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Assessment of genetic variation and relationships within the varieties of four *Brassica* species by RAPD markers

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Abstract:

This investigation was aimed at exploring the genetic diversity and relationship among nine *Brassica* varieties of four different species using Random Amplified Polymorphic DNA (RAPD) markers. In total, 59 reproducible DNA bands were generated by four arbitrarily selected primers, of which 58 bands were proved to be polymorphic. These bands have had range from 212 to 2272 bp in size. The highest proportion of polymorphic loci (37.29 %) was found in BARI sarisha-12 (*B. rapa*) and the lowest was 8.47 % for both in BINA sarisha-4 (*B. napus*) and Rai-5 (*B. juncea*). The highest intra-varietal similarity index was found in Alboglabra while the lowest value was found in BARI sarisha-12 which suggested that the former one is the least diversified variety and the later is the most diversified variety among the studied varieties. UPGMA cluster analysis indicated that nine accessions were capable of being classified into two major groups. One group consists of BARI sarisha-12, Agrani and Sampad of *B. rapa* (2n =20, AA); Daulot and Rai-5 of *B. juncea* (2n =36, AABB) and Alboglabra (*B. oleracea,* 2n =18 CC) where Daulot and Rai-5 showed the lowest genetic distance of 0.049. Another group contains BINA sarisha-4, BINA sarisha-5 and BARI sarisha-13 all of which represent *B. napus* where BINA sarisha-5 and BARI sarisha-13 showed genetic distance of 0.071. The highest level of genetic distance was found between the varieties of BINA sarisha-4 and Rai-5 followed by the varietal pairs BINA sarisha-4 and Daulot.

Key words: *Brassica,* genetic distance, genetic variation, RAPD markers, relationship.

Introduction

Brassica is one of the major crop genuses that comprise different kinds of economically important food crops like oilseed, vegetables and condiments. It contained six economically important species with great genetic and morphological diversity which are distributed all over the world. The elementary species are *Brassica nigra* (n = 8, genome B), *B. oleracea* (n = 9, genome C) and *B. rapa* (n = 10, genome A)

Table 1. Genomic constituents, some morpho-physiological characteristics, seed source and approval year of the nine *Brassica* varieties included in this study

Species	Variety	Days to flowering	Days to maturity	No. of seeds/ silique.	1000-seed weight	Seed color	Source	Approval year
B. rapa	Agrani	29-30	84-90	>21	3.55	Y	BINA	1991
(2n = 20, AA)	BARI sa.-12	24-25	78-85	11-20	2.90	B	BARI	2002
	Sampad	30-32	85-95	>21	2.85	Y	BAU	1981
B. nupus	BARI sa.-13	38-39	110-115	>21	3.05	B	BARI	P
(2n=38,AACC)	BINA sa.-4	24-25	80-85	>21	3.65	DrB	BINA	1997
	BINA sa. 5	27-28	110-115	>21	2.95	Bl	BINA	2002
B. juncea	Daulot	27-28	90-105	11-20	2.25	RB	BAU	1988
(2n=36, AABB)	Rai-5	24-25	90-100	<10	1.80	RB	BARI	1976
B. oleracea (2n=18, CC)	Alboglabra	50-55	100-110	>20	3.4	B	Germany	2002a

B.: *Brassica*, sa.: Sarisha, Y: Yellow, B: Brown, Dr: Dark, Bl: Black, R: Reddish, BINA: Bangladesh Institute of Nuclear Agriculture, BARI: Bangladesh Agricultural Research Institute, BAU: Bangladesh Agricultural University, P: Proposed, a: Exotic line.

