The Agriculturists 14(1): 122-133 (2016) ISSN 2304-7321 (Online), ISSN 1729-5211 (Print)
A Scientific Journal of Krishi Foundation ***Indexed Journal***
Impact Factor: 0.402 (GIF, 2014)

Use of RAPD Markers to Analyze Genetic Variability of Introgressed *Brassica* Lines

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Received: 28 March 2016 Accepted: 12 June 2016

Abstract

Seven individuals of introgressed *Brassica* lines (Binasarisha-5/Daulot) and two of their parental lines were used for this study to estimate genetic variability using three randomly amplified polymorphic DNA (RAPD) markers (61AB10G1, 72AB10G12 and 73AB10T13). A total of 23 clear bands were scored, of which 21 (91.30%) bands were proved to be polymorphic. The highest proportion of polymorphic loci and gene diversity values were 43.48% and 0.187, respectively in the line five of Binasarisha-5/Daulot. The lower proportion of polymorphic loci and gene diversity values were 8.70% and 0.034; 8.70% and 0.026 in the line seven of the cross and one parent, Daulot, respectively. The co-efficient of gene differentiation (G_{st}) and gene flow (N_m) values were 0.677 and 0.237, found respectively from the Popgene analysis. Result of cluster analysis indicated that the nine accessions were capable of being classified into two major groups - one consists of only one parent Daulot (*Brassica juncea*) while another consists of Binasarisha-5 (*Brassica napus*) and all introgressed lines of C_6 generation (treated with colchicine in C_1 generation) resulted from the cross *B. napus* and *B. juncea*. Introgressed line seven and Binasarisha-5 showed the lowest genetic distance of 0.077. Higher similarity was found between Binasarisha-5 and introgressed progenies. Introgressed line one and Daulot showed the highest genetic distance of 0.709, which can be used as germplasm for breeding program that aim to improve *Brassica*. It was concluded that RAPD markers can be used for the study of molecular characterization and diversity in *Brassica.*

Keywords: RAPD, Genetic Variability, Introgressed *Brassica*

1. Introduction

The genus *Brassica* includes two major groups of crop plants viz. the vegetable group and the oilseed group. The vegetable group includes the species *B. oleracea* (CC, 2n= 18) while the oilseed group includes five major species viz. *B. nigra* Koch (BB, 2n=16), *B. campestris* L. (AA, 2n=20), *B. carinata* Braun (BBCC, 2n=34), *B. juncea* Czern and Coss (AABB, 2n=36) and *B. napus* L. (AACC, 2n=38). In *Brassica*, the three diploid species *Brassica rapa* (AA), *Brassica nigra* (BB), *Brassica oleracea* (CC) are elemental to the species of *B. juncea* (AABB), *B. carinata* (BBCC) and *B. napus* (AACC), which are amphidiploid (Prakash and Hinata, 1980). Among the oleiferous *Brassica* species the varieties of *B. campestris* and *B. napus* are commonly known as rapeseed, while those of *B. nigra*, *B. carinata* and *B. juncea* as mustard

(Yarnell, 1956). RAPD technique requires only the presence of a single 'randomly chosen' oligonucleotide. Individual RAPD primers are able to hybridize to several hundred sites within the target DNA. A relatively small number of primers can be used to generate a very large number of fragments which are usually generated from different regions of the genome and hence multiple loci may be examined very quickly (Edwards, 1998). RAPD technique is widely applicable for analysis of most organisms because universal sets of primers are used without any need for primer sequence information (Hallden *et al*., 1996, Hasan and Raihan, 2014). This marker system is being used in many different applications involving the detection of DNA sequence polymerisms, mapping different types of populations (Carlson *et al*., 1991; Reiter *et al*., 1992), isolation of markers linked to various traits or specific targeted intervals (Giovannoni *et al*., 1991; Michelmore *et al*., 1991) and applications such as variety identification and analysis of parentage (Tinker *et al*., 1993; Mailer *et al*., 1994, Hasan and Raihan, 2015). In recent studies on *Brassica* crops using molecular markers, RAPDs have been widely used in variety identification (Qiao *et al*., 1998), analysis of genetic relatedness (Warwick *et al*., 2001; Geraci *et al*., 2001; Matsui *et al*., 2002), genetic diversity analysis (Yuan *et al*., 2004; Dan *et al*., 2003; Machao *et al*., 2003; Rabbani *et al*., 1998), estimation of genetic variations (Das *et al*., 1998), measurement of genetic distance (Furguth *et al*., 2000), DNA fingerprinting (Duan *et al*., 2002) and genetic mapping (Sharma *et al*., 2002). The general objective of this study was to create a base for genetic analysis of the cross product and their parents of *Brassica* using Random Amplified Polymorphic DNA (RAPD) markers. Some works based on phenotypic characters have been performed. However, 'characterizing the C_6 population of *Brassica* hybrids along with their parents at morphological traits level' and 'determining the genetic variation among the introgressed progenies and their parents' were carried out to attain the specific aims.

