Egypt. J. Exp. Biol. (Bot.), 11(2): 245 – 256 (2015)

RESEARCH ARTICLE

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GENETIC DIVERSITY AND PHYLOGENETIC RELATIONSHIPS OF *BRASSICA NAPUS* L. AS REVEALED BY PROTEIN PROFILING AND SSR MARKERS

ABSTRACT:

Brassica napus L. (AACC, 2n = 38) is one of the most economically important crop species worldwide. Evaluating the genetic diversity of this crop species is essential for establishing efficient conservation and breeding practices. This study aimed to assess the genetic diversity, genetic structure and relationships of 26 *Brassica napus* accessions of different origins using SDS-PAGE of total seed proteins and microsatellite (SSR) markers. The percentage of protein polymorphism observed among accessions was 61%. SDS-PAGE results also revealed that the total amount of variation accounted for the first three principal components was 73%. Cluster analysis based on the protein data divided the 26 accessions into 4 groups. The 11 SSR primer pairs used revealed 14 loci of a total of 50 alleles. The observed heterozygosity (0.47) was higher than the expected one (0.298). Polymorphic information content (PIC) varied from 0.141 to 0.743, with an average of 0.59 per SSR primer pair. SSR results also showed that 58.8% of the total variation was found among accessions, and 41.2% of the SSR variation resided within accessions. Cluster analysis based on SSR data revealed that the two winter oilseed rape cultivars CGN06870 and CGN17374 were grouped with the spring oilseed accessions. The spring oilseed rape cultivar CGN11019 was grouped with the winter oilseed accessions. The genetically diverse spring and winter oilseed genotypes identified could be useful resources for oilseed rape breeding. The useful results of this study could be used for enhancing and broadening the genetic base of *Brassica napus* gene pool.

KEY WORDS:

Brassica napus, genetic diversity, phylogenetic relationships, SDS-PAGE, SSR.

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ARTICLE CODE: 27.02.15

INTRODUCTION:

Brassica napus L. (AACC, 2n = 38) is an important crop species that originated in a limited geographic region through spontaneous interspecific hybridizations of *Brassica rapa* (AA, 2n = 20) and *Brassica oleracea* (CC, 2n = 18), followed by chromosome doubling (UN, 1935; Hasan *et al*., 2006; Fu and Gugel, 2010; El-Esawi, 2015; El-Esawi *et al*., 2012). This species includes oilseed rape, swede or rutabaga, vegetable types and fodder crops (Snowdon *et al*., 2007; Wu *et al*., 2014). *Brassica napus* is predominantly self-compatible (McNaughton, 1995; Fu and Gugel, 2010), with interplant out-crossing rates varying between 20-45% under field conditions (Olsson, 1960; Rakow and Woods, 1987; Becker *et al*., 1992; Damgaard and Loeschcke, 1994). Nowadays, *Brassica napus* is the world's third most important source of vegetable oil after soybean and palm (Moghaieb *et al*., 2014). Plant breeders seek to develop new *Brassica napus* cultivars of nutritionally beneficial high-oleic acid oil to replace harmful saturated palm oil in food applications (Spector, 1999; Stoutjesdijk *et al*., 2000). In Egypt, the two cultivars of Serw-3 and Serw-4 seem to be promising for their high seed oil contents (40-42%) and should be used to increase *Brassica napus* production (Moghaieb *et al*., 2014). However, the limited geographic range and intensive breeding of *Brassica napus* have led to a narrow genetic basis in its current breeding material (Hasan *et al*., 2006; Fu and Gugel, 2010). The gene pool of *Brassica napus* breeding material has been further eroded by an emphasis on oil and seed quality traits (Hasan *et al*., 2006). Consequently, genetic variation in *Brassica napus* is limited regarding many valuable characters for breeding purposes. Therefore, assessment of genetic diversity and phylogenetic relationships in this important crop is essential for establishing efficient conservation and future breeding practices (Hasan *et al*., 2006; Moghaieb *et al*., 2014).

Over the last years, efforts have been made to evaluate the genetic diversity and relationships of *Brassica napus* germplasm using a variety of biochemical and molecular techniques such as total seed proteins using