which are believed to be secondarily balanced polyploids (Prakash and Hinata, 1980). *Brassica carinata* (n = 17, genome BC), *B. juncea* (n = 18, genome AB) and *B. napus* (n = 19, genome AC) each originated by hybridization and polyploidization of two different elementary species (U N, 1935). The oleiferous *Brassica* represented by rapeseed and mustard plays a pivotal role in vegetable oil production in Bangladesh. Undoubtedly, it is the most important edible oil crop, which covers the highest acreage of 66% of the total oil seed acreage of Bangladesh (BBS, 2006). Although the area of production has been declined in recent year (0.24, 0.22 and 0.21 million hectares in 2004, 2005 and 2006, respectively), total production has been increased through the increase in yield (BBS 2006). However, this crop is mostly grown under residual soil moisture in winter season and due to the lack of using improved varieties as well as poor cultural practices, the average yield is quite lower than that in the developed countries (Hasanuzzaman and Karim, 2007). Despite the probable origin of *Brassica* in the Mediterranean-middle eastern area, a secondary centre of origin and differentiation of the species *B. rapa* and *B. juncea* appears to be in China (Nishi, 1980). Since their introduction into China thousands of years ago, these two species have remarkably been changed in forms, structure and productivity by domestication. As a result of the allogamous breeding system in *Brassica*, there is a large amount of morphological variability in the many sub-species, botanical varieties and cultivar groups of *B. rapa* and *B. juncea* (Li, 1981; Lee, 1982; Opena et al., 1988). Therefore, knowledge of genetic variation has become more critical and deciding factor in any future breeding program of these complex crop species. Improvements through genetical manner have largely been contributed to the high productivity of many crops. However, breeders tend to concentrate on specific genotypes, which combine traits of interest and could be use as progenitors in several breeding programs. With the development of a wide range of molecular techniques, marker assisted breeding is now used to enhance flexibility, choice of the breeding materials of conventional breeding programs in crop improvement (Frey et al., 2004; Lu et al., 2004). Among them, RAPD markers, generated by the polymerase chain reaction (PCR) has widely been used to assess intra-specific genetic variation at molecular level (Welsh and McClelland, 1990; Williams et al., 1990). In some recent studies on *Brassica* crops, RAPD technique has successfully been used to determine the genetic relationships with different related species (Thormann et al., 1994; Ren et al., 1995); to identify cultivars (Hu and Quiros, 1991); to determine the percentage of hybridity (Marshall et al., 1994); and to estimate genetic relationships and diversity among the crop

Table 2. Parameters of the Operon random primers used in the present study for screening

Primer Code	Sequence (5´ - 3´)	GC content (%)	References
OPA-02	TGCCGAGCTG	70	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPA-05	AGGGGTCTTG	60	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPA-07	GAAACGGGTG	60	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPA-09	GGGTAACGCC	70	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPB-03	CATCCCCCTG	60	Cartea *et al*. (2005)
OPB-04*	GGACTGGAGT	60	Cartea *et al*. (2005)
OPB-05	TGCGCCCTTC	70	Cartea *et al*. (2005)
OPB-10	CTGCTGGGAC	70	Cartea *et al*. (2005)
OPC-05*	GATGACCGCC	70	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPC-09*	CTCACCGTCC	70	Jain *et al*. (1994) and Cartea *et al*. (2005)
OPD-02*	GGACCCAACC	70	Cartea *et al*. (2005)
OPK-10	GTGCAACGTG	60	Rabbani *et al*. (1998)
OPL-07	AGGCGGGAAC	70	Rabbani *et al*. (1998)
OPL-14	GTGACAGGCT	60	Rabbani *et al*. (1998)

* Selected for RAPD analysis for all samples of the nine varieties

germplasm (Divaret and Thomas, 1998; Teklewold and Becker, 2006; Ahmad et al., 2007). Although the use of RAPD technique for the study of genetic variation has been demonstrated as suitable in many species, less attention has actually been devoted to the selection of the most suitable approaches for genetic distance coefficients calculation. Specific amplified products (RAPDs) segregated in Mendelian fashion (Williams et al., 1990), therefore, can effectively be used as genetic markers. The advantages of this technique are its ability to detect extensive polymorphisms, simplicity, rapidity and need for very small amounts of genomic DNA, which allows the analysis of single seeds and young seedlings. Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species and replication to ensure that only the reproducible bands are scored. Since *Brassica* contributed largely on the oilseed production of Bangladesh, studies on *Brassica* have generally been limited to varietal differences, agronomical practices such as seed rate, sowing date, spacing, effects of irrigation that affect yield and yield parameters. However, no report is available on genetic variability and relationships among the locally developed *Brassica* varieties in Bangladesh. As a developing country, due to lack of much research facilities and sophisticated technique, very few numbers of oil seed *Brassica* varieties have been developed in Bangladesh. Hence, this study was conducted to provide information on genetic variation and relatedness among the *Brassica* varieties those are commonly grown in Bangladesh.

