Evolution of the *Centaurea Acrolophus* subgroup

[*Evolució del subgrup* Acrolophus *del gènere* Centaurea]

Andreas Hilpold

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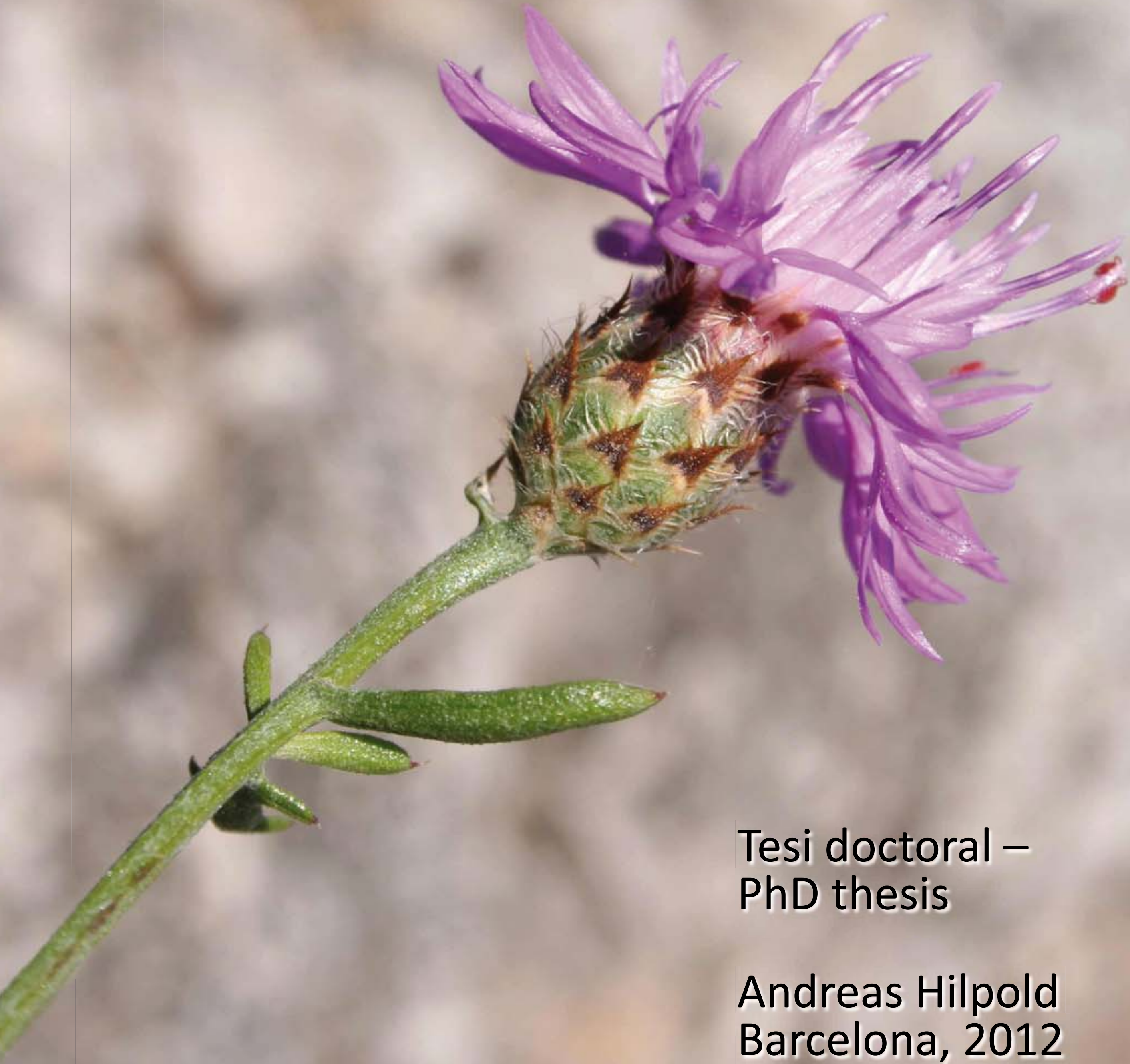
Universitat de Barcelona
Facultat de Farmàcia
Departament de Productes Naturals,
Biologia Vegetal i Edafologia
Secció de Botànica

Evolution of the *Centaurea Acrolophus* subgroup (Compositae)



Tesi doctoral –
PhD thesis

Andreas Hilpold
Barcelona, 2012

UNIVERSITAT DE BARCELONA FACULTAT DE FARMÀCIA
Departament de Productes Naturales, Biologia Vegetal i Edafologia,
Secció Botànica
Programa de Doctorat: Biodiversitat 2009–2012

INSTITUT BOTÀNIC DE BARCELONA (CSIC-ICUB)

Evolution of the *Centaurea Acrolophus* subgroup

[*Evolució del subgrup* Acrolophus *del gènere* Centaurea]

Memòria presentada per Andreas Hilpold per a optar al títol de Doctor per la Universitat de Barcelona

Amb el vist-i-plau dels directors de tesi:

Dra. Núria Garcia Jacas   Dra. Roser Vilatersana Lluch

i del tutor de tesi:
Dr. Cèsar Blanché Vergés

Andreas Hilpold
Barcelona, 2012

Jag frågade Jonatan, varför han måste ge sej ut på nånting som var så farligt. Han kunde ju lika gärna sitta hemma vid elden i Ryttargården och ha det bra. Men då sa Jonatan, att det fanns saker som man måste göra, även om det var farligt.
"Varför då", undrade jag.
"Annars är man ingen människa utan bara en liten lort" sa Jonatan.

[*I asked Jonathan, why he had to go away to something so dangerous. He could just sit at home by the fire in Ryttargården and have it good. But then Jonathan said it was something he must do, even if it was dangerous. ‘Why?’ I wondered. ‘Otherwise you’re not a human being but just a piece of dirt’ said Jonathan.*]

From Bröderna Lejonhjärta / The Brothers Lionheart,
Astrid Lindgren (1973)

4238A

Aragorn Hilpold
post doc

Smeagol Nylinder
Doktorand

Finrod de Sousa
Doktorand

The concrete highway was edged with a mat of tangled, broken, dry grass, and the grass heads were heavy with oat beards to catch on a dog's coat, and foxtails to tangle in a horse's fetlocks, and clover burrs to fasten in sheep's wool; sleeping life waiting to be spread and dispersed, every seed armed with an appliance of dispersal, twisting darts and parachutes for the wind, little spears and balls of tiny thorns, and all waiting for animals and for the wind, for a man's trouser cuff or the hem of a woman's skirt, all passive but armed with appliances of activity, still, but each possessed of the anlage of movement.
From: The Grapes of Wrath
John Steinbeck (1939)

Aquest treball ha estat possible gràcies a la concessió d'una beca predoctoral JAE del *Consejo Superior de Investigaciones Científicas* (CSIC) d'Espanya. La recerca ha estat parcialment finançada pels projectes de recerca del *Ministerio de Ciencia y Inovación* (CGL2007-60781/BOS i CGL2010-18631) i de la *Generalitat de Catalunya* (Ajuts a Grups de Recerca Consolidats 2009/SGR/00439), i per dues bosses de viatge del CSIC.

Acknowledgements – Agradecimientos

Quiero a agradecer a mis directoras de tesis **Núria Garcia-Jacas** y **Roser Vilatersana** por darme su apoyo incondicional desde el principio. Durante muchas horas de laboratorio, de campo y de discusiones cientificas me introdujieron con mucha paciencia en el mundo de la ciencia sistemática, equipándome con una base sólida de conocimientos botánicos y moleculares. A **Alfonso Susanna** por acompañar mi estancia en el Instituto Botánico, dándome mucha ayuda y consejos, pero también por la instrucción profunda en el mundo español.
Agradezco a mi tutor **Cèsar Blanché Vergés** por su gran ayuda en todos los asuntos burocráticos.

Muchas gracias a mis **compañeros de trabajo**, a Piotr Kosinsky y Konstantin Romaschenko por muchas horas de diversión deportiva, a Laia Barres que me acompañó durante todo el estudio, a Sara López por su ayuda, a Javier López que compartió mis dificultades con su grupo plantas, a María Sanz, que me mostró que vale la pena luchar para acabar una tesis. Además agradezco a Ismael Sánchez Jiménez, Noemi Montes, Almudena Galian, Jordi López, Cristina Roquet, Mercè Galbany Casals por acompañarme durante partes de mi vida laboral en Barcelona. Moltes gràcies a tots!

Special thanks go to **Peter Schönswetter**, who joined my way into science from the beginning. In a research stay in Vienna I got introduced to phylogeographical methods and could improve my working efficiency. His working group, namely Pablo Escobar, Ruth Flatscher, Clemens Pachschwöll, Katharina Bardy, Manuela Winkler and Gerald Schneeweiss, made my stay immediately familiar.

My thanks go also to **Bengt Oxelman**, University of Gothenburg, for integrating me into his working group and for opening my eyes for new horizons in biology. To Bernard Pfeil, of the same working group, I owe a large part of my systematic skills, but also the fundamental insight that the hard science, as dry as it may seem, can be fun. Thomas Marcussen is acknowledged for his openness to discuss whatever botanical and not botanical question emerged. Finally, only the active role of Filipe Sousa, Yann Bertrand, Elisabeth Sjöquist, Elisabeth Gondel, Anna Petri, Ulrika Rydberg, Atefeh Pirani, Zeynep Toprak Aydın, Henrik Nilson and Stephan Nylinder rendered the highly prosperous atmosphere at Botaniska possible. Tack så mycket för en ganska bra tid och jag hoppar att vi ser oss snart!

Agradezco a **Isabel Sanmartín** que me recibió con los brazos abiertos en el Real Jardín Botánico de Madrid durante una estancia, donde pude terminar mi tesis de doctorado mientras podía aprovechar de una atmosfera científica muy productiva. Agradezco a Javier Fuertes Aguilar por la compañía en el Fomento, a Jose Luis Blanco-Pastor, Mario Fernández-Mazuecos, Isabel Liberal, Ricarda Riina, Angélica Bello y Marieta Aguilar por su compañía y muchos impulsos científicos.

Estoy muy agradecido a **Andrea Sánchez-Meseguer** que me acompañó los últimos dos años de mi doctorado compartiendo muchas horas difíciles y felices conmigo. Además me ayudó mucho en el desarrollo de este trabajo.

Ganz besonders möchte ich auch jenen danken die mich im Laufe meines Studiums tatkräftig unterstützt haben. Ein besonderer Dank geht an meine **Eltern** und an meine **Geschwister**. Als ich vor mehr als vier Jahren aufbrach um meine Doktoratsstudium in Spanien zu beginnen, war ich mir stets ihrer Unterstützung gewiss.
Ein Dank auch all meinen **Freunden** in Südtirol, Innsbruck und Wien – viele von ihnen haben mich im Laufe der Jahre mit ihrem Besuch beehrt und vielen von ihnen verdanke ich meinerseits unvergessliche Urlaubstage. Stets gaben sie mir das Gefühl, dass es einen Platz in den Alpen gibt, an dem ich immer willkommen bin.

Finally, I want also to thank the many newly gained **friends** who joined my way in **Barcelona**, sharing flats, hanging out, accompanying me for excursions by foot or by bike, namely are they, Alex, Richard, Marta, Georg, Marco, Laia, Maria, Jürgen, Magdalena, Christine, Kamil, Josep, Roberto, Estela, Ixone, Francesca, Corinna, Clothilde, Ewelina, Borja, Angela, Miriam, Sophie, Marion, Flavio, Ana Carolina, Raquel, Raphael and many more. Thank you all, muchas gracias, moltes gracies, grazie mille, vielen Dank, dziękuję bardzo, muito obrigado, merci beaucoup, donkschian.

Index

1.

Introduction

[*Introducció*]

1.1 – *Centaurea* within Cardueae

The genus *Centaurea* L., part of the Compositae family and an important member of tribe Cardueae Cass., is one of the most emblematic groups of the Mediterranean. The centre of distribution of the genus lies in fact around the Mediterranean Sea, with the Balkan, the Anatolian, the Apennine and the Iberian Peninsula with hundreds of species. Their purple, blue, yellow or orange flower heads are colour spots in many Mediterranean habitats and appreciated photo subjects for many travellers. The contrast between lively colored flowers and a very protective form of the vegetative parts of the plant is a common feature in most Cardueae. This tribe contains many species commonly known as thistles, for example the genera *Carduus* L. and *Cirsium* Mill. with extremely thorny leaves and therefore avoided both by most grazing animals and collecting botanists. Many species of the tribe are perennial or biennial herbs or small shrubs (Susanna and Garcia-Jacas, 2009). The tribe contains about 2400 species in 72 genera (Susanna and Garcia-Jacas, 2007, 2009) and is one of the largest in Compositae. It includes five subtribes: Carlininae Dumort., Echinopsinae (Cass.), Carduinae (Cass.), Centaureinae (Cass.) Dumort. (Bentham, 1873; Hoffmann, 1894; Bremer, 1994). Only recently subtribe Cardopatiinae Less. was included into Cardueae (Susanna and Garcia-Jacas, 2007). Most species included in the family belong to Carduinae and Centaureinae (Susanna *et al.*, 2006). Whilst Carduinae is a paraphyletic group, Centaureinae is clearly monophyletic (Susanna and Garcia-Jacas, 2009). Centaureinae encompass more than 650 species in 31 genera. Most of them have unarmed leaves but many show spines in their bract appendages. Likewise Cardueae, Centaureinae are mainly perennial or biennial herbs. The outer flowers of the capitula are frequently sterile and radiant. Systematic relationships within the group were subject of exhaustive molecular surveys (Garcia-Jacas *et al.*, 2000, 2001) dealing mainly with the delimitation of the genus *Centaurea*. *Centaurea*, as defined traditionally by Cassini (1819) or Dostál (1969), was polyphyletic. These molecular studies showed that some clades, including the former section *Centaurea* with the original type species, *Centaurea centaurium* L., had to be excluded in order to make the genus monophyletic. To prevent the renaming of hundreds of species, the type species was changed: the new type is now *C. paniculata* L. (Greuter *et al.*, 2001). The old section *Centaurea* acquired the rank of genus on its own, namely *Rhaponticoides* Vaillant (Greuter, 2003; Greuter *et al.*, 2005).

1.2 – The genus *Centaurea*

1.2.1 – Characterization, distribution and subdivision

Centaurea consists of annual, biennial or perennial herbs or shrubs with usually unarmed leaves (Susanna and Garcia-Jacas, 2007). They are also characterized by a lateral seed hilum (Dittrich, 1968), and a specialized floral morphology with showy sterile peripheral florets without staminodes (Wagenitz and Hellwig, 1996). The main morphological character used for systematics within *Centaurea* is the form of the scarious bract appendages. The genus occurs mainly in the Mediterranean and Irano-Turanian Region. *Centaurea* is the largest genus within subtribe Centaureinae encompassing between 250 and 500 species, highly depending on the taxonomic treatment (Susanna and Garcia-Jacas, 2007, 2009).

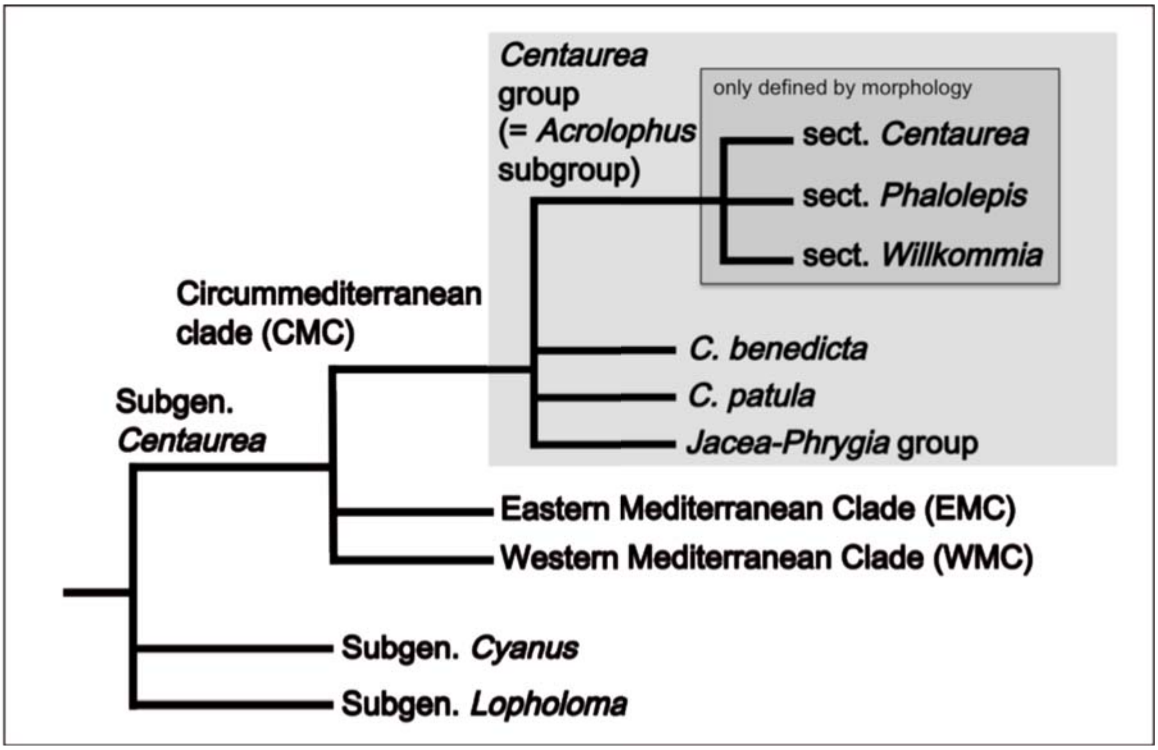


Figure 1: Scheme of the systematic relationships within genus *Centaurea*, focussing on the subgenus *Centaurea* and therein on the CMC and the *Centaurea* group. All clades are supported by molecular data except the sectional division within the *Centaurea* group.

The genus *Centaurea* contains three well delimited subgenera (Fig. 1): *Lopholoma* (Cass.) Dobrocz (often named *Acrocentron*), *Cyanus* (Juss.) Hayek and *Centaurea* (corresponds to *Jacea* group sensu Garcia-Jacas *et al.*, 2006). The three are sometimes also treated as separate genera (e.g., Greuter, 2008).

Lopholoma (Fig. 2: a) is monophyletic after removing sect. *Crocodilium* (Vaill.) DC. and sect. *Aegialophila* (Boiss. & Heldr.) O. Hoffm., which were merged to the separate genus *Crocodilium* Vaill. (Font *et al.*, 2002). It is also morphologically well differentiated by presenting a unique and characteristic pollen type (Wagenitz, 1955). Subgenus *Lopholoma* comprises about 100 species (Font *et al.*, 2002) and is distributed all over the Mediterranean with one species, *Centaurea scabiosa* L., reaching the high north of Europe. This subgenus was studied with molecular tools by Font *et al.* (2002, 2009). The flower heads of *Lopholoma* are usually larger than in the other subgenera. Bract appendages possess frequently a long spine.

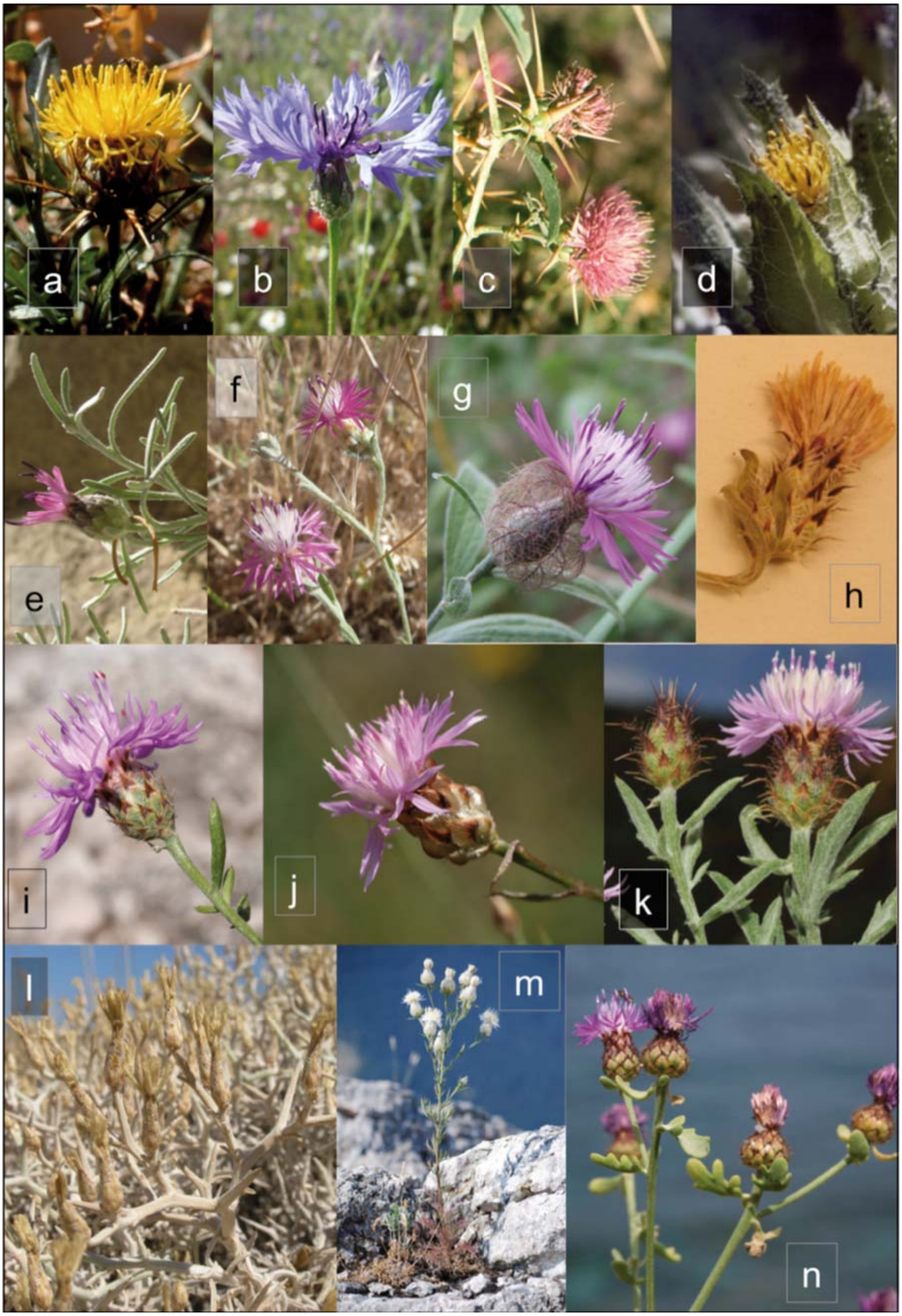


Figure 2: Photos of the genus *Centaurea*, with special emphasis on subgenus *Centaurea* and the *Centaurea* group. a) Subgen. *Lopholoma*: *C. saxicola*, Murcia, Spain, Photo: A. Susanna; b) Subgen. *Cyanus*: *Centaurea cyanus*, Soria, Spain, Photo: A. Susanna; c–n) Subgen. *Centaurea*: c) EMC: *C. iberica*, Armenia, Photo: A. Susanna; d–n) CMC. d) *C. benedicta*, Barcelona Botanical

Garden, Photo: A. Susanna; e) *C. akamantis*, Avakas gorge, Cyprus, Photo: M. Galbany; f) *C. hierapolitana*, Afon lake, Turkey, Photo: A. Susanna; g) *C. hyrcanica* (*Jacea-Phrygia* group), Iran, Photo: A. Pirani. h) *C. ammocyanus*, Herbarium GB. i–n) *Centaurea* group. i) *C. tenorei*, Minori, Italy, Photo: A. Hilpold; j) *C. alba*, Sierra de Aracena, Spain, Photo: L. Barres; k) *C. pulvinata*, Sierra de Abrucena, Spain, Photo: G. Blanca López; l) *C. spinosa*, Crete, Photo: M. Galbany; m) *C. princeps*, Mt. Tymfristos, Greece, Photo: T. Constantinidis; n) *C. panormitana,* Sferracavallo, Sicily, Photo: A. Hilpold.

The second subgenus, *Cyanus* (Fig. 2: b), sticks out through its predominantly bright blue flowers, as shown by its most prominent member, the cornflower *Centaurea cyanus* L. Further characters for this group are two unique pollen types (Wagenitz, 1955) and the pectinate-ciliate, decurrent appendages of the phyllaries (Wagenitz and Hellwig, 1996). The subgenus comprises about 40 species, also concentrated in the Mediterranean, reaching central Asia and the Caucasus. Molecular studies of the group were carried out by Boršić *et al.* (2011), Olšavská *et al.* (2011) and Löser *et al.* (in prep.).

The third subgenus is the most species-rich of the genus and harbours also the study group of this work. The subgenus *Centaurea* (= *Jacea* group; Fig. 2: c–n) is represented by several hundreds of species mainly in the Mediterranean Region but also in western Asia and central and eastern Europe. The most unique trait of the group is the characteristic pollen type, called *Jacea* pollen type (Wagenitz, 1955; Garcia-Jacas *et al*., 2000). New systematic insights on molecular grounds of this group were provided by Garcia-Jacas *et al.* (2006). One of the main result of this study, based on ITS (Internal Transcribed Spacer), was the separation of subgen. *Centaurea* into three clades (Fig. 1), which where named after their main geographic distribution: the Eastern Mediterranean Clade (short EMC; Fig. 2: c) comprises species often with spiny bract appendages from the eastern Mediterranean, for example the widely distributed weed *Centaurea calcitrapa* L.; the Western Mediterranean Clade (WMC), the members of which show also pronounced spiny bract appendages, well visible in the common weeds *Centaurea solstitialis* L. or *C. sulphurea* Willd.; and the Circum-Mediterranean Clade (CMC), whose members are predominantly not or only slightly spiny in their bract appendages. Despite their wide distribution, their importance as weeds, and the high species diversity, knowledge about systematics within the EMC and the WMC is poor and would need a more comprehensive study with molecular methods. The CMC, however, which includes the study group of this work, was already the centre of attention of profound molecular work (see below), however mainly restricted to the Western Mediterranean and especially the Iberian Peninsula.