sodium dodecyl sulphate polyacrylamide gel electrophoresis "SDS-PAGE" (Curn, 1997; Mukhlesur and Hirata, 2004; Sadia *et al*., 2009; Turi *et al*., 2010; Khurshid and Rabbani, 2012; Khan *et al*., 2014; Choudhary *et al*., 2015), allozyme (Becker *et al*., 1992), random amplified polymorphic DNA "RAPD" (Lazaro and Aguinaglde, 1998; Shengwu *et al*., 2003; Cartea *et al*., 2005; Asghari *et al*., 2011), restriction fragment length polymorphism "RFLP" (Diers and Osborn, 1994; Diers *et al*., 1996), amplified fragment length polymorphism "AFLP" (Lombard *et al*., 2000), inter-simple sequence repeat "ISSR" (Chao-zhi *et al*., 2003) and simple sequence repeat "SSR" (Hasan *et al*., 2006; Wang *et al*., 2009; Fu and Gugel, 2010; Li *et al*., 2011; Moghaieb *et al*., 2014; Wu *et al*., 2014) markers. In comparison with other molecular techniques, SSR markers (alternatively known as microsatellites) have been more useful and efficient for genetic diversity and structure studies of *Brassica* because they are numerous, highly polymorphic, informative, codominant, technically simple, reproducible and relatively inexpensive (Hasan *et al*., 2006; Qu *et al*., 2012). Moreover, SSR markers often occur in gene-rich genome regions, enhancing their potential relevance for allele-trait association studies in well-characterized genome regions containing quantitative trait loci (Hasan *et al*., 2006; Qu *et al*., 2012). Hasan *et al*. (2006) used SSR markers to assess the genetic diversity of *Brassica napus* germplasm. Cluster analysis identified four main groups comprising spring oilseed and fodder, winter oilseed, winter fodder, and vegetable genotypes. Fu and Gugel (2010) studied the genetic diversity of *Brassica napus* using 22 SSR primer pairs, and detected a total of 33 loci. The polymorphic information content per SSR primer pair ranged from 0.01 to 0.99 with an average of 0.43. The percentage of the total SSR variation among cultivars was higher than that resided within cultivars. Li *et al*. (2011) used SSR and AFLP markers to assess the genetic diversity of 25 *Brassica napus* hybrids. The expected heterozygosity and the genetic differentiation detected by SSRs were higher than those revealed by AFLPs. Moghaieb *et al*. (2014) assessed the genetic diversity among four *Brassica napus* cultivars (namely, Serw-3, Serw-4, Misser L-16 and Semu 249), using SSR, RAPD and AFLP markers. The data indicated that all of the three molecular markers gave different levels of polymorphism. The above studies have provided useful information for understanding the genetic diversity and phylogenetic relationships of *Brassica napus* germplasm, however most of them have investigated a limited range of genotypes (Hasan *et al*., 2006; Fu and Gugel, 2010). Therefore, more information on genetic diversity and relationships of *Brassica napus* genotypes of different origins is still required in order to help select diverse promising parents for yield and quality improvement (Hasan *et al*., 2006; Fu and Gugel, 2010). Consequently, the objectives of the present study were to assess the genetic diversity, genetic structure and relationships of *Brassica napus* genotypes using SDS-PAGE of total seed proteins and SSR markers.

MATERIAL AND METHODS:

Plant material:

The present study covered 26 accessions of *Brassica napus* (Table 1), out of which 22 accessions were obtained from the Centre for Genetic Resources (CGN) in the Netherlands, and 4 genotypes, namely Serw-3, Serw-4, Semu 249 and Misser L-16 were kindly provided by Agricultural Research Center, Ministry of Agriculture in Egypt. Those 26 accessions represented diverse geographical origins worldwide.

Total seed protein extraction and electrophoresis:

For total seed protein extraction, *Brassica* seeds were ground to a fine powder and 10 mg of each sample was added to 400 µl of protein extraction buffer (0.05 M Tris-Hcl (pH 8.0), 0.2% SDS, 5 M Urea, 1% 2-mercaptoethanol, and 0.002% bromophenol blue) and mixed thoroughly by vortexing and then centrifuged at 15000 rpm for 5 minutes at room temperature. The extracted crude proteins were recovered as supernatant for using in electrophoresis. SDS-PAGE of the extracted protein solutions was performed in 12.25% polyacrylamide gel according to the protocol of Laemmli (1970). Electrophoresis was carried out in an electrode buffer (0.025M Tris, 0.129M glycine and 0.125% SDS) at 100V for 3 hours until the bromophenol blue dye reached the gel bottom. BenchMark™ prestained protein ladder (Thermo Scientific), within a range of 6 to 180 kDa, was used as a protein molecular weight standard. At the end of electrophoresis, protein bands were revealed by Comassie Brilliant Blue R-250 staining and distained by methanol and acetic acid solution for overnight. The gels were then photographed.

DNA extraction:

The plant seeds were grown in pots in the laboratory at 20ºC for 2 weeks. The genomic DNA was separately extracted from 3-5 individual plants from each accession using the DNeasy Plant Mini Kit (Qiagen, UK) according to the manufacturer's instructions. The quality and quantity of the extracted DNA were measured using nano-drop 2000C (Thermo Scientific) and its integrity was tested in 1% agarose gel. DNA concentration of all samples was adjusted to 25 ng/µl for PCR reactions.

Table 1. Accession numbers, taxonomic group and origin country of the accessions of *Brassica napus* L. studied.

No.	Accession Number	Taxonomic group and accession name	Country of origin
1	CGN06892	Fodder Rape (Potem)	France
2	CGN07231	Fodder Rape (Akela)	Netherlands
3	CGN06888	Fodder Rape (Vysokopol'skij 12)	USSR
4	CGN15178	Fodder Rape (Elsoms Giant)	United Kingdom
5	CGN06887	Fodder Rape (Ragged Jack Kale)	New Zealand
6	CGN19964	Spring Oilseed Rape (Altex)	Canada
7	CGN12014	Spring Oilseed Rape (Esora)	Germany
8	CGN19968	Spring Oilseed Rape (Hanna)	Netherlands
9	CGN11019	Spring Oilseed Rape (Bronowsky)	Poland
10	CGN19967	Spring Oilseed Rape (Global)	Sweden
11	CGN06900	Swede (York)	Germany
12	CGN06899	Swede (York)	Germany
13	CGN15779	Swede (Friese Gele)	Netherlands
14	CGN07237	Swede (Hollandse Gele)	Netherlands
15	CGN06902	Swede	New Zealand
16	CGN06870	Winter Oilseed Rape (Slapska)	Czechoslovakia
17	CGN17305	Winter Oilseed Rape (Shen-Li Jutsaj)	China
18	CGN17324	Winter Oilseed Rape (Fertodi)	Hungary
19	CGN17374	Winter Oilseed Rape (Olimpiade)	Italy
20	CGN17306	Winter Oilseed Rape	Morocco
21	CGN17308	Winter Oilseed Rape (Kombainer)	Ukraine
22	CGN13915	Winter Oilseed Rape (Bridger; US 8500171)	USA
23	-	Serw-3	Egypt
24	-	Serw-4	Egypt
25	-	Semu 249	Egypt
26	-	Misser L-16	Egypt

