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The Use of SSR DNA Markers to Reveal the Polymorphism at Ploidy Level in Cultivated Rapeseed Varieties in Bangladesh

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Abstract: SSR genotyping may provide useful method for characterization, conservation and utilization of agricultural crop diversity. The SSR are usually used widely for the analysis of the variations within and between populations. In this work a set of three previously developed microsatellite loci B.n.12A, B.n.38A and B.n.59A1 were used to amplify genomic DNA of the seven rapeseed (*Brassica napus* L.) varieties, BINA sarisha-3, BINA sarisha-4, BINA sarisha-5, BARI sarisha-7, BARI sarisha-8, BARI sarisha-13 and BINA sarisha-6. Among the three primers B.n.12A was found to be most polymorphic as five alleles were identified at this locus. BINA sarisha-3, BINA sarisha-5 and BARI sarisha-7 showed triploid at 248 bp, 271 bp and 312 bp and BARI sarisha-8 and BARI sarisha-13 varieties showed tetraploid allelic positions at 248, 271, 280, 312 bp. The results are possible due to accumulation of micro-mutants in the genome with modifiers. However, these issues will further be studied to clarify the positions of the diploids and the amphidiploids. But in all possibility the position indicated by the specific SSR primers for the particular variety shall remain unchanged providing further no mutation take place.

Key words: *Brassica* · SSR markers · Ploidy level

INTRODUCTION

The Brassicas are a diverse range of species of considerable global economic importance, having been cultivated for use as a variety of oilseed, vegetable and fodder crops. In Bangladesh there are extensive *Brassica* breeding programs from which large number of variants is being selected every year. These along with the varieties already developed and registered for cultivation are very good sources of variable genes. It is not possible to differentiate all these materials only through morphological traits so attempts have been taken to use microsatellites for analysis of variation within and between populations.

The distance calculations are based usually on the proportion of shared alleles [1,2]. This method followed implicitly the infinite allele model, thus assuming independence of alleles and ignoring mutational processes, which can result in a biased distances especially when alleles are highly polymorphic. The identified SSR primers sometimes may not only indicate the locus of a single genome when the genomic constitution of the variety or the species is of long association and might have the possibility of sharing the gene(s) of one genome with that of the other accrued in the same genome through mutation of any dimension. This is more common when the tetraploidy happens in a population where the number of locus of the same gene(s) became double the number in diploid and may also became three instead of four because of the nature of gene balance and gene recombination in the tetraploidy, which is part of the mutation by number of genome. As the SSR system determines the diploid situation of a gene locus and when the triploid and tetraploids are involved the same soft ware cannot determine the distance [3].

This species when crossed with *Brassica oleracea* having 2n=2x=18 has produced *Brassica napus* after doubling of the chromosome numbers. This species contains varieties, which are mostly self incompatible

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and have undergone changes in the Mediterranean area to be of leafy kales, leafy cabbage, cauliflower as well as broccoli and many more eco-types in further north.

In case of *Brassica napus,* the product of these two basic species having same genome sets from two species as in the amphidiploid will usually show variable results in identifying the locus by the SSR, which usually does the perfect identification for diploids. In some of the varieties the triploid loci has also been identified by the SSR. The results of the present study are possible due possibly to accumulation of micro-mutants in the genome with modifiers. The objective of the work reported here was to reveal the polymorphism at ploidy level in cultivated rapeseed varieties in Bangladesh using microsatellite DNA markers.

MATERIALS AND METHODS

Collection of Samples and Isolation of Genomic DNA: Seeds of seven rape seed (*Brassica napus* L.) varieties were collected from Oilseed Research Center of Bangladesh Agricultural Research Institute (BARI sarisha-7, BARI sarisha-8 and BINA sarisha-13) and Plant Breeding division of Bangladesh Institute of Nuclear Agriculture (BINA sarisha-3, BINA sarisha-4, BINA sarisha-5 and BINA sarisha-6) Seeds were germinated and grown at aseptic condition. Fresh leaf samples of 12-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°C with intermittent swirling, the mixture was emulsified with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v). DNA w as precipitated using two volume of absolute alcohol in presence of 0.3 M sodium acetate and pelleted by centrifugation. The pellets were then washed with 70% ethanol, air dried and re-suspended 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: A set of five microsatellite loci (B.n.12A, B.n.35D, B.n.38A, B.n.59A1 and B.n.68/1) have been selected from the literature cited by Szewc-McFadden *et al.* [4] to estimate the potential of these marker for variety identification. Finally three primers, B.n.12A, B.n.38A and B.n.59A1 were selected based on their performance for SSR data analysis (Table 1). Polymerase Chain Reactions were done in a volume of 10 µl containing 10X PCR Buffer, 0.25 mM each of the dNTPs, 2.5 µM of each primer, 1 unit ampli *Taq* DNA polymerase, 50 ng template DNA and a suitable amount of sterilize deionized water. Amplification were carried out in a oil free thermal cycler (Thermal cycler gradient, Eppendorf) with the following program: Initial denaturation at 94°C for 3 min followed by 35 cycles at 95°C for 30 sec, 58°C for 45 sec and 72°C for 1 min and a final cycle at 72°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 7 M urea. Electrophoresis was carried out on Sequi Gen GT eletrophoresis cell (Bio-Rad laboratories, USA). Gels were stained with silver using the Promega Silver Stquence™ protocol with some modifications. Digital images of gels were made using an A4 scanner. The size (in nucleotides) of the most intensely amplified band for each microsatellite marker was determined based on its migration relative to molecular weight (mw) size markers (100bp DNA ladder, Genei, India).