2. Materials and Methods

2.1. Plant material

Twenty introgressed progenies (C_6 generation) were developed from the cross between Binasarisha-5 (*B. napus*) and Daulot (*B. juncea*). The seeds of Daulot, introgressed progeny lines and Binasarisha-5 were collected from the Department of Genetics and Plant Breeding of Bangladesh Agricultural University (BAU) and Bangladesh Institute of Nuclear Agriculture, Mymensingh, Bangladesh, respectively. Also the present research was conducted in Department of Genetics and Plant Breeding of BAU during the year 2006-2007.

2.2. DNA extraction

Approximately 2 cm of fresh leaf tissues were cut into small pieces in 1.5 ml eppendorf tube and digested with extraction buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 25 mM EDTA, 1% Sodium Dodecyl Sulphate). DNA was purified by successive extraction with phenol:chloroform:isoamyl alcohol (25:24:1; v:v:v). DNA was precipitated with 800 µl of absolute ethanol where DNA became visible as white strands by flicking the tube several times with fingers. DNA was pelleted by centrifugation and reprecipitation of the DNA solution was done by adding 400 µl of 70% ethanol with 20 µl 3M sodium acetate and pelleted by centrifugation. The pellets were then air dried and dissolved in 50 µl of TE buffer (10 mM Tris. HCl, 1 mM EDTA, pH 8.0). DNA quality was checked by electrophoresis in 1% agarose gel and quantified using a spectrophotometer at 260 nm wave length (Spectronic® GENESIS™).

2.3. Primer selection

Eleven primers (63AB10A3, 68AB10A8, 62AB10C2, 69AB10C9, 70AB10C10, 61AB10G1, 64AB10G4, 66AB10G6, 71AB10G11, 72AB10G12, 73AB10T13) random sequence were screened on a sub-sample of two randomly chosen individuals from two different

varieties to evaluate their suitability for amplification of the DNA sequences, which could be scored accurately. Primers were evaluated based on intensity of bands, consistency within individual, presence of smearing and potential for population discrimination. The details of the primers are given in Table 1. A final subset of three primers (61AB10G1, 72AB10G12 and 73AB10T13) exhibiting good quality banding patterns and sufficient variability were selected for further analysis.

2.4. DNA amplification by PCR and electrophoresis

The amplification conditions were based on Williams *et al*. (1990) with some modification. PCR reactions were performed on each DNA sample in a 10 µl reaction mix containing 1 µl of 10X Ampli *Taq* polymerase buffer, 2 µl of 10 µM primer, 1 µl of 250 µM dNTPs, 1 unit of Ampli *Taq* DNA polymerase (Bangalore Genei, India) and 100 ng (4 µl) genomic DNA and a suitable amount of sterile deionized water. The PCR buffer, dNTPs, primer and DNA samples solutions were thawed from frozen stocks, mixed by vortexing and kept on ice. DNA templates were pipetted first into PCR tubes compatible with the thermocycler used (0.2 ml). A pre-mix was then prepared in the course of the following order: reaction buffer, primer, dNTPs, sterile distilled water. *Taq* polymerase enzyme was then added to the pre-mix. The pre-mix was then mixed up well and aliquoted into the tubes that already contain DNA. The tubes were then sealed and placed in a thermocycler and the cycling was started immediately. DNA amplification was performed in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf). The reaction mix was preheated at 94°C for 3 minutes followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 37°C and elongation or extension at 72°C for 2 minutes. After the last cycle, a final step of 7 minutes at 72°C was added to allow complete extension of all amplified fragments. After completion of cycling program, reactions were held at 4°C. PCR products from each sample were confirmed by running 1.4% agarose gel containing 6µl Ethidium bromide in 1X TBE buffer at 120V for 1hr. Loading dye (2.5 µl) was added to the PCR products and loaded in the wells. Molecular weight marker DNA (100bp DNA ladder) was also loaded on either side of the gel. RAPD bands were observed under ultra violet light on a transilluminator (Bio-Rad Gel Doc) and documented by taking photograph using a Gel documentation system.