SSRs analysis:

A total of 16 SSR primer pairs were selected from available literatures (Lowe *et al*., 2004; Piquemal *et al*., 2005; Choi *et al*., 2007; Long *et al*., 2007; Rahman and Peter, 2007; Cheng *et al*., 2009), and were screened for polymorphisms. Following the pre-screening, only 11 primer pairs revealed polymorphism and were used to analyse all the accessions of the current study. The PCR reactions were carried out in a final volume of 25 µl containing 2 µl of genomic DNA (25 ng/µl), 1.5 µl of forward primer (50 ng/µl), 1.5 µl of reverse primer (50 ng/µl), 12.5 µl of GoTaq® green master mixture and 7.5 µl of nuclease-free water. The PCR reactions were then amplified in a Bio-Rad thermocycler programmed as follows: 94ºC for 4 min as initial denaturation followed by 35 cycles of 94ºC for 45 sec., 55ºC for 45 sec. and 72ºC for 1 min. The PCR products were then left at 72ºC for 15 min for final extension. The amplified PCR products were resolved in 2% (w/v) agarose gels stained with ethidium bromide. A 50 bp DNA ladder was used as a DNA molecular size standard. Bands were detected and photographed using a UVP gel documentation system.

Data analysis:

For protein data, molecular weight of visually clear protein bands was calculated using LabImage software version 2.7 produced by Kapelan GmbH, Germany. The protein bands were then scored as 0 for absence and 1 for presence. The obtained binary data set was analysed using SYSTAT version 7 software package, and cluster and factor analyses were conducted to evaluate the genetic diversity among accessions. The dendrogram was constructed based on Nei's (1978) genetic distance using unweighted pair group method with arithmetic average (UPGMA).

For SSR data, microsatellites were scored as homozygotic and heterozygotic genotypes due to their codominance. For SSR primer pairs that amplified more than one locus, each allele was assigned to a specific locus. The number of loci recorded for each SSR primer pair was determined by the observed allelic pattern along with the information available from related articles (Lowe *et al*., 2004; Piquemal *et al*., 2005; Fu and Gugel, 2010). The SSR data were analysed using GenAlEx version 6 (Peakall and Smouse, 2006) and POPGENE version 1.31 (Yeh *et al*., 1999), and a dendrogram was constructed based on Nei's genetic distance using UPGMA (Nei, 1978). The partitioning of total genetic diversity (H_T) into within- (H_S) and among- (D_{ST}) accession components was examined using Nei (1973 & 1978) genetic diversity statistics ($H_T = H_S+D_{ST}$). F-statistics were calculated under the infinite allele model:

$1-F_{IT} = (1-F_{IS})\ (1-F_{ST})$, whereas, F_{ST} is the inter-accession genetic differentiation due to genetic drift, F_{IS} is the fixation index related to non-random mating within accessions, and F_{IT} is the mean inbreeding coefficient of a set of accessions. The polymorphic information content (PIC) of each SSR primer pair was also calculated, as described in Roussel *et al*. (2004).

RESULTS:

Total seed protein analysis:

Total seed proteins of 26 accessions of *Brassica napus* L. were analyzed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Table 2 shows the presence and absence of SDS-PAGE protein bands in the 26 accessions of *Brassica napus* analyzed. In total, 18 protein bands ranging from 16 kDa to 46 kDa were observed; 11 bands (61%) were polymorphic and 7 bands (39%) were monomorphic (Table 2). Variation was also detected in the density or sharpness of protein bands.

Table 2. Presence and absence of SDS-PAGE protein bands in the 26 *Brassica napus* accessions studied.

Protein bands	Molecular weight (kDa)	No. of accessions	
		Presence	Absence
1	46	20	6
2	43	21	5
3	40	26	0
4	37	17	9
5	35	26	0
6	32	12	14
7	30	14	12
8	28	26	0
9	26	19	7
10	25	23	3
11	24	16	10
12	23	16	10
13	22	4	22
14	22	20	6
15	21	26	0
16	20	26	0
17	17	26	0
18	16	26	0

A dendrogram was constructed based on protein data using Euclidean distance matrix on average linkage (Fig. 1). An arbitrary genetic distance of 0.39 divided the 26 accessions into 4 groups i.e. Group I, II, III and IV. The four Egyptian cultivars (Serw-3, Serw-4, Misser L-16 and Semu 249) were clustered in Group I. All accessions of winter oilseed rape and fodder rape could not be distinguished and were distributed within Group II. Group III comprised 4 accessions of spring oilseed rape (CGN12014, CGN19968, CGN11019, CGN19967) and one swede accession (CGN06902). Group IV consisted of the remainder of swede accessions (CGN06900, CGN06899, CGN15779, CGN07237) and one accession of spring oilseed rape (CGN19964).

