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Polymorphism in Seed Protein Electrophoretic Pattern and Species Relationships in the Genus *Orobanche* L.

Sherif M. Sharawy

Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

Abstract: Polymorphism in the banding profiles of the electrophoretic separation of seed proteins are used to reassess the taxonomic relationships between 21 samples representing eight species of the genus *Orobanche* L. (Orobanchaceae) that were collected from different cultivated and wild host in Egypt and Saudi Arabia. A high degree of similarity in the banding profile of samples of the same species is revealed in the studied taxa confirming the validity of seed proteins as a source of taxonomic criteria. Moreover, nine bands are common to all samples of the studied 21 species which may be indicative of their common origin and support the view that the genus *Orobanche* is a monophyletic group. The analysis of results using the NTSYS-pc program and the UPGMA and NJ clustering methods support the delimitation of species of *Orobanche* in two sections *Orobanche* Wallr. and *Trionychon* wallr. Also, supports delimitation of the studied species in section *Orobanche* in three groups. Moreover the delimitation of the studied species belonging to section *Trionychon* in two groups supports the previous delimitation of this section.

Key words: *Orobanche*, Orobanchaceae, Taxonomy, Seed protein electrophoresis, Morphology

INTRODUCTION

Orobanche L. is the largest achlorophylls annual or perennial plants that parasitizes on roots of different plant species, and comprises approximately 170 species distributed predominantly in the Old and New World. Parasitism has led to a simplification in their morphology and therefore a reduction in features which are used to distinguish species (Roman *et al.* 2003). The intrinsic taxonomic difficulties in *Orobanche* are further compounded by the fact that important differential characters can be observed only with great difficulty or not observed, in dried specimens, and the features used to distinguish species are poorly defined (Musselman, 1986).

Following the treatment of Beck-Mannagetta (1930), most authors divide *Orobanche* into four section: *Gymnocaulis* Nutt., *Myzorrhiza* (Phil.) Beck, *Trionychon* Wallr. and *Osproleon* Wallr.. Section *Osproleon* is now treated as a synonym of section *Orobanche* according to the rules of the International Code Botanical Nomenclature (Greuter *et al.* 2000). Other authors treat these sections as the separate genera *Orobanche*, *Phelipanche*, *Aphyllon* and *Myzorrhiza* respectively (Sojak, 1972; Holub, 1977, 1990; Teryokhin *et al.,* 1993 and Bennett & Mathews 2006).

The species of the greatest agronomic importance are found in sections *Orobanche* and *Trionychon* that occurred in the Old World. These are distinguished by characters of the bracts, placentation, inflorescence type, cytology and distribution. Relationships among *Orobanche* species have mainly been investigated by means of morphological studies and ifrageneric systematics has been the subject of disagreement (Roman *et al.* 2003).

Recently the uses of more accurate methods have contributed to a better understanding of the systematic relationships within genus. Light and scanning electron microscope have been used to study pollen morphology and seed micromorphology (Abu Sbaih *et al.* 1994) and the chemotaxonomic techniques have been used to measure phenolic compounds and fatty acids (Andary, 1994; Georguieva & Edrewa, 1994 and Velasco *et al.* 2000). Schneeweiss *et al.* (2004) recorded three basic chromosome numbers, x=19, x= 12 and x= 24 in the genus *Orobanche*. The first is confined to members of section *Orobanche*, the second occurs in sections Myzorrhiza and *Trionychon* and the third in section *Gymnocaulis*.

The polymorphism in the seed protein electrophoretic profile has been found useful in the study of systematics and evolution of plant species (Ladizinsky & Hymowitz, 1979; Vaughan, 1983; Jenesen & Lixue, 1991). The major storage of seed proteins in plants have been used to understand the relationships in some

Corresponding Author: Sherif M. Sharawy, Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

genera. Forexample in *Trifolium* (Badr, 1995), *Phaseolus* (Schmit *et al.* 1996), Sesbania (Badr *et al.* 1998), *Astragalus* (Al-Nowaihi *et al.* 2002), *Mentha* (Badr *et al.* 2003), *Artemisia* (Mohamed, 2004), Lupinus (El-Shazly *et al*. 2006) and *Erodium* (Sharawy & Bdar 2008).

