C	Temnykh et al, 2000
3.	RM 153	$(GAA)_9$	5	GCC TCG AGC ATC ATC ATC AG	ATC AAC CTG CAC TTG CCT GG	55^0C	Temnykh et al, 2000

Among 18 alleles detected, three were specific to three rice cultivars. One specific allele was detected in the variety BR-11 (RM-11/147), BR-19 (RM-153/178) and Badshabhog (RM-151/ 289) (Table 3). The three microsatellite primer pairs were able to identify and discriminate fourteen local cultivars and a wild cultivar, wild rice-7. The local cultivar Dular and wild rice-2 was not identified. Among the HYVs, six varieties (Binadhan-4, Binadhan-6, BRRI dhan30, Binasail, Iratom 24 and BRRI dhan28) were not identified but the rest of the 11 HYVs were identified by the three microsatellite markers (Table 3).

The PIC value which is the reflection of allele diversity was also estimated. The average PIC value was 0.69 and it ranged from 0.67 (RM11) to 0.71(RM 151). The average PIC value for 17 HYVs was 0.59 which was lower than that of 17 local cultivars (0.70) suggested that local cultivars are more diverse than the HYVs.

Discussion

Utilization of three microsatellite markers in the analysis of rice varieties revealed a high level of genetic polymorphism which allowed unique

Table 3. Analysis of 3 microsatellite loci for 34 rice cultivars

No	Cultivars	Band positions due to primers (bp)																	
M Satellite		RM11					RM151									RM153			
		A	**B**	**C**	**D**	**E**	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**A**	**B**	**C**	**D**
1.	Binadhan-4					143						220				223			
2.	Binadhan-5		172									220				223			
3.	Binadhan-6					143						220				223			
4.	Binasail	180										220				223			
5.	Iratom-24	180										220				223			
6.	BR-11				**147**							220					212		
7.	BR-14		172									220					212		
8.	BR-19		172												**178**		212		
9.	BR-21		172											204			212		
10.	BR-26	180										220					212		
11.	BRRI dhan-27			165								220					212		
12.	BRRI dhan-28	180										220				223			
13.	BRRI dhan-29					143					234					223			
14.	BRRI dhan-30					143						220				223			
15.	BRRI dhan-36		172								234					223			
16.	BRRI dhan-38					143				244							212		
17.	BRRI dhan-40					143						220					212		
18.	Badshabhog					143		**289**									212		
19.	Dular		172										214			223			
20.	Hashikalmi					143			265									201	
21.	Hizaldigha					143								204					181
22.	Kataktara					143						220							181
23.	Kataribhog					143	347										212		
24.	Khaiyaboro			165		143						220				,	212	201	
25.	Maliabhanger		172											204					181
26.	Nizersail	180										220							181
27.	Nonasail		172										214						181
28.	Pajam		172			143				244						223			
29.	Pakhbiroi		172			143				244									181
30.	Pashusail					143						220						201	
31.	Razasail			165			347										212		
32.	Tepiboro					143					234							201	
33.	Wild rice-2		172										214			223			
34.	Wild rice-7	180		165					265					214		223			
PIC Value		0.67									0.71					0.70			
Number of Alleles		5					9									4			

genotyping of 82.4% of the studied varieties and only these three markers were sufficient for unambiguous identification of twenty eight rice varieties which includes thirteen high yielding, fourteen local and a wild rice cultivars. In the set of twenty eight cultivars, eighteen alleles were detected which multiplied into a number of observed genotypes at each locus, giving high discrimination value for varietal identification. Most of the unique genotypes used for varietal identification were found at locus RM 151. Our results represent one of the first attempts to find out a small set of microsatellite makers to discriminate rice cultivars of Bangladesh providing meaningful data that can be enlarged by additional rice cultivars and new microsatellite markers.

Microsatellites are not very demanding technically, and a particularly important advantage is that microsatellite data can be easily compared among laboratories and are suitable for computer databases, which is not always the case with other markers, such as RAPD. These results can be of practical use in rice variety identification.

Microsatellites are considered appropriate for variety identification because of their ability to detect large numbers of discrete alleles repeatedly, accurately and efficiently (Smith et al., 1996). In a study, a minimum number of three microsatellite markers was sufficient for rapid and unambiguous discrimination of olive varieties (Dunja et al., 2002). In another study by Olufowote et al. (1997), as few as six, well chosen SSLPs were sufficient to discriminate between 71 related lines of rice.

In the present experiment, larger number of local cultivars was discriminated at the same loci compared with the modern high yielding rice varieties. It is due to the fact that local varieties posses more genetic variability than that of the HYV's.

In conclusion, the set of microsatellite markers used here provides a positive assessment to the ability of SSR marker to produce unique DNA profiles of rice genotypes. The data obtained can be used for varietal survey and the construction of a database of all rice varieties grown in Bangladesh, providing also additional genetic information of the agronomic and quality characteristics of rice varieties.

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