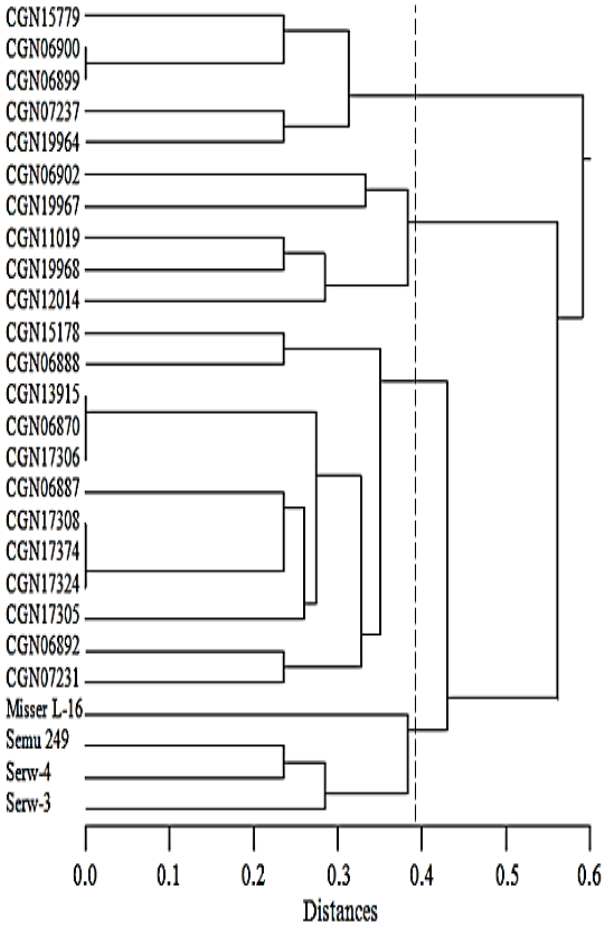

Fig. 1. UPGMA dendrogram showing the genetic relationships among 26 *Brassica napus* L. accessions based on SDS-PAGE of total seed proteins.

The principal component analysis (PCA) of the protein data shows that the first three components accounted for 73% of the total variation (Table 3). Principal Component 1 (PC1), PC2 and PC3 contributed 39.738%, 18.630%, and 14.636% of the total seed protein banding patterns variation, respectively. The genotypes contributed most to the first principal component included all accessions of fodder rape and winter oilseed rape, 3 accessions of spring oilseed rape (CGN12014, CGN19968, CGN19967), one swede accession (CGN06902), and one Egyptian cultivar (Misser L-16). The genotypes contributed most to the second principal component comprised one accession of spring oilseed rape (CGN11019) and 3 Egyptian cultivars (Serw-3, Serw-4, Semu 249). The genotypes contributed most to the third principal component included one accession of spring oilseed rape (CGN19964) and 4 swede accessions (CGN06900, CGN06899, CGN15779, CGN07237).

Table 3. Matrix of eigenvectors and values of the principal components for protein data of *Brassica napus* L. accessions.

Accession number	Principal components		
	C1	C2	C3
Fodder Rape CGN06892	0.606	-0.154	-0.313
Fodder Rape CGN07231	0.686	0.160	-0.166
Fodder Rape CGN06888	0.826	0.190	-0.212
Fodder Rape CGN15178	0.788	0.128	0.063
Fodder Rape CGN06887	0.776	0.043	-0.133
Spring Oilseed Rape CGN19964	0.533	-0.290	0.631
Spring Oilseed Rape CGN12014	0.717	0.374	0.368
Spring Oilseed Rape CGN19968	0.749	0.468	0.259
Spring Oilseed Rape CGN11019	0.395	0.685	0.434
Spring Oilseed Rape CGN19967	0.666	0.504	-0.059
Swede CGN06900	0.376	-0.609	0.673
Swede CGN06899	0.376	-0.609	0.673
Swede CGN15779	0.495	-0.471	0.575
Swede CGN07237	0.497	-0.367	0.726
Swede CGN06902	0.667	0.341	0.294
Winter Oilseed Rape CGN06870	0.772	-0.119	-0.367
Winter Oilseed Rape CGN17305	0.503	-0.302	-0.408
Winter Oilseed Rape CGN17324	0.781	-0.414	-0.341
Winter Oilseed Rape CGN17374	0.781	-0.414	-0.341
Winter Oilseed Rape CGN17306	0.772	-0.119	-0.367
Winter Oilseed Rape CGN17308	0.781	-0.414	-0.341
Winter Oilseed Rape CGN13915	0.772	-0.119	-0.367
Serw-3	0.040	0.603	0.084
Serw-4	0.236	0.784	0.178
Semu 249	0.371	0.815	0.050
Misser L-16	0.588	0.263	0.082
Variance Explained by Components	10.332	4.844	3.805
Percent of Total Variance Explained	39.738	18.630	14.636
Accumulated Eigenvectors	39.738	58.368	73.004

Microsatellites (SSRs) analysis:

SSR loci and alleles scored:

The 11 SSR primer pairs used in this study revealed a total of 14 loci which were found to be polymorphic (Table 4). Only three SSR primer pairs (BN35D, CNU-SSR149, CNU-SSR223) revealed more than one locus. A total of 50 SSR alleles were observed across all loci. The number of alleles amplified by each SSR primer pair varied from 3 to 7, with an average of 4.6 alleles per SSR primer pair. The primer pairs CN78 and MR47MR32 amplified the lowest number of alleles (3). The primer pair CNU-SSR149 amplified the highest number of alleles (7). The polymorphic information content (PIC) values varied from 0.141 to 0.743, with an average of 0.59 per SSR primer pair. The most informative SSR primer pairs were CN17 for linkage group N13, followed by primer pairs ENA6 and CB10196 for linkage groups N7 and N4, respectively. Nine SSR primer pairs had PIC values above 0.5.

Table 4. SSR primer pairs, number of alleles and loci, and polymorphic information content (PIC) of each primer set amplified in the 26 accessions of *Brassica napus*.

