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Determination of Genetic Diversity of Some Species of Brassicaceae Using SDS-PAGE of Seed Protein and ISSR Markers

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EVALUATION of genetic diversity among 10 species of Brassicaceae have been performed based on variations in seed protein electrophoretic patterns as revealed by SDS-PAGE and ISSR analyses, separately and in combination. The maximum genetic similarity was observed between *Brassica nigra* and *B. rapa*, while the lowest genetic similarity was observed between *B. nigra* and *Sisymbrium irrio.* Cluster analysis generated a dendrogram that separated the studied taxa in two main clusters; the five species of tribe Brassiceae were grouped close to each other in one cluster with *Sisymbrium irrio*. The remaining studied species; *Capsella bursa-pastoris*, *Erysimum cheiranthoides*, *Mathiola longipetale* and *Thlaspi arvense*, were grouped in a second cluster.

Keywords: Brassicaceae, SDS-PAGE, ISSR, Genetic diversity.

The Brassicaceae (Cruciferae or mustard family) includes several crop plants grown worldwide, some of which have been cultivated since prehistoric times. Various species are grown for oil, mustard condiment, fodder and forage for animals, or as vegetables (Crisp, 1976 and Simmonds, 1986). Several classification systems were proposed from the early 19th to the mid 20th century. According to these systems, the Brassicaceae divided into anywhere from 4 to 19 tribes and 20 to 30 sub-tribes (Schulz, 1936 and Janchen, 1942). Mark *et al.* (2006) suggested that Brassicaceae includes two important model systems; the first included the *Arabidopsis thaliana* (L.) Heynh. The second model system is the agriculturally important *Brassica oleracea* complex (*B. oleracea* L., *B. rapa* L., *B. nigra* L.), and their three reciprocal hybrids, which has provided into the genetics of flowering time, hybridization and gene silencing. The genus *Brassica* is the most economically important genus within the Brassicaceae tribe that shares with other 18 tribes a wide gene pool, which over time has been utilized directly or indirectly to improve several crops. A molecular study by Koch *et al*. (2001) suggested that these taxonomic subdivisions mostly do not reflect phylogenetic relationships. However, different species of the sub-tribes Raphaninae and Moricandinae were as confirmed by a long series of investigations on the chloroplast DNA (cp-DNA) and restriction sites (Warwick and Black, 1991; Warwick *et al*., 1992; Warwick and Sauder, 2005).

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Laboratory based biochemical and molecular methods were developed for identification and differentiation of plant species and cultivars of a crop. Among these methods, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is commonly used (Cooke, 1984) due to its easiness and usefulness (Badr, 1995; Badr *et al.,* 2002; Siddiqui *et al*., 2010; Khan *et al.*, 2013 and Sultan *et al*., 2013). Seed storage proteins are highly stable as these are not affected by environmental conditions (Neto *et al.,* 2002). Sadia *et al.* (2009) conducted SDS-PAGE to distinguish cultivars of *B. rapa, B. napus, B. carinata* and *B. juncea.* Among the various molecular markers, Inter-Simple Sequence Repeat (ISSR) is a simple and a quick technique. Nowadays, ISSR is widely being used for line identification and genetic diversity among various plant groups (Li *et al.,* 2005; Pezhmanmehr *et al*., 2009; Nan *et al*., 2003; Zhang and Dai, 2010, Pradeep *et al.,* 2005; Xavier *et al*., 2011 and Abdel Khalik *et al.,* 2012). The present study describes the genetic variability observed after SDS-PAGE and ISSR analyses of, separately and in combination, would be more credible to analyze the genetic diversity of five tribes from family Brassicaceae.

Material and Methods

Seeds were obtained from the Botanischer Garten Berlin-Dahlem, Germany and collected from different localities in Egypt (Table 1).

TABLE 1.Tribes and origin of the studied taxa of the family Brassicaceae.

NO.	Taxa	Tribe	Origin
1	*Brassica nigra* L.	Brassiceae	Botanischer Garten Berlin-Dahlem Germany
2	*Brassica rapa* L.	Brassiceae	Al Sharkia – Egypt
3	*Capsella bursa-pastoris* L.	Lepidieae	Cairo- Suez road – Egypt
4	*Eruca sativa* L.	Brassiceae	Cairo-Alex-Desert road – Egypt
5	*Erysimum cheiranthoides* L.	Hesperideae	Botanischer Garten Berlin-Dahlem Germany
6	*Moricandia sinaica* L.	Brassiceae	North costal, Alex.- Egypt
7	*Mathiola longipetale* L.	Matthioleae	Cairo-Ismailia road - Egypt
8	*Raphanus raphanistrum* L.	Brassiceae	Cairo- Suez road – Egypt
9	*Sisymbrium irrio* L.	Sisymbrieae	Al Sharkia – Egypt
10	*Thlaspi arvense* L.	Lepidieae	Botanischer Garten Berlin-Dahlem Germany

Protein extraction and electrophoresis

For protein extraction, seeds were grounded to a fine powder using mortar and pestle. About 0.1 g seed powder was suspended in 400 µl protein extraction buffer in 1.5 ml Eppendorf tubes for 30 min. The extraction buffer composed of 0.5 M Tris-HCl (pH 8.0), 0.2% SDS, 5 M Urea and 1%. βmercaptoethanol. Dye (Bromophenol blue) was added to display the movement of protein. Eventually samples were mixed carefully by vigorous shaking and centrifugation at 15.000 rpm for 10 min. at room temperature (RT), and kept at - 4°C till gel electrophoresis process.

Electrophoresis was carried out in polyacrylamide slab gels in discontinuous buffer system. The separating gel solution contained 20% acrylamide and 0.135% N.N-methylene-acrylamide in 0.5 M Tris-HCl buffer (pH 8.8) and 0.27% SDS. The gel was polymerized by adding 10% APS (Ammonium persulphate) and 15 µl TEMED. Ten to 12 µl of protein sample was added into the wells. Electrophoresis was carried out at 90V for about 3 hr till blue marker reached at the bottom of gel. The molecular weights of separated protein bands were compared with standards protein ladder ranging from 15 to 160 kDa. Later, the gels were stained with Coomassie blue solution for 40 to 60 min. Gels were then destained in a solution composed of acetic acid, methanol and distilled water in the ratio of 5:20:75 (v/v) for more than 2 hr. They were photographed and scanned by BIO-RAD Video documentation system, Model Gel Doc 2000 (Fig. 1).

Plant genomic DNA extraction and ISSR fingerprinting

Total genomic DNA was extracted from young leaves of germinated seedlings, following the steps of CTAB protocol (Doyle and Doyle,1990 and Doyle, 1991). PCR amplification was performed according to the protocol of Zielkiewicz *et al.* (1994). ISSR amplification reactions were carried out in 25 µl volume containing 0.75 µl $MgCl_2$ (50 mM), 0.5 µl dNTP (10 mM), 2.5 µl PCR buffer (10 x), 1 µl Taq DNA polymerase, 0.5 µl primer (100 µM), 1 µl template DNA (10 ng/µl) and 19.5 µl ddH_2O. Twelve ISSR primers were purchased from Metabion (Planneg, Germany). Amplified products were electrophoresed on 2% agarose in 0.5 x TBE buffer. The gels stained with ethidium bromide (1.0 µg /ml). A marker of 1 Kb plus DNA ladder 1 µg/µl (Thermoscientific, USA) that contain a total of twelve bands ranging from 3,000 to 100 bp was used. Bands were detected on UV- Trans-illuminator and photographed then analyzed using BIO-RAD Video documentation system, Model Gel Doc 2000 (BioRad, Germany). Five primers which gave reproducible fingerprints (DNA bands) were considered for the data analysis (Table 2).

TABLE 2. Similarity matrices based on jaccard similarity coefficients of protein profiles of the studied species (Taxa names in table, 1).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.000									
2	0.429	1.000								
3	0.267	0.235	1.000							
4	0.188	0.400	0.250	1.000						
5	0.083	0.071	0.167	0.167	1.000					
6	0.143	0.385	0.214	0.417	0.100	1.000				
7	0.200	0.000	0.182	0.083	0.167	0.000	1.000			
8	0.250	0.294	0.400	0.400	0.154	0.385	0.273	1.000		
9	0.143	0.200	0.417	0.417	0.222	0.400	0.250	0.636	1.000	
10	0.000	0.000	0.000	0.100	0.250	0.000	0.333	0.091	0.143	1.000

Data analysis

For data analysis, each amplified band was treated as a unit character regardless of its intensity and scored in terms of a binary code, based on presence (1) and absence (0) of bands. Only clear and reproducible bands were considered for scoring. Genetic distance trees were constructed using the NTSYS-pc version 2.1 statistical packages (Rohlf, 2000). The binary qualitative data matrices were used to construct similarity matrices based on Jaccard similarity coefficients (Jaccard, 1908). The similarity matrices were then used to construct dendrograms using unweighted pair group method with arithmetic average (UPGMA) as described by Sneath and Sokal (1973).

Results and Discussion

Protein electrophoresis

A total of 21 protein bands were observed among the ten studied species with 100% polymorphism (Fig. 1). Size varied from 14 to 160 kDa. Few bands were observed in *Thlaspi arvense* L., *Mathiola longipetale* L. and *Erysimum cheiranthoides* L. While *Brassica rapa* L. and *Raphanus raphanistrum* L. showed maximum of 11 bands. The other five species showed 7 to 10 protein bands. In each of *Moricandia sinaica* and *R. raphanistrum* banding pattern, one band was found to be species specific markers. Large differences in the number of bands were observed, where some specific bands that can be used to distinguish such taxa from each other.

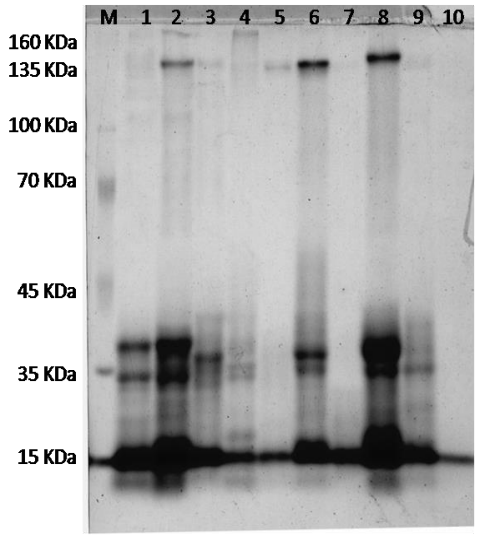

Fig. 1. Electrophoretic banding pattern produced by SDS-PAGE total seed protein of studied species, 1.*Brassica nigra*; 2.*Brassica rapa*; 3.*Capsella bursa-pastoris*; 4. *Eruca sativa*; 5. *Erysimum cheiranthoides*; 6.*Moricandia sinaica*; 7. *Mathiola longipetale*; 8. *Raphanus raphanistrum*; 9. *Sisymbrium irrio*; 10. *Thlaspi arvense.* M: Standard protein marker, kDa: Kilo Dalton.

The highest similarity (0.636) was obtained between *R. raphanistrum* and *Sisymbrium irrio* with 7 similar and 4 dissimilar bands. The lowest similarity (0.00) was obtained between *T. arvense* and each of *B. nigra, B. rapa, Capsella bursa* and *R. raphanistrum* (Table 2). The dendrogram based on protein variation separated the ten studied taxa into two main clusters (G1&G2). Moreover, *T. arvense, M. longipetale* (sub-cluster I) and *E. cheiranthoides* (sub-cluster II) were separated into G1. Cluster G2 was further divided into 2 sub-clusters (I & II). Sub-cluster I included *Eruca sativa, M. sinaica* and *R. raphanistrum* of tribe Brassiceae with *C. bursa-pastoris* and *S. irrio* (Fig. 2a) separate the three figures from each other. These results are in accordance with Koch *et al.,* (2001); O'Kane and Al-Shehbaz (2003) who found the genera *Capsella, Lepidium* and *Thlaspi*, which have been placed in tribe Lepidieae in every system of classification of Brassicaceae, are unrelated; *Capsella* showed more affinity to *Arabidopsis* than to either *Lepidium* or *Thlaspi*.

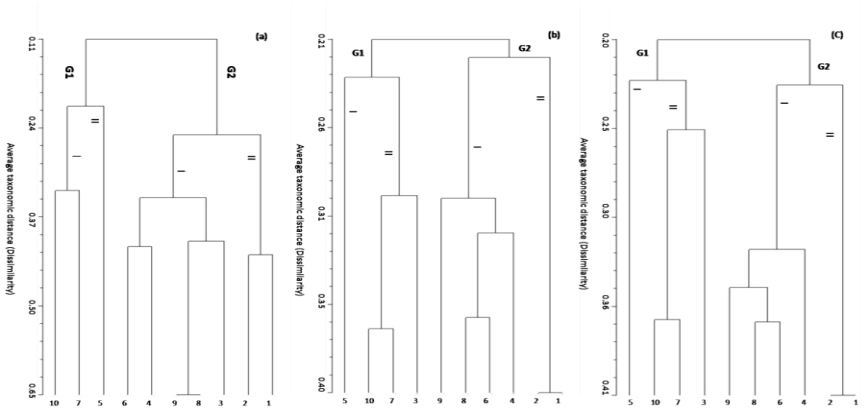

Fig. 2. Dendrograms obtained from 10 studied species with UPGMA based on similarity coefficient. (a) Protein data-based dendrogram; (b) ISSR data-based dendrogram; (c) Combined (protein and ISSR) data based dendrogram. 1. *B. nigra*; 2. *B. rapa*; 3. *C. bursa-pastoris*; 4. *E. sativa*; 5. *E. cheiranthoides*; 6. *M. sinaica*; 7. *M. longipetale*; 8. *R. raphanistrum*; 9. *S. irrio*; 10. *T. arvense*.

ISSR analysis

Five ISSR primers produced a total of 88 bands in the profiles of the ten examined species; only one monomorphic band was revealed by primer 5 (Fig. 3 and Table 3). The results indicated the presence of wide genetic variability as a result of high polymorphism among the studied species of *Brassicaceae.* This high level of polymorphism indicates the existence of high level of genetic diversity among the genotypes under investigation. Primer 1 produced the highest total number of bands (24) while primer 5 revealed the lowest total number of

amplified fragments (10). Ten specific markers for some *Brassicaceae* species across ISSR analysis are listed in Table 3. These results are in accordance with that of Khalil *et al.* (2010) who found that 8 out of 123 ISSR markers were specific markers among ten *Brassicaceae* species using 14 ISSR primers.

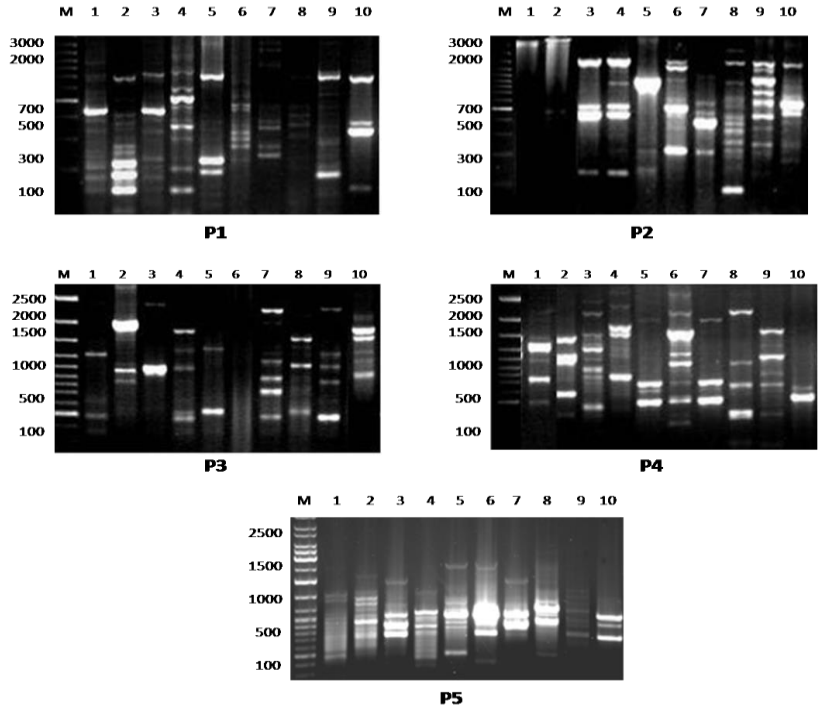

Fig. 3. DNA polymorphism using ISSR with primers P1, P2, P3, P4 and P5 in 10 taxa of Brassicaceae. (M) Marker. 1. *B. nigra*; 2. *B. rapa*; 3. *C. bursa-pastoris*; 4. *E. sativa*; 5. *E. cheiranthoides*; 6. *M. sinaica*; 7. *M. longipetale*; 8. *R. raphanistrum*; 9. *S. irrio*; 10. *T. arvense*.

TABLE 3. Primer sequences, total bands number of monomorphic bands, number of polymorphic bands and polymorphism percentages of ISSR primers among the studied species.

	Primer Sequences	Monomorphic	Polymorphic		Total	Polymorphism %
			Uniq	Non-Uniq		
P1	$(GT)_6GG$	0	4	20	24	100
P2	$ATTA(CA)_5$	0	3	14	17	100
P3	$(GTG)_3GC$	0	2	17	19	100
P4	$(CTC)_3GC$	0	1	17	18	100
P5	$GG(CA)_6$	1	0	9	10	90
Total		1	10	77	88	98

In the present study, similarity coefficients revealed close relationships between some taxa (Table 4 and Fig. 2b). The maximum genetic similarity was 0.40 between *B. nigra* and *B. rapa*, while a lowest genetic similarity of 0.074 was between *B. nigra* and *S. irrio*. Data obtained in this work presented a medium level of similarity between *Eruca* and *Raphanus*. Similarities in nuclear and morphological characters such as flower size between *Eruca* and *Raphanus* led Song *et al.* (1990) to suggest that both had evolved from a common ancestor. Cluster analysis based on ISSR data generated a dendrogram that separated the studied taxa into two main clusters (Fig. 2b). In this grouping, the five species of tribe Brassiceae were located close to each other with *S. irrio* in cluster G2 while the remaining studied species were located in cluster G1. Koch *et al.* (1999a) showed that some taxa from tribe Lepidieae such as *Capsella* are integrated in tribe Arabideae. Moreover, it has been suggested that taxa from tribe Thelypodieae should be included in tribe Lepideae (Zunk *et al.*, 1996)

TABLE 4. Similarity matrices based on Jaccard similarity coefficients of ISSR of the studied species (Taxa name as in table, 1).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.000									
2	0.400	1.000								
3	0.239	0.239	1.000							
4	0.241	0.288	0.216	1.000						
5	0.205	0.232	0.200	0.231	1.000					
6	0.250	0.300	0.222	0.333	0.170	1.000				
7	0.184	0.184	0.311	0.211	0.310	0.264	1.000			
8	0.180	0.229	0.177	0.296	0.192	0.360	0.196	1.000		
9	0.074	0.208	0.255	0.278	0.122	0.314	0.224	0.298	1.000	
10	0.200	0.200	0.279	0.204	0.186	0.260	0.366	0.164	0.273	1.000

Clustering of studied species based on the combined protein and ISSR data revealed similar results with those of ISSR alone (Table 5 and Fig. 2c). The dendrogram based on combined data separated the ten taxa into two main clusters, the first cluster (G1) was divided into two sub-clusters, *E. cheiranthoides* formed a separate taxon sub-cluster I showing the least similarity distance (0.228) with the other species, sub-cluster II comprised *T. arvense* and *M. longipetale* with a similarity distance of 0.364 and *C. bursa-pastoris* which recorded a distance of 0.249 with them. The second cluster (G2) was divided into two sub-clusters at a distance of 0.235 similarity level. Sub-cluster II included *B. nigra* and *B. rapa* with the highest similarity (0.407). Sub-cluster I comprised *E. sativa*, *M. sinaica*, *R. raphanistrum* and *S. irrio*. Molecular and hybridization data indicate that close relatives of *Brassica* crop species also include species currently placed in separate genera in three different subtribes, the Brassicinae, Raphaninae and Moricandiinae (Warwick and Sauder, 2005). The most closely related genera to the *Brassica* crop species include *Eruca*, *Moricandia*,

Raphanus, *Sinapidendron*, *Sinapis* and *Trachystoma* (Harbed, 1972). ISSR is proved to be an efficient and inexpensive way to generate molecular data. The ISSR method has been reported to be more reproducible (Goulao and Oliveira, 2001) and produces complex marker pattern (Chowdhury *et al*., 2002), which is advantageous when differentiating closely related cultivars.

TABLE 5. Similarity matrices based on Jaccard similarity coefficients of protein+ ISSR of the studied species (Taxa name as in Table 1).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1.000									
2	0.407	1.000								
3	0.246	0.238	1.000							
4	0.229	0.313	0.239	1.000						
5	0.179	0.193	0.193	0.219	1.000					
6	0.227	0.318	0.221	0.348	0.159	1.000				
7	0.186	0.143	0.286	0.188	0.292	0.222	1.000			
8	0.197	0.246	0.227	0.319	0.183	0.365	0.210	1.000		
9	0.088	0.206	0.288	0.303	0.138	0.328	0.228	0.362	1.000	
10	0.164	0.158	0.222	0.188	0.192	0.224	0.364	0.150	0.255	1.000

Conclusion

The overall dendrograms based on seed protein profile and ISSR markers indicated that there were considerable diversity and relationships. In the light of the above results, it may be concluded that biochemical and molecular markers can be used effectively to estimate genetic distances among investigated genotypes and they are useful in exploring the genetic diversity and relationships among examined species that add new dimension to plant taxonomy. However, it is suggested that a greater number of species and molecular markers are required to have better understanding of the presence of genetic variability in Brassicaceae and consequently, more efficient utilization of existing variability for improvement of these important crops.

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تحديد التنوع الجينى لبعض الأنواع من جنس براسيكا بإستخدام التفريد الكهربى لبروتينات البذرة وتكرار التتابع الداخلى البسيط

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تمت دراسة بذور عشرة وحدات تصنيفية تابعة للفصيلة الصليبية جمعت من مواقع مختلفة فى مصر وجلب بعضها من الخارج باستخدام الفصل الكهربى لبروتينات البذرة ودراسة البصمة الوراثية للحامض النووى (الدنا) باستخدام طريقة تكرارالتتابع الداخلى البسيط لإيجاد التشابهات والاختلافات بين الوحدات التصنيفية قيد الدراسة. أمكن باستعمال هذة التقنيات التوصل إلى مصفوفة التشابهات بين الوحدات المدروسة مما سهل إيجاد العلاقات التصنيفية بينهم. كما أظهرت الدراسة نتائج التحليل العددى لأنماط بروتينات البذور والحامض النووى أهمية خاصة كمعايير ودلائل تصنيفية أدت الى إيضاح العلاقات بينهم.