

Two additions to the *Jacea-Lepteranthus* complex: parallel adaptation in the enigmatic species *Centaurea subtilis* and *C. exarata*

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Abstract

Centaurea subtilis from south east Italy and *C. exarata* from south west Iberia were classified in the *Acrolophus-Phalolepis* group and therein in section *Maculosae*. A molecular survey based on ITS sequence data indicates that both species should rather be placed in the *Jacea-Lepteranthus* group instead. This placement is consistent with the chromosome number of the two species, which is $x = 11$ like the rest of species of the *Jacea-Lepteranthus* group, and differs from the $x = 9$ of the other taxa included in sect. *Maculosae*. These results confirm previous suggestions on the unnaturality of sect. *Maculosae*. *Centaurea exarata* and *C. subtilis* are quite different from the other species of *Jacea-Lepteranthus* in some striking morphological characters, which we hypothesize to be the result of parallel adaptation to dryer climates. The lack of competitors for pollination might be a good explanation for the partial or even total loss of showy flowers in these two species.

Key words: *Acrolophus*; Compositae; ITS; *Jacea-Lepteranthus*; karyology; *Maculosae*; morphology; showy flowers.

Resumen

Dos adiciones al complejo *Jacea-Lepteranthus*: adaptaciones paralelas en las enigmáticas especies *Centaurea subtilis* y *C. exarata*. - *Centaurea subtilis* del sureste de Italia y *C. exarata* del suroeste de la Península Ibérica fueron clasificadas anteriormente en el grupo *Acrolophus-Phalolepis* y dentro de él en la sect. *Maculosae*. Una revisión molecular basada en secuencias de la región ITS indica que ambas deberían clasificarse en el grupo *Jacea-Lepteranthus*. Este cambio es coherente con el número cromosómico de las dos especies, que tienen $x = 11$ como el resto de las especies del grupo *Jacea-Lepteranthus* y no $x = 9$ como las especies del grupo *Acrolophus-Phalolepis*. Estos resultados confirman advertencias anteriores sobre el carácter artificial de la sect. *Maculosae*. *Centaurea exarata* y *C. subtilis* son bastante diferentes de las otras especies de *Jacea-Lepteranthus* en algunos caracteres morfológicos importantes, resultado, según nuestra hipótesis, de adaptaciones a un clima más árido. La reducción o total pérdida de las flores estériles radiantes podría ser una adaptación a la falta de especies competidoras en la polinización.

Palabras clave: *Acrolophus*; cariológia; *Compositae*; flores estériles; ITS; *Jacea-Lepteranthus*; *Maculosae*; morfología.

Resum

Dues addicions al complex *Jacea-Lepteranthus*: adaptacions paral·leles en les enigmàtiques espècies *Centaurea subtilis* i *C. exarata*. - *Centaurea subtilis* del sud-est d'Itàlia i *C. exarata* del sud-oest de la Península Ibèrica han estat classificades en el grup *Acrolophus-Phalolepis* i, dins d'aquest grup, a la secció *Maculosae*. Una revisió molecular basada en seqüències de la regió ITS indica que ambdues espècies haurien de classificar-se en el grup *Jacea-Lepteranthus*. Aquest canvi és coherent amb el nombre cromosòmic de les dues espècies, que tenen $x = 11$ com la resta de les espècies del grup *Jacea-Lepteranthus* i no $x = 9$ com les espècies del grup *Acrolophus-Phalolepis*. Aquests resultats confirmen advertències anteriors sobre el caràcter artificial de la sect. *Maculosae*. *Centaurea exarata* i *C. subtilis* són bastant diferents de les altres espècies de *Jacea-Lepteranthus* en alguns caràcters morfològics importants, degut, segons la nostra hipòtesi, a adaptacions a un clima més àrid. La reducció o la total pèrdua de les flors estèrils radiants podria ser una adaptació a la falta d'espècies competidores en la pol·linització.

Paraules clau: *Acrolophus*; cariológia; *Compositae*; flors estèrils; ITS; *Jacea-Lepteranthus*; *Maculosae*; morfologia.