2.5. RAPD data analysis

All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. This was used to estimate polymorphic loci (Nei's, 1972), gene diversity, population differentiation (F_{st}), gene flow (N_m), genetic distance (D) and to construct a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using a computer program, POPGENE (Version 1.31) (Yeh *et al.,* 1999). The similarity index values (SI) between the RAPD profiles of any two individuals on the same gel were calculated from RAPD markers according to the following formula: Similarity index (SI) = $2 N_{xy}/N_x + N_y$ Where, N_{xy} is the total number of RAPD bands shared by individuals x and y (Chapco *et al*., 1992) respectively, and N_x and N_y are the number of bands in individual x and y, respectively. The SI values range from 0 to 1. When SI=1.0, the two DNA profiles are identical and when SI is 0.0, there are no common bands between the two profiles. Population similarity within individuals (S_i) was calculated as the average of SI across all possible comparisons between individuals within a population. Population similarity between individuals (S_{ij}) was calculated as the average similarity between each paired individuals of population i and j (Lynch, 1991).

3. Results and Discussion

Seven introgressed individuals from C_6 materials (Binasarisha-5/Daulat) and two individuals of their parent including species of *B. nupus* and *B. juncea* were analyzed as a group and a total of 23 RAPD bands were scored. Each of the 11 primers varied greatly in their ability to resolve variability among the accessions. Eleven decamer primers were initially screened for their ability to produce polymorphic patterns and three decamer primers (61AB10G1, 72AB10G12 and 73AB10T13) which gave reproducible and distinct polymorphic amplified products were selected for evaluation of diversity across all the accessions. A total of 21 polymorphic amplification products were obtained by using these arbitrary primers. The size of the amplification products ranged from 246-3683bp (Table 1). The selected three primers produced comparatively maximum number of high intensity band with minimal smearing. The three primers showing good technical resolution and sufficient variation among different samples produced a total of 23 RAPD markers of which 21 (91.3%) were considered as polymorphic (either occurring in or absent in less than 23 of all individuals). The primer 61AB10G1 amplified maximum number of polymorphic bands (42.86%) and the primer 72AB10G12 generated 23.80% polymorphic bands which were minimum in number. The polymorphic amplification bands ranged from 5-9 and averaged to 7 (Table 1).The banding patterns of seven individuals from C_6 materials (Binasarisha-5/Daulat) and their two parents of *Brassica sp.* using the primers 61AB10G1, 72AB10G12 and 73AB10T13 are shown in using primers 61AB10G1, 72AB10G12 and 73AB10T13, the banding patterns of seven individuals from C_6 materials (Binasarisha-5/Daulat) and their parents of *Brassica* are shown in Figure 1-3.

