TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2019) 43: 366-385
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doi:10.3906/bot-1806-53

Hybridization among three *Cirsium* (Asteraceae) species and important evidence for three new hybrids from Turkey

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Received: 25.06.2018 • **Accepted/Published Online:** 21.01.2019 • **Final Version:** 06.05.2019

Abstract: In this study, three new hybrids from the genus *Cirsium* are described for the first time. *Cirsium* ×*nezaketiae* Yıldız, Dirmenci & Arabacı, *C.* ×*kelkitensis* Yıldız, Arabacı & Dirmenci, and *C.* ×*erzincanicum* Yıldız, Dirmenci & Arabacı are investigated in detail morphologically, palynologically, and molecularly. The hybrid individuals were detected on field trips due to their intermediate morphological characters. Their morphological properties were analyzed to compare with their parents. The pollen grain morphologies of the hybrids and their allies were investigated using light and a scanning electron microscope. The pollen grains were radially symmetrical, isopolar, trizonocolporate, and with echinate or scabrate sculpturing. The tectum is psilate/punctate, microreticulate, or ornate in sculpture. Dimorphic pollen grains were encountered in *C. leucocephalum* (Willd.) Spreng. subsp. *leucocephalum*. Two nuclear DNA regions were used to find single nucleotide polymorphisms. nrITS DNA data gave more information than nrETS data about polymorphism. In conclusion, *C.* ×*nezaketiae*, *C.* ×*kelkitensis*, and *C.* ×*erzincanicum* exhibit some significant morphological, palynological, and molecular differences from their parents and present evidence of potential introgression hybridization. Since the hybrids live in a complex with their parents, and some parents have polymorphic loci like their putative hybrids, it can be presented as evidence for potential introgression since backcrossing may be possible among taxa.

Key words: Endemic, Erzincan, hybrid, introgression, ITS, Turkey

1. Introduction

The genus *Cirsium* Mill. (Asteraceae, Cardueae) is one of the largest genera in Asteraceae. It contains about 250 species, which are mainly distributed in Europe, North Africa, East Asia, Central Asia, SW Asia, and North and Central America (Charadze, 1963; Davis and Parris, 1975; Petrak, 1979; Kadereit and Jeffrey, 2007). Most of the species are distributed in Europe-Russia-Turkey and the Caucasus. Turkey is an important gene center for the genus. It is represented by 68 species (80 taxa, 33 endemics) and two hybrids. These 80 taxa belong to sect. *Epitrachys* (50 species, 52 taxa), sect. *Cirsium* (17 species, 27 taxa, and 2 hybrids), and sect. *Cephalonoplos* (Neck.) DC. (1 species), 33 (41%) of which are indigenous to Turkey (Davis and Parris, 1975; Davis et al., 1988; Yıldız et al., 2012, 2016; Duman et al., 2017).

Palynological studies of *Cirsium* are very limited in number. Pollen morphology of some *Cirsium* species from Turkey and 23 species from Iran have been investigated (Yıldız et al., 2011; Nouroozi et al., 2012; Erşen Bak and Özcan, 2018).

Cirsium species have generally diploid or tetraploid chromosome numbers with $2n = 2x = 34$ and $2n = 4x = 68$. Other chromosome numbers of 2n = 16, 18, 20, 22, 24, 26, 28, 51, and 102 have also been reported (Werner, 1976; Bureš et al., 2004; Rotreklová et al., 2004; Ozcan et al., 2008, 2011; Yüksel et al., 2013; Polat et al., 2018). Tetraploids and hexaploids occur much more rarely in Europe, Turkey, and East Asia (Bureš et al., 2004; Ozcan et al., 2008, 2011; Nouroozi et al., 2011; Yüksel et al., 2013, 2018; Bureš et al., 2018). In Turkey, 2n =32, 34, 60, and 68 chromosome numbers have been reported (Ozcan et al., 2008, 2011; Yüksel et al., 2013, 2018). Meiotic abnormalities including chromosome stickiness, multipolar cell formation, and cytomixis have been observed in some of the species, and this may result in some degree of pollen sterility in *Cirsium* (Nouroozi et al., 2011). Homoploid hybrids occur between diploid species among Old World thistles (Czapik, 1958;

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Bureš et al., 2004; Rotreklová et al., 2004). However, in North America, more than half of the known hybrids are between species having different chromosome numbers (cf. Keil, 2006).

nrITS sequences have been used in many studies in the literature. They provide good information about the relationships of some Cardueae members (Susanna et al., 1995; Garcia-Jacas et al., 2001; Kelch and Baldwin, 2003; Slotta et al., 2012). In addition, external transcribed spacer (ETS) sequences have also been used in some significant phylogenetic studies about some Compositae tribes (Baldwin and Markos, 1998; Clevinger and Panero, 2000; Linder et al., 2000; Chan et al., 2001; Markos and Baldwin, 2001; Lee et al., 2002; Kelch and Baldwin, 2003; Slotta et al., 2012). ITS and ETS sequences are very useful for phylogenetic studies about Compositae members (Kelch and Baldwin, 2003).

The genus *Cirsium* exhibits a large number of interspecific and intersubspecific hybrids. This hybridization potential is considered a result of the sympatric occurrence of different taxa in the genus (Segarra-Moragues et al., 2007). Interspecific hybridization occurs among the closely related species by the occasional breakdown of sterility barriers that cause many morphological differences (Ownbey, 1951, 1964; Davidson, 1963; Bloom, 1977; Dabydeen, 1987).

Interspecific hybridization has been seen less intensively in North America with 29 hybrids belonging to 62 species, while there are 21 hybrids belonging to 64 species in Japan. However, hybridization is common in the Central European species. Seventy hybrids belonging to 17 native Central European species have been observed (Bureš et al., 2010). This intensive interspecific hybridization makes this region attractive for the study of possible factors that limit hybridization, such as reproductive isolation between some species pairs (Bureš et al., 2010).

As mentioned above, the number of hybrids in Turkey is expected to be higher due to the number of species there. *The Flora of Turkey* mentioned three possible hybrids but did not define them (Davis and Parris, 1975). Then two hybrids were recorded from Sect. *Cirsium* (Yıldız et al., 2016). However, there are not enough studies on *Cirsium* hybrids in Turkey. As a result of the field studies conducted since 2006 for revision of the work on Turkish *Cirsium* taxa, many hybrids were detected with their putative parents. This number is around 30, and they are all of the *Epitrachys* sections except for three (previously described hybrids of the sect. *Cirsium* species), and they have not been identified yet.

This is an initial study about *Cirsium* hybrids in Turkey. Our goal moving on from this study is to describe the three hybrids formed between the three species of *Cirsium* sect. *Epitrachys* by presenting morphological, molecular, and palynological evidence together with a general overview of the world's hybridization of the genus, and by discussing the importance of hybridization in the speciation of the genus in this and future studies.

2. Materials and methods

2.1. Morphological studies

The materials of this study were collected from field studies in Erzincan Province made in the years 2006 and 2017 (Figure 1, yellow star area). The hybrids and their parents were photographed in their natural habitats (Figures 2A–2F and 3A–3F). The specimens were identified using *The Flora of Turkey* (Davis and Parris, 1975) and supplements (Davis et al., 1988; Güner et al., 2000). In addition, the literature concerning the genus and hybridization was checked (Charadze, 1963; Petrak, 1979; Segarra-Moragues et al., 2007; Bureš et al., 2010; Yıldız et al., 2016; Duman et al., 2017). The voucher specimens were kept in the ANK, GAZI, ISTE, and Balıkesir University Education Faculty in Balıkesir (Hb. Dirmenci) herbaria, Turkey.

2.2. Morphological characters and data analyses

A data matrix was constructed according to the distinctive morphological characters mentioned in Table 1. Principal

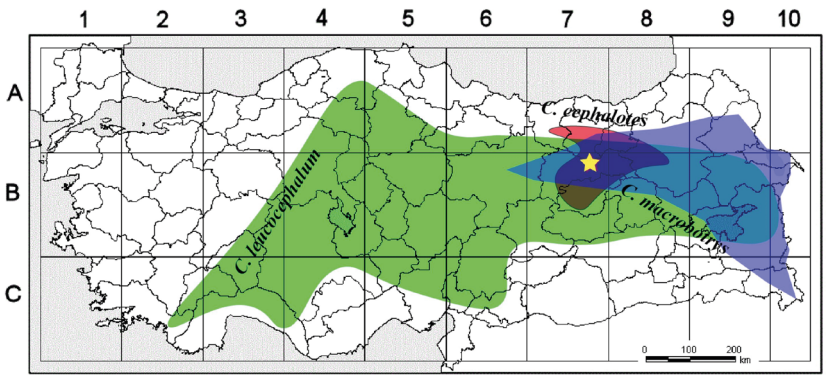

Figure 1. General distribution of the studied parental species and hybrids (yellow star).

component analysis (PCA) was applied based on the constructed data matrix (Jolliffe, 2002). In addition, we used the UPGMA clustering method based on the Manhattan similarity index (Romesburg, 2004). All analyses were carried out with PAST (Hammer et al., 2001) (Figures 4A and 4B).

2.3. Palynological studies

Slides were prepared following the Wodehouse (1959) method for light microscope (LM) studies. The following parameters were measured: polar axis (P), equatorial diameter (E), exine thickness and length, and thickness of the spines. At least twenty measurements were taken for P and E, and 10 for the other parameters per population (see Appendix I). The measurements and scanning electron microscope (SEM) examinations of the grains are presented in Tables 2–4. A histogram (Figure 5) and a box plot (Figure 6) for P/E ratios of pollen grains are also given. Micrographs of pollen grains were taken using the SEM and are shown in Figures 7–9.

For the SEM examinations, pollen grains were mounted on stubs using double-sided adhesive tape and were then coated with gold-palladium for 2 min in a BAL-TEC SCD 005 sputter-coater. The micrographs were obtained using an XL-30 ESEM-FEG/PHILIPS microscope. The measurements from spine/spinules were taken 15–20 grains from each specimen from SEM micrographs. The pollen morphological descriptions followed the terminology used by Erdtman (1952), Faegri and Iversen (1989), Punt (2007), and Hesse et al. (2009).

For comparison of the P-E axis, analysis of variance (ANOVA) and Tukey's test were performed using SPSS 23.00. For all of these tests, a value of $P < 0.001$ was accepted as the level of statistical significance.

2.4. Molecular studies

The specimens used for molecular studies were selected from the hybrids and the closest parental individuals of hybrids, and also two different species from sect. *Epitrachys* and two species from sect. *Cirsium* were used as outgroups (Appendix I). DNA isolations were performed using the DNeasy Plant Mini Kit (QIAGEN, Germany), following the manufacturer's instructions with some modifications. Solutions of the powdered plant tissues were kept in an incubator at 65 °C for 30 min and then placed on ice for 30 min, and 100 µL of elution buffer was used for the first and the second elutions.

The polymerase chain reactions (PCRs) were mostly performed using the second elution of DNA extractions. Molecular analysis of the rearranged new hybrid and its parental species was carried out using two different nrDNA regions: the internal transcribed spacer (ITS) region and the external transcribed spacer (ETS) region. PCR amplification of the ITS nrDNA were performed using ITS5a (5'-CCT TAT CAT TTA GAG GAA GGA G-3') (Stanford et al., 2000) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White et al., 1990) primers, and 18S-ETS (5'-ACT TAC ACA TGC ATG GCT TAA-3') (Baldwin and Markos, 1998) and ETS-Car-1 (5'-TTC GTA TCG TTC GGT-3') (Baldwin and Markos, 1998) primers were used for amplifying the ETS region. The QIAGEN Taq DNA polymerase kit was used with some modifications for PCR amplification. During the PCR amplification, a thermal cycle machine (Techne-Prime, USA) was used for routine amplification. The PCR protocol developed by Shaw et al. (2007) was applied with 25 cycles.

2.5. Data analysis and editing of the ITS and ETS nrDNA data

The PCR products were sent to Genoks (Gene Research and Biotechnology Company, Turkey) for sequencing. The sequenced DNA was edited using Sequencer version 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA). Some nucleotides from the 5' end of ITS1 (ETS-Car-1) and 3' end of ITS2 (18S-ETS) were cut to avoid doubtful base callings and redundant gaps. Finally, 642 nucleotides in length were produced from the nrITS region (574 nucleotides for the ETS region) of the studied taxa. Polymorphic sequence regions of *C. cephalotes* Boiss., *C. leucocephalum* (Willd.) Spreng. subsp. *leucocephalum*, and *C. macrobotrys* (K.Koch) Boiss. and their putative hybrids were identified, and the polymorphism of the different samples of the new hybrids was demonstrated by comparison with their parents and outgroups. Parsimony analyses were constructed using PAUP* (Swofford, 2002) ver. 4.0a159. All the DNA sequences edited in this study were deposited in DDBJ/EMBL/GenBank with their accession numbers (see Appendix II).

3. Results

3.1. Morphological results

3.1.1. *Cirsium* ×*nezaketiae* Yıldız, Dirmenci & Arabacı nothosp. nov. (Figures 2D–3D)

(*Cirsium cephalotes* Boiss. × *Cirsium macrobotrys* (K.Koch) Boiss.)

Type: Turkey B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4659, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK)

Diagnosis: *Cirsium* ×*nezaketiae* is similar to its parents, *Cirsium cephalotes* and *C. macrobotrys*. It can be distinguished from *C. cephalotes* as follows: its life form is biennial (not perennial), its main stem is single (not branched), lengths of phyllaries are 15–18 mm with 2–3 mm apical spine (not 23–29 mm with 4–8 mm apical spine), its corolla is pinkish and measurement is 25–32 mm (not pinkish-purple to purple and 30–37 mm), its lobe's measurements are 4–8 mm (not 10–13 mm) (Figures 2A, 2D, 3A, 3D). It differs from *C. macrobotrys* in its inflorescence raceme to paniculate, capitula distinctly pedunculate to 5 cm (not spicate, subsessile and 5–12 capitula aggregate at stem

apex), corolla pinkish (not ochroleucous) (Figures 2B, 2D, 3B, 3D).

Description: Biennial herbs. Stem to 80 cm, stout and single, unbranched, unwinged, striate, densely arachnoid with sparsely multicellular hairs, glaucous. Basal leaves unknown. Stem leaves diminishing from base to inflorescence, 8–20 × 5–8 cm, pinnatifid to pinnatisect, oblong in outline, lateral lobes oblong to lanceolate, apical spine stout, 5–15 mm, glaucous, sparsely arachnoid above, densely white tomentose below, spinose-strigose above, setae erecto-patent, 0.5–1 mm, more than 5 in 2 mm square; middle and upper leaves auriculate. Involucral leaves 7–10, 4–9 cm with to 10 mm apical spine, shorter or longer than capitula. Inflorescence raceme to paniculate. Capitula 30–40 × 30–40 mm, distinctly pedunculate to 5 cm, globose; phyllaries lanceolate to linear-lanceolate, densely arachnoid at apex, imbricate, 7–8 seriate; median phyllaries 15–18 mm with 2–3 mm apical spine, recurved. Corolla pinkish, 25–32 mm, unequally 5-lobed to 1/4, 4 lobes ±equal 4–5 mm, other lobe 7–8 mm; anthers 9–11 mm, glabrous; filaments densely hairy; style exserted from corolla, 2-lobed. Pappus 22–25 mm, dirty white, plumose (Figures 2D, 3D).

Flowering time: August to September.

Etymology: This hybrid was named in honor of the Turkish botanist Prof. Dr. Nezaket Adıgüzel.

3.1.2. *Cirsium* ×*kelkitensis* Yıldız, Arabacı & Dirmenci nothosp. nov. (Figures 2E, 3E)

(*Cirsium cephalotes* Boiss. × *Cirsium leucocephalum* (Willd.) Spreng. subsp. *leucocephalum*)

Type: Turkey B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4656, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK).

Diagnosis: *Cirsium* ×*kelkitensis* is similar to its parents *C. cephalotes* and *C. leucocephalum* subsp. *leucocephalum*. It can be distinguished from *C. cephalotes* as follows: capitula 25–35 × 25–30 mm (not 30–55 × 30–50 mm), median phyllaries 12–18 mm with 2–5 mm apical spine (not 23–29 mm with 4–8 mm apical spine), corolla 20–25 mm (not 30–37 mm), pappus 18–20 mm (not 23–26 mm) (Figures 2A, 2E, 3A, 3E). It differs from *C. leucocephalum* subsp. *leucocephalum* in its capitula 25–35 × 25–30 mm (not 20–25 × 15–20 mm), median phyllaries 12–18 mm with 2–5 mm apical spine (not 6–13 with 1–2 mm apical spine), corolla 20–25 mm (not 12–20 mm), pappus 18–20 mm (not 10–17 mm) (Figures 2C, 2E, 3C, 3E).

Description: Perennial herbs. Stem 50–70 cm, stout, a few to many stemmed from the same root, much branched above, unwinged, striate, densely arachnoid with sparsely multicellular hairs, glaucous. Basal leaves unknown. Stem leaves diminishing from base to inflorescence, 7–16 × 2–12 cm, pinnatifid to pinnatisect, oblong to lanceolate in outline, dark green to glaucous, lateral lobes oblong to triangular, apical spine stout, 5–15 mm, ±glabrous above, densely white tomentose below, spinose-strigose above, setae erecto-patent, 0.5–1.5 mm, more than 5 in 2 mm square, auriculate. Inflorescence raceme to paniculate. Involucral leaves 5–8, 4–8 cm with 5–12 mm apical spine, clearly longer than capitula. Capitula 25–35 × 25–30 mm, ±globose, pedunculate to 10 cm; phyllaries ovate to lanceolate, densely arachnoid at apex, imbricate, 8–10 seriate; median phyllaries 12–18 mm with 2–5 mm apical spine, recurved. Corolla purple, 20–25 mm, unequally 5-lobed to 1/4, 4 lobes ±equal 4–5 mm, other lobe 6–7 mm; anthers absent, if present 6–9 mm, glabrous; filaments densely hairy; style exserted from corolla, 2-lobed. Pappus 18–20 mm, dirty white, plumose (Figures 2E, 3E).

Flowering time: August to September.

Etymology: The species epithet is derived from the name of the Kelkit district, where the type specimen was collected.

Paratype: Turkey B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 09.08.2017, Dirmenci 4906 & Arabacı (GAZI).

3.1.3. *Cirsium* ×*erzincanicum* Yıldız, Dirmenci & Arabacı nothosp. nov. (Figure 2F, 3F)

(*Cirsium leucocephalum* (Willd.) Spreng. subsp. *leucocephalum* × *Cirsium macrobotrys* (K.Koch) Boiss.)

Type: Turkey B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4661, Yıldız & Arabacı (Holotype: ISTE, isotype: ANK)

Diagnosis: *Cirsium* ×*erzincanicum* is similar to its parents, *Cirsium leucocephalum* subsp. *leucocephalum* and *C. macrobotrys*. It can be distinguished from *C. leucocephalum* subsp. *leucocephalum* as follows: its life form is biennial (not perennial), its stems are single and short branched above (not many stemmed from base or many branched above), involucres ±globose (not ovate to obovate), phyllaries are densely arachnoid at apex, median phyllaries measuring 8–10 mm with 2–3 mm apical spine (not 6–13 mm with 1–2 mm apical spine), pappus of 17–18 mm (not 10–17 mm) (2C, 2F, 3C, 3F). It differs from *C. macrobotrys* in its inflorescence raceme, capitula subsessile to distinctly pedunculate to 5 cm (not spicate, subsessile and 5–12 capitula aggregate at stem apex), median phyllaries 8–10 mm with 2–3 mm apical spine (not 13–19 mm with 1–2 mm apical spine), corolla pinkish, 18–20 mm (not ochroleucous, 23–28 mm), pappus 17–18 mm (not 22–24 mm) (Figures 2B, 2F, 3B, 3F).

Description: Biennial herbs. Stem to 75 cm, stout and single, branched above, unwinged, striate, densely arachnoid with sparsely multicellular hairs, grayish-green. Basal leaves unknown. Stem leaves diminishing from base to inflorescence, 7–15 × 2–8 cm, pinnatifid to pinnatisect, oblong in outline, lateral lobes oblong to lanceolate, apical spine stout, 5–15 mm, green ±glabrous above, densely

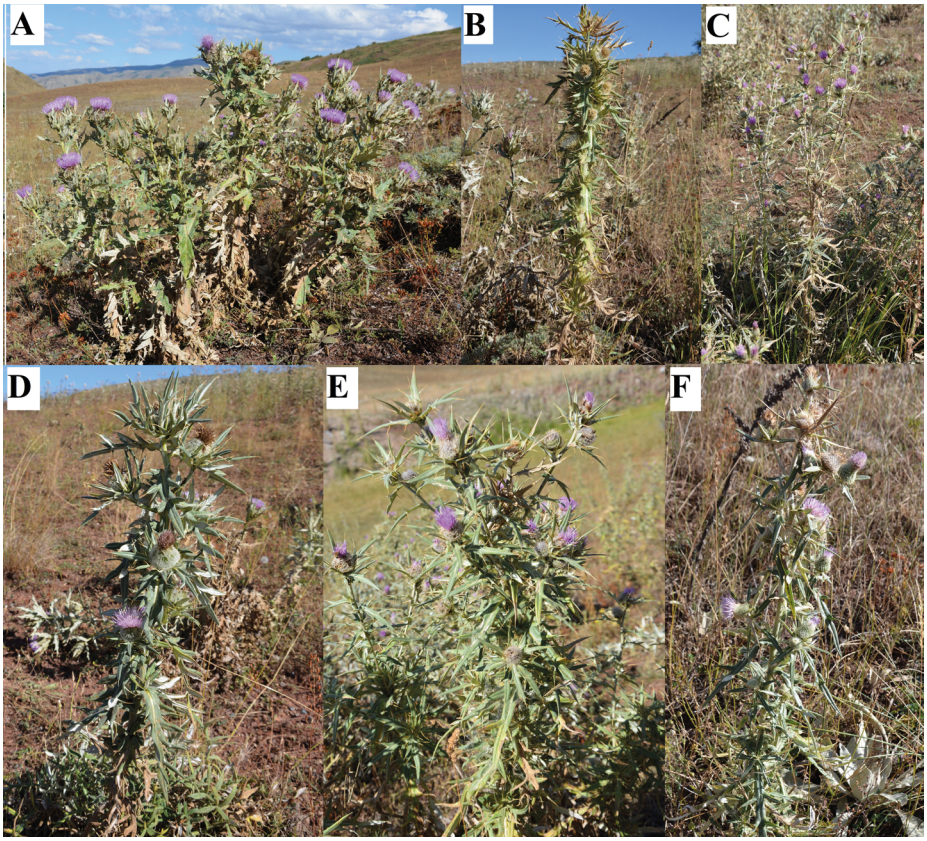

Figure 2. Habit and habitat of *Cirsium cephalotes* (A), *C. macrobotrys* (B), *C. leucocephalum* subsp. *leucocephalum* (C), *C.* ×*nezaketiae* (D), *C.* ×*kelkitensis* (E), and *C.* ×*erzincanicum* (F).

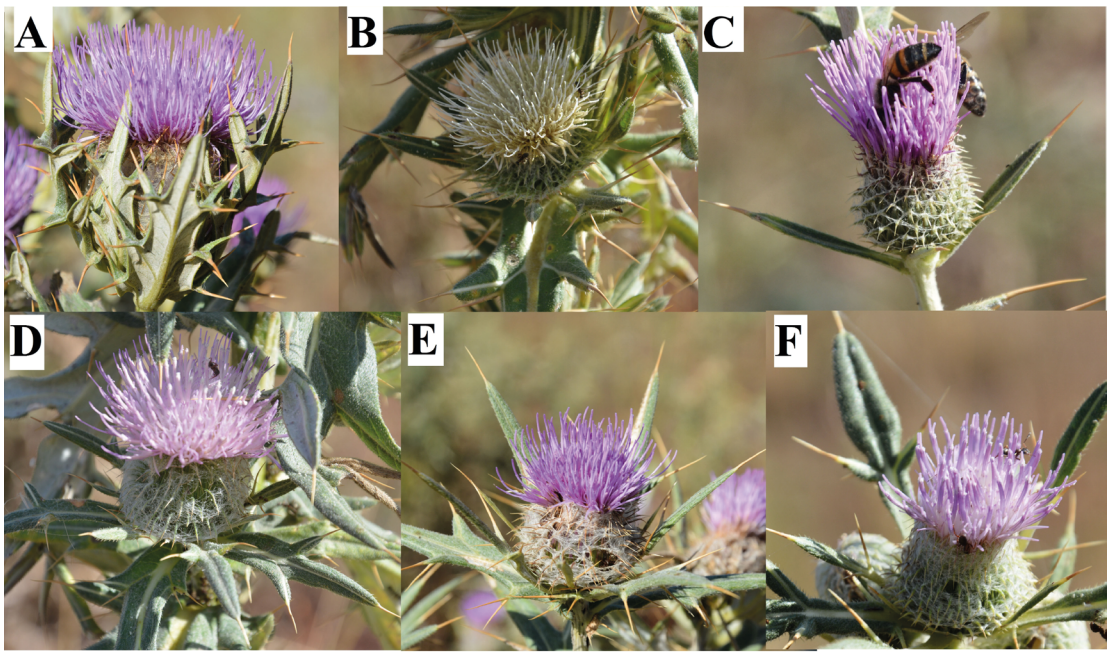

Figure 3. Capitula of *C. cephalotes* (A), *C. macrobotrys* (B), *C. leucocephalum* subsp. *leucocephalum* (C), *C.* ×*nezaketiae* (D), *C.* ×*kelkitensis* (E), and *C.* ×*erzincanicum* (F).

Table 1. Detailed morphological comparison between hybrids and their parents. Species/hybrids characters.

	C. cephalotes	*C. ×nezaketiae*	*C. ×kelkitensis*	*C. leucocephalum* subsp. *leucocephalum*	*C. ×erzincanicum*	*C. macrobotrys*
Life form	Perennial	Biennial	Perennial	Perennial	Biennial	Biennial
Habit/stem	Many stemmed from base, branched above, glaucous	Single branched above, glaucous	Few to many stemmed from base, branched above , glaucous	Many stemmed from base, branched above, green to grayish green	Single, branched above, grayish green	Single, unbranched above, light green
Leaves	Pinnatifid to pinnatisect, glaucous	Pinnatifid to pinnatisect, glaucous	Pinnatifid to pinnatisect, dark green to glaucous	Pinnatifid to pinnatisect, green to dark green	Pinnatifid to pinnatisect, green	Pinnatisect, green
Involucral leaves	6–12, as long as to longer than involucre	7–10, 4–9 cm with to 10 mm apical spine, shorter or longer than involucre	5–8, 4–8 cm with 5–12 mm apical spine, clearly longer than involucre	3–6, longer or shorter than involucre	3–5, 2.5–5 cm with to 6 mm apical spine, longer than involucre	6–10, longer than involucre
Involucre	Raceme	Raceme	Raceme to paniculate	Raceme or paniculate	Raceme	Spicate
Capitula	Pedunculate, 30–55 × 30–50 mm, globose to broadly obovate, densely arachnoid	Pedunculate, 30–40 × 30–40 mm, globose, densely arachnoid	Pedunculate, 25–35 × 25–30 mm, ±globose, densely arachnoid	Pedunculate, 20–25 × 15–20 mm, ovoid-globose to obovoid, sparsely arachnoid	Pedunculate, 20-30 × 20-25 mm, globose, densely arachnoid	Sessile, 25–35 × 20–30 mm, ovoid-globose to obovoid, densely arachnoid
Phyllary indumentum	Densely arachnoid	Densely arachnoid at apex	Densely arachnoid at apex	±Arachnoid at apex	Densely arachnoid at apex	Densely arachnoid at apex
Median phyllary	23–29 mm with 4–8 mm apical spine, erecto-patent	15–18 mm with 2–3 mm apical spine, recurved	12–18 mm with 2–5 mm apical spine, recurved	6–13 mm with 1–2 mm apical spine, recurved	8–10 mm with 2–3 mm apical spine, recurved	13–19 mm with 1–2 mm apical spine, recurved
Corolla	Pinkish to purple, 30–37 mm	Pinkish, 25–32 mm	Purple, 20–25 mm	Pinkish-purple, 12–20 mm	Pinkish, 18–20 mm	Ochroleucous, 23–28 mm
Pappus	23–26 mm	22–25 mm	18–20 mm	10–17 mm	17–18 mm	22–24 mm

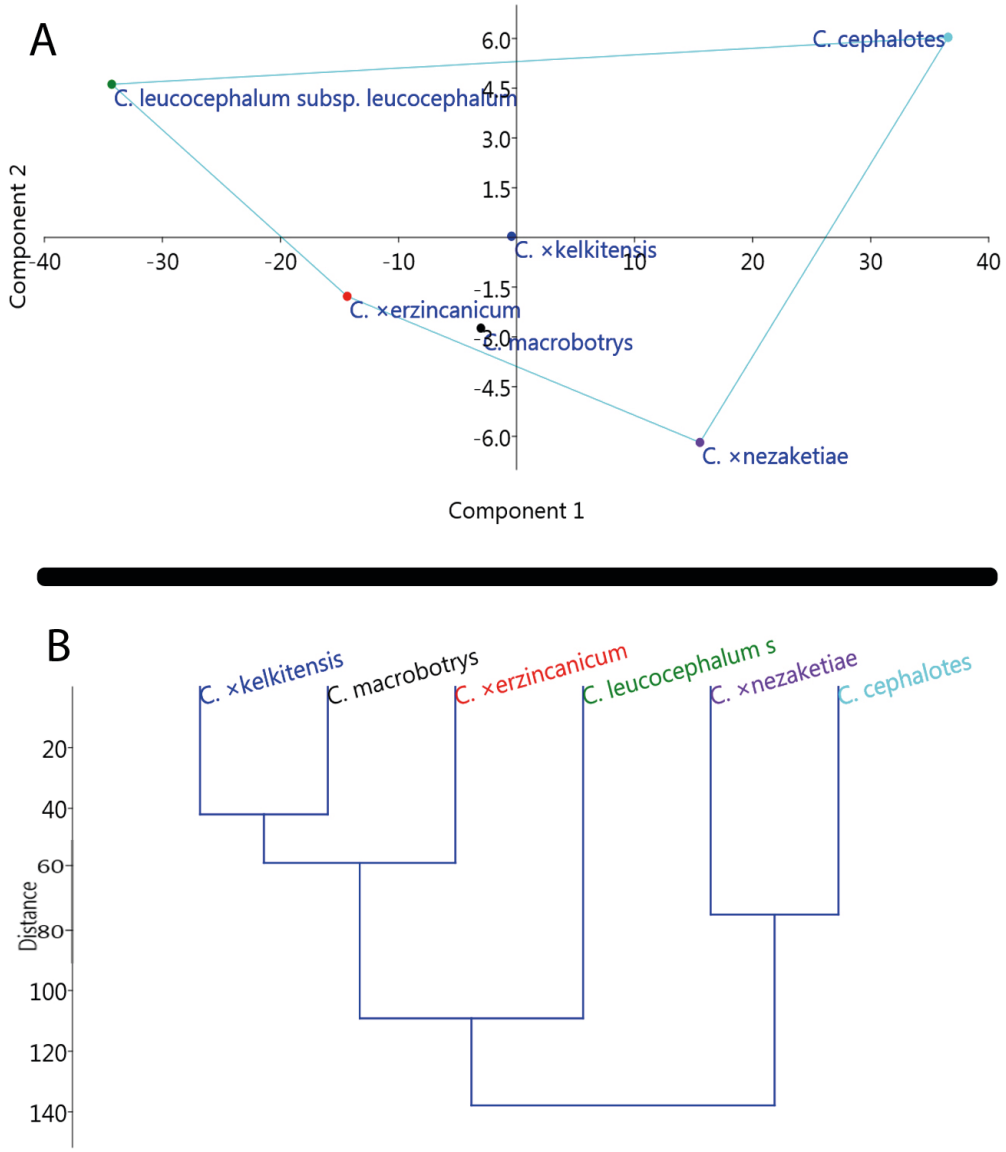

Figure 4. PCA scatter plot diagram (A) and UPGMA dendrogram (B) based on morphometric analysis.

white tomentose below, spinose-strigose above, setae erecto-patent, 0.5–1 mm, more than 5 in 2 mm square; middle and upper leaves auriculate. Inflorescence raceme. Involucral leaves 3–5, involucrate, 2.5–5 cm with apical spine to 6 mm, longer than capitula. Capitula 20–30 × 20–25 mm, ±globose, subsessile to pedunculate, peduncle to 5 cm; phyllaries ovate-lanceolate, densely arachnoid at apex, imbricate, 7–10 seriate; median phyllaries 8–10 mm with 2–3 mm apical spine, recurved. Corolla pinkish, 18–20 mm, unequally 5-lobed to 1/4, 4 lobes ±equal c. 4 mm, other lobe c. 6 mm; anthers 7.5–9 mm, glabrous; filaments densely hairy; style exserted from corolla, 2-lobed. Pappus 17–18 mm, dirty white, plumose.

Flowering time: August to September.

Etymology: The hybrid epithet is derived from the name of Erzincan Province, where the type specimen was collected.

Paratype: Turkey B7 Erzincan: between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 09.08.2017, Dirmenci 4909 & Arabacı (GAZI).

Habitat: All the new hybrids grow in mountain steppe together with their parents and meadows grass.

3.2. Palynological results

Pollen grains of *C. cephalotes* are tricolporate, 73% of pollen oblate spheroidal (Figure 5). The range of P/E is 0.82–1.01 (Figure 6), amb circular, exine 1.35 µm, thinner in poles, intine 1.36 µm. Ornamentation echinate, tectum completely structured, the surface of tectum psilate with supratectal spines. Spines conic, curve-ended, number of

100 μm² 2–3; 2.33 μm in length, base diameter of 2.02 μm. Colpi are 35.74 μm in length and 9.64 in width (Figures 7A, 7B, 9A, 9B; Tables 2–4).

Pollen grains of *C.* ×*kelkitensis* are tricolporate, 54% of pollen oblate spheroidal and 46% suboblate (Figure 5). The range of P/E is 0.80–0.98 (Figure 6), amb circular. Exine 1.38 μm, thinner in poles, intine 1.34 μm. Ornamentation echinate, tectum completely structured, surface of tectum ornate with supratectal spines. Spines conic, pointed-ended, number of 100 μm² 3; 2.73 μm in length, base diameter of 3.46 μm, colpi margins distinct with pointed ends and 27.61 μm in length and 8.45 in width. Distances between colpi ends 19.37 μm (Figures 7C, 7D, 9C, 9D; Tables 2–4).

Pollen grains of *C. leucocephalum* subsp. *leucocephalum* have dimorphic characters and two different sizes. Large-size pollen grains are tricolporate, 77% of pollen oblate spheroidal (Figure 5), amb circular. Exine 0.58 μm, thinner in poles, intine 0.68 μm, ornamentation scabrate, tectum completely structured, surface of tectum ornate with supratectal spines. Spines conic, blunt-ended, number of 100 μm² 4–6; 0.93 μm in length, base diameter of 1.4 μm (Figures 8A, 8B; Tables 2–4). Small-size pollen grains of *C. leucocephalum* subsp. *leucocephalum* are tricolporate, 82% of pollen oblate spheroidal (Figure 5), amb circular. Exine 0.58 μm, thinner in poles, intine 0.65 μm, ornamentation echinate, tectum completely structured, surface of tectum is ornate with supratectal spines. Spines conic, blunt-ended, number of 100 μm² 4–5; 2.54 μm in length, base diameter of 3.14 μm (Figures 8C, 8D; Tables 2–4).

Pollen grains of *C. macrobotrys* tricolporate, 95% of pollen oblate spheroidal (Figure 5). The range of P/E is 0.88–0.98 (Figure 6), amb circular. Exine 1.06 μm, thinner in poles, intine 1.02 μm, ornamentation echinate, tectum completely structured, surface of tectum is psilate with supratectal spines. Spines conic, pointed-ended, number of 100 μm² 4; 3.14 μm in length, base diameter of 3.93 μm. Colpi are 27.13 μm in length and 6.12 in width, distances between colpi ends 17.07 μm (Figures 7E, 7F, 9A, 9B; Tables 2–4).

Pollen grains of *C.* ×*nezaketiae* are tricolporate, 88% of pollen suboblate (Figure 5), the range of P/E is 0.79–0.93 (Figure 6), amb circular. Exine 1.33 μm, thinner in poles, intine 1.29 μm, ornamentation echinate, tectum completely structured, the surface of tectum is microreticulate with supratectal spines, reticules regular and small. Spines conic, pointed-ended, number of 100 μm² 4; 3.05 μm in length, base diameter of 4.48 μm, colpi are 27.37 μm in length and 8.47 in width, distances between colpi ends 18.79 μm (Figures 7G, 7H, 9I; Tables 2–4).

Pollen grains of *C.* ×*erzincanicum* are tricolporate, 69% of pollen oblate spheroidal (Figure 5), the range of P/E is 0.83–0.99 (Figure 6), amb circular, exine 1.29 μm, thinner in poles, intine 1.28 μm, ornamentation echinate, the surface of tectum is psilate with supratectal spines. Spines conic, pointed-ended, number of 100 μm² 4; 1.95 μm in length, base diameter of 2.21 μm, colpi are 24.25 μm in length and 6.53 in width, distances between colpi ends 15.8 μm (Figures 7I, 7J, 9G, 9H; Tables 2–4).

3.3. Molecular results

The following three new hybrids were examined in this study: *C.* ×*nezaketiae* (between *C. cephalotes* and *C. macrobotrys*), *C.* ×*kelkitensis* (between *C. cephalotes* and *C. leucocephalum* subsp. *leucocephalum*), and *C.* ×*erzincanicum* (between *C. leucocephalum* subsp. *leucocephalum* and *C. macrobotrys*). Their nrITS and nrETS DNA sequences gave some significant information about their molecular differentiation.

3.3.1 nrITS results

A total of 642 nucleotides in length were produced from the nrITS region. According to PAUP* parsimony analysis, 636 characters of 642 were constant, five variable characters were parsimony-uninformative, and only one character was parsimony-informative between *C.* ×*nezaketiae* and one of its putative ancestors, *C. cephalotes.* The similarity

Table 2. Pollen morphological characters (polar axis and equatorial axis) of *Cirsium* hybrids and parental species (mean, SD, min and max values; all of the measurements given in μm).

Taxa	Polar axis	Equatorial axis
C. cephalotes	38.15 ± 4.68 (36.43–60.51)	52.06 ± 3.93 (44.25–61.45)
C. ×*kelkitensis*	39.39 ± 4.50 (33.78–49.86)	46.06 ± 4.48 (38.06–55.84)
C. leucocephalum subsp. *leucocephalum**	33.91 ± 3.28 (28.82–39.70)	36.61 ± 3.07 (32.43–43.06)
C. leucocephalum subsp. *leucocephalum* *†	27.29 ± 1.94(23.91–31.14)	25.19 ± 1.76 (25.79–34.08)
C. macrobotrys	37.51 ± 3.04 (33.20–42.69)	40.23 ± 2.56 (35.50–44.90)
C. ×*nezaketiae*	36.03 ± 1.93 (33.06–40.79)	42.01 ± 2.13 (36.09–46.91)
C. ×*erzincanicum*	34.51 ± 4.30 (27.05–43.49)	38.38 ± 3.78 (29.66–47.06)

*Dimorphic pollen, † small-sized grains.

Table 3. Ornamentation of pollen grains from SEM micrographs of *Cirsium* hybrids and parental species.

Taxa	Type	Tectum surface
Cirsium cephalotes	Echinate	Psilate/punctate
C. ×kelkitensis	Echinate	Ornate
Cirsium leucocephalum subsp. *leucocephalum**	Scabrate	Ornate
Cirsium leucocephalum subsp. *leucocephalum*†*	Echinate	Ornate
Cirsium macrobotrys	Echinate	Psilate/punctate
Cirsium ×nezaketiae	Echinate	Microreticulate
C. ×erzincanicum	Echinate	Psilate/punctate

*Dimorphic pollen, † small-sized grains.

Table 4. Pollen morphological characters from SEM micrographs of *Cirsium* hybrids and parental species.

	Spine/spinule characters					
Taxa	Tip shape	Base shape	Base length	Tip length	Number in 100 μm^2	Distance between two spine tips
Cirsium cephalotes	Curve	Narrow	2.02	2.33	2–3	10.7
C. ×kelkitensis	Pointed	Narrow	3.46	2.73	3	9.94
Cirsium leucocephalum subsp. *leucocephalum**	Blunt-ended	-	1.4	0.93	4–6	6.68
Cirsium leucocephalum subsp. *leucocephalum *†*	Blunt-ended	-	3.14	2.54	4–5	7.85
Cirsium macrobotrys	Pointed	Extremely wide	3.93	3.14	4	8.74
Cirsium ×nezaketiae	Pointed	Extremely wide	4.48	3.05	4	8.25
C. ×erzincanicum	Pointed	Narrow	2.21	1.95	4	9.95

*Dimorphic pollen, † small-sized grains.

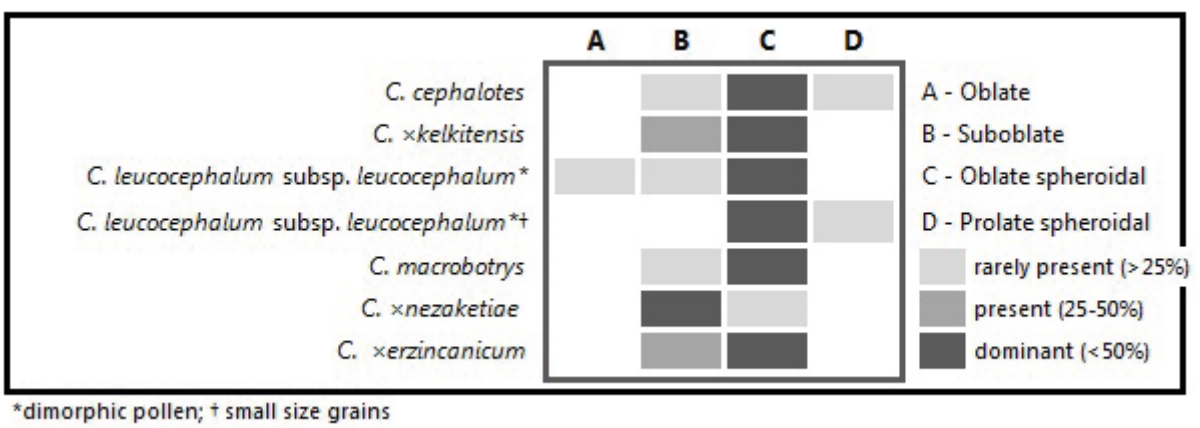

Figure 5. Histograms of frequency of pollen grains shape in *Cirsium* hybrids and parental species (*dimorphic pollen,, † small size grains).

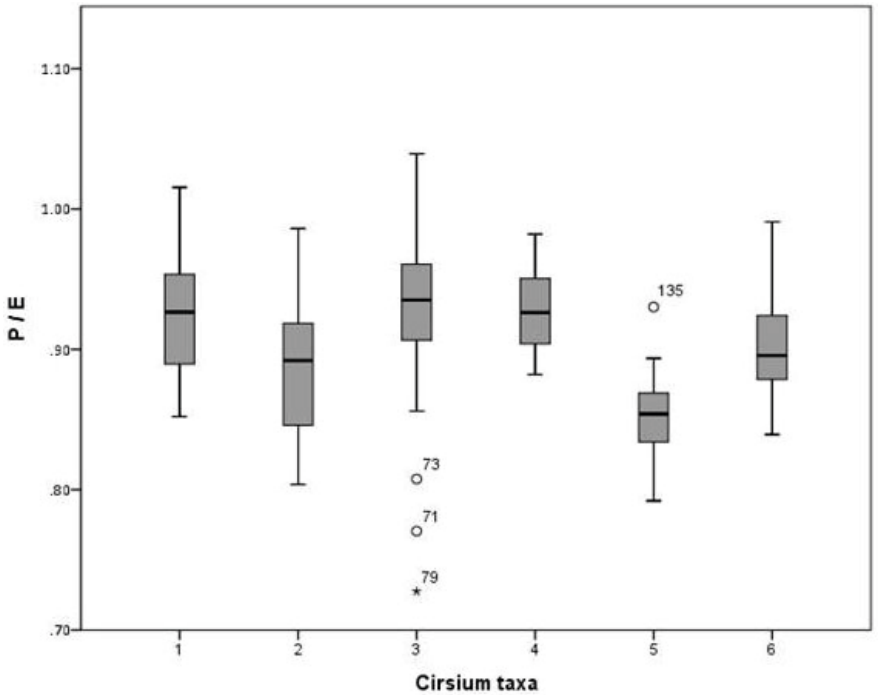

Figure 6. P/E rate of pollen grains in *Cirsium* hybrids and parental species: 1- *C. cephalotes*, 2- *C.* ×*kelkitensis*, 3- *C. leucocephalum* subsp. *leucocephalum*, 4- *C. macrobothrys*, 5- *C.* ×*nezaketiae*, 6- *C.* ×*erzincanicum*. Numbers on the figure are outliers. Bars represent minimum and maximum values, the middle bar represents the median (95% CI).

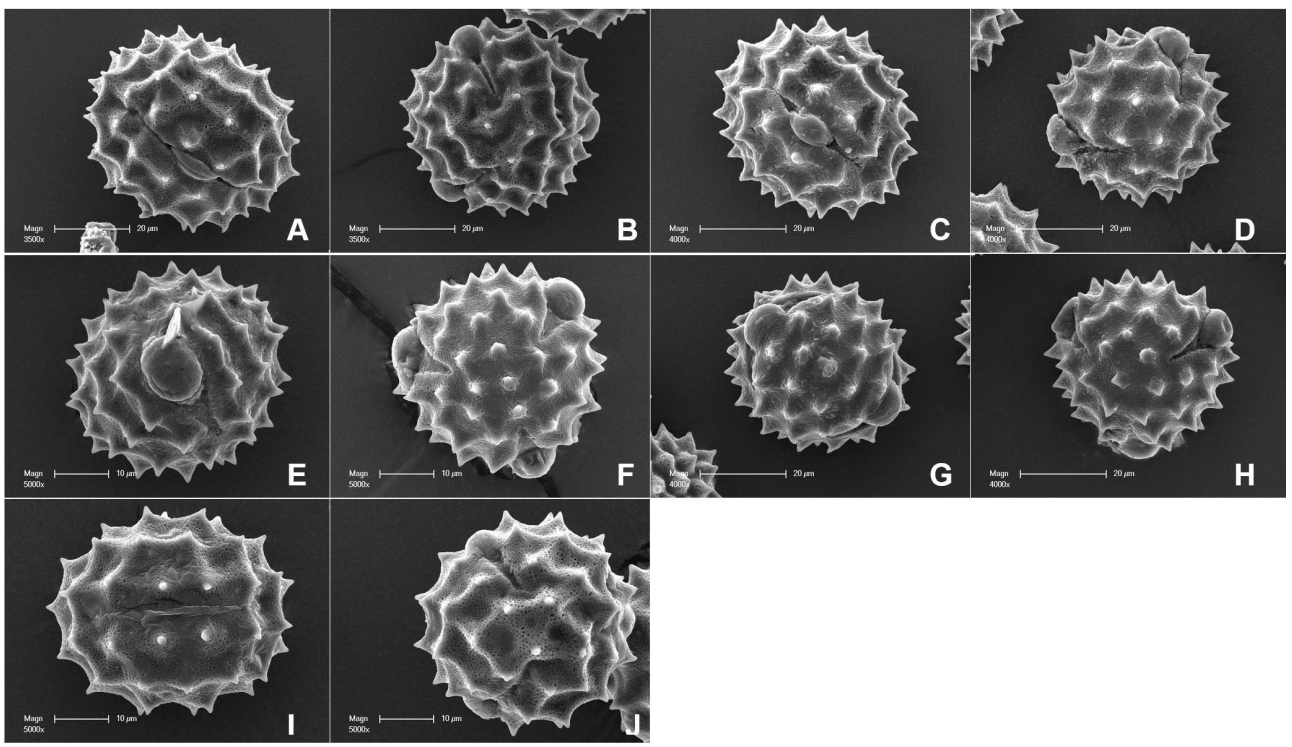

Figure 7. Pollen micrograph of *C. cephalotes* (A, B), *C.* ×*kelkitensis* (C, D), *C. macrobothrys* (E, F), *C.* ×*nezaketiae* (G, H), and *C.* ×*erzincanicum* (I, J). A, C, E, G, I- equatorial view; B, D, F, H, J- polar view.

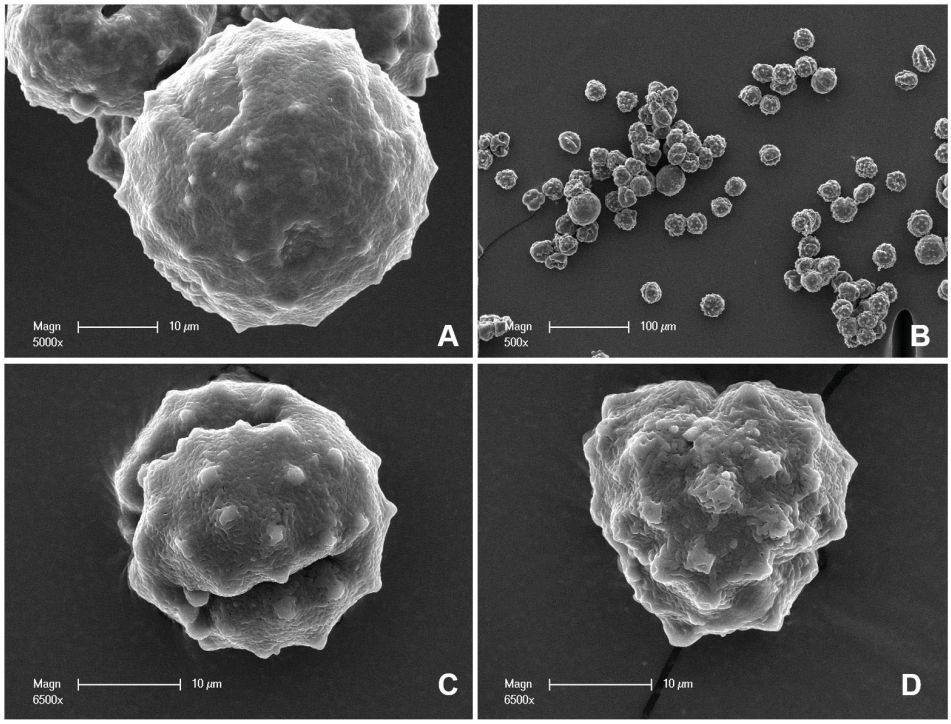

Figure 8. Dimorphic pollen grains of *Cirsium leucocephalum* subsp. *leucocephalum*. A- Big pollen grains; B- pollen grains of different sizes; C- small pollen grains in equatorial view; D- small pollen grains in polar view.

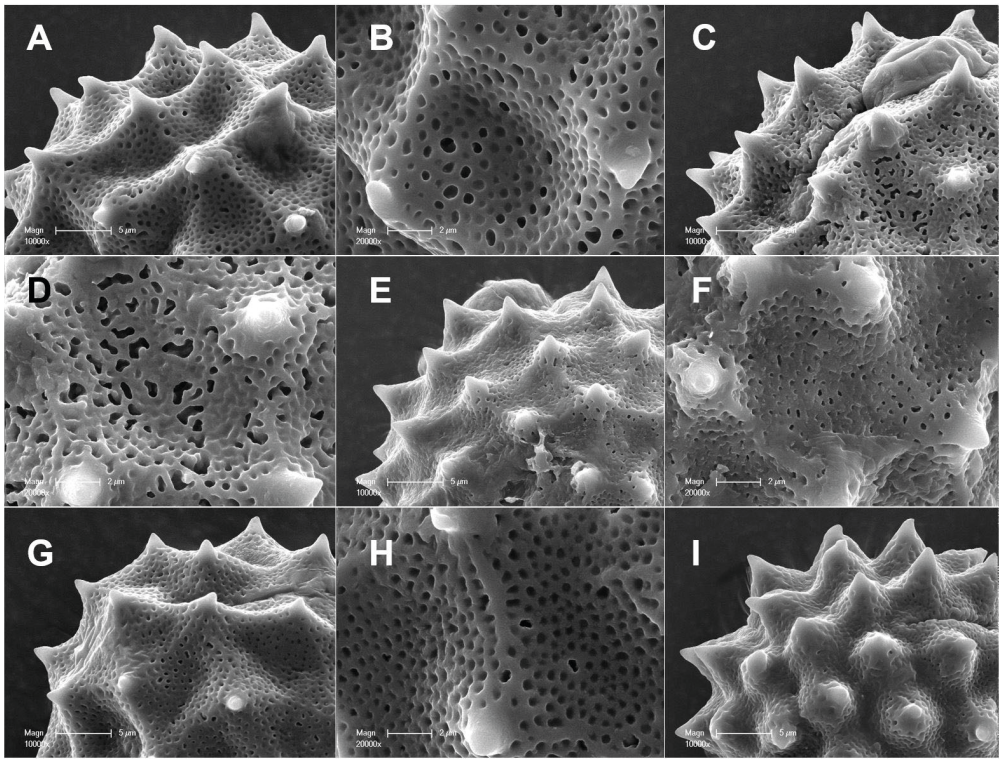

Figure 9. Pollen micrograph of *C. cephalotes* (A, B), *C.* ×*kelkitensis* (C, D), *C.macrobotrys* (E, F), *C.* ×*erzincanicum* (G, H), *C.* ×*nezaketiae* (I). A, C, E, G, I- detail of spines; B, D, F, H- exine structure.

proportion of *Cirsium* ×*nezaketiae* and *C. cephalotes* was 0.992. Furthermore, 642 of 642 characters were constant between *Cirsium* ×*nezaketiae* and *C. macrobotrys*, and so the similarity proportion was 1.0 between these individuals. Also, because the polymorphic loci are significant when defining the hybrid specimens, we carefully analyzed these regions with respect to polymorphic loci. The *Cirsium* ×*nezaketiae* hybrid specimens had polymorphism at nucleotides 9, 18, 36, 86, 97, 215, 219, 422, 445, and 587. The single nucleotide polymorphism (SNP) at positions 18, 36, 86, 215, 219, 422, 445, and 587 fully revealed two ancestors, and these SNPs caused *Cirsium* ×*nezaketiae* to be in a molecularly intermediate form between its parents (Figure 10; Table 5). Also, *C. macrobotrys* individuals had no polymorphism while *C. cephalotes* had eight polymorphic loci. In addition, *C. cephalotes* had some individual differences among its different specimens. The occurrence of polymorphism and individual differences in *C. cephalotes* might be formed by introgression and backcrossing (Table 5).

According to PAUP* parsimony analysis, the other hybrid, *C.* ×*kelkitensis*, had 634 (in total 642) constant characters with *C. cephalotes*, four variable characters were parsimony-uninformative, and the number of parsimony-informative characters was four. The similarity proportion between *C.* ×*kelkitensis* and *C. cephalotes* was 0.987. Furthermore, the similarity proportion between *C.* ×*kelkitensis* and *C. leucocephalum* subsp. *leucocephalum* was 0.996, and only two characters out of 642 characters were parsimony-uninformative, the others being constant. When we analyzed the polymorphic loci, nucleotide positions 9, 18, 36, 219, 586, and 587 were polymorphic in all *C.* ×*kelkitensis*, and four of them, 18, 36, 219, and 587, were SNPs between two ancestors, meaning that the hybrid might have two different datasets in that loci. Since there were no differences in *C. leucocephalum* subsp. *leucocephalum* and *C. macrobotrys*, *C.* ×*erzincanicum* had no polymorphic loci, making it an intermediate form between its parents. When the ancestors were compared, the similarity proportions of *C. cephalotes*-*C. macrobotrys* and *C. cephalotes*-*C. leucocephalum* subsp. *leucocephalum* were 0.98 with four parsimony-uninformative and seven parsimony-informative characters. In addition, the similarity proportion between *C. macrobotrys* and *C. leucocephalum* subsp. *leucocephalum* was 0.996 with two parsimony-uninformative characters.

In addition to this information, *C. ciliatum* (Murray) Moench. subsp. *szovitzii* (K.Koch) Petr. (TD4446) and *C. sintenisii* Freyn. (TD4917A) were chosen as outgroups from sect. *Epitrachys*. The nucleotide loci of 18, 36, 86, 89, 215, 219, 422, 445, 452, 587, and 609 distinguished *C. macrobotrys*, *C. cephalotes*, *C. leucocephalum* subsp. *leucocephalum*, *C. ciliatum* subsp. *szovitzii*, and *C. sintenisii*, although these different species belong to the same section. *C. ciliatum* subsp. *szovitzii* and *C. sintenisii* were randomly selected as outgroups; on the other hand, these species had good hybridization potential according to our observations (unpublished data).

3.3.2 nrETS results

A total of 574 nucleotides were obtained in the nrETS region. According to PAUP* analysis, the similarity proportion of *C.* ×*nezaketiae* was 1.0 with *C. cephalotes* and 0.998 with *C. macrobotrys*. Although there were no parsimony-informative/uninformative characters between *Cirsium* ×*nezaketiae* and *C. cephalotes*, a parsimony-informative character was obtained between *Cirsium* ×*nezaketiae* and *C. macrobotrys*.

C. ×*kelkitensis*, the most polymorphic hybrid, had four polymorphic nucleotide sites at positions 237, 255, 261, and 282. In addition, according to PAUP* analysis, the similarity proportion of *C.* ×*kelkitensis* with *C. cephalotes* was 1.0 and all 574 characters were constant. However, there was one variable parsimony-uninformative character between *C.* ×*kelkitensis* and *C. leucocephalum* subsp. *leucocephalum* and the similarity proportion was 0.998.

C. ×*erzincanicum* had the lowest polymorphic loci among these hybrids (two loci at the positions 255 and 306), and the similarity proportion was 1.0 with *C. leucocephalum* subsp. *leucocephalum* and 0.998 with *C. macrobotrys*.

According to nrITS and nrETS data, *C. leucocephalum* subsp. *leucocephalum* is generally closer to *C. macrobotrys* than *C. cephalotes* (Figure 10; Table 6). *C. ciliatum* subsp. *szovitzii* (TD4446) and *C. sintenisii* (TD4917A) were chosen as outgroups from sect. *Epitrachys* like the ITS data.

4. Discussion

4.1. Morphological evaluation

Hybrids frequently occur in fields where the distribution areas of close relatives or two or three species groups (complex mutually morphologically very different species) overlap. Hybrids generally grow with their parents in the same habitats. Sometimes, one of the ancestral species is not in the same field, or at least the hybrids may be distributed in a different field than their parents. If only one of the parents is with the hybrids, the possibility of it being a female ancestor is very high. When hybrids coexist with parental species in the same area, even if the hybrid is more similar to one of the parents in terms of morphological features, it will show a lot of intermediated characters (Bureš et al., 2010; Metzgar et al., 2016; Dirmenci et al., 2018a, 2018b, unpublished data; Jaźwa et al., 2018). Also, they have different characters from their parents. The new hybrids identified in this study and the three morphologically dissimilar species are distributed in the same area and all three species hybridize with each other: *Cirsium cephalotes* × *C. macrobotrys* (*C.* ×*nezaketiae*), *C. cephalotes* × *C.*

leucocephalum subsp. *leucocephalum* (*C.* ×*kelkitensis*), and *C. leucocephalum* subsp. *leucocephalum* × *C. macrobotrys* (*C.* ×*erzincanicum*). All the hybrids have intermediate forms, and they are measured in terms of habit, stem, and leaf indumentum; involucres size; and phyllary, corolla, and pappus lengths. Only the hybrids that were formed by *C. macrobotrys* are biennial, and biennialism is a feature of *C. macrobotrys*. *Cirsium* ×*erzincanicum* is similar to its parents in some characteristics. *C.* ×*erzincanicum* is similar to *C. leucocephalum* subsp. *leucocephalum* by stems branched above, leaves green, inflorescence raceme, involucral leaves longer than capitula, capitula pedunculate, corolla pinkish, and similar to *C. macrobotrys* by its life form biennial, stem single, involucral leaves longer than capitula, phyllaries densely arachnoid at apex, median phyllaries with 2–3 mm apical spine at apex. *Cirsium* ×*nezaketiae* is similar to its parents in some characteristics. It is similar to *C. macrobotrys* by its life form biennial, stem single, leaves characters, phyllaries densely arachnoid at apex and median phyllaries with 2–3 mm apical spine and recurved, and similar to *C. cephalotes* its stems branched above, leaves glaucous, capitula pedunculate, phyllaries densely arachnoid, corolla pinkish.

Cirsium ×*kelkitensis* is similar to its parents in some characteristics. *C.* ×*kelkitensis* is similar to *C. cephalotes* by its life form perennial, few to many stemmed from base and branched above, leaves glaucous, involucral leaves longer than involucre, capitula pedunculate and globose, phyllaries densely arachnoid at the apex. *C.* ×*kelkitensis* is similar to *C. leucocephalum* subsp. *leucocephalum* by its life form perennial, few to many stemmed from the base and branched above, leaves green, capitula pedunculate, phyllaries recurved, corolla pinkish-purple.

The morphological measurements of the studied taxa were used to evaluate morphological quantitative characters of PCA. Component 1 explained the significant part of the variation in species as 93.845%. The structure of capitula, pappus, corolla, and phyllary are the most distinctive characters in explaining the variation among these taxa according to PCA. As seen from Figure 4A, 4B *C.* ×*nezaketiae* is more similar to *C. cephalotes* than *C. macrobotrys*. Although *C. cephalotes*, *C. leucocephalum* subsp. *leucocephalum*, and *C. macrobotrys* generate hybrid specimens with each other, *C. leucocephalum* and *C. macrobotrys* are more similar to each other than *C. cephalotes*. Also, *C.* ×*kelkitensis* and *C.* ×*erzincanicum* have a complex structure with their parents.

Detailed morphological comparisons between hybrids and their parents are given in Table 1.

4.2. Palynological evaluation

There are a few studies about pollen morphologies of *Cirsium* (Yıldız et al., 2011; Nouroozi et al., 2012). Yıldız et al. (2011) identified a new species and used pollen morphology as supporting evidence. Nouroozi et al. (2012) studied the pollen morphology of Iranian *Cirsium* species, but they did not study any of the species in our article, and they did not report any hybrid species.

The size of pollen grains falls within the range of the pollen grain size of the parents in all investigated hybrids except *C.* ×*nezaketiae*. Measurements of pollen grains for both polar and equatorial axes were evaluated statistically to ascertain the value of pollen characters in the taxonomy of *Cirsium* hybrids. The results of Tukey tests showed statistically significant differences for the polar axis in *C.* ×*kelkitensis* ($P < 0.001$) and the equatorial axis for *C.* ×*nezaketiae* from both parental taxa. The other remaining hybrid taxa are statistically different from at least one parental species ($P < 0.001$, Table 7). This feature is characteristic for hybrid pollen and has been reported in other studies (Dirmenci et al., 2018a, 2018b). *C.* ×*kelkitensis* also differs from its parental species in terms of pollen surface characters. Its ornate tectum surface distinguishes it from *C. cephalotes* (Table 3; Figures 7 and 9). Dimorphic pollen grains were encountered in other parents of C. ×*kelkitensis*, named *C. leucocephalum* subsp. *leucocephalum* (Figure 8). Dimorphic pollen grains are not common within the family Asteraceae and only *C. leucocephalum* subsp. *leucocephalum* has dimorphic pollen grains within the investigated taxa. Some other families such as Rubiaceae and Lythraceae have dimorphic pollen with different aperture numbers and ornamentation (Baker, 1955; Kim et al., 1994). Kim et al. (1994) investigated pollen dimorphism in the genus *Lagerstroemia* L., and they stated that pollen dimorphism occurs in the dimorphic stamens. Baker (1955) published a paper on the dimorphic pollen of *Rudgea jasminoides* (Cham.) Müll. Arg. and concluded that pollen dimorphism in the species is associated with heterostyly. Baker (1955) stated that dimorphism has provided an extremely useful tool for the elucidation of genetic, gene-ecological, phytogeographical, and evolutionary problems. In our study, we observed dimorphic pollen grains in *C. leucocephalum* subsp. *leucocephalum*, but we did not observe either heterostyly or dimorphic stamen. Dimorphic pollen grains of *C. leucocephalum* subsp. *leucocephalum* can be evidence of hybrid characters for this species, or of introgression. We found some molecular data in *C. leucocephalum* subsp. *leucocephalum* that support the palynological data in the case of hybridization. Scabrate ornamentation was identified in larger pollen grains of *C. leucocephalum* subsp. *leucocephalum*, but the other smaller grains had echinate ornamentation (Figure 8). This hybrid differs from the other species in that it has different spin properties (Figure 8; Table 2).

The hybrids of *C.* ×*nezaketiae* are separated from the parental species by microreticulate tectum surface (Figure 9I). *C.* ×*erzincanicum* is similar to *C. macrobotrys* in terms of character of tectum surface (Figures 9E, 9F, 9G, 9H),

but differs from *C. leucocephalum* subsp. *leucocephalum* due to its microreticulate tectum surface (Table 3).

4.3. Cytological evaluation

In this work, chromosome studies could not be performed because mature seeds were not collected. However, in previous studies, analyses of parent species were conducted, and the chromosome numbers of *C. cephalotes*, *C. leucocephalum* subsp. *leucocephalum*, and *C. macrobotrys* were found as 2n = 34. Polyploidy was also reported from some Turkish *Cirsium* materials (*C. pubigerum* (Desf.) DC. var. *caniforme* Petr., *C. pubigerum* var. *glomeratum* (Freyn & Sint.) PH.Davis & Parris, and *C. pubigerum* var. *paphlagonicum* Petr.). *C. sintenisii* Freyn. and *C. vulgare* (Savi) Ten. have the somatic chromosome number 2n = 4x = 68 (Ozcan et al., 2011; Yüksel et al., 2013; Polat et al., 2018).

4.4. Molecular evaluation

The genus *Cirsium* has considerable hybridization potential. The sympatric occurrence of different taxa, the overlapping of the flowering terms, and the close molecular similarity have all been considered some of the potential outcomes of the extraordinary hybridization occurrence. Molecular phylogenetic studies have contributed to defining the relationship between hybrids and their parents and have been used to show larger hybridization potentials among *Cirsium* species. Although some hybrids of the genus *Cirsium* are easily distinguished from their parents in the sympatric distribution area by intermediate morphological characters (Werner, 1976), some significant molecular markers (AFLPs, RAPDs, ISSRs, nuclear rDNA, cpDNA) could be useful for identifying different taxa in the genus *Cirsium* (Jump et al., 2002; Kelch and Baldwin, 2003; Kaplan and Fehrer, 2007; Segarra-Moragues et al., 2007; Seif et al., 2012; Sheidai et al., 2012; Nouroozi et al. 2013; Sheidai et al., 2016).

Cirsium cephalotes, *C. leucocephalum* subsp. *leucocephalum*, and *C. macrobotrys*, examined in this study, form a natural complex living in a close/overlapping natural habitat. Since interspecific hybridization is well known in the genus *Cirsium*, and since these three species live in a complex, the possibility of hybridization occurring was the main factor in conducting this study. We therefore

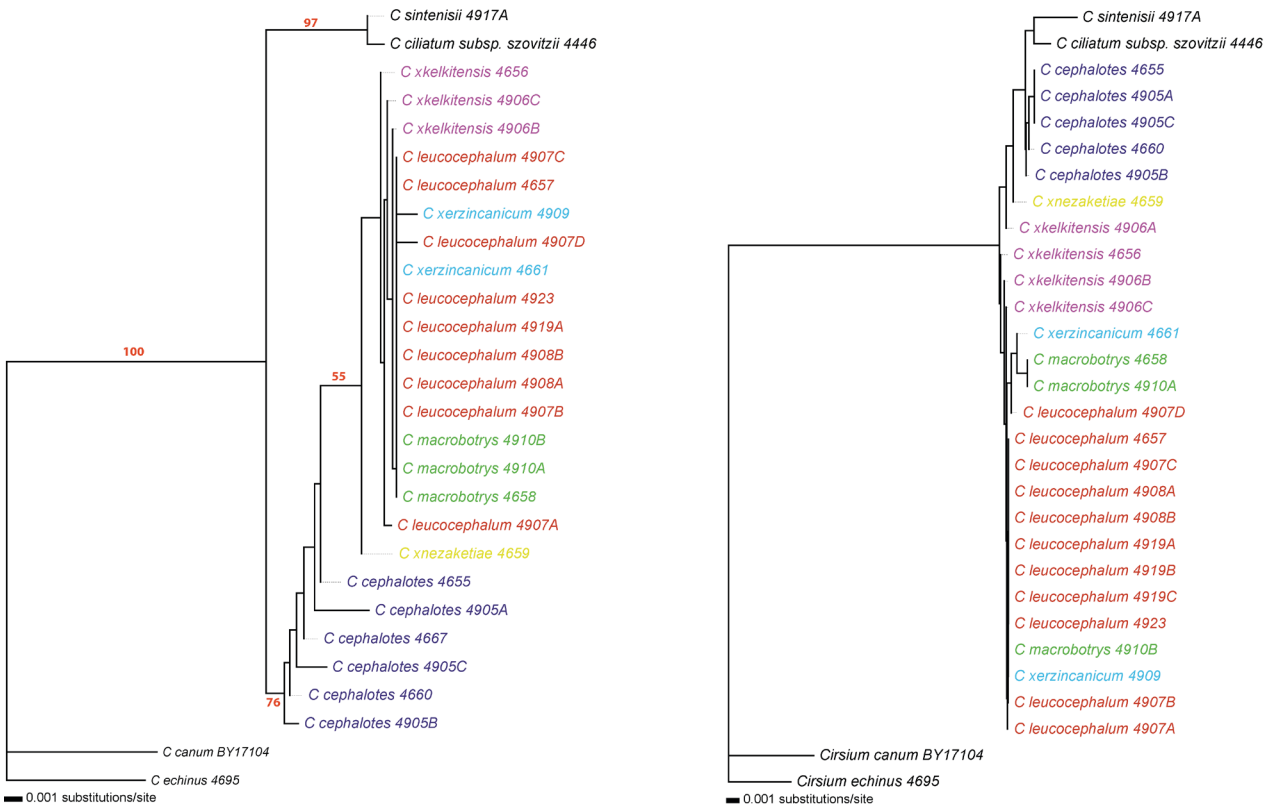

Figure 10. Phylograms of the new hybrids and their parents based on nrITS sequences (left side) and nrETS sequences (right side).

Table 5. The nrITS sequences of all the successfully sequenced individuals (with voucher numbers).

ITS	9	18	36	86	89	97	202	215	219	422	445	452	586	587	609
C. macrobotrys 4658	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. macrobotrys 4910A	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. macrobotrys 4910B	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. ×nezaketiae 4659	C/T	A/T	G/T	C/T	G	G/T	C	C/T	C/T	A/T	C/T	G	C	C/T	C
C. cephalotes 4660	C/T	A	G	C/T	G	G/T	C/T	C/T	C	T	C/T	A/G	C	C	C
C. cephalotes 4667	C/T	A	G	C/T	G	G/T	C	C/T	C/T	T	C/T	G	C/T	C	C
C. cephalotes 4905A	C	A	G	C	G	G	C	T	C	T	T	G	C	T	C
C. cephalotes 4905B	C/T	A	G	C/T	G	G	T	C	C	T	C	G	C	C	C
C. cephalotes 4905C	C	A	G	C	G	T	C	T	C	T	C	G	C	T	C
C. cephalotes 4655	C	A	G	C/T	G	G/T	C	C/T	C	T	C	G	C	C	C
C. ×kelkitensis 4656	C/T	A/T	G/T	T	G	G	C	T	C/T	A	T	G	C/T	C/T	C
C. ×kelkitensis 4906B	C/T	A/T	G/T	T	G	G	C	T	C/T	A	C/T	G	C/T	C/T	C
C. ×kelkitensis 4906C	C/T	A/T	G/T	T	G	G	C	T	C/T	A	T	G	C/T	C/T	C
C. leucocephalum subsp. *leucocephalum* 4657	C	T	T	T	G	G	C/T	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4907A	C	A	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4907B	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4907C	C	T	T	T	G	G	C/T	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4907D	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4908A	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4908B	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4919A	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. leucocephalum subsp. *leucocephalum* 4923	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. ×erzincanicum 4661	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. ×erzincanicum 4909	C	T	T	T	G	G	C	T	T	A	T	G	C	T	C
C. ciliatum subsp. *szovitzii* 4446	C	A	G	C	**A**	G	C	C	C	C	C/T	**A**	C	C	**A**
C. sintenisii 4917A	C	A	G	C	**A**	G	C	C	C	C	C/T	**A**	C	C	**A**
C. canum BY17104	T	A	A	T	G	G	C	C	C	C	C	G	C	T	C
C. echinus 4695	T	A	A	C	G	G	C	C	C	C	C	G	C	T	G

investigated the possible occurrence of interspecific hybridization among *C. cephalotes*, *C. leucocephalum* subsp. *leucocephalum*, and *C. macrobotrys* species.

Introgression could be considered a kind of genetic invasion (Mallet, 2005; Currat et al., 2008). The direction of introgression is commonly considered to be from the native species over to the invaders (Currat et al., 2008; Valtuena et al., 2011). Two parental species can have different abundances or parental species, and hybrids can display spatial segregation in the population of some distribution areas. Asymmetric introgression can occur in these regions, so that F1 hybrids can mate more frequently than expected with the most abundant parent, or with the closest parental species (Jacquemyn et al., 2012).

Sheidai et al. (2016) investigated interspecific hybridization between *Cirsium aduncum* (Fisch. & C.A.Mey. ex DC.) and *C. haussknechtii* Boiss. using the ISSR markers. They found that some of the studied specimens of *C. aduncum* had some intermixed characters with the samples of *C. haussknechtii*. Their results strongly supported introgressive hybridization between *C. aduncum* and *C. haussknechtii* and that meant the hybrid individuals backcrossed with the parental species. In this study, new three *Cirsium* hybrids were hybridized, possibly via introgression forming backcrosses. According to nrITS data, and as can be seen from Table 5, *C. ×nezaketiae* specimens had polymorphism at the 9th, 18th, 36th, 86th, 97th, 215th, 219th, 422nd, 445th, and 587th nucleotide

Table 6. The nrETS sequences of all the successfully sequenced individuals (with voucher numbers).

ETS	237	255	261	282	306
C. macrobotrys 4658	A	C	T	G	A
C. macrobotrys 4910A	A	C	T	G	A
C. macrobotrys 4910B	A	C/T	T	G	A/G
C. ×nezaketiae 4659	A/G	C	A/T	A/G	G
C. cephalotes 4655	G	C	A	A	G
C. cephalotes 4660	G	C	A	A/G	G
C. cephalotes 4905A	G	C	A	A	G
C. cephalotes 4905B	G	C	A	A/G	G
C. cephalotes 4905C	G	C	A	A	G
C. ×kelkitensis 4656	A/G	C/T	A/T	A/G	G
C. ×kelkitensis 4906A	A/G	C	A/T	A/G	G
C. ×kelkitensis 4906B	A/G	C/T	A/T	A/G	G
C. ×kelkitensis 4906C	A/G	C/T	A/T	A/G	G
C. leucocephalum subsp. *leucocephalum* 4657	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4907A	A	C/T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4907B	A	C/T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4907C	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4907D	A	C/T	T	G	A/G
C. leucocephalum subsp. *leucocephalum* 4908A	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4908B	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4919A	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4919B	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4919C	A	T	T	G	G
C. leucocephalum subsp. *leucocephalum* 4923	A	T	T	G	G
C. ×erzincanicum 4661	A	C/T	T	G	A/G
C. ×erzincanicum 4909	A	C/T	T	G	G
C. ciliatum subsp. *szovitzii 4446*	G	C	A	A	A
C. sintenisii 4917A	G	C	A	A	G
C. canum BY17104	A	A	A	G	G
C. echinus 4695	A	T	A	G	G

positions. *C. cephalotes* also had some polymorphic regions at positions 9, 86, 97, 202, 215, 219, 445, and 586, despite being a parental species. *C. ×nezaketiae* and its parents live in a complex, and *C. cephalotes* has more living individuals than *C. macrobotrys*. Thus, backcrossing between *C. ×nezaketiae* and *C. cephalotes* is presumably more possible. Polymorphic *C. cephalotes* individuals could be formed after these backcrosses. *C. ×kelkitensis*, the second most polymorphic hybrid, has six polymorphic nucleotide sites at positions 18, 36, 219, 445, 586, and 587, but *C. ×kelkitensis* is in a more intermediate position between *C. cephalotes* and *C. leucocephalum* subsp. *leucocephalum* in comparison to *C. ×nezaketiae* (Figure 10).

Kaplan and Fehrer (2007) studied molecular evidence for a natural primary triple hybrid in *Potamogeton* L. They used the nrITS region and *trn*L-*trn*F and *rpl*20-*rps*12 from cpDNA. *P. ×torssanderi* (Tselius) Dörfl. was the studied hybrid and *P. lucens* L., *P. gramineus* L., and *P. perfoliatus* L. were the putative triple parents. Two samples of *P. gramineus* were polymorphic, like *Cirsium cephalotes*

Table 7. The results of ANOVA and Tukey tests for all hybrids taxa.

Hybrid		P	E
C. ×*kelkitensis*			
	F	165.50	256.52
	Sig.	0.000	0.000
	Tukey groups	*	**
C. ×*nezaketiae*			
	F	42.38	93.27
	Sig.	0.000	0.000
	Tukey groups	**	*
C. ×*erzincanicum*			
	F	17.92	40.73
	Sig.	0.000	0.000
	Tukey groups	**	**

* Statistically significant from parental species ($P < 0.001$).
** Statistically significant from at least one parental species ($P < 0.001$).

examined in this study. The specimens of *Cirsium cephalotes* showed polymorphism at six positions (mentioned above) while *C. leucocephalum* subsp. *leucocephalum* had only three polymorphic loci and *C. macrobotrys* had none. *C. cephalotes* differed at six positions from the other two species according to nrITS data and differed at three positions based on nrETS data (Tables 5 and 6).

Segarra-Moragues et al. (2007) investigated a new Pyrenean hybrid, *Cirsium* ×*vivantii* L.Villar, Segarra, J.López, Pérez-Coll. & Catalán, using morphological and molecular analysis. They used the nrITS region and *trn*L-F of cpDNA, and AFLP analysis. The 5.8S nrDNA region was preserved in both parental taxa and the hybrids according to their findings and ours. The hybrid specimens showed polymorphism at 29 nucleotide sites (in total 647 bp) where the parents separated from each other. These polymorphic sequences are very important when defining hybrid specimens. In this study, the most polymorphic hybrid was *C.* ×*nezaketiae* and it had ten nucleotide loci where the two parents of *C.* ×*nezaketiae* totally differed at a specific level. nrETS sequences did not have as many SNPs as the nrITS sequences according to our investigations.

ITS sequences for *Cirsium* species may have some positional polymorphisms. According to Schilling (2013), *C. arvense* (L.) Scop., *C. discolor* Spreng., *C. muticum* Michx., and *C. vulgare* (Savi) Ten. had eight or more polymorphic loci in their nrITS sequences, and only *C. altissimum* (L.) Hill. had fewer than three polymorphic nucleotides. Six different specimens of *C. cephalotes*, three different specimens of *C. macrobotrys*, and nine different specimens of *C. leucocephalum* subsp. *leucocephalum* were compared in this study using nrITS data. In addition, the nrETS sequences of five individuals of *C. cephalotes*, three different individuals of *C. macrobotrys*, and eleven individuals of *C. leucocephalum* were obtained in this study. The examined parental species based on the nrITS region had more polymorphisms than the nrETS sequences. *C. cephalotes* (4667) was the species having the most polymorphic sites based on nrITS data. *C. cephalotes* individuals were also distinct from the other parental species, while *C. leucocephalum* and *C. macrobotrys* were closer to each other, according to the obtained diagrams and phylograms in this study (Tables 5 and 6).

Kelch and Baldwin (2003) used ITS and ETS rDNA sequences to gain information about phylogeny and the ecological spread of New World *Cirsium* species. They concluded that the ETS and ITS regions complemented each other, and that ETS and ITS-1 +ITS-2 had evolved at almost similar rates. rDNA transcribed spacers in *Cirsium* can be used for some wide-spread species in phylogeographical studies to define ongoing concerted evolution. In this study, we also studied *trn*L-F and matK markers from cpDNA but were unable to obtain adequate parsimony-informative characters. The ITS and ETS sequences helped us to distinguish the putative hybrids and their parents, but both ITS sequences and ETS data made little contribution in distinguishing *C. macrobotrys*-*C. leucocephalum* subsp. *leucocephalum* and their hybrids, there being one nucleotide for ITS and two for ETS (Tables 5 and 6).

Consequently**,** it should not be forgotten that DNA data alone are not sufficient to identify hybrids; DNA data should be supported using different approaches (morphological distinguishing characters, palynological features, cytological structure). Furthermore, polymorphism in the DNA data gives only some information about hybridization and backcrossing among the hybrids and their parents (introgression). It is not always very easy to find out which individual is a parental species or which individual does not particularly come from a mixed complex. When polymorphic loci (or SNPs) are found in some individuals based on nuclear or chloroplast DNA data (or mitochondrial DNA), hybrid individuals may be suspected. For example, Kokubugata et al. (2011), Dirmenci et al. (2018a, 2018b, unpublished data), and Segarra-Moragues et al. (2007) found some polymorphic loci in DNA data, and they showed that some morphologically indistinct specimens separated from their parents with some SNPs that meant they could possibly have two DNA datasets belonging to their parents. Zalewska-Gałosz et al. (2018) carried out reinterpretation of *Potamogeton* ×*nerviger* Wolfg. using nrITS and rpl32 DNA region from cpDNA and examined the polymorphic loci.

In this study, we described three new hybrids living in a complex with three parental species. We reviewed the literature and decided to use nrITS and nrETS DNA regions. These DNA regions helped us to define the hybrid individuals and their parents. Obtained PCA results (based on morphological analysis) support the molecular results. *C.* ×*kelkitensis*, *C.* ×*erzincanicum*, and *C.* ×*nezaketiae* live in a complex with their parents and they have some intermediate morphological characters. In addition, molecular and palynological results support this intermediate situation. Molecular data show it with the polymorphic loci, and palynological data show it with the dimorphic pollen characters.

References

Baker HG (1955). Pollen dimorphism in the Rubiacae. Evolution 10: 23-31.

Baldwin BG, Markos S (1998). Phylogenetic utility of the external transcribed spacer (ETS) of 18S-26S rDNA: congruence of ETS and ITS trees of *Calycadenia* (Compositae). Mol Phylogenet Evol 10: 449-463.

Bloom W (1977). Chromosomal differentiation between *Cirsium discolor* and *C. muticum* and the origin of supernumerary chromosomes. Syst Botany 2: 1-13.

Bureš P, Smarda P, Rotreklová O, Oberreiter M, Buresova M, Konecny J, Knoll A, Fajmon K, Smerda J (2010). Pollen viability and natural hybridization of Central European species of *Cirsium*. Preslia 82: 391-422.

Bureš P, Smerda J, Michalkova E, Smarda P, Knoll A, Vavrinec M (2018). *Cirsium greimleri*: a new species of thistle endemic to the Eastern Alps and Dinarides. Preslia 90: 105-134.

Bureš P, Wang YF, Horova L, Suda J (2004) Genome size variation in Central European species of *Cirsium* (Compositae) and their natural hybrids. Ann Bot London 94: 353-363.

Chan R, Baldwin BG, Ornduff R (2001). Goldfields revisited: a molecular phylogenetic perspective on the evolution of *Lasthenia* (Compositae: Heliantheae sensu lato). Int J Plant Sci 162: 1347-1360.

Charadze AL (1963). Cirsium Mill. In: Bobrov EG, Cherepanov SK (editors). Flora of the USSR, Vol. XXVIII. Moscow, USSR: Izdatel'stvo Akademii Nauk SSSR, pp. 63-270.

Clevinger JA, Panero JL (2000). Phylogenetic analysis of *Silphium* and subtribe Engelmanniinae (Asteraceae: Heliantheae) based on ITS and ETS sequence data. Am J Botany 87: 565-572.

Currat M, Ruedi M, Petit RJ, Excoffier L (2008). The hidden side of invasions: massive introgression by local genes. Evolution 62: 1908-1920.

Czapik R (1958). Karyological studies in species of *Cirsium* Mill. em. Scop. occurring in Poland. Acta Soc Bot Pol 27: 483-489.

Dabydeen S (1987). Natural hybridization in the genus *Cirsium*: *C. flodmanii* × *C. undulatum*. Rhodora 89: 369-373.

Davidson R (1963). Initial biometric survey of morphological variation in *Cirsium altrissimum-C. discolor* complex. Brittonia 15: 222-241.

Davis PH, Parris SB (1975). *Cirsium* Mill. In: Davis PH (editor). Flora of Turkey and the East Aegean Islands, Vol. 5. Edinburgh, UK: Edinburgh University Press, pp. 370-412.

Davis PH, Tan K, Mill RR (editors) (1988). Flora of Turkey and the East Aegean Islands, Vol. 10 (Suppl. 1). Edinburgh, UK: Edinburgh University Press.

Dirmenci T, Özcan T, Yazıcı T, Arabacı T, Martin E (2018a). Morphological, cytological, palynological and molecular evidence on two new hybrids: an example of homoploid hybridization in *Origanum* (Lamiaceae). Phytotaxa 371: 145-167.

Dirmenci T, Yazıcı T, Özcan T, Çelenk S, Martin E (2018b). A new species and a new natural hybrid of *Origanum* L. (Lamiaceae) from the west of Turkey. Turk J Bot 42: 73-90.

Erdtman G (1952). Pollen Morphology and Plant Taxonomy—Angiosperms. Stockholm, Sweden: Almqvist and Wiksell.

Erşen Bak F, Özcan M (2018). Pollen morphology of endemic NE Anatolian *Cirsium* taxa (Asteraceae). Pak J Bot 50: 1181-1185.

Faegri K, Iversen J (1989). Textbook of Pollen Analysis. 4th ed. Chichester, UK: Wiley.

Garcia-Jacas N, Garnatje T, Susanna A, Vilatersana R (2002). Tribal and subtribal delimitation and phylogeny of the Cardueae (Asteraceae): a combined nuclear and chloroplast DNA analysis. Mol Phylogenet Evol 22: 51-64.

Güner A, Özhatay N, Ekim T, Başer KHC (editors) (2000). Flora of Turkey and the East Aegean Islands, Vol. 11 (Suppl. 2). Edinburgh, UK: Edinburgh University Press.

Hammer Ø, Harper DAT, Ryan PD (2001) PAST: Paleontological statistics software package for education and data analysis. Palaeontol Electronica 4: 1-9.

Hesse M, Halbritter H, Zetter R, Weber M, Buchner R, Frosch-Radivo A, Ulrich S (2009). Pollen Terminology. An Illustrated Handbook. New York, NY, USA: Springer.

Jacquemyn H, Brys R, Honnay O, Roldán-Ruiz I, Lievens B, Wiegand T (2012). Nonrandom spatial structuring of orchids in a hybrid zone of three *Orchis* species. New Phytol 193: 454-464.

Jaźwa M, Jedrzejczak E, Klichowska E, Pliszko A (2018). Predicting the potential distribution area of *Solidago* ×*niederederi* (Asteraceae). Turk J Bot 42: 51-56.

Jolliffe IT (2002). Principal Component Analysis. New York, NY, USA: Springer Verlag.

Jump AS, Dawson DA, James CM, Woodward FI, Burke T (2002). Isolation of polymorphic microsatellites in the stemless thistle (*Cirsium acaule*) and their utility in other *Cirsium* species. Mol Ecol Notes 2: 589-592.

Kadereit JW, Jeffrey C (editors) (2007). Flowering Plants. Eudicots: Asterales. Berlin, Germany: Springer.

Kaplan Z, Fehrer J (2007). Molecular evidence for a natural primary triple hybrid in plants revealed from direct sequencing. Ann Bot 99: 1213-1222.

Keil DJ (2006). *Cirsium* Mill. In: Flora of North America Editorial Committee (editors). Flora of North America North of Mexico, Vol. 19. New York, NY, USA: Oxford University Press, pp. 95-164.

Kelch DG, Baldwin BG (2003). Phylogeny and ecological radiation of New World thistles (*Cirsium*, Cardueae Compositae) based on ITS and ETS rDNA sequence data. Mol Ecol 12: 141-151.

Kim SC, Graham SA, Graham A (1994). Palynology and pollen dimorphism in the genus *Lagerstroemia* (Lythraceae). Grana 33: 1-20.

Kita Y, Ueda K, Kadota Y (1995). Molecular phylogeny and evolution of the Asian *Aconitum* subgenus *Aconitum* (Ranunculaceae). J Plant Res 108: 429-442.

Kokubugata G, Kurihara T, Hirayama Y, Obata K (2011). Molecular evidence for a natural hybrid origin of *Ajuga* ×*mixta* (Lamiaceae) using ITS sequence. Bulletin of the National Museum of Natural Science 37: 175-179.

Lee J, Baldwin BG, Gottlieb LD (2002). Phylogeny of *Stephanomeria* and related genera (Compositae–Lactuceae) based on analysis of 18S-26S nuclear rDNA ITS and ETS sequences. Am J Botany 89: 160-168.

Linder CR, Goertzen LR, Heuvel BV, Francisco-Ortega J, Jansen RK (2000). The complete external transcribed spacer of 18S-26S rDNA: amplification and phylogenetic utility at low taxonomic levels in Asteraceae and closely allied families. Mol Phylogenet Evol 14: 285-303.

Mallet J (2005). Hybridization as an invasion of the genome. Trends Ecol Evol 20: 229-237.

Markos S, Baldwin BG (2001). Higher-level relationships and major lineages of *Lessingia* (Compositae, Astereae) based on nuclear rDNA internal and external transcribed spacer (ITS and ETS) sequences. Syst Botany 26: 168-183.

Nouroozi M, Sheidai M, Attar F, Noormohammadi Z (2011). B-Chromosome and cytomixis in *Cirsium* (Asteraceae). Cytologia 76: 41-47.

Nouroozi M, Sheidai M, Attar F, Noormohammadi Z (2012). Pollen morphological studies on the genus *Cirsium* Mill. (Asteraceae) in Iran. J Japanese Bot 87: 268-279.

Nouroozi M, Sheidai M, Attar F, Noormohammadi Z (2013). ISSR and RAPD analyses of species and their relationships in the genus *Cirsium* (Asteraceae) in Iran. Phytologia Balcanica 19: 225-232.

Ownbey G (1951). Natural hybridization in the genus *Cirsium*-I. *C. discolor* (Mubl. ex Willd.) Spreng. × *C. muticum* Mich. Bulletin of the Torrey Botanical Club 37: 541-547.

Ownbey G (1964). Natural hybridization in the genus *Cirsium*-II. *C. altissimum* × *C. discolor*. Michigan Bot 1: 87-97.

Ozcan M, Hayirlioglu-Ayaz S, Inceer H (2008). Chromosome counts of some *Cirsium* (Asteraceae, Cardueae) taxa from Turkey. Caryologia 6: 375-382.

Ozcan M, Hayirlioglu-Ayaz S, Inceer H (2011). Chromosome reports in some *Cirsium* (Asteraceae, Cardueae) taxa from north-east Anatolia. Caryologia 64: 55-66.

Petrak F (1979). *Cirsium* Mill. In: Rechinger KH (editor). Flora Iranica. Compositae III-Cynareae, Vol. 139a. Graz, Austria: Akademische Druck-u Verlagsanstalt, pp. 231-280.

Polat N, Kıran Y, Şahin A, Yıldız B, Arabacı T (2018). Chromosome counts and karyotype analysis of some representatives of genus *Cirsium* Mill. (Asteraceae) in Turkey. Caryologia 71: 133-138.

Punt W, Hoen PP, Blackmore S, Nilsson S, Le Thomas A (2007). Glossary of pollen and spore terminology. Rev Palaeobot Palynol 143: 1-81.

Romesburg HC (2004). Cluster Analysis for Researchers. Raleigh, NC, USA: Lulu Press.

Rotreklová O, Bureš P, Grulich V (2004). Chromosome numbers for some species of vascular plants from Europe. Biologia 59: 425-433.

Segarra-Moragues JG, Villar L, Lopez J, Perez-Collazos E, Catalan P (2007). A new Pyrenean hybrid *Cirsium* (Asteraceae) as revealed by morphological and molecular analyses. Bot J Linn Soc 154: 421-434.

Seif E, Sheidai M, Nouroozi M, Noormohammadi Z (2012). Biosystematic studies of *Cirsium arvense* populations in Iran. Phytologia Balcanica 18: 279-288.

Shaw J, Lickey EB, Schilling EE, Small RL (2007). Comparison of whole chloroplast genome sequences to choose noncoding regions for phylogenetic studies in angiosperms: the tortoise and the hare III. Am J Bot 94: 275-288.

Sheidai M, Seif M, Nouroozi M, Noormohammadi Z (2012). Cytogenetic and molecular diversity of *Cirsium arvense* (Asteraceae) populations in Iran. J Japanese Bot 87: 193-205.

Slotta TAB, Horvath DP, Foley ME (2012). Phylogeny of *Cirsium* spp. in North America: host specificity does not follow phylogeny. Plants 1: 61-73.

Stanford AM, Harden R, Parks CR (2000). Phylogeny and biogeography of *Juglans* (Juglandaceae) based on matK and ITS sequence data. Am J Bot 87: 872-882.

Susanna A, Garcia-Jacas N, Soltis DEPS, Soltis PS (1995). Phylogenetic relationships in tribe Cardueae (Asteraceae) based on ITS sequences. Am J Bot 82: 1056-1068.

Swofford DL (2002). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Sunderland, MA, USA: Sinauer Associates.

Valtuena FJ, Preston CD, Kadereit JW (2011). Evolutionary significance of the invasion of introduced populations into the native range of *Meconopsis cambrica*. Mol Ecol 20: 4318-4331.

Werner K (1976). *Cirsium* Mill. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Web DA (editors). Flora Europaea, Vol. 4. Cambridge, UK: Cambridge University Press, pp. 232-242.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Gelfand DH, Sninsky JJ, White TJ (editors). PCR Protocols: A Guide to Methods and Applications. New York, NY, USA: Academic Press, pp. 315-322.

Wodehouse RP (1935). Pollen Grains. New York, NY, USA: McGraw-Hill.

Yıldız B (2012). *Cirsium* Mill. In: Güner A, Aslan S, Ekim T, Vural M, Babaç. MT (editors). Türkiye Bitkileri Listesi (Damarlı Bitkiler). İstanbul, Turkey: Nezahat Gökyiğit Botanik Bahçesi ve Flora Araştırmaları Derneği Yayını, pp. 141-146 (in Turkish).

Yıldız B, Arabacı T, Dirmenci T, Çelenk S (2011). *Cirsium sivasicum* sp. nov. and *C. peshmenianum* sp. nov. (Asteraceae) and their allies from Turkey. Nord J Bot 29: 26-37.

Yıldız B, Arabacı T, Dirmenci T, Köstekçi S (2016). A taxonomic revision of the genus *Cirsium* Mill. sect. *Cirsium* (Asteraceae: Cardueae) in Turkey. Turk J Bot 40: 514-530.

Yüksel E, Kıran Y, Şahin A, Yıldız B, Arabaci T (2013). Karyological studies of 10 *Cirsium* sect. *Epitrachys* (Asteraceae) species from Turkey. Turk J Bot 37: 1085-1092.

Zalewska-Gałosz J, Kaplan Z, Kwolek D (2018). Reinterpretation of *Potamogeton* ×*nerviger*: solving a taxonomic puzzle after two centuries. Preslia 90: 135-149.

Appendix I

C. cephalotes: Turkey A7 Giresun: Between Şebinkarahisar and Giresun, 26.5 km, Eğribel pass, 1200 m, 02.09.2016, Dirmenci 4667*,**, Yıldız & Arabacı (Hb. Dirmenci); B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 2100 m, 02.09.2016, Dirmenci 4655*,** (Hb. Dirmenci), 4660*,**, Yıldız & Arabacı (Hb. Dirmenci): ibid. 09.08.2017, Dirmenci 4905*,**,*** & Arabacı (Hb. Dirmenci).

C. canum: A6 Sivas: Suşehri, 3–4 km west of Değirmentaş village, 1300 m, 23.08.2009, Yıldız 17104** (Hb. Dirmenci).

C. ciliatum subsp. szovitzii: Ardahan: between Posof and Türkoğlu border gate, 2 km from Eminbey village to Türkoğlu, 1461 m, 04.08.2015, Dirmenci 4446** & Kahraman (Hb. Dirmenci).

C. echinus: A9 Artvin: 25 km from Şavşat to Ardahan, Kocabey plateau, 04.09.2016, Dirmenci 4695**, Yıldız & Arabacı (Hb. Dirmenci).

C. ×erzincanicum: B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 2100 m, 02.09.2016, Dirmenci 4661*,**,*** Yıldız & Arabacı (ANK, ISTE); Ibid, 09.08.2017, Dirmenci 4909*,**,*** & Arabacı (GAZI).

C. leucocephalum subsp. leucocephalum: B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 2100 m, 02.09.2016, Dirmenci 4657*,**, Yıldız & Arabacı (Hb. Dirmenci); ibid., 09.08.2017, Dirmenci 4907*,**,*** (Hb. Dirmenci), 4908*,**,*** & Arabacı (Hb. Dirmenci). C4 Konya: 30 km from Seydişehir to Akseki, 1850 m, 17.08.2017, Dirmenci 4919 (Hb. Dirmenci), Konya: Bozkır, Sorgun district, 1 km from Dikilitaş plateau to Keklicek plateau, 2060 m, Dirmenci 4923 & Tugay (Hb. Dirmenci).

C. ×kelkitensis: B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 39.89030 N 39.36216, 2100 m, 02.09.2016, Dirmenci 4656*,**, Yıldız & Arabacı (ANK, ISTE); ibid., 09.08.2017, Dirmenci 4906*,**,*** & Arabacı (GAZI).

C. macrobotrys: B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 39.89030°N, 39.36216°E, 2100 m, 02.09.2016, Dirmenci 4658*,**,***, Yıldız & Arabacı; ibid. 09.08.2017, Dirmenci 4910*,** & Arabacı.

C. ×nezaketiae: B7 Erzincan: Between Erzincan and Kelkit, Pöske pass, steppe, 2100 m, 02.09.2016, Dirmenci 4659*,**,***, Yıldız & Arabacı (ANK, ISTE).

C. sintenisii: C4 Konya: 30 km from Seydişehir to Akseki, 1850 m, 17.08.2017, Dirmenci 4917-A ** (Hb. Dirmenci).

Note: "*" indicates the specimens examined in morphological analysis, "**" indicates the specimens examined in molecular analysis, "***" indicates the specimens examined in palynological analysis.

Appendix II

GenBank accession numbers of the obtained ITS sequences

C. cephalotes 4655 (MK298354), 4905A (MK298355), 4905C (MK298356), 4667 (MK298358), 4905B (MK298359), 4660 (MK298360); **C. canum** BY17104 (MK298361); **C. ciliatum subsp. szovitzii** 4446 (MK298336); **C. echinus** 4695 (MK298362); **C. ×erzincanicum** 4661 (MK298353), 4909 (MK298344); **C. leucocephalum subsp. leucocephalum** 4907A (MK298340), 4907C (MK298341), 4657 (MK298342), 4907D (MK298343), 4907B (MK298348), 4908A (MK298349), 4908B (MK298350), 4919A (MK298351), 4923 (MK298352); **C. ×kelkitensis** 4656 (MK298337), 4906C (MK298338), 4906B (MK298339); **C. macrobotrys** 4658 (MK298345), 4910A (MK298346), 4910B (MK298346); **C. ×nezaketiae** 4659 (MK298357); **C. sintenisii** 4917-A (MK298335).

GenBank accession numbers of the obtained ETS sequences

C. cephalotes 4655 (MK301515), 4905A (MK301516), 4905C (MK301517), 4905B (MK301519), 4660 (MK301518); **C. canum** BY17104 (MK301520); **C. ciliatum subsp. szovitzii** 4446 (MK301493); **C. echinus** 4695 (MK301521); **C. ×erzincanicum** 4661 (MK301501), 4909 (MK301513); **C. leucocephalum subsp. leucocephalum** 4907A (MK301511), 4907C (MK301504), 4657 (MK301503), 4907D (MK301502), 4907B (MK301512), 4908A (MK301505), 4908B (MK301506), 4919A (MK301507), 4919B (MK301508), 4919C (MK301509), 4923 (MK301510); **C. ×kelkitensis** 4656 (MK301494), 4906A (MK301495), 4906B (MK301496), 4906C (MK301497); **C. macrobotrys** 4658 (MK301499), 4910A (MK301500), 4910B (MK301498); **C. ×nezaketiae** 4659 (MK301514); **C. sintenisii** 4917-A (MK301492).