SSR primer pairs	Linkage group	Sequences	No. of alleles detected	No. of loci	PIC
CN17	N13	F: CACCATCACCACCTTCACAA R: TGGTTCACTCATGTCTCCGA	4	1	0.743
CN78	N9/N18	F: AGTCGGGCTCGTATATCTCG R: GTTTCGTGGCGGAAATTAGA	3	1	0.560
CB10196	N4	F: TTGTAGGCAATGATGAGGA R: GAGAGAAGGGCTCCTTTG	4	1	0.717
ENA6	N7	F: CTCGTCTTCTTCACCTACAAC R: CTGACATCTTTCTCACCCAC	4	1	0.739
EJU4	N8	F: CACCTTATCATCTCTCTATCCC R: CCTCTGTTTCTCTCCTTGTG	5	1	0.684
Ra2-A01	N7	F: TTCAAAGGATAAGGGCATCG R: TCTTCTTCTTTTGTTGTCTTCCG	4	1	0.597
Niab_ssr013	N5	F: GGAACCGTCCTTACTTTCTCTGT R: AGGATTGTGTTTTCCACATTGTC	5	1	0.563
MR47MR32	SCAR	F: TGAACTGTGGAAGCCAAGC R: TCACCACTACGCGGTAACTG	3	1	0.647
BN35D	N1/N11	F: GCAGAAGGAGGAGAAGAGTTGG R: TTGAGCCGTAAAGTTGTCACCT	5	2	0.423
CNU-SSR149	N6	F: GGAAGCCTCTGTGCGAAAAA R: TGCCGACGATTTGATAGAGGA	7	2	0.689
CNU-SSR223	N3	F: ACCCGAAAAGAGAATATGGCCT R: ACAGTGGCGTTAGGTGGGG	6	2	0.141
Total			50	14	
Mean ± Standard Deviation			4.6 ± 1.2		0.59 ± 0.18

Genetic diversity and accession-level heterozygosity:

The genetic diversity of each accession was quantified using the proportion of polymorphic SSR loci, SSR allelic count, heterozygosity, and fixation index (Table 5). The proportion of polymorphic loci (P) was variable among accessions, and varied between 28.6-85.7%, with an average of 51.1%. The mean number of alleles per locus (A) for each accession varied from 1.286 to 1.857, with an average of 1.51. The effective number of alleles per locus (A_e) ranged from 1.257 for fodder rape (CGN07231) to 1.857 for winter oilseed rape (CGN06870), with an average of 1.49. The observed heterozygosity (H_o) varied from 0.238 for fodder rape (CGN07231) to 0.857 for winter oilseed rape (CGN06870), with an average of 0.47. The expected heterozygosity (H_e) ranged from 0.162 for fodder rape (CGN07231) to 0.514 for winter oilseed rape (CGN06870), with an average of 0.298. The average fixation indices values (F) were lower than zero for all the accessions studied, indicating an excess of heterozygotes.

Genetic structure:

Eleven out of the 14 SSR loci detected were statistically significant ($p < 0.001$) for discriminating among the 26 accessions of *Brassica napus* studied (Table 6). F-statistics showed varying fixation indices among SSR loci (Table 6). The estimates of fixation indices (F_{IS}) showed that all SSR loci (except CNU-SSR149-2) were with excess of heterozygotes, as revealed by the negative average values of their fixation indices. The locus CNU-SSR149-2 exhibited heterozygote deficiencies. The F_{IS} values varied from -1.000 to 0.268, with an average of -0.886. Moreover, the mean inbreeding coefficient (F_{IT}) ranged from -0.275 (locus CN17) to 0.902 (locus CNU-SSR149-2), with an average of 0.224. The inter-accession genetic differentiation (F_{ST}) values for all accessions ranged from 0.340 (locus CN17) to 0.877 (locus CNU-SSR223-1), with an average of 0.588.

The estimates of accessions genetic structure using Nei's (1973) genetic diversity statistics are shown in Table 6. The total genetic diversity (H_T) ranged from 0.345 to 0.743, with an average value of 0.603. The average values of intra-accessional genetic diversity (H_S) and inter-accessional genetic diversity (D_{ST}) were 0.248 and 0.355, respectively.

Table 5. Percentage of polymorphic SSR loci, estimates of heterozygosity, number of alleles per locus, and fixation index of the 26 accessions of *Brassica napus*.

Accessions	P	A	A_e	H_o	H_e	F
Fodder Rape CGN06892	28.6	1.286	1.271	0.262	0.167	-0.569
Fodder Rape CGN07231	28.6	1.286	1.257	0.238	0.162	-0.469
Fodder Rape CGN06888	28.6	1.286	1.271	0.262	0.167	-0.569
Fodder Rape CGN15178	28.6	1.286	1.271	0.262	0.167	-0.569
Fodder Rape CGN06887	35.7	1.357	1.343	0.285	0.210	-0.357
Spring Oilseed Rape CGN19964	64.3	1.643	1.643	0.643	0.386	-0.666
Spring Oilseed Rape CGN12014	64.3	1.643	1.614	0.595	0.376	-0.583
Spring Oilseed Rape CGN19968	71.4	1.714	1.671	0.595	0.414	-0.437
Spring Oilseed Rape CGN11019	35.7	1.357	1.357	0.357	0.214	-0.668
Spring Oilseed Rape CGN19967	64.3	1.643	1.643	0.643	0.386	-0.666
Swede CGN06900	50.0	1.500	1.471	0.452	0.291	-0.553
Swede CGN06899	50.0	1.500	1.471	0.452	0.291	-0.553
Swede CGN15779	50.0	1.500	1.471	0.452	0.291	-0.553
Swede CGN07237	50.0	1.500	1.457	0.429	0.286	-0.500
Swede CGN06902	57.1	1.571	1.529	0.452	0.329	-0.374
Winter Oilseed Rape CGN06870	85.7	1.857	1.857	0.857	0.514	-0.667
Winter Oilseed Rape CGN17305	50.0	1.500	1.500	0.500	0.300	-0.667
Winter Oilseed Rape CGN17324	50.0	1.500	1.500	0.500	0.300	-0.667
Winter Oilseed Rape CGN17374	85.7	1.857	1.842	0.833	0.510	-0.633
Winter Oilseed Rape CGN17306	50.0	1.500	1.500	0.500	0.300	-0.667
Winter Oilseed Rape CGN17308	57.1	1.571	1.543	0.500	0.316	-0.582
Winter Oilseed Rape CGN13915	50.0	1.500	1.500	0.500	0.300	-0.667
Serw-3	42.9	1.429	1.429	0.429	0.257	-0.669
Serw-4	42.9	1.429	1.400	0.381	0.248	-0.536
Semu 249	42.9	1.429	1.414	0.405	0.252	-0.607
Misser L-16	64.3	1.643	1.506	0.429	0.302	-0.421
Mean ± Standard Deviation	51.1 ± 15.9	1.511 ± 0.16	1.490 ± 0.16	0.470 ± 0.16	0.298 ± 0.09	- 0.578 ± 0.1