Materials and Methods

Plant materials

Seeds of nine *Brassica* varieties, four from Bangladesh Agricultural Research Institute (BARI), three from Bangladesh Institute of Nuclear Agriculture (BINA) and two from Bangladesh Agricultural University (BAU), were used in this study (Table 1). The plants were grown in green house in March 2006 at Bangladesh Agricultural University, Mymensingh, Bangladesh. Fresh and young leaves of 12-15 days old seedlings were used as the sources of genomic DNA.

Extraction of genomic DNA

Total genomic DNA was extracted from young and actively growing fresh leaf tissues collected from a total of 27 individual plants (three individuals per-

Table 3. List of selected RAPD primers with corresponding bands scored and their size ranges together with polymorphic bands observed in *Brassica* varieties

Primer codes	Sequences (5´ - 3´)	Total number of bands scored	Size range (bp)	Number of polymorphic bands
OPB-04	GGACTGGAGT	11	300-1929	10
OPC-05	GATGACCGCC	19	376-2068	19
OPC-09	CTCACCGTCC	14	212-2034	14
OPD-02	GGACCCAACC	15	396-2272	15
Total		59		58

variety) following the method of Weining and Langridge (1991) with slight modification. Approximately 0.5 g of leaf tissue was homogenized and digested with a total of 800 µL DNA extraction buffer (50 mM tris-HCl, 25 mM EDTA, 300 mM NaCl and 1% SDS, pH 8.0) at 65°C for 5 min and following 20-30 sec vortex another 10 min in a hot water bath. The extract was centrifuged at 13,000 rpm for 10 min and the upper aqueous phase of about 600 µL was collected in another tube. An equal volume of phenol: chloroform: isoamyl alcohol at the ratio of 25:24:1(v/v/v) respectively, was added to it. The mixture was vortexed for about 20 sec and aqueous phase was recovered by centrifugation in the same condition. The supernatant was carefully transferred to a fresh tube and the DNA was precipitated using 1/10 volume of ice-cold isopropanol and 20 µL 3M sodium acetate (pH 5.2). Finally, the DNA was pelleted by centrifugation at 13,000 rpm for 10 min. The pellet was then washed twice with ice-cold 70% ethanol, air dried and dissolved in 40-50 µL of TE buffer (10mM tris-HCl, 1mM EDTA, pH 8.0) and stored at -20ºC. The DNA concentration was monitored by visual assessment of the band intensity in comparison with the λ DNA of known concentration using 1.4% agarose gel.

Primer selection

Fourteen decamer arbitrary primers (Operon Technologies Inc., California, USA) were initially screened on a sub-sample of two randomly chosen individuals to test their suitability for amplification of the DNA sequences, which could be scored accurately (Table 2). Primers were evaluated based on clear, easily-detectable bands, consistency within individual, presence of smearing, and potential for population discrimination. Easily- detectable, well-resolved bands were regarded as reproducible over repeated runs, with sufficient intensity to determine presence or absence in samples with the same relative band intensity. A final subset of four primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis (Table 3). Banding patterns produced from other primers were either faint or hard to detect or not reproducible.

PCR amplification

The polymerase chain reaction was carried out following the protocol of Williams et al. (1990) with some modifications. Amplification reactions were performed on each DNA sample in a total volume of 10 µL that contain 1 µL of 10x Ampli Taq polymerase buffer, 2µL of 10 µM primer, 1µL of 10 mM each of dNTPs (Bangalore Genei, India), 1 unit of Ampli Taq DNA polymerase (Bangalore Genei, India), 100 ng genomic DNA and a required amount of sterile deionized water. DNA amplification was performed in a programmable thermal cycler (Master Cycler Gradient, Eppendorf, Germany), with initial denaturation for 3 min at 94ºC followed by 45 cycles each of 1 min at 94ºC, 1 min at 37ºC and 2 min at 72ºC, for denaturing, annealing and DNA extension or elongation, respectively. The reaction was terminated by incubation at 72ºC for 7 min.

Agarose gel electrophoresis

Amplification products were separated by electrophoresis on 1.4% agarose gels run at 120V for about 1 h in 1x tris-borate-EDTA (TBE) buffer. Molecular sizes of the amplification products were estimated using a known molecular weight marker DNA (1000 bp DNA ladder) (Fig 1 and 2).