INTRODUCTION

Centaurea L. is one of the largest genera of the Asteraceae with ca. 250 species (Susanna & Garcia-Jacas, 2007). Only thanks to recent studies (Garcia-Jacas *et al.*, 2000; Garcia-Jacas *et al.*, 2006), which widely modified former classifications (Dostál, 1976; Wagenitz, 1955), a natural delineation of the genus and an outline of the classification were established. Several species and groups formerly considered as *Centaurea* are now classified as independent genera, and *Centaurea* is reduced to three groups. The first two are *Acrocentron* and *Cyanus*, and conform well delimited subgenera (Susanna & Garcia-Jacas, 2007). The third group, which encompasses the remaining of the genus, is a wide group of species of manifold morphological characters, the *Centaurea jacea* group. A comprehensive molecular survey of the *Jacea* group revealed two large complexes: a first group of taxa mostly with spiny involucre appendages (Eastern and Western Mediterranean clades, cf. Garcia-Jacas *et al.*, 2006), and a second extense group comprising sections *Acrolophus* (Cass.) DC., *Phalolepis* (Cass.) DC., *Willkommia* G. Blanca, *Jacea* (Mill.) DC., and *Lepteranthus* (Neck.) DC. The first three sections build up the monophyletic *Acrolophus-Phalolepis-Willkommia* group clearly separated from the *Jacea-Lepteranthus* group. These two diverse groups await further classification, although some approaches have been already done, a difficult task because of intense hybridization (Suárez-Santiago *et al.*, 2007a, 2007b).

Centaurea subtilis was described by Bertoloni (1853) and it has been traditionally placed in sect. *Acrolophus*, more precisely into the direct proximity of *C. stoebe* L. or *C. paniculata* L. Fiori (1927) included it in sect. *Acrolophus* as part of his broad concept of *C. paniculata* together with many other taxa, which are nowadays considered either independent species or subspecies of *C. aplolepa* Moretti. Dostál (1976) placed *C. subtilis* into subg. *Acrolophus* (Cass.) Dobrocz. sect. *Maculosae* Dostál, together with *C. calvescens* Pančić, *C. corymbosa* Pourr., *C. exarata* Coss., *C. filiformis* Viv., *C. glaberrima* Tausch, *C. peucedaniifolia* Boiss. & Orph., *C. reichenbachii* DC., *C. triniifolia* Heuff. and the whole *C. stoebe* complex (including former *C. maculosa* Lam. and *C. rhenana* Boreau, both with several subspecies, recently partly recognized as

different species, and *C. coziensis* Nyár.). Pignatti (1982) omitted to place it in any taxonomic group below the genus level.

Centaurea subtilis is a dwarf shrub 10–30 cm high with little branched or simple stems. The whole plant is white tomentose. Lower leaves are 3–5 cm long, 1-pinnatisect into linear laciniae (1×10 mm) ending in a small mucro; middle leaves are smaller, often trifid, while the uppermost are often simple. The ovoid capitula are solitary and distant from the leaves, and the involucre is 7–11 mm wide. The bracts are green, with outstanding yellowish nerves, and pubescent on the margin. The brownish appendages have an appressed apical spine of 0.5–1 mm, and lateral fimbriae of 0.5 mm. The florets are of about 17 mm length, purple to wine red, the outer ones are sterile and showy (Fig. 1A). The achenes are ca. 3 mm long, with a pappus 1–2 mm long, 1/3–1/2 as long as the achene, purple in our collection. The species is a narrow endemic from south-eastern Italy and grows in the Puglia and Basilicata regions (Fig. 2; Conti *et al.*, 2005). It occurs only in the southern part of the Gargano peninsula, near Monte San Angelo and near Matera (Pignatti, 1982). The species gives name to two phytosociological societies, *Centaureetum subtilis*, which settles in dry, sunny limestone rocks at low altitudes (Bianco *et al.*, 1988) and *Centaureo subtilis-Thymetum capitati*, which grows in garrigues on shallow, calcareous soils (Terzi & D'Amico, 2006).

Centaurea exarata was described by Cosson (1851) and it was placed by Dostál (1976), as mentioned above, in subg. *Acrolophus* sect. *Maculosae*, mainly because of the similarity of the involucre appendages to those of other species from this group.

Centaurea exarata is a 30–60 cm high erect perennial. The stem is simple or sparingly branched. The whole plant is arachnoid-pubescent. Leaves are undivided, the lower ones oblong-lanceolate and the upper ones linear-lanceolate and semi-amplexicaul or auriculate. The capitula are solitary with a bract-like leaf on their base. The involucre is ca. 14–18 mm long, ovoid. The bracts are adpressed with outstanding nerves and narrowly triangular, reddish-brown, erect, long fimbriate appendages, without apical spine. The florets are purple, all fertile and non-radiant (Fig. 1B). The achenes are 3–4 mm long, with a pappus 1.2–2 mm long, half as long as the achene. Like *C. subtilis*, it is also a



Figure 1. Detail of capitula. (A) *Centaurea subtilis*; (B) *C. exarata*.

narrow endemic and occurs along the Atlantic coast of occidental Andalusia and central Portugal (Fig. 2; Coutinho, 1939; Franco, 1984; Talavera, 1987). It gives the name to the association *Centaureo exaratae-Armerietum gaditanae* (Allier & Bresset, 1977). Its ecology is quite different from the preferences of *C. subtilis*: *Centaurea exarata* inhabits there sandy beaches, dunes and sandy depressions which get frequently inundated and dry out later.

