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Full Length Research Paper

Genetic analysis of Ethiopian mustard genotypes using amplified fragment length polymorphism (AFLP) markers

Tsige Genet[1], C.D. Viljoen[2] and M.T. Labuschagne[2*]

[1]Amhara Agricultural Research Institute, Adet Research Center, P.O.Box 1626, Bahir Dar, Ethiopia.
[2]University of Free State, Department of Plant Sciences, P.O. Box 339 Bloemfontein 9300, Republic of South Africa.

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Genetic diversity within *Brassica carinata* A. Braun has not been extensively examined with molecular markers. The objective of this study was to investigate the genetic relationships among 39 *B. carinata* genotypes using amplified fragment length polymorphisms (AFLPs). Thirty-nine genotypes of *B. carinata* were analyzed using six AFLP primer combinations. A total of 189 polymorphic fragments were scored, with an average of 32 fragments per primer combination. Genetic distance estimates (GDEs) based on AFLPs was calculated and found to range from 0.346 to 0.639 with a mean of 0.504 ± 0.002. Polymorphic rates ranged from 50 to 80%. The unweighted pair group method of arithmetic averages (UPGMA) cluster analysis group these genotypes into seven distinct clusters. These data demonstrate that AFLP is a reliable tool and permits greater insights into the genetic diversity of *B. carinata*.

Key words: AFLPs, *Brassica carinata* A. Braun, cluster analysis, DNA fingerprinting, genetic distance.

INTRODUCTION

The genus *Brassica* includes a total of 41 species (Gladis and Hammer, 1990). Six of these are economically important species, namely, *Brassica rapa* (AA), *B. oleraceae* (CC), *B. nigra* (BB), *B. juncea* (AABB), *B. napus* (AACC) and *B. carinata* (BBCC). Ethiopian or Abyssinian mustard (*B. carinata)* is an important oil crop in Ethiopia. It is the third most important oil crop next to niger seed (*Guizotia abyssinica* Cass.) and linseed (*Linum usitatissimum* L) (CSA, 1998). It is also eaten as a vegetable, a condiment or salad, and even used as green manure. It is mainly self-pollinating.

B. carinata evolved as a natural cross between *B. nigra* (BB) (n=8) and *B. oleracea* (CC) (n=9), in the highlands of the Ethiopian plateau and the adjoining portion of East Africa and the Mediterranean coast and underwent further chromosomal doubling (2n=34) (UN, 1935; Gomez-Campo and Prakash, 1999). In Ethiopia, *B. carinata* is higher yielding, more resistant to diseases, insect pests, and seed shattering than *B. napus* with the additional agronomic advantages of better tolerance for semi-arid conditions (Knowles et al., 1981; Fereres et al., 1983; Malik, 1990). Hence, *B. carinata* can serve as an important source of genes, which are rare in other oilseed *Brassicas*.

For an efficient breeding program, information concerning the extent and nature of genetic diversity within a crop species is useful for characterizing individual accessions and cultivars in the selection of parents for hybridization (Rabbani et al., 1998a). There are various techniques for studying the genetic variability of crop germplasm, including morphological traits, total seed proteins, isozymes and various types of molecular markers (Rabbani et al., 1998a).

Different heterotic groups have been assigned on the basis of morphological traits and biochemical parameters such as isozyme analysis and seed storage proteins (Nucca et al., 1978; Arus et al., 1985). However, these

*Corresponding author. E-mail:Tsigegenet@yahoo.com; Fax: +251-08-280235; Tel: +251-08-280591.

markers reveal only limited polymorphisms among closely related genotypes and are influenced by prevailing environmental conditions. Therefore, a new technique that detects more polymorphisms than morphological traits and seed proteins must be explored for genetic characterization. DNA marker systems have been effectively used for genetic variation analysis (Lee, 1995). These markers can identify many genetic loci simultaneously with an excellent coverage of an entire genome, are phenotypically neutral, and can be applied at any development stage (Jones et al., 1997b). Furthermore, molecular markers are not subject to environmental change, making them especially informative and superior to traditional methods of genotyping such as the use of morphological traits and biochemical markers (Tanksley et al., 1989; Messmer et al., 1993; Melchinger et al., 1994).