1.2.2 – Palynology of the genus *Centaurea*

The genus *Centaurea* can be well identified by its pollen. Like all Compositae, it is tricolporate and the ectexine is formed by two layers of columellae (Wagenitz, 1955). Within the genus, four clearly distinct pollen types can be found, which correspond to the tree subgenera (Fig. 3; Wagenitz, 1955; Martín-Villodre and Garcia-Jacas 2000; Garcia-Jacas *et al*., 2006): The *Centaurea scabiosa*-type in subgenus *Lopholoma*, and the *Cyanus*- and the closely related *Montana*-type both in subgenus *Cyanus* and the

Jacea-type in subgenus *Centaurea*. The *Centaurea scabiosa*-type includes rhomboidal, subprolate to prolate pollen grains with scabrate or less often microechinate sculpture, and also a caveate exine (Fig. 3: b). The two pollen types present in *Cyanus* are also subprolate to prolate elliptic in shape with little surface sculpture but the exine is not caveate, but forms a two well-differentiated layers of collumelae (Fig. 3: b). The pollen of subgenus *Centaurea* is prolate-subprolate to subprolate-spheroidal with scabrate, scabrate-echinate or verrucate sculpture and a caveate exine (Fig. 3: c). The palynologic study of Tormo-Molina and Ubera-Jiménez (1988) found additionally two poorly characterized groups within the *Jacea*-type. These correspond vaguely to the separation between the CMC and the rest of subgen. *Jacea*. Information to the pollen of individual species of the *Centaureinae* can be found in Wagenitz (1955), Tormo-Molina and Ubera-Jiménez (1988), Pehlivan (1995) and Martín-Villodre and Garcia-Jacas (2000).

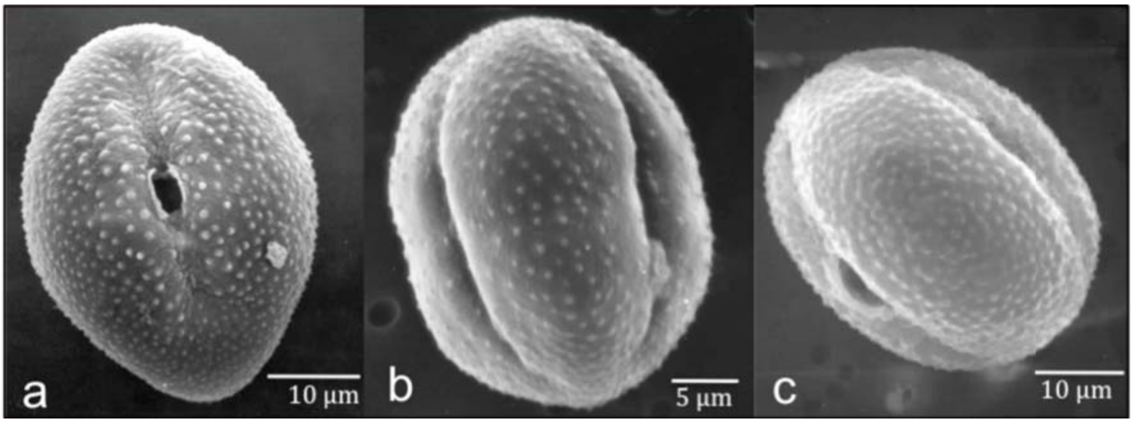


Figure 3: Three of the four main pollen types in *Centaurea*. a. *Centaurea scabiosa* type: *Centaurea prolongi*; b. Cyanus type: *Centaurea cyanus*; c. *Jacea* type: *Centaurea calcitrapa.*

1.2.3 – Caryology of the genus *Centaurea*

Centaureinae show a dysploid chromosome series, with basic chromosome numbers ranging between $x = 16$ and $x = 7$ (Garcia-Jacas *et al.*, 1996). Low numbers can be found in more evolved groups and have been interpreted as an adaptation to dry conditions (Susanna and Garcia-Jacas, 2009). Chromosome numbers in *Centaurea* range from $x = 7$ to $x = 12$ (Table 1). Whereas subgenera *Cyanus* and *Centaurea* have a broad range of chromosome numbers, subgenus *Lopholoma* is more conservative, showing only two chromosome numbers. Chromosome numbers in the EMC and in the WMC follow largely the sectional division. Lowest chromosome numbers can be found in the annual *Centaurea patula* with $x = 7$.

1.3 – The Circum-Mediterranean Clade (CMC) of the genus *Centaurea*

1.3.1 – Characterization and subdivision

The most distinctive characters within this group are also the bract appendages. There exist three extreme forms: bracts membranaceous, bracts long ciliate-fimbriate and bracts without any appendage. According to Garcia-Jacas *et al.* (2006) there are two main clades (Fig. 1): the *Jacea-Phrygia* group (Fig. 2: e) and the *Centaurea* group (Fig. 2: i–n; formerly *Acrolophus* subgroup; Wagenitz and Hellwig, 1996). Holub (1972a,b), who split the genus *Centaurea* into a series of separate genera, raised these two groups to genus rank, namely *Acosta* Adans. and *Jacea* Mill.

Table 1: Chromosome numbers in *Centaurea*, focussing on the CMC of subgenus *Centaurea* (for details see Appendix 1).

Genus *Centaurea*	Basic chromosome numbers *x* =
Subgenus *Cyanus*	8, 9, 10, 11 & 12
Subgenus *Lopholoma*	10 & 11
Subgenus *Centaurea* (corresponds to the *Jacea* group)	
Eastern Mediterranean Clade (EMC)	8, 9 & 10
Western Mediterranean Clade (WMC)	8, 10, 11 & 12
Circum-Mediterranean Clade (CMC)	
Jacea-Phrygia group	11
Centaurea group	9
C. benedicta	11
C. patula	7
C. ammocyanus	8
C. hierapolitana	8

1.3.2 – The *Jacea-Phrygia* group

The members of the *Jacea-Phrygia* group can be distinguished from the *Centaurea* group by (1) the basic chromosome numbers 11 vs. 9 respectively, (2) their leaf forms: *Jacea-Phrygia* leaves are mostly entire, while in the *Centaurea* group at least the upper leaves are always deeply divided; and (3) the showy flowers: in the *Centaurea* group they are often reduced, in *Jacea* they are usually present (Fig. 2: g). *Jacea-Phrygia* also has a different ecology and distribution: the *Centaurea* group is more dry adapted and much more bound to Mediterranean climates. In contrast, the vast majority of *Jacea-Phrygia* species are typical elements of montane and subalpine meadows, influenced by frequent hay cut, grazing or avalanches. They are dependent on a good and constant water supply and are quite resistant to cold periods. These adaptations permitted the group also to disperse into the high north of Europe, with species like *Centaurea nigra* L. or *C. jacea* L. Some nemoral species are morphologically clearly distinct from other *Centaurea* species since they have broad and soft, mesophyllous leaves. Systematic classification within the *Jacea-Phrygia* group is highly complex, and the latest attempts to elucidate it (Koutecký *et al.*, 2011; López-Alvarado, 2012) are only partially satisfying because of the very low levels of variation found. The apparent lack of any intrinsic breeding barriers led to the description of many hybrids on one hand (Vanderhoeven *et al.*, 2002; Koutecký, 2007; Vonica and Cantor, 2011). On the other hand, a bisection of the breeding communities into diploid and tetraploid lineages, connected by frequent polyploidization events, was observed (Hardy *et al.*, 2000; Koutecký, 2007; Koutecký *et al.* 2011). Traditional systematics based in morphology within this group uses merely the shape of the bract appendages for the subdivision into two sections: section *Phrygia* with long fimbriate bract appendages and section *Jacea* with either short ciliate or membranaceous bract appendages. Intermediate forms between these extremes are commonly attributed to hybridization events (Vanderhoeven *et al.*, 2002; Koutecký, 2007; Vonica and Cantor, 2011).

1.4 – The *Centaurea* group (= *Acrolophus* subgroup)

1.4.1 – Distribution and ecology

The *Centaurea* group is the main objective of this work. It has its centre of distribution around the Mediterranean and Black Sea (Fig. 4). Highest species numbers can be found in the Balkan Peninsula, Italy, Turkey and the Iberian Peninsula. Almost all African species are concentrated in the NW of the continent, in the Atlas mountain ranges. A few widespread species reach central Europe and the Baltic Sea (*C. stoebe* L.) and Inner-Asia, Afghanistan and Pakistan (*C. virgata* Lam.).

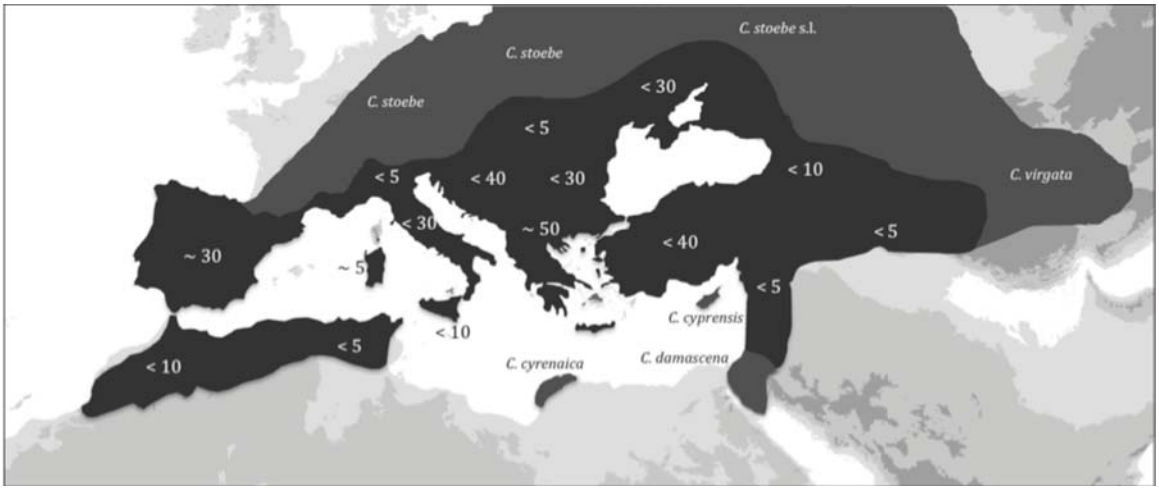


Figure 4: Distribution of the *Centaurea* group (without introduced populations). Dark grey: areas with more than one species occurring. Light grey: only one species occurring, species name is given. Numbers show the approximate species number in the area. Note that the species numbers reflect, besides real differences in diversity, also the species concepts used in the different areas.

All members of the *Centaurea* group grow in dry, open vegetation. Many species can be found in frequently pastured garrigues, on the coast or in the interior. Furthermore, open rocks are inhabited. Only exceptionally, high mountain areas were colonized. Some species are specialized in sandy beaches (e.g., *C. spinosa* L.). Both substrates, calcareous and siliceous soils are inhabited with predominance on the first ones.

1.4.2 – Traditional, morphology based treatments and their limitations

The *Centaurea* group (= *Acrolophus* subgroup) is traditionally divided into two sections (Fig. 1) based on the morphology of their bract appendages: section *Phalolepis* (Cass.) DC. (Fig. 2: j) with membranaceous appendages, and section *Centaurea* (formerly *Acrolophus* (Cass.) DC.; Fig. 2: i) with ciliate to fimbriate ones. These two groups are treated as subgenera in Dostál's treatment for Flora Europaea (1976). The appendage morphology is the only character used for their delimitation (Dostál, 1976), but within these two groups, however, a broad range of morphological characters can be found to perform further delimitations

(Fig. 2: l–n): leaf shape (divided), character of the indumentum (tomentose, glabrous), flower colors (rarely yellow, mostly purple), plant size (a few centimetres in annual and biennial species up to 1.90 m in both biennial and perennial species, but mostly between 20 and 50 cm). A third section was described in literature 30 years ago: under the name of section *Willkommia* Blanca (Blanca López, 1981a; Fig. 2: k) a group of species was defined which shows perennial life form (frequently dwarf shrubs) and usually fimbriate bract appendages ending in a small spine. This group, contrarily to the other two sections, was defined also geographically as including only individuals from NW Africa and the Iberian Peninsula, but leaving behind morphologically similar forms from the eastern Mediterranean. The delimitation between these three sections is more than problematic. The distinction between the two sections *Phalolepis* and *Centaurea* based on one single morphological character is highly questionable if this single character shows intermediate forms or is not present at all (as in some species from the central Mediterranean, where the bract appendages are totally or almost totally reduced). The presence of intermediate forms was demonstrated by Wagenitz (1989) and appears in many species descriptions (e.g., López and Devesa, 2008; Breitwieser and Podlech, 1986). Intermediate forms were commonly attributed to hybridization between the two sections, without doubting the monophyly of the two sections (Garcia-Jacas *et al.*, 2006; Suárez-Santiago *et al.*, 2007b). Also the delimitation and monophyly of section *Willkommia*, is not clear at all. Towards east, *C. attica* Nyman from Greece shows similar bract appendages. Similar species are also found in NE Libya (*Centaurea cyrenaica* Bég. & A. Vacc.) and on the easternmost shore of the Mediterranean, for example *C. dumulosa* Boiss., *C. damascena* Boiss. and also *C. virgata*. All these species are assigned to section *Centaurea*. Intermediates between sections *Willkommia* and *Centaurea* are frequent on the Iberian Peninsula, where most members of section *Centaurea* show somewhat fimbriate and at least slightly spiny bract appendages. The membership of species like *Centaurea cordubensis* and *C. monticola* to any of these two sections is unclear, which can also be seen in incongruencies between taxonomic treatments (Blanca López, 1981a vs. López and Devesa, 2008). But also the delimitiation between *Willkommia* and *Phalolepis*, following sole morphological observations, is not precise. Partly or entirely membranaceous bract appendages within *Willkommia* are found in NW Africa (*C. debdouensis* Breitw. & Podlech, *C. pomeliana* Batt.) and on the Iberian Peninsula (*C. avilae* Pau). *Centaurea tougourensis* Boiss. & Reut., one of two *Phalolepis* species from NW Africa, has a perennial, subshrubby life form and a small spine on their otherwise membranaceous bract appendages – very similar to members of section *Willkommia*, of which it is surrounded by.

Besides the separation of these three subgenera, Dostál (1975, 1976) distinguished between two subgroups within his subgenus *Phalolepis* and 11 within *Acrolophus*, stressing for their delimitation upon morphology of leaves, indumentum and bract appendages. Further groupings within the three sections are used in local treatments, for example in Pignatti (1982).

1.4.3 – Hybridization

Hybridization is the breeding of two different species, but also between two separate lineages within the same species (Arnold, 1997). Both events might have happened frequently in the *Centaurea* group. The question of hybridization between species or within, is depending on the species concept used for species delimitiation (see discussion). The morphologically described species of the *Centaurea* group are not separated through intrinsic breeding barriers. Reports about hybrids within the *Centaurea* group are abundant (e.g., Halácsy, 1902, Georgiadis, 1981; Blanca, 1984). These hybrids are frequently fertile and homoploid (Blanca López, 1981b; Ochsmann, 1998; Pisanu *et al.*, 2011), sometimes also polyploid (Blair and Hufbauer, 2010; Mráz *et al.*, 2012). Also hybrids with not closely related species – for example from the WMC – were reported (Pau, 1914; Prodan, 1930; A. Susanna pers. obs.), these however, produce usually no fertile offspring due to their distinct chromosome numbers and may therefore not lead to reticulations. An important aim of the presented work is to understand the role that hybridization plays in the *Centaurea* group and to distinguish it from other biological processes, which may produce similar patterns in the molecular data.

1.4.4 – Systematic treatments of the *Centaurea* group in light of molecular approaches

From the late 90 onwards, a series of molecular investigations in the *Centaurea* group (= *Acrolophus* subgroup) has been conducted, using mainly DNA sequence data (Ochsmann, 2000; Garcia-Jacas *et al.*, 2001, 2006; Wagenitz *et al.*, 2006; Suárez-Santiago *et al.*, 2007b; Beltrame, 2007; Mráz *et al.*, 2012). Additionally, some work has been done, considering small species groups and using microsatellites techniques (Marrs *et al.*, 2006; Suárez-Santiago *et al.*, 2007a), isozymes (Bancheva *et al.*, 2006, 2011), RAPD (Tornadore *et al.*, 2000; Sozen and Ozaydin, 2010) and SDS-PAGE (Uysal *et al.*, 2010). Interestingly, the traditional sectional division based on morphological traits is not confirmed in any of these molecular works. However, despite all these efforts, the insights about groupings within the *Centaurea* group are still insufficient. Therefore, one of the main aims of the presented work is to improve the knowledge within the CMC and, especially, within the *Centaurea* group.

1.4.5 – Species delimitation within the *Centaurea* group

As taxonomic treatments within the *Centaurea* group (= *Acrolophus* subgroup) are highly incongruent, a really crucial question arises: what has to be considered as a species and what not? From the answer of this question depend many other investigations, like the diagnosis of the conservation status and conservation strategies (e.g., Townsend-Peterson and Navarro-Sigüenza, 1999) or the calculation of diversification rates (e.g., Magallón and Sanderson, 2001; Ryberg *et al.*, 2011). Modern, proposed species concepts agree in the point that species are separately evolving metapopulation lineages (de Queiroz, 2007), but members of the genus *Centaurea*, like most other plant species as well, were and are still described following a purely phenetic species concept (Michener, 1970), i.e. only by assessing morphological characters in the hope that all populations bearing these characters would correspond to a single lineage. This approach itself may be prone to produce inaccurate results, but even more difficult is the question how much morphological divergence is enough to separate two morphologically distinct plant groups as separate species. Additionally, the same or similar characters may arise separately and therefore not be synapomorphies but rather homoplasic traits. Differences in species numbers produced by different modi operandi are huge. Examples can be easily seen if we compare different taxonomic treatments in *Centaurea*. It is highly welcoming that this problem is matter of large scientific dispute (cf., Isaac *et al.*, 2004; Harris and Froufe, 2006; Knapp *et al.*, 2006) – finally all other scientific disciplines are dependent on this question. In the presented work, the species definition and delimitation problem is explicitly treated, using molecular tools and comparing the results with traditional classifications.

1.4.6 – Reproduction and pollination biology of the *Centaurea* group

Centaurea is insect pollinated, mainly by bees and bumblebees (Harrod and Taylor, 1995; Bilisik *et al.*, 2008; Albrecht *et al.*, 2009; McIver *et al.*, 2009). Members of *Centaurea* group are only rarlely selfing or are even obligate outcrossers (Harrod and Taylor 1995; Hardy *et al.* 2004). Reproduction is usually sexual (Noyes, 2007), although facultative apospory has been reported (Cela Renzoni and Viegi, 1982).

The average seed dispersal in the *Centaurea* group might be rather short. Colas *et al.* (1997) reported only 32 cm for *Centaurea corymbosa* Pourr. Notwithstanding the presence of a pappus in most species, it is too short to promote efficient wind dispersal. Further aid for seed dispersal is given by an elaiosome (Dittrich, 1968).

1.4.7 – Caryology of the *Centaurea* group

The *Centaurea* group has a basic chromosome number of x = 9. Most counted populations are diploid (2n = 18). A certain amount of populations, however, have been counted as tetraploid (2n = 36), one as hexaploid (2n = 6x = 54 in *C. cithaeronea*, Phitos and Constantinidis, 1993). Two species, *Centaurea exarata* Boiss ex Coss. and *C. subtilis* Bertol., traditionally assigned to the *Centaurea* group with divergent chromosome numbers (x = 11) possessed the *Jacea* ribotype and had to be removed from the *Centaurea* group (Garcia-Jacas *et al.*, 2006; Hilpold *et al.*, 2009). Tetraploids seem not to compose separate lineages but arise independently through polyploidization from diploid parents (Španiel *et al.*, 2008). If these parents belong to the same species (autopolyploidization) or to different ones (allopolyploidization) is centre of recent scientific work (Mráz *et al.*, 2012). Both mechanisms are possible. Counts of chromosome numbers differing from the basic number x = 9 are extremely rare: 2n = 44 (in *C. affinis* Friv.; Baden, 1983), 2x = 16 (in *C. deustiformis* Adamović; Strid, 1983; in *C. diffusa* Lam., Bancheva and Greilhuber, 2006), 2n = 32 (in *C. arenaria* Willd., Bancheva and Greilhuber, 2006). The presented work is not primarily focussed on caryological questions. Chromosome numbers, however, give evidence for questionable taxonomic assignations and can be helpful to delimiting species – the control of the chromosome number is therefore an important tool in the presented work.

1.4.8 – Phytochemistry of the *Centaurea* group

Whoever collected *Centaurea* in the field using the bare hands to eradicate them, may have noticed that the skin of the hands gives a very bitter taste if it gets in contact with the mouth. *Centaurea* is indeed rich in secondary metabolites, which may mostly be a protection against herbivores (Olson and Kelsey, 1997; Susanna and Garcia-Jacas, 2009) or have antimicrobial activity (Karioti *et al.*, 2001; Ugur *et al.*, 2009). As in the entire tribe Cardueae, these metabolites are predominantly lipophilic compounds, especially sesquiterpene lactones (e.g., Tarasov *et al.*, 1975; Koukoulitsa *et al.*; 2005; Karamenderes *et al.*, 2007). Among other compounds there are flavonoids (Zapesochnaya *et al.*, 1978; Nacer *et al.*, 2006), essential oils (Altintas *et al.*, 2004) and phenols (Bubenchikov *et al.*, 1992). Many phytochemical studies have been published over the last decade (Table 2) but only very few treat explicitly systematic questions (Yildirim *et al.*, 2009). The presented work won't treat any further phytochemical questions.

Table 2: Species of the *Centaurea* group which have been investigated phytochemically and the correspondent citation.

Species	Published articles
C. affinis	Tešević *et al.*, 2007
C. aggregata	Zapesochnaya *et al.*, 1978; Yildirim *et al.*, 2009
C. arenaria	Tešević *et al.*, 2007; Csapi *et al.*, 2010
C. aplolepa	Tava *et al.*, 2010 (subsp. *carueliana*)
C. attica	Koukoulitsa *et al.*, 2005
C. austro-anatolica	Ugur *et al.*, 2009
C. besseriana (*C. ovina* Aggr.)	Formisano *et al.*, 2011
C. cadmea	Karamenderes *et al.*, 2007
C. calolepis	Tekeli *et al.*, 2010, 2011; Erel *et al.*, 2011
C. cariensis	Tekeli *et al.*, 2010, 2011 (subsp. *maculiceps* & *macrolepis*); Ugur *et al.* 2010 (subsp. *niveotomentosa*)
C. cristata	Formisano *et al.*, 2010
C. cuneifolia	Tešević *et al.*, 2007; Rosselli *et al.*, 2009
C. deusta	Karioti *et al.*, 2001; Koukoulitsa *et al.*, 2005; Tešević *et al.*, 2007 (subsp. *splendens*)
C. dichroa	Altintas *et al.*, 2004
C. diffusa	Tharayil *et al.*, 2009
C. euxina	Rosselli *et al.*, 2009
C. glaberrima	Tešević *et al.*, 2007
C. gracilenta	Formisano *et al.*, 2011
C. pelia	Lazari *et al.*, 2000
C. pseudomaculosa	Zapesochnaya *et al.*, 1978; Bubenchikov *et al.*, 1992
C. spinosa	Saroglou *et al.*, 2011
C. spinosociliata	Formisano *et al.*, 2010
C. stoebe	Perry *et al.*, 2005; Broz and Vivanco, 2006; Tešević *et al.*, 2007; Tharayil and Triebwasser, 2010; Pollock *et al.*, 2011
C. thessala subsp. *drakiensis*	Lazari *et al.*, 2000; Koukoulitsa *et al.*, 2005
C. tougourensis	Nacer *et al.*, 2006
C. virgata	Tarasov *et al.*, 1975 (subsp. *squarrosa*); Tešević *et al.*, 2007 (subsp. *squarrosa*); Yildirim *et al.*, 2009; Tekeli *et al.*, 2011
C. zuccariniana	Lazari *et al.*, 2000

1.4.8 – Economic importance of the *Centaurea* group

A few members of the *Centaurea* group are used as ornamentals, first and foremost the "Velvet Centaurea" *Centaurea cineraria* (Ellis, 1999). Because of its secondary compounds, some species in *Centaurea* are used as medicinals, especially in folk medicine (Nacer *et al.*, 2006; Akkol *et al.*, 2009). The sesquiterpene lactone cnicin, found in *Centaurea benedicta* (the name of the compound derives from its former name *Cnicus benedictus* L.) but also in members of the *Centaurea* group (e.g., Olson *et al.*, 1997; Erel *et al.*, 2011) is sometimes used for bitter tonics (Wikipedia, 2012).

Most important, however, are not the benefits that members of the *Centaurea* group provide, but their negative impacts on agriculture and landscape. *Centaurea stoebe* (= *C. maculosa* Lam.) and to a lesser extent *C. diffusa* and *C. virgata* subsp. *squarrosa* (Boiss.) Gugler, are tremendous invasive weeds in pastures of the US and Canada. *Centaurea stoebe*, 'one of North America's most devastating invasive plants' (Blair and Hufbauer, 2010), diminishes the fodder value of pastures infesting about 3 million ha (Di Tomaso, 2000) producing in this way economic damage of hundreds of million dollars every year. The estimated damage for the agriculture of Montana amounts to 42 million dollars (Duncan *et al.*, 2001). The fight against these weeds is also the motor of most of the research conducted in *Centaurea* (e.g., Hufbauer and Sforza, 2008; Collins *et al.*, 2011; Pollock *et al.*, 2011; Reinhart and Rinella, 2011). The work presented here is an important contribution for further research in these fields.

1.5 – Justificación del trabajo presente [*justification for the realization of this PhD*]

Centaurea es un género de plantas perteneciente a la familia de las Compuestas y a la tribu *Cardueae*, y da nombre a la subtribu *Centaureinae*. El género es emblemático para la flora mediterránea, de hecho está distribuido alrededor del Mar Mediterráneo y el Mar Negro con algunos cientos de especies. Con una diversidad tan alta, el género constituye un complejo muy importante para la conservación del patrimonio natural de esta zona. Además hay muchas especies de *Centaurea* y sobre todo del grupo *Centaurea* que están presentes con poblaciones muy grandes, haciéndolas componentes importantes de los ecosistemas correspondientes. Otras, en cambio, tienen áreas muy reducidas, y se trata de especies incluidas en los libros rojos de distintos países.

El género *Centaurea* tiene un potencial notable para la medicina. Se viene investigando en muchos estudios sobre sustancias secundarias con posible aplicación práctica, sobre todo antibacterial, pero también hasta antitumoral o hipoglucemiante. Además *Centaurea* contiene algunas especies que se consideran malas hierbas altamente dañinas para la agricultura. Debido a que estas malas hierbas producen daños económicos valorados en cientos de millones de euros cada año, se invierten también millones en la investigación de estas especies problemáticas. Como base para estos estudios, es fundamental entender las relaciones sistemáticas entre las diferentes especies.

Antes de este trabajo, ya se han publicado importantes contribuciones al estudio molecular del género *Centaurea* y del grupo *Centaurea*. Garcia-Jacas *et al.* (2001, 2006) aclararon las relaciones sistemáticas dentro el género entero. Font *et al.* (2002, 2009) y Boršić *et al.* (2011) elucidaron la sistemática de los subgéneros *Lopholoma* y *Cyanus.* Ochsmann (2000) y Suárez-Santiago *et al.* (2007a,b) aportaron conocimientos importantes sobre grupos del grupo *Centaurea*.