First doubts on the systematic position of these two species came from their base chromosome number, $x = 11$ in both cases (Damboldt & Matthäs, 1975; Tornadore & Marcucci, 1988; Valdés-Bermejo, 1980), instead of $x = 9$ found in members of the *Acrolophus-Phalolepis* group. Damboldt & Matthäs (1975) already pointed out that *C. subtilis* had no close relationship with the *C. stoebe* complex due to the difference in the chromosome number and also due to morphological differences. The doubts were confirmed for *C. exarata* by Garcia-Jacas *et al.* (2006) through molecular data, where it was shown that this species is placed within the clade *Jacea-Lepteranthus*. The phylogenetic position of *C. subtilis* was still unknown and further studies were still wanting, which led to the present work.

With the aim of exploring and confirming the phylogenetic and systematic relationships of *C. exarata* and *C. subtilis*, we carried out new chromosome counts, a phylogenetic analysis of molecular data (ITS sequences) and a morphological survey.

MATERIALS AND METHODS

Plant material

During summer 2008 vouchers, seeds and leaves of one population of *C. subtilis* were collected and stored in silica gel. Vouchers are deposited in Herbaria BC and BOZ.

Chromosome counts

To count the chromosome number we used the squash technique on somatic metaphases of root meristems from germinating seeds collected in the wild.

After a pretreatment with 0.002 M 8-hydroxyquinoline at 4°C for 8 h, the material was fixed with Carnoy at low temperatures for 24 h. Then it was hydrolysed with 5N HCl at room temperature for



Figure 2. Distribution of *Centaurea subtilis* (pointed area) and *Centaurea exarata* (area with stripes).

1 h. The staining was done with 1% acetic orcein at room temperature for 2 to 12 hours, and the root tips were mounted in 45% acetic acid. Five metaphase plates from different individuals were examined. Preparations were made permanent by freezing with CO₂, dehydrating in ethanol and mounting in Canada balsam. Digital photographs were taken with an Olympus 3030 camera, mounted on an Olympus microscope U-TV1 X.

Molecular phylogeny study

For the molecular phylogeny, we selected several species from the western and widely-distributed clades showed in Garcia-Jacas *et al.* (2006): sections *Acrolophus*, *Jacea* (incl. *Lepteranthus*), *Phalolepis*, and *Willkommia*. All the sequences were taken from that previous study, with the only exception of *C. subtilis*, which has been generated for the present study, and *C. aeolica* Lojac., which has been recovered from the GenBank (Table 1). Outgroup species (*C. mollis* Waldst. & Kit. and *C. napulifera* Rochel) were chosen among the sister subgenus of the *Jacea* group, *Centaurea* subg. *Cyanus*, according to Garcia-Jacas *et al.* (2001).

DNA extraction, amplification and sequencing strategies

Total genomic DNA was extracted following the CTAB method of Doyle & Doyle (1987) as modified by Cullings (1992) from silica-gel-dried leaves collected in the field. Double-stranded DNA of the ITS region was amplified using the 17SE, forward, and the 26SE, reverse, primers (Sun *et al.*, 1994). The PCR was executed with the following conditions: 2 min denaturing at 94°C, followed by 30 cycles of 94°C denaturing for 1 min 30 s, 57°C annealing for 2 min and 72°C extension for 3 min, with an additional 15 min at 72°C. Double-stranded PCR products were purified with QIAquick® Purification Kit (Qiagen Inc., Valencia, CA, USA) and sequenced with the primers 17SE as forward primer, and 26SE as reverse. Direct sequencing of the amplified DNA segments was performed with a “Big Dye® Terminator v3.1 kit” (Applied Biosystems, Foster City, CA, USA), following the protocol recommended by the manufacturer. Nucleotide sequencing was carried out at the “Serveis Científico-Tècnics” of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (Applied Biosystems).

Table 1. Origin of materials, herbaria where vouchers are deposited and GenBank accession numbers (the new sequence is boldfaced).