P, percentage of polymorphic loci (%); A, the mean number of alleles per locus; A_e, the effective number of alleles per locus; H_o, the observed heterozygosity; H_e, the expected heterozygosity; F, Wright's fixation index [$F = (1 - H_o/ H_e)$].

Table 6. F-statistics and Nei (1973) genetic diversity indices of the 26 accessions of *Brassica napus*.

SSR loci	F-statistics			Nei's genetic diversity indices			X^2	P
	F_{IS}	F_{IT}	F_{ST}	H_T	H_S	D_{ST}		
CN17	-0.930	-0.275	0.340	0.743	0.490	0.253	139.7	0.000*
CN78	-0.801	0.760	0.867	0.560	0.074	0.486	83.59	0.000*
CB10196	-0.822	0.162	0.540	0.717	0.330	0.387	97.62	0.000*
ENA6	-0.957	-0.060	0.459	0.739	0.400	0.339	110.5	0.000*
EJU4	-0.891	-0.090	0.424	0.684	0.394	0.290	68.71	0.000*
Ra2-A01	-0.865	0.026	0.478	0.597	0.312	0.285	51.07	0.000*
niab_ssr013	-0.972	0.220	0.604	0.563	0.223	0.340	91.94	0.000*
MR47MR32	-1.000	0.043	0.521	0.647	0.310	0.337	12.88	0.005
BN35D-1	-1.000	0.461	0.730	0.500	0.135	0.365	17.36	0.000*
BN35D-2	-1.000	-0.189	0.405	0.345	0.205	0.140	5.12	0.163
CNU-SSR149-1	-0.699	0.451	0.677	0.723	0.233	0.490	98.34	0.000*
CNU-SSR149-2	0.268	0.902	0.866	0.655	0.088	0.567	133.9	0.000*
CNU-SSR223-1	-1.000	-0.189	0.405	0.345	0.205	0.140	5.12	0.163
CNU-SSR223-1	-1.000	0.754	0.877	0.627	0.077	0.550	104.4	0.000*
Mean ± Standard Deviation	-0.886 ± 0.33	0.224 ± 0.39	0.588 ± 0.19	0.603± 0.13	0.248± 0.13	0.355 ± 0.13	72.88	

F_{IS}, the fixation index related to non-random mating within accessions; F_{IT}, the mean inbreeding coefficient of a set of accessions; F_{ST}, the inter-accession genetic differentiation due to genetic drift; H_T, the total genetic diversity; H_S, the genetic diversity within accessions; D_{ST}, the genetic diversity among accessions; X^2, Chi-square value to test F_{ST} for significant difference from zero; and P, the probability value (*significant F_{ST} at $P < 0.001$).

Genetic relationships:

The UPGMA dendrogram constructed based on SSR data using Euclidean distance matrix on average linkage, showed the phylogenetic relationships among the 26 accessions of *Brassica napus* studied (Fig. 2). It revealed 2 major groups. The first major group included 2 genetically distinct clusters; the first distinct cluster contained all swede accessions, whereas the second one comprised 4 accessions of spring oilseed rape (CGN12014, CGN19964, CGN19967, CGN19968) and only 2 accessions of winter oilseed rape (CGN06870, CGN17374). The second major group split into 3 distinct clusters; the first distinct cluster contained the four Egyptian cultivars (Serw-3, Serw-4, Misser L-16 and Semu 249), whereas the second one included all the accessions of fodder rape. The third distinct cluster included 4 accessions of winter oilseed rape (CGN17305, CGN17324, CGN17306, CGN17308, CGN13915) and only one accession of spring oilseed rape (CGN11019).

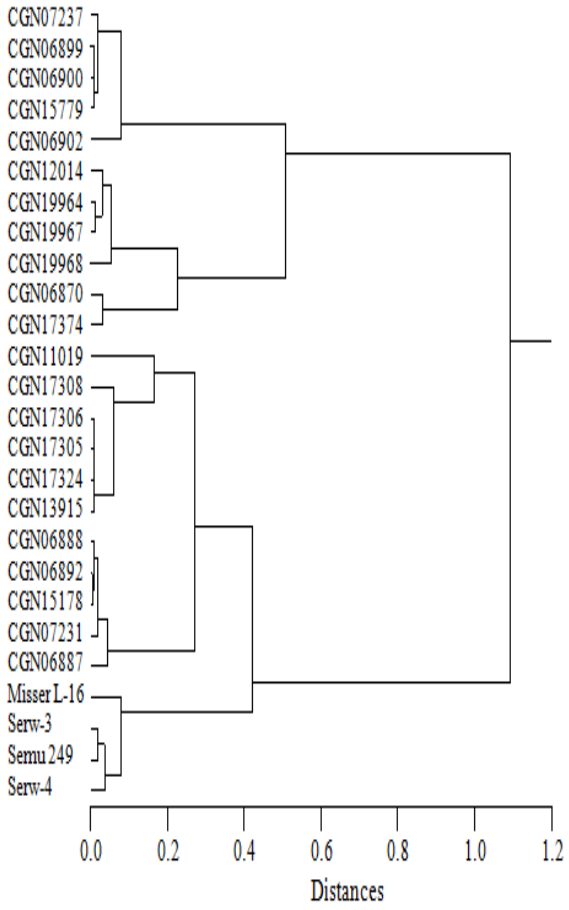

Fig. 2. UPGMA dendrogram showing the genetic relationships among 26 accessions of *Brassica napus* L. based on SSR data.

DISCUSSION:

Evaluation of the genetic diversity and phylogenetic relationships in crop plants is essential for establishing efficient conservation and breeding practices, resulting in developing more productive crops (Hasan *et al*., 2006; Moghaieb *et al*., 2014). SDS-PAGE is a useful tool to reveal genetic variations in seed storage proteins. In the present study, a reasonable genetic variability was observed in the electrophoretic patterns of the seed storage protein of *Brassica napus* accessions. This study revealed 11 (61%) polymorphic bands out of the 18 protein bands observed among the 26 accessions. These values were lower than those reported by Khan *et al*. (2014) who recorded 16 (76.19%) polymorphic bands out of a total of 21 protein bands observed among *Brassica napus* genotypes. Moreover, the percentage of protein polymorphism of the present study was higher than that was earlier reported by Mukhlesur and Hirata (2004) and Choudhary *et al*. (2015) for *Brassica napus* seed protein (6.3% and 50%, respectively). These differences in values could be attributed to the difference in the genotypes studied and the percentage of the gel used.

The dendrogram constructed based on differences in protein data divided the 26 accessions into 4 groups, and showed a reasonable level of genetic variation among them. SDS-PAGE results also showed that the total amount of variation accounted for the first three principal components was 73%, indication that the accessions studied show a relatively good association. This reasonable level of variability within the studied accessions agrees with the previous studies based on SDS-PAGE of the total seed proteins of *Brassica* germplasm (Curn, 1997; Sadia *et al*., 2009; Turi *et al*., 2010; Khurshid and Rabbani, 2012; Khan *et al*., 2014; Choudhary *et al*., 2015). The genetic variation in the accessions studied based on SDS-PAGE of the seed storage proteins is associated with the expression of the genome. However, to express high levels of the genetic variation of *Brassica napus* gene pools, more studies based on molecular traits such as SSR markers are recommended.

Microsatellite markers have been more useful and efficient for characterization of genetic diversity and phylogenetic relationships of *Brassica* genetic resources due to their codominance, high polymorphism and reproducibility (Hasan *et al*., 2006; Fu and Gugel, 2010; Wu *et al*., 2014). The 14 SSR loci detected in this study were polymorphic and useful for differentiation among *Brassica napus* accessions. The 11 SSR primer pairs revealed a total of 50 SSR alleles, with an average of 4.6 alleles per primer pair. This average value was higher than that was reported by Fu and Gugel (2010) and Qu *et al*. (2012) for *Brassica napus* (4), but lower than that was reported by

Hasan *et al*. (2006) for *Brassica napus* (7.3). Furthermore, the polymorphic information content (PIC) values classified 9 SSR primer pairs as informative markers (PIC > 0.5). The PIC values varied from 0.141 to 0.743, with an average of 0.59 per SSR primer pair, indicating the ability of the used SSR markers to differentiate among *Brassica napus* accessions studied. This average value of PIC was higher than that was reported by Fu and Gugel (2010) and Wu *et al*. (2014) for *Brassica napus* (0.43 and 37, respectively). The differences in the above values could be attributed to differences in the analyzed *Brassica* species and the used SSR markers.

The proportion of polymorphic SSR loci was variable among accessions analyzed in this study, and showed an average value of 51.1%. This polymorphism percentage was relatively higher than that was recorded by previous studies on *Brassica napus* (Cheung *et al*., 1997; Kresovich *et al*., 1995; Li *et al*., 2011). Moreover, the observed heterozygosity was higher than the expected one for all the 26 *Brassica napus* accessions. Consequently, the fixation indicx (F) for each accession showed negative average value, indicating an excess of heterozygotes in all the accessions studied. This data was consistent with that was reported by Wu *et al*. (2014) for *Brassica napus* hybrids. This result suggests that outcrossing rate might be reasonable within these accessions (Rakow and Woods 1987), or that the breeding technologies used to develop them did not reduce the intra-accession variation (Fu and Gugel, 2010). The estimates of observed and expected heterozygosity of all accessions analyzed in this study (H_o= 0.47, H_e= 0.298) were lower than those were reported by Wu *et al*. (2014) for *Brassica napus* hybrids (H_o= 0.68, H_e= 0.48), but showed high mean values compared to the accepted mean values for all plant species (Soltis and Soltis, 1989). The mean number of alleles per SSR locus (1.51) and the effective number of alleles per locus (1.49) were lower than those were reported by Li *et al*. (2011) for *Brassica napus* (2.55 and 2.01, respectively). The difference in this data could be attributed to differences in *Brassica napus* cultivars analyzed or the SSR markers used.