El trabajo que presentamos aquí profundiza estos conocimientos incluyendo nuevos métodos moleculares y ampliando el muestreo. Además de las puras cuestiones sistemáticas, hemos usado el grupo *Centaurea* para elaborar preguntas biológicas más generales en el campo evolutivo, biogeográfico y de delimitación de especies, explicado en los siguientes tres párrafos.

La evolución del grupo *Centaurea* dentro del subgénero *Centaurea* es el centro de este trabajo. Por su diversificación reciente este género nos permite estudiar la evolución casi contemporáneamente. El grupo tiene tendencia a desarrollar una gran variabilidad morfológica a poca distancia, un hecho que comportó y sigue comportando la descripción de muchísimos taxones. Sin embargo, estas entidades no desarrollaron barreras que impidan su intercambio genético. Una fase parecida existía probablemente durante la especiación de muchos otros géneros de plantas. El grupo *Centaurea* se puede considerar por eso como un grupo modelo para estudiar esta etapa primaria de diversificación.

El grupo *Centaurea* esta ligado estrictamente a ambientes mediterráneos, que constituyen una zona altamente importante para la conservación del patrimonio mundial de la naturaleza. Dentro de la historia evolutiva del grupo se puede observar patrones biogeográficos parecidos a muchos otros grupos de organismos del mismo ambiente. En conjunto, entender estos patrones nos ayuda a manejar y conservar la diversidad en el Mediterráneo con más efectividad. Los patrones observados dentro del Mediterráneo son comparables con los de otras zonas del mundo, y en conjunto el estudio filogeográfico en *Centaurea* ayuda a entender cómo se ha desarrollado la diversidad en nuestro planeta en perspectiva temporal y geográfica.

El número de especies dentro del grupo *Centaurea* varía muchísimo dependiendo del tratamiento taxonómico que se consulta. Ya que la especie es la unidad fundamental en la biología, esta situación tiene consecuencias tremendas para muchas otras disciplinas como la ecología y la conservación. Por este motivo, alcanzar una clasificación clara debe ser un objetivo central en la sistemática de los organismos. Pero para lograr este fin tendremos que ser conscientes del concepto de especies que estamos usando y refinarlo; así, no tendremos que temer el uso de información molecular para su delimitación.

2.

Aims

[*Objectius*]

The main aims of this PhD are:

- To increase the sampling in comparison to former studies, including new plant material from all over the Mediterranean
- To elucidate the systematic relationships within the *Centaurea* group with further molecular markers
- To test if the main groups within the CMC are monophyletic. These groups are the *Centaurea* group with its three sections *Centaurea*, *Phalolepis* and *Willkommia*.
- To trace back the evolutionary past of the study group by conducting dating analyses and comparing the results with the geological past.
- To use further molecular tools (i.e. AFLP) for improving knowledge about systematics and biogeography of the *C. cineraria* group.
- To understand the role of hybridization in the study group, by cloning ITS sequences and sequencing nuclear low copy regions.
- To improve the knowledge about species delimitation within the study group and use recently developed methods for testing different hypotheses of delimitation in a coalescence framework.
- To study the identity of some extremely rare species discovered during field collections carried out in the preparatives of this work.

3.

Informe dels directors de la Tesi Doctoral referent al factor d'impacte i a la contribució del doctorand a cadascun dels articles publicats

[*document providing information about the impact factor of the publications and the contribution made by the PhD student for every single article*]

Núria Garcia Jacas i Roser Vilatersana Lluch, investigadors de l'Institut Botànic de Barcelona, directors de la Tesi Doctoral elaborada per Andreas Hilpold, amb el títol Evolució del subgrup *Acrolophus* del gènere *Centaurea* (*Evolution of the Acrolophus subgroup of the genus Centaurea*)

INFORMEN

Que els treballs de recerca duts a terme per Andreas Hilpold com a part de la seva formació predoctoral i inclosos a la seva Tesi Doctoral han donat lloc a 2 publicacions i 3 manuscrits (1 enviat a revisió i 2 més pendents d'enviar en el moment del dipòsit de la tesi). A continuació es detalla la llista d'articles així com els índexs d'impacte (segons el JCR de la ISI web of Knowledge) de les corresponents revistes.

1- Hilpold A., N. Garcia-Jacas, R. Vilatersana & A. Susanna (2009). Two additions to the *Jacea-Lepteranthus* complex: Parallel adaptation in the enigmatic species *Centaurea subtilis* and *C. exarata*. *Collectanea Botanica (Barcelona)* 28: 47-58.

Collectanea Botanica (Barcelona) no té índex d'impacte però és una revista internacional amb accés obert (*open access*) y procés de revisió externa (*peer reviewed*), inclosa a la categoria de *Plant Sciences*. Aquest és el primer treball del doctorand. El resultat obtingut, dins un estudi més gran, va donar lloc a una publicació independent degut a la seva importància taxonòmica. La responsabilitat de A. Hilpold en el treball ha sigut la següent: recol·leccions de camp, treball de laboratori (seqüenciació de la regió ITS del ADN ribosòmic), anàlisis filogenètiques i co-redacció final.

2- Hilpold, A., P. Schönswetter, A. Susanna, N. Garcia-Jacas & R. Vilatersana (2011). Evolution of the central Mediterranean *Centaurea cineraria* group (Asteraceae): evidence for relatively recent, allopatric diversification following trans-oceanic seed dispersal. *Taxon* 60: 528-538.

L'índex d'impacte de la revista *Taxon* és, en l'actualitat, 2.364. Aquesta revista està inclosa en el primer quartil (Q1) a la categoria *Plant Sciences*. Tenint en compte l'índex d'impacte, *Taxon* ocupa el 46è lloc de la seva categoria, que inclou 188 revistes.

La publicació és fruit de la col·laboració amb el Dr P. Schönwetter, gràcies a una estada de tres mesos del doctorand a la Universitat de Viena durant el seu període de formació.

La responsabilitat del doctorand A. Hilpold en aquest treball ha estat l'aprenentatge i realització dels marcadors AFLP, la seqüenciació de la regió cloroplàstica *rpl32*, la co-realització de les anàlisis filogeogràfiques i la co-redacció final.

3- Hilpold A., J. López-Alvarado, N. Garcia-Jacas & E. Farris (revisió). Tackling taxonomic ambiguity with an integrative approach: the case of *Centaurea corensis*.
Plant Systematics and Evolution

S'ha enviat a la revista *Plant Systematics and Evolution* amb índex d'impacte de 1.369, en l'actualitat està inclosa en el segon quartil (Q2) a la catagoria de *Plant Sciences*. Tenint en compte l'índex d'impacte, *PSE* ocupa el 83è lloc de la seva categoria, que inclou 188 revistes.

La responsabilitat del doctorand en aquest treball ha estat les recol·leccions de les mostres, la definició de la metodologia i la redacció final.

4- Hilpold A., R.Vilatersana, A. Susanna, K. Romaschenko, I. Boršić, R. Filigheddu, K. Ertuğrul, T. Constantinidis, V. Suárez-Santiago & N. Garcia-Jacas. Phylogeny of the *Centaurea* group (*Centaurea*, Compositae) – geography is a better predictor than morphology

Es preveu enviar aquest article a la revista *Annals of Botany* amb índex d'impacte de 3.388 en l'actualitat. Aquesta revista està inclosa en el primer quartil (Q1) a la categoria *Plant Sciences*. Tenint en compte l'índex d'impacte, ocupa el 21è lloc de la seva categoria, que inclou 188 revistes.

5- Hilpold, A., N. Garcia-Jacas, A. Susanna, C. Löser, R. Vilatersana & B. Oxelman. Current taxonomy in light of a species coalescence approach in the *Centaurea alba* complex.

Es preveu enviar aquest article a la revista *Systematic Biology* amb índex d'impacte de 9.532 en l'actualitat. Tenint en compte l'índex d'impacte, aquesta revista ocupa el 3r lloc de la seva categoria *Evolutionary Biology*, que compren 45 revistes i està inclosa en el primer quartil (Q1).

Aquest treball s'ha fet amb col·laboració del Dr. B. Oxelman de la Universitat de Göteborg (Suecia) gràcies a una estada del doctorand durant la seva etapa de formació.

A més, CERTIFIQUEN:

Que Andreas Hilpold ha participat activament en el desenvolupament del treball de recerca associat a cadascun dels articles, així com en la seva elaboració. En concret, la seva participació a cadascuna de les tasques ha estat la següent:

- Plantejament inicial dels objectius de cadascun dels treballs.
- Recol·leccions dels materials estudiats en els treballs
- Realització de les seqüències de DNA i les anàlisis filogenètiques.
- Desenvolupament i posada a punt dels marcadors AFLP (amb una estada de tres mesos a la Universitat de Viena en el laboratori del Dr. P. Schönswetter).
- Càlcul de resultats i anàlisi de dades. Especialment anàlisi de coalescència (amb una estada a la Universitat de Göteborg amb el Dr. B. Oxelman)
- Redacció dels articles i seguiment del procés de revisió dels mateixos.

Atentament,

Barcelona, 4 de Juny de 2012

Núria Garcia Jacas
Roser Vilatersana Lluch

4.

Discussion

[*Discussió dels resultats obtinguts*]

Taxonomical implications

We consider the Circum-Mediterranean Clade (CMC) as monophyletic, confirming thereby the study of Garcia-Jacas *et al.* (2006). The clade is well delimited in the ITS. In the cpDNA the situation is less clear: the three species *Centaurea benedicta*, *C. hierapolitana* and *C. tossiensis* are sister to the rest of the CMC and to one of the outgroup species (*C. cheirolopha*; EMC). *C. benedicta* (formerly *Cnicus benedictus*) possess fimbriate bract appendages with a spiny tip, similar to those in section *Ammocyanus* or some members of the *Centaurea* group (= *Acrolophus* subgroup). The ribbed achenes in *C. benedicta* (Dittrich, 1968) were considered an autapomorphy of the genus *Cnicus*. However, strongly ribbed achenes can also be found in *C. akamantis* (Georgiadis and Chatzikyriakou, 1993), and some species of the *Centaurea* group from the Iberian Peninsula have faintly ribbed achenes (López and Devesa, 2008). These two morphological characters point also to a membership in the CMC. The basic chromosome number $x = 11$ is consistent with those in the CMC ($x = 7–11$; see Table 1). *C. hierapolitana* and *C. tossiensis*, which group together in both markers (hereafter referred to as *C. hierapolitana* group) were earlier considered as members of section *Phalolepis* of the *Centaurea* group (= *Acrolophus* subgroup), due to their membranaceous bract appendages and their narrow leaves. Morphology points therefore towards a membership in the CMC, following the ITS tree. Their basic chromosome numbers ($x = 8$ in *C. hierapolitana* and $x = 9$ in *C. tossiensis*; Uysal *et al*., 2009) are also consistent with a membership in the CMC. The *C. hierapolitana* group and *C. benedicta* group together in the ITS tree, but there is no morphological evidence that supports this clade. A further species that is quite isolated in the CMC in both markers is *Centaurea akamantis*, endemic to the island of Cyprus (basic chromosome number $x = 9$; Georgiadis and Chatzikyriakou, 1993). It shows divided leaves and ciliate bract appendages, which led to its inclusion within the section *Centaurea* of the *Centaurea* group. The two annual species *C. ammocyanus* and *C. patula*, traditionally included in section *Ammocyanus*, are another well separated group in the ITS tree, but not in the *rpl32*, where they fall into the *Centaurea* group. Their divergent chromosome numbers ($x = 7$ in *C. patula* and $x = 8$ in *C. ammocyanus*; Ghaffari, 1989; Garcia-Jacas *et al*., 1996), however, supports the idea that they are distinct from the rest of the *Centaurea* group (hereafter referred to as *C. ammocyanus* group). Apart from these isolated species, there are two main groups, which include the majority of the described species of the CMC. They are the *Jacea-Phrygia* group, with a very uniform basic chromosome number of $x = 11$ and the *Centaurea* group (= *Acrolophus* subgroup) with a likewise uniform basic chromosome number $x = 9$. These two groups appear clearly monophyletic in the ITS. In the *rpl32* tree, however, some species of the *Jacea-Phrygia* group are nested within the *Centaurea* group. The morphological separation between the two groups is, with some exceptions, quite clear (*Jacea-*

Phrygia with undivided leaves, whereas members of the *Centaurea* group exhibit deeply divided leaves), and supports the topology of the ITS tree. We consider therefore these two clades as monophyletic. Best predictor for the membership in any of these two clades is the basic chromosome number: *C. subtilis* and *C. exarata*, both counted as $x = 11$, but with deeply divided leaves in the first case and narrow leaves in the second one – a fact which led to their assignation to section *Acrolophus* (= *Acrolophus* subgroup) – must be considered as members of the *Jacea-Phrygia* group, following the ITS tree (see publication 2). In summary, there are six well delimited groups in the CMC (Fig. 5). The exact relationships between these groups, however, could not be resolved in our studies, but further research is under way.

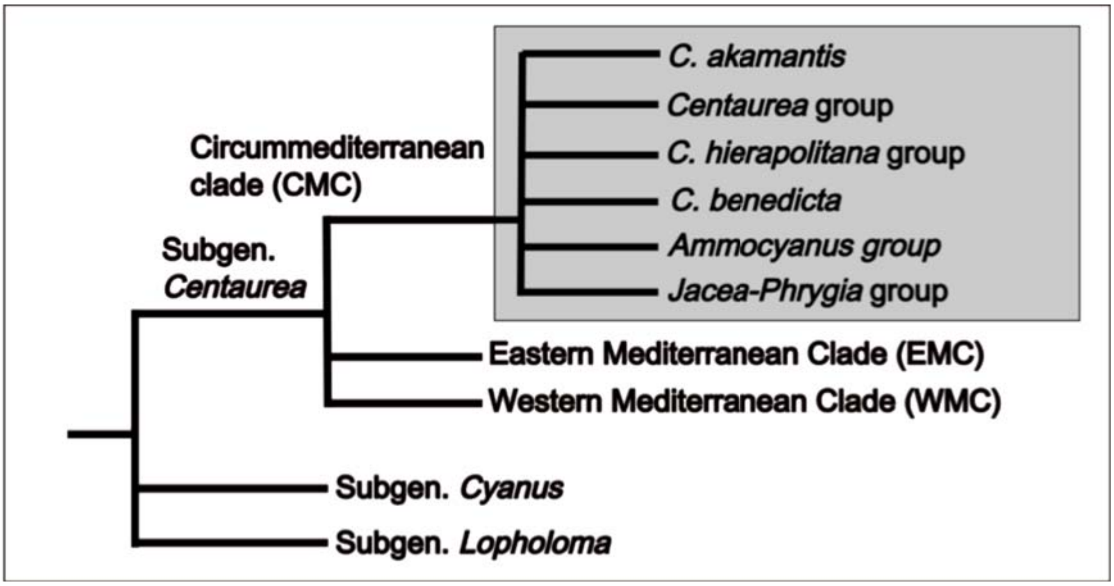


Figure 5: Scheme of the systematic relationships within genus *Centaurea*, focussing on the *Jacea*-Group and therein on the CMC. All clades are supported by molecular data.

Incongruences and geographical patterns

Within the *Centaurea* group, it is impossible to establish clearly monophyletic groups. Despite the fact that both gene trees show well supported clades, these clades are not congruent between the two markers. They do not follow the traditional subdivision in three sections (based on the morphology of the bract appendages), but follow much better their geographical distribution. A geographical pattern is very well visible in the ITS and in the cpDNA (see publication 1). The Iberian Peninsula is quite uniform in both markers, suggesting that the Iberian populations conform a monophyletic clade. The populations of the central and the eastern Mediterranean, however, show many different ribo- and haplotypes. The NW African populations are quite uniform in the cpDNA but show very different ribotypes in the ITS. Also the study on the *Centaurea alba* complex (publication 4), including additionally five nuclear, single copy markers, shows a clear geographical structure, supporting the presence of only three different groups: an eastern Mediterranean one, including the Turkish populations; a central Mediterranean one, including the Greek and most of the Italian populations; and a western Mediterranean one, including all NW African and Iberian populations and one population from NW Italy. Once again, these groupings do not reflect the traditional sectional division. Clear support for geography as better predictor for molecular relationships was also given by an AFLP study about central Mediterranean members of the *Centaurea cineraria* group (member of the *Centaurea* group; publication 3). In this study, all populations from the Sicilian archipelago are grouped within one clade, including the quite aberrant *Centaurea parlatoris*, and clearly separated from populations from the Italian mainland, traditionally included in the *C. cineraria* group.

Hybridization

In the study of Suárez-Santiago *et al.* (2007b) hybridization was already declared as major driver for incongruences between morphology and molecular results in the *Centaurea* group. Also in our studies, hybridization could be shown by means of cloning of the ITS and subsequent detection of different ribotypes within the same individual (publications 1 and 5). Also the classification of two alleles of the same individual in two different clades in single copy regions (publication 4) might be best explained by hybridization. Furthermore, the incongruence between the ITS and the cpDNA maybe caused by hybridizations, since their inheritance is different (but we cannot exclude incomplete lineage sorting as cause). Hybridizations between different lineages of the *Centaurea* group might have happened frequently, especially between geographically adjacent populations. The existence of hybrid zones (cf. Barton, 1979; Barton and Hewitt, 1989) was reported in some cases and could also be observed during collection campaigns for this work. Hybridization between the major, geographically separated clades might have been less frequent, since achene and pollen dispersal is fairly limited in the *Acrolophus* subgroup. However, the Pleistocene is characterized by frequent climatic oscillations and subsequent, vegetational changes, and thereafter the distribution of the major clades of the *Centaurea* group might have changed frequently: this may have allowed even the more distant clades to become sympatric. In the some extreme cases, hybridization may have led to a total disappearance of a clear morphological and molecular separation between two populations.

Species delimitation

The description of a plethora of species in the *Centaurea* group is based on the presence of morphologically well distinct populations or groups of populations. Their morphological separation, however, is frequently based on only one single character, which may be the color of the bract appendages (e.g. *C. brulla* vs. *C. deusta*), the shape of the bract appendages (e.g. *C. japygica* vs. *C. leucadea*) or the growth form (e.g. *C. giardinae* vs. *C. parlatoris*). The question of what amount of morphological difference in such cases is sufficient to describe or accept separate species is totally unresolved. Our studies cast serious doubts about species delimitation in the *Centaurea* group. Species, which were included with more than one population, in any of the two markers, are almost never monophyletic in the resulting gene trees (publication 1). The variation in both markers is atypically low for a group encompassing more than 200 described and accepted (see Greuter, 2008) species. Most species seem to be interfertile – migration between geographically adjacent and morphologically well distinct populations may have blurred the species boundaries. Coalescence models give high evidence that in *Centaurea* group the actual species concept is too narrow (publication 4).

Biogeographical insights

The CMC seems to derive from the eastern Mediterranean. All six clades (see Fig. 5) occur in Turkey or Cyprus. Within the *Jacea-Phrygia* group, the Turkish species *C. inexpectata* is sister to all other species included in our study. Four of the six clades are restricted to Turkey or Cyprus (the dispersal of the medicinal herb *C. benedicta* into the central and western Mediterranean may have happened in the Quaternary with human help, like many other archaeophytes too). First ancestral area reconstruction analyses (A. Hilpold, unpubl. research) with the program Lagrange v. 2.0.1 (Ree and Smith, 2008) support this hypothesis. The diversification into these six clades is likely to have happened between 6 and 9 Mya (publication 1), following the dating analyses of both the ITS and the *rpl32*, also in the eastern Mediterranean. Starting from the eastern Mediterranean, the dispersal into the central and western Mediterranean begun probably between 4 and 6 Mya. This allows the interpretation that the Messinian Salinity Crisis (5.96–5.33 Mya; Hsü, 1972) may have favored the dispersal into the west. However, transoceanic seed dispersal may have happened as well, as evidenced for the *Centaurea cineraria* group (publication 3). For reaching the westernmost part of the Mediterranean, two pathways are possible: via south, over Greece, southern Italy and NW Africa; or via north, crossing the Balkans, the north of Italy, the south of France for reaching finally the Iberian Peninsula. The distribution of the haplotypes and ribotypes along these two pathways suggests that both corridors have been used for dispersal in different epochs. The steppe climates during cold periods of the Pleistocene may have fostered the northern pathway.

The richness in different ribotypes in NW Africa suggests that this area may have played an important role for the diversification within the *Centaurea* group. Further diversification within the new settled areas may have occurred mainly during the Pleistocene. In Sicily, the diversification into several “species” is supposed to have happened during the last few hundred thousand years (publication 3). Finally, in *C. corensis*, we found evidence for very recent polyploid speciation and dispersal (publication 5).

5.

Conclusions

[*Conclusions finals*]

- The used markers were not sensitive enough to trace back the exact evolutionary history within the *Centaurea* group (= *Acrolophus* subgroup). The use of further markers may not improve these results.
- AFLP-Markers were sensitive enough to show the evolutionary history on a small geographical scale, but did not give enough resolution on a large scale.
- Topologies and branch lengths between all used markers were incongruent. Both hybridization and ILS might be responsible for this.
- Diverging basic chromosome numbers are a good signal for wrong taxonomical assignation. The basic chromosome number in the *Centaurea* group is almost totally consistently $x = 9$, with both diploid and tetraploid populations occurring.
- In order to establish the *Centaurea* group (formerly *Acrolophus* subgroup) as a monophyletic clade, *Centaurea akamantis*, *C. tossiensis* and *C. hierapolitana* have to be excluded.
- Caryological, morphological and molecular evidence suggests that the *Jacea-Phrygia* group is a monophyletic clade within the CMC
- *Centaurea benedicta* (formerly *Cnicus benedictus*) is very likely part of the CMC, even though its position within the group is not totally clear.
- The sectional division within the CMC should be reorganized. We propose a subdivision into seven sections, which are the following: section *Centaurea* (= *Acrolophus* subgroup), section *Akamantis*, section *Hierapolitana* (including *C. hierapolitana* and *C. tossiensis*), section *Ammocyanus,* section *Cnicus*, section *Jacea* and section *Phrygia.* Monophyly of the latter two is not proved.
- The character of the bract appendages is not reliable for taxonomic deduction because it is too variable and prone to convergent evolution.
- Diversification within the *Centaurea* group is rather recent and started about 5 Ma years ago, diversification took place during Plio- and Pleistocene.
- The *Centaurea* group might have its origin in the eastern Mediterranean. The Iberian Peninsula is genetically the most uniform area.
- The young age of diversification and migrations within the group, mainly after the MSC, suggests that transoceanic dispersal took place several times, between Greece and SE Italy, between Sicily and NW Africa and between NW Africa and the Iberian Peninsula.
- Species delimitation in the *Centaurea* group is highly critical. Many described species may not be “good species” in sense of a independently evolving metapopulation lineage.

6.

Resumen en castellano

[*detailed summary in Spanish*]

Resumen castellano

Centaurea dentro de las Cardueae

Centaurea es un género importante de la tribu *Cardueae* (familia Compuestas) y es uno de los grupos más emblemáticos de la flora mediterránea por su gran variedad y su valor ornamental. La tribu *Cardueae* tiene unas 2400 especies repartidas en 72 géneros y es uno de los grupos más grandes dentro de las Compuestas. Incluye cinco subtribus: *Carlininae*, *Echinopsinae*, *Carduinae*, *Centaureinae* y *Cardopatiinae*, la última redescubierta hace poco tiempo. La mayoría de las especies de las *Cardueae* pertenecen a las subtribus *Carduinae* y *Centaureinae*. Las *Centaureinae* son claramente un grupo monofilético e incluyen unas 650 especies en 31 géneros. La mayoría de estos tienen hojas no espinosas, pero muchos tienen espinas en las brácteas. Igual que las *Cardueae*, las *Centaureinae* son también en su mayoría hierbas perennes o bienales. Dentro de este grupo, el mayor problema sistemático ha sido tradicionalmente la delimitación del género *Centaurea*, ya que investigaciones moleculares mostraron que el antiguo género *Centaurea* era polifilético. Por eso se excluyeron del género algunos grupos, entre otros el que incluía la especie tipo *C. centaurium*. Para el género *Centaurea* restante, se eligió *C. paniculata* como nueva especie tipo.

Caracterización, distribución y subdivisión del género *Centaurea*

El género *Centaurea* incluye hierbas anuales, bienales o perennes con hojas normalmente no espinosas. Las semillas tienen un hilo lateral. Las flores periféricas son normalmente estériles y vistosas. El carácter morfológico más importante para la subdivisión sistemática es la forma de los apéndices de las brácteas. El género está distribuido principalmente en el Mediterráneo y en la Región Irano-Turania y contiene entre 250 y 500 especies.

Centaurea tiene tres subgéneros bien delimitados también por métodos moleculares: *Lopholoma* (sinónimo *Acrocentron*), *Cyanus* y *Centaurea* (corresponde al grupo *Jacea* en Garcia-Jacas *et al.*, 2006).

El subgénero *Centaurea* está representado en el Mediterráneo por algunos cientos de especies. El carácter más importante de este grupo es su tipo de polen, llamado tipo de polen *Jacea*. Este polen es prolado-subprolado hasta subprolado-esferoidal con una escultura escábrida y la exina caveada. Los resultados de estudios moleculares dividen el grupo *Centaurea* en tres grandes clados nombrados según su distribución geográfica: el clado mediterráneo oriental (*Eastern Mediterranean Clade*; EMC), el clado del mediterráneo occidental (*Western Mediterranean Clade*; WMC) y el clado circum-mediterráneo (*Circum-Mediterranean Clade*; CMC). El grupo objeto de este trabajo pertenece al último de estos tres clados. La mayoría de las especies de los primeros dos grupos tienen los apéndices de las brácteas espinosos. El CMC tiene los apéndices membranáceos o ciliados-fimbriados. Un carácter importante dentro del género *Centaurea* es el número cromosómico básico, que varía entre siete y doce. Los grupos más evolucionados tienen normalmente el número cromosómico reducido. La anual *C. patula* tiene el número cromosómico mas bajo encontrado hasta la fecha con el número básico de $x = 7$. La reducción del número cromosómico durante la evolución del género se interpretó como adaptación a condiciones secas.

El clado circum-mediterráneo (CMC)

Dentro de este grupo, la forma de los apéndices de las brácteas es el carácter más distintivo. Hay tres formas extremas: apéndices fimbriados/ciliados, apéndices lacerados membranáceos y por último, sin apéndice. Existen dos grupos principales dentro del clado: el grupo *Centaurea* (=subgrupo *Acrolophus*) y el grupo *Jacea-Phrygia*. El primer grupo tiene las hojas divididas y la brácteas ciliadas o membranáceas, y el segundo tiene las hojas enteras y las brácteas fimbriadas/ciliadas o también membranáceas. Además de estos dos grupos se comprobó en estudios moleculares previos que *Centaurea benedicta* (= *Cnicus benedictus*) y *C. patula*, que tradicionalmente se incluye dentro de la sección *Ammocyanus*, forman parte del CMC.