Species	Range	Voucher	ITS accession
<i>Centaurea aggregata</i> Fisch. & C. A. Mey. ex DC.	Caucasus, Iran, Turkey (weed)	Turkey, Adana: Ala Dağ above Dağdibi, 2000 m, 03.08.2002, <i>Ertuğrul, Garcia-Jacas, Susanna 2305 & Uysal</i> (BC)	DQ319077
<i>Centaurea alba</i> L. subsp. <i>costae</i> (Willk.) Dostál	Iberian Peninsula endemic	Spain, Huesca: Peña de Oroel, 15.07.1947, <i>Fernández-Galiano & Rivas Goday 23733</i> (GDA)	AM114325
<i>Centaurea alba</i> L. subsp. <i>latronum</i> (Pau) Dostál	Iberian Peninsula endemic	Spain, Ávila: La Adrada, 27.07.1982, <i>Sánchez-Mata & Cantó 24946</i> (GDAC)	AM114326
<i>Centaurea aeolica</i> Guss. ex DC.	Italy, endemic	GenBank	AM117057
<i>Centaurea avilae</i> Pau	Iberian Peninsula endemic	Spain, Ávila: Sierra de Gredos, 30.07.1979, <i>Blanca 6087</i> (GDAC)	AM114309
<i>Centaurea aziziana</i> Rech. f.	Caucasus endemic	Iran, Azarbayjan-e-Sharghi: between Tatar and Golfā, 85 km from Golfā, 07.08.1996, <i>Garcia-Jacas, Mozaffarian, Susanna 1680 & Vallès</i> (BC)	DQ319089
<i>Centaurea boissieri</i> DC. subsp. <i>boissieri</i>	Iberian Peninsula endemic	Spain, Granada: Sierra de Cázulas, 08.06.1979, <i>Blanca 6597</i> (GDAC)	AM114278
<i>Centaurea cadmea</i> Boiss.	Turkey endemic	Turkey, Burdur: 4 km from Burdur on the road to Sparta, mountains above Burdur, 1200 m, 28.07.2002, <i>Ertuğrul, Garcia-Jacas, Susanna 2249 & Uysal</i> (BC)	DQ319094
<i>Centaurea calolepis</i> Boiss.	Turkey endemic	Turkey, Burdur-Muğla: Dirimli mountain pass, 1600 m, 29.07.2002, <i>Ertuğrul, Garcia-Jacas, Susanna 2254 & Uysal</i> (BC)	DQ319095
<i>Centaurea cariensis</i> Boiss.	Turkey endemic	Turkey, Antalya: 40 km from Elmali on the road to Korkuteli, N slopes of the Karamanbeli mountain pass, 1400 m, 30.07.2002, <i>Ertuğrul, Garcia-Jacas, Susanna 2258B & Uysal</i> (BC)	DQ319097
<i>Centaurea carratracensis</i> Lange	Iberian Peninsula endemic	Spain, Málaga: Carratraca, Sierra de Aguas, 04.07.1998, <i>Blanca 42802</i> (GDAC)	AM114302
<i>Centaurea corymbosa</i> Pourr.	S France endemic	France, Narbonne: La Clappe, 1995, <i>M. Riba s. n.</i> (BC)	DQ319103
<i>Centaurea debdouensis</i> Breitw. & Podlech	Morocco endemic	Morocco, Debdou: Gaada de Debdou, 18.06.1954, <i>Pasquier & Ch. Rungs s. n.</i> (MPU)	AM114317
<i>Centaurea deusta</i> Ten.	Italy endemic	Italy, Calabria: Crotona, Torrente Matassa near Caccuri, 360 m, <i>Vogt 15531</i> , Berlin Botanical Garden, Index Seminum 1997	DQ319107
<i>Centaurea diffusa</i> Lam.	Widespread (weed)	Armenia, Talin: between vil. Pokr Arthik and Bagravan, 26.08.1995, <i>Fajvush, Gabrielyan, Garcia-Jacas, Guara, Hovhannisyanyan, Susanna 1589, Tamanyan & Vallès</i> (BC)	DQ319108
<i>Centaurea donetzica</i> Klokov	Ukraine endemic	Ukraine, Donetskaya: Krasny Liman, 12.08.2002, <i>Romashchenko s. n.</i> (BC)	DQ319110
<i>Centaurea exarata</i> Coss.	Iberian Peninsula endemic	Spain, Huelva: road A-983, Almonte to Matalascañas km 25, 09.07.1999, <i>Roché & Susanna 1909</i> (BC)	DQ319113

Table 1. (Cont.)