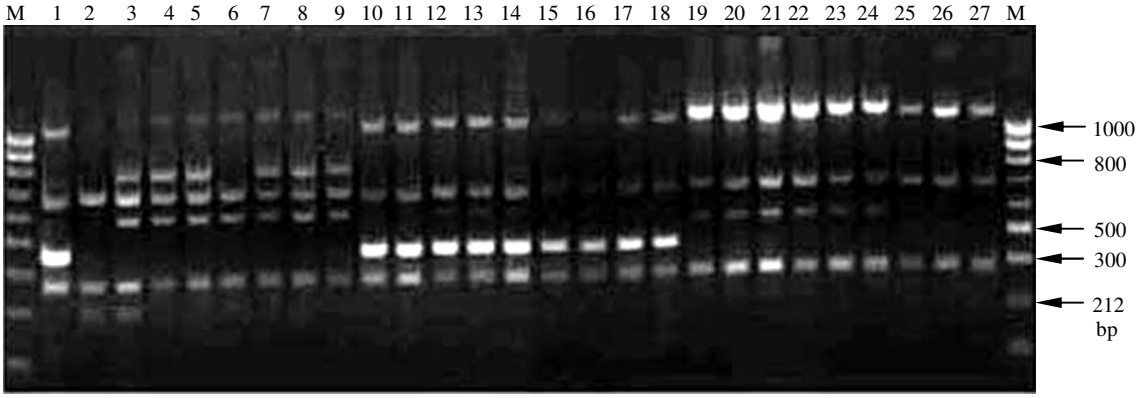

Fig 1. RAPD profiles of nine *Brassica* varieties using primer OPB-4; Lane 1-3: BARI sarisha-12, Lane 4-6: Agrani, Lane 7-9: Sampad, Lane 10-12: BINA sarisha-4, Lane 13-15: BINA sarisha-5, Lane 16-18: BARI sarisha-13, Lane 19-21: Daulot, Lane 22-24: Rai-5, Lane 25-27: Alboglabra M: Molecular weight marker (1000 bp DNA ladder in both sides)

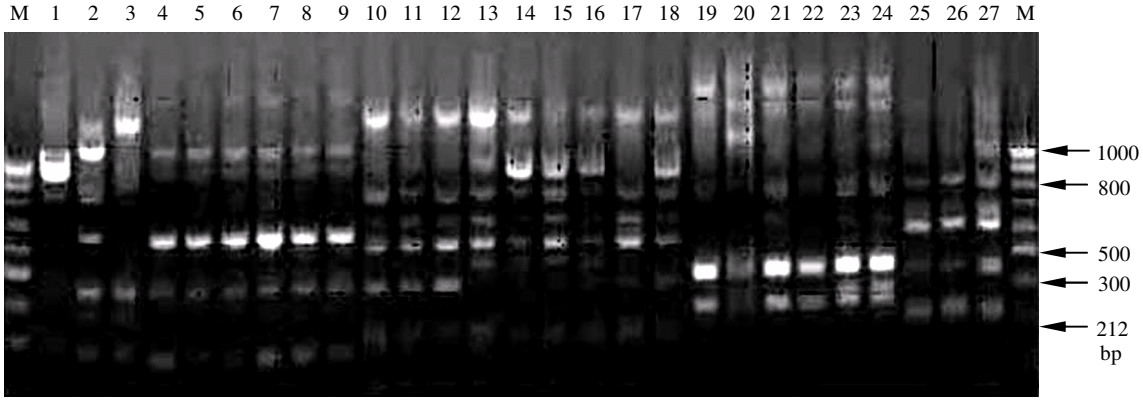

Fig 2. RAPD profiles of nine *Brassica* varieties using primer OPD-2; Lane 1-3: BARI sarisha-12, Lane 4-6: Agrani, Lane 7-9: Sampad, Lane 10-12: BINA sarisha-4, Lane 13-15: BINA sarisha-5, Lane 16-18: BARI sarisha-13, Lane 19-21: Daulot, Lane 22-24: Rai-5, Lane 25-27: Alboglabra M: Mo

DNA bands were visualized by staining with ethidium bromide and photographed on a UV transilluminator using gel documentation system.

Data analysis

RAPD bands were scored visually on the basis of their presence (+) or absence (-), separately for each individual variety against each primer (Table 4).

The scores obtained using all primers in the RAPD analysis were then pooled for constructing a single data matrix. This was used for estimated polymorphic loci, Nei (1973) gene diversity, co-efficient of gene differentiation (G_{ST}), genetic distance and for constructing a UPGMA (unweighted pair group method of arithmetic means) dendrogram using computer program POPGENE (Verson 1.31) (Yeh et al., 1999). The same program was also used to perform pair-wise homogeneity test across different

Table 4. An example of fragments that distinguish among the varieties and the species

Fragment	BARI-12	Agrani	Sampad	*B. rapa*	*B.napus*	*B juncea*	Alboglabora
OPC5-1	-	+	+	+	-	-	+
OPC5-2	-	+	+	+	-	-	+
OPC5-3	-	+	+	+	-	+	-
OPC5-6	-	+	+	+	-	+	-
OPC5-7	+	-	-	+	-	-	+
OPC5-12	+	+	-	+	+	+	+
OPC5-19	-	-	+	+	-	-	-
Fragment	BINA 4	BINA-5	BARI-13				
OPC 5-18	+	+	-	-	+	-	-
OPC9-1	-	+	-	-	+	-	-
OPC 9-4	+	+	-	-	+	+	-
OPD2-10	-	+	+	-	+	+	+
OPD2-11	+	-	+	+	+	+	-
Fragment	Daulot	Rai-5					
OPC9 -4	+	-		-	+	+	-
OPC9-11	+	-		+	+	+	-
OPD2 - 4	+	-		+	-	+	+
OPD2-11	-	+		-	+	+	+

(-) = fragments never existed in the varieties or species, (+) = fragments always existed in the varieties or species

loci. The similarity index (SI) values between the RAPD profiles of any two individuals on the same gel were calculated from RAPD markers according to the formula (Nei and Li, 1979): Similarity Index (SI) = 2 N_{xy} / (N_x + N_y) .where, N_{xy} is the number of RAPD bands shared between the individuals x and y, and N_x and N_y are the number of bands in individual x and y, respectively. Within population, similarity (S_i) was calculated as the average of S_i across all possible comparisons between individuals within a population. Between population, similarity (S_{ij}) was calculated as the average similarity between randomly paired individuals from populations i and j (Lynch, 1991).

Results and Discussion

Fourteen primers were initially screened for their ability to produce polymorphic patterns and only four of them were selected which gave reproducible and distinct polymorphic amplified products (Table 2 and 3). A total of 59 RAPD bands were scored using these primers. The sizes of these bands were ranged from 212 to 2272 base pairs which was similar to the previously reported range in *Brassica* germplasm (Rabbani et al., 1998; Cartea et al., 2005).

However, of the 59 scored band, 58 or about 98% were considered as polymorphic which is higher than some previous studies such as 81.72% in Chinese mustard (Fu et al., 2006) and 76% in *B. napus* germplasm (Cartea et al., 2005). The reason might be the *Brassica* varieties used in the present study are different in morphology, ploidy level and genome constituents. All of the varieties could be distinguished from each other by means of a combination of fragments that always present in one variety and always absent in the other (Figs. 1 and 2; Table 4). Presence of bands in all varieties known as monomorphic and absence of bands in any variety was regarded as polymorphic.

Absence of bands may be caused by failure of primer to anneal a site in some individuals due to nucleotide sequence differences or by insertions or deletions between two conserved primer sites (Clark and Lanigan, 1993). Therefore, the probable reason of higher average scorable and polymorphic bands could be attributed that the primers used in this study consist of 60 – 70% GC content.

Table 5. Inter-varietal similarity indices (%) (above diagonal) and pair wise genetic distance values (below diagonal) in different *Brassica* varieties)

Variety	BARI sarisha-12	Agrani	Sampad	BINA sarisha-4	BINA sarisha-5	BARI sarisha-13	Daulot	Rai-5	Alboglabra
BARI sarisha-12	***	51.99	49.04	50.75	45.89	49.69	23.24	22.14	21.88
Agrani	0.236	***	82.88	36.71	33.02	33.87	36.96	37.82	29.70
Sampad	0.293	0.071	***	34.41	31.58	33.34	42.11	40.37	29.29
BINA sarisha-4	0.356	0.672	0.722	***	82.19	75.78	24.91	26.55	25.25
BINA sarisha-5	0.261	0.556	0.613	0.126	***	77.56	25.84	27.11	31.24
BARI sarisha-13	0.189	0.447	0.483	0.150	0.071	***	25.31	28.03	27.66
Daulot	0.411	0.450	0.438	0.764	0.614	0.506	***	84.82	36.76
Rai-5	0.461	0.495	0.490	0.768	0.598	0.528	0.049	***	44.19
Alboglabra	0.307	0.473	0.487	0.579	0.403	0.354	0.394	0.332	***