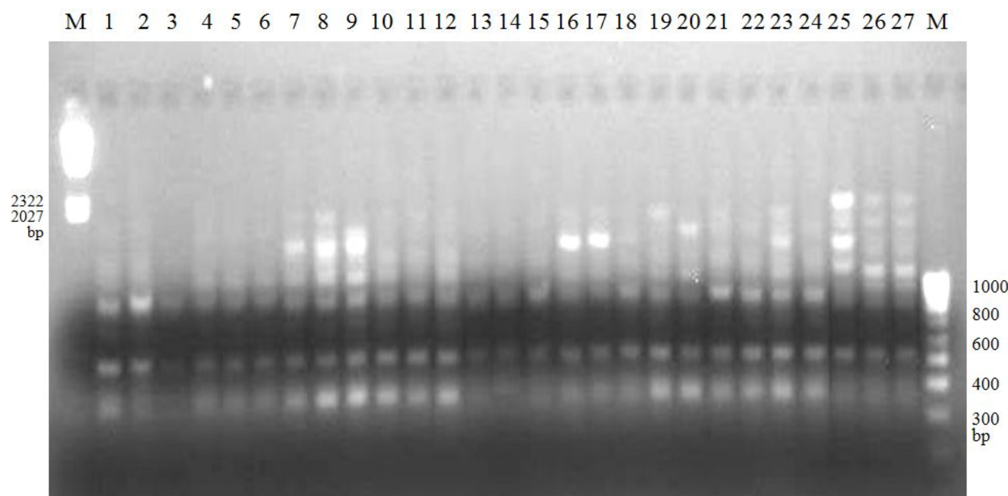

Figure 1. RAPD profiles of seven introgressed progeny lines and two parents using primer 61AB10G1. Lane 1-21: Binasarisha-5 / Daulot, lane 22-24: Binasarisha-5 and lane 25-27: Daulot. Ms: Molecular weight markers (λ DNA *Hind* III digest/100 bp DNA ladder)

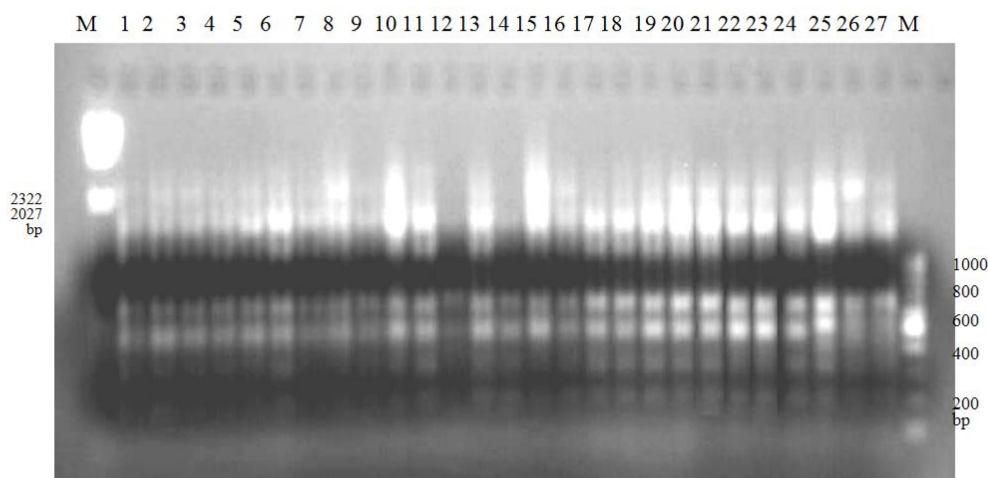

Figure 2. RAPD profiles of seven introgressed progeny lines and two parents using primer 72AB10G12. Lane 1-21: Binasarisha-5 / Daulot, lane 22-24: Binasarisha-5 and lane 25-27: Daulot. Ms: Molecular weight markers (λ DNA *Hind* III digest/100 bp DNA ladder)

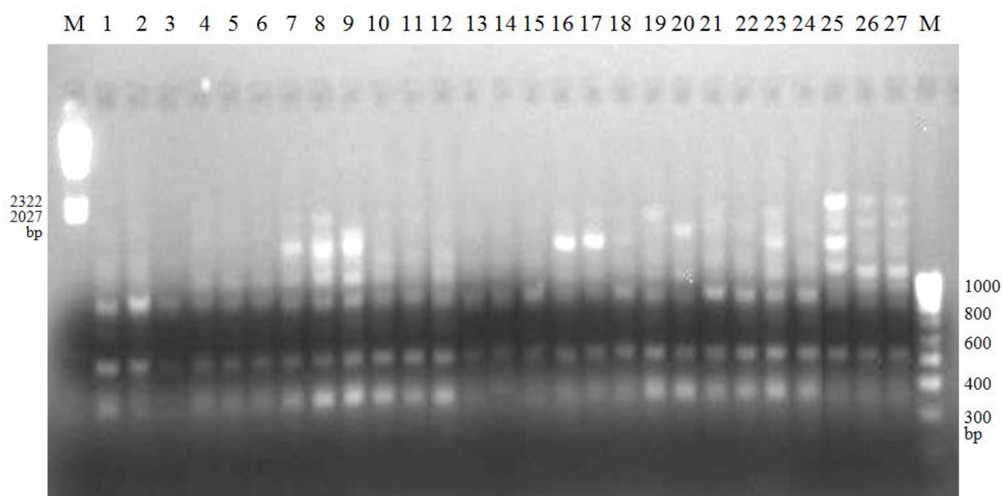

Figure 3. RAPD profiles of seven introgressed progeny lines and two parents using primer 73AB10T13Lane 1-21: Binasarisha-5 / Daulot, lane 22-24: Binasarisha-5 and lane 25-27: Daulot. Ms: Molecular weight markers (λ DNA *Hind* III digest/100 bp DNA ladder)