Species	Range	Voucher	ITS accession
<i>Centaurea gadorensis</i> Blanca	Iberian Peninsula endemic	Spain, Almería: Sierra de Gádor, Pico La Estrella, 1730 m, 29.07.1996, <i>Martínez Lirola & Salinas 44171</i> (GDAC)	AM114298
<i>Centaurea hyssopifolia</i> Vahl	Iberian Peninsula endemic	Spain, Toledo: near Ontígola, 500 m, 22.06.1996, <i>García-Jacas, Susanna 1600 & Vilatersana</i> (BC)	DQ319119
<i>Centaurea inexpectata</i> Wagenitz	Turkey endemic	Turkey, Antalya: Gevne valley, high of village Küçükklü, 1750 m, 30.06.2004, <i>Uysal 598</i> (KNYA)	DQ319122
<i>Centaurea jacea</i> subsp. <i>vinyalsii</i> (Sennen) O. Bolòs, Vígo & J. M. Panareda	Iberian Peninsula endemic	Spain, Barcelona: La Garrotxa, Pass of Bracons, 1100 m, 04.11.1995, <i>García-Jacas & Susanna 1593</i> (BC)	DQ319125
<i>Centaurea jaennensis</i> Degen & Debeaux	Iberian Peninsula endemic	Spain, Jaén: Pozo Alcón, La Bolera dam, 19.06.1978, <i>Blanca & Varo 6724</i> (GDAC)	AM114287
<i>Centaurea linifolia</i> L.	Iberian Peninsula endemic	Spain, Tarragona: St. Magí de Brufaganya, 10.06.1995, <i>Vilatersana 3</i> (BC)	DQ319129
<i>Centaurea monticola</i> Boiss. ex DC.	Iberian Peninsula endemic	Spain, Granada: Pantano del Cubillas, 06.06.1977, <i>Blanca 6750</i> (GDAC)	AM114313
<i>Centaurea nigra</i> L.	Eurosiberian	Spain, La Coruña: near Carballo, 03.08.1992, <i>García-Jacas & Susanna 1446</i> (BC)	DQ319138
<i>Centaurea pectinata</i> L.	W Mediterranean	Spain, Barcelona: Montseny, Santa Fe to Sant Marçal, 1300-1400 m, 06.07.1994, <i>García-Jacas & Susanna 1469</i> (BC)	DQ319144
<i>Centaurea pinae</i> Pau var. <i>pinae</i>	Iberian Peninsula endemic	Spain, Teruel: Puerto Ragudo, 900 m, 15.07.1978, <i>Blanca, Socorro & Valle 6768</i> (GDAC)	AM114310
<i>Centaurea protogerberi</i> Klokov	Ukraine endemic	Ukraine, Luganskaya: Stanichno-Lugansk, 05.09.2002, <i>Romashchenko s. n.</i> (BC)	DQ319149
<i>Centaurea pseudoleucolepis</i> Kleopow	Ukraine endemic	Ukraine, Donetskaya: Kamennye Mogily national reservation, 01.08.2002, <i>Romashchenko s. n.</i> (BC)	DQ319150
<i>Centaurea resupinata</i> Coss. subsp. <i>resupinata</i>	Iberian Peninsula endemic	Spain, Albacete: between Elche de la Sierra and Hellín, Cenajo dam, 06.07.1977, <i>Blanca & Varo 6714</i> (GDAC)	AM114288
<i>Centaurea sarandinakiae</i> N. B. Illar	Ukraine endemic	Ukraine, Crimea: Planerskoe, Kara-Dag mountain, 16.08.2002, <i>Romashchenko s. n.</i> (BC)	DQ319160
<i>Centaurea semijusta</i> Juz.	Ukraine endemic	Ukraine, Crimea: Simferopol, Chatyr-Dag mountain, 01.09.2002, <i>Romashchenko s. n.</i> (BC)	DQ319162
<i>Centaurea spinosa</i> L.	Aegean	Greece, Thrakia: Nomos Evrou, Samothraki, 2 m, <i>Raus/Sch 18942</i> , Berlin Botanical Garden, Index Seminum 1997	DQ319165
<i>Centaurea sterilis</i> Stev.	Ukraine endemic	Ukraine, Crimea: Planerskoe, Kara-Dag mountain, 16.08.2002, <i>Romashchenko s. n.</i> (BC)	DQ319167
<i>Centaurea subtilis</i> Bertol.	Italian endemic	Italy, Gargano peninsula: road from Madonna delle Grazie to Monte Sant'Angelo, about 0.5 km N of Madonna delle Grazie, 280 m, 03.06.2008, <i>Hilpold, García-Jacas & Vilatersana 1208</i> (BC, BOZ)	FJ572057

Table 1. (Cont.)

Species	Range	Voucher	ITS accession
<i>Centaurea vankovii</i> Klokov	Ukraine endemic	Ukraine, Crimea: Alupka, Ai-Petri mountain, 30.08.2002, <i>Romashchenko s. n.</i> (BC)	DQ319173
<i>Centaurea virgata</i> Lam.	Turkey endemic	Turkey, Muğla: Köyceğiz district, Sandras Dag range 13 km from Ağla, 1700 m, 29.07.2002, <i>Ertuğrul, Garcia-Jacas, Susanna 2252 & Uysal</i> (BC)	DQ319174
<i>Centaurea wiedemanniana</i> Fisch. & C. A. Mey.	Turkey endemic	Turkey, Bilecik: Selimiye, between Osmaneli and Bilecik, 100 m, 01.07.1962, <i>Davis & Coode</i> (E)	DQ319175
Outgroup			
<i>Centaurea mollis</i> Waldst. & Kit.	E Europe	Ukraine, Podolia: Lysa Hora, 2 km E of Vilshanitsa near Zolochiv, 05.06.2000, <i>Boratyński & Romo 0506D</i> (BC)	DQ319133
<i>Centaurea napulifera</i> Rochel subsp. <i>thirkei</i> (Sch. Bip.) Stoj. & Acht.	E Europe	Romania, Constanta: Dobrogea, N from Cheia village, Cheia gorges of the Casimcea river, 130 m, 02.04.1996, <i>Badarau</i> (BC)	DQ319136
Vouchers used for figures 1 and 3			
<i>Centaurea subtilis</i> Bertol.	Italian endemic	as above	
<i>Centaurea exarata</i> Coss.	Iberian Peninsula endemic	as above	
<i>Centaurea linifolia</i> L.	Iberian Peninsula endemic	as above	
<i>Centaurea jacea</i> subsp. <i>vinyalsii</i> (Sennen) O. Bolòs, Vigo & J. M. Panareda	Iberian Peninsula endemic	as above	
<i>Centaurea jacea</i> subsp. <i>gaudinii</i> (Boiss. & Reut.) Greml	Europe, Mediterranean	Italy, Südtirol, Samtaler Alps, Feldthurns, 1 km WNW Gamer Wetterkreuz, 1400 m, 16.08.2008, <i>Hilpold</i> (BC, BOZ)	

Phylogenetic analyses

Nucleotide sequences were edited using Chromas 2.0 (Technelysium Pty. Ltd., Tewantin, Australia) and aligned visually by sequential pairwise comparison (Swofford & Olsen, 1990). Data matrices are available on request from the corresponding author. Parsimony analysis of the ITS dataset involved heuristic searches conducted with PAUP 4.0b10 (Swofford, 2002) using TBR branch swapping with character states specified as unordered and

unweighted. The indels were coded as missing data. All most-parsimonious trees (MPTs) were saved. To locate other potential islands of MPTs (Maddison, 1991), we performed 1000 replications with random taxon addition, also with TBR branch swapping. Bootstrap analyses (BS) (Felsenstein, 1985) were performed using 1000 replicates of heuristic search with the default options. For the strict consensus tree, consistency index (CI) and retention index (RI) are given, excluding uninformative characters.

Bayesian inference estimation of the ITS dataset was calculated using MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best available model of molecular evolution required for Bayesian estimations of phylogeny was selected using hierarchical likelihood ratio tests (hLRT) and Akaike information criteria (AIC) as implemented in the software MrModeltest 2.2 (Nylander, 2004), which considers only nucleotide substitution models that are currently implemented in PAUP and MrBayes.

The symmetrical model, with equal base frequencies with variable sites assumed to follow a discrete gamma distribution SYM+G (Zharkikh, 1994), was selected as the best-fit model of nucleotide substitution. The Bayesian inference analyses were initiated with random starting trees and were run for 1×10^6 generations. Four Markov chains were run using the Markov Chain Monte Carlo (MCMC) principles to sample trees. We saved one out of every 100 generations, resulting in 10000 sample trees. Data from the first 1000 generations were discarded as the “burn-in” period, after confirming that likelihood values had stabilized prior to the 1000th generation. Internodes with posterior probabilities $\geq 95\%$ were considered statistically significant.

Morphological survey

To prepare Fig. 3, the middle involucre bracts of five different species, all taken from herbarium specimens (Table 1), were glued onto a black paper and scanned using a scanner Epson Perfection 4990 Photo. The background of the resulting image was underlain in black using Adobe-Photoshop 7.0.

RESULTS AND DISCUSSION

Chromosome counts

Centaurea subtilis is a diploid with $2n = 2x = 22$ chromosomes (Fig. 4). The basic chromosome number is $x = 11$. This result is coincident with previous reports (see introduction and references therein).

Molecular phylogeny

The results of both analyses are commented together because the topology of the trees produced

by parsimony and Bayesian approaches was coincident. The parsimony analysis resulted in 30 equally parsimonious trees of 76 steps. Descriptive statistics: the consistency index (CI) was 0.750 and the retention index (RI) was 0.932, excluding uninformative characters.

Figure 5 shows the Bayesian phylogram with the addition of Bayesian posterior probabilities (PP) above branches, and parsimony bootstrap values (BS) below branches. The results are coincident with the ones in Garcia-Jacas *et al.* (2006) for the clade that was named “widely distributed clade”. Within this group, our analyses showed three subclades: one which comprised species of the *Acrolophus-Phalolepis* sections (BS = 91%, PP = 1.00), a second one which comprised species of sect. *Willkommia* (BS = 97%, PP = 1.00), and a third subclade which comprised the taxa from the *Jacea-Lepteranthus* group plus *Centaurea subtilis* with high support values (BS = 93%, PP = 1.00).

