

Determination of the relationship between some *Centaurea* species based on SDS-PAGE

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Abstract: The protein patterns of 47 *Centaurea* species from Turkey were obtained using SDS-PAGE methods. Relationships between *Centaurea* species and related genera were evaluated via use of a dendrogram. SDS-PAGE provided protein profiles for an important and applicable selection within *Centaurea*, particularly for the section *Cheirolepis* and its relatives. We determined that the technique could be used to resolve taxonomic and evolutionary problems in the genus *Centaurea*, as most of the *Centaurea* species studied exhibited special protein profiles and patterns.

Key words: *Centaurea*, endemic, molecular analysis, SDS-PAGE, taxonomy

SDS-PAGE metodu kullanılarak bazı *Centaurea* türleri arasındaki akrabalıkların belirlenmesi

Özet: Bu çalışmada Türkiye'den 47 *Centaurea* türüne ait protein örnekleri SDS-PAGE metodu ile elde edildi. *Centaurea* türleri ve ilgili cinsler arasındaki akrabalıklar bir dendrogram kullanılarak değerlendirildi. SDS-PAGE *Centaurea* içinde uygulanabilir ve önemli bir ayırım için, özellikle *Cheirolepis* seksiyonu ve akrabalarıyla ilgili protein profilleri sağladı. *Centaurea* türlerinin büyük çoğunluğunun kendilerine özgü protein örneklerini ve profillerini sergilemeleri nedeniyle, cinsin taksonomik ve evrimsel problemlerinin çözümünde bu tekniğin kullanılabileceğine karar verdik.

Anahtar sözcükler: *Centaurea*, endemik, moleküler analiz, SDS-PAGE, taksonomi

Introduction

Turkey is renowned throughout the world for its floristic richness. It has numerous endemic and rare plants, most of which are known as locally growing species. In Turkey, Asteraceae is an important large family that includes some genera of superior quality, such as the genus *Centaurea*, the third largest floral genus in Turkey. *Centaurea* has one of the highest rates of endemism in Turkey, with 112 endemics among 181 total species, 18 endemics among 32 subspecies, and 16 of the 28 varieties (1).

The taxonomy of the genus *Centaurea* has been debated since it was first described. However, some realistic suggestions and regulations for the taxonomy of the genus have been submitted in recent years by modern taxonomists (2-6), and *Centaurea* experts have readily adopted them. Recent taxonomic studies based on morphology, karyology, palynology, and molecular sequencing have investigated *Centaurea*. As a result of these studies, new taxonomic divisions have been made in the *Cardueae* tribes: *Rhaponticoides* and *Psephellus* have been separated from the genus

Centaurea (5,6) and have therefore been elevated to their own genera.

The genus *Centaurea* in Turkey comprises a taxonomically complex group of plants with systematic problems yet to be solved. Previously, no extensive study of Turkish endemic *Centaurea* based on molecular analysis had been performed. The genus *Centaurea* was recently revised via molecular sequencing analysis by Garcia-Jacas et al. (4). The present study predominately used samples from Turkey, Europe, Iran, the Iberian Peninsula, and Armenia. The aim of the study was to confirm or refute what were essentially biogeographical groupings proposed in a previous study.

Polyacrylamide gel electrophoresis (PAGE) is a widely used biochemical method, favored for its accuracy and simplicity in describing the genetic structure of plant collections. Seed protein patterns obtained using the PAGE method have been successfully used to elucidate the taxonomy and evolutionary relationships of several species, because banding patterns are species specific and completely dependent on the genotype (7). Seed storage proteins have been used as genetic markers in the analysis of

genetic distances within and between species in Turkey in order to clarify their taxonomic relationships (8,9).

