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Cephalaria duzceënsis (Dipsacaceae), a new species from the western Black Sea region, Turkey

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A new species, *Cephalaria duzceënsis* N. Aksoy & R. S. Göktürk (Dipsacaceae) from western Black Sea region of Turkey is described. It is illustrated in line drawings and the morphology of the new species is compared with *Cephalaria speciosa*. Molecular and morphological methods were used to separate the two species. Both species were first identified morphologically and then studied by randomly amplified polymorphic DNA (RAPD) markers. Genetic similarities were calculated based on the RAPD data and used to construct a UPGMA dendrogram. According to the result, two main clusters were observed using the character differences. It is concluded that *C. duzceënsis* sp. nov. is different from *C. speciosa*.

During vegetation fieldwork on the Elmacik mountain, material of an interesting plant was collected by the senior author (NA) in July 2004, Gölyaka, (Düzce). On further visits to the same and nearby localities in August 2005, more material was gathered providing a range of specimens bearing good flowers and fruits. These specimens were compared with material in the herbaria of ANK, GAZI, HUB, ISTF, ISTE, AKDU (herbarium of the Biology Dept of Akdeniz Univ.) (Appendix 1) and the following literature: Flora of Turkey (Matthews 1972, Davis et al. 1988, Duman 2001), Flora Europaea (Ferguson 1976), Flora of the USSR (Shishkin and Bobrov 1957), Flora Iranica (Lack 1991), Monographia genus *Cephalaria* (Szabó 1940), the Flora of Turkey check-list II (Özhatay et al. 1999), the Flora of Turkey check-list III (Özhatay and Kültür 2006) and Revision of the Turkish *Cephalaria* (Göktürk 2003). However, no matching specimen or descriptions were found and we came to the conclusion that our sample was a new species.

RAPD profiling is a useful means of determining the genetic distinctness and variation of rare and endangered plants using minuscule amounts of leaf tissue. RAPD profiling also distinguishes between population level and species-level genetic variation economically and rapidly (Stewart and Porter 1995). Apart from identification, proteins and DNA-based data can also be used in tests of parentage, in genetic mapping and in the measurement of genetic diversity (Chapparo et al. 1994, Iqbal et al. 1997).

Material and methods

The new species was collected from an open rock area in *Pinus sylvestris* forest, on disturbed ground, Gölyaka in Düzce province, (A3), 644 m, in 2004 and deposited at ISTO. The other plant species used in this study was collected from steppe, Tercan in Erzincan province (B8), 1765 m, in 2000 and deposited at AKDU (unregistered acronym, herbarium of the Biology Dept of Akdeniz Univ.). In total 17 herbarium specimens (22 individuals) of the presumed new species were collected from two adjacent localities and deposited in the herbaria of ISTO, GAZI, AIBU and AKDU.

Extraction of DNA

Plant DNA extraction was carried out using the methods of Doyle and Doyle (1987), with minor modifications. DNA was isolated from dried leaves. Dried leaves (0.05 g) were ground using mortar. The grindate was added to 1 ml extraction buffer (2% (w/v) CTAB; 100 mM Tris-Cl buffer (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, (1% 8w/v) PVP-40) and incubated at 65°C for 90 min. The homogenate was mixed with 500 24:1 µl chloroform: isoamyl alcohol (v/v) and mixed well by gentle inversion. Following centrifugation at 10 000 rpm for 10 min, the upper aqueous layer was transferred to a fresh tube containing 600 µl of isoproponal, the mixture was allowed to sit at room temperature for 40 min. Following centrifugation at

Table 1. Primers used in the investigation.

Primer no.	Primer sequence (5 →3′)	Primer no.	Primer sequence (5 →3′)
A2	TGCCGAGCTG	OPW 10	TCGCATCCCT
LA12	ACGACCCACG	OPR 03	ACACAGAGGGT
LA13	CACCACGCCT	OPB 10	CTGCTGGGAC
SC1023	GGCTCGTACC	B6	TGCTCTGCCC
OPC 02	GGTCTACACC	B4	GGACTGGAGT
M13	GAGGGTGGCGGTTCT	0PA07	GAAACGGGTG
OPU16	CTGCGCTGGA	B7	GGTGACGCAG

5000 rpm for 3 min the samples were washed twice with 76% ethanol. The pellets were allowed to sit overnight at room temperature, and resuspended in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA pH 8.0).

RAPD Analysis

The selected thirty arbitrary primers were purchased from Operon Technologies (Table 1). Amplification reactions were performed using the protocol of Williams et al. (1990). Two samples representing each taxon were used for amplification. Amplification was repeated twice for each sample. For the DNA amplification, Biometra thermocycler was programmed for 45 cycles at 96°C for 30 s, 35°C for 30 s and 72°C for 30 s, for denaturing, annealing and primer extension, respectively. Following the procedure, 20 µl of the samples were loaded onto 2% agarose gels in 1 × TAE buffer and run at 75 V for about 4 h. A 1 kb DNA ladder was used as molecular weight marker. The gel was stained with ethidium bromide and photographed under UV light (Maniatis et al. 1982). The images were used for the analysis of the amplification products. Only bands reproducible in multiple runs were considered. Out of 30, only 14 primers which showed the best readability were used in the calculations (Table 1).

Data analysis

Bands on RAPD gels were scored as either present (1) or absent (0) for all subspecies studied. Common band analysis was conducted using the computer programme POPGEN (dendrogram based Nei's 1978, genetic distance: method = UPGMA – modified from NEIGHBOR procedure of PHYLIP ver. 3) to determine the genetic distance between them. The values for genetic distance were then used in a cluster analysis to generate UPGMA-dendrograms (Nei 1978).

The new species described bellow is relatively close to *Cephalaria speciosa*, but differs from it by characters listed in Table 2.

Cephalaria duzceënsis N. Aksoy & R. S. Göktürk sp. nov. (Fig. 1)

Cephalariae speciosae affinis sed ab ea planta rhizomatosa, foliis inferioribus deflexis, capitula globosa, florifera 2–2.5 cm in diametro, fructifera 2.5–3 cm in diametro (non ovato-globosa, florifera 2.5–4.5 cm in diametro, fructifera 2–3.5 cm in diametro), corollis cremeis (non dilute luteis vel cremeis), bracteis involucralibus ovatis–oblongis, 8–12.5 × 3.5–5 mm, omnino nigris vel stramineis, mucronatis (non ovatis vel triangulari–anceolatis, 7–15 × 3–7 mm, omnino stramineis, acuminatis vel subacuminatis), bracteis receptacularibus late oblongo–lanceolatis, 13–15.5 × 1.5–2.5 mm, omnino stramineis vel apice brunneis, subacuminatis (non triangulari–lanceolatis, 12–20 × 3.5–6 mm, omnino stramineis, acuminatis), involucris in fructo 6.1–7.1 mm longis, sericeis (non 7–13 mm longis, pilosis), nec non habitatione pinetosa (nec stepposa) differt.

Holotype: Turkey, western Black Sea, A3 Düzce: Gölyaka, Elmacik mountain, Balikli area, behind of Konaş, Melik deresi, open rock area in *Pinus sylvestris* forest, on disturbed ground, 644 m, 30 Aug 2004, 40°41′924″N, 31°02′452″ E, N. Aksoy 5339 (holotype ISTO 31000; isotypes GAZI, AIBU, AKDU).

Paratype: Turkey, western Black Sea: A3: Düzce, Gölyaka, Elmacik Dağı, Kardüz Area, upper side of Melik deresi, Karaardiç open rock area of *Pinus sylvestris* and *P. nigra* forest, on disturbed ground, 1265 m, 6 Aug 2005, N. Aksoy 5978 (Düzce Univ. Herbarium.

Slender, erect, rhizomatous, perennial herb. Stem up to 1.5 m tall, simple, branched from the middle or upper part, solid or hollow, sparsely stellate hairy throughout and retrorse hairy in lower part. Leaves coriaceous, stellate hairy

Table 2. Morphological comparasion of *Cephalaria duzceënsis* sp. nov. with *C. speciosa*.

Taxon character	*C. duzceënsis*	*C. speciosa*
Cauline	rhizomatous	non rhizomatous
Lower leaves	deflexed from petiole	not deflexed from petiole
Capitula	globose, 2–2.5 cm diameter in flower, 2.5–3 cm diameter in fruit	ovate–globose, 2.5–4.5 cm diameter in flower, 2–3.5 cm diameter in fruit
Corolla	cream	pale yellow–cream
Involucral bracts	narrowly ovate–oblong, 8–12.5 × 3.5–5 mm, completely black or straw-coloured, mucronate at apex	ovate to triangular–lanceolate, 7–15 × 3–7 mm, completely straw-coloured, acuminate or subacuminate at apex
Receptacular bracts	narrowly oblong–lanceolate, 13–15.5 × 1.5–2.5 mm, completely straw-coloured or basal and dorsal surface straw-coloured and brown only at apex, subacuminate	triangular–lanceolate, 12–20 × 3.5–6 mm, completely straw-coloured, acuminate
Involucel	6.1–7.1 mm long in fruit, sericeous	7–13 mm long in fruit, pilose
Habitat	*Pinus sylvestris* forest	steppe