By showing *C. subtilis* within the *Jacea-Lepteranthus* group, our results confirm that sect. *Maculosae* is an artificial assemblage. After *C. exarata* (moved to the *Jacea-Lepteranthus* group by Garcia-Jacas *et al.*, 2006), this is the second species of sect. *Maculosae* which belongs to a different clade and is placed within the *Jacea-Lepteranthus* group. Most of the remaining species of sect. *Maculosae* are part of the *Acrolophus-Phalolepis* group as verified by previous molecular studies [*Centaurea corymbosa* (Garcia-Jacas *et al.*, 2006), *C. filiformis* (Mameli, 2008), *C. stoebe* and *C. triniifolia* (Ochsmann, 2000)], and they do not form a natural group in any of these studies. The delimitation of *Maculosae* from some other sections, namely sections *Arenariae* Dostál, *Paniculatae* Dostál and *Dissectae* Dostál, is doubtful and the whole taxonomic treatment by Dostál (1976) for the *Jacea* group needs a deep revision.

Morphology

Centaurea subtilis shows intriguing morphological parallels with *C. exarata*. Their appendages of the involucre bracts are very similar (Fig. 3). When compared with other members of the *Jacea-Lepteranthus* group, the outer showy flowers are much smaller in *C. subtilis*, while they are totally reduced in *C. exarata* (Fig. 1). Most members of the *Jacea-Lepteranthus* group have broad, undivided, mesophyllitic, mostly glabrous or sparsely hirsute leaves, whereas *C. subtilis* and *C. exarata* have narrow, tomentose, more



Figure 3. Middle involucral bracts of five different taxa of the *Jacea* Group. (A) *Centaurea linifolia* L.; (B) *C. subtilis*; (C) *C. exarata*; (D) *C. jacea* subsp. *vinyalsii* (Sennen) O. Bolòs, Vigo & J. M. Panareda; (E) *C. jacea* subsp. *gaudinii* (Boiss. & Reut.) Greml.

xerothermic leaves. *Centaurea subtilis* is hitherto the only species of the group with divided leaves. All these morphological characters, which in part led to their wrong taxonomic classification in former times, can be considered as adaptations to dryer micro- and macroclimatic environments. The distributional centre of the *Jacea-Lepteranthus* group are the mountain ranges in the northern Mediterranean region, especially the southern Alps, the northern Balkanic mountains, the Pyrenees, the Apennines and the mountains of central Iberia. Its members usually grow in mountain meadows with deep soil and good water supply or in some cases in well water-provided crevices. In contrast, *C. subtilis* and *C. exarata* grow in drier habitats, like calcareous rocks, garrigues or dunes, which share the tendency to suffer water deficiencies, induced by their local ecology on one hand and by the mediterranean climate on the other. Strong droughts can occur in late spring and early summer, at a time when this two species have still not concluded their reproduction cycle. Thus, it was evolutionary essential to develop adaptations to avoid water loss. Ecological differences of these two species relative to other taxa of the *Jacea-Lepteranthus* group might be a good explanation for their morphological divergence.

This adaptation to a drier climate would also contribute to explain the secondary loss of the showy peripheral sterile florets in both species, much reduced in *C. subtilis* and totally absent in *C. exarata*. According to Lack (1976), the presence of radiant peripheral florets in the related *C. nigra*

L. is directly related to competition for pollinators (mainly *Bombus* sp., cf. Hegland & Totland, 2004) with a larger species of *Centaurea* with similar ecological preferences, *C. scabiosa* L. Radiate heads in *C. nigra* predominate in populations which grow in presence of *C. scabiosa*, while non-radiate heads predominate in stands of *C. nigra* alone. According to Font *et al.* (2009), *C. scabiosa sensu latissimo* is an Euro-Siberian species and thereafter totally absent from the areas where *C. exarata* and *C. subtilis* grow. There is a species of *Centaurea* with very large radiate heads which grows in the vicinity of *C. exarata*, *C. polyacantha* Willd., but its flowering period is much earlier (Talavera, 1987).

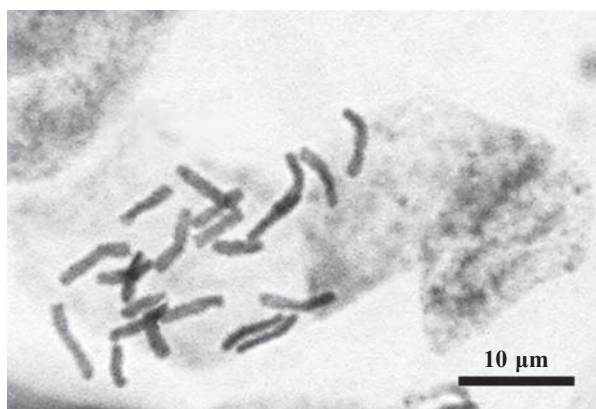


Figure 4. Orcein-stained metaphase plate of *Centaurea subtilis*. $2n = 22$.