The present study aimed to evaluate the reliability of SDS-PAGE profiles as tools for the classification of Turkish *Centaurea* species. Generally, taxonomists assume that different environmental conditions affect seed protein patterns in flowering plants. Contrary to general consensus, we suggest that SDS-PAGE should be used in xerophytes like *Centaurea*, many species of which are found in arid regions. Moreover, we wondered how much resolution SDS-PAGE would provide for *Centaurea* taxonomy and to what degree these results would support previous molecular analyses, such as those reported by Garcia-Jacas et al. (4).

Materials and methods

Plant material

The seeds of *Centaurea* species used in this study were obtained from the KNYA Seed Bank at the Selçuk University Herbarium. The names of the studied species and their locations are given in the Table.

Table. The names and the locations of *Centaurea* and closely related species used for protein analyses.

Band no.	Species	Locations
2	<i>Rhaponticoides mykalea</i>	C1 Aydın: between Kuşadası and Söke, 5 km, 50 m, 29 vi 2006, O. Tugay 4122, Ertuğrul & Uysal
3	<i>R. iconiensis</i>	C4 Konya: Seydişehir-Bozkır road 20 km, 1050 m, Ertuğrul 2480, 14 vii 2001.
4	<i>Centaurea balsamita</i>	C4 Konya, between Çumra and Bozkır, 25 km, 1100 m, 24 vii 2002, O. Tugay 2868 & Uysal.
5	<i>C. coronopifolia</i>	A4 Çankırı: between Çankırı and Bayat, 42 km, 600 m, 16 vii 2005, O. Tugay 3712 & Uysal
6	<i>C. polyclada</i>	B1 Balıkesir: Zeytinli, Akçay, 11 m, 31 vii 2006, O. Tugay 4330 & Uysal
7	<i>C. yozgatensis</i>	B3 Eskişehir, between Eskişehir and Sarıcakaya, on rocks, 200 m, 4 viii 2006, O. Tugay 4408 & Uysal
8	<i>C. pinetorum</i>	C4 Mersin: between Mut and Mersin, 4 km, 210 m, 17 vi 2006, O. Tugay 4087
9	<i>C. lycaonica</i>	C4 Konya: between Konya and Seydişehir, 50 km, 1580 m, 22 vii 2006, O. Tugay 4146 & Uysal
10	<i>C. hierapolitana</i>	B2 Denizli: between Denizli and Afyon, beside Acıgöl, 1100 m, 30 vi 2006, O. Tugay 4131, Ertuğrul & Uysal
11	<i>C. inexpectata</i>	C4 Antalya: Gevne Valley, around Küçükklü village, 1750 m, 30 vi 2004, Uysal, 598
12	<i>C. cheirollopha</i>	C5 Adana: Osmaniye, Yarpuz road, forest openings, 800 m, 26 vii 2003, Uysal 534
13	<i>C. cheirolepidoides</i>	C3 Antalya: Elmalı, Teke-Çiğlikara, 1500 m, 28 vi 2004, Uysal 1000
14	<i>C. cf. isaurica</i>	C4 Karaman: Ayrancı, northern slopes of Avdan Mountain, 1500 m, Ertuğrul 2309.

Table. (Continued).