Intra-variety similarity indices (S_i) were higher (ranged from 78.22 - 97.21%) than inter variety similarity indices (S_{ij}) (ranging from 44.75 - 90.85%) indicating high genetic variation. For the population of introgressed line seven (97.21%) and Daulot (96.26%), intra-varietal similarity indices were found to be higher than the other introgressed progeny lines and Binasarisha-5. On the other hand, population of introgressed line five showed the lowest

similarity indices (78.22%). Inter-varietal means the pair-wise similarity indices (S_{ij}) ranged from (44.75 - 90.85%). The highest similarity indices of 90.85% was found between introgressed line seven - Binasarisha-5, while introgressed line one - Daulot showed least inter-variety similarity indices 44.75%. Band sharing based intra-varietal similarity indices were higher (Average 97.21%) than inter-varietal similarity indices (90.85%).

The highest number and proportion of polymorphic loci was found in the introgressed progeny line five, which were 10 and 43.48%, respectively, whereas lower values (2 and 8.70%) of these traits were recorded in both the varieties Daulot and introgressed progeny line seven (Table 3). However, the highest and lowest Nei's (1972) gene diversity values were found in introgressed line five and Daulot, respectively.

The Nei's original measures of genetic distance (Nei, 1972) were calculated from combined data sets for 3 primers ranging from 0.077 to 0.709. The genetic distance value between introgressed progeny line of (Binasarisha-5/Daulot)1 and variety Daulot was found to be the highest (0.709). This high genetic similarity supports the theory that they share a common origin. The lowest genetic distance (0.077) was found in introgressed progeny lines pair of two and four (Table 4).

Table 1. Selected primers and genetic variations of introgressed *Brassica* lines in RAPD analysis

Primer codes	Sequences (5´ – 3´)	Total number of bands scored	Size range (bp)	Number of polymorphic bands
61AB10G1	ACCGCGAAGG	9	246-2493	9
72AB10G12	AGGGCGTAAG	7	250-2478	5
73AB10T13	CTGGGGACTT	7	359-3683	7
Total		23		21

Table 2. Summary of band sharing based similarity indices within and between individuals of nine different *Brassica* lines A. Intra-varietal similarity indices (S_i)

Introgressed progeny lines/ Variety	Band sharing values (%)			
	61AB10G1	72AB10G12	73AB10T13	Average
(Binasarisha-5/Daulot)1	62.24	100	77.77	80.00
(Binasarisha-5/Daulot)2	90.47	86.66	100	92.37
(Binasarisha-5/Daulot)3	91.64	100	93.93	95.19
(Binasarisha-5/Daulot)4	91.66	62.24	100	84.63
(Binasarisha-5/Daulot)5	74.53	73.48	86.66	78.22
(Binasarisha-5/Daulot)6	81.67	88.88	93.93	88.16
(Binasarisha-5/Daulot)7	91.64	100	100	97.21
Binasarisha-5	84.91	90.90	86.66	87.49
Daulot	94.86	93.93	100	96.26