El grupo *Jacea-Phrygia* está distribuido sobre todo en el Mediterráneo meridional y se extiende también por el centro y norte de Europa. En comparación con el grupo *Centaurea*, está mas adaptado a condiciones algo húmedas, sus hojas tienen un carácter más mesófilo. Hasta este trabajo no se podían resolver las relaciones sistemáticas dentro de este grupo debido a la falta de resolución de los marcadores moleculares y a la fuerte presencia de hibridación. Tradicionalmente se divide el grupo en dos secciones, la sección *Phrygia* (o *Lepteranthus*) y la sección *Jacea*. Esta división, sin embargo, no se refleja en los resultados de los estudios moleculares preliminares.

El grupo *Centaurea* (= *Acrolophus* subgroup)

Este grupo es el objetivo principal del trabajo aquí presentado. Tiene su área de distribución alrededor del Mar Mediterráneo y el Mar Negro, con muchas especies en las Penínsulas Ibérica, Apenina, Balcánica y Anatólica. La mayoría de las especies son diploides, pero también se cuentan algunas poblaciones tetraploides. Todas las especies del grupo crecen en condiciones más o menos áridas y en vegetación abierta, más a menudo en suelos calcáreos que silíceos. Tradicionalmente se distinguen tres secciones dentro de este grupo: la sección *Centaurea* (= sección *Acrolophus*) con brácteas ciliadas/fimbriadas a veces totalmente reducidas, la sección *Phalolepis* con brácteas laceradas membranáceas y la sección *Willkommia* con brácteas fimbriadas terminadas en una espina y con un hábito subarbustivo. Mientras las primeras dos secciones ocurren en todo el Mediterráneo, la sección *Willkommia* tiene un área de distribución restringida a África noroccidental y la Península Ibérica. La separación entre estas tres secciones, sobre todo entre la sección *Phalolepis* y *Centaurea*, se cuestionó ya antes de la aparición de estudios moleculares, basándose en observaciones de poblaciones con caracteres intermedios. Con los primeros estudios moleculares se confirmaron estas dudas, ya que no se encontró ninguna conexión entre los resultados moleculares y las subdivisión seccional. Este hecho se explicó en parte por la falta de barreras de cruzamiento y, a consecuencia de esto, a la presencia de hibridaciones frecuentes entre las secciones. De hecho, se han descrito muchos ejemplos de hibridación dentro del grupo *Centaurea*, con o sin duplicación del genoma.

Otro problema dentro del grupo es la incongruencia extrema entre diferentes tratamientos taxonómicos. La definición de qué se debe considerar una especie es problemática, con consecuencias tremendas para líneas de investigación relacionadas como la ecología o la conservación. Cada año se vienen describiendo especies nuevas dentro del grupo basándose en caracteres puramente morfológicos sin poder resolver la cuestión de si son linajes bien separados y monofiléticos.

El género *Centaurea* es rico en metabolitos secundarios, sobre todo lactonas sesquiterpénicas. La presencia de muchos metabolitos se interpreta como protección contra los herbívoros. Dentro del grupo *Centaurea* los intentos de usar la fitoquímica para objetivos sistematicos no ha dado resultados significativos.

La importancia económica que tiene el grupo *Centaurea* se basa sobre todo en que es una planta perjudicial para la agricultura, ya que algunas especies son malas hierbas, sobre todo en Estados Unidos. *Centaurea stoebe* produce daños por milliones de dólares por año al empeorar la calidad de los pastos. A este hecho se debe la inversión de mucho dinero para la investigación del grupo *Centaurea.*

Los objetivos principales de esta tesis son:

- Aumentar el muestreo en comparación con estudios previos, incluyendo nuevo material de especímenes distribuidos en todo el Mediterráneo.
- Aumentar el conocimiento sobre las relaciones filogenéticas dentro del grupo *Centaurea* con más marcadores moleculares.
- Verificar la monofilia de los grupos principales dentro del clado del Mediterráneo Central del género *Centaurea* (CMC) formado por el grupo *Centaurea* con tres secciones, *Centaurea*, *Phalolepis* y *Willkommia*; el grupo *Jacea-Phrygia* con las dos secciones homónimas, y el grupo *Ammocyanus*.
- Elucidar la historia evolutiva haciendo análisis de datación y discutiendo los resultados en un marco paleogeográfico.
- Probar nuevos marcadores (AFLP) para resolver la posición sistemática y biogeográfica del grupo *C. cineraria* del Mediterráneo Central.
- Estudiar el papel de la hibridación, llevando a cabo clonajes de la región ITS de algunas especies sospechosas de ascendencia híbrida, y usando genes nucleares de copia única.

- Profundizar en el estudio del concepto de especie y la delimitación de especies dentro del grupo de estudio entero y dentro del complejo de *Centaurea alba* de la Península Ibérica, y comprobar hipótesis con métodos de coalescencia recientemente desarrollados.
- Estudiar la identidad de algunas especies extremadamente raras encontradas en el curso de las recolecciones llevadas a cabo para la preparación de este trabajo.

Discusión

Taxonomía del CMC

El CMC aparece claramente monofilético en ITS pero en cpDNA *Centaurea benedicta*, *C. hierapolitana, C. tossiensis* y *C. cheirolopha* (EMC) forman grupos hermanos de todo el resto del CMC. Teniendo en cuenta las similitudes morfológicas y cariológicas entre estos tres especies y el resto del CMC damos más valor a los resultados de ITS y consideramos el CMC como grupo monofilético. Podemos distinguir seis grupos dentro del CMC (incluidas las tres especies anteriores) que son los siguentes: *Centaurea benedicta*, *C. akamantis*, el grupo de *C. hierapolitana* (formado por *C. hierapolitana* y *C. tossiensis*), el grupo *Ammocyanus* (*C. ammocyanus* y *C. patula*), el grupo *Centaurea* (= *Acrolophus subgroup*) y el grupo *Jacea-Phrygia*. Estos seis grupos se diferencian, en su mayoría, por sus números cromosómicos. Sobre todo los dos grupos *Jacea-Phrygia* y el grupo *Centaurea* tienen un número cromosómico muy uniforme. Gracias al número cromosómico divergente (x = 11) y su ribotipo, detectamos que *Centaurea exarata* y *C. subtilis* forman parte del grupo *Jacea-Phrygia* y no del grupo *Centaurea*, como antes se creía.

Incongruencia con la taxonomía tradicional y congruencia con la geografía

Dentro del grupo *Centaurea* no pudimos establecer grupos claramente monofiléticos. Aunque en los dos marcadores hay grupos bien resueltos, estos grupos no son congruentes entre marcadores. La segregación de estos grupos es más acorde con la geografía que con la división seccional tradicional. Creemos que esta incongruencia se debe en parte a procesos hibridación, y en parte a la distribución incompleta de linajes génicos (*incomplete lineage sorting*). Asimismo, el estudio del complejo de *Centaurea alba*, en el cual usamos además de ITS y *rpl32*, otros cinco marcadores nucleares de copia única, nos da una división acorde con la geografía y no con la división seccional. El estudio hecho con AFLP sobre el grupo centro-mediterráneo de *C. cineraria* nos da también un grupo monofilético que sigue la geografía.

Delimitación de especies

La descripción de una multitud de especies dentro del grupo *Centaurea* (= *Acrolophus* subgroup) se basa en diferencias morfológicas claras, pero a menudo basadas en un solo carácter de las brácteas o del hábito. No está claro si estos caracteres son de verdad apropiados para demostrar un proceso de especiación. En el caso de especies de las cuales analizamos más de una población, en muy pocos casos formaban clados monofiléticos en los árboles de genes. La variación genética en los marcadores ITS y *rpl32* es relativamente baja para un grupo de más de 200 especies descritas y aceptadas. El estudio basado en modelos de coalescencia demuestra que en el grupo *Centaurea* el concepto de especies es demasiado estrecho.

Biogeografía

El CMC parece que tiene su origen en el este del Mediterráneo. Los seis grupos del CMC ocurren en esta área y, de estos seis, cuatro solo se encuentran allí (al mismo tiempo, parece que también *Centaurea benedicta* tenga su origen en esta área, como muchas otras malas hierbas). La diversificación desde un ancestro común que originó estos seis grupos parece haber ocurrido entre hace 6 y 9 millones de años. La dispersión hacia el oeste dentro del grupo *Centaurea* parece haber ocurrido entre 4 y 6 millones de años quizás relacionada con la Crisis de Salinidad del Mesiniense. Pero también se observan dispersiones transoceánicas, demostradas para el grupo de *C. cineraria*. Para llegar hasta el extremo occidental del Mediterráneo hay dos vías: a través de Grecia y el sur de Italia hasta el nordoeste de África o, alternativamente, a través los Balcanes, el norte de Italia, el sur de Francia hasta la Península Ibérica. Parece que la dispersión del grupo *Centaurea* ha podido producirse por estas dos rutas. El desarrollo de estepas en el sur de Europa durante el Pleistoceno podría haber ayudado la migración a través la ruta del norte.

7.

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8.

Compendium of publications

[*Compendi de publicacions*]

1. **Phylogeny of the *Centaurea* group (*Centaurea*, Compositae) – geography is a better predictor than morphology**
2. **Two additions to the *Jacea-Lepteranthus* complex: parallel adaptation in the enigmatic species *Centaurea subtilis* and *C. exarata***
3. **Evolution of the central Mediterranean *Centaurea cineraria* group (Asteraceae): Evidence for relatively recent, allopatric diversification following transoceanic seed dispersal**
4. **Current taxonomy in light of a species coalescence approach in the *Centaurea alba* complex**
5. **Tackling taxonomic ambiguity with an integrative approach: the case of *Centaurea corensis***

Capítol 1: s'enviarà a *Annals of Botany*
[*first publication, will be sent to Annals of Botany*]

Títol original [*original title*]:

Phylogeny of the *Centaurea* group (*Centaurea*, Compositae) – geography is a better predictor than morphology

[*Filogènia del grup* Centaurea *(*Centaurea*,* Compositae*) – la geografia és millor predictor que la morfologia*]
[*Filogenia del grupo* Centaurea *(*Centaurea*,* Compositae*) – la geografía es mejor predictor que la morfología*]

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Resum en català [*abstract in Catalan*]:

El grup *Centaurea* forma part del clade Circum-Mediterrani del subgènere *Centaurea* del gènere homònim. Aquest grup, predominantment mediterrani amb més de 200 espècies descrites, es divideix tradicionalment en tres seccions basades en la morfologia: *Centaurea*, *Phalolepis* i *Willkommia*. Aquesta divisió, tanmateix, és dubtosa, especialment des de la perspectiva molecular. En aquest estudi intentem resoldre aquest problema filogenètic i consolidar la circumscripció i delimitació de tot el grup. Incloem la majoria de les espècies descrites, fent ús d'una regió nuclear, l'espaidor transcrit internament (ITS), i una regió cloroplàstica, l'espaidor intergènic *rpl32-trnL*. Els resultats confirmen la monofilia del grup si excloem algunes espècies, però no recolzen la divisió seccional tradicional. Pel fet que hi ha fortes incongruències entre els dos marcadors i entre les dades genètiques i la morfologia no s'ha pogut establir una divisió taxonòmica clara dins del grup. Trobem signes clars de que la hibridació és una de les raons d'aquesta incongruència, però considerem la distribució de llinatges incompleta (*incomplete lineage sorting*) com un factor addicional. Finalment posem en dubte la delimitació d'espècies actual.

Resumen en castellano [*abstract in Spanish*]:

El grupo *Centaurea* es parte del clado Circum-Mediterráneo del subgénero *Centaurea* del género homónimo. Este grupo, predominantemente mediterráneo con más de 200 especies descritas se divide tradicionalmente en tres secciones basadas en la morfología: *Centaurea*, *Phalolepis* y *Willkommia*. Esta división, sin embargo, es dudosa, especialmente desde la perspectiva molecular. En este estudio intentamos resolver este problema filogenético y consolidar la circunscripción y delimitación del grupo entero. Incluimos la mayoría de las especies descritas, usando una región nuclear, el espaciador transcrito internamente (ITS), y una región cloroplástica, el espaciador intergénico *rpl32-trnL*. Nuestros resultados no apoyan la división seccional tradicional. No hemos podido establecer una división taxonómica clara dentro del grupo debido a las fuertes incongruencias entre los dos marcadores y entre los datos genéticos y la morfología. Encontramos signos claros de hibridación como una

de las razones de incongruencia, pero consideramos que la separación incompleta de linajes (*incomplete lineage sorting*) es un factor adicional. Finalmente ponemos en entredicho la delimitación de especies actual.

Phylogeny of the *Centaurea* group (*Centaurea*, Compositae) – geography is a better predictor than morphology

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Abstract:

- *Background and Aims* The *Centaurea* group is part of the Circum-Mediterranean Clade of genus *Centaurea* subgenus *Centaurea*. This mainly Mediterranean plant group with more than 200 described species is traditionally split on morphological basis into three sections: *Centaurea*, *Phalolepis* and *Willkommia*. This division, however, is doubtful, especially in light of molecular approaches. In this study we try to resolve these phylogenetic problems and to consolidate the circumscription and delimitation of the entire group against other closely related groups.
- *Methods* We included the majority of the described species using the internal described spacer (ITS) and a cpDNA region (*rpl32-trnL*). We checked the data for recombination, performed dating analyses and created phylogenetic trees and networks.
- *Key results* Monophyly of the group is confirmed if a few species are removed. Our results do not support the traditional sectional division. Due to high incongruence between the two markers and between genetic data and morphology, no clear taxonomical division within the group could be established.
- *Conclusions* The large incongruencies, which hamper phylogenetic reconstruction, may be produced by hybridization or convergent evolution of morphological traits, but probably also by incomplete lineage sorting as an additional factor. Our results also cast major doubts about current species delimitation.

Key words: Hybridization, incomplete lineage sorting, incongruence, Mediterranean, morphology.

INTRODUCTION

In the genus *Centaurea* great efforts have been made over the last fifteen years to get a phylogeny based on molecular evidence (Garcia-Jacas *et al.*, 2001, 2006). The genus itself was newly delimited to exclude many of the old sections (e.g., *Centaurea*, *Plectocephalus* and *Psephellus* among others) usually recognized in classic works (Bentham, 1873; Hoffmann, 1890–94; Dittrich, 1977). A new type, *Centaurea paniculata*, had to be chosen to conserve the name *Centaurea* (Greuter *et al.*, 2001). The remaining several hundreds of species are nowadays divided into the three subgenera *Centaurea s. str.*, *Lopholoma* and *Cyanus* (Garcia-Jacas *et al.*, 2001, 2006; Susanna and Garcia-Jacas, 2007), with the latter one often treated as a genus on its own (for example Greuter, 2008). All the subgenera have been the subject of detailed studies (subgenus *Lopholoma* by Font *et al.*, 2009; subgenus *Cyanus* by Boršić *et al.*, 2011; subgenus *Centaurea* by Garcia-Jacas *et al.*, 2006). The study in subgenus *Centaurea* (Table 1) showed that most of the spiny members, such as *C. calcitrapa* or *C. aspera,* were part of two well delimited groups: the Eastern and Western Mediterranean Clades (shortly EMC and WMC), leaving behind a high number of species with circum-Mediterranean distribution: the Circum-Mediterranean Clade (or CMC), characterized by ciliate or lacerate-membranaceous bract appendages. This group itself consists of two separated clades with many species and a few isolated taxa, among them the very divergent *Centaurea benedicta*, formerly *Cnicus benedictus.* The two large groups are the *Jacea-Phrygia* group (formerly *Jacea-Lepteranthus* group), encompassing plants with mostly entire leaves and a unique basic chromosome number of $x = 11$, and the *Centaurea* group (in former publications *Acrolophus* subgroup, with sections *Centaurea*, formerly *Acrolophus*, *Phalolepis* and *Willkommia*), with divided leaves and a basic chromosome number of $x = 9$. The morphological delimitation between these two groups, however, is not totally clear. The studies of Garcia-Jacas *et al.* (2006) and Hilpold *et al.* (2009) also showed that some species fall out from the *Centaurea* group on the basis of molecular data and chromosome numbers.

The *Jacea-Phrygia* and the *Centaurea* groups have given continuous taxonomic problems ever since they began to be studied due to high morphological variability, often on a very small geographical scale but often with unclear borders and intermediate forms leading to the description of a wealth of species and subspecies. Attempts to force this highly diverse complex of taxa into a hierarchical taxonomic system have been plentiful. Within the *Centaurea* group, which is the main topic of this publication, the most striking morphological feature is the shape of the bract appendages, which has led to the quite traceable description of two sections within the group: on one hand membranaceous lacerate appendages (section *Phalolepis*) and on the other hand ciliate to fimbriate appendages (section *Centaurea* =[*Acrolophus*]). The latter are well visible in the two redoubtable weeds *Centaurea diffusa* and *C. stoebe*. These bract characters, however, were shown to be relatively unreliable because of the frequent intermediate forms that exist between the two (Wagenitz, 1989; Wagenitz and Hellwig, 1996; A. Hilpold, pers. obs.). Dostál (1976) accepted this subdivision in his revision of the genus for Flora Europaea, assigned them the rank of subgenera, and divided them into several sections using for their delimitation morphological characters such as shape and indument of the leaves and bract appendages. Later on, the presence of a group of species with relatively continuous characters in the western Mediterranean led to the restoration of section *Willkommia* (Blanca López, 1981a), described by Willkomm (1870) as sect. *Acrocentroides*.

Species of this section show ciliate bract appendages with the final cilium strengthened into a small spine, and a subshrubby life-form.

TABLE 1. *Placement of the Centaurea group within the genus* Centaurea**. Groups in bold are also based on molecular information* (*Garcia-Jacas et al., 2006*)

Genus *Centaurea*

- **Subgenus *Cyanus***
- **Subgenus *Lopholoma***
- **Subgenus *Centaurea* (corresponds to the *Jacea* group)**
  - **Eastern Mediterranean Clade (EMC)**
  - **Western Mediterranean Clade (WMC)**
  - **Circum-Mediterranean Clade (CMC)**
    - ***Centaurea benedicta***
    - ***Jacea-Phrygia* group (formerly *Jacea-Lepteranthus* group)**
      - Section *Jacea*
      - Section *Phrygia* (formerly sect. *Lepteranthus*)
    - ***Centaurea* group (formerly *Acrolophus* subgroup)**
      - Section *Centaurea* (formerly sect. *Acrolophus*)
      - Section *Phalolepis*
      - Section *Willkommia*

* only sections of the CMC are shown.

However, morphology-based classifications were only poorly reflected in studies using molecular tools (Ochsmann, 2000; Garcia-Jacas *et al.*, 2006; Suárez-Santiago *et al.*, 2007; Hilpold *et al.*, 2011). Two studies based on the ITS region (Garcia-Jacas *et al.*, 2006, Suárez-Santiago *et al.*, 2007) showed the existence of two main ribotypes, one occuring mainly in the eastern (the Eastern ribotype or *Acrolophus-Phalolepis* ribotype) and another one mainly in the western Mediterranean (the Western ribotype or *Willkommia* ribotype). Hard incongruence between morphology and molecular data was explained in two ways. In the first case, the suggested cause was frequent hybridization and gene flow between sections *Centaurea, Phalolepis* and *Willkommia* within the Western ribotype (Suárez-Santiago *et al.*, 2007). In the second case, within the Sicilian group, a small central Mediterranean clade of recent origin that is nested within the *Centaurea* group, geographical proximity was much better correlated to molecular results than to morphology (Hilpold *et al.*, 2011). In contrast with the Iberian group, the Sicilian group did not show evidence of hybridization but showed that some characters, such as the shape of bract appendages and leaves, are prone to undergo rapid changes and are therefore unreliable for classification at higher levels. This may be a hint for the artificiality of the traditionally used taxonomy, which is mainly based on these rather unreliable characters.

We use a fast-evolving nuclear marker, the ITS, and a slowly evolving chloroplast marker, the *rpl32-trnL* to get new insights into the systematic relationships within the *Centaurea* group. The first aim of the study is (1) to delimit once again the *Centaurea* group from the *Jacea-Phrygia* group and the outlying species, this time sampling the majority of all described species, including many new described taxa. Furthermore we want to (2) check if there are clear subdivisions in the molecular data within the *Centaurea* group and (3) whether they correspond with either morphology and/or to geography and finally (4) we want to estimate when diversification of the main clades may have started.

MATERIAL AND METHODS

Plant Material

The aim of the study was to include the highest possible number of more than 200 described species of the *Centaurea* group. As a reference, we used the Euro+Med Plantbase (Greuter, 2008), which is the most complete list of all described taxa within *Centaurea*. For the Iberian species the new treatments of López and Devesa (2011) and López *et al.* (2011) were taken into account. Most specimens used for this study were new collections made during the last 5 years. In a few cases herbarium specimens and plants from botanical gardens were used. Vouchers are deposited in herbaria ANK, ATHU, BC, BOZ, GB, KNYA, SS and ZA.

Plant identification was an important part of this study. Collection in the type localities when possible together with the original description of the species made identification easier. Broader treatments (Hayek, 1931; Dostál, 1976) of the genus helped only in few cases since these works are relatively outdated and incomplete. Most of the time regional floras were consulted. Also taxonomic treatments of smaller clades within the *Centaurea* group were of great help (e.g., Blanca López, 1981a; Breitwieser and Podlech, 1986; Arrigoni, 2003). In total, we included 278 populations of 157 species from the CMC in our study (Table 2). Outgroup sequences were downloaded from Genbank. Selection of the outgroups followed studies of Garcia-Jacas *et al.* (2006) and Susanna *et al.* (2006). For Bayesian analyses, sequences of two members of subgenus *Cyanus* (only in *rpl32*; C. *triumfetti* subsp. *stricta*, *C. napulifera* subsp. *thirkei*), two of the WMC (*C. involucrata* and *C. ensiformis*) and two of the EMC (*C. cheirolopha* and *C. ensiformis*) were included. In addition, for the dating analyses *Psephellus pulcherrimus* (Willd.) Wagenitz, *Zoegea leptaurea* L. and *Rhaponticoides hajastana* (Tzvelev) M.V. Agab. & Greuter were included in both data matrices as the outgroups (in accordance with Barres *et al.,* in press).

DNA Extraction and PCR

The extraction of DNA from the plant material followed the CTAB method of Doyle and Dickson (1987) with the modification of Tel-Zur *et al.* (1999), including three washing steps with sorbitol buffer. Total genomic DNA was extracted from silica gel-dried tissue of one plant per population.

The investigated regions were the nuclear Internal Transcribed Spacer (ITS; primers ITS1 and ITS4 White *et al.*, 1990; and primers 17SE and 26SE; Sun *et al.*, 1994) and the plastid *rpl32-trnL* intergenic spacer (hereafter *rpl32*; primers rpl32-F, trnL(UAG); Shaw *et al.*, 2007). The PCR conditions for the ITS were the following: 4 min denaturing at 95 °C, followed by 30 cycles of 94 °C denaturing for 1.5 min, 55 °C annealing for 55 s (57 °C in case of primers 17SE and 26SE) and 72 °C extension for 3 min, with additional 15 min at 72 °C. For the *rpl32* the following PCR profile was used: 3 min of denaturing at 95 °C, followed by 35 cycles of 95 °C denaturing for 40 s, 54 °C annealing for 40 s and 72 °C extension for 1 min 40 s with additional 10 min at 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 at the University of Florida ICBR Core Facility on an ABI 3730xl capillary sequencer (Applied Biosystems).