Band no.	Species	Locations
15	<i>C. isaurica</i>	C3 Konya: on the Seydişehir-Antalya road, around Tinaztepe, 1500 m, 12 vii 2003, Uysal 506
16	<i>C. drabifolia</i> subsp. <i>detonsa</i>	C4 Karaman: Ermenek-Kazanç village, Kırkkuyu, 1800m, 13 vii 2003, Uysal 517
17	<i>C. drabifolia</i> subsp. <i>cappadocica</i>	B5 Yozgat: Deveci Mountain, Artova Yeşilyurt road, Kunduz Deresi, 1120 m, 26 vii 2003, Uysal 525
18	<i>C. kotschy</i> var. <i>kotschy</i>	C4 Konya: Karapınar, Protection Research Area, 1800 m, 27 vii 2003, Uysal 535
19	<i>C. kotschy</i> var. <i>persica</i>	B5 Sivas: Hafik, Celali district, Süleymaniye Bakımlı village road, 1400 m 26 vii 2003, Uysal 527
20	<i>C. kotschy</i> var. <i>decumbens</i>	C4 Karaman: between Ermenek and Tekeçatı, Akpınar, roadside c. 1500 m, 13 vii 2003, Uysal 514
21	<i>C. derderiifolia</i>	B6 Kayseri: Görün-Divriği road, 18 km, 1500-1600 m, 26 vii 2003, Uysal 530
22	<i>C. deflexa</i>	C4 Konya: Taşkent-Ermenek road, 22 km, 1660 m, 13 vii 2003, Uysal 512
23	<i>C. nivea</i>	B3 Eskişehir: Mihalçık, Mihalpu-Alpu road, 19-20 km, c. 900-950 m, 15 vii 2003, Uysal 519
24	<i>C. sericea</i>	B3 Eskişehir: between Eskişehir and Kütahya, 1140 m, 14 viii 2005, O. Tugay 3647 & Uysal
25	<i>C. cankiensis</i>	A4 Çankırı: Kalfat-Atkaracalar road, 1455 m, 26 vii 2003, Uysal 524
26	<i>C. armena</i>	B9 Van: Gürpınar, Kurubaş, 2237 m, 7 viii 2003, Ertuğrul 2940
27	<i>C. macrocephala</i>	A9 Ardahan : Ardahan-Şavşat road, 16. km, 2140 m, 07.08.2007, 41 11 677 N, 42 33 309 E, O.Tugay 5127 & Uysal.
28	<i>C. glastifolia</i>	A9 Ardahan: between Ardahan and Şavşat, 1810 m, 7 viii 2007, O. Tugay 5123 & Uysal
29	<i>C. pterocaula</i>	C4 Konya: Cihanbeyli, between Gölyazı and Günyüzü, 930 m, 09 vii 2005, O Tugay 3533 & Uysal
30	<i>C. spectabilis</i> var. <i>spectabilis</i>	B9 Van: Van-Gürpınar, Kurubaş pass, 2237 m, 7 viii 2003, Ertuğrul 2941a
31	<i>C. spectabilis</i> var. <i>microlophus</i>	B9 Van: Van-Gürpınar, Kurubaş pass, 2237 m, 7 viii 2003, Ertuğrul 2941b
32	<i>C. stapfiana</i>	B8 Diyarbakır: Diyarbakır-Silvan, Yeşilköy village, 627 m, 1 viii 2004, Uysal 900
33	<i>C. sclerolepis</i>	B8 Diyarbakır: Diyarbakır- Silvan, 38 km, 751 m, 1 viii 2004, Uysal 898
34	<i>C. fenzi</i>	B8 Erzurum: Erzurum-Varto, 1 km, 1650 m, 31 vii 2004, Uysal 895
35	<i>C. tomentella</i>	B7 Malatya: Doğanşehir-Polat road 4 km, 1000 m (Garcia-Jacas, Susana, Uysal 2353)
36	<i>C. babylonica</i>	C6 Kahramanmaraş: Süleymanlı district road, 750 m, 26 vii 2003, Uysal 533
37	<i>C. thracica</i>	B2 Balıkesir: Dursunbey, 502 m, 29 vi 2004, Uysal 539
38	<i>C. athoa</i>	B1 Balıkesir: Edremit, KazDağları, Sarıkız hill, 1650 m, 15 vii 2003, Uysal 521
39	<i>C. carduiiformis</i> subsp. <i>carduiiformis</i>	A4 Çankırı: between Bayanpınar and Çankırı, 3. km, 635 m, 16 vii 2005, O.Tugay 3720 & Uysal
40	<i>C. carduiiformis</i> subsp. <i>orientalis</i>	B5 Yozgat: Yeşilyurt between, Kunduz Deresi, rock on the roadside, 1120 m, T. Uysal 348, 27.7.2003
41	<i>C. pseudoscabiosa</i> subsp. <i>pseudoscabiosa</i>	B8 Erzurum, Tercan, Aşkale road, 1880 m, O. Tugay 5076 Uysal, 05.08.2007
42	<i>C. pseudoscabios</i> subsp. <i>araratica</i>	B8 Erzurum: 23 km before to Varto, 1700 m, 31 vii 2003, Uysal 894
43	<i>C. aegialophila</i>	C3 Antalya: Side, ancient city, beach, 15 m, 22 vii 2007, O.Tugay 4158 & Uysal
44	<i>Psephellus mucronifer</i>	B6 Sivas: between Gürün and Kangal, 1880 m, 04 viii 2007, O.Tugay 5050 & Uysal
45	<i>P. hadimensis</i>	C4 Konya: Hadim, Tosmur Plateau, Gevne Valley, 1700 m, 21 vi 2001, O.Tugay 1754 & Uysal
46	<i>P. pulcherrimus</i>	A9 Ardahan: between Ardahan and Şavşat, 2150 m, 07 viii 2007, O.Tugay 5126 & Uysal
47	<i>C. triumfetti</i>	C4 Konya: Hadim, Çalca, 1780 m, 31 v 2001, O.Tugay 1421
48	<i>C. tchihatcheffii</i>	B4 Ankara: Gölbaşı, field sides, 990 m, 17 vii 2005, O.Tugay 3727 & Uysal

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Seed storage protein isolation was carried out in the manner described by Saraswati et al. (10). The SDS-PAGE methods used followed Laemmli (11,12). Proteins on the gel were fixed and stained overnight, according to Demiralp et al. (13). Seeds were ground to a fine powder with a mortar and pestle. Sample buffer was added to 0.04 g of seed flour as extraction liquid and mixed thoroughly in an Eppendorf tube with vortex. The extraction buffer contained the following final concentration: 0.5 M Tris-HCl (pH 6.8), 10% SDS, urea, and 5% 2-merkaptoethanol. Before centrifugation at 10,000 ×g for 5 min (4 °C), the sample buffer was boiled for 5 min. Standard SDS-PAGE was performed on a vertical slab gel. Bromophenol blue was added to the supernatant as tracking dye in order to observe the movement of protein in the gel. Seed protein was analyzed via slab-type SDS-PAGE, using 10% polyacrylamide gel. Following electrophoresis, the protein bands were visualized with Coomassie brilliant blue G-250. The seed storage protein profile patterns of the genus *Centaurea* were scored automatically. A dendrogram was created to determine genetic distance via the Bio-Profiles analysis system.

Results and discussion

The 47 species protein patterns obtained with SDS-PAGE analysis are shown in Figure 1. Relationships between *Centaurea* species and their relatives were evaluated using a dendrogram (Figure 2). Analysis of the seed proteins showed that all the studied genotypes had a specific protein pattern.

The dendrogram differentiated 2 main groups (Figure 2). The first group was divided further into 3 subgroups, with genetic distances between 0% and 90%. The upper cluster consisted of *Rhaponticodes mykalea*, *R. iconiensis* (genus *Rhaponticoides*), *Centaurea balsamita*, and *C. coronopifolia* (sect. *Stizolophus*). The middle cluster consisted of *C. polyclada*, *C. yozgatensis*, *C. pinetorum*, *C. lycaonica* (sect. *Phalolepis*), *C. hierapolitana* (sect. *Ammocyanus*), *C. inexpectata* (sect. *Jacea*), and *C. macrocephala* (sect. *Grossheimia*). The lower cluster included *C. cheirollopha*, *C. cheirolepidoides*, *C. cf. isaurica*, *C. isaurica* (sect. *Pseudoseridia*), *C. drabifolia* subsp. *detonsa*, *C. drabifolia* subsp. *cappadocica*, *C. kotschy* var. *kotschy*, *C. kotschy* var. *persica*, *C. kotschy* var. *decumbens*, *C. derderiifolia*, *C. deflexa*, *C. nivea*, *C. sericea*, *C. cankiensis* (sect. *Cheirolepis*), *C. armena* (sect. *Rhizocalathium*), *C. glastifolia*, *C. pterocaula* (sect. *Chartolepis*), *C. spectabilis* var. *spectabilis*, and *C. spectabilis* var. *microlophus* (sect. *Phaeopappus*).

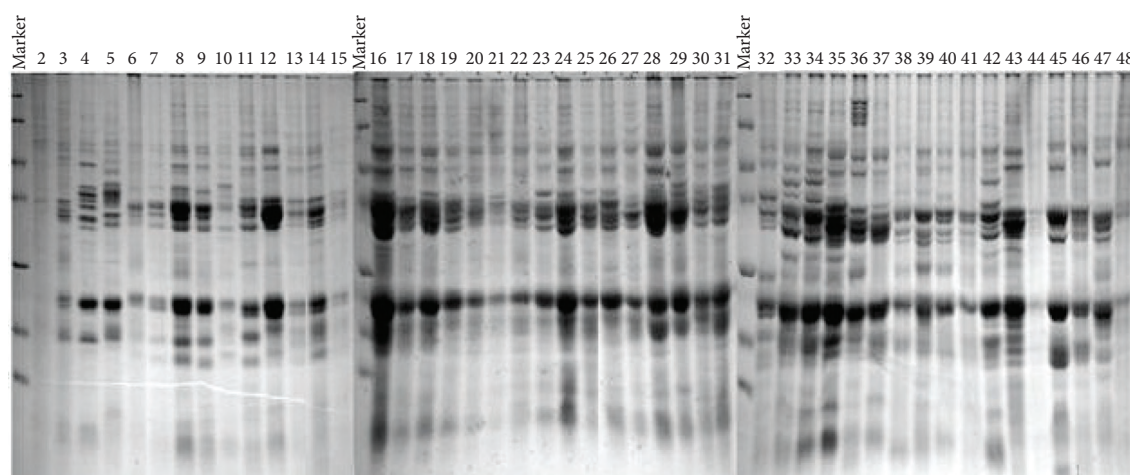


Figure 1. *Centaurea* species protein profiles obtained via SDS-PAGE methods.