B. ***Inter-varietal similarity indices (S_{ij})***

Variety and introgressed progeny lines pair	Band sharing values (%)			
	61AB10G1	72AB10G12	73AB10T13	Average
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)2	74.47	93.33	88.88	85.56
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)3	69.99	100.0	75.53	81.84
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)4	69.99	73.12	88.88	77.33
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)5	67.45	82.56	68.67	72.89
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)6	66.66	78.10	72.76	72.50
(Binasarisha-5/Daulot)1-(Binasarisha-5/Daulot)7	71.70	72.72	78.30	74.24
(Binasarisha-5/Daulot)1-Binasarisha-5	67.84	72.72	82.59	74.38
(Binasarisha-5/Daulot)1- Daulot	00.00	85.92	48.33	44.75
(Binasarisha-5/Daulot)2-(Binasarisha-5/Daulot)3	89.26	93.33	85.92	89.50
(Binasarisha-5/Daulot)2-(Binasarisha-5/Daulot)4	89.07	75.67	100.0	88.24
(Binasarisha-5/Daulot)2-(Binasarisha-5/Daulot)5	81.65	80.86	73.00	78.50
(Binasarisha-5/Daulot)2-(Binasarisha-5/Daulot)6	84.10	80.86	82.96	82.64
(Binasarisha-5/Daulot)2-(Binasarisha-5/Daulot)7	85.85	79.24	88.88	84.65
(Binasarisha-5/Daulot)2-Binasarisha-5	78.61	79.24	93.33	83.72
(Binasarisha-5/Daulot)2-Daulot	71.23	86.73	60.00	72.65
(Binasarisha-5/Daulot)3-(Binasarisha-5/Daulot)4	94.41	73.12	85.92	84.48
(Binasarisha-5/Daulot)3-(Binasarisha-5/Daulot)5	84.90	82.57	60.58	76.01
(Binasarisha-5/Daulot)3-(Binasarisha-5/Daulot)6	82.19	78.10	94.94	85.07
(Binasarisha-5/Daulot)3-(Binasarisha-5/Daulot)7	92.83	72.72	83.63	83.06
(Binasarisha-5/Daulot)3-Binasarisha-5	82.98	72.72	88.59	81.43
(Binasarisha-5/Daulot)3- Daulot	80.46	85.92	76.25	80.87
(Binasarisha-5/Daulot)4-(Binasarisha-5/Daulot)5	84.95	72.83	73.00	76.92
(Binasarisha-5/Daulot)4-(Binasarisha-5/Daulot)6	81.89	76.29	82.96	80.38

Continued				
(Binasarisha-5/Daulot)4- (Binasarisha-5/Daulot)7	92.93	78.91	88.88	86.90
(Binasarisha-5/Daulot)4- Binasarisha-5	83.12	78.91	93.33	85.12
(Binasarisha-5/Daulot)4- Daulot	80.62	76.48	60.00	72.36
(Binasarisha-5/Daulot)5- (Binasarisha-5/Daulot)6	76.19	83.55	58.06	72.60
(Binasarisha-5/Daulot)5- (Binasarisha-5/Daulot)7	82.88	81.81	63.09	75.92
(Binasarisha-5/Daulot)5- Binasarisha-5	79.42	81.81	67.18	76.13
(Binasarisha-5/Daulot)5- Daulot	67.92	81.11	31.48	60.17
(Binasarisha-5/Daulot)6- (Binasarisha-5/Daulot)7	76.87	94.44	87.26	86.19
(Binasarisha-5/Daulot)6- Binasarisha-5	79.20	94.44	87.62	87.08
(Binasarisha-5/Daulot)6- Daulot	59.60	85.42	79.78	74.93
(Binasarisha-5/Daulot)7- Binasarisha-5	82.98	100.0	89.585	90.85
(Binasarisha-5/Daulot)7- Daulot	80.46	86.31	72.72	79.83
Binasarisha-5(E)- Daulot	68.05	86.31	67.77	74.04
Average	76.74	82.17	77.24	78.71

Table 3. Number and proportion of polymorphic bands, gene diversity obtained in different *Brassica* lines

Introgressed progeny lines/ Variety	Number of polymorphic Loci	Proportion of polymorphic loci (%)	Gene diversity
(Binasarisha-5/Daulot)1	7	30.43	0.140
(Binasarisha-5/Daulot)2	4	17.39	0.052
(Binasarisha-5/Daulot)3	3	13.04	0.047
(Binasarisha-5/Daulot)4	7	30.43	0.132
(Binasarisha-5/Daulot)5	10	43.48	0.187
(Binasarisha-5/Daulot)6	6	26.09	0.119
(Binasarisha-5/Daulot)7	2	8.70	0.034
Binasarisha-5	5	21.74	0.081
Daulot	2	8.70	0.026

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) values among studied *Brassica* progeny lines

Introgressed progeny lines/ Variety	E×G 1	E×G 2	E×G 3	E×G 4	E×G 5	E×G 6	E×G 7	E	G
E×G 1	****	0.881	0.789	0.777	0.838	0.769	0.663	0.733	0.491
E×G 2	0.126	***	0.915	0.925	0.847	0.839	0.771	0.790	0.633
E×G 3	0.235	0.088	***	0.901	0.788	0.860	0.758	0.736	0.727
E×G 4	0.251	0.077	0.104	***	0.833	0.798	0.827	0.802	0.646
E×G 5	0.176	0.165	0.237	0.182	***	0.758	0.690	0.751	0.508
E×G 6	0.262	0.174	0.150	0.225	0.275	***	0.779	0.881	0.631
E×G 7	0.410	0.259	0.276	0.189	0.371	0.249	***	0.875	0.686
E	0.310	0.234	0.305	0.220	0.285	0.126	0.132	***	0.602
G	0.709	0.456	0.317	0.436	0.677	0.459	0.376	0.507	****

**** Introgressed progeny lines/ Variety
E×G = Binasarisha-5/Daulot
E= Binasarisha-5
G= Daulot

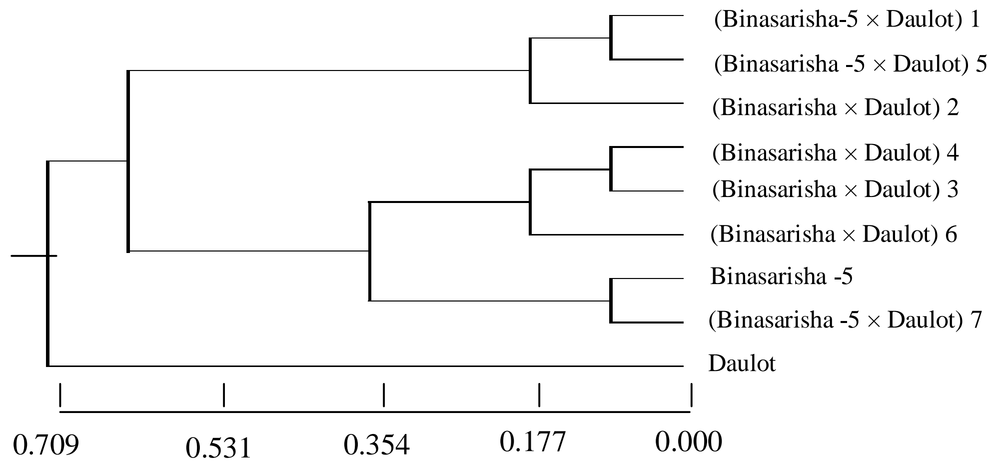

Figure 4. A UPGMA dendrogram based on Nei's (1972) genetic distance, summarizing the data on differentiation between nine *Brassica* lines according to RAPD analysis

Dendrogram based on Nei's (1972) genetic distance using Unweighted Pair Group Method of Arithemetic Means (UPGMA), the nine accessions were segregated into two major clusters (Fig. 4). As a result, one to seven introgressed progeny lines and Binasarisha-5

clustered into one group that had two sub-clusters. Introgressed lines of one, two and five were grouped in sub-cluster I and three, four, six, seven and Binasarisha-5 were grouped in sub-cluster II. In sub-cluster I, introgressed lines one and five showed closer relationship with minimal genetic distance (0.176). Again sub-cluster II divided into two sub sub-cluster. Introgressed lines three, four and six were grouped in sub sub-cluster I. Line three and four showed closer relationship with minimal genetic distance (0.104). In sub sub-cluster II, Binasharisha-5 and introgessed line seven clustered into another group and also showed closer relationship with minimal genetic distance (0.132). Daulot, which was *B. juncea* variety and formed one major cluster.

4. Conclusions

The present study indicated high level of genetic diversity among the nine accessions and proved that RAPD is an effective molecular marker in differentiating different varieties and introgressed progenies. The results suggest that the introgressed progenies could be used as source for breeding line and improved *Brassica* varieties. Though, larger number of samples and higher number of primers would be necessary to generate and construct an appropriate genetic relationship, sample identification and analysis of genetic variation among different varieties, cultivars and introgressed progenies is widely acceptable by all concern. Using larger number of samples and higher number of primers could be useful in future research.

5. Acknowledgement

Authors express thanks to the DANIDA for supporting this study through the Agriculture Extension Component/ Seed Wing of the Ministry of Agriculture (MoA), Government of the People's Republic of Bangladesh and to Mr. Anwar Faruqe, Joint Secretary MoA and Director-General of the Seed Wing, for his support and keen interest in the project activities.

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