TABLE 2. *Number of populations, taxon name, origin of the materials with herbaria where the vouchers are deposited and GenBank accession numbers.*

	Taxon	Locality and collection-number (or collectors and date if no collection numbers given), herbarium acronyme.	Genbank accession ITS	Genbank accession *rpl32*
1	*Centaurea acarnanica* (Matthäs) Greuter	Greece, West Greece: Mt. Akarnanika Ori, *Karamplianis 1513* (ATHU).		
2	*Centaurea aeolica* Lojac. subsp. *aeolica*	Italy, Messina: Lipari, *Hilpold V-1179* (BC).		
3	*Centaurea aeolica* subsp. *pandataria* (Fiori & Bég.) Anzal.	Italy, Latina: Ventotene Island, *Hilpold V-1113* (BC).		
4	*Centaurea aetaliae* (Sommier) Bég.	Italy, Livorno: Elba Island, Madonna del Monserrato, *Guidi s.n.*, 9.6.2008 (BC).		
5	*Centaurea affinis* Friv.	Bulgaria, Karlovo: Bogdan, *Kiebacher s.n.*, 5.5.2008 (BC).		
6		Bulgaria, Plovdiv: Kuklen, *Kiebacher s.n.*, 3.5.2008 (BC).		
7		Greece, Central Greece: Mount Parnassos, *Sánchez-Jiménez 28 et al.* (BC).		
8		Greece, Central Greece: Mt. Panetoliko, *Susanna 2754 et al.* (BC).		
9		Greece, Central Macedonia: Kato Olympos, *Susanna 2730 et al.* (BC).		
10		Greece, Thessaly: Mt. Kerketion, *Susanna 2735 et al.* (BC).		
11	*Centaurea aggregata* DC. subsp. *aggregata*	Turkey, Niğde: Çiftehan-Pozantı, *Tugay 4944 et al.* (KNYA).		
12	*Centaurea akamantis* T. Georgiadis & Hadjik.	Cyprus, Paphos: Avakas Gorge, *Galbany-Casals 2046 et al.* (BC).		
13	*Centaurea alba* L. subsp. *alba*	Spain, Ávila: El Barco de Ávila, *Hilpold 20093089 et al.* (BC).		
14		Spain, Segovia: El Burgo, *Hilpold 20093103 et al.* (BC).		
15		Spain, Segovia: Puerto de Guadarrama, *Hilpold 20093100 et al.* (BC).		
16		Spain, Soria: Hoz de Arriba, *Romaschenko 641 et al.* (BC).		
17		Spain, Cádiz: Arcos de la Frontera, *Susanna 2631 et al.* (BC).		
18		Portugal, Santarém: Abrantes, *Hilpold 20093068 et al.* (BC).		
19	*Centaurea alba* subsp. *tartesiana* Talavera	Spain, Huelva: between Marines and Fuenteheridos, *Barres s.n. et al.,* 4.5.2008 (BC).		
20	*Centaurea amaena* Boiss. & Balansa	Turkey, Kayseri: Kayseri, *Bağcı 1529* (KNYA).		
21	*Centaurea ambigua* Guss.	Italy, Caserta: Lago Matese, *Hilpold 20094009* (BC).		
22		Italy, L'Aquila: Roccaraso, *Garcia-Jacas et al. V-1220* (BC).		
23	*Centaurea ammocyanus* Boiss.	Iraq, Al-Anbar: Haswa desert, 20 km W Rutba, *Rechinger s.n.,* 8.6.1957 (GB).		
24	*Centaurea antalyensis* H. Duman & A. Duran	Turkey, Antalya: Akseki, way to Güzelsu, *Bağcı s.n.*, 2004 (KNYA).		
25	*Centaurea anthemifolia* Hub.-Mor.	Turkey, Konya: Aydos Dağı, *Tugay 5520 et al.* (KNYA)		
26	*Centaurea aphrodisea* Boiss.	Turkey, İzmir: Kayak Merkezi, *Bağcı 1530* (KNYA).		
27	*Centaurea aplolepa* Moretti subsp. *aplolepa*	Italy, Savona: Spotorno, *Vilatersana 1265 et al.* (BC).		
28	*Centaurea aplolepa* subsp. *bertolonii* (Arrigoni) Greuter	Italy, Genova: Chiavari, *Vilatersana 1263 et al.* (BC).		
29	*Centaurea aplolepa* subsp. *carueliana* (Micheletti) Dostál	Italy, Grosseto: Lago dell'Accesa, *Vilatersana 1236 et al.* (BC).		
30	*Centaurea aplolepa* subsp. *cosana* (Fiori) Dostál	Italy, Grosseto: Argentario, *Vilatersana 1228 et al.* (BC).		
31	*Centaurea aplolepa* subsp. *levantina* (Arrigoni) Greuter	Italy, Genova: Sestri Levante, *Vilatersana 1262 et al.* (BC).		
32	*Centaurea aplolepa* subsp. *lunensis* (Fiori) Dostál	Italy, La Spezia: Termine di Roverano, *Vilatersana 1258 et al.* (BC).		
33	*Centaurea aplolepa* subsp. *maremmana* (Fiori) Dostál	Italy, Livorno: Castiglioncello, *Vilatersana 1243 et al.* (BC).		
34	*Centaurea aplolepa* subsp. *subciliata* (DC.) Arcang.	Italy, Pisa: Marina di Vecchiano, *Vilatersana 1247 et al.* (BC).		
35	*Centaurea arenaria* Willd.	Turkey, Kırklareli: İğneada, *Tugay 4486* (KNYA).		
36	*Centaurea argentea* L. subsp. *argentea*	Greece, Crete: Falassarna, *Galbany-Casals 2031 et al.* (BC).		
37		Greece, Crete: Kavousi, *Galbany-Casals 2008 et al.* (BC).		
38		Greece, Crete: Topolia gorge, *Galbany-Casals 2030 et al.* (BC).		
39	*Centaurea argentea* subsp. *macrothysana* (Rech. f.) Turland & L. Chilton	Greece, Crete: Samaria gorge, *Galbany-Casals 2028 et al.* (BC).		
40	*Centaurea aristata* Hoffmanns & Link	Spain, Soria: Ucero, *Hilpold 20093104 et al.* (BC).		
41		Spain, León: Murias de Rechivaldo, *Hilpold 20093021 et al.* (BC).		
42		Spain, Madrid: Vallefresnos, *Hilpold 20093099 et al.* (BC).		
43		Spain, Soria: Torrubia de Soria, *Hilpold 20093105 et al.* (BC).		
44		Spain, Valladolid: Peñaflor de Hornija, *Hilpold 20093020 et al.* (BC).		
45	*Centaurea arrigonii* Greuter	Italy, Lucca: Levigliani, *Garcia-Jacas et al. V-1250* (BC).		
46		Italy, Reggio Nell'Emilia: Bondolo, *Hilpold s.n.,* 29.8.2008 (BC).		
47	*Centaurea aspromontana* Brullo & al. (*C. deusta* s.l.)	Italy, Reggio Calabria: S. Cristina Aspromonte, *Vilatersana 1137 et al.* (BC).		
48	*Centaurea austroanatolica* Hub.-Mor.	Turkey, Muğla: Balan Dağı, *Tugay 4267 et al.* (KNYA).		
49	*Centaurea avilae* Pau	Spain, Ávila: Prados de Roza, *Hilpold 20093093 et al.* (BC).		
50	*Centaurea aziziana* Rech. f. (*C. ovina* aggr.)	Iran, Azarbayjan-e-Sharghi: Golfa, *Susanna 1680 et al.* (BC).		
51	*Centaurea benedicta* (L.) L.	Nancy Botanical Garden (1993).		
52	*Centaurea bethurica* E. López & Devesa	Portugal, Portalegre: Alter do Chão, *Hilpold 20093072 et al.* (BC).		
53		Spain, Cáceres: Arroyo de la Vid, *Hilpold 20093080 et al.* (BC).		
54	*Centaurea boissieri* DC.	Spain, Tarragona: Ulldecona, *López-Alvarado s.n. et al.*, 4.6.2009 (BC).		
55	*Centaurea boissieri* subsp. *atlantica*	Morocco, Meknès-Tafilalt: Ifrane, *Hilpold 20103093 et al.* (BC).		

	(Font Quer) Blanca	
56	*Centaurea boissieri* subsp. *calvescens* (Maire) Greuter	Morocco, Meknès-Tafilalt: Col de Zad, *Hilpold 20103085 et al.* (BC).
57		Morocco, Meknès-Tafilalt: Tizi-n-Talrhemt pass, *Hilpold 20103075 et al.* (BC).
58	*Centaurea boissieri* subsp. *funkii* (Willk.) Blanca	Spain, Granada: Puerto de la Mora, *Hilpold 1029 et al.* (BC).
59	*Centaurea boissieri* subsp. *mariolensis* (Rouy) Dostál	Spain, Alicante: Serra de Mariola, *Vilatersana 1808 et al.* (BC).
60	*Centaurea boissieri* subsp. *transmalvana* (Emb. & Maire) Breitw. & Podlech	Morocco, Oriental: Debdou, *Hilpold 20103115 et al.* (BC).
61	*Centaurea breviceps* Iljin (*C. margaritacea* aggr.)	Ukraine, Dnipropetrovsk: Left bank of river Dnieper, *Derkach s.n.,* 16.8.2006 (BC).
62	*Centaurea brulla* Greuter (*C. deusta* s.l.)	Italy, Bari: Gorgofreddo, *Vilatersana 1206* (BC).
63	*Centaurea brunnea* (Halácsy) Halácsy	Greece, Epirus: 2.7 km N of Kamarina, *Constantinidis s.n. et al.*, 29.10.2008 (ATHU).
64	*Centaurea busambarensis* Guss.	Italy, Palermo: Isnello, *Vilatersana 1144 et al.* (BC).
65	*Centaurea cadmea* Boiss.	Turkey, Denizli: Honaz, *Bağcı 1524* (BC).
66	*Centaurea calolepis* Boiss.	Turkey, Bilecik: Bozhüyük-Eskişehir road, *Tugay 4400 et al.* (KNYA)
67		Turkey, Burdur-Muğla: Dirimli pass, *Susanna 2254 et al.* (BC).
68	*Centaurea cariensiformis* Hub.-Mor.	Turkey, Maraş: Elbistan-Malatya road, *Tugay 5032 et al.* (KNYA).
69	*Centaurea cariensis* Boiss.	Turkey, Antalya: Karamanbeli pass, *Susanna 2258 et al.* (BC).
70	*Centaurea cariensis* Boiss. subsp. *cariensis*	Turkey, Antalya: Finike-Elmalı, *Tugay 4230 et al.* (KNYA).
71	*Centaurea cariensis* subsp. *longipapposa* Wagenitz	Turkey, Konya: Akşehir-Isparta, *Tugay 4981 et al.* (KNYA).
72	*Centaurea cariensis* subsp. *microlepis* (Boiss.) Wagenitz	Turkey, Niğde: Çiftehan-Pozantı, *Tugay 4939* (KNYA).
73	*Centaurea cariensis* subsp. *niveotomentosa* (Hub.-Mor.) Wagenitz	Turkey, Antalya: Fethiye-Elmalı, *Tugay 4239* (KNYA).
74	*Centaurea carratracensis* Lange	Spain, Málaga: Carratraca, *Vilatersana 2151 et al.* (BC).
75	*Centaurea castellanoides* Talavera	Spain, Granada: SE Tocón, *Hilpold 20081017 et al.* (BC).
76		Spain, Granada: Tocón, *Hilpold 20081014 et al.* (BC).
77	*Centaurea chrysocephala* Phitos & T. Georgiadis	Greece, Thessaly: Meteora, monastery of Varlaam, *Constantinidis s.n. et al.*, 28.10.2007 (ATHU).
78	*Centaurea cineraria* L. subsp. *cineraria*	Italy, Latina: Gaeta, *Vilatersana 1112 et al.* (BC).
79		Italy, Latina: Sperlonga, *Vilatersana 1111 et al.* ((BC).
80		Italy, Latina: Torre Capovento, *Vilatersana 1107 et al.* ((BC).
81		Italy, Potenza: Acquafredda, *Vilatersana 1120 et al.* ((BC).
82		Italy, Salerno: Minori, *Vilatersana 1118 et al.* (BC).
83	*Centaurea cineraria* subsp. *circae* (Sommier) Cela Renz. & Viegi	Italy, Latina: S. Felice Circeo, *Vilatersana 1104 et al.* ((BC).
84	*Centaurea cithaeronea* Phitos & Constant.	Greece, Attica/Central Greece: Mt. Kithairon, *Constantinidis 2690* (ATHU).
85	*Centaurea consanguinea* DC.	Turkey, Karabük: İsmetpaşa-Çerkeş, *Tugay 4534* (KNYA).
86	*Centaurea cordubensis* Font Quer	Spain, Badajoz: Almendralejo, *Hilpold 20093076 et al.* (BC).
87		Spain, Badajoz: Puerto Peña, *Escobar-García 117/08* (BC).
88	*Centaurea corensis* Vals. & Filigh.	Italy, Sardinia: Scala di Giocca, *Filigheddu & Mameli s.n.,* 11.2008 (BC).
89	*Centaurea corymbosa* Pourr.	France, Languedoc-Roussillon: La Clappe, *Riba 002* (BC).
90	*Centaurea costae* Willk.	Spain, Huesca: Viacamp castle, *Hilpold s.n.,* 23.5.2008 (BC).
91		Spain, Huesca: Sobrarbre, *Hilpold 20095109 et al.* (BC).
92		Spain, Lleida: Pallars Sobirà, *Hilpold 20104074 et al.* (BC).
93		Spain, Huesca: Sopeira, *Hilpold 20104055 et al.* (BC).
94		Spain, Lleida: Montsec, *Hilpold 20104063 et al.* (BC).
95	*Centaurea cristata* Bartl. (*C. spinosociliata* aggr.)	Italy, Trieste: Duino castle, *Hilpold s.n.,* 31.8.2008 (BC).
96		Italy, Trieste: San Giovanni al Timavo, *Hilpold s.n.,* 31.8.2008 (BC).
97	*Centaurea crithmifolia* Vis.	Croatia, Split-Dalmatia: Jabuka Island, *Boršić 33bis* (ZA).
98	*Centaurea cuneifolia* Sm.	Turkey, Tekirdağ: Saray-Kıyıköy, *Tugay 4448 et al.* (KNYA).
99	*Centaurea cyprensis* (Holub) T. Georgiadis	Cyprus, Paphos: Akamas peninsula, *Galbany-Casals 2047 et al.* (BC).
100	*Centaurea debdouensis* Breitw. & Podlech	Morocco, Souss-Massa-Draâ: Djebel Sarhro, *Hilpold 20103061 et al.* (BC).
101		Morocco, Suárez-Santiago *et al.* (2007)
102	*Centaurea delicatula* Breitw. & Podlech	Morocco, Suárez-Santiago *et al.* (2007)
103		Tunisia, Kasserine: Jebel ech Chambi, *Vilatersana 1355 et al.* (BC).
104		Tunisia, Siliana: Jebel Seri, *Vilatersana 1346 et al.* (BC).
105	*Centaurea delucae* C. Guarino & Rampone	Italy, Chieti: Passo Lanciano, *Garcia-Jacas et al. V-1227* (BC).
106	*Centaurea deusta* Ten.	Bosnia and Herzegovina, Republika Srpska: G. Jagodina, *Frajman12395 et al.* (BC).
107		Bulgaria, Karlovo: Bogdan, *Kiebacher s.n.,* 5.5.2008 (BC).
108		Croatia, Dubrovnik-Neretva: Ploče, *Boršić 35* (ZA).
109		Italy, Caserta: San Gregorio Matese, *Hilpold 20094008 et al.* (BC).
110		Italy, Foggia: Monte Sant'Angelo, *Vilatersana 1210 et al.* (BC).
111		Italy, Lecce: Porto Badisco, *Vilatersana 1198 et al.* (BC).
112		Italy, Napoli: Procida Island, *Hilpold 20094007 et al.* (BC).
113		Italy, Potenza: Pollino, *Vilatersana 1131 et al.* (BC).
114		Italy, Trieste: Mt. Spaccato, *Martini s.n.*, 7.7.2009 (BC).
115		Italy, Verona: Monte Pastello, *Bertolli & Prosser s.n.*, 20.5.2010 (BC).
116		Montenegro, Bar: Limljani, *Sánchez-Jiménez 15 et al.* (BC).
117	*Centaurea deustiformis* Adamović	Greece, Nomos Kastorias: Mt. Vernon (Vitsi), *Constantinidis 11205 et al.*

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		(ATHU).	
118	*Centaurea dichroa* Boiss. & Heldr.	Turkey, Antalya: Çıralı, *Tugay 4215 et al.* (KNYA).	
119	*Centaurea diffusa* Lam.	Armenia, Aragatsotn: Bagravan, *Susanna 1589 et al.* (BC).	
120		Turkey, Çanakkale: Kurşunlu, *Tugay 4345 et al.* (KNYA).	
121	*Centaurea diomedea* Gasp.	Italy, Foggia: San Domino Island, *Vilatersana 1211* (BC).	
122	*Centaurea donetzica* Klokov (*C. margaritacea* aggr.)	Ukraine, Donetzk region, Krasny Liman, *Romaschenko 648* (BC).	JF913986-87
123	*Centaurea ertugruliana* Uysal	Turkey, Bursa: Orhaneli, 13.8.2008, *Tugay 5628 et al.* (KNYA).	
124	*Centaurea erycina* Raimondo & Bancheva	Italy, Trapani: Erice, 28.5.2008, *Vilatersana 1160bis et al.* (BC).	
125	*Centaurea exarata* Coss.	Spain, Garcia-Jacas *et al.* (2006)	DQ319113
126	*Centaurea ferulacea* Martelli	Italy, Sardinia: Baunei, *Mameli* & *Pisanu s.n.*, 12.10.2007 (SS).	
127	*Centaurea filiformis* Viv.	Italy, Sardinia: Monte Maccione, Oliena, *Pisanu s.n.*, 28.5.2007 (SS).	
128	*Centaurea friderici* subsp. *jabukensis* (Ginzb. & Teyber) Greuter	Croatia, Split-Dalmatia: Jabuka Island, *Boršić 33* (ZA).	
129	*Centaurea friderici* Vis. subsp. *friderici*	Croatia, Dubrovnik-Neretva: Palagruža Island, *Boršić 15* (ZA).	
130	*Centaurea gadorensis* Blanca	Spain, Almería: Castala, *Hilpold 20081028 et al.* (BC).	
131		Spain, Almería: Láujar de Andarax, *Hilpold 20081027 et al.* (BC).	
132		Spain, Granada: Puerto de la Ragua, *Hilpold 20081023 et al.* (BC).	
133	*Centaurea giardinae* Raimondo & Spadaro	Italy, Catania: Linguaglossa, *Vilatersana 1177 et al.* (BC).	
134	*Centaurea* cf. *glaberrima* Tausch	Croatia, Dubrovnik-Neretva: Slano, *Boršić 34* (ZA).	
135	*Centaurea grisebachii* (Nyman) Heldr.	Greece, Central Greece: Mt. Timfristos, *Susanna 2755 et al.* (BC).	
136	*Centaurea gymnocarpa* Moris & De Not.	Italy, Livorno: Capraia Island, *Vilatersana 1248 et al.* (BC).	
137	*Centaurea hanrii* Jord.	Spain, Girona: Cap de Creus, *Hilpold s.n.,* 22.6.2008 (BC)	
138		Spain, Girona: Espolla, *Hilpold s.n.*, 10.7.2008 (BC).	
139		Spain, Tarragona: Porrera, *Hilpold s.n.*, 5.5.2008 (BC).	
140	*Centaurea heldreichii* Halácsy	Greece, West Greece: Varasova mountain, *Constantinidis10993* (ATHU).	
141	*Centaurea hierapolitana* Boiss.	Turkey, Afyonkarahisar: Çıkışı, *Bağcı1523* (KNYA).	
142	*Centaurea horrida* Badarò	Italy, Sardinia: Valle della Luna, Stintino Peninsula, *Pisanu s.n.*, 8.5.2007 (SS).	
143	*Centaurea ilvensis* (Sommier) Arrigoni (*C. aetaliae* s.l.)	Italy, Livorno: Isola d'Elba, Monte Capanne, *Guidi s.n.*, 8.6.2008 (BC).	
144	*Centaurea incompleta* Halácsy	Greece, Nomos Pierias: Mt. Olympos, *Constantinidis 11251 et al.* (ATHU).	
145	*Centaurea inermis* Velen.	Turkey, İstanbul: Aydos Dağı, *Tugay 4421 et al.* (KNYA).	
146	*Centaurea inexpectata* Wagenitz	Turkey, Antalya: Gevne valley, *Uysal 598* (BC).	
147	*Centaurea ionica* Brullo (*C. deusta* s.l.)	Italy, Reggio Calabria: Pazzano, *Vilatersana 1191 et al.* (BC).	
148	*Centaurea jacea* subsp. *gaudinii* (Boiss. & Reut.) Gremli	Italy, Reggio Nell'Emilia: Toano, *Hilpold s.n.*, 3.9.2008 (BC).	
149	*Centaurea japygica* (Lacaita) Brullo (*C. diomedea* s.l.)	Italy, Lecce: Cagliano del Capo, *Vilatersana 1204 et al.* (BC).	
150	*Centaurea kartschiana* Scop. subsp. *dalmatica* Nyman	Croatia, Primorje-Gorski kotar: Cres Island, *Boršić 37* (ZA).	
151	*Centaurea kartschiana* subsp. *lubenicensis* (Trinajstić& Zi. Pavletić) Greuter	Croatia, Primorje-Gorski kotar: Cres Island, *Boršić 14* (ZA).	
152	*Centaurea kilaea* Boiss.	Turkey, İstanbul: Yeniköy, *Tugay 4526 et al.* (KNYA).	
153	*Centaurea langei* Nyman subsp. *langei*	Portugal, Guarda: Ratoeira, *Hilpold 20093055 et al.* (BC).	
154		Portugal, Vila Real: Vila Real, *Hilpold 20093049 et al.* (BC).	
155		Portugal, Viseu:Caramulo, *Hilpold 20093052 et al.* (BC).	
156		Portugal, Viseu: Lamego, *Hilpold 20093051 et al.* (BC).	
157		Spain, Lugo: Becerreá, Bullan, *López-Alvarado s.n.*, 7.8.2009 (BC).	
158		Spain, Lugo: Becerreá, *Hilpold 20093027 et al.* (BC).	
159		Spain, Pontevedra: Los Baños de Caldela de Tui, *Hilpold 20093033 et al.* (BC).	
160	*Centaurea langei* subp. *geresensis* (Arènes) Franco	Portugal, Braga: Portela de Leonte, *Hilpold 20093041 et al.* (BC).	
161		Portugal, Viana do Castelo: Cidadelhe de Lindoso, *Hilpold 20093040 et al.* (BC).	
162	*Centaurea langei* subsp. *coutinhoi* (Franco) E. López, Devesa & Arnelas	Portugal, Portalegre: Castelo de Vide, *Hilpold 20093070 et al.* (BC).	
163	*Centaurea langei* subsp. *exilis* (Arènes) E. López, Devesa & Arnelas	Portugal, Castelo Branco: Termas de Monfortinho, *Hilpold 20093086 et al.* (BC).	
164	*Centaurea langei* subsp. *kheilii* (Pau) E. López, Devesa & Arnelas	Spain, Cáceres: Hervás, *Hilpold 20093087 et al.* (BC).	
165	*Centaurea langei* subsp. *rothmalerana* (Arènes) E. López, Devesa & Arnelas	Portugal, Cova de Beira: Serra da Estrela, *Hilpold 20093056 et al.* (BC).	
166	*Centaurea leonidia* Kalpoutz. & Constantin.	Greece, Nomos Arkadias: Mt Parnonas, *Constantinidis 10674 et al.* (ATHU).	
167	*Centaurea leucadea* Lacaita	Italy, Lecce: S. Maria di Leuca, *Vilatersana 1205 et al.* (BC).	
168	*Centaurea leucophaea* Jord. subsp. *leucophaea*	Italy, Imperia: Airole, *Vilatersana 1271 et al.* (BC).	
169		Italy, Savona: Albenga, *Vilatersana 1268 et al.* (BC).	
170		Spain, Barcelona: Prats de Lluçanès, *Hilpold et al. s.n.*, 17.5.2008 (BC).	
171		Spain, Barcelona: Rupit, *Hilpold 20100008 et al.* (BC).	
172		Spain, Barcelona: Valcàrquera, *Hilpold 20090054 et al.* (BC).	
173		Italy, Savona: Toirano, *Vilatersana 1266 et al.* (BC).	
174	*Centaurea leucophaea* subsp. *controversa* (Briq. & Cavill.) Kerguélen	France, Alpes-Maritimes: Eze-Bord de Mer, *Vilatersana 1274 et al.* (BC).	
175	*Centaurea limbata* Hoffmanns. & Link subsp. *limbata*	Spain, A Coruña: A Coruña, *Hilpold 20093029 et al.* (BC).	
176	*Centaurea limbata* subsp. *lusitana* (Arenès) E. López & Devesa	Portugal, Viseu: Caramulho, *Hilpold 20093053 et al.* (BC).	

177	*Centaurea litigiosa* (Fiori) Arrigoni (*C. aetaliae* s.l.)	Italy, Grosseto: Monte Argentario, *Vilatersana 1231 et al.* (BC).		
178	*Centaurea litochorea* T. Georgiadis & Phitos	Greece, Thessaly: Mount Olympos, *Susanna 2671 et al.* (BC)		
179	*Centaurea luschaniana* Heimerl ex Stapf	Turkey, Antalya: Elmali–Korkuteli, *Bağcı s.n.*, 2004 (KNYA).		
180	*Centaurea lycia* Boiss.	Turkey, Antalya: Kazdaği, *Bağcı s.n.*, 2004 (KNYA).		
181	*Centaurea magistrorum* Arrigoni & Camarda	Italy, Sardinia: Villagrande, Monte Luas, *Camarda et al. s.n.*, 28.7.1995 (Camarda pers. herb.).		
182	*Centaurea margarita-alba* Klokov (*C. margaritacea* aggr.)	Ukraine, Mykolaiv Oblast: Mikhailo-Larino *Derkach s.n.*, 7.2004 (BC).		
183	*Centaurea margaritacea* Ten.	Ukraine, Mykolaiv Oblast: Mishkovo-Pogorelovo, *Derkach s.n.*, 15.8.2006 (BC).		
184	*Centaurea messenicolasiana* T. Georgiadis & al.	Greece, Karditsa: Messenikolas village, *Constantinidis s.n. et al.* (ATHU).		
185	*Centaurea monticola* DC.	Spain, Granada: Prado Llano, *Hilpold 20081007 et al.* (BC).		
186	*Centaurea musakii* T. Georgiadis	Greece, Central Greece: Proussos monastery, *Susanna 2741 et al.* (BC).		
187	*Centaurea musarum* Boiss. & Orph.	Greece, Nomos Viotias: Mt. Parnassos, *Vassiliades s.n.*, 28.07.1995 (ATHU).		
188	*Centaurea niederi* Heldr.	Greece, West Greece: Frangouleika, *Susanna 2753 et al.* (BC).		
189	*Centaurea nigra* L.	Spain, La Coruña: Carballo, *Susanna 1446 et al.* (BC).		
190	*Centaurea nobilis* (H. Groves) Brullo (*C. deusta* s.l.)	Italy, Lecce: Otranto, *Vilatersana 1197 et al.* (BC).		
191	*Centaurea olympica* (DC.) K. Koch (*C.cuneifolia* s.l.)	Turkey, Bursa: Soğukpınar Köyü, *Tugay 4356 et al.* (KNYA).		
192	*Centaurea paczoskii* Klokov (*C. margaritacea* aggr.)	Ukraine, Kherson: Novogrebnevo, *Moysienko s.n.*, 20.09.2008 (BC).		
193	*Centaurea paniculata* L. s.l.	France, Alpes-Maritimes: Coll de la Faye, *Molero 224/09 et al.* (BC).		
194		France, Alpes-Maritimes: La Turbie, *Vilatersana 1273 et al.* (BC).		
195		Italy, Pisa: Migliarino, *Vilatersana 1245 et al.* (BC).		
196	*Centaurea paniculata* L. subsp. *paniculata*	Italy, Imperia: Roverino, *Vilatersana 1272 et al.* (BC).		
197		Spain, Girona: Santa Madalena de Terrades, *Hilpold s.n.*, 10.7.2008 (BC).		
198	*Centaurea paniculata* subsp. *esterellensis* (Briq.) Dostál	France, Alpes-Maritimes: Forêt domainale de l'Esterell, *Vilatersana 1275 et al.* (BC).		
199	*Centaurea panormitana* Lojac.	Italy, Palermo: Sferracavallo, *Vilatersana 1163 et al.* (BC).		
200	*Centaurea panormitana* subsp. *seguenzae* (Lacaita) Greuter	Italy, Messina: Tindari, *Vilatersana 1173 et al.* (BC).		
201	*Centaurea papposa* (Coss.) Greuter	Tunisia, Nabeul: Cap Bon, *Vilatersana 1384 et al.* (BC).		
202	*Centaurea parlatoris* Heldr.	Italy, Messina: Novara di Sicilia, *Vilatersana 1175 et al.* (BC).		
203		Italy, Palermo: Madonie, *Vilatersana 1147 et al.* (BC).		
204	*Centaurea patula* DC.	Turkey, Eskişehir: Sivirihisar, *Ilarslan 4305* (ANK).		
205	*Centaurea paui* Willk.	Spain, Castellon: Eslida, *Vilatersana 1803 et al.* (BC).		
206	*Centaurea pawlowskii* Phitos & Damboldt	Greece, Ioannina: Mt. Timfi, Vikos gorge, *Constantinidis s.n. et al.*, 29.10.2008 (ATHU).		
207	*Centaurea pectinata* L.	Spain, Barcelona: Sant Marçal, *Susanna 1469 et al.* (BC).		
208	*Centaurea pelia* DC.	Greece, Central Greece: Delphi, *Susanna 2741 et al.* (BC).		
209	*Centaurea pentadactyli* Brullo & al. (*C. deusta* s.l.)	Italy, Reggio Calabria: Pentidattilo, *Vilatersana 1183 et al.* (BC).		
210	*Centaurea pinae* Pau	Spain, Teruel: Calatayud, *Hilpold 20093108 et al.* (BC).		
211	*Centaurea pinetorum* Hub.-Mor.	Turkey, Mersin: Mut road, *Tugay 4957 et al.* (KNYA).		
212	*Centaurea poculatoris* Greuter	Greece, Crete: Asfendos gorge, *Galbany-Casals 2024 et al.* (BC).		
213	*Centaurea poeltiana* Puntillo (*C. deusta* s.l.)	Italy, Reggio Calabria: Montalto, *Vilatersana 1184 et al.* (BC).		
214	*Centaurea polyclada* DC.	Turkey, Balıkesir: Zeytinli-Akçay, *Tugay 4330 et al.* (KNYA).		
215	*Centaurea pomeliana* Batt.	Morocco, Souss-Massa-Draâ: Igherm, *Hilpold 20103028 et al.* (BC).		
216	*Centaurea pomeliana* Batt. subsp. *pomeliana*	Morocco, Souss-Massa-Draâ: Tizi-n-Bachkoum pass, *Escobar-García 363/08* (BC).		
217		Morocco, Souss-Massa-Draâ: Tizi-n-Bachkoum pass, *Hilpold 20103046 et al.* (BC).		
218		Morocco, Suárez-Santiago *et al.* (2007)	AM114315	-
219	*Centaurea pomeliana* subsp. *rouxiana* (Maire) Breitw. & Podlech	Algeria, Suárez-Santiago *et al.* (2007)	AM1114316	-
220	*Centaurea princeps* Boiss. & Heldr.	Greece, Central Greece: Mt. Tymfristos, *Constantinidis s.n. et al.*, 1.1.2008 (ATHU).		
221	*Centaurea protogerberi* Klokov (*C. margaritacea* aggr.)	Ukraine, Luhansk Oblast: Stanichno-Lugansk, *Romaschenko 1* (BC).		
222	*Centaurea protomargaritacea* Klokov (*C. margaritacea* aggr.)	Ukraine, Mykolaiv Oblast: Forest Gorge "Balabanivka", left bank of the river Bug, *Derkach s.n.*, 8.2006 (BC).		
223	*Centaurea pseudocadmea* Wagenitz	Greece, Nomos Lakonias: Eparchia Epidavrou-Limiras, Mt. Chionovouni, *Constantinidis 11038 et al.* (ATHU).		
224	*Centaurea pseudoleucolepis* Kleopow (*C. margaritacea* aggr.)	Ukraine, Donetsk Oblast: Kamennye Mogily national reservation, *Romaschenko 5* (BC).		
225	*Centaurea pseudomaculosa* Dobrocz. (*C. stoebe* s.l.)	Russia, Volgograd Oblast: Ostroukhov, (Muséum National d'Histoire Naturelle Paris, Index Seminum 2009).		
226	*Centaurea pulvinata* (Blanca) Blanca	Spain, Granada: La Calahorra, *Hilpold 20081020 et al.* (BC).		
227	*Centaurea resupinata* Coss.	Morocco, Suárez-Santiago *et al.* (2007)	AM114293	-
228	*Centaurea resupinata* subsp. *rifana* (Emb. & Maire) Breitw. & Podlech	Morocco, Taza-Al-Hoceima-Taounate: Ketama, *Hilpold 20103104 et al.* (BC).		
229		Morocco, Taza-Al-Hoceima-Taounate: Targuist, *Hilpold 20103106 et al.* (BC).		
230	*Centaurea resupinata* subsp. *simulans* (Emb. & Maire) Breitw. & Podlech	Morocco, Souss-Massa-Draâ: Djebel Sarhro, *Hilpold 20103054 et al.* (BC).		