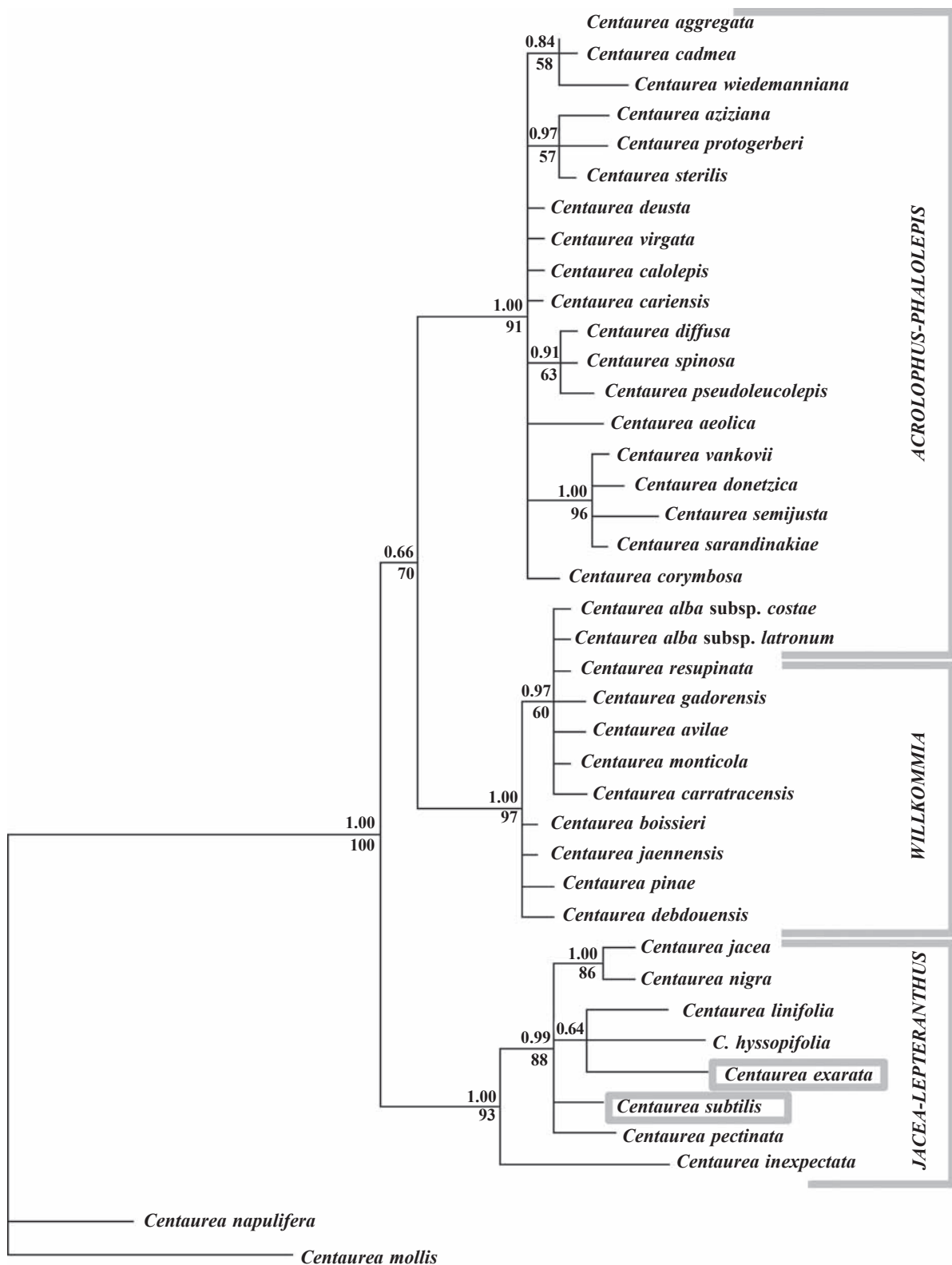


Figure 5. Phylogram obtained from the Bayesian analysis of ITS sequences. Numbers above branches indicate Bayesian-credibility values (PP); numbers below branches indicate bootstrap values (BS) from parsimony analysis.

Concluding remarks

This study emphasizes once again the importance of chromosome numbers as a predictor of the systematic relationships in the genus *Centaurea*, especially in the *Jacea* group. In the case of a species where the base chromosome number does not correspond with the one of its systematic group, caution is required: it may be a sign of a mistake in its taxonomic classification, as demonstrated by molecular phylogeny analysis in the case of *C. exarata* and *C. subtilis*.

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