The objectives of this study were to evaluate the usefulness of the polymorphism in seed protein electrophoretic profiles as revealed by SDS-PAGE to reassess the taxonomic relationships of 21 samples of the genus *Orobanche* which are found on cultivated and wild plants in Egypt and Saudi Arabia in the light of their previous taxonomic treatments.

MATERIALS AND METHODS

Twenty one samples representing eight *Orobanche* L. species were collected from different localities in Egypt and Saudi Arabia. The sectional delimitation and localities of the examined materials are given in Table 1. The species were identified according to Täckholm (1974) and Boulos (2000) for the species collected in Egypt and according to Migahid (1989) for species collected in Saudi Arabia.

Table 1: A list of the studied taxa of the *Orobanche* species and their localities.

Taxa	Locality
I- Section: *Orobanche* Wallr.	
1- *O. cernua* Loefl. 1	On *Lycium showii, Sinai,* Egypt
O. cernua Loefl. 2	On *Lycopersicum esculentum*, Sharquia, Egypt
O. cernua Loefl. 3	On *Lycopersicum esculentum*, Hail, Saudi Arabia
2- *O. crenata* Forssk. 1	On *Trifolium alexandrinum*, Cairo, Egypt
O. crenata Forssk. 2	On *Trifolium alexandrinum*, Sharquia, Egypt
3- *O. minor* Smith.	On *Vicia faba*, Cairo, Egypt
4- *O. pubescens* Urv. 1	On *Tropaeolum majus*, Cairo, Egypt
O. pubescens Urv. 2	On *Tropaeolum majus*, Alexandria, Egypt
O. pubescens Urv. 3	On *Tropaeolum majus*, Hail, Saudi Arabia
O. pubescens Urv. 4	On *Anthemis pseudocotula* , Hail, Saudi Arabia
II- Section: *Trionychon* Wallr.	
5- *O. aegyptiaca* Pers. 1	On *Lycopersicum esculentum* , Sharquia, Egypt
O. aegyptiaca Pers. 2	On *Lycopersicum esculentum*, Alexandria, Egypt
O. aegyptiaca Pers. 3	On *Lycopersicum esculentum*, Hail, Saudi Arabia
O. aegyptiaca Pers. 4	On *Brassica oleraceae* spp. *botrytis*, Hail, Saudi Arabia
6- *O. lavandulacea* Reichenb	On *Cucurbita pepo*, Sharquia, Egypt
7- *O. mutelii* F.G.Schultz. 1	On *Malva parviflora*, Hail, Saudi Arabia
O. mutelii F.G.Schultz. 2	On *Lycopersicum esculentum*, Hail, Saudi Arabia
O. mutelii F.G.Schultz. 3	On *Rumex vesicarius*, Hail, Saudi Arabia
8- *O. ramosa* L. 1	On *Lycopersicum esculentum*, Sharquia, Egypt
O. ramosa L. 2	On *Trifolium alexandrinum*, Sharquia, Egypt
O. ramosa L. 3	On *Lycopersicum esculentum*, Hail, Saudi Arabia

For protein extraction, 0.2g dry seeds were powdered and homogenized with 0.2M Tris-HCl buffer, pH=8 for 1h. The mixture was centrifuged at 12000g for 10 min and 50 µl of the extracted were mixed with an equal volume of a sample buffer (0.125 M Tris-HCl, pH=6.8, 2%SDS, 10% sucrose, 0.5% β-mercaptoethanol), denaturated by boiling for 5 min in a water bath, cooled and 0.1% bromophenol blue as a dye was added. For separation of protein components, 20 µl of this mixture were electrophoresed in 12.5% gel slabs, which was prepared as described by Laemmli (1970). Electrophoresis was carried out in Tris-Glycine buffer (pH=8.3) at 4°C and 125 volt for 2h in a Consort Vertical Slab Gel Apparatus using a Pharmacia low-molecular weight protein mixture as a standard marker. Gels were then stained in sufficient amount of staining solution (Coomassie brilliant blue) for about 16hrs at room temperature. The dye was then replaced by the distaining solution, which was changed regularly until clear background was observed.

The banding profile of the examined species was photographed while gels were wet. The number of bands was scored by direct observation of gels and photographs. Each band was considered as a character and its presence or absence was coded for analysis. A total of 66 protein bands were scored in the examined 21 samples of Orobanche, nine of which were presented in all samples were excluded from the analysis. Protein bands were coded as 0 for absence and 1 for presence in the computer analysis. For the numerical analysis the NTSYS-pc, version 2.2 program (Rohlf, 2000) was used. The trees illustrating the relationships between the studied samples were performed using the unweighted Pair group Method using Arithmetic Average (UPGMA) proposed by Sokal and Michener (1958) and the Neighbor Joining (NJ) method (Saitou & Nei, 1987) as implemented in the NTSYS-pc program (Rohlf, 2000).

RESULTS AND DISCUSSION

The electrophoretic patterns of proteins extracted in Tris-HCl are illustrated in Figs 1 & 2. The codes given to the band revealed by these patterns are given in Table 2.

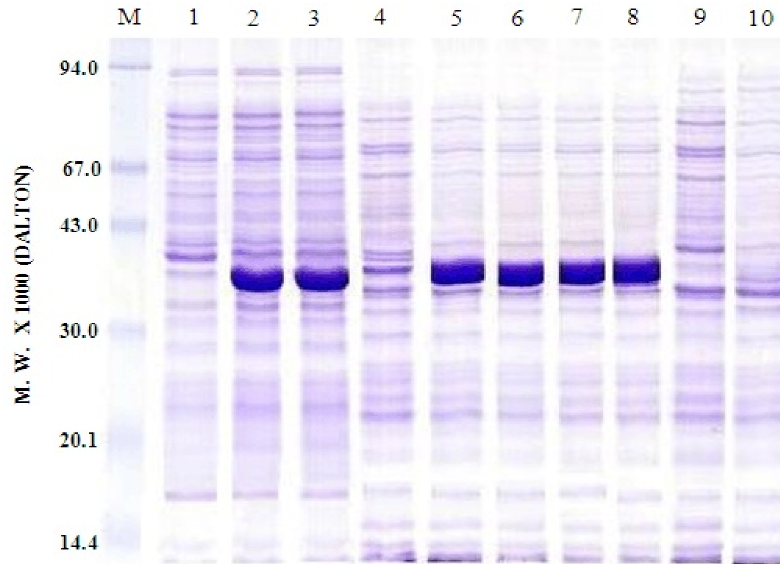

Fig. 1: SDS-PAGE profile of the seed storage protein for the studied taxa of the genus *Orobanche* L. belonging to section *Orobanche* Wallr., M refers to the Pharmicia low molecular weight and the No. 1-10 refer to the Orobanche taxa as follows: 1= *O. cernua* 1, 2= *O. cernua* 2, 3= *O. cernua* 3, 4= *O. minor,* 5= *O. pubescens* 1, 6= *O. pubescens* 2, 7= *O. pubescens* 3, 8= *O. pubescens* 4, 9= *O. crenata* 1, 10= *O. crenata* 2.

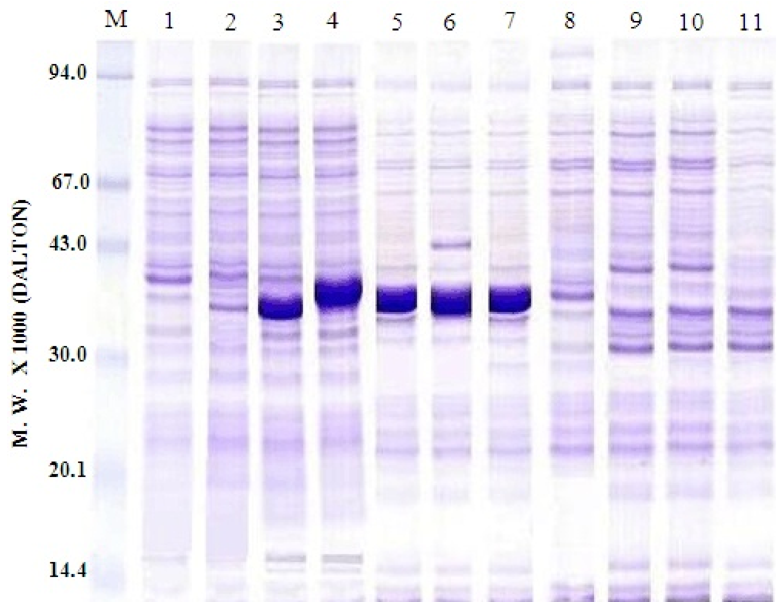

Fig. 2: SDS-PAGE profile of the seed storage protein for the studied taxa of the genus *Orobanche* L. belonging to section *Tionychon* Wallr., M refers to the Pharmicia low molecular weight and the No. 1-11 refer to the *Orobanche* taxa as follows: 1= *O. aegyptiaca* 1, 2= *O. aegyptiaca* 2, 3= *O. aegyptiaca* 3, 4= *O. aegyptiaca* 4, 5= *O. mutelii* 1, 6= *O. mutelii* 2, 7= *O. mutelii* 3, 8= *O. lavandulacea* 4, 9= *O. ramosa* 1, 10= *O. ramosa* 2, 11= *O. ramosa* 3.

Table 2: Data matrix for the codes given to the seed protein characters used for the analysis by using NTSYS-pc program.

OUT's → / ↓ Bands NO.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
9	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	1	1	1	1
14	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
15	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
16	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	1	1	1	1	0	0	1	0	0	0	1	1	1	0	0	0	0
18	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0
19	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	1
20	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
21	1	1	1	1	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	0	0
22	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
24	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0
25	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
27	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
28	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
29	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
30	1	0	0	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
32	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0
33	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
34	0	0	0	1	1	1	0	0	1	1	0	1	0	0	0	0	0	0	1	1	1
35	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0
36	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0
37	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0
38	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1
39	0	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0
40	1	1	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1
42	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0	0
44	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
45	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
46	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
47	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
48	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	1
50	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
51	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
52	0	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	0	0	1	1	0
53	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0
54	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
55	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
56	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
57	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	1	0	0	0

The tree constructed by the UPGMA method (Fig. 3) shows that the examined species are distinguished in two groups at a UPGMA distance coefficient of about 1.38; the first group comprising the four species of section *Orobanche* Wallr. and the other group including the four species of section *Trionychon* Wallr. In the former group the three samples of *O. cernua* are delimited as a separate cluster at a distance coefficient of about 1.10. At a distance coefficient of about 0.90 the *O. minor* is distinguished from the other two species of section Orobanche. The remaining two species (*O. pubescens* and *O. crenata*) are separated from each other at a distance coefficient of about 0.75. With regard to the four samples of *O. pubescens*, the samples 1&2 that were collected from Egypt are clearly distinguished from the other two samples (3&4) that were collected from Saudi Arabia, including evident polymorphism in this species in the components of the seed storage proteins.