231	*Centaurea rouyi* Coincy	Spain, Alicante: Serra de l'Assafor, *Vilatersana 1807 et al.* (BC).		
232		Spain, Alicante: Simat de la Valldigna, *Vilatersana 1804 et al.* (BC).		
233	*Centaurea saccensis* Raimondo & al.	Italy, Agrigento: Lago Arancio, *Vilatersana 1159 et al.* (BC).		
234	*Centaurea sarandinakiae* N.B. Illar. (*C. sterilis* aggr.)	Ukraine, Crimea: Planerskoe, Kara-Dag mountain, *Romaschenko 3* (BC).		
235	*Centaurea scannensis* Anzal. & al.	Italy, L'Aquila: Gole del Sagittario, *Hilpold et al. V-1225* (BC).		
236	*Centaurea schousboei* Lange	Spain, Badajoz: Monesterio, *Hilpold 20093077 et al.* (BC).		
237	*Centaurea scillae* Brullo (*C. deusta* s.l.)	Italy, Reggio Calabria: Bagnara, *Vilatersana 1140 et al.* (BC).		
238	*Centaurea semijusta* Juz. (*C. sterilis* aggr.)	Ukraine, Crimea: Chatyr-Dag, *Romaschenko 2* (BC).		
239	*Centaurea sipylea* Wagenitz	Turkey, Manisa: Spil Dağı, *Tugay 4322 et al.* (KNYA).		
240	*Centaurea sivasica* Wagenitz	Turkey, Sivas: Sarıoğlan road, *Tugay 5011 et al.* (KNYA).		
241	*Centaurea spinosa* L.	Greece, Crete: Georgioupoli, *Galbany-Casals 2023 et al.* (BC).		
242		Greece, Crete: Kolimbari beach, *Galbany-Casals 2032 et al.* (BC).		
243	*Centaurea spinosociliata* aggr.	Bosnia and Herzegovina, Republika Srpska: Trebinje, *Frajman 12388* (BC).		
244		Croatia, Dalmatia: Biokovo, *Frajman 12382* (BC).		
245		Croatia, Island Hvar: Velo Grablje, *Frajman 12636* (BC).		
246		Croatia, Primorje-Gorski kotar: Krk Island, *Boršić 33* (ZA).		
247		Croatia, Rab: Rab, *Frajman 12696* (BC).		
248		Croatia, Šibenik-Knin: Skradin, *Boršić 31* (ZA).		
249	*Centaurea spinosociliata* Seenus	Croatia, Lika-Senj: Karlobag, *Boršić 35* (ZA).		
250	*Centaurea sterilis* Steven	Ukraine, Crimea: Planerskoe, Kara-Dag mountain, *Romaschenko 4* (BC).		
251	*Centaurea stoebe* L.	Austria, Niederösterreich: Marchegg, Schloss Hof, *Flatscher s.n.*, 10.10.2009 (BC).		
252		Italy, Bolzano: Feldthurns, Trumbühel, *Hilpold s.n.*, 18.8.2008 (BOZ).		
253		Italy, Udine: Tagliamento bridge near Tolmezzo, *Hilpold s.n. et al.*, 31.8.2008 (BC).		
254		Kosovo: Ceraje, N of Kosovska Mitrovica, *Kiebacher s.n.*, 31.10.2008 (BC).		
255		Serbia, Šumadija: Mt. Maljen, *Frajman 12646 et al.* (BC).		
256		Slovenia, Dolenjska: Mrtvice, *Frajman 12415* (BC).		
257	*Centaurea stoebe* subsp. *australis* (A. Kern.) Greuter	Hungary, Central Hungary: Budapest, *Boršić 22 et al.* (ZA).		
258	*Centaurea subciliaris* Boiss. & Heldr.	Greece, Ionian Islands: Kefallinia Island, Mt. Enos, *Constantinidis s.n. et al.*, 11.5.2007 (BC).		
259	*Centaurea subtilis* Bertol.	Italy, Foggia: Madonne delle Grazie, *Vilatersana 1208 et al.* (BC).		
260	*Centaurea tenacissima* (H. Groves) Brullo (*C. deusta* s.l.)	Italy, Lecce: San Foca, Porto, *Tomaselli s.n. et al.,* 12.6.2008 (BC).		
261	*Centaurea tenoreana* Willk.	Italy, Chieti: Majella, Rifugio Puntilio, *Garcia-Jacas et al.V-1221* (BC).		
262	*Centaurea tenorei* Lacaita	Italy, Salerno: Capo d'Orso, *Vilatersana* 1116 *et al.* (BC).		
263	*Centaurea tommasinii* A. Kern. (*C. spinosociliata* aggr.)	Italy, Rovigo: Porto Caleri, *Hilpold s.n. et al.*, 30.8.2008 (BC).		
264	*Centaurea tossiensis* Freyn & Sint.	Turkey, Kastamonu: Tosya, *Bağcı s.n.*, 2004 (KNYA).		
265	*Centaurea tougourensis* Boiss. & Reut.	Algeria, Batna: Tighanimine, *Lidén 660* (GB).		
266	*Centaurea tuzgoluensis* Aytaç & H. Duman	Turkey, Aksaray: Eskil, *Tugay 3543* (KNYA).		
267	*Centaurea tymphaea* Hausskn.	Greece, Nomos Florinis: Erarchia Florinis, *Constantinidis 10305 et al.* (ATHU).		
268	*Centaurea ulrichiorum* Wagenitz & al.	Turkey, Antalya: Alanya, Beldibi Köyü, *Tugay 5368 et al.* (KNYA).		
269	*Centaurea vankovii* Klokov (*C. sterilis* aggr.)	Ukraine, Crimea, Mt. Demerdji, 1200 m, *Romaschenko 646* (BC).	JF914073-74	
270	*Centaurea veneris* (Sommier) Bég.	Italy, La Spezia: Palmaria, *Garcia-Jacas et al. V-1255* (BC).		
271	*Centaurea virgata* Lam.	Turkey, Konya: Beyşehir-Avşar road, *Tugay 4965* (KNYA).		
272		Turkey, Muğla: Sandras Dağ, *Susanna 2252 et al.* (BC).		
273	*Centaurea wagenitzii* Hub.-Mor.	Turkey, Antalya: Adrasan, *Bağcı 1520* (KNYA).		
274	*Centaurea wiedemanniana* Fisch. & C.A. Mey.	Turkey, Bilecik: Selimiye, *Davis 201483 et al.* (E).		
275		Turkey, Bilecik: Vezirhan road, *Tugay 4391 et al.* (KNYA).		
276	*Centaurea yozgatensis* Wagenitz	Turkey, Eskişehir: Sündiken mountains, *Tugay 4408 et al.* (KNYA).		
277	*Centaurea zeybekii* Wagenitz	Turkey, İzmir: Nif Dağı, *Tugay 4304 et al.* (KNYA).		
278	*Centaurea zuccariniana* DC.	Greece, Epirus: Aristi, *Vilatersana 546 et al.* (BC).		
Outgroups				
	Centaurea aspera L. (**WMC**)	Portugal, Garcia-Jacas *et al.* (2006)	DQ319086	
	Centaurea involucrata Desf. (**WMC**)	Algeria, Susanna *et al.* (2006)	AY826256	
	Centaurea cheirolopha (Fenzl) Wagenitz (**EMC**)	Turkey, Garcia-Jacas *et al.* (2006)	DQ319101	
	Centaurea ensiformis P. H. Davis (**EMC**)	Turkey, Garcia-Jacas *et al.* (2006)	DQ319112	
	Centaurea triumfettii All. subsp. *stricta* (Waldst. & Kit.) Dostál (**subgenus *Cyanus***)	Ukraine, Garcia-Jacas *et al.* (2006) as *C. mollis*	DQ319133	
	Centaurea napulifera Rochel subsp. *thirkei* (Sch. Bip.) Stoj. & Acht. (**subgenus *Cyanus***)	Romania, Garcia-Jacas *et al.* (2006)	DQ319136	
	Rhaponticoides hajastana (Tzvelev) M.V. Agab. & Greuter	Armenia, Hidalgo *et al.* (2006); Susanna *et al.* (2011)	DQ310922	JF754895
	Zoegea leptaurea L.	Iran, Susanna *et al.* (2006, 2011)	AY826349	JF754906
	Psephellus pulcherrimus (Willd.) Wagenitz	Armenia, Susanna *et al.* (2006, 2011)	AY826317	JF754894

Cloning

We selected to clone the following taxa because of previously detected polymorphisms in direct sequences: *Centaurea aeolica* subsp. *aeolica*, *C. aeolica* subsp. *pandataria*, *C. cineraria* subsp. *cineraria*, *C. corensis, C. ferulacea*, *C. filiformis.* We added several additional species (*C. horrida, C. paniculata* subsp. *maremmana* and *C. veneris*) that did not display polymorphisms in direct sequences in order to check for the presence of additional ribotypes that may be poorly amplified, compared to the originally sequenced copy. Eventually, only a few clearly distinct sequences per individual were included into the analysis. The selected PCR products were cloned using TOPO TA Cloning kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, with the difference that only half reactions were used. Eight positive colonies from each reaction were screened with direct PCR using the universal primers T7 and M13R and under the following conditions: 10 min denaturing at 94 °C, followed by 30 cycles of 94 °C denaturing for 30 s, 55 °C annealing for 1 min and 72 °C extension for 2 min, with an additional 10 min at 72 °C. If necessary, the sampling was increased with more colonies until all the bands present in the products were found. Purification and sequencing were performed as described above.

Phylogenetic analyses

Nucleotide sequences were edited using Bioedit v7.0.5.3 (Hall, 1999) and aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990). Data matrices are available on request from the author. The ITS matrix has an aligned length of 645 bp and the *rpl32* of 1029 bp. Parsimony analysis of the ITS and the *rpl32* dataset involved heuristic searches conducted with PAUP v4.0b10 (Swofford, 2002) using TBR branch swapping with character states specified as unordered and unweighted. The indels were coded as missing data in the ITS data matrix since there existed almost no indel characters within the *Centaurea* group. In the *rpl32* the few indels were coded manually as additional character states following the methods outlined in Simmons and Ochoterena (2000) and were included in the matrix.

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. Internodes with BS ≥ 0.75 were considered statistically significant. For the strict consensus tree consistency index (CI) and homoplasy index (HI) are given, excluding uninformative characters.

Bayesian inference of the two datasets was calculated using MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The best available model of molecular evolution required for Bayesian estimations of phylogeny was selected using Akaike information criteria (AIC) for both datasets as implemented in the software MrModeltest v2.2 (Nylander, 2004). The best fitting model was GTR + G + I for both regions. The simplest possible model, i.e. the Jukes Cantor model, was used for Bayesian analysis of coded indels.

Bayesian inference analyses were initiated with random starting trees and were run for 10×10^6 generations. Four Markov chains were run using the Markov Chain Monte Carlo (MCMC) principles to sample trees. We saved one out of every 1,000 generations, resulting in 10,000 sample trees. Data from the first 1,000 generations were discarded as the “burn-in” period, after confirming that likelihood values had stabilized prior to the 1,000th generation. Internodes with posterior probabilities ≥ 0.95 were considered statistically significant.

Recombination tests

To check the ITS data for possible recombination the program RDP v3.44 was used (Martin *et al.*, 2010) following the default settings of the package with a highest acceptable P value of 0.05 and using the Bonferoni correction. We scanned the sequence alignment with the following methods: (a) the RDP method (Martin and Rybicki, 2000) with internal and external reference, (b) the Sister Scanning method implemented in SiScan (Gibbs *et al.*, 2000), (c) the gene conversion method in GENECONV (Padidam *et al.*, 1999), (d) the bootscanning method in Bootscan (Martin *et al.*, 2005), (e) the maximum v2 method in MaxChi (Maynard Smith, 1992), and (f) a modification of this method implemented in CHIMAERA (Posada and Crandall, 2001).

Dating

As large polytomies seem to change node dates towards older ages (B. Pfeil *et al.*, unpubl. res.), we ran analyses with the entire matrix and a reduced subset of specimens, including only two sequences of any polytomy resulting from the Bayesian analysis. These reduced matrices encompassed 98 specimens in case of the ITS and 109 in case of the *rpl32.* We performed dating analyses with BEAST v1.7 (Drummond and Rambaut, 2007). The validity of a strict molecular clock compared to a relaxed clock was tested in advance with the program Tracer v1.5.0 (Rambaut and Drummond, 2007) using Bayes factor method. Because of the lack of fossils from the *Centaurea* group we used a secondary calibration from a phylogeny of tribe Cardueae calibrated with six fossils as calibration points (Barres *et al.*, in press). The monophyly of all *Centaurea* sequences was constrained. To ease treefinding in BEAST, the main groups resulting from the MrBayes analysis were also constrained as monophyletic. Within the *Centaurea* group, no clades were constrained to be monophyletic.

The split between the ingroup comprising all *Centaurea* species and the three outgroups *Zoegea*, *Rhaponticoides* and *Psephellus* was estimated at 13.06 Ma (Barres *et al.*, in press) and was used as a calibration point. The normal distribution prior we used was given a standard deviation of ± 1.6 Ma, as published in Barres *et al.* (in press). For this analysis we performed model selection with MrModelTest v2.2 (Nylander, 2004) following the AIC. The best-fit model selected was GTR + G + I. For the tree prior we assumed a Yule-Process and an uncorrelated lognormal distribution for the molecular clock model (Drummond *et al.*, 2006; Ho, 2007). The other parameters were not changed from the default adjustment. The MCMC chains were run for 20 million generations saving one out of 1,000 trees. 10 % of the first sampled trees were removed as burn-in, and posterior probability density was summarized with TreeAnnotator v1.6.1 (Drummond and Rambaut, 2007). The estimated parameters and their 95 % highest posterior density of the most important nodes are shown in Table 3. The dated gene trees resulting from the BEAST analyses can be found in the supplementary material (S1-S2).

Network analyses

We conducted a network analysis for the entire ITS dataset using the Neighbor-Net (NN) algorithm (Bryant and Moulton, 2004) as implemented in SplitsTree4 v4.10 software (Huson and Bryant, 2006). As this method is sensitive to missing data, which can produce reticulations similar to those caused by conflicting characters, we excluded columns with missing bases. The criterion was set to uncorrected pair-wise (p) distances, excluding non-informative characters. For the *rpl32* chloroplast region of all members of the *Centaurea* group TCS v1.21 (Clement *et al.*, 2000) was used for constructing a statistical parsimony haplotype network. Insertions and deletions longer than one base pair were re-coded as single base pair mutations and were treated as fifth character state.

RESULTS

Phylogenetic and network analyses

Both the ITS and the *rpl32* region showed good resolution in the internal branches and insufficient resolution in external ones. The phylogenetic trees for the two markers resulting from the Bayesian analyses are given in Figs. 1 and 2. The trees given by the two different markers are not congruent (Fig. 3). In the ITS, support for two main clades (i.e. the Western and the Moroccan ribotypes) within the *Centaurea* group is good. A third group, the Eastern ribotype, shows only moderate support. These three groups are also clearly visible in the network resulting from the Neighbor-Net analysis (Fig. 4). Sequences of eight individuals, however, fall outside these three main groups, and they do not form a clade. They are discussed under the name "Central Mediterranean Clade". The geograhic distribution of the main ribotypes, and a few smaller ones nested therein, is shown in Fig. 5. The Bayesian and the Network analysis gave also clear groups for the cpDNA that follow mostly a geographic distribution (Figs. 2 and 6).

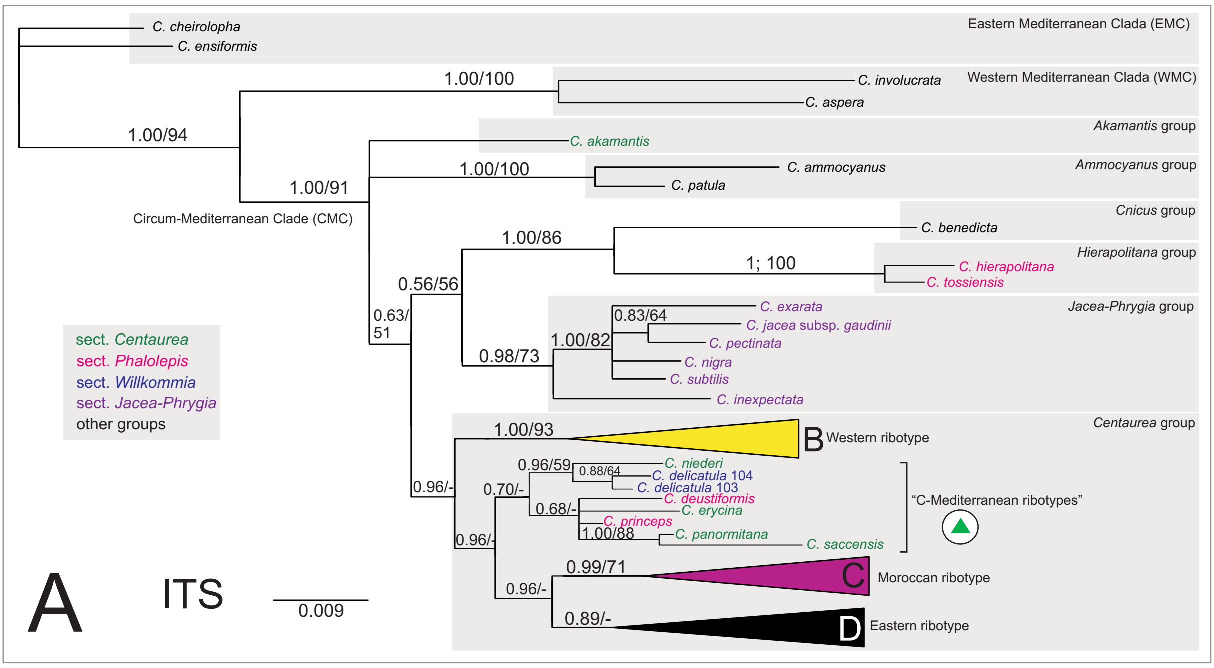

FIG. 1. A. Majority rule consensus tree obtained from Bayesian analysis of the ITS data. First number on branches indicates Bayesian posterior probabilities, second numbers after shlash indicate Bootstrap values (CI=0.596; HI=0.404). In cases, where more than one population per taxon was included, the population number (Table 2) is given. The distribution of the clades marked with a white circle is shown in Fig. 5.

FIG. 1. B–D. ITS tree, continuation from previous page.

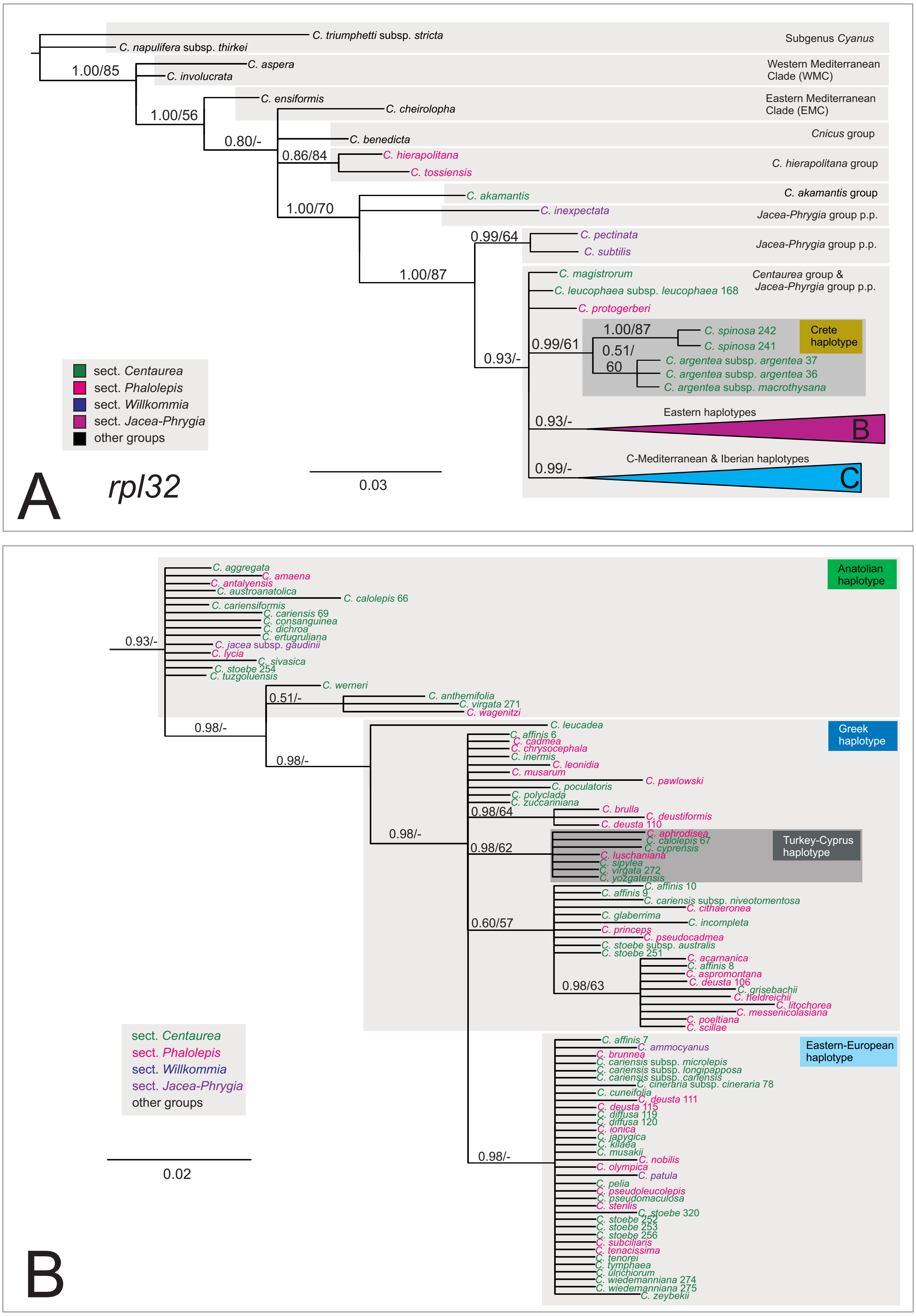

FIG. 2. A–B. Majority rule consensus tree obtained from Bayesian analysis of the cpDNA region *rpl32-trnL*. First number on branches indicates Bayesian posterior probabilities, second numbers after slash indicate Bootstrap values of the Parsimony tree (CI=0.908; HI=0.092). In cases where more than one population per taxon were included, the population number (Table 2) is given.