Fukuoka et al. (1992) observed an increase in the number of bands with increasing GC content of the primer and explained that the stability of base complementation is higher when G is pairing with C by three hydrogen bonds than that of the complementation of A with T by two hydrogen bonds. Relationship between species and varieties were determined by UPGMA cluster analysis based on of Nei's (1972) genetic distance. The values for intra-variety similarity indices (Si) were higher (average 81.40) than inter-variety similarity indices (Sij, average 40.55) (Fig. 3 & Table 5).

The highest S_i value was observed in Alboglabra, a very recent introduction of CC genomic constitution and the lowest was in BARI sarisha-12, a yellow seeded variety of *B. rapa*. The higher S_i value reflects lower genetic variability while the lower degree indicates the presence of higher genetic variability in these two varieties. Hence, based on the Nei's (1973) gene diversity it could therefore be pointed out that BARI sarisha-12 was likely to be the most diversified variety while Alboglabra was the least diversified variety compared to the others. The possible reason may be that Alboglabra was a recent introduction while BARI sarisha -12 is a selection from endemic sources. The highest genetic distance (0.768) and lower level of inter-variety similarity indices (26.55) were observed between BINA sarisha-4 and Rai-5 among the verities (Table 5) which clearly indicated the existence of greater genetic distance between these two population. It could be noted that the former is a variety representing *B. napus* (AACC) while the latter represents *B. juncea* (AABB) and the RAPD markers have possibly tagged the alleles of the DNA of AA genome from both the species which has been differentiated over a long time. Similarly, BINA sarisha-4 and Daulot varietal pairs also showed higher genetic distance (0.764) and lower inter-variety similarity (24.91). On the other hand, BINA sarisha-5 vs BARI sarisha-13, both are *B. napus*; Agrani vs Sampad, both are *B. rapa* and Daulot vs Rai-5, both are *B. juncea*; exhibited lower level of genetic distance between these cultivar-pairs (Table 5). Based on Nei's (1972) genetic distance, a dendrogram was prepared using UPGMA where the nine accessions were segregated into two groups (Fig. 4). The major group could be divided into two subgroups in which first subgroup included three *Brassica* varieties of *B. rapa* namely BARI sarisha-12, Agrani and Sampad while the second subgroup included two *B. juncea* varieties Daulot and Rai-5 and one *B. oleracea* variety Alboglabra. The reason for two species belonged to same subgroup is likely that the nearly introduced genotype might have accepted some genes from the AA genomic base of other varieties which have been used in this study. It may also likely that the alleles of CC genome that have been marked by the primers have had original gene base of the species close to that of the other two elemental species. Another group included BINA sarisha-4, BINA sarisha-5 and BARI sarisha-13 all of which are *B. napus* species where BINA sarisha-5 and BARI sarisha-13 showed closer relationship with a minimal