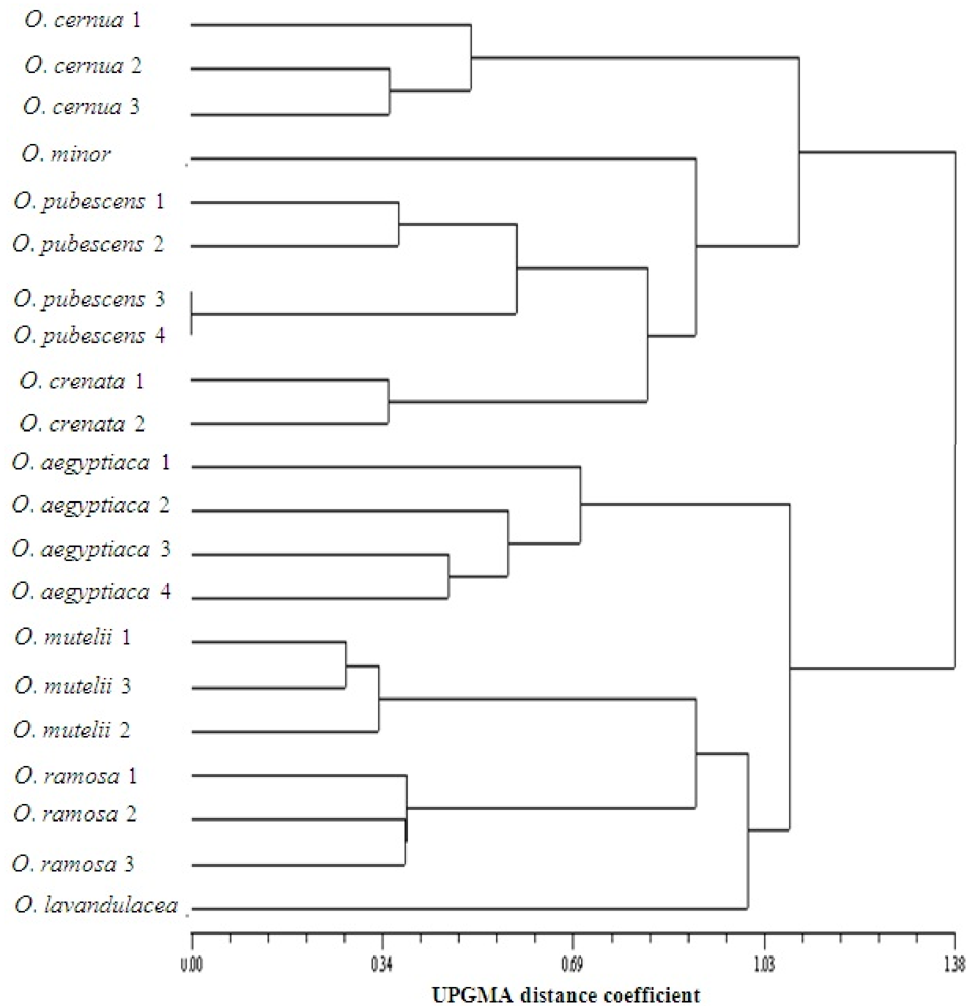

Fig. 3: UPGMA tree illustrating the relationships between the studied samples of the genus Orobanche, based on variation in seed protein electrophoresis.

In the latter group that comprises the four species of section *Trionychon*; the four samples of *O. aegyptiaca* are delimited at a distance coefficient of about 1.10 from the other three species of the same section. At a UPGMA distance coefficient of about 0.91 *O. lavandulacea* is distinguished from the remaining two species *O. ramosa* and *O. mutelii*. The samples of the both latter two species (*O. ramosa* and *O. mutelii*) that were collected from different localities are separated at a low distance coefficient (about 0.34) indicating similarity in the components of the seed storage proteins of these samples.

The tree produced by the analysis of SDS-PAGE profiles of seed using the Neighbor Joining method (NJ) is shown in Fig. 4. The topology of this tree generally resembles that of the tree constructed by the UPGMA method (Fig. 3) in the examined species that were delimited in two groups, one comprising the four species of section *Orobanche* and the other including the four species of section *Trionychon*. In the former group the three samples of *O. cernua* are clearly distinguished from the remaining species of section *Orobanche* at a NJ distance coefficient of about 6.00. The three samples of *O. cernua* are clustered at a distance coefficient (about 5.00) indicating evident polymorphism in this species in the components of the seed storage protein. The remaining three species (*O. minor*, *O. crenata* and *O. pubescens*) are separated into two groups; the first group comprises *O. minor* and two samples of *O. crenata*. The second group comprises the four samples of *O. pubescens*. With regard to the four samples of the latter species, samples 1&2 that were collected from Egypt are clearly distinguished from the other two samples (3&4) that were collected from Saudi Arabia indicating evident polymorphism in this species in the components of the storage protein.

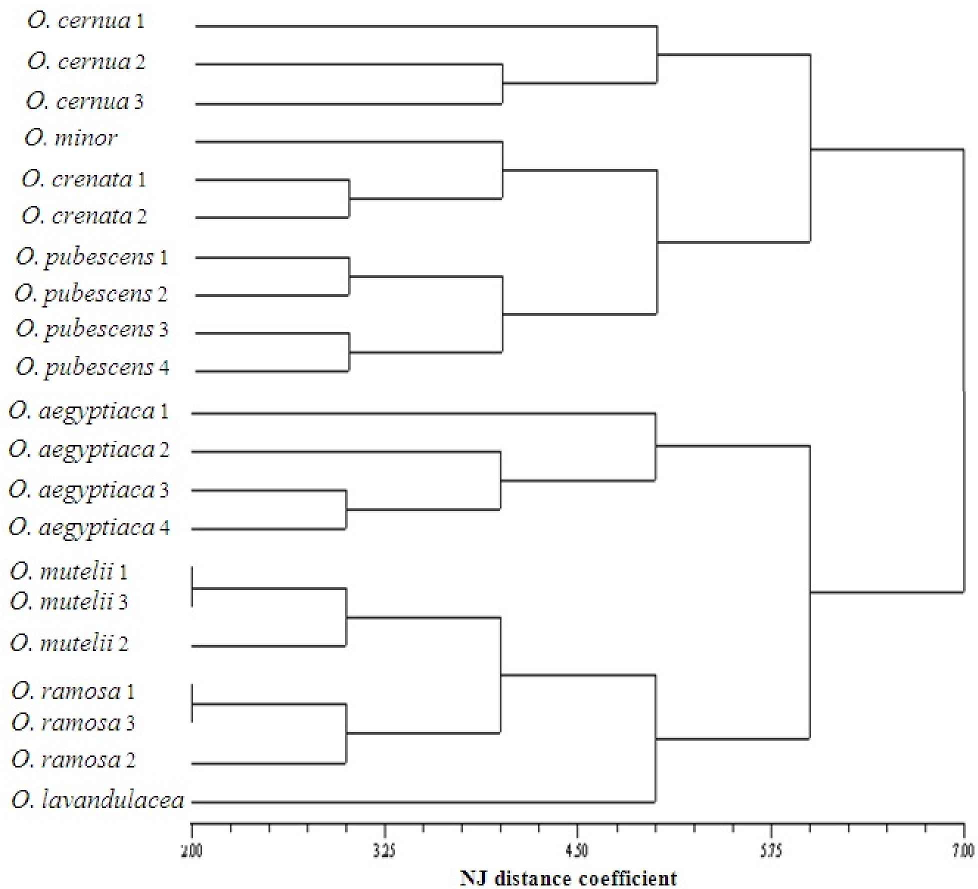

Fig. 4: NJ tree illustrating the relationships between the studied samples of the genus Orobanche, based on variation in seed protein electrophoresis.

In the second group of the NJ tree based on polymorphism in seed protein electrophoretic profiles (Fig. 4). The four studied species of section *Trionychon* are separated into two clusters. The first one comprises the four samples of *O. aegyptiaca*, while the second cluster comprises the remaining three species. The two samples of *O. aegyptiaca* (1&2) that were collected from Egypt are distinguished from the other two samples (3&4) that were collected from Saudi Arabia, indicating the polymorphism in the components of the seed storage protein in this species (*O. aegyptiaca*). At a distance coefficient of about 5.00 the *O. lavandulacea* is separated from the other two species (*O. mutelii* and *O. ramosa*). The latter two species are separated at a distance coefficient of about 4.00. With regard to the *O. ramosa*; samples 1&3 that were found on the same host (*Lycopersicum esculentum*) are separated from sample 2 that was found on the other host (*Trifolium alexandrinum*). At the same time, the studied samples of this species (*O. ramose*) show a close similarity in the components of the storage seed protein that is indicated by the clustering of the three samples at a low distance coefficient (about 3.00). Also the three samples of *O. mutelii* are clustered at a low distance coefficient (about 3.00) indicating close similarity in the components of the seed storage protein. Although the two samples 1&3 of *O. mutelii* that were found parasitized on wild plants (*Malva parviflora* and *Rumex vesicarinus*) respectively are separated from sample 2 that was found on cultivated plants (*Lycopersicum esculentum*).

The difference between the two sections *Orobanche* Wallr. and *Trionychon* Wallr. observed in this study is corroborate with the taxonomic classification established by Beck-Mannagetta (1930) on the basis of morphological traits. Micromorphological studies of seed and pollen (Andary, 1994) have also confirmed the division of Old World *Orobanche* into two sections: seed testa cells with circular homogeneous perforations and spherical non-aperturate pollen in section *Orobanche*, and seed testa cells with a large perforation and tricolpate or triapeturate pollen in section *Trionychon*. Heckard and Chuang (1975) and Schneeweiss *et al.* (2004) supported the separation of the Old World *Orobanche* into two sections *Orobanche* and *Trionychon* by

using chromosome number. They recorded the basic chromosome number of x=19 in section *Orobanche* and x=12 in section *Trionychon*.

According to Abu Sbaih *et al*. (1994) and Abu Sbaih and Jury (1994), members of section *Trionychon* can easily be delimited from members of section *Orobanche* by their exine and seed coat sculpturing. The composition of Caffeic glycoside esters (CGEs) was also found to differentiate between the two sections (Andary, 1994). The genetic relationships among *Orobanche* species as revealed by RAPD analysis separated the species of the Old World *Orobanche* into two sections *Orobanche* and *Trionychon* (Roman *et al*., 2003 and Banett & Mathews 2006). In the present study, the polymorphism in seed protein characters supports the separation of the Old World species of the genus *Orobanche* into the two previous sections.

The four studied species of section *Orobanche* are separated into three groups; group one including *O. cernua*, group two including *O. minor* and group three including *O. pubescens* and *O. crenata*. Previous taxonomical chlorological and ecological aspects carried by Pujadas-Salva *et al*. (1994) separated *O. minor* in subsection *Minores*, *O. crenata* and *O. pubescens* in subsection *Speciosae* and *O. cernua* in subsection *Coerulescentes*. Recent molecular studies and the contrasting seed fatty acid profiles of section *Orobanche* clearly support the separation of *O. cernua* from the other species of section *Orobanche* (Paran *et a*l. 1997; Roman *et al.* 2003; Benett & Mathews, 2006; Pujadas-Salva & Velasco, 2000). The present study also supports these finding since the UPGMA and NJ dendogram clearly separated the species of the section *Orobanche*.

Within section *Trionychon*, the UPGMA and NJ trees (Figs. 3&4) show *O. aegyptiaca* is clearly delimited from the other studied members of the same sections: *O. mutelii*, *O. ramosa* and *O. lavandulacea*. Previous studies have also supported the differentiation between *O. aegyptiaca* and the other studied species (section *Trionychon*). Novopokrovsky and Tzvelev (1958) divided the section *Trionychon* into two subsections, *Holoclada* Novopokr. containing *O. aegyptiaca* and *Pleioclada* Novopokr. containing *O. mutelii*, *O. ramosa* and *O. lavandulacea*. Andary (1994) suggested the division of section *Trionychon* into two subsections: subsection *Ramosa* (type *O. ramosa* L.) including *O. ramosa*, *O. minor* and *O. lavandulacea*, where these species were characterized by branched stem and testa cells with large perforation and subsection Arenaria (type *O. arenaria*) including *O. aegyptiaca* in addition to *O. arenaria* that were usually with simple stems and testa cell walls with reticulate perforations. Also Velasco *et al.* (2000) separated the *O. ramosa* and *O. mutelii* from the other species of the section *Trionychon* by studying the fatty acid and Tocochromanol pattern in *Orobanche* seeds. However, recent molecular study (Roman *et al*. 2003) clearly supports the separation of the species of section *Trionychon* into two groups; one group containing *O. aegyptiaca* the other group containing *O. ramosa*, *O. mutelii* and *O. lavandulacea*.

In conclusion, the relationships between the studied species of *Orobanche* based on variation in polymorphism in seed protein components, agree with their delimitation in the two sections *Orobanche* and *Trionychon*. The delimitation of the studied species in section *Orobanche* in three groups is in agreement with their classification proposed by Rumsey & Jury (1991) and Pujadas-Salva *et al*. (1994). Moreover the delimitation of the studied species belonging to section *Trionychon* in two groups supports the previous delimitation of this section as proposed by Novopokrovsky and Tzvelev (1958), Andary (1994) and Roman *et al*. (2003).

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