

## MORPHOLOGICAL HOMOPLASY AND SECTIONAL POLYPHYLY IN *CENTAUREA* SECT. *CYNAROIDES* AND RELATED TAXA

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Building natural classifications for genera with complex morphological variations such as *Centaurea* needs the support of molecular analysis and knowledge of morphological character diversification. Here we use parsimony and Bayesian analyses of nuclear ribosomal ITS sequences to gain insight into the sectional relationships and character evolution in *Centaurea* sections *Chartolepis*, *Cheirolepis*, *Cynaroides*, *Grosheimia*, *Microlophus*, *Paraphysis*, *Phaeopappus*, *Plumosipappus*, *Pseudoserida*, *Pseudophaeopappus*, *Ptheracantha*, *Ptosimopappus*, and *Rhizocalathium*, from Turkey. In particular, we increase sampling in Turkish endemic species of *Centaurea* sect. *Cynaroides* and related taxa. The new ITS sequences for 26 taxa from 48 locations are given. Sequences of 17 taxa are given here the first time. The phylogeny derived from ITS data provides a reasonably resolved and reasonably supported phylogenetic framework for the genus. Our analysis shows characters traditionally used in *Centaurea* taxonomy such as flower colour, appendage width, spine length, and pappus length evolved several times independently and show a lot of homoplasies. However, we can confirm the validity of character combinations for a reliable subgeneric classification of *Centaurea*.

*Key words:* Asteraceae, character reconstruction, ITS, phylogeny, taxonomy

### INTRODUCTION

The family Asteraceae is a big and important family that has 24,000–30,000 species placed within 1600 genera belong to 12 subgenera and 43 tribes (FUNK *et al.*, 2009).

The systematics of the tribe *Cardueae* has been progressed considerably based on molecular phylogenetic studies (GREUTER, 2003a; FUNK *et al.*, 2009; HERRANDO-MORAIRA *et al.*,

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2019). The tribe *Cardueae* has been divided into 12 monophyletic subtribes: *Carlininae*, *Cardopatiinae*, *Echinopsinae*, *Dipterocominae*, *Xerantheminae*, *Berardiinae*, *Stahelininae*, *Onopordinae*, *Carduinae*, *Arctiinae*, *Saussureinae*, and *Centaureinae* (Herrando-Moraira *et al.* 2019).

Numerous studies have focused on molecular taxonomy, morphology, and anatomy of the subtribe *Centaureinae* (WAGENITZ and HELLWIG, 1996, 2000; GARCIA-JACAS *et al.*, 2000; GREUTER, 2003a, 2003b; FUNK *et al.*, 2009; HERRANDO-MORAIRA *et al.*, 2019). Recently, the subtribe *Centaureinae* is accepted as a monophyletic group comprising 32 genera (HERRANDO-MORAIRA *et al.*, 2019).

*Centaurea* L. is formed by annual, biennial, or perennial herbs, less often shrubs, with usually unarmed leaves (WAGENITZ, 1980; SUSANNA and GARCIA-JACAS, 2007). Lateral hilum and sterile marginal flowers without staminodes have also characterized the genus *Centaurea* (DITTRICH, 1968; WAGENITZ and HELLWIG, 1996). The main morphological character used for systematics within *Centaurea* is the form of the scarious bract appendages (WAGENITZ, 1980; HILPOLD *et al.*, 2014a). The genus has ca. 250 species placed into 40 sections distributed mainly in the Mediterranean and Irano-Turanian regions (HILPOLD *et al.*, 2014a). In addition, the total species number is increasing with the description of many new species (INVERNON *et al.*, 2012; RANJBAR *et al.*, 2012; BANCHEVA and RAIMONDO, 2013; CARUSO *et al.*, 2013; BONA, 2015a, 2015b).

*Centaurea* subg. *Centaurea* sect. *Cynaroides* Boiss. includes biennial, long-stemmed plants with large capitula and entire hastate leaves (GARCIA-JACAS *et al.*, 2006; NEGARESH and RAHIMINEJAD, 2014; 2018). It is mainly distributed the Irano-Turanian and Mediterranean regions, except *C. charreltii* Halácsy & Dörfl. which is distributed in Greece (WAGENITZ, 1975, 1980; CONSTANTINIDIS *et al.*, 2002; NEGARESH and RAHIMINEJAD, 2018). Recently some proposals have been made about the classification of the section according to molecular and morphological studies (HILPOLD *et al.*, 2014a; NEGARESH and RAHIMINEJAD, 2014; 2018) but Turkish specimens especially endemics were not studied comprehensively. Turkey is renowned throughout the world for its floristic richness, and it is one of the main centres of diversity for the genus *Centaurea* (WAGENITZ, 1986). Endemic species are more susceptible to extinction for a variety of reasons and local or narrow endemic taxa are the first to experience these threats (BIZOUX *et al.*, 2004; BERNARDOS *et al.*, 2006; BONA and GHAZAL ASSWAD, 2014; PETIT *et al.*, 2015). Clear species delimitation is important to identify narrow endemics and outline their distribution (GARCIA-JACAS *et al.*, 2000, 2001, 2006; THOMAS *et al.*, 2011; AYDIN *et al.*, 2013; UYSAL *et al.*, 2016). In Turkey, *Centaurea* has one of the highest rates of endemism and species number has been increasing recently (ÖZHATAY and KÜLTÜR, 2006; ÖZHATAY *et al.*, 2009, 2011; BANCHEVA *et al.*, 2014; NEGARESH *et al.*, 2015; YÜZBAŞIOĞLU *et al.*, 2015; KÜLTÜR *et al.*, 2016; UYSAL and HAMZAOĞLU, 2016; UYSAL *et al.*, 2016, 2017; BEHÇET *et al.*, 2017; OREIZI *et al.*, 2017). Previous studies on *Centaurea* did not sample *Cynaroides* and allies comprehensively and members of the section, especially Turkish endemics, were poorly known taxonomically and phylogenetically. This paper adds to the knowledge of the delimitation and morphological evolution in *Centaurea* sect. *Cynaroides* and related taxa by combining new data from Turkish endemic *Centaurea* with currently available data.