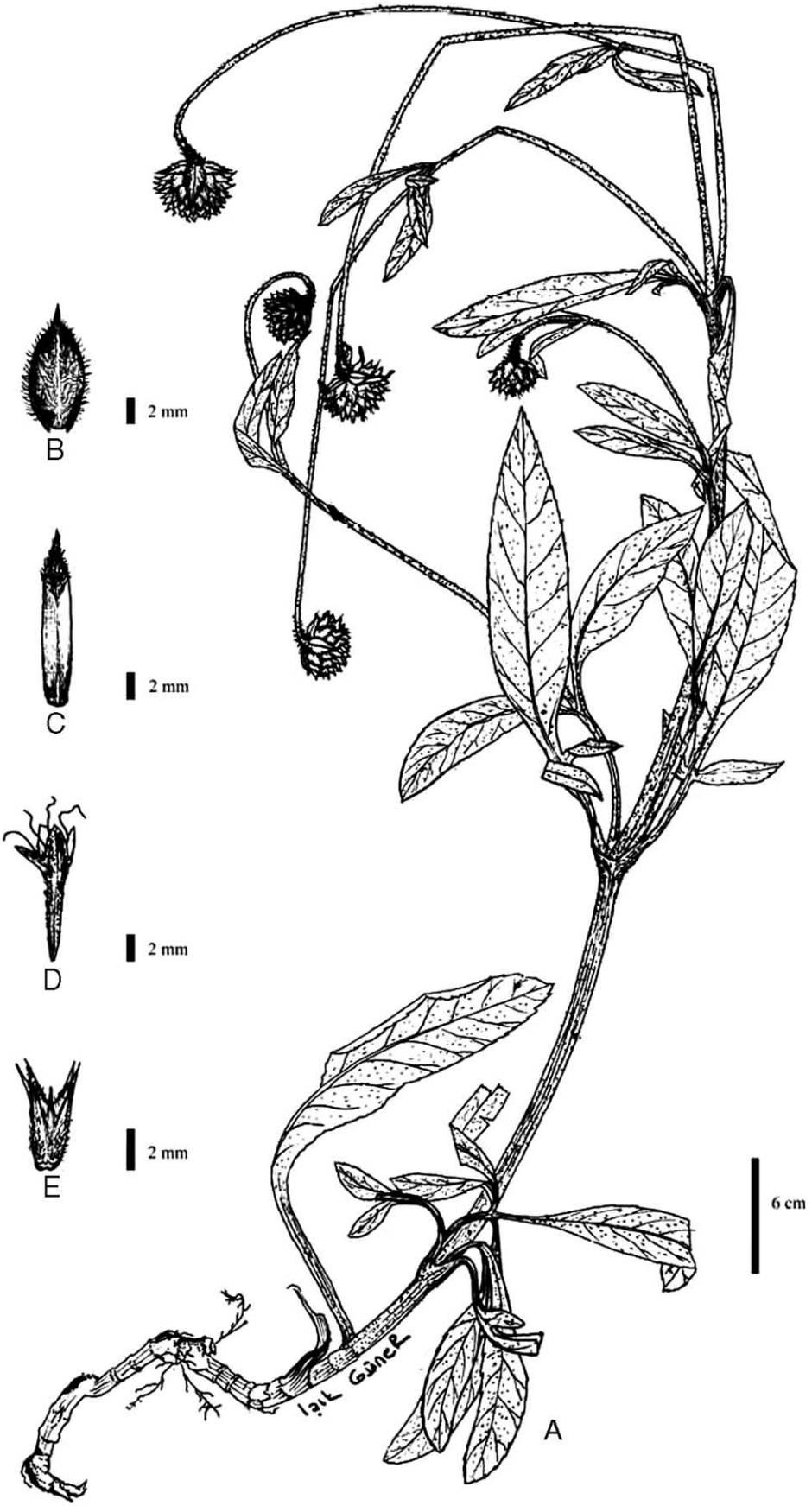

Fig. 1. *Cephalaria duzceënsis* (from the holotype). (A) habit, (B) involucral bract, (C) receptacular bract, (D) corolla, (E) involucel.

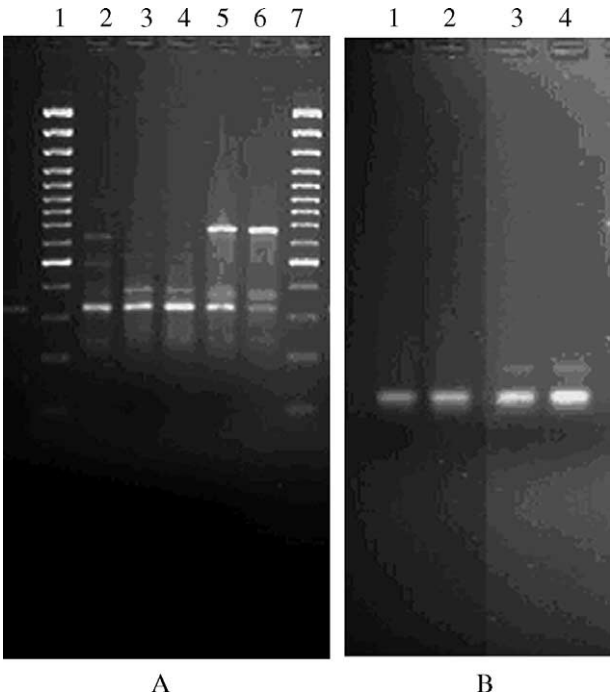

Fig. 2. (A) RAPD profiles of *Cephalaria* species. 1,7: marker (100 bp DNA ladder, Fermentas SMO321), (1–4: primer OPW10); 2: *C. speciosa*, 3: *C. speciosa*, 4: *C. speciosa*, 5: *C. duzceënsis*, 6: *C. duzceënsis*. (B) (1–4: primer M13); 1: *C. speciosa*, 2: *C. speciosa*, 3: *C. duzceënsis*, 4: *C. duzceënsis*.

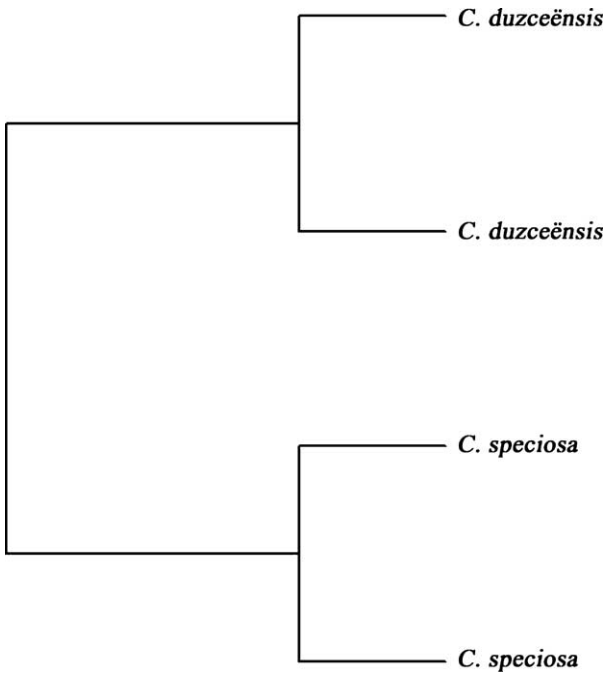

Fig. 3. Dendrogram based on genetic distances between *Cephalaria* species.

on both surfaces. Lower leaves simple, deflexed from petiole, oblong–lanceolate or ovate–lanceolate, 15.5–22 × 2.8–4.7 cm, crenate or crenate–serrate at margin, acute at apex. Cauline leaves simple or rarely lyrate, lanceolate; simple leaves and segments of lyrate leaves entire or crenate-serrate at margin, acute at apex, 7.8–13.5 × 1.5–4 cm; lyrate leaves 8–13 × 1.3–4 cm, with 2 linear–lanceolate segments, 1.1–2 × 0.3–0.5 cm, terminal segment larger than lateral ones, lanceolate, 6–12 × 1.3–4.2 cm. Upper cauline leaves simple or very rarely lyrate, linear–lanceolate or linear; simple leaves 1–5.5 × 0.2–0.7 cm, acute or subacuminate at apex; lyrate leaves with 2 linear segments, 1.1–2 × 0.3–0.5 mm, terminal segment larger than lateral ones, narrowly lanceolate, 0.8–4.5 × 0.3–0.6 cm. Capitula globose, 2–2.5 cm diameter in flower, 2.5–3 cm in diameter in fruit; corolla 9.14–12.05 mm long, cream, pilose with densely adpressed hairs on the outside. Involucral bracts narrowly ovate–oblong, completely black or straw-coloured, 8–12.5 × 3.5–5 mm, pilose with densely adpressed hairs, margin ciliate, mucronate at apex. Receptacular bracts narrowly oblong–lanceolate, completely straw-coloured or basal and dorsal surface straw-coloured but brown at apex, 13–15.5 × 1.5–2.5 mm, densely pilose with adpressed hairs, margin ciliate, subacuminate at apex. Involucel 6.1–7.1 mm long in fruit, 4- angled, sericeous, with 4 short and 4 long teeth at apex. Flowering time middle July to middle August. Fruting time middle August to end of September.

Distribution and ecology

Endemic in Euro-Siberian flora region and western Black Sea part of Anatolia (Fig. 4). Euxine element. *Pinus sylvestris* L. in forest on stony slopes at 1000–1200 m. This plant grows with *Pinus sylvestris* L., *P. nigra* Arnaud, *Abies nordmanniana* (Steven) Spach ssp. *bornmuelleriana* (Mattf.) Coode & Cullen, *Daphne pontica* L., *Frangula alnus* Mill. ssp. *alnus*, *Laurocerasus officinalis* A. Roem., *Lonicera caucasica* Pall. ssp. *orientalis* (Lam.) Chamb. & G. Long, *Rubus ideaus* L., *Linum aroanium* Boiss. & Orph., *Genista tinctoria* L. ssp. *tinctoria*, *Galium fissurense* Ehrend. & Schönb.-Tem., *Thlaspi jaubertii* Hedge, *Hypericum montbretii* Spach, *Ferulago confusa* Velen., *Jurinea alpigena* K. Koch, *Teucrium chamaedrys* L., *Silene vulgaris* (Moench) Garcke var. *vulgaris*, *Cirsium hypoleucum* DC., *Anthemis tinctoria* L., *Brachypodium sylvaticum* (Huds.) P. Beauv. and *Melica ciliata* L.

Table 3. Nei's genetic distance based on RAPD results of *Cephalaria* species.

Pop ID	*C. duzceënsis*	*C. duzceënsis*	*C. speciosa*	*C. speciosa*
C. duzceënsis	0.00001			
C. duzceënsis	0.00001	0.00001		
C. speciosa	0.6650	0.00001	0.00001	
C. speciosa	0.6650	0.6650	0.0588	0.00001

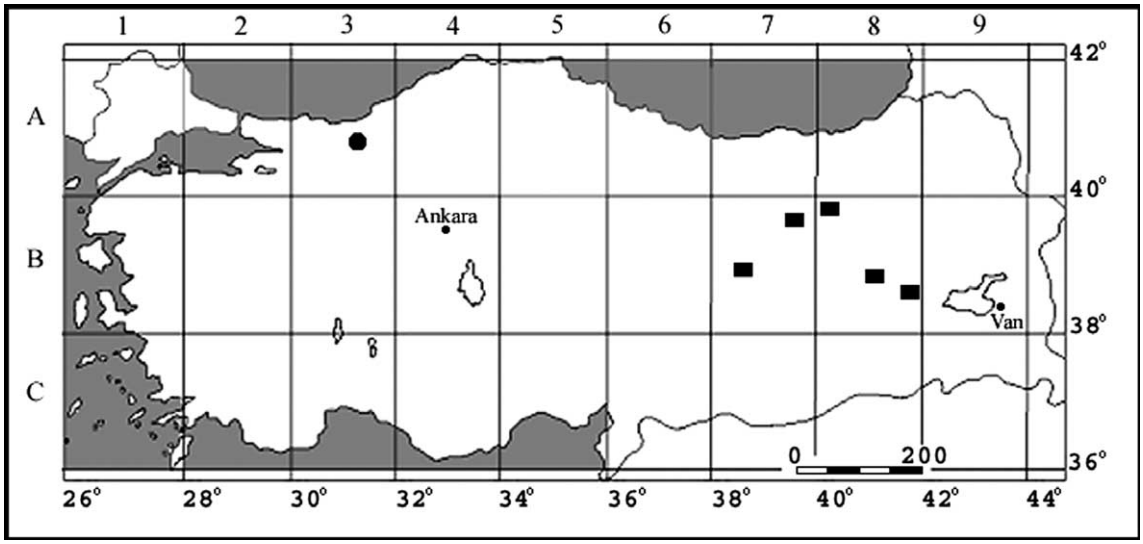

Fig. 4. Geographical distribution of *Cephalaria duzceënsis* (●) and *C. speciosa* (■) in Turkey.

Conservation status

Cephalaria duzceënsis is endemic and only known from two adjacent localities with small populations. It is suggested that this new species should be placed under the IUCN threat category critically endangered (CR) (IUCN 2001), because the estimated area of occupancy is less than 10 km^2 (criterion B2) and it is known from only two adjacent localities (criterion B2a). The population size of the new species is estimated to be less than 250 mature individuals (criterion C).

Etymology

The specific epithet is derived from the name of the city Düzce, where the holotype of *Cephalaria duzceënsis* was collected.

Results of the molecular analysis

For the RAPD-PCR analysis 33 random primers were tested in the amplification reactions. Among them, 14 primers were chosen for further analysis. The 14 RAPD primers (M13, B4, B6, OPC02, OPW10, SC1023, LA12, LA13, OPB10, OPR03, OPU16, OPA07, B7 and A2) generated a total of 100 fragments in the two *Cephalaria* species. The approximate size of the fragments ranged from 100 to 1500 bp. The total number of amplified bands per primer varied from 1 to 13 with an average of 6.7 fragments per primer.

Figure 2A and B show RAPD profiles of *Cephalaria* species. A dendrogram was constructed with 100 fragments generated from RAPD data as shown in Fig. 3. The genetic distances based on RAPD analysis are presented in Table 3.

A considerable genetic distance (66%) was found between the species *C. duzceënsis* and *C. speciosa* wheras the two samples of *C. duzceënsis* were found to be identical. The distance matrix based on RAPD data is graphically represented as a dendrogram using the UPGMA method in Fig. 3. The dendrogram was divided into two main groups. The upper cluster consists of two samples of *C. duzceënsis*, while the lower cluster consists of two samples of *C. speciosa*.

Discussion

Since *Cephalaria* was revised by Matthews (1972) for the Flora of Turkey (29 species), eight new species have been described from Turkey: *Cephalaria scoparia* Contandr. & Quézel, *C. dirmilensis* Hub.-Mor. (Davis et al. 1988). *C. gazipashensis* Sümbül, *C. peshmenii* Sümbül (Sümbül 1991), *C. ekimiania* R. S. Göktürk & Sümbül (Göktürk and Sümbül 1997), *C. elazigensis* R. S. Göktürk & Sümbül (Göktürk et al. 2003), C. *aytachii* R. S. Göktürk & Sümbül (Göktürk and Sümbül 2003), *C. tuteliana* S. Kuş & R. S. Göktürk (Kuş and Göktürk 2005), The total number of species of *Cephalaria* reported from Turkey is 38, of which including *C. duzceënsis* belong in Euxine element.

RAPD-PCR profiles plus morphological data provides taxonomic distinctions at the species levels. Although the method has been widely used for distinction of intraspecies, it has rarely been used as a taxonomic tool. In conclusion, RAPD markers seem to be quite effective differentiating two taxa from each other in this study.

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Appendix 1

Representative specimens examined of the other species: *C. speciosa*: B7 Erzincan: Keşiş mountain, Cimin, rocky slopes, ca 2300 m, 28 Aug 1957, Davis 31828. (ANK); Yaylabaşi village, Kazankaya mountain, 1500–2300 m, 7 Aug 1980, Ş. Yildirimli 3861. (HUB); Kemah, above Kömürköy, steppe, 1850 m, 31 Jul 1996, A. A. Dönmez 5367. (HUB). B7 Tunceli: Ovacik, Munzur mountain, Aksu stream, ca 1700 m, 21 Jul 1957, Davis (31462) & Hedge. (ANK); B7 Tunceli: Ovacik, Munzur mountain, Karagöl valley, 1350–1500 m, 8 Apr 1979, Ş. Yildirimli 2443. (HUB). B8 Erzincan: Aşkale-Tercan, dry rocky slopes, ca 1700 m, 25 Aug 1957, Davis (32657) & Hedge. (ANK); Tercan, between Tercan and Aşkale, steppe, 1765 m, 17 Aug 2000, R. S. Göktürk (4528), F. Göktürk. (AKDU); 30 km of Erzincan-Sivas road, steppe, 1500 m, 17 Aug 2000, R. S. Göktürk (4531), F. Göktürk. (AKDU). B8 Muş: Muş-Solhan, hillsides, ca 1350 m, 31 Aug 1954, Davis (24783) & Polunin (ANK); Varto, between Muş and Varto, dry rocky slopes, steppe, 1350 m, 2 Aug 2001, R. S. Göktürk (4727), M. Göktürk. (AKDU); Varto, Varto-Erzurum road 12 km, Seferek pass, steppe, 1800 m, 2 Aug 2001, R. S. Göktürk (4730), M. Göktürk. (AKDU); between Muş and Solhan, stepe, 1600 m, 2 Aug 2001, R. S. Göktürk (4732), M. Göktürk. (AKDU).