Analysis of Microsatellite Data: The bands representing particular alleles at the microsatellite loci were scored manually and designated the bands as A, B, C etc. from the top to the bottom of the gel. The genotypes of different strains were scored as AA, BB, CC, etc. for homozygous or as AB, AC, BC etc. for heterozygous. A single genotypic data matrix was constructed for all loci. The software DNA FRAG version 3.03 [5] was used to estimate marker length and allelic length.

Table 1: Details of the microsatellite markers used in this study

Sl.	Locus	Forward Primer	Reverse Primer	Ann.T.	Ref
1.	B.n.12A*	gcc-gtt-cta-ggg-ttt-gtg-gga	gag-gaa-gtg-aga-gcg-gga-aat-ca	58°C	Szewc-McFadden *et al.* 1996
2.	B.n.19A	cac-agc-tca-cac-caa-aca-aac-cta	ccc-cgg-gtt-cga-aat-cg	58°C	Szewc-McFadden *et al.* 1996
3.	B.n.38A*	caa-ggc-caa-aag-tgt-cca-t	acg-ctg-tct-tca-ggt-ccc-act	58°C	Szewc-McFadden *et al.* 1996
4.	B.n.59A-1*	tgg-ctc-gaa-tca-acg-gac	ttg-cac-caa-caa-gtc-act-aaa-gtt	58°C	Szewc-McFadden *et al.* 1996
5.	B.n.68A-1	tcg-cat-gct-cct-cta-gac-tcg	ttt-agc-acg-gga-atg-tca-gg	58°C	Szewc-McFadden *et al.* 1996

* Used under this study

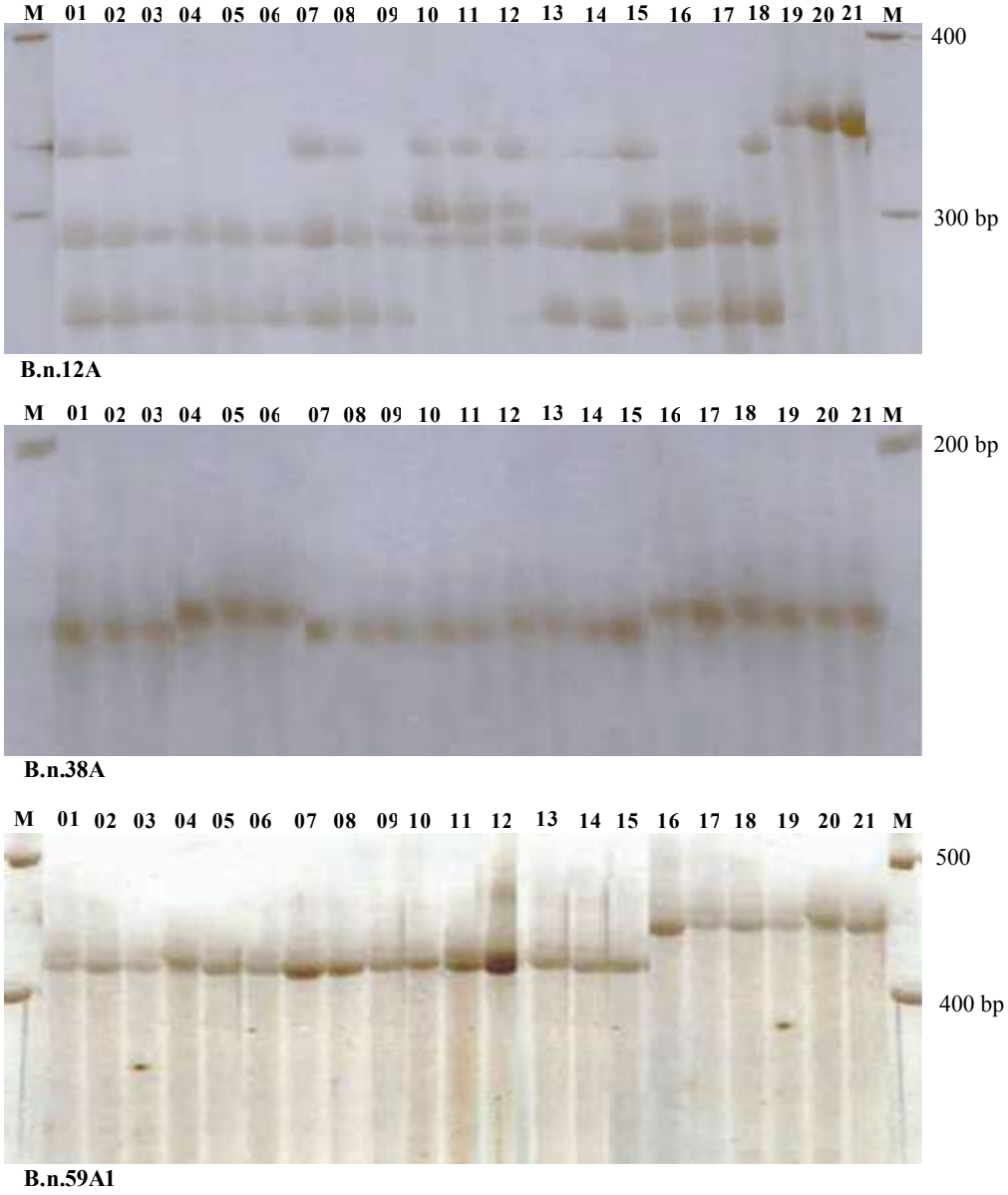

Fig. 1: Microsatellite profiles of nine rape seed (*Brassica napus* L.) varieties across three loci, B.n.12A, B.n.38A and B.n.59A1. Lanes, 1-3 = BINA sarisha-3; 4-6 = BINA sarisha-4; 7-9 = BINA sarisha-5; 10-12 = BARI sarisha-7; 13-15 = BARI sarisha-8; 16-18 = BARI sarisha-13; 19-21 = BINA sarisha-6. M: Molecular wt. Marker (100 bp DNA ladder)

RESULTS AND DISCUSSION

All the seven rapeseed (*Brassica napus* L.) varieties were successfully amplified with the three microsatellite primer pairs (B.n.12A, B.n.38A and B.n.59A1). Based on previous results [6] 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 9 alleles with an average number of 3 alleles per locus in the seven rape seed (*B. napus*) varieties (Table 2). At the B.n.12A locus, a total of 5 different alleles were identified among the seven rapeseed (*B. napus*) varieties ranging in size from 248 bp to 321 bp. Likewise, 2 alleles (size ranging from 151 bp-153 bp) and 2 alleles (size ranging from 431 bp-450 bp) were detected at the locus B.n.38A and B.n.59A1 respectively (Table 2). Banding patterns generated by primer pairs B.n.12A, B.n.38A and B.n.59A1in seven rapeseed (*B. napus*) varieties are shown in Fig. 1.

Table 2: Analysis of three microsatellite loci for seven rape seed (*Brassica rapa* L.) varieties

		Band positions due to primers (bp)									
Sl. No.	Cultivars			B.n.12A				B.n.38A		B.n.59A1	
01	BINA sarisha-3	312		271	248	BDE		151	BB	431	CC
02	BINA sarisha-4	271			248	DE	153		AA	431	CC
03	BINA sarisha-5	312		271	248	BDE		151	BB	431	CC
04	BINA sarisha-6	321				AA	153		AA	450	AA
05	BARI sarisha-7	312		271	248	BDE		151	BB	431	CC
06	BARI sarisha-8	312	280	271	248	BCDE		151	BB	431	CC
07	BARI sarisha-13	312	280	271	248	BCDE	153		AA	450	AA
	Number of Alleles			5			2			2	
	Allele Range (bp)			248-321			151-153			431-450	

SSR genotypic data from a number of loci have the potential to provide unique allelic profiles or DNA fingerprints for precisely establishing genotypic identity. Comparisons between SSR band positions against each marker in this study are shown in Table 2. The band patterns corresponding to individual variety may help to recognize the variety in question. When one primer would not distinguish individual variety from others, another primer should be considered and sometimes combination of more than one primer should be taken into account. Thus additional primer or set of primers might be needed to test to identify all expected varieties.

Among nine alleles detected, one specific allele was detected in the variety BINA sarisha-6 (B.n.12A/321) (Table 2). The three microsatellite primer pairs were able to identify four rapeseed varieties. Except BINA sarisha-3, BINA sarisha-5 and BARI sarisha-7 other four varieties showed unique and differential DNA banding patterns across one and/or combination of three primers. The variety BARI sarisha-8 and BARI sarisha-13 could b e easily identified by the primer B.n.12A and B.n.38A, in which locus B.n.12A showed tetraploid and locus B.n.38A showed diploid condition (Table 2). Microsatellites are considered appropriate for variety identification because of their ability to detect large numbers of discrete alleles repeatedly, accurately and efficiently [7]. In a study, a minimum number of three microsatellite markers were sufficient for rapid and unambiguous discrimination of olive varieties [8]. The unidentified three varieties BINA sarisha-3, BINA sarisha-5 and BARI sarisha-7 showed triploid banding position in the locus B.n.12A.

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