## MATERIALS AND METHODS

*Plant material*

Sampling was based on the sections *Chartolepis*, *Cheirolepis*, *Cynaroides*, *Grosheimia*, *Microlophus*, *Paraphysis*, *Phaeopappus*, *Plumosipappus*, *Pseudoserida*, *Pseudophaeopappus*, *Ptheracantha*, *Ptosimopappus*, and *Rhizocalathium*, from Turkey. Specimens used for this study were collected in 2014 and dried in silica gel. In addition, we used herbarium materials from Istanbul University Faculty of Pharmacy, Department of Pharmaceutical Botany Herbarium (ISTE) and Gazi University, Science Faculty Herbarium (GAZI). We included multiple samples for each species where possible. In total, we extracted 48 different populations of 26 species in our study. Vouchers are deposited in herbaria GAZI and ISTE. 13 outgroup species were selected from genera *Carthamus*, *Centaurea*, *Rhaponticoides*, *Psephellus*, *Serratula*, *Klasea*, and *Zoegea* according to previous studies on *Centaurea* (GARCIA-JACAS *et al.*, 2001; FONT *et al.*, 2002; BARRES *et al.*, 2013; HILPOLD *et al.*, 2014a). Totally 127 taxa and 203 sequences were used. GenBank sequence accession numbers for the newly studied taxa are given in Table 1. GenBank sequence accession numbers of the taxa from GenBank are given in Table 2.

Table 1. GenBank sequence accession numbers for the newly studied taxa

Taxa	Accession numbers
<i>Centaurea aladaghensis</i>	EDNA15_0039140
<i>Centaurea aladaghensis</i>	EDNA15_0040183
<i>Centaurea aladaghensis</i>	EDNA15_0040182
<i>Centaurea amanicola</i>	EDNA15_0039095
<i>Centaurea amanicola</i>	EDNA15_0039131
<i>Centaurea amanosensis</i>	EDNA15_0039029
<i>Centaurea antitauri</i>	EDNA15_0039741
<i>Centaurea arifolia</i>	EDNA15_0038283
<i>Centaurea arifolia</i>	EDNA15_0039743
<i>Centaurea cataonica</i>	EDNA15_0038284
<i>Centaurea cheirolepidioides</i>	EDNA15_0038279
<i>Centaurea cheirolopha</i>	EDNA15_0038276
<i>Centaurea cheirolopha</i>	EDNA15_0040177
<i>Centaurea cheirolopha</i>	EDNA15_0039742
<i>Centaurea cynarocephala</i>	EDNA15_0038272
<i>Centaurea doddsii</i>	EDNA15_0040174
<i>Centaurea drabifolioides</i>	EDNA15_0038278
<i>Centaurea fenzi</i>	EDNA15_0039139
<i>Centaurea zaferii</i>	EDNA15_0038234
<i>Centaurea zaferii</i>	EDNA15_0039129
<i>Centaurea zaferii</i>	EDNA15_0040175
<i>Centaurea gigantean</i>	EDNA15_0039099
<i>Centaurea haradjiannii</i>	EDNA15_0040173
<i>Centaurea haussknechtii</i>	EDNA15_0039132
<i>Centaurea haussknechtii</i>	EDNA15_0039027

Table 1. continued

Taxa	Accession numbers
<i>Centaurea haussknechtii</i>	EDNA15_0039133
<i>Centaurea haussknechtii</i>	EDNA15_0039740
<i>Centaurea haussknechtii</i>	EDNA15_0038236
<i>Centaurea haussknechtii</i>	EDNA15_0039135
<i>Centaurea haussknechtii</i>	EDNA15_0039137
<i>Centaurea hermanii</i>	EDNA15_0038277
<i>Centaurea kurdica</i>	EDNA15_0039126
<i>Centaurea kurdica</i>	EDNA15_0039128
<i>Centaurea kurdica</i>	EDNA15_0039130
<i>Centaurea lycopifolia</i>	EDNA15_0038275
<i>Centaurea lycopifolia</i>	EDNA15_0039739
<i>Centaurea ptosimopappoides</i>	EDNA15_0040180
<i>Centaurea ptosimopappoides</i>	EDNA15_0040178
<i>Centaurea regia</i>	EDNA15_0038235
<i>Centaurea sclerolepis</i>	EDNA15_0038273
<i>Centaurea spicata</i>	EDNA15_0038233
<i>Centaurea spicata</i>	EDNA15_0039127
<i>Centaurea spicata</i>	EDNA15_0039744
<i>Centaurea stevenii</i>	EDNA15_0038274
<i>Centaurea yaltirikii</i> subsp. <i>dumanii</i>	EDNA15_0038281
<i>Centaurea yaltirikii</i> subsp. <i>yaltirikii</i>	EDNA15_0039023
<i>Centaurea yaltirikii</i> subsp. <i>yaltirikii</i>	EDNA15_0038280
<i>Centaurea ziganensis</i>	EDNA15_0038282

Table 2. GenBank sequence accession numbers of the taxa from GenBank.

Taxa	Accession numbers
<i>Carduus kartschiana</i> subsp. <i>lubenicensis</i>	KJ666019
<i>Carduus oxyacanthus</i>	GU969639
<i>Carduus oxyacanthus</i>	GU969641
<i>Carduus oxyacanthus</i>	GU969640
<i>Carduus oxyacanthus</i>	AY826248
<i>Carduus oxyacanthus</i>	HM009326
<i>Carduus palaestinus</i>	HQ407426
<i>Carduus palaestinus</i>	GU969642
<i>Carduus tinctorius</i>	GU969652
<i>Carduus tinctorius</i>	JQ230977
<i>Carduus tinctorius</i>	EF483946
<i>Carduus tinctorius</i>	EF483943
<i>Carduus tinctorius</i>	GU724280
<i>Centaurea aeolica</i>	KJ665886

Table 2. continued

Taxa	Accession numbers
<i>Centaurea aeolica</i>	AM117057
<i>Centaurea aeolica</i>	KJ665885
<i>Centaurea aetaliae</i>	KJ665889
<i>Centaurea akamantis</i>	KJ665897
<i>Centaurea aladaghensis</i>	DQ319078
<i>Centaurea albonitens</i>	DQ319081
<i>Centaurea amadanensis</i>	DQ319083
<i>Centaurea antalyensis</i>	KJ665909
<i>Centaurea aphrodisea</i>	KJ665911
<i>Centaurea aplolepa</i>	JF913981
<i>Centaurea aplolepa</i> subsp. <i>aplolepa</i>	KF032416
<i>Centaurea aplolepa</i> subsp. <i>caruealiana</i>	KJ665913
<i>Centaurea aplolepa</i> subsp. <i>cosana</i>	KJ665914
<i>Centaurea argentea</i>	KJ665921
<i>Centaurea argentea</i>	KJ665922
<i>Centaurea armena</i>	DQ319085
<i>Centaurea arrigonii</i>	KC969536
<i>Centaurea aucheri</i>	DQ319087
<i>Centaurea austroanatolica</i>	KJ665933
<i>Centaurea aziziana</i>	DQ319089
<i>Centaurea behen</i>	DQ319090
<i>Centaurea behen</i>	AY826250
<i>Centaurea bethurica</i>	KJ665936
<i>Centaurea bethurica</i>	KJ665935
<i>Centaurea boissieri</i>	DQ319092
<i>Centaurea busambarensis</i>	KJ665947
<i>Centaurea cachinalensis</i>	JF754804
<i>Centaurea calolepis</i>	DQ319095
<i>Centaurea cariensiformis</i>	KJ665950
<i>Centaurea carratracensis</i>	AM114302
<i>Centaurea carratracensis</i>	KJ665955
<i>Centaurea carratracensis</i>	DQ319098
<i>Centaurea castellanoides</i>	KJ665957
<i>Centaurea cataonica</i>	DQ319099
<i>Centaurea cheirolepidioides</i>	DQ319100
<i>Centaurea cheirolopha</i>	DQ319101
<i>Centaurea chrysocephala</i>	KJ665958
<i>Centaurea cineraria</i>	KC969537
<i>Centaurea cordubensis</i>	KJ665967
<i>Centaurea cristata</i>	KJ665974
<i>Centaurea cristata</i>	KJ665975

Table 2.continued

Taxa	Accession numbers
<i>Centaurea crithmifolia</i>	KF032408
<i>Centaurea debdouensis</i>	KJ665978
<i>Centaurea deflexa</i>	DQ319105
<i>Centaurea delicatula</i>	AM114318
<i>Centaurea derderiifolia</i>	DQ319106
<i>Centaurea donetzica</i>	DQ319110
<i>Centaurea donetzica</i>	JF913987
<i>Centaurea donetzica</i>	JF913988
<i>Centaurea donetzica</i>	JF913986
<i>Centaurea ensiformis</i>	DQ319112
<i>Centaurea ertugruliana</i>	KJ665998
<i>Centaurea fenzi</i>	DQ319114
<i>Centaurea ferulacea</i>	KF032424
<i>Centaurea ferulacea</i>	KF032425
<i>Centaurea friderici</i> subsp. <i>friderici</i>	KJ666000
<i>Centaurea friderici</i> subsp. <i>jabukensis</i>	KJ666001
<i>Centaurea gadorensis</i>	KJ666002
<i>Centaurea gadorensis</i>	KJ666004
<i>Centaurea gadorensis</i>	DQ319115
<i>Centaurea glastifolia</i>	DQ319116
<i>Centaurea hanrii</i>	KJ666009
<i>Centaurea hanrii</i>	KJ666011
<i>Centaurea hermanni</i>	DQ319118
<i>Centaurea imperialis</i>	DQ319121
<i>Centaurea kotschy</i>	DQ319127
<i>Centaurea kurdica</i>	DQ319128
<i>Centaurea langei</i>	KJ666025
<i>Centaurea langei</i>	KJ666026
<i>Centaurea langei</i>	KJ666027
<i>Centaurea limbata</i>	KJ666043
<i>Centaurea litigiosa</i>	KJ666045
<i>Centaurea luschaniana</i>	KJ666047
<i>Centaurea lycopifolia</i>	DQ319130
<i>Centaurea macrocephala</i>	DQ319131
<i>Centaurea messenicolasiana</i>	KJ666051
<i>Centaurea nemecii</i>	DQ319137
<i>Centaurea niederi</i>	KJ666055
<i>Centaurea nigra</i>	DQ319138
<i>Centaurea odyssei</i>	DQ319140
<i>Centaurea paczoskii</i>	KJ666058
<i>Centaurea paphlagonica</i>	DQ319142

Table 2. continued

Taxa	Accession numbers
<i>Centaurea parlatoris</i>	KJ666069
<i>Centaurea parlatoris</i>	KC969540
<i>Centaurea parlatoris</i>	KJ666068
<i>Centaurea paui</i>	AM114307
<i>Centaurea pectinata</i>	DQ319144
<i>Centaurea pentadactyli</i>	KF032417
<i>Centaurea pinae</i>	DQ319145
<i>Centaurea pinae</i>	AM114310
<i>Centaurea pinetorum</i>	KJ666074
<i>Centaurea polypodiifolia</i>	DQ319148
<i>Centaurea pomeliana</i>	AM114315
<i>Centaurea pomeliana</i>	KJ666078
<i>Centaurea pomeliana</i>	KJ666077
<i>Centaurea pomeliana</i>	KJ666079
<i>Centaurea protogerberi</i>	KJ666081
<i>Centaurea protogerberi</i>	DQ319149
<i>Centaurea protomargaritacea</i>	KJ666082
<i>Centaurea pterocaula</i>	DQ319151
<i>Centaurea ptosimopappa</i>	DQ319152
<i>Centaurea ptosimopappoides</i>	DQ319153
<i>Centaurea pulvinata</i>	AM114296
<i>Centaurea resupinata</i> subsp. <i>resupinata</i>	DQ319156
<i>Centaurea resupinata</i> subsp. <i>simulans</i>	AM114292
<i>Centaurea rhizantha</i>	DQ319157
<i>Centaurea rigida</i>	DQ319158
<i>Centaurea rouyi</i>	KJ666090
<i>Centaurea saccensis</i>	KJ666092
<i>Centaurea saligna</i>	DQ319159
<i>Centaurea sarandinakiae</i>	JF913992
<i>Centaurea sarandinakiae</i>	JF913994
<i>Centaurea sarandinakiae</i>	JF913993
<i>Centaurea sarandinakiae</i>	DQ319160
<i>Centaurea scannensis</i>	KJ666094
<i>Centaurea semijusta</i>	DQ319162
<i>Centaurea sipylea</i>	KJ666097
<i>Centaurea spectabilis</i>	DQ319164
<i>Centaurea spinosociliata</i>	KJ666107
<i>Centaurea stapfiana</i>	DQ319166
<i>Centaurea stevenii</i>	DQ319168
<i>Centaurea stoebe</i>	JF914045
<i>Centaurea subciliaris</i>	KJ666115

Table 2.continued

Taxa	Accession numbers
<i>Centaurea subtilis</i>	FJ572057
<i>Centaurea tomentella</i>	DQ319172
<i>Centaurea tougourensis</i>	KJ666118
<i>Centaurea vankovii</i>	JF914073
<i>Centaurea virgata</i>	DQ319174
<i>Centaurea xylobasis</i>	DQ319176
<i>Centaurea zuccariniana</i>	KJ666128
<i>Klasea serratuloides</i>	AY826295
<i>Klasea serratuloides</i>	JX274517
<i>Psephellus pulcherrimus</i>	AY826317
<i>Rhapanticoides hajastana</i>	DQ310922
<i>Rhapanticoides hajastana</i>	AY826235
<i>Rhapanticoides iconiensis</i>	DQ310923
<i>Serratula coronata</i>	JX274515
<i>Serratula coronata</i>	AY826327
<i>Serratula coronata</i> subsp. <i>manshurica</i>	JX274514
<i>Zoegea leptaura</i>	AY826349

#### DNA extraction and PCR

The extraction of DNA from the silica gel dried plant material using QIAGEN Plant DNeasy Kit according to the manufacturer's protocols. The entire ITS region (ITS1, 5.8S, and ITS2) was amplified by Polymerase Chain Reaction (PCR). The amplification primers were ITS1 and ITS4 (WHITE *et al.*, 1990). The PCR program run with the following conditions: warm start at 94°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C 1 minute and 30 seconds and at the end 5 minutes for 72°C.

Purification of the PCR product was conducted with ExoSAP-IT (USB Corp., Cleveland, Ohio, USA). The amplified DNA segments were sequenced directly using BigDye Terminator Cycle Sequencing v.3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol.

Three computer programs were used for editing and alignment of the sequences. Sequences were assembled and edited by using Sequencher (Gene Codes Corporation), then, the sequences were pre-aligned using the multiple sequence alignment software Mega6 (TAMURA *et al.*, 2013), and sequences were aligned visually using Bioedit v7.0.5.3 (Hall, 1999). The ITS matrix has an aligned length of 620 bp.

#### Phylogenetic analyses

We prepared two different analyses, with the first analysis used to assess species monophyly with multiple samples; a second analysis was conducted with a reduced data set where multiple samples of the same species were removed when they proved monophyletic. Outgroups were also simplified. We used *C. austroanatolica* Hub.-Mor., *C. cariensiformis* Hub.-Mor., *Psephellus pulcherrimus* (Willd.) Wagenitz, *Rhaponticoides hajastana* (Tzvelev)



M.V. Agab. & Greuter, *R. iconiensis* (Hub.-Mor.) M.V. Agab. & Greuter, and *Zoegea leptaurea* L. as outgroups.

Parsimony analysis of the ITS datasets involved heuristic searches conducted with PAUP v4.0b10 (SWOFFORD, 2002) using TBR branch swapping with character states specified as unordered and unweighted. Under the heuristic search option, tree bisection-reconnection (TBR) branch-swapping, and 10 random-addition sequence replicates. Branch support was estimated with the bootstrap (BS) method, using 10000 replicates.

Bayesian inference of the dataset was calculated using MrBayes v3.2.4 (HUELSENBECK and RONQUIST, 2001; RONQUIST and HUELSENBECK, 2003; HUELSENBECK and RANNALA, 2004). The best available model of molecular evolution required for Bayesian estimations of phylogeny was selected using the software jModelTest (POSADA, 2008). The best fitting model was SYM G for our data set, based on a Bayesian Information Criterion weight of 0.80.

Bayesian inference analyses were initiated with random starting trees and were run for  $1 \times 10^7$  generations in two independent runs of four Metropolis-coupled or Markov chains. We saved one out of every 1000 generations, resulting in 10,000 sampled trees. Data from the first 2500 sampled trees were discarded as the “burn-in” period, after confirming that log-likelihood values had stabilized prior to the 2500th sampled tree. Internodes with posterior probabilities  $\geq 0.9$  were considered well supported.

#### *Ancestral character state reconstructions*

Parsimony character reconstruction was performed with Mesquite 3.01 version using a single most parsimonious tree. (MADDISON and MADDISON, 2010). Ancestral character states of seven characters have been used classification in *Centaurea* including stem length, inflorescence, capitula size, flower colour, appendage broad, spine and pappus length were reconstructed. These character states are known as diagnostic characteristics for classification of the genus *Centaurea* in both species and sectional levels. Two character states were differentiated for the ancestral character state reconstruction for each of the seven characters. The character matrix can be found in Table 3.

Stem length: Character states were differentiated as long stem (more than 1 m) and short stem (up to 1 m).

Inflorescence: Character states were differentiated as spike (spike, spike like, racemose) or not spike like.

Capitula size: Character states were differentiated as big capitula (more than 25 mm) and small capitula (up to 25 mm).

Flower colour: Character states were differentiated as purple (purple, reddish purple, bluish) and yellow (yellow, cream, and whitish).

Appendage broad: Character states were differentiated as large (totally concealing basal part of phillaries) and small (not totally concealing basal part of phillaries).

Spine length: Character states were differentiated as long (more than 6 mm) and short (up to 6 mm).

Pappus length: Character states were differentiated as long (more than 5 mm) and short (up to 5 mm).

Table 3. The character matrix for taxa studied. Stem length more than 1 m (0), up to 1 m (1); inflorescence spike, spike like, racemose (0), not spike like (1); capitula size more than 25 mm (0), up to 25 mm (1); flower colour purple, reddish purple, bluish (0), yellow, cream, whitish (1); appendage totally concealing basal part of phyllaries (0), not totally concealing basal part of phyllaries (1); spine length more than 6 mm (0), up to 6 mm (1); pappus length more than 5 mm (0), up to 5 mm (1); data missing (?)

Taxon name	Stem length	Inflorescences	Capitula size	Flower color	Apendage broad	Spine length	Pappus length
<i>C_aladaghensis</i>	0	0	0	0	1	0	0
<i>C_albonitens</i>	1	1	0	1	1	1	0
<i>C_amadanensis</i>	0	0	0	0	1	1	0
<i>C_amanicola</i>	0	0	0	0	0	0	0
<i>C_amanosensis</i>	1	1	1	1	1	1	1
<i>C_antalyensis</i>	?	?	?	?	?	?	?
<i>C_antitauri</i>	1	1	0	1	1	1	0
<i>C_arifolia</i>	0	0	0	0	0	1	0
<i>C_armena</i>	1	1	1	1	1	1	1
<i>C_aucheri</i>	1	1	1	1	1	1	0
<i>C_austroanatolica</i>	?	?	?	?	?	?	?
<i>C_behen</i>	0	1	1	1	1	1	0
<i>C_cariensisformis</i>	?	?	?	?	?	?	?
<i>C_cataonica</i>	0	0	0	0	0	0	0
<i>C_cataonica</i>	0	0	0	0	0	0	0
<i>C_cheirolepidioides</i>	1	1	1	1	1	1	1
<i>C_cheirolopha</i>	1	1	1	1	1	1	1
<i>C_cynarocephala</i>	0	0	0	0	0	0	0
<i>C_deflexa</i>	1	1	1	1	1	1	0
<i>C_derderiifolia</i>	1	1	1	1	1	1	0
<i>C_doddsii</i>	0	0	0	0	1	0	0
<i>C_drabifolioides</i>	1	1	1	1	1	1	1
<i>C_ensiformis</i>	1	1	1	1	0	0	0
<i>C_fenzlii</i>	0	0	0	1	0	0	0
<i>C_zuferii</i>	0	0	1	1	0	0	0
<i>C_gigantea</i>	0	0	0	0	1	0	0
<i>C_glastifolia</i>	0	1	1	1	0	1	0
<i>C_haradjianii</i>	0	0	0	0	1	0	0
<i>C_haussknechtii</i>	0	0	0	0	0	0	0
<i>C_hermanii</i>	1	1	1	1	1	1	0
<i>C_imperialis</i>	0	0	0	0	0	1	0
<i>C_kotschyi</i>	1	1	1	1	1	0	0
<i>C_lycopifolia</i>	1	1	1	1	1	1	1
<i>C_macrocephala</i>	1	1	0	1	0	1	0

Table 3.continued							
Taxon name	Stem length	Inflorescences	Capitula size	Flower color	Apendage broad	Spine length	Pappus length
<i>C_nemecii</i>	0	0	0	0	1	1	0
<i>C_odyssei</i>	1	1	1	1	1	0	1
<i>C_paphlagonica</i>	1	1	1	1	0	1	0
<i>C_polypodiifolia</i>	1	1	1	1	1	1	0
<i>C_pteroaula</i>	0	1	1	1	1	1	0
<i>C_ptosimopappa</i>	0	1	1	1	1	1	1
<i>C_ptosimopappoides</i>	1	1	1	1	1	1	1
<i>C_regia</i>	0	0	0	0	0	0	0
<i>C_rhizantha</i>	1	1	1	1	1	1	1
<i>C_rigida</i>	1	1	1	1	1	1	1
<i>C_saligna</i>	1	1	0	1	0	1	0
<i>C_sclerolepis</i>	0	0	0	0	0	0	0
<i>C_spectabilis</i>	1	1	1	1	1	1	0
<i>C_spicata</i>	0	0	0	0	1	0	0
<i>C_stapfiana</i>	1	1	1	1	1	1	0
<i>C_stevenii</i>	1	1	1	1	1	1	1
<i>C_xylobasis</i>	1	1	1	1	1	0	1
<i>C_yaltirikii_dumanii</i>	1	1	1	1	1	1	1
<i>C_yaltirikii_yaltirikii</i>	1	1	1	1	1	1	1
<i>C_ziganensis</i>	1	1	1	1	1	1	1
<i>P_pulcherrimus</i>	?	?	?	?	?	?	?
<i>R_hajastana</i>	?	?	?	?	?	?	?
<i>R_ioniensis</i>	?	?	?	?	?	?	?
<i>Z_leptaurea</i>	?	?	?	?	?	?	?

## RESULTS AND DISCUSSION

### Phylogeny

A consensus of the trees resulting from the parsimony analysis gave us a monophyletic group for in-group sections *Cheirolepis*, *Chartolepis*, *Cynaroides*, *Grossheimia*, *Microlophus*, *Paraphysis*, *Phaeopappus*, *Plumosipappus*, *Pseudophaeopappus*, *Pseudoseridia*, *Pteracantha*, *Ptosimopappus*, and *Rhizocalathium*. We highlight three main clades (Fig. 1).

Clade 1 (PP=0.9; BS=50) includes *C. amanosensis* and *C. cheirolopha* from sect. *Pseudoseridia*, *C. ptosimopappoides* from sect. *Ptosimopappus* and *C. zaferii* from sect. *Cynaroides*.

Clade 2 (PP=1; BS=83) includes *C. cheirolepidioides*, *C. hermannii*, *C. yaltirikii* subsp. *dumanii*, *C. yaltirikii* subsp. *yaltirikii*, *C. drabifolioides*, *C. lycopifolia* and *C. ziganensis* from section *Pseudoseridia* and *C. odyssei* and *C. xylobasis* from sect. *Pteracantha*, *C. kotschyi*, *C. derderiifolia* and *C. deflexa* from sect. *Cheirolepis*, *C. paphlagonica* from sect. *Plumosipappus* and *C. ensiformis* which was not placed into a certain section before.



Clade 3 (PP=0.99) covers *C. aladaghensis*, *C. amanicola*, *C. cataonica*, *C. cynarocephala*, *C. haradjiannii*, *C. haussknechtii*, *C. tomentella*, *C. doddsii*, *C. spicata*, *C. arifolia*, *C. fenzlii*, *C. gigantea*, *C. kurdica*, *C. sclerolepis*, *C. regia*, *C. imperialis* from sect. *Cynaroides*, *C. behen*, *C. rigida*, and *C. polypodiifolia* from sect. *Microlophus*, *C. aucheri* and *C. albonitens* from sect. *Phaeopappus*, *C. amadenensis* and *C. nemecii* from section *Paraphysis*, *C. spectabilis* and *C. stapfiana* from sect. *Phaeopappus*, and *C. macrocephala* from sect. *Grossheimia*, *C. stevenii* from sect. *Pseudoseridia*, *C. rhizantha* and *C. armena* from sect. *Rhizocalathium*, and *C. antitauri* from sect. *Pseudophaeopappus*, *C. pterocaula* and *C. glastifolia* from sect. *Chartolepis* and *C. saligna* from sect. *Cheirolepis*.

#### Ancestral character state reconstructions

The parsimony analysis with reduced taxa sampling which gave us a tree 8,202 equally most parsimonious trees. There are three main clades in the tree; Clade a, Clade b and Clade c (Fig. 2).

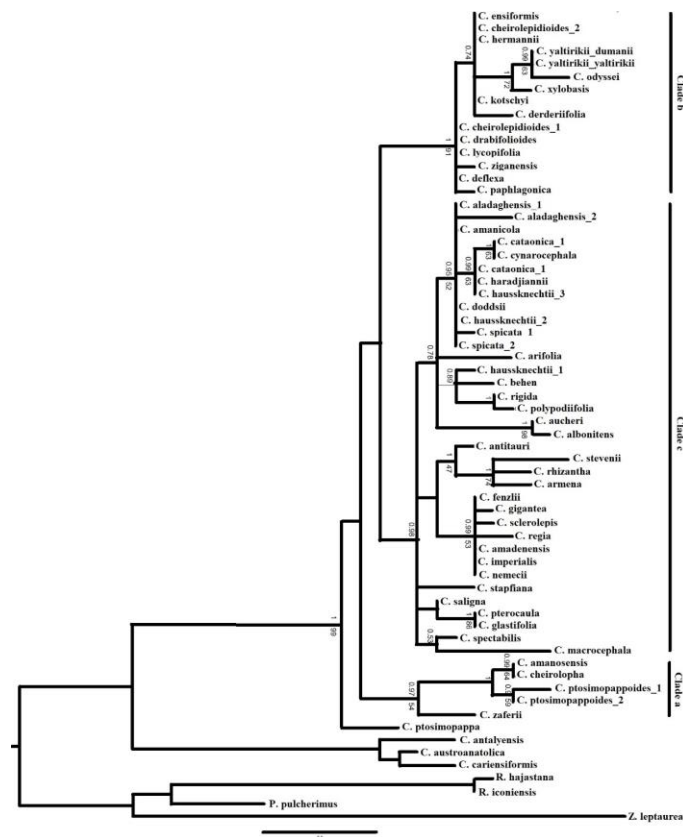


Figure 2. Consensus tree of Parsimony analysis with reduced taxa sampling.

Clade a (PP=0.97; BS=54) contains *C. amanosensis* and *C. cheirolopha* from sect. *Pseudoseridia*, *C. ptosimopappoides* from sect. *Ptosimopappus* and *C. zaferii* from sect. *Cynaroides*. Clade b (PP=1; BS=91) includes *C. cheirolepidioides*, *C. hermanii*, *C. yaltirikii* subsp. *dumanii*, *C. yaltirikii* subsp. *yaltirikii*, *C. drabifolioides*, *C. lycopifolia* and *C. ziganensis* from section *Pseudoseridia* and *C. odyssei* and *C. xylobasis* from sect. *Pteracantha*, *C. kotschyi*, *C. derderiifolia* and *C. deflexa* from sect. *Cheirolepis*, *C. paphlagonica* from sect. *Plumosipappus* and *C. ensiformis* which is not placed into a certain section. Clade c (PP=0.98) *C. aladaghensis*, *C. amanicola*, *C. cataonica*, *C. cynarocephala*, *C. haradjiannii*, *C. haussknechtii*, *C. doddsii*, *C. spicata*, *C. arifolia*, *C. fenzlii*, *C. gigantea*, *C. sclerolepis*, *C. regia*, *C. imperialis* from sect. *Cynaroides*, *C. behen*, *C. rigida*, and *C. polypodiifolia* from sect. *Microlophus*, *C. aucheri* and *C. albonitens* from sect. *Phaeopappus*, *C. amadenensis* and *C. nemecii* from section *Paraphysis*, *C. spectabilis* and *C. stapfiana* from sect. *Phaeopappus*, and *C. macrocephala* from sect. *Grossheimia*, *C. stevenii* from sect. *Pseudoseridia*, *C. rhizantha* and *C. armena* from sect. *Rhizocalathium*, and *C. antitauri* from sect. *Pseudophaeopappus*, *C. pterocaula* and *C. glastifolia* from sect. *Chartolepis* and *C. saligna* from sect. *Cheirolepis*.

Ancestral character states of seven characters frequently used in the classification in *Centaurea* including stem length, inflorescence, capitula size, flower colour, appendage broad, spine and pappus length were reconstructed.

Stem length: Consistency Index is 0.14 for this character and a short stemmed state has arisen several times (Fig. 3). The ancestral state is long-stemmed, changing to short-stemmed in Clade a (except *C. zaferii*) and Clade b. Clade c members are long-stemmed apart from *C. stapfiana*, *C. macrocephala*, *C. spectabilis*, *C. saligna*, *C. antitauri*, *C. armena*, *C. stevenii*, *C. rhizantha*, *C. albonitens*, *C. aucheri*, *C. polypodiifolia*, *C. rigida* which are short-stemmed.

Inflorescence: Consistency Index is 0.2 for this character with the ancestral state being non spike-like inflorescences; this character has changed five times on our tree (Fig. 4). Spike like inflorescences appear on *C. zaferii* in Clade a, and also on *C. regia*, *C. sclerolepis*, *C. fenzlii*, *C. gigantea*, *C. imperialis*, *C. arifolia*, *C. haussknechtii*, *C. doddsii*, *C. aladaghensis*, *C. spicata*, *C. amanicola*, *C. haussknechtii*, *C. haradjiannii*, *C. cataonica*, *C. cynarocephala*, *C. amadenensis*, *C. nemecii* in Clade c.

Capitula size: Consistency Index is 0.125 for this character, with the ancestral state being small capitula, changing to large capitula seven times (Supplement 1). The ancestral state of small capitula is found throughout Clade a and Clade b. The large capitula character state has been seen in Clade c and changes again on *C. stapfiana*, *C. spectabilis*, *C. glastifolia*, *C. pterocaula*, *C. armena*, *C. stevenii*, *C. rhizantha*, *C. aucheri*, *C. behen*, *C. polypodiifolia*, *C. rigida*.

Flower colour: Consistency Index is 0.2 for this character and the ancestor was yellow-flowered, with changes to purple occurring five times (Supplement 2). Purple flower colour is found on *C. regia*, *C. sclerolepis*, *C. gigantea*, *C. imperialis*, *C. arifolia*, *C. haussknechtii*, *C. doddsii*, *C. aladaghensis*, *C. spicata*, *C. amanicola*, *C. haussknechtii*, *C. haradjiannii*, *C. cataonica*, *C. cynarocephala*, *C. amadenensis*, *C. nemecii* in Clade c.

Appendage broad: Consistency Index is 0.06 for this character and appendage character states change 16 times (Supplement 3). The ancestral state is a small appendage, with the character changing several times in each clade.

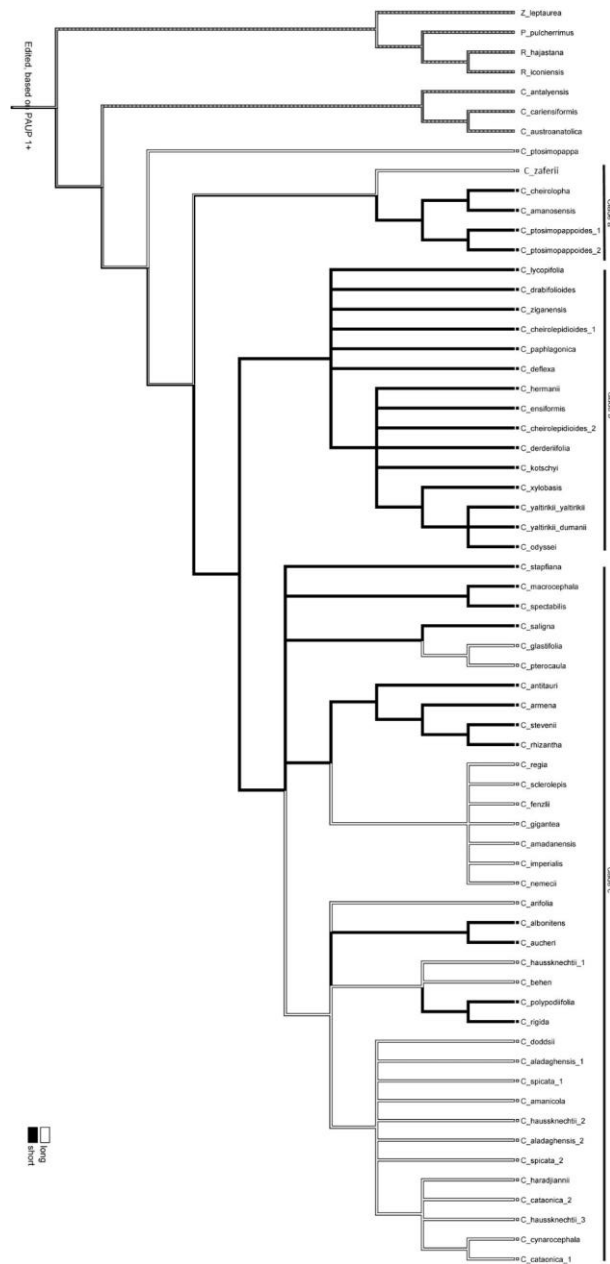


Figure 3. Parsimony ancestral character reconstruction: Consistency Index is 0.14 for stem length.

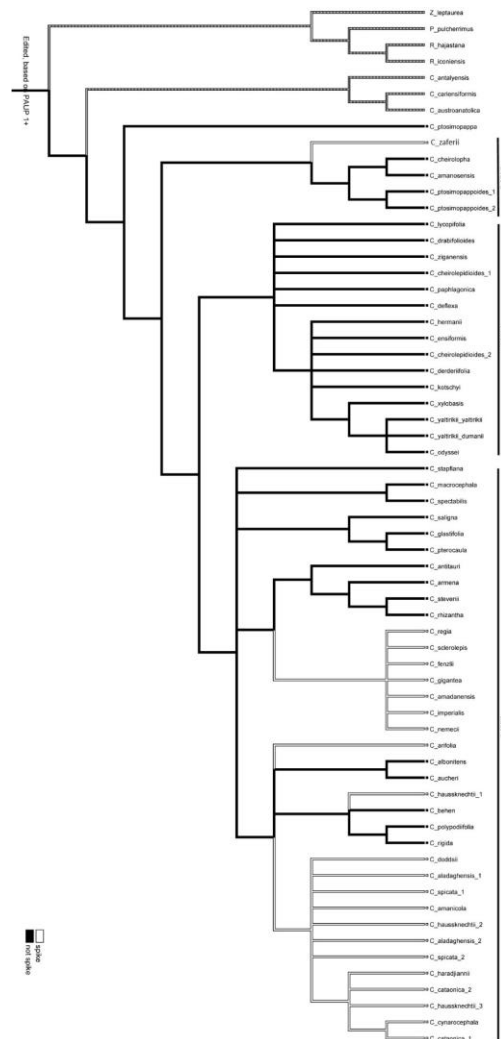


Figure 4. Parsimony ancestral character reconstruction: Consistency Index is 0.2 for inflorescence.

Spine length: Consistency Index is 0.09 for this character and spine length character states also change 11 times (Supplement 4). The ancestral state is short spines, with the long spine character state seen in *C. zaferii* in Clade a, *C. xylobasis*, *C. odyssei*, *C. ensiformis* and *C. kotschy* in Clade b. *C. regia*, *C. sclerolepis*, *C. fenclii*, *C. gigantea*, *C. imperialis*, *C. haussknechtii*, *C. doddsii*, *C. aladaghensis*, *C. spicata*, *C. amanicola*, *C. haussknechtii*, *C. haradjiannii*, *C. cataonica*, *C. cynarocephala*, *C. amadenensis*, *C. nemecii* have long spines in Clade c.



Pappus length: Consistency Index is 0.11 for this character and the short pappus character state is ancestral, changing nine times (Supplement 5). The short pappus character state is seen in Clade a (except *C. zaferii*). *C. hermannii*, *C. ensiformis*, *C. derderiifolia*, *C. kotschyi*, *C. deflexa* and *C. paphlagonica* have long pappus in Clade b. Clade c members have long pappus except *C. rigida*, *C. stevenii*, *C. armena* and *C. rhizantha*.

The biogeographic approach seems of the highest interest for the phylogeny of *Centaurea* (HILPOLD *et al.*, 2014b). However, the phylogeny derived from ITS data provides a reasonably resolved and reasonably supported phylogenetic framework for the genus. Ancestral character state reconstructions showed important characters to the grouping of members of genus *Centaurea*.

#### *Sections Psudoseridia and Ptosimopappa*

Section *Ptosimopappus* has two species *C. ptosimopappa* and *C. ptosimopappoides* (WAGENITZ, 1975). A previous phylogenetic study which included these two taxa indicated that the section should be monotypic and include only *C. ptosimopappa* (GARCIA-JACAS *et al.*, 2006). Our results confirm that *C. ptosimopappa* should be placed in the section *Ptosimopappus*. The ITS region of *C. amanosensis* is sequenced for the first time here. The results also show *C. ptosimopappoides* should be placed in section *Pseudoseridia* with *C. cheirolopha* and *C. amanosensis*. These three taxa grouped with strong support (PP=1; BS=90).

Having a short stem, non-spike inflorescence, small capitulum, yellow flower, narrow and short spiny appendage and short pappus are common character states for *C. amanosensis*, *C. cheirolopha* and *C. ptosimopappoides*.

#### *Sections Pseudoseridia (pro parte), Pteracantha, Cheirolepis, and Plumosipappus*

It has been proposed to combine sections *Pseudoseridia*, *Pteracantha*, *Cheirolepis*, and *Plumosipappus* (HILPOLD *et al.*, 2014a). Our results also confirm this proposal because the sections are grouped in one clade with PP=1 and BS=83 values, respectively.

This group of species have a short stem, non-spike inflorescence, small capitulum and yellow flower. They generally have narrow appendage except for broad appendages of *C. ensiformis* and *C. paphlagonica*. Spine and pappus vary and can be short or long.

#### *Sections Pseudoseridia, Rhizocalathium and Pseudophaeopappus*

Previous studies on *C. armena* and *C. rhizantha* from section *Rhizocalathium* and *C. stevenii* from section *Pseudoseridia* showed that they represent a natural group with strong support and they need to be placed in the section *Rhizocalathium* (GARCIA-JACAS *et al.*, 2006, HILPOLD *et al.*, 2014a). Our results contain the first ITS sequences for the taxa, *C. drabifolioides*, *C. hermannii*, *C. yaltirikii* subsp. *dumanii*, *C. yaltirikii* subsp. *yaltirikii*, and *C. ziganensis*, also confirm this relationship with PP=0.99 and BS=73. The section *Pseudophaeopappus* and its only representative *C. antitauri* were reported as problematic or need confirmation as the affinities and relationships were unclear (GARCIA-JACAS *et al.*, 2006). Our study shows *C. antitauri* has affinity with *C. armena*, *C. rhizantha* and *C. stevenii* with PP=0.99 and BS=47 and therefore must be placed in this section.

The character suite for this group of species includes short stem, non-spike inflorescence, small capitulum, yellow flower, narrow and short spiny appendage, and short pappus is common character states for the group except big capitulum and a long pappus of the *C. antitauri*.

#### *Sections Cynaroides, Microlophus, Paraphysis, and Phaeopappus*

The species of these four sections are placed into five clades: the first comprises *C. aladaghensis*, *C. amanicola*, *C. cataonica*, *C. cynarocephala*, *C. haradjianii*, *C. haussknechtii*, *C. tomentella*, *C. doddsii*, *C. spicata* from section *Cynaroides* (PP=0.94; BS=51), the second included only *C. arifolia* from section *Cynaroides* (PP=1; BS=95), the third covered *C. fenzlii*, *C. gigantea*, *C. kurdica*, *C. sclerolepis*, *C. regia*, and *C. imperialis* from section *Cynaroides* and *C. amadenensis* and *C. nemecii* from section *Paraphysis* (PP=0.94; BS=51), the fourth covered *C. haussknechtii* from section *Cynaroides* and *C. behen*, *C. rigida* and *C. ploypodiiifolia* from section *Microlophus* (PP=0.7; BS=38) and fifth clade *C. aucheri* and *C. albonitens* from section *Phaeopappus* (PP=1; BS=97). These five clades combine into a larger clade with PP=0.9 support. These results support the idea of combining section *Cynaroides* and *Paraphysis* (GARCIA-JACAS *et al.*, 2006; HILPOLD *et al.*, 2014a; NEGARESH and RAHIMINEJAD, 2018). ITS region of *C. amanicola*, *C. amanosensis*, *C. arifolia*, *C. cataonica*, *C. cynarocephala*, *C. doddsii*, *C. haradjianii*, *C. haussknechtii*, *C. regia*, and *C. sclerolepis* is sequenced here the first time. Our results show section *Cynaroides* is related to the other two sections, *Microlophus* and *Phaeopappus*, but neither support value nor morphological characteristics support any change in sectional classification.

Having a long stem, spike inflorescence, big capitulum, purple flower, long spine (except short spined *C. arifolia*), and long pappus are common character states for sections *Cynaroides* and *Paraphysis*. The broad appendage is important for delimitation of species but it is not useful for the sectional classification.

#### *C. zaferii*

*C. zaferii* is distributed only in Amanos Mountains, Province Hatay in Turkey (BONA and GHAZAL ASSWAD, 2014). The sectional classification of the species into section *Cynaroides* is based only on morphological examination of the type specimens (WAGENITZ, 1975). Negaresh and Rahiminejad (2018) kept *C. zaferii* in section *Cynaroides*. The ITS sequence of *C. zaferii* and *C. amanosensis* are used here the first time. *C. zaferii* is placed apart from section *Cynaroides* and closes with *C. amanosensis*, *C. cheirolopha* (section *Pseudoseridia*) and *C. ptosimopappoides* (Section *Ptosimopappa*) (PP=0.9; BS=51) in the present study; we suggest *C. zaferii* should be placed in a separate section.

Long stem, spike inflorescence, big capitulum with yellow flowers and broad appendage with a short spine are defining characters for *C. zaferii* and potentially a separate section.

#### *C. ensiformis*

*C. ensiformis* is endemic to Turkey, with sectional placement not clear (WAGENITZ, 1975; GARCIA-JACAS *et al.*, 2006). The previous results showed a relationship with the sections

*Cheirolepis* and *Pseudoseridia* (GARCIA-JACAS *et al.*, 2006) and our results also confirm this situation, so it is possible to add the taxon in this group.

The new ITS sequences for 26 taxa from 48 locations are given in this study. Sequences of 17 taxa (*C. amanicola*, *C. amanosensis*, *C. arifolia*, *C. cataonica*, *C. cynarocephala*, *C. doddsii*, *C. drabifolioides*, *C. zaferii*, *C. haradjiannii*, *C. haussknechtii*, *C. hermannii*, *C. regia*, *C. sclerolepis*, *C. spicata*, *C. yaltirikii* subsp. *dumanii*, *C. yaltirikii* subsp. *yaltirikii*, and *C. ziganensis*) are given here the first time. The phylogeny derived from ITS data provides a reasonably resolved and reasonably supported phylogenetic framework for the genus.

#### CONCLUSION

Although some problems remain for sectional level classification, our results give new insights for into character combinations for the sections and place some local endemic taxa for the first time using molecular data.

The results of the phylogenetic analyses and ancestral character reconstructions presented here indicate that stem length, inflorescence, capitula size, flower colour, appendage width, spine length and pappus length have highly homoplastic character states. However, there are reliable character combinations to be found for the classification of *Centaurea*. Combining these characters in light of the molecular analysis can give us a well-defined new section classification.

*Centaurea* is one of the largest and taxonomically most difficult genera of the Asteraceae. We produce some character combinations for the reliable subgeneric classification of *Centaurea*. This is a step toward making a new and clear sectional classification.

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