Molecular marker techniques include restriction fragment length polymorphisms (RFLPs) (Beckman and Soller, 1983), simple sequence repeats (SSRs) or microsatellites (Tautz, 1989), random amplification of polymorphic DNA (RAPDs) (Williams et al., 1990; Welsh and McClelland, 1990; Karp et al., 1997) and amplified fragment length polymorphisms (AFLPs) (Vos et al., 1995). These techniques differ in their principles and applications and generate different amounts of data points (Das et al., 1999). RFLPs are well suited for the construction of linkage maps because of their high specificity (Chyi et al., 1992) and their codominant nature and have also been used for the analysis of genetic diversity (Song et al., 1988a; 1988b). Although RFLPs have been used to study the taxonomic status of a number of *Brassicaceae* species using nuclear and chloroplast DNA (Song et al., 1988a; 1988b; Pradhan et al., 1992; Lanner et al., 1997) this technique is labor intensive, time consuming, and expensive. Therefore, RFLPs is not a technique of choice for studying new or alternative crops where little prior data is available.

With the advent of the polymerase chain reaction (PCR) (Mullis et al., 1986) resulting in the further development of RAPDs, SSRs and AFLPs, most of the problems associated with RFLPs were overcome. The RAPD technique gained importance due to its simplicity and efficiency, and requiring no prior sequence knowledge (Karp et al., 1997). However, the RAPD technique has been shown to be not reproducible especially between laboratories as it is highly influenced by experimental conditions (Jones et al., 1997a; Karp et al., 1997; Virk et al., 2000; Devos and Gale, 1992; Staub et al., 1996). In *Brassica*, the RAPD assay has been employed to develop genome specific markers (Quiros et al., 1991) and to resolve taxonomic relationships (Demeke et al., 1992; Rabbani et al., 1998b). It has also been used for cultivar identification (Hu and Quiros, 1991; Kresovich et al., 1992; Das et al., 1999). However, finding stable RAPD polymorphic markers in rapeseed has proven difficult (Mailer et al., 1994). Simple sequence repeats (SSRs), which are based on microsatellite sequences, have been shown to detect very high levels of polymorphism (Karp et al., 1997). However, prior information about the genome is necessary before SSR markers can be exploited to their fullest potential.

AFLPs combine the advantage of time efficiency of PCR-based markers with the reliability of RFLP markers (Vos et al., 1995). The AFLP assay requires no prior sequence knowledge and detects at least 10 times more genetic loci than RFLPs, RAPDs and SSRs in most crops (Tohme et al., 1996; Maughan et al., 1996; Hill et al., 1996). Therefore, the AFLP assay has the ability to detect thousands of independent genetic loci in a short time. The aim of this study was to determine the feasibility of using AFLP markers to evaluate the genetic relationships between Ethiopian mustard cultivars and advanced breeding lines in order to maximize selection of diverse parents in a breeding program.

MATERIALS AND METHODS

Plant material

Thirty-nine B. carinata cultivars/landraces of diverse geographic origin were used in this study (Table 1). The material consisted of two nationally released cultivars (Yellow Dodolla and S-67), 18 advanced breeding lines, which were obtained from the Ethiopian national *B. carinata* breeding program and 20 landraces collected from different parts of the country by the Institute of Biodiversity Conservation and Research, Ethiopia. Plants were grown from seeds in pots in the greenhouse with 20ºC night temperature and 26ºC day temperature, during August 2001, at the University of the Free State, Bloemfontein, South Africa.