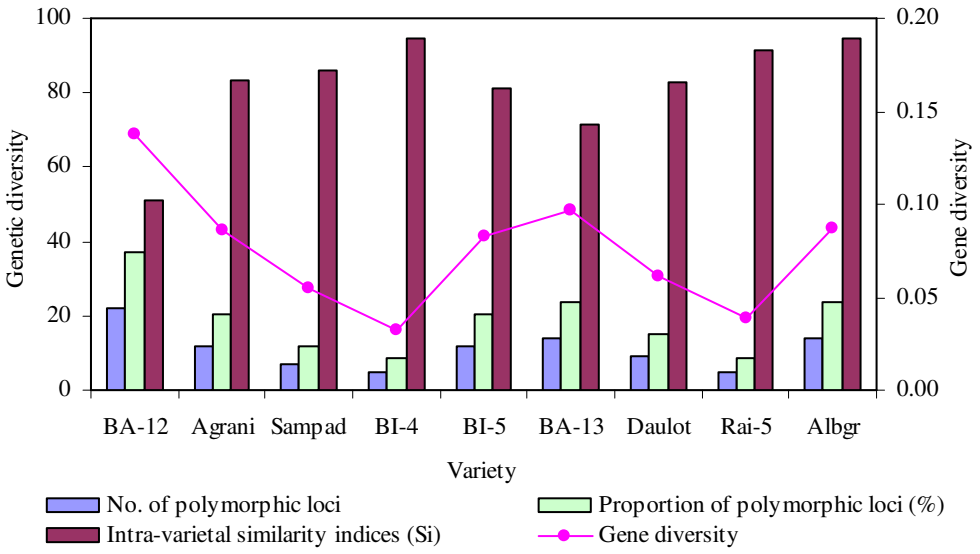

Fig 3. Estimated genetic diversity (number and proportion of polymorphic loci, intra-varietal similarity indices) as well as gene diversity obtained in different *Brassica* varieties. Legend: BA-12=BARI sarisha-12, BI-4=BINA sarisha-4, BI-5=BINA sarisha-5, BA-13=BARI sarisha-13, Albrg=Alboglabra

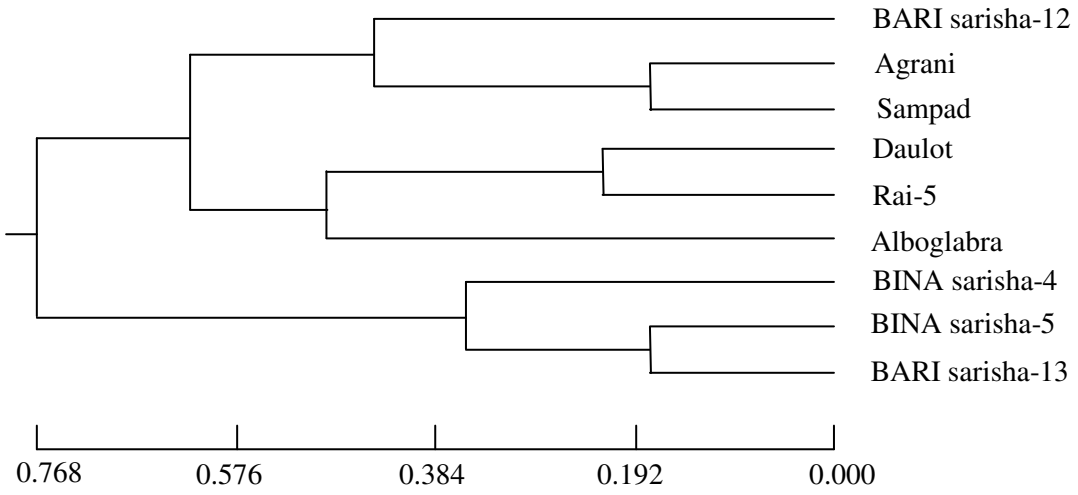

Fig 4. UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between nine *Brassica* varieties according to RAPD analysis

genetic distance of 0.071. in the first group the morphological characteristics such as seed colors are identical in Agrani and Sampad (yellow), Daulot and Rai-5 (reddish brown) (Table 1). It seems that yellow seeded cultivars were separated from brown seeded cultivar BARI sarisha-12 through cluster analysis. Similarly, Das et al. (1999) reported that yellow seeded *Brassica* cultivars clearly separated from brown seeded cultivars through cluster analysis even when the introgressive hybridization was done between *B. juncea* and *B. rapa.* (yellow sarson ecotype). In the second group, BINA sarisha-4, BINA sarisha-5 and BARI sarisha-13 have almost the same seed color and other characteristics, such as days to flowering and days to maturity (Table 1). High genetic variability within varieties and significant differentiation between varieties indicate rich genetic resource of a species. The varieties of BINA sarisha-4 and Rai-5 contain the highest genetic variation, while Daulot and Rai-5 contain the lowest genetic variation, among the varieties used in this study. Varieties having close proximity in their origin, breeding strategy and morphological traits are likely to have less genetic distance from each other. Hence, the results of this study suggested that the varieties of BINA sarisha-4, Rai-5 and Daulot could provide the opportunities for selecting as parental source in future breeding program to improve *Brassica* varieties in Bangladesh. Further research is needed for a detail investigation on genetic assessment of *Brassica* germplasm in Bangladesh including newly released varieties/ lines using more primers not only of RAPD but also of the SSR or microsatellite markers.

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