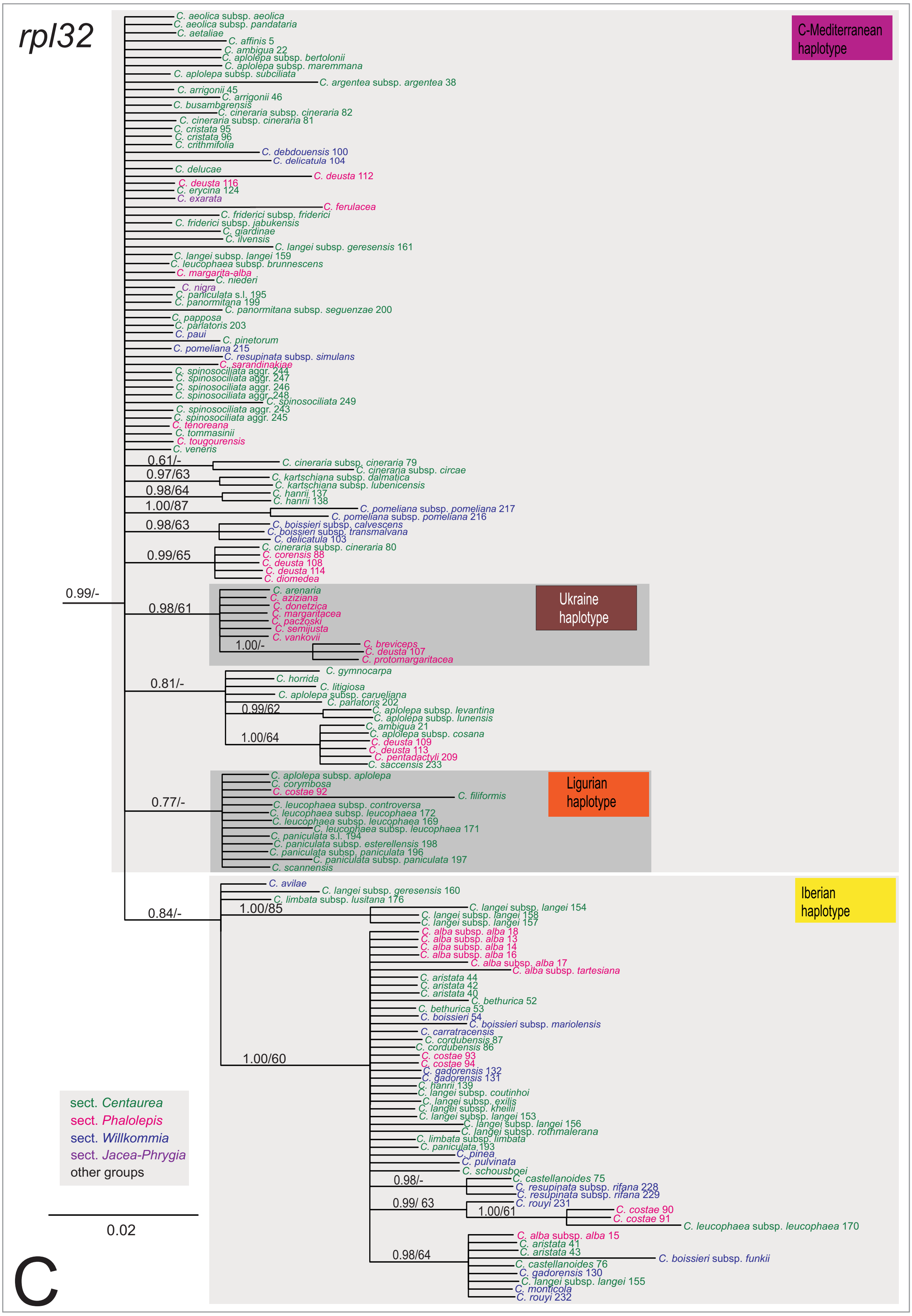

FIG. 2. C. *rpl32* tree, continuation from previous page.

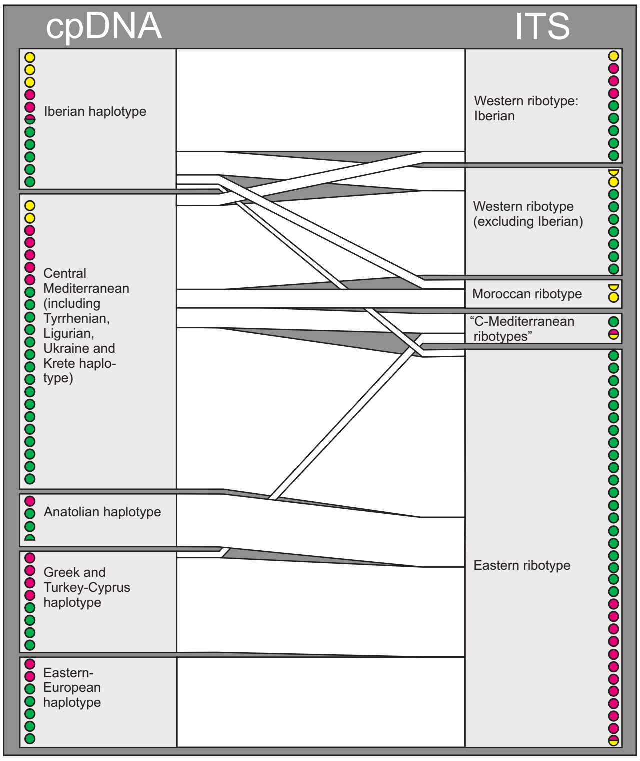

FIG. 3. Flow chart of the incongruences between the main clades of the *rpl32* and the ITS within the *Centaurea* group. Small circles amount to five populations, colors correspond to one of the three traditional sections: green = *Centaurea*; red = *Phalolepis*; yellow = *Willkommia*.

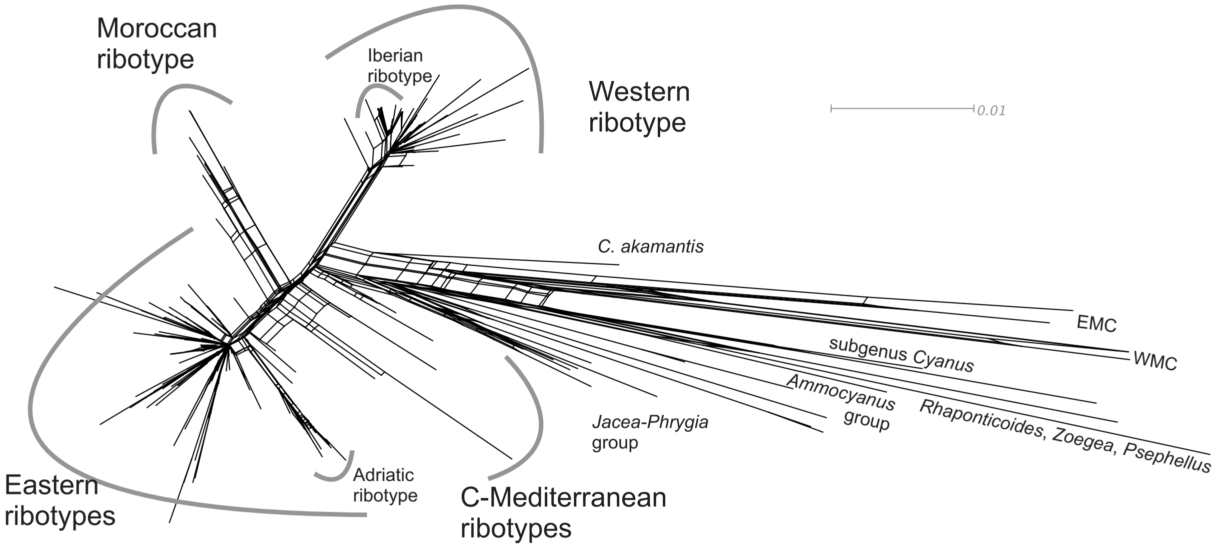

FIG. 4. Neighbor-Net network of ITS sequences.

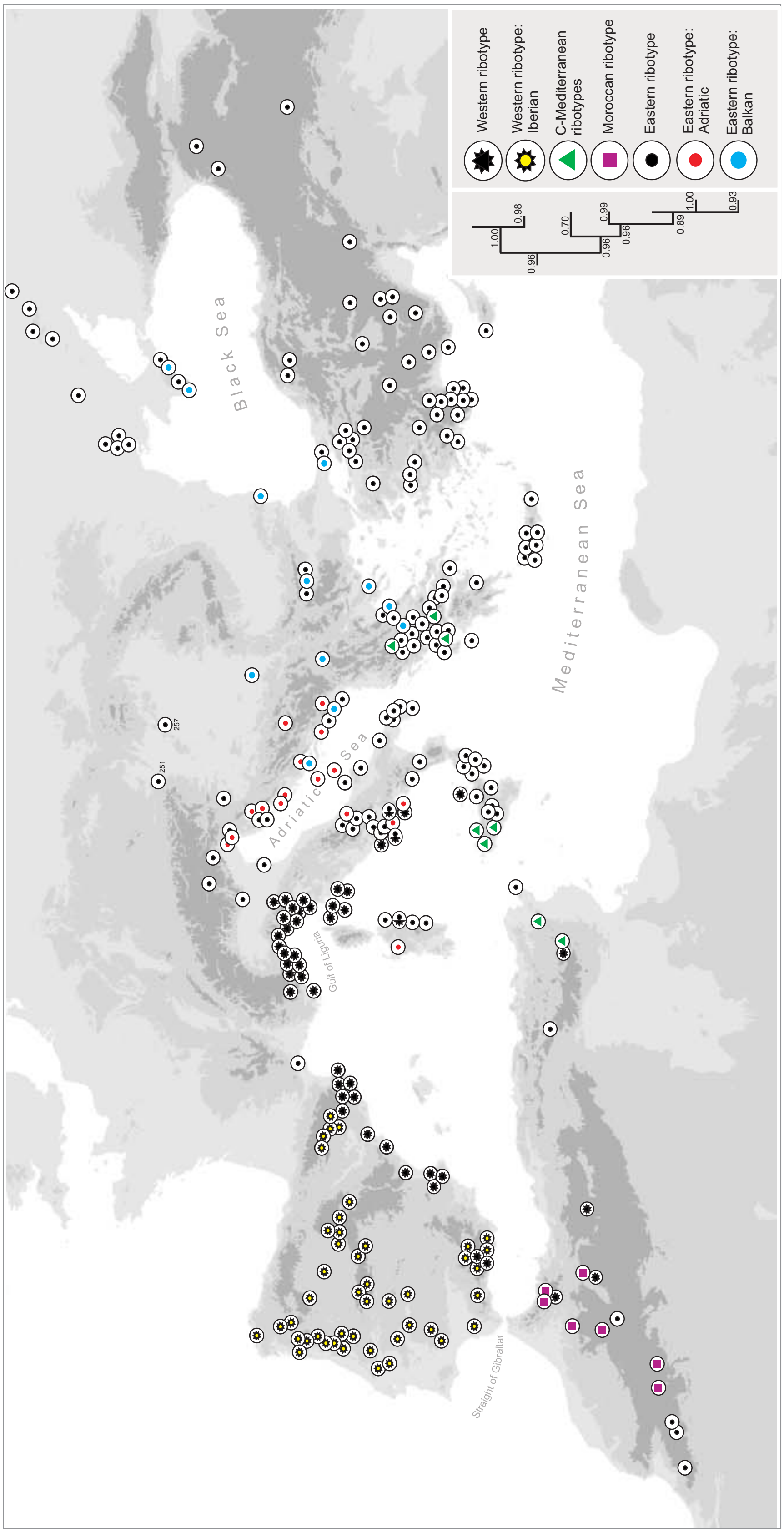

FIG. 5. Geographical distribution of ITS ribotypes derived from Bayesian analysis. The small tree shows a simplified version of the ITS tree, as shown in Fig. 1.

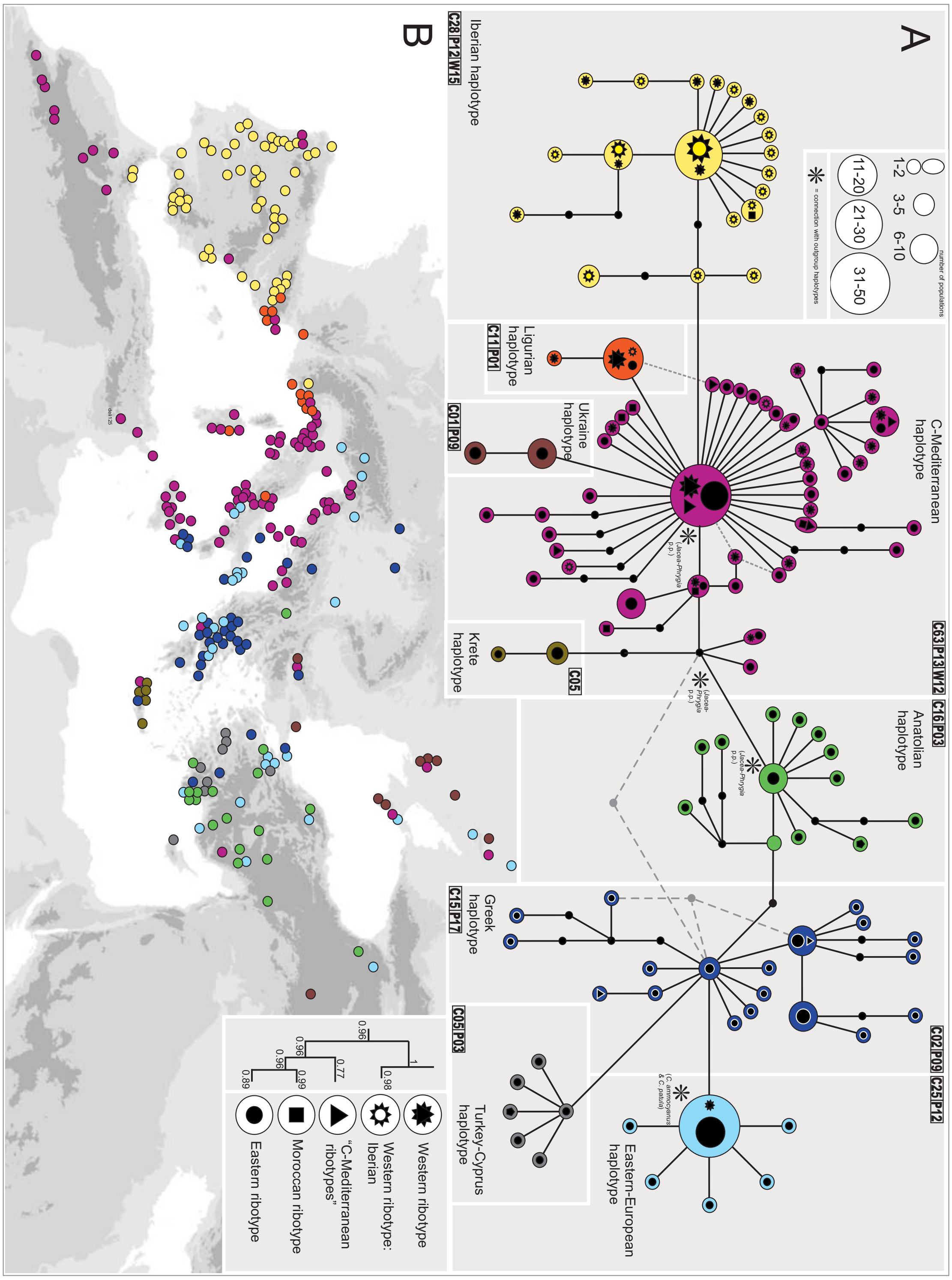

FIG. 6. A. Statistical parsimony network of plastid DNA haplotypes. A mutational step not sampled is given as small black dot. The asterisk shows the connection with haplotypes sampled in the outgroups, name of the outgroup in brackets. If the connection between two haplotypes were equally parsimonious, two lines are shown – in these cases the interrupted one was regarded as less likely due to geographic evidence. Size of the circles give the approximate quantity of populations showing the same haplotype. The symbols within the circles correspond to different ribotypes from the Bayesian tree (Figure 1) – symbols are explained on the right bottom side of the figure. The numbers in the corner of every haplotype field give the portion of every of the three sections of the haplotype group. A= *Centaurea*, P= *Phalolepis*, W= *Willkommia*. B. Geographical distribution of plastid DNA haplotypes derived from *rpl32* region.

Recombination tests

The recombination tests conducted with RDP gave no evidence of recombination and therefore allows us to use a tree model for the individual gene data sets without removing any sequences from the alignments. Also the Neighbor-Net analysis did not show clear evidence of recombination (Fig. 4).

Dating

The results of the Bayes Factor calculation (Newton and Raftery, 1994; Suchard *et al.*, 2001) for the ITS were significantly better with a relaxed clock than with a strict clock (–3758.75 vs. –3769.72), whereas they were almost equally good for the *rpl32* (–2516.91 for a relaxed and –2517.72 for a strict clock), therefore we used a strict clock for *rpl32* and a relaxed one for ITS. Age estimations conducted with BEAST for the main ITS and *rpl32* groups (Table 3) have a high uncertainty, expressed in a huge difference between lower and upper 95% Bayesian highest posterior density (HPD).

As the main groups in the two regions are incongruent, a direct comparison between the datings is not possible. Both markers, however, suggest that the *Centaurea* group started to diversify between approximately 3 and 8 Mya. The clades within the *Centaurea* group are estimated to have arisen between few hundred thousand years ago and 7 Mya, with the highest probability between 1.2 and 5 Mya.

TABLE 3. *Results of the ITS and rpl32 BEAST dating analyses with reduced data matrices; only node ages for resolved clades are shown*

DNA region	Crown age of different groups (95% HPD lower and upper)
ITS	
Circum-Mediterranean Clade	8.40 (5.67; 11.37)
Jacea-Phrygia group	3.73 (1.96; 5.77)
Centaurea group	5.74 (3.70; 8.09)
Western ribotype	2.73 (1.53; 4.33)
Iberian ribotype	1.31 (0.57; 2.26)
Moroccan ribotype	2.28 (1.10; 3.80)
Eastern ribotype*	3.39 (2.10; 4.91)
Adriatic ribotype	2.00 (0.61; 2.25)
Balkan ribotype	0.37 (0.03; 0.97)
rpl32	
Centaurea group, *Jacea-Phrygia* group, *C. akamantis* & *C. ammocyanus* group	7.7 (4.99; 10.40)
Centaurea group, *Jacea-Phrygia* group p.p. & *C. ammocyanus* group	6.41 (4.15; 8.87)
C-Mediterranean & Iberian haplotype	5.60 (3.48; 7.77)
Iberian haplotype (with intermediates)**	4.39 (2.48; 6.35)
Iberian haplotype (without intermediates)	3.33 (1.85; 4.96)
Eastern-European haplotype	1.70 (0.40; 3.28)
Turkey-Cyprus haplotype	1.55 (0.32; 3.13)
Crete haplotype	1.74 (0.29; 3.76)
Ukraine haplotype	1.60 (0.26; 3.30)

*** clade shows only a significant posterior probability in the BEAST analysis, but not in the MrBayes analysis.**

**** clade shows a posterior probability of 0.91 in the BEAST analysis.**

DISCUSSION

The interpretation of our results is by no means straightforward. Both cpDNA and nrDNA gene trees group many sequences into large poorly resolved clades and there is not enough information to distinguish between different populations or closely related *Centaurea* taxa. Only the most internal branches are partly resolved and supported. We shall discuss our results in terms of the classification and evolution of the group.

Overall classification

The Circum-Mediterranean Clade (CMC) of subgenus *Centaurea* consists of two core groups comprising the majority of the species of sections *Centaurea*, *Phalolepis*, *Willkommia*, *Jacea* and *Phrygia*, and a few species placed outside these core groups by ITS (Fig. 1) and partly by *rpl32* sequence data (Fig. 2).

The isolated species. *Centaurea akamantis*, *C. ammocyanus*, *C. benedicta*, *C. hierapolitana*, *C. patula* and *C. tossiensis* do not share any morphological characters. The chromosome numbers of some of them also deviate from the two core groups (Table 4). These species consist of (1) a small group of very similar, annual taxa from the easternmost Mediterranean region traditionally classified as sect. *Ammocyanus* (represented in our study by *C. ammocyanus* and *C. patula*). Chromosome numbers in this group are x = 7 in *C. patula* (Garcia-Jacas *et al.*, 1996) and x = 8 in *C. ammocyanus* (Ghaffari and Chariat-Panahi, 1985); (2) *Centaurea akamantis* from Cyprus, up to now member of section *Centaurea* (basic chromosome number x = 9; Georgiadis and Chatzikyriakou, 1993); (3) *C. hierapolitana* and *C. tossiensis* from S Turkey, classified in section *Phalolepis* (chromosome number of *C. hierapolitana* is $x = 8$ and of *C. tossiensis* $x = 9$; Uysal *et al.*, 2009); and (4) the morphologically very distinct *C. benedicta* (formerly *Cnicus benedictus*; basic chromosome number x = 11; Morton, 1981; Vogt and Aparicio, 1999). The last two groups are sister in the ITS-tree, and not in the cpDNA-tree, but do not share morphological traits.

Jacea-Phrygia and *Centaurea groups*. The remaining taxa consist of two major clades: the *Jacea-Phrygia* group including the morphologically defined sections *Jacea* and *Phrygia*, and the *Centaurea* group, comprising sections *Centaurea*, *Phalolepis* and *Willkommia*. Clear support for the monophyly of these two groups is only given by the ITS (Fig. 1). In the cpDNA, however, the *Jacea-Phrygia* group cannot be distinguished clearly from the *Centaurea* group (Fig. 2). Some of the species from sections *Jacea-Phrygia* are grouped together with members of the *Centaurea* group whereas others fall outside. However, the clear separation between the two clades is also supported by their chromosome numbers (x = 11 in the *Jacea-Phrygia* group and x = 9 in the *Centaurea* group) and by morphology. Most of the *Jacea-Phrygia* species have undivided leaves and showy outer flowers, whereas the *Centaurea* group usually has divided leaves and reduced showy outer flowers. There are, however, exceptions, which are also reflected in a few incorrect taxonomical assignments detected recently in one of the groups (Garcia-Jacas *et al.*, 2006; Hilpold *et al.*, 2009).

With respect to basic chromosome numbers, in *Centaurea* and in the entire subtribe Centaureinae there is a trend to descending disploid reduction (Garcia-Jacas and Susanna, 1992; Wagenitz and Hellwig, 1996; Garcia-Jacas *et al.*, 1996, 2000, 2001). Selvi and Bigazzi (2002) hypothesized that descending dysploidy was associated with shortening life cycles in *Nonea* (Boraginaceae) as an adaptation to arid habitats. Watanabe *et al*. (1999) also found a relationship between low chromosome numbers, annual habit and dry habitats in *Pogonolepis, Sondottia* and *Trichantodium* (Compositae) and in fact annual *C. patula* is the species within the *Centaurea* group with the lowest chromosome number. The higher chromosome number in the *Jacea-Phrygia* and *Cnicus* group would be therefore a plesiomorphic character.

Incongruences

The strong incongruence between the nuclear and chloroplast markers between *Jacea-Phrygia* and *Centaurea* is not an exception. Topology and branch lengths are highly incongruent between the two gene trees within the *Centaurea* group too (Fig. 3). Such a strong incongruence is usually explained by three different hypotheses: introgression, incomplete lineage sorting and duplication (Doyle, 1992; Wendel and Doyle, 1998; Degnan and Rosenberg, 2009). Other causes exist, but we can exclude some of them from consideration on the following grounds: recombination, because we tested for and failed to detect any such events using a permissive *p*-value; phylogenetic inference problems including choice of methods and models, because of the overall low sequence variation and because the parsimony networks, the parsimony analysis and Bayesian trees included the same groups; sample misidentification, because the same samples were used for both nuclear and chloroplast regions (i.e. the incongruence remains, irrespective of the species to which a sample is assigned).

Incongruences and ITS. The evolutionary behaviour of the ITS region is complex due to concerted evolution among its multiple copies (Álvarez and Wendel, 2003). Indeed, more than one ITS sequence type is frequently visible in the ITS of the *Centaurea* group (cf. also Suárez-Santiago *et al*., 2007), often easily detectable through double peaks in the chromatograms. We cloned a few examples and detected several different sequence types. However, these sequence types usually only differed by a few bases and were placed in the same clade. In some cases, two very different ribotypes occurred within one plant, but this happens mainly in geographical areas where two ribotypes occur syn- or parapatrically. Two examples are found in central Italy and Sardinia, where Eastern and Western ribotype meet. In *Centaurea cineraria* and *C. filiformis* these two ribotypes have been found within the same individual. In these cases recent hybridization between species carrying each of these two main ribotypes might be the reason for this co-occurrence.

Incomplete lineage sorting vs. hybridization. Hybridization between different species or between genetically divergent populations or races within a species (cf. Arnold, 1997) may indeed be a driving force in the genus *Centaurea*, as already emphasized in Suárez-Santiago *et al.* (2007). Many hybrid individuals or populations have been already described (e.g., Wagenitz, 1975; Blanca López, 1981a; Ochsmann, 1998, 2000). Homoploid hybridization with fertile offspring is frequent in *Centaurea* (Kummer, 1977; Fernández Casas and Susanna, 1986; Pisanu *et al*., 2011), probably as a consequence of the low genetic divergence between parental species (Paun *et al*., 2009). Homoploid hybridization may connect two parental lineages and subsequent introgressions may hamper reconstruction of the correct phylogeny. Moreover, in case of hybridization, gene trees derived from cpDNA and nrDNA may show strongly divergent topologies since their inheritance is different (uniparental vs. biparental; Birky, 2001) after several generations, the ITS copies may usually end up with only one type due to concerted evolution – either the same as in one of the parentals or an intermediate one due to recombination

(Soltis and Soltis, 2009). The cpDNA, however, would always remain the same as in the maternal chloroplasts since it does not undergo recombination (Gillham *et al.*, 1991; Kuroiwa, 1991). Due to higher intraspecific gene flow in nuclear genes than in the (typically) maternally inherited cpDNA, hybridization will lead more frequently to detectable introgressions in latter one (Rieseberg *et al.*, 1996; Currat *et al.*, 2008; Petit and Excoffier, 2009), this might be responsible for the somewhat more clear topology in the ITS compared to the *rpl32*.

Hybridization, however, may not be the only cause for the strong incongruence between the two gene trees. Hybridization is expected to occur mainly where two different lineages meet in the same geographical area, but not over large distances, since pollen and seed dispersal in *Centaurea* is fairly limited (Colas *et al.* , 1997; Bancheva *et al.*, 2006; Albrecht *et al.*, 2009). In addition, concerted evolution in many species of *Centaurea* goes at a slow pace because of the long generation time due to their rhizomatous habit (cf. Sang *et al.*, 1995; Devos *et al.*, 2005) and traces of such a hybridization event should be visible over a long time. Poor separation between the *Jacea*-*Phrygia* and the *Centaurea* groups in the cpDNA can hardly be explained by hybridization either, since the difference in chromosome numbers should inhibit gene flow (Baker and Bickham, 1986; Basset *et al.*, 2006). Finally a study with nuclear low copy genes (A. Hilpold *et al.*, unpubl. res.) showed likewise strong incongruence between the different markers, probably more than sole hybridization would explain.