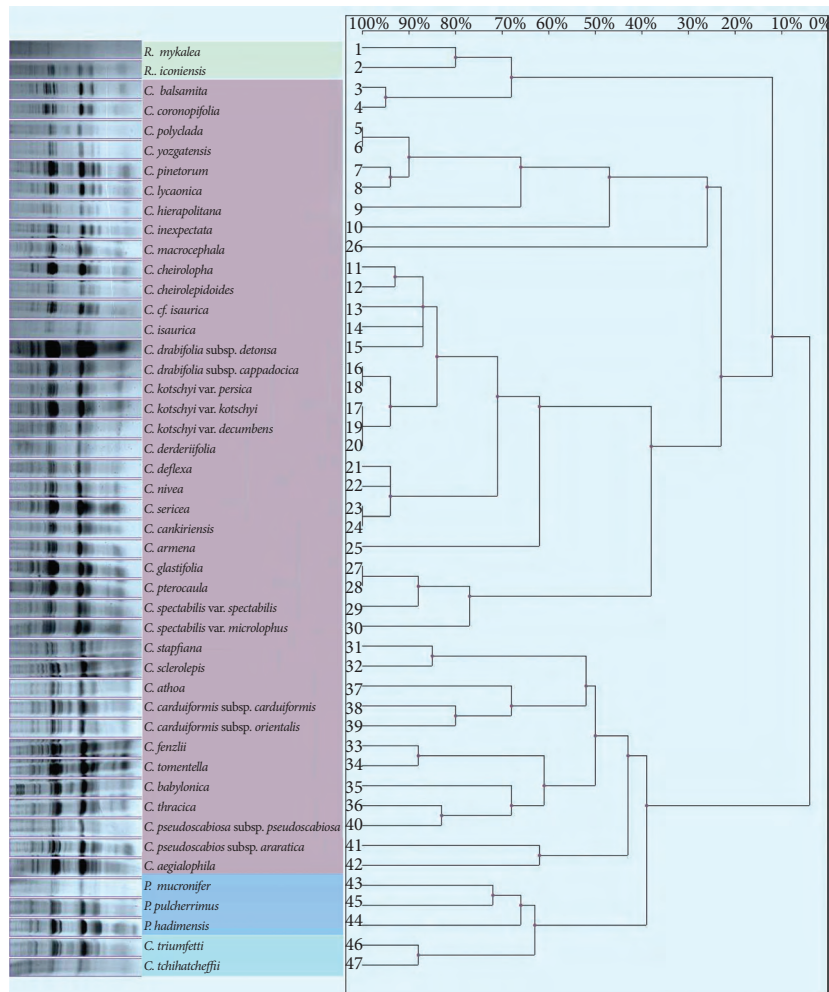


Figure 2. The genetic relationships of *Centaurea* and related species according to protein profiles.

First, *R. mykalea* and *R. iconiensis* were placed in the first main clade as an outer group, the group most genetically distinct from the other species in the dendrogram. The genetic distance between *R. mykalea* and *R. iconiensis* was 20%. *Stizolophus* spp. were surprisingly connected to this outer group, showing a genetic distance of only 32%. The 2 endemic taxa of *Rhaponticoides* were placed outside the genus *Centaurea*, as expected based on previous analyses (2-4,6,14); however, the result for *Stizolophus* was not anticipated. Second, 3 groups were placed together: *Acrolophus*, *Ammocyanus*, and *Chartolepis*. The 4 endemic taxa of the *Acrolophus* group were positioned very close together, according to protein

patterns that determined genetic distance was at most 10%. The protein patterns for this group were fairly similar, but they were slightly different from those of the related species. *C. polyclada* exhibited no difference from *C. yozgatensis* (genetic distance was 0%). The other 2 endemic species, *C. lycaonica* and *C. pinetorum*, had slightly distinct protein profiles (with a genetic distance of 5%). The clade that included the *Acrolophus* group was connected to *C. hierapolitana* of the *Ammocyanus* group. The genetic distance between these 2 groups was about 30%. As these 2 groups are considered to be closely related morphologically, the similarity in their protein patterns was to be expected. The other 2 species

placed within first main clade (*C. macrocephala* and *C. inexpectata*) were connected to the first main clade with a very low degree of similarity in protein patterns (genetic distances between 50% and 68%). The high genetic distance at this intrasectional level is not surprising, as these 2 species represent their own groups and had the protein profiles unique to those groups. Finally, the largest group within the first main clade contained *Cheirolepis* and its relatives.

We propose that protein profiles can help explain the taxonomy specific to the *Cheirolepis* group and related species. These profiles, for the most part, confirmed the results of the molecular analysis reported by Garcia-Jacas et al. (4). The protein profiles did not taxonomically differentiate between the *Cheirolepis* and *Pseudoseridia* groups. Moreover, taxa belonging to these groups were placed very close together in a single group, and genetic distances between the *Cheirolepis*-*Pseudoseridia* complex were very low (between 0% and 25%). *C. cheirolopha* and *C. cheirolepidoides* were placed together in the center of this large group, with a genetic distance of 10%. This group also included 1 undefined specimen placed near *C. isaurica*, based on a genetic distance of 12%. Two morphologically very similar species (*C. drabifolia* and *C. kotschyi*) and their taxa were placed close together, with a genetic distance of 5%. *C. sericea* and *C. cankiriensis* were completely identical with respect to their protein patterns (genetic distance was 0%). *C. deflexa* and *C. nivea* also had largely similar protein patterns; the genetic distance between them was about 10%. Furthermore, all species within the large group comprising the *Cheirolepis*-*Pseudoseridia* complex shared a basic chromosome number of $x = 9$ (15). Even *C. armena* was closely related to this group, with a genetic distance of 35%, and protein profiles were separated within a linked group on a sectional level. In addition, 2 taxonomically closely related species (*C. glastifolia* and *C. pterocaula*) had identical protein patterns (genetic distance of 0%) and were connected to *C. spectabilis* from the *Phaeopappus* group in the last branch, with a genetic distance of 55%.

The second main clade was also divided into 4 small subgroups, with genetic distances between 0% and 60%. The 1st and 2nd subgroups could be considered a complicated subclade. The 4 taxa of the

Acrocentron group included in this study (*C. carduiformis* subsp. *carduiformis*, *C. carduiformis* subsp. *orientalis*, *C. pseudoscabiosa* subsp. *pseudoscabiosa*, and *C. athoa*) were linked to the complicated clade with a genetic distance of 55%. The similarity of the protein patterns among *Acrocentron* species was not very high (genetic distances between 20% and 50%). In this complicated clade the *Acrocentron* spp. were positioned next to the genus *Psephellus* and the *Cyanus* (*Centaurea*) spp. In addition, 3 representatives of the *Phaeopappus* group within the complex clade were included (*C. fenzlii*, *C. tomentella*, and *C. sclerolepis*). Interestingly, the taxa belonging to the *Phaeopappus* group were placed in different positions and on different branches of the dendrogram. The genetic distance between *C. stapfiana* and *C. sclerolepis* was about 15%; however, these 2 species were placed far from *C. fenzlii* and *C. tomentella*, with a genetic distance of 50%. *C. stapfiana* also showed some clear morphological differences from the other species of the *Phaeopappus* group. For example, the appendages of *C. stapfiana* had distinct hyaline borders without ciliate, but the appendages of the other taxa examined within the group had ciliate borders. Finally, 2 species of the *Microlophus* group were positioned next to an undetermined species in this complex.

In *The Flora of Turkey*, *C. babylonica* and *C. thracica* are considered very closely related species, taxonomically. However, recent molecular sequencing studies suggest that *C. thracica* should be placed in the same clade as the *Cheirolepis*-*Pseudoseridia* complex (4). According to the protein patterns observed, *C. thracica* and *C. babylonica* had protein patterns that were less similar, with a genetic distance of 32%. Furthermore, according to their protein patterns, these 2 species did not exhibit any relationship with the *Cheirolepis*-*Pseudoseridia* complex, as the genetic distance between *Microlophus* and the *Cheirolepis*-*Pseudoseridia* complex was about 95%. Although the undetermined species (*C. pseudoscabiosa*) was placed near *C. thracica*, with a genetic distance of 22%, morphologically it primarily resembled *C. pseudoscabiosa*. Although this undetermined species could be seen as a new taxon of the Turkish flora, more research is needed.

In the last subgroup of the second main clade in the present study, *Psephellus* was represented by 3 endemic species. The *Psephellus* spp. studied were similar with respect to their protein patterns, with genetic distances between 30% and 35%. The genetic distance between *P. mucronifer* and *P. pulcherrimus* was 30%. *P. hadimensis* differed from *P. mucronifer* and *P. pulcherrimus* by a genetic distance of 35%. The last 2 species within the second main clade belonging to the *Cyanus* group were placed the farthest from the rest of the group, as though they were a different genus. These species, along with *Psephellus*, were clearly separated from the core of the *Centaurea* complex, with a genetic distance of at least 60%.

In conclusion, SDS-PAGE facilitated important and applicable selection in the sectional or interspecies level within *Centaurea*, particularly for *Cheirolepis* and its relatives. We think that the

technique could be used to resolve the taxonomic and evolutionary problems in the genus *Centaurea*, since most of the *Centaurea* spp. studied had unique protein profiles and patterns. Consequently, the related species and sections were placed meaningfully into clades using a dendrogram. Moreover, SDS-PAGE confirmed previous findings and supports a proposed separation in the level of the genera, such as *Psephellus* and *Rhaponticoides*.

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