DNA extraction

DNA was extracted from fresh young leaves using a modified monocot extraction protocol (Edwards et al., 1991). Young plant leaf tissue was collected on ice, from one plant of each cultivar and ground into a fine powder in liquid nitrogen. The macerated tissue was incubated in 10 ml of extraction buffer (0.5 M NaCl, 0.1 M Tris-HCl pH 8, 0.25 M EDTA and 20% SDS) at 65ºC for 30 min, and with shaking every 10 min. One ml of 1% CTAB buffer (w/v) (1 M Tris-CHl, 0.25 M EDTA, and 1% CTAB) and 2 ml 5 M NaCl were added to the homogenate and incubated at 65ºC for one hour with mixing every 10 min. Chloroform-isoamyl alcohol (24:1 v/v) (10 ml) was added in a 1:1 ratio and mixed gently. Cell debris and proteins were removed by centrifugation for 15 min at 10 000 rpm. After centrifugation, the supernatant was retained and the DNA was precipitated by adding two volumes (v/v) of cold absolute ethanol (1:2) (%) followed by overnight incubation at 4ºC. The precipitated DNA was spooled with a sterile Pasteur pipette and washed twice in 1 ml 70% ethanol (EtOH) to remove residual salt. The DNA pellet was resuspended in 1 ml of sterile PCR grade water and stored at -20ºC.

DNA concentration and purity was determined spectrophotometerically at 260 and 280 nm (Hitachi U-2000). The DNA concentration was calculated using the formula, [DNA] = Optical density x dilution factor x constant (50 µg/ml). Purity was determined using the 260/280 nm ratio. DNA samples were diluted to a working concentration of 250 ng µlL-1 and stored at 4ºC. The integrity and concentration of the DNA was confirmed by 1%

Table 1. List of Ethiopian mustard genotypes used for genetic analysis.

No	Genotypes	No	Genotypes	No	Genotypes	No	Genotypes
1	Yellow Dodolla-03	11	Merawi	21	PGRC/E 21156	31	Yellow Dodolla
2	S-67-02	12	PGRC/E 20059	22	PGRC/E 20112/2	32	S-67
3	PGRC/E 21261-03	13	PGRC/E 21207	23	PGRC/E 200413	33	Adet
4	PGRC/E 203221-03	14	PGRC/E 20080	24	(4DxZem-1)x(Zem-1-AD/88)	34	PGRC/E 21358/2
5	C94-S-67	15	PGRC/E 207929	25	(4DxZem-1)x(Zem-1-F5/10)	35	PGRC/E 207928
6	C94-Dodolla	16	PGRC/E 207975	26	PGRC/E 21162/1	36	PGRC/E 21320/5
7	Yellow Dodolla-01	17	PGRC/E 20168/1	27	PGRC/E 20163/1	37	PGRC/E 20113
8	PGRC/E 203221-01	18	PGRC/E 20095/1	28	PGRC/E 20076/2	38	PGRC/E 20165
9	PGRC/E 21261-02	19	PGRC/E 21356/1	29	PGRC/E 20147/1	39	PGRC/E 21051
10	PGRC/E 20130	20	PGRC/E 21237	30	PGRC/E 208404		

Table 2. Adaptors and primers used for AFLP preamplification and selective amplification.

Name of Adapters/Primers	Sequence (5'-3')
(1) Adaptor:	
EcoR1 adaptor	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'
Mse1 adaptor	5'-GACGATGAGTCCTGAG-3' 3'-TACTCAGGACTCAT-5'
(2) Primers:	
Mse1 primer:	GATGAGTCCTGAGTAA
Mse1 +CTT	GATGAGTCCTGAGTAACTT
Mse1 +CAC	GATGAGTCCTGAGTAACAC
Mse1 +CTC	GATGAGTCCTGAGTAACTC
EcoR1 primer	GACTGCGTACCAATTC
EcoR1 + AAC(Ned)	GACTGCGTACCAATTCAAC
EcoR1 + ACT (Fam)	GACTGCGTACCAATTCACT
EcoR1 +ACA(Fam)	GACTGCGTACCAATTCACA

agarose electrophoresis for 45 min at 80 volts in 0.5X TAE (0.438 g/l Tris, 0.09 ml/L acetic acid, and 0.022 g/L acid EDTA) with visualization under UV light after staining with ethidium bromide.

AFLP procedure

The AFLP reactions were done according to the manufacturers instructions (Gibco BRL, 1996) using commercial adaptor and primer sequences (Table 2).

Restriction endonuclease digestion and ligation of adaptors

Genomic DNA (250 ng) was mixed with 5X reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM Mg-acetate, and 250 mM K-acetae) and 2 µl of the restriction enzyme EcoR1 and Mse1 and incubated for 2 h at 37ºC. The digested DNA fragments were then ligated to EcoR1 and Mse1 adaptors (Table 2) with 1 µl T4 DNA ligase (1 unit/µl in 10 mM Tris-HCl pH 7.5) for 2 h at 20ºC. The ligated DNA template was diluted 10 times with TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at -20ºC.

Polymerase chain reaction

Pre-selective PCR reaction were performed in 51 µl reactions with 5 µl (1:10) diluted ligation product, 40 µl pre-amp primer mix, 10X PCR buffer (100 mM Tris-HCl [pH 8.3], 15 mM MgCl2, and 500 mM KCl) and Taq DNA polymerase (1 unit/µl) (Gibco BRL). A touchdown Hybaid thermal cycler was used to perform the reaction for 20 cycles with the following profile: 30 s at 94ºC, 60 s at 56ºC and 60 s at 72ºC. Pre-selective PCR products were diluted 1:50 in TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at 4ºC.

Selective PCR-reactions were performed in 20 µl reactions containing 5 µl of diluted (1:50) pre-selective template DNA, 4.5 µl of Mse + primer (6.7 ng/l, and dNTPs) (Table 2), 1 µl Eco+ primer (27.8 ng/µl), 2 µl of 10X PCR buffer and 5 U of Ampli Taq DNA polymerase (unit/µl). Reactions were performed for 30 cycles with the following cycle profile: 30 s at 94ºC, 30 s at 65ºC and 60 s at 72ºC. The 65ºC annealing temperature was reduced by 0.7ºC per cycle for 12 consecutive cycles and then maintained at 56ºC for the remaining 18 cycles. Six primer combinations were tested. EcoR1 primers (PE Biosystems) were labeled with NED or FAM. After amplification, 5 µl of each selective reaction was added to 24 µl Formamide, and 1 µ l Rox standard size marker, denatured at 94ºC for 5 min, cooled on ice and resolved on a Perkin Elmer ABI Prism 310 Automated capillary sequencer (PE Biosystems).

Estimates of genetic distance

Genotypes were scored for presence and absence (1 for presence and 0 for absence) of AFLP bands and the data entered into a binary data matrix as discrete variables. Bands present in all accessions were not scored. Fragments smaller than 80 bp were excluded from the data matrix. Distance matrices for all pairs of genotypes were constructed from the AFLP data matrix using the Euclidean distance method (Kaufman and Rousseeuw, 1990). The Euclidean distances were calculated as follows:

$$GD = \sqrt{\sum [(X_i - Y_i)^2 / N]},$$

Table 3. Analysis of the level of polymorphism with AFLP primer combinations among 39 *B. carinata* genotypes.

Primer	Total number of fragments (a)	Polymorphic fragments	
		Number (b)	Polymorphism (%) (=b/a x (100))
M-CTT/E-ACC	39	28	72
M-CTT/E-ACT	80	64	80
M-CAC/E-AAC	47	30	64
M-CAC/E-ACA	28	14	50
M-CTC/E-ACC	30	18	60
M-CTC/E-ACT	54	35	65
Total	278	189	68
Average	46	32	

Where, GD is the genetic distance between individual X and individual Y ; $i = 1$ to N ; N is the total number of bands; X_i and Y_i are the i[th] band scores (1 or 0) for individuals X and Y .

Cluster analysis

Cluster analysis was performed using the genetic distance matrices generated by the Euclidean distance method to reveal the patterns of genetic relationships among genotypes. The unweighted pair group method of arithmetic averages (UPGMA) (Hintze, 2001), which minimizes within-cluster variance, was used. The results of cluster analysis were presented in the form of dendrograms to infer relationships among genotypes. The cophenetic correlation (Kaufman and Rousseeuw, 1990; Hintze, 2001) for each dendrogram was also computed as a measure of 'goodness of fit' for each dendrogram. The two delta goodness of fit statistics, delta (0.5) and delta (1.0) were calculated to determine which clustering configuration fits the data better (Mather, 1976).

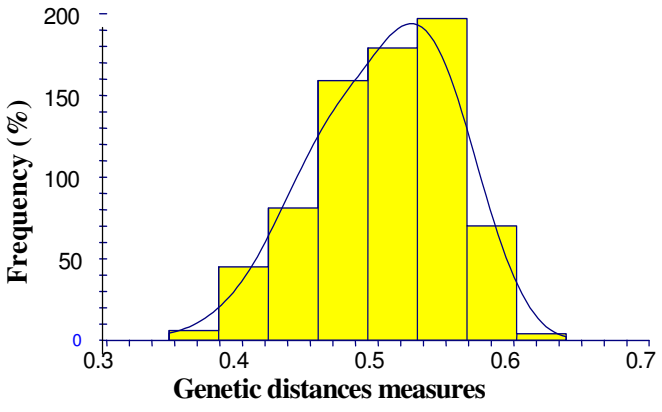


Figure 1. Frequency distribution of pair wise AFLP based genetic distance estimates (GDEs) among 39 *B. carinata* genotypes. Genetic distance estimates were calculated for all combinations (n=741).

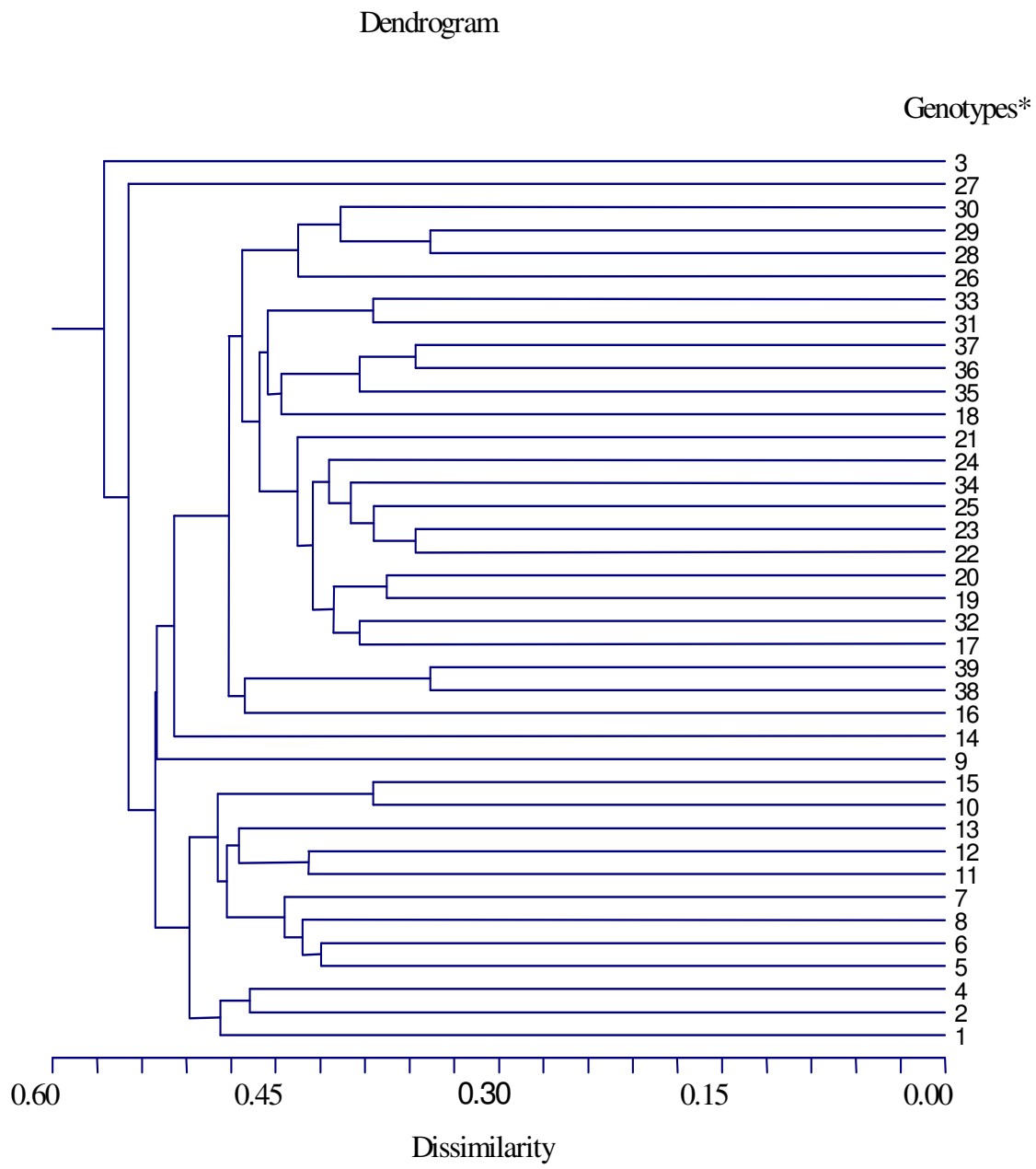
RESULTS AND DISCUSSION

Genetic diversity among *B. carinata* genotypes was assayed with AFLP techniques using six AFLP primer combinations. The number of scorable fragments amplified for each AFLP primer pair varied from 28 to 80 with an average of 46 across all the genotypes (Table 3). The number of polymorphic fragments for each primer pair varied from 14 to 64 with an average of 32 polymorphic fragments. A total of 189 AFLP fragments were polymorphic across all the genotypes for the six primer pairs assayed (Table 3). The 189 polymorphic fragments accounted for 68% of the total amplified fragments.

The fragments generated range in size from 56 to 497 bp. The number of fragments decreased as the size increased. Fragments longer than 400 bp were rarely detected. The number of fragments varied for different primer combinations (Table 3). The primer combination of M-CAC/E-ACA gave the smallest number of fragments (28) while all other primer combinations detected ≥ 30 fragments. The M-CTT/E-ACT, primer combination generated the highest number of polymorphic fragments, with an average of 80 fragments amplified to each (Table 3).