F-statistics revealed varying fixation indices among SSR loci detected in this study. The estimates of fixation indices (F_{IS}) of all SSR loci showed a negative average value (-0.886), indicating an excess of heterozygotes. This result was consistent with that was reported by Wu *et al*. (2014) for *Brassica napus* hybrids (-0.46). The 26 accessions studied contained a considerable level of genetic variation, but the distribution of this variation was not homogenous. The proportion of the total SSR variation residing within the accessions studied was 41.2% and the among-accession proportion was 58.8%. Therefore, the majority of the total genetic variation resided among accessions. These results were in agreement with the previous studies which assessed the genetic diversity of *Brassica napus* based on SSR markers (Fu and Gugel, 2010; Li *et al*., 2011). The distribution of SSR variation among accessions studied might be due to interactions among numerous evolutionary factors including selection, effective population size and the ability of the species to disperse pollen and seeds. Furthermore, the total genetic diversity (H_T) of the accessions studied was 0.603, and was found to be higher than that was reported by Wu *et al*. (2014) and Li *et al*. (2011) for *Brassica napus* (0.44 and 0.45, respectively). This difference in data could again be attributed to the different *Brassica napus* accessions analyzed and the different SSR markers used.

The dendrogram constructed based on SSR data revealed a considerable level of genetic variation among the accessions of *Brassica napus*. Unexpectedly, the two winter oilseed rape cultivars 'Slapska CGN06870' from Czechoslovakia and 'Olimpiade CGN17374' from Italy were more closely related to the spring oilseed rape material than the other winter oilseed rape accessions. Similarly, the spring oilseed rape cultivar 'Bronowsky CGN11019' from Poland was grouped with the winter oilseed rape accessions. This result was in agreement with that was reported by Hasan *et al*. (2006) and Diers and Osborn (1994). The dendrogram constructed based on protein data as well as the one based on SSR data showed that the Egyptian cultivars formed a distinct cluster and were more closely related to the winter oilseed and fodder rapes than to spring oilseed rapes. Moreover, the cultivars of spring oilseed rape and swede were more closely related to each other. In contrast to protein data, SSR data was able to distinguish among accessions of winter oilseed and fodder rapes, and all fodder rape cultivars formed a distinct cluster.

The genetically diverse spring and winter oilseed genotypes identified in this study could be useful resources for improving heterotic potential in spring and winter oilseed rape through establishing promising breeding programs. In this regard, the highly genetically diverse winter oilseed rape cultivars 'Slapska CGN06870' from Czechoslovakia and 'Olimpiade CGN17374' from Italy could be potential resources for winter oilseed rape breeding, whereas the spring oilseed rape cultivar 'Bronowsky CGN11019' from Poland could be potentially used for diversifying the spring oilseed rape gene pool. Furthermore, the Egyptian *Brassica napus* cultivars could be promising resources for increasing *Brassica napus* production in Egypt. In conclusion, this study demonstrated the efficiency of SSR markers

for assessing the genetic diversity and relationships of *Brassica napus* genotypes. The useful results of this study could be used for broadening the genetic base of improved *Brassica napus* gene pool, selecting genetically diverse genotypes for hybrid combinations to develop more productive crops, and for managing and conserving *Brassica napus* germplasm.

ACKNOWLEDGEMENTS:

I would like to thank anonymous reviewers for their valuable comments and constructive suggestions. I thank the Centre for Genetic Resources (CGN) in The Netherlands and the Agricultural Research Centre of the Ministry of Agriculture in Egypt for providing *Brassica napus* material examined in the present study. I would also like to thank Prof. Dr. Abdelfattah Badr, Dr. Margaret Ahmad and Jerome Bodin for their advice and support during conducting this work and data analysis. This work was supported by Tanta University in Egypt and Pierre and Marie Curie University in Paris, France.

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دراسة التنوع الوراثي والعلاقات التطورية في نوع البراسيكا نابس باستخدام التباين في أنماط بروتينات البذرة وواسمات الميكروستاليت

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يعد نوع البراسيكا نابس (السلجم) واحدا من النباتات الهامة إقتصاديا في العالم. وسيساعد التوصيف الوراثي لهذا النبات علي الحفاظ عليه وتحسينه وراثيا وزيادة انتاجيته من خلال برامج التهجين المختلفة. تهدف هذه الدراسة الي تقييم التنوع الوراثي والعلاقات التطورية في نوع البراسيكا نابس باستخدام التباين في أنماط بروتينات البذرة والواسمات الجزيئية من نوع الميكروستاليت . حيث أجريت الدراسة علي 26 مدخل وراثي من نوع "البراسيكا نابس" تم جمعهم من مركز حفظ الأصول الوراثية في هولندا و مركز البحوث الزراعية في مصر وقد استخدمت طرائق ووسائل مختلفة لتقييم التنوع الوراثي منها تقنية التفريد الكهربي (الإلكتروفوريسيز) لدراسة الأنماط البروتينية لبذور هذه المدخلات الوراثية وأيضا الواسمات الجزيئية من نوع الميكروستاليت وقد أجريت تحليلات احصائية للنتائج التي تم الحصول عليها. و أظهرت نتائج هذه الدراسة أن واسمات بروتينات البذرة أعطت 18 حزمة من البروتينات منها 11 حزمة كانت متابينه بين المدخلات الوراثية المختلفة واستطاعت التمييز بينها. وقد قسم الشكل العنقودي لنتائج هذه الروتينات المدخلات الوراثية محل الدراسة الي أربعة مجموعات. كما أظهرت النتائج أن 11 من واسمات الميكروستاليت قد كشفت عن نسبة كبيرة من التباين الوراثي بين المدخلات الوراثية وكانت نسبة التنوع الوراثي بين المدخلات الوراثية أكبر من مثيلاتها داخل المدخلات الوراثية. كما أوضح التحليل العنقودي لنتائج الميكروستاليت بعض المدخلات الوراثية المتباعدة في القرابة مع مثيلاتها والتي يمكن استغلالها في برامج التهجين من أجل تحسين انتاجية المحصول.