Another hypothesis for interpreting strong incongruences between gene trees is incomplete lineage sorting (ILS; Tateno *et al.*, 1982; Pamilo and Nei, 1988; Maddison, 1997; Doyle and Gaut, 2000; Nichols, 2001). The patterns it produces can be very similar to those produced by hybridization (Holder *et al.*, 2001). The most important preconditions for ILS are big population sizes in the ancestral populations and a low number of generations between speciation events (Pamilo and Nei, 1988) both leading to short branch lengths (when measured in coalescent units) in the species tree (Edwards *et al.*, 2007). For distinguishing between hybridization and ILS, the assessment of the population sizes of the different clades is fundamental. This can be done by using methods from population genetics by analyzing DNA polymorphisms from sequence data. Large population sizes would favour ILS as explication while small ones would rather favour sole hybridization. Future work in this direction is planned.

When a high degree of ILS exists, an increase of genetic data alone (i.e. simply including more gene regions) is no guarantee for a more accurate species tree: concatenation, ‘democratic vote’ or consensus trees are likely to provide misleading results (Gadagkar *et al.*, 2005; Edwards *et al.*, 2007; Kubatko and Degnan, 2007; Degnan and Rosenberg, 2009). Only the additional use of multispecies coalescent approaches (for example Kubatko *et al.*, 2009; Heled and Drummond, 2010) including several individuals per population can help.

Incongruence between molecular data and morphology

The virtual absence of congruence between morphology and molecular data suggests that the morphological characters that have been used taxonomically are not reliable with respect to the true relationships. Some morphological traits may have developed convergently in several cases – a high plasticity of morphological characters is also likely. The development of equal or similar morphological characters may have partly been due to an adaptive response to similar ecological conditions, for example the development of small divided tomentose leaves as a response to dry conditions (cf. Larcher, 2003), or of spiny bract appendages as a response to herbivores (cf. Cooper and Owen-Smith, 1986).

Ancestral polymophisms for morphological characters. If we consider that ILS may have played a crucial role in the genetic evolution of the *Centaurea* group, ancestral polymorphisms in morphological characters are also a possible explanation for the occurrence of the same characters in different lineages. Thus, the two different bract appendages may have already been present in the common ancestor of the entire *Centaurea* group. The difference between membranaceous-lacerate and not membranaceous bract appendages, the main character used in traditional taxonomy for the sectional division, exists in both the *Jacea-Phrygia* group and the *Centaurea* group, and is even present in some taxa from CMC that fall outside these two groups. That means that these characters may have existed already in the common ancestor of these different clades or, alternatively, they have been developed separately several times – being a proof of the characters' plasticity. A similar evolution of the bracts was suggested for the Turkish sects. *Cheirolepis-Plumosipappus* (Ertuğrul *et al.*, 2004).

TABLE 4. *A proposal for a new classification based on molecular grounds within the Circum-Mediterranean Clade* (CMC) and basic chromosome numbers*

Genus *Centaurea*	**Basic chromosome numbers *x* =**
Subgenus *Cyanus*	8, 9, 10, 11 & 12
Subgenus *Lopholoma*	10 & 11
Subgenus *Centaurea* (corresponds to the *Jacea* group)	
Eastern Mediterranean Clade (EMC)	8, 9 & 10
Western Mediterranean Clade (WMC)	8, 10, 11 & 12
Circum-Mediterranean Clade (CMC)	
Section *Akamantis*	9
Section *Ammocyanus*	7 & 8
Section *Centaurea***	9
Section *Cnicus*	11
Section *Hierapolitana*	8 & 9
Section *Jacea*	11
Section *Phrygia*	11

* only sections of the CMC are shown.

** including sections *Phalolepis* and *Willkommia*, which are not supported molecularly

How to interpret ribo- and haplotypes

Because of the assumed existence of these three phenomena, i.e. hybridization, ILS and a high plasticity in morphological characters, it is complicated to define on molecular grounds the limits of taxonomical entities within the *Centaurea* group above the species level. Even though some of the groupings found in the ITS (Figs. 1, 4, 5) and in the *rpl32* (Figs. 2, 6) may correspond to real evolutionary lineages, for example the split between western Mediterranean and Moroccan, Central and Eastern Mediterranean ribotypes in the ITS tree (Fig. 1), it is impossible to verify whether they are really monophyletic lineages in the species tree. Their exact delimitation might be blurred by hybridization and ILS. The traditional morphology-based taxonomy, i.e. the sectional division into *Centaurea*, *Phalolepis* and *Willkommia* and the additional division into many small groups by Dostál (1976) are not reflected at all. We hence suggest that section *Centaurea* should be redefined as including sections

Phalolepis and *Willkommia*. This redefined section *Centaurea* would be characterized by its uniform basic chromosome number $x = 9$, a perennial, biennial or rarely annual life form, divided leaves (at least the basal ones), usually small flower heads with lacerate-membranaceous to ciliate-fimbriate, sometimes spiny bract appendages and reduced outer showy flowers. Other sections of subgenus *Centaurea* would be *Akamantis, Ammocyanus*, *Cnicus*, *Hierapolitana, Jacea* and *Phrygia* (Table 4).

Biogeography

Even though molecular data are not sufficient for any taxonomical subdivisions within the *Centaurea* group, it provides still information about biogeographical history of the group. We can actually see a clear geographical structure in our data if we draw them on a map (Figs. 5 and 6). The geographical patterns of cpDNA and nuclear DNA, however, are incongruent. For example, the western Mediterranean group in one marker is differently delimited than in the other. In many cases it can be stated that the closer two populations occur, the tighter is their molecular relationship, totally independent of their taxonomical affiliation. Geography is again a better predictor than morphology for relationships within the *Centaurea* group, as already stated in Suárez-Santiago *et al.* (2007) and Hilpold *et al.* (2011). The most obvious interpretation for these patterns is a stepwise dispersal with subsequent diversification. Genetic admixture between the main clades may have been mainly restricted to the contact zones between these clades, otherwise the clear geographical patterns would have been blurred.

Dispersal

The main dispersal direction in the *Centaurea* group may have been from east to west, since the closest related lineages of the *Centaurea* group exist in the eastern Mediterranean. In fact, the haplotypes of the central and eastern Mediterranean can also be found in the *Jacea-Phrygia* group and the *Ammocyanus* group (Fig. 2). For reaching the westernmost part of the Mediterranean two pathways are possible: via south, over Greece, S-Italy, and Sicily to Tunisia; and via north, coming from the Balkans traversing N-Italy and S-France.

The southern pathway connecting Greece and S-Italy is especially obvious from the cpDNA network (Fig. 6), where the predominantly E-Mediterranean Greek and Eastern-European haplotypes were also found in Apulia and in Calabria on the southernmost tips of the Apennine Peninsula, whereas they are absent in all of Central Italy. The connection between S-Italy and NW-Africa is shown by similar or identical haplo- and ribotypes on both landmasses (Figs. 5 and 6). The same southern dispersal way with "landmark species" on the Island of Sicily and northern Algeria and Morocco was shown for *Centaurea* subgen. *Acrocentron* (Font *et al.,* 2009).

The northern pathway finds support in the presence of the Greek and the Eastern European haplotypes in northen Italy, Austria and Hungary. The wide distribution of the Greek haplotype from the eastern Black See coast till the Alps might testify an expansion on the Balkan Peninsula.

For reaching the Iberian Peninsula again two pathways are possible: by crossing the Strait of Gibraltar from the south or over northern Italy and southern France. The connection between Africa and the Iberian Peninsula is given in both markers (Figs. 5, 6). Morphologically, members of the *Centaurea* group from these two areas are hard to distinguish and in some cases they were even merged into one single species (*Centaurea boissieri* DC. and *C. resupinata* Coss.). The northern pathway, however, cannot be excluded and in fact there is a continuum of the Ligurian haplotype from northwest Italy to northeast Spain (Fig. 6). Species presenting this haplotype belong to the *Centaurea paniculata* complex, which would be the group from which all Iberian members have derived, as suggested by Blanca López (1981b), if we accept the northern pathway as predominant. However, the interpretation of the populations in the Ligurian gulf is ambiguous. On the one hand they possess the Western ribotype, suggesting a close relation with Iberian populations, on the other hand they possess the Central Mediterranean haplotype, alike populations in the rest of Italy. One possibility would be that this entire group is product of hybridization between representatives of the Iberian and Italian clades. In this sense, the presence of the Iberian haplotype in northern Italy reinforces the hypothesis of a bidirectional migration between the extreme north-east Iberia and north-west Italy.

As the interior of the Iberian Peninsula was colonized, a separate ribosomal lineage could develop, only found in the continental parts of the peninsula. The distribution of the Iberian haplotype (Fig. 6) corresponds mainly with the Iberian ribotype (Fig. 5), and it may reflect the same evolutionary event.

As already mentioned, the picture in the eastern Mediterranean is more confusing. Groupings, as the Anatolian haplotype or the Adriatic ribotype (Figs. 5 and 6), are likely to reflect some shared evolutionary processes, but their delimitation and monophyly is highly uncertain. The Adriatic ribotype (Fig. 5) has its center on the eastern shore of the Adriatic sea, encompassing mainly members of the *C. spinosociliata* aggr., a group inhabiting calcareous coastal rocks. Today these species are in tight contact with other Balkan members of the *Centaurea* group, like *C. stoebe* and *C. deusta*. But there may have been a period of isolation during Quaternary cold times, when the mountainous inner parts of the Western Balkans might have been a strong barrier towards the Eastern Balkans. Although this group is not visible in the *rpl32*, its eastern edge coincides more or less with the eastern limit of the Central Mediterranean haplotype (Fig. 6).

Interpretation of the Balkan ribotype is even more difficult (Fig. 5). The remarkable connection of ribotypes between the southeastern Balkans and Crimea is similar to that of the Ukraine haplotype (Fig. 6) and may testify a dispersal from the Mediterranean to the northern Black Sea along its western shore.

When did all these processes happen?

Datings for the ITS and the *rpl32* (Table 3) have very high uncertainty. Nevertheless, they contribute to place the data into a biogeographical context. The crown age of the entire *Centaurea* group in the ITS is 5.7 Mya. In the *rpl32* the node age for the group containing sections *Centaurea* and *Ammocyanus*, and part of the *Jacea* group is about 6.4 Mya. Even considering the broad uncertainty, both ages allow the interpretation that the dispersal into the western Mediterranean took place during the Messinian Salinity Crisis (5.96–5.33 Mya; Hsü, 1972). The main clades inside these two groups have an age between 1 and 5 Mya. The success of the *Centaurea* group would have been positively influenced by the onset of the Mediterranean climate around 3.2–2.8 Mya (Suc, 1984; Thompson, 2005). The pronounced droughts during the Mediterranean summer, especially in the eastern and southern parts, may have provided the group with a new range of habitats and may have fostered its diversification. A large amount of the diversity, however, evolved during the last 2 Mya during the Quaternary, when cold periods also lowered precipitation, allowing steppe-like vegetation to dominate in the continental interiors of the Mediterranean – a fact which might have been highly beneficial for a group of plants which can only be successful in open vegetation. The steppe vegetation in large parts of Europe may have favored the dispersal from east to west via the northern Mediterranean. The dating, however, is not precise enough to correlate evolutionary events with single climatic events, for example with a certain glacial period. With the frequently changing environment during the Quaternary, the distribution areas may also have changed frequently, providing new possibilities for isolation and merging as suggested in another section of *Centaurea*, sect. *Acrocentron* (Font *et al*., 2009). As the last ice age has just finished, this process may not have stopped yet. The development of intrinsic breeding barriers, an important property that has to be adopted for the completion of the speciation process (de Queiroz, 2007), is totally lacking in most members of the *Centaurea* group. We can therefore assume that the speciation in the *Centaurea* group is recent or even contemporary, a situation where the velocity of evolution may outpace the sensitivity of molecular markers (Ramdhani *et al*., 2011) – introducing flaws into the molecular results through lack of monophyly, high incongruence and huge polytomies.

CONCLUDING REMARKS

Hybridization between recently diverged lineages, which may be morphologically distinct, has surely created further confusion between morphological and molecular sources of evidence. However, it should not be considered the only reason for the virtual absence of clear congruence between the cpDNA, the nuclear ITS marker and morphology-based taxonomy. Also important is the fact that some morphological changes in the *Centaurea* group, such as leaf shape or shape of the bract appendages, may happen quickly (cf. Hilpold *et al*., 2011) and that very different molecular and morphological character traits may have already co-occurred within existing breeding communities and have led to incongruencies through incomplete lineage sorting. These breeding communities or syngameons may be larger than the described species. We conclude that not only the existence of the three morphologically defined sections is highly doubtful, but also that the delimitation between at least some of the many described species is questionable. In any case, unraveling the evolutionary history of *Centaurea* will remain a big challenge for botanists in the future.

Concluding remarks

Financial support from the Spanish Ministry of Science and Innovation (Projects CGL2007-60781/BOS and CGL2010-18631) and the Generalitat de Catalunya (Ajuts a Grups de Recerca Consolidats 2009/SGR/00439) is gratefully acknowledged. A. Hilpold benefited from a predoctoral grant of the JAE program of the CSIC. Special thanks go also to the Parco Nazionale della Majella (prot. n. 1763), to the Parco Naturale Regionale di Porto Venere (prot. n. 2804), to the Parco Nazionale Arcipelago Toscano (prot. n. 3363), to the Parco della Madonie (prot. n. 2195), to the Parco Nazionale delle Cinque Terre (prot. n. 2137), to the Parco Nazionale del Circeo (prot. n. PNC/2008/196) and to the Riserva Naturale delle Gole del Sagittario (prot. n. 1081) and to the Junta de Andalucía (Ref. SGYB/FAO/CRH Re-519-08) for the permission to collect plants in their territory. We also thank A. Sánchez-Meseguer for the help with BEAST dating; Y. Bağcı, A. Bertolli, O. Derkach, P. Escobar, R. Flatscher, B. Frajman, M. Galbany-Casals, T. Garnatje, T. Guidi, T. Karamplianis, T. Kiebacher, J. López-

Alvarado, B. Medagli, J. Molero, I. Moysienko, S. Pisanu, L. Poldini, F. Prosser, A. Romo, I. Sánchez-Jiménez, P. Schönswetter, S. Tomaselli and O. Tugay for their help in collecting specimens.

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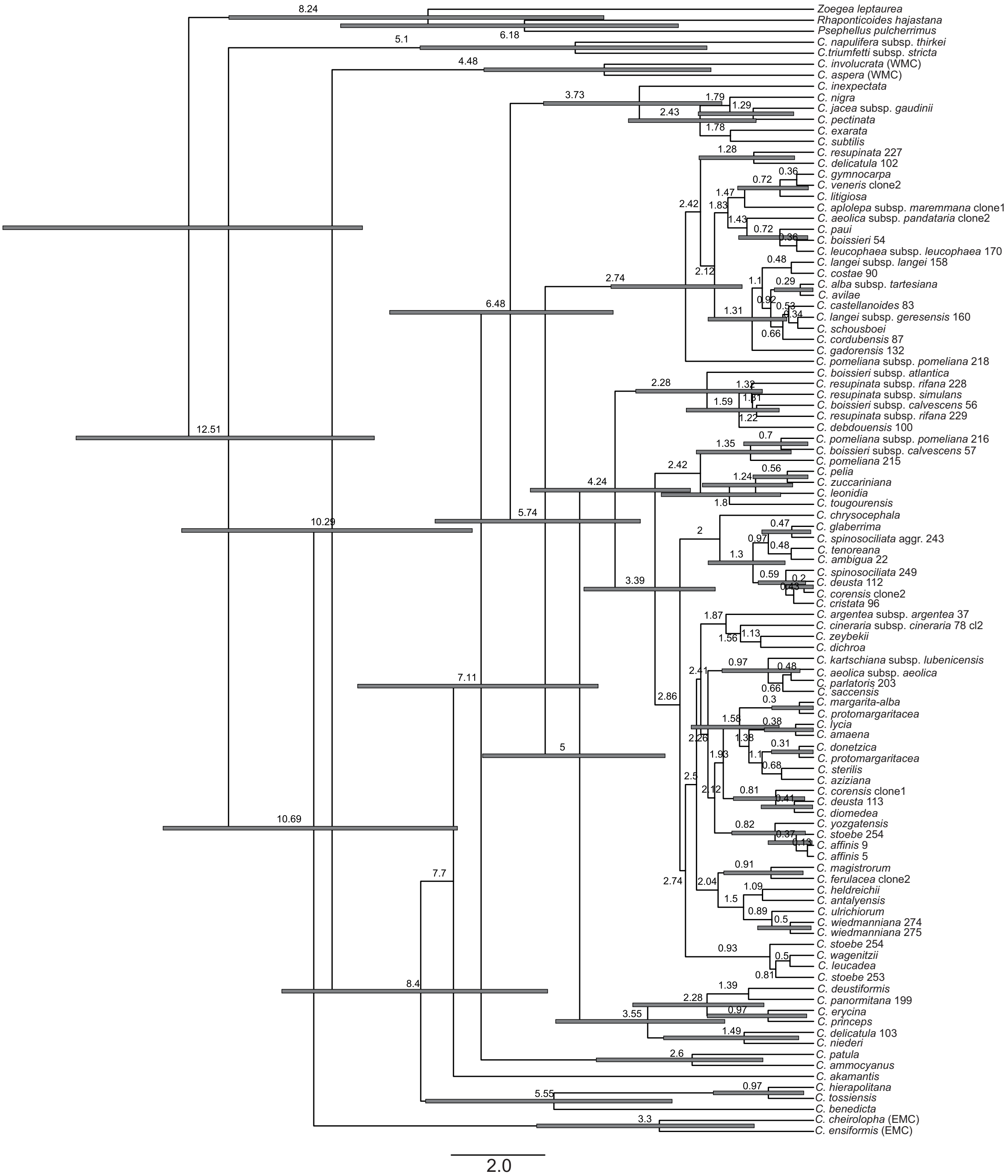
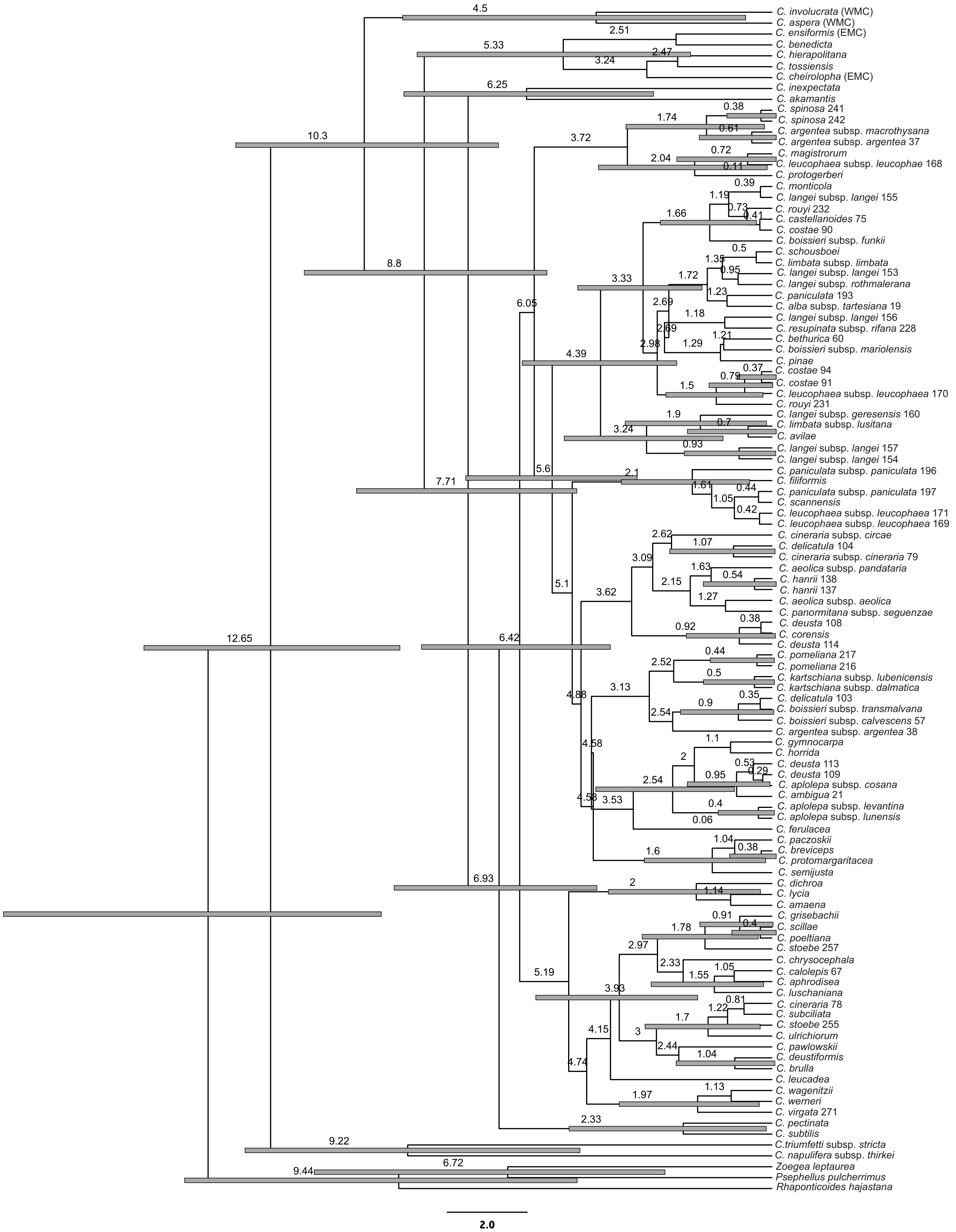


SUPPLEMENTARY MATERIAL S1. BEAST tree of the ITS with reduced data matrix. Values next to nodes show node ages. Numbers give millions of years.



SUPPLEMENTARY MATERIAL S2. BEAST tree of *rpl32* with reduced data matrix. Values next to nodes show node ages. Numbers give millions of years.

Capítol 2: s'ha publicat en *Collectanea Botanica (Barcelona)*
[*second publication, has been published in* Collectanea Botanica (Barcelona)]

Títol original [*original title*]:
Two additions to the *Jacea-Lepteranthus* complex: parallel adaptation in the enigmatic species *Centaurea subtilis* and *C. exarata*
[*Dues addicions al complex* Jacea-Lepteranthus*: adaptacions paral·leles en les enigmàtiques espècies* Centaurea subtilis *i* C. exarata]
[*Dos adiciones al complejo* Jacea-Lepteranthus*: adaptaciones paralelas en las enigmáticas especies* Centaurea subtilis *y* C. exarata]

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Resum en català i castellà [*abstract in Catalan and Spanish*]: pàgina següent [*see below*]

Collectanea Botanica (Barcelona)
vol. **28** (2009): 19-30
ISSN: 0010-0730
doi: 10.3989/collectbot.2008.v28.003

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

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Received 2 February 2009; Accepted 29 May 2009

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*; cariología; *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*; cariologia; *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 involucral 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.*, 2007*a*, 2007*b*).

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 involucral 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 4ºC 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 CO_2, 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 94ºC, followed by 30 cycles of 94ºC denaturing for 1 min 30 s, 57ºC annealing for 2 min and 72ºC extension for 3 min, with an additional 15 min at 72ºC. 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
Centaurea aggregata Fisch. & C. A. Mey. ex DC.	Caucasus, Iran, Turkey (weed)	Turkey, Adana: Ala Dağ above Dağdibi, 2000 m, 03.08.2002, *Ertuğrul, Garcia-Jacas, Susanna 2305 & Uysal* (BC)	DQ319077
Centaurea alba L. subsp. *costae* (Willk.) Dostál	Iberian Peninsula endemic	Spain, Huesca: Peña de Oroel, 15.07.1947, *Fernández-Galiano & Rivas Goday 23733* (GDA)	AM114325
Centaurea alba L. subsp. *latronum* (Pau) Dostál	Iberian Peninsula endemic	Spain, Ávila: La Adrada, 27.07.1982, *Sánchez-Mata & Cantó 24946* (GDAC)	AM114326
Centaurea aeolica Guss. ex DC.	Italy, endemic	GenBank	AM117057
Centaurea avilae Pau	Iberian Peninsula endemic	Spain, Ávila: Sierra de Gredos, 30.07.1979, *Blanca 6087* (GDAC)	AM114309
Centaurea aziziana Rech. f.	Caucasus endemic	Iran, Azarbayjan-e-Sharghi: between Tatar and Golfa, 85 km from Golfa, 07.08.1996, *Garcia-Jacas, Mozaffarian, Susanna 1680 & Vallès* (BC)	DQ319089
Centaurea boissieri DC. subsp. *boissieri*	Iberian Peninsula endemic	Spain, Granada: Sierra de Cázulas, 08.06.1979, *Blanca 6597* (GDAC)	AM114278
Centaurea cadmea Boiss.	Turkey endemic	Turkey, Burdur: 4 km from Burdur on the road to Sparta, mountains above Burdur, 1200 m, 28.07.2002, *Ertuğrul, Garcia-Jacas, Susanna 2249 & Uysal* (BC)	DQ319094
Centaurea calolepis Boiss.	Turkey endemic	Turkey, Burdur-Muğla: Dirimli mountain pass, 1600 m, 29.07.2002, *Ertuğrul, Garcia-Jacas, Susanna 2254 & Uysal* (BC)	DQ319095
Centaurea cariensis Boiss.	Turkey endemic	Turkey, Antalya: 40 km from Elmalı on the road to Korkuteli, N slopes of the Karamanbeli mountain pass, 1400 m, 30.07.2002, *Ertuğrul, Garcia-Jacas, Susanna 2258B & Uysal* (BC)	DQ319097
Centaurea carratracensis Lange	Iberian Peninsula endemic	Spain, Málaga: Carratraca, Sierra de Aguas, 04.07.1998, *Blanca 42802* (GDAC)	AM114302
Centaurea corymbosa Pourr.	S France endemic	France, Narbonne: La Clappe, 1995, *M. Riba s. n.* (BC)	DQ319103
Centaurea debdouensis Breitw. & Podlech	Morocco endemic	Morocco, Debdou: Gaada de Debdou, 18.06.1954, *Pasquier & Ch. Rungs s. n.* (MPU)	AM114317
Centaurea deusta Ten.	Italy endemic	Italy, Calabria: Crotone, Torrente Matassa near Caccuri, 360 m, *Vogt 15531*, Berlin Botanical Garden, Index Seminum 1997	DQ319107
Centaurea diffusa Lam.	Widespread (weed)	Armenia, Talin: between vil. Pokr Arthik and Bagravan, 26.08.1995, *Fajvush, Gabrielyan, Garcia-Jacas, