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Genetic relationships of the genera *Onobrychis, Hedysarum,* and *Sartoria* using seed storage proteins

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Abstract: Fifteen species belonging to 3 genera (*Onobrychis, Hedysarum* and *Sartoria*), collected from different geographical regions of Turkey were studied for the polypeptide patterns of their seed storage proteins. The variability of seed storage proteins was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). As many as 72 bands were scored and a dendrogram was constructed using UPGMA (unweighted pair group method with arithmetic mean). The dendrogram of the electrophoretic protein profiles of seeds showed 2 main clusters. While the first cluster with further subclusters included *Onobrychis* species, the second cluster included *Sartoria* and *Hedysarum* species present in 2 separate subclusters. The results showed that *Sartoria* and *Hedysarum* are closer to each other than they are to *Onobrychis*. It is also suggested that *Sartoria hedysaroides* should be included in the genus *Hedysarum*. Additionally, it is concluded that seed storage protein profiles could be useful markers in studies of genetic diversity, genetic relationships, and classification of adapted cultivars.

Key words: *Onobrychis*, *Hedysarum*, *Sartoria*, SDS-PAGE, seed storage protein

Tohum depo proteinleri kullanılarak *Onobrychis*, *Hedysarum* ve *Sartoria* cinslerinin genetik akrabalıkları

Özet: Bu çalışmada Türkiye'nin farklı coğrafik bölgelerinden toplanan, üç farklı cins *Onobrychis, Hedysarum* ve *Sartoria*'ya ait olan on beş türün tohum depo proteinlerinin polipeptid örnekleri çalışılmıştır. Tohum depo proteinlerinin çeşitliliği sodium dodesil sülfat-poliakrilamit jel elektroforez (SDS-PAGE) ile analiz edilmiştir. 72 kadar bant skorlanmış ve UPGMA (unweighted pair group method with arithmetic mean) kullanılarak bir dendrogram yapılmıştır. Tohumların elektroforetik proteinlerinin dendrodramında iki ana grup görülmüştür. Daha fazla altgruplu ile ilk grup, *Onobrychis* türlerini içerirken, ikinci grup, iki ayrılmış altgrupta *Sartoria* ve *Hedysarum* türlerini içermektedir. Bu sonuçlar *Sartoria* ve *Hedysarum*'un birbirlerine, *Onobrychis*'den daha yakın olduğunu göstermiştir. *Sartoria hedysaroides*'in *Hedysarum* cinsi içinde olması gerektiği de önerilmiştir. İlave olarak, tohum depo protein profillerinin genetik çeşitlilik, genetik akrabalık ve adapte olmuş kültürlerin sınıflandırılması çalışmalarında faydalı markırlar olacağı sonucu çıkarılmıştır.

Anahtar sözcükler: *Onobrychis*, *Hedysarum*, *Sartoria*, SDS-PAGE, tohum depo protein

Introduction

Fabaceae, represented by 750 genera and more than 18,000 species, is one of the largest dicot families, having species of profound economic importance (1). The tribe Hedysareae DC. is represented by 4 genera in Turkey. These are *Ebenus* L., *Hedysarum* L., *Onobrychis* Mill., and *Sartoria* Boiss. & Heldr. The genus *Ebenus* has its centre in Anatolia, where all species are endemic. Only 6 species are known outside Turkey. The genera *Onobrychis* and *Hedysarum* constitute the main part of the tribe Hedysareae in the sense adopted by Polhill (2). The genus *Onobrychis* is represented by 162 species in the world, and is densely distributed in the Anatolia-Iran-Caucasian triangle. The genus *Hedysarum* is represented by 154 species and it has a distribution especially in Middle Asia (the genus centre of *Hedysarum*). This genus has also an important distribution in the Anatolian-Iran-Caucasian triangle. In Turkey, the genera *Onobrychis* and *Hedysarum* are represented by 52 species and 22 species, respectively (3). The genus *Hedysarum* is regarded as the closest relative of the genus *Onobrychis* in various studies, and the *Onobrychis* species are usually confused with the *Hedysarum* species during identification (4-8). Traditionally, the fruit shape has been basically used to separate these 2 genera. Most *Hedysarum* species have 2 or more segments to the lomentum whereas in *Onobrychis* the fruit is constantly 1-segmented; in the few species of *Hedysarum* that have only 1 segment to the fruit, this is a result of abortion of other segments. The 2 genera are also separated based on the floral characters of wing size and ovary shape (6). On account of seed number, *Onobrychis* includes less seed than the genus *Hedysarum* (9).

Sartoria is a monotypic endemic genus of Turkey. Hedge (6) indicated that, although allied to *Onobrychis* and *Hedysarum*, *Sartoria* is distinct from both of them. According to Hedge, *Sartoria* differs from *Onobrychis* in the 3 ovulate ovary and 2-3-seeded, large, ovate-oblong unarmed fruit, and from *Hedysarum* it is distinguished by the non-lomentoid fruit, small corollas, and dwarf habit.

The first specimens of *Sartoria hedysaroides* Boiss Heldr. were collected by Heldreich from the area around Geyikdag, South Anatolia, in 1845. Unfortunately, this species was not collected again. During an expedition for a floristic project, *Sartoria* was rediscovered by Ertuğrul et al. (10) and they reported a high degree of morphological variation in the 2 newly discovered populations of the genus *Sartoria*. According to the reports, plant stems showed distinctly very high variation between 2 localities. While some plants had short procumbent stems, others had conspicuous erect stems.

The genera analysed are economically important and, based on their beautiful flowers and habit, these genera could be used as ground cover in gardens. Animals that graze grass are also observed to eat *Sartoria*, and so after evaluation of its nutritional value it could also be utilised as fodder. Additionally, it could be used for grassland improvement and to help combat soil erosion (10). *Onobrychis*, which is another perennial and economically important genus, is used to improve the quality of the soil. It is also harvested as dried, fresh, and purebred fodder (11). The fruit and leaves of *Hedysarum syriacum* are used as animal feed (12). Some species, i.e. *H. varium* (Pied Spaniard trefoil), *Onobrychis armena* (Anatolia trefoil), and *O. sativa*, are described as legumes of high quality in the law concerning pastures.

Seed storage proteins are synthesized and accumulated to high levels in seeds during seed development. During seed germination, these proteins are degraded and the resulting amino acids are utilised by the developing seedlings as a nutritional source. Seed storage proteins are the major proteins in grains and, of the plant proteins, represent those that are the most abundantly consumed by humans and animals. Studies on the expression of seed storage proteins genes have contributed greatly to the development of plant molecular biology (13). Seed protein profiles are a powerful tool to ascertain genetic homology at the molecular level and to resolve taxonomic and phylogenetic problems (14). Sammour (15) used electrophoretic markers of the seed storage proteins to identify cultivars, to check species identification, to assist biosystematic analysis, to study phylogenetic relationships of the species, and to generate pertinent information to complement evaluation and passport data and thereby increase the knowledge of the genetic diversity of the materials in the germplasm collections in the laboratory.

This study aimed to detect the certain limitations and relationship degree of problematic genera in the tribe Hedysareae by SDS-PAGE methods.

Materials and methods

Materials: For seed protein profiles, specimens of 11 species belonging to 3 sections of the genus *Onobrychis*, 2 species belonging to 2 sections of the genus *Hedysarum*, and 2 different representatives of the genus *Sartoria* (short procumbent stems and conspicuous erect stems) were examined. The studied species and their locations are given in the Table.

Protein extraction and SDS-PAGE: Protein extraction was performed according to Saraswati et al. (16). Seeds were ground to fine powder with a pestle and mortar. Sample buffer was added to 0.04 g of seed flour as extraction liquid and mixed thoroughly in an Eppendorf tube by vortex. The extraction buffer contained the following final concentration: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS,

Table. Different taxa of *Onobrychis, Hedysarum*, and *Sartoria* and their localities (E - stands for endemic to Turkey).

Taxa	Section	Plant numbers	Locality
O. altissima Grossh.	*Onobrychis* Adans.	Ertuğrul 3456 Tugay	C6 Hatay: between İskenderun and Arsuz 15 km. 2 m. 36°31′524′N-36°02′035′E. 21.05.2005
O. oxyodonta Boiss.	*Onobrychis* Adans.	Ertuğrul 3166 Bağcı, Dural	C4 Konya: between Hadim and Taskent steppe. 1600 m. 14.07.2004
O. armena (E) Boiss. & Huet	*Onobrychis* Adans.	Bağcı 3099 Ertuğrul	C4 Konya to Karaman 60-70 km road side steppe, 1075 m. 37°26′907′N,-32°45′506′E, 10.06.2003
O. lasiostachya Boiss.	*Onobrychis* Adans.	Bağcı 3001 Ertuğrul	C4 İçel: Mut to Ermenek 5-6 km, Quercus scrub, 173 m. 36°39′681′N-33°23′168′E 10.06.2003
O. hajastana Grossh.	*Onobrychis* Adans.	Ertuğrul 3588 Uysal	C4 Karaman: Taşkale Avdan Mountain, Northern stony slopes. 1880 m. 37°02′459′N-33°40′859′E. 25.06.2005
O. oxyodonta Boiss.	*Onobrychis* Adans.	Tugay 1848	C4 Konya; Çumra to Bozkır 4 km, field side, 1000 m, 30.06.2001.
O. argyrea Boiss. subsp. *argyrea*	*Heliobrychis* Bunge.	Ertuğrul 2696 Tugay	C8 Diyarbakır Silvan to Diyarbakır Oğuzlar village, field sides, slopes. 38°03′680′N-40°34′580′E. 700-750 m. 15.06.2002
O. subacaulis Boiss.	*Heliobrychis* Bunge.	Ertuğrul 2610 Tugay	B9 Iğdır: Aralık-Kazım Karabekir Tarım İşletmesi. 1000 m. 37°47′160′N-44°37′200′E. 12.06.2002
O. galegifolia Boiss.	*Hymenobrychis* DC.	Ertuğrul 2911 Tugay	C8 Mardin: Bakırkırı Yusufdağ vineyards 1000 m. 37°18′997′N-40°46′790′E. 04.07.2003
O. tournefortii (Willd.) Desv.	*Hymenobrychis* DC.	Tugay 2864	C4 Konya; Bozkır, Ördek Bogazı, sandy and stony place, 1230 m, 24.07.2002
S. hedysaroides Boiss. & Heldr. (Erect)		Ertuğrul 3237	C4 Karaman: Ermenek above Göktepe Cedrus forest openings 36°36′444′N-32°36′494′E 1710 m. 16.07.2004
S.hedysaroides Boiss. & Heldr. (Prostrate)		Ertuğrul 3713	C4 Konya: Taşkent to Gevne Valley, Çukuryurt, Kızılgedik steppe. 1800 m. 30.05.2008
H. syriacum Boiss.	*Multicaulia* (Boiss.) B. Fedtsch.	Ertuğrul 2979 Dural	C4 Karaman: Karadağ–around Akkaya, debris 1300 1500 m. 12.07.2003
H. pannosum (Boiss.) Boiss.	*Crinifera* (Boiss.) B. Fedtsch.	Ertuğrul 2973 Dural	C4 Karaman: Karadağ-above Akkaya stony slopes 37°21′773′N-33°10′090′E 1496 m 12.07.2003

5% urea, and 5% 2-merkaptoethanol. Before centrifugation at 10,000 ×g for 5 min (4 °C), the sample buffer was boiled for 5 min.

SDS-PAGE was performed by a standard method on a vertical slab gel. Bromophenol blue was added to the supernatant as tracking dye to watch the movement of protein in the gel. Seed proteins were analysed by SDS-PAGE using 10% polyacrylamide gel. After electrophoresis, the protein bands were visualised by staining with Coomassie brilliant blue G-250 (17). Marker proteins (Fermentas) were used as references. Molecular weights of protein bands were estimated from their relative mobilities.

Data analysis: The polymorphic bands were scored visually as present [1] or absent [0]. Genetic dissimilarity among taxa was estimated using B/01D++ computer programs. Cluster analysis was performed using the UPGMA.

Result and discussion

In all, 72 polypeptide bands of different molecular weights ranging from 18.4 to 116 kDa were observed in 15 species belonging to the 3 genera as analysed on SDS gels (Figure 1). Protein profiles were basically similar among *Onobrychis* species and the most similarity occurred between 66.2 and 116 kDa. Some minor differences in protein patterns for *Onobrychis* species were particularly seen among 18.4-66.2 kDa. The observed protein patterns of different *Hedysarum* species were quite similar but minor differences were also observed in the region of 35-45 kDa. Although 2 populations of *S. hedysaroides* usually had common protein patterns, they had slightly different patterns for polypeptides in the region of 55-65 kDa. With this study, we also found data supporting previous studies concerning morphological differences and variation of the *Sartoria* populations (10). As a result, it may be

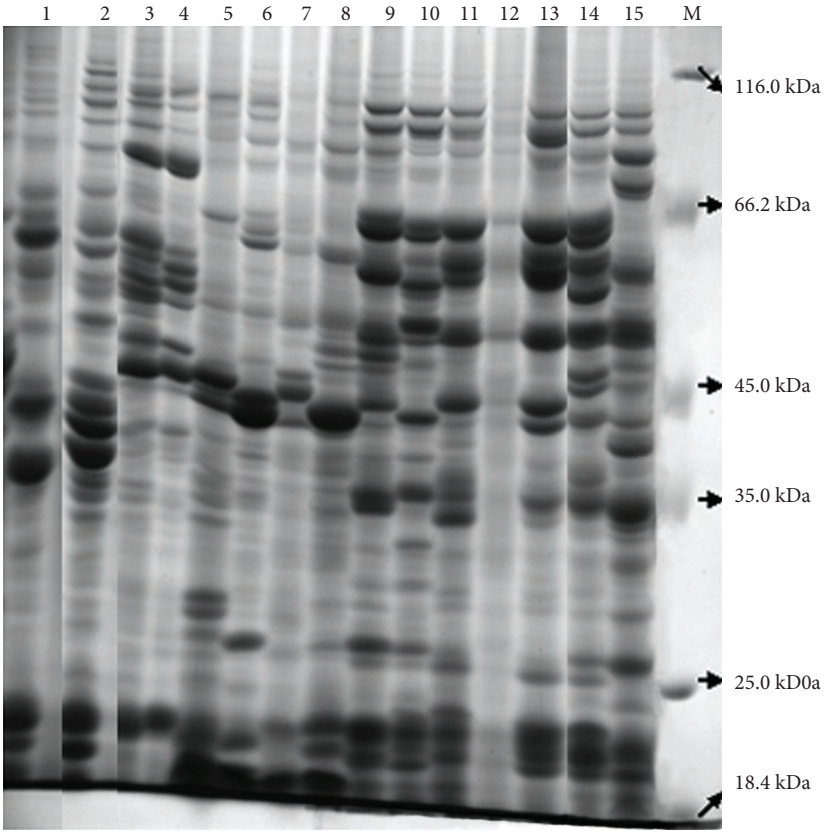

Figure 1. SDS-PAGE protein profiles of different taxa of *Onobrychis, Hedysarum*, and *Sartoria*. 1: *H. pannosum*, 2: *H. syriacum*, 3: *S. hedysaroides* (prostrate), 4: *S. hedysaroides* (erect), 5: *O. tournefortii*, 6: *O. galegifolia*, 7: *O. subacaulis*, 8: *O. argyrea* subsp. *argyrea*, 9: *O. oxyodonta*, 10: *O. hajastana*, 11:, 12: *O. armena*, 13: *O. lasiostachya*, 14: *O. oxyodonta*, 15: *O. altissima*, M: Marker.

stated that SDS-PAGE also supported the earlier studies on 2 *Sartoria* populations showing morphological variations.

When the protein patterns are considered for all species among subclades, a high similarity index of protein profiles (more than 80%) was noted for *Sartoria* and *Hedysarum*. Moreover, *Onobrychis* species were clearly clustered as a different genus with rate of dissimilarity rising to 38% within the A clade.

Cluster analysis of seed storage proteins of 15 species was performed on the results of SDS-PAGE using the B/01D++ computer program to find out the relationship degree among the species belonging to the tribe Hedysareae. The results of the cluster analysis are given in the dendrogram (Figure 2).

The dendrogram of total seed protein based on the dissimilarity matrix using UPGMA showed that all the species of the genera formed 2 main groups. Group A consisted of 2 subclades that belonged to different species of the genus *Onobrychis*. The first clade consisted of the section *Onobrychis*. The section has genetic distances between 10% and 22%. The second subclade consisted of 4 species belonging to the sections *Hymenobrychis* and *Heliobrychis*. The genetic distance in the second subclade ranged between 13% and 21%. Group B also consisted of 2 subclades. The first subclade consisted of 2 genotypes of *S. hedysaroides*. The genetic distance was around 11% for the 2 *Sartoria* genotypes. The second subclade was represented by *H. syriacum* and *H. pannosum*. The genetic distance was around 12% for the 2 *Hedysarum* species.

O. altissima species belonging to the section *Onobrychis* were far from other section members by 22%, while other members of the section *Onobrychis* were close to the others by a variety of rates ranging between 83% and 90%. Our results support the sectional classification given in the *Flora of Turkey* (6). According to the *Flora of Turkey*, the section *Hymenobrychis* is closer to *Heliobrychis* than the section *Onobrychis*.

Also in this study these 2 sections (*Hymenobrychis* and *Heliobrychis*) are placed in the same group and the distance between them is 21%. While the genetic distance between the 2 populations of *O. oxyodonta* is determined to be 13%, the 2 members of *O. lasiostachya* belonging to the same population are only 10% far from each other, which

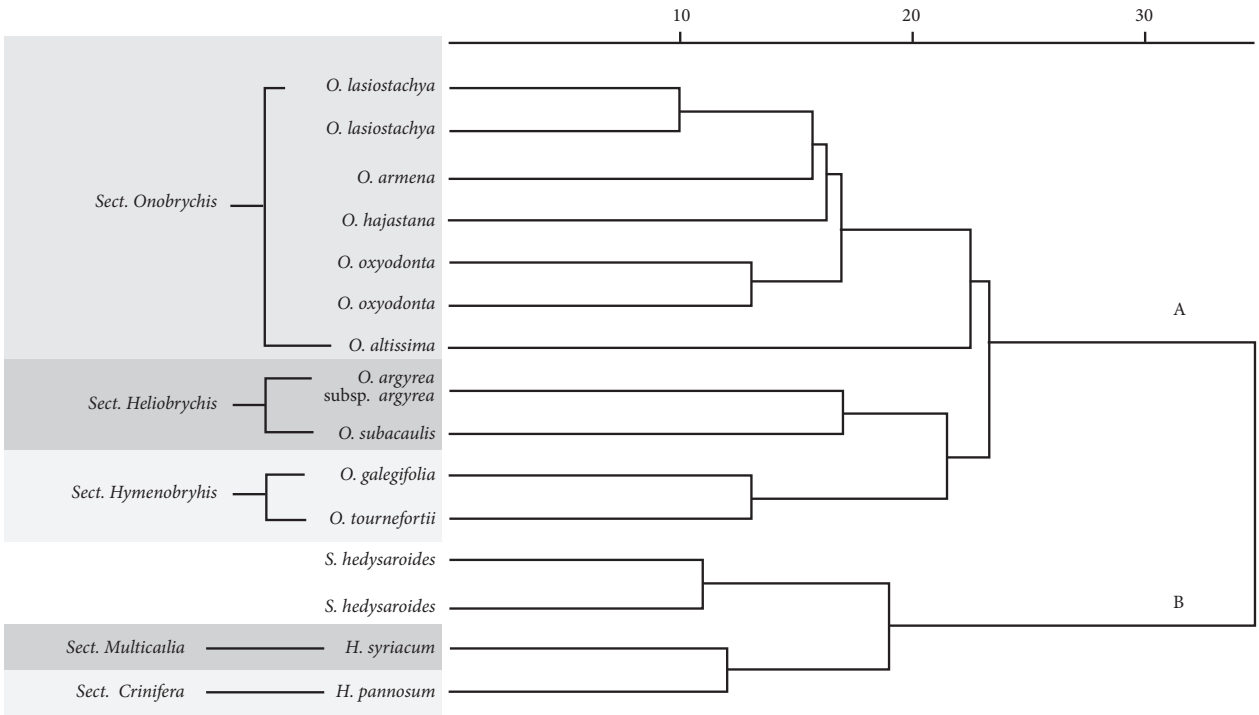

Figure 2. UPGMA dendrogram showing genetic distances among taxa of the genera *Onobrychis*, *Hedysarum*, and *Sartoria*.

is an expected result. While *Hedysarum* and *Sartoria* are placed in a group in which the distance is 19%, the distance of group B to group A containing *Onobrychis* species is determined to be 38%. Therefore, it is clear that the genus *Sartoria* is closer to *Hedysarum* than *Onobrychis*. In this study, the protein profiles of 2 vertical and horizontal forms of *S. hedysaroides* are also displayed. As a result of this protein profile, both populations have the same major bands, whereas the minor bands have differences. Taking the genetic distance between the different populations of *O. oxydonta* to be 13% into consideration, the 11% genetic distance in the 2 different forms of *Sartoria* expresses the genetic diversity in 2 different populations. Cluster analysis based on SDS-PAGE proved to be a powerful tool for differentiating *Onobrychis*, *Sartoria*, and *Hedysarum*.

Although there have been a limited number of studies on seed proteins belonging to the genus *Onobrychis*, no study on electrophoresis of seed storage proteins in the genera *Hedysarum* and *Sartoria* has been reported. As a result of the electrophoretic study carried out on seed storage proteins, Emre et al. (18) obtained 2 different groups with 36% similarity. While the first group was constituted by the species of the sections *Lophobrychis* and *Onobrychis*, the second group included members of the section *Hymenobrychis*. In that study, the rate of similarity between species of *Onobrychis* was 36%-63%. In our study, the sections *Onobrychis* and *Hymenobrychis* occur in 2 different groups with a similarity rate of 77%, which is higher than in previous studies (18). The rate of similarity between all species of *Onobrychis* was higher (77%-90%) compared to previous studies (18).

Although Emre et al. (18) noted in their study that the most similar protein bands were between 116 and 35 kDa, in our study the protein bands that showed the highest similarity among species of *Onobrychis* were observed between sizes 116 and 66.2 kDa. Cenci and Mizianty (19) reported that fruit characters were not sufficient for the discrimination of 3 species belonging to the genus *Onobrychis,* but they formed groups that showed similarity more than 70% with total seed proteins, and thus they confirmed that the 3 *Onobrychis* species show high similarity. The similarity rates of 77%-90% between species obtained in our study support the results of the previous study. Although the similarity rates (77%-90%) in our study do not support the similarity rates (38%-54%) detected by Cenci et al. (20) among species of *Onobrychis*, intra-species similarity rates (87%-90%) were close to the results of the previous study. Abou-El-Enain (21) reported that the seed protein data of different species of the section *Lophobrychis* revealed variations in the protein profiles that were species specific and were compatible with other taxonomic traits in demonstrating interspecific variability among the species of the section *Lophobrychis.* At the same time, these data confirm similarity more than 60% among species belonging to the genus *Onobrychis* as in the study by Abou-El-Enain (22).

The electrophoretic analysis of seed proteins of the 3 genera provided highly useful information regarding the relationships of different species of these genera. Based on these studies it may be suggested that of the 3 genera *Sartoria* and *Hedysarum* are closely related to each other, with *Onobrychis* being distant from the 2. This is in contrast to the previous reports, which mentioned a close taxonomical relationship among the genera *Onobrychis*, *Hedysarum*, and *Sartoria*. Studies using a number of nucleic acid markers like AFLP, SSP, and ribosomal gene analysis may help in further elucidation of their relationships.

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