Genetic distance was determined for all 741 pair wise comparisons of genotypes. The range of dissimilarities ranged between 0.346 PGRC/E 20165 and PGRC/E 21051 as well as between PGRC/E 20076/1 and PGRC/E 20147/1 to 0.639 between PGRC/E 21261-03 and PGRC/E 20163/1. The mean genetic distance across all the samples was 0.504±0.002. The frequency of distribution of (Figure 1) indicated a normal distribution.

The dendrogram from UPGMA clustering method of 39 *B. carinata* genotypes based on Euclidean genetic distances matrix is shown in Figure 2. At a cut off 0.5 the dendrogram revealed seven major clusters consisting of three clusters with more than one genotype and four singletons. The correlation between the cophenetic



*1=Yellow Dodolla-03, 2=S-67-02, 3=PGRC/E 21261-03, 4=PGRC/E 203221-03, 5=C94-S-67, 6=C94-Dodolla, 7=Yellow Dodolla-01, 8=PGRC/E 203221-01, 9=PGRC/E 21261-02, 10=PGRC/E 20130, 11=Merawi, 12=PGRC/E 20059, 13=PGRC/E 21207, 14=PGRC/E 20080, 15=PGRC/E 207929, 16=PGRC/E 207975, 17=PGRC/E 20168/1, 18=PGRC/E 20095/1, 19=PGRC/E 21356/1, 20=PGRC/E 21237, 21=PGRC/E 21156, 22=PGRC/E 20112/2, 23=PGRC/E 200413, 24=(4DxZem-1) x (Zem-1-AD/88), 25=(4DxZem-1) x (Zem-1-F5/10), 26=PGRC/E 21162/1, 27=PGRC/E 20163/1, 28=PGRC/E 20076/2, 29=PGRC/E 20147/1, 30=PGRC/E 208404, 31=Yellow Dodolla, 32=S-67, 33=Adet, 34=PGRC/E 21358/2, 35=PGRC/E 207928, 36=PGRC/E 21320/5, 37=PGRC/E 20113, 38=PGRC/E 20165, 39=PGRC/E 21051.

Figure 2. Dendrogram generated based on UPGMA clustering method depicting genetic relationships among 39 *B. carinata* genotypes based on AFLP data.

values was very high (0.82) indicating a very good fit of the cluster analysis performed. The two delta goodness of fit statistics, delta (0.5) and delta (1.0) were (0.04) and (0.06), respectively (Mather, 1976). Cluster analysis separated the 39 genotypes into seven major clusters. The first cluster consists of 23 genotypes with a genetic distance value ranging from 0.437 between PGRC/E 20112/2 and PGRC/E 200413 to 0.664 between PGRC/E 21156 and PGRC/E 20165 with a mean value of 0.557±0.002. This cluster further divided into two distinct

sub clusters. The first sub cluster consisted of genotypes PGRC/E 207975, PGRC/E 20165, and PGRC/E 21051. The second sub cluster consisted of 20 other genotypes. Cluster two consisted of three genotypes, Yellow Dodolla, S-67-02, and PGRC/E 203221-03. Cluster three had three sub clusters. The first sub cluster had C94-S-67, C94-Dodolla, PGRC/E 203221-01 and Yellow Dodolla-01.

The four genotypes, PGRC/E 21261-03, PGRC/E 21261-02, PGRC/E 20080 and PGRC/E 20163/1 clustered independently (Figure 2). Divergent genotypes may have good breeding values. Genotypes in the same cluster may represent members of one heterotic group. Maximum variability for selection in segregating populations may be achieved by utilizing genotypes from different clusters as parents for crosses.

Knowledge of genetic relationships among genotypes is useful in plant breeding programs because it permits the organization of germplasm and provides information for more efficient parental selection. The breeder can use genetic distance information to make informed decisions regarding the choice of genotypes to cross for the development of populations, or to facilitate the identification of diverse parents to cross in hybrid combinations in order to maximize the expression of heterosis (Smith et al., 1990). Furthermore, fingerprinting genotypes offers the opportunity for the removal of any duplicate germplasms. In B. carinata, information on genetic diversity and/or genetic relationships among genotypes is currently limited. Therefore, the AFLP DNA pattern, or fingerprint should be a valuable tool for breeders.

Lombard et al. (2000) in their study of the genetic relationships and fingerprinting of 83 rapeseed (*B. napus* L.) cultivars by AFLP showed that AFLP markers functioned well in the assessment of genetic relatedness between rapeseed cultivars in the context of plant registration and protection. A total of 324 polymorphic fragments were generated with 17 primer combinations. The number of markers per primer combination ranged from 12 to 30, with an average of 19.1. The most polymorphic primer combinations were M-CAA+E-AAC, M-CTT+E-AAC, and M-CTT+E-AAG, which produced 30 markers each. The fragment sizes ranged from 100 to 750 bp. Das et al. (1999) assessed the genetic variation of B. campestries cultivars with AFLP and RAPD markers. RAPDs generated a total of 125 bands using 13 decamer primers (an average of 9.6 bands per assay) of which nearly 80% were polymorphic. The percent polymorphism ranged from 60-100%. AFLPs, on the other hand generated a total of 319 markers, an average of 64 bands per assay. Of these, 213 were polymorphic in nature (66.8%). AFLP methodology detected polymorphism more efficiently than the RAPD approach due to greater numbers of loci assayed per reaction. However, the level of polymorphism was higher with RAPD (80 %) than with AFLP (66.8 %). Although AFLPs do not reveal a high percentage of polymorphism but are more efficient as they produce more number of bands per reaction as compared to RAPD. Thus, AFLP has a higher marker index, an overall measure of marker efficiency (Powell et al., 1996; Nakajima et al., 1998). AFLP has also been reported to be highly reproducible with low error rates, which provides a definite advantage over RAPD (Jones et al., 1997a).

In conclusion, AFLP markers exhibited a high level of efficiency for detecting DNA polymorphisms among B. carinata genotypes. Knowledge of diversity patterns and specific genetic distance estimates may increase the efficiency of B. carinata genetic improvement in Ethiopia among adapted parents used for cultivar development, and providing predictive measure of such important parameters as population genetic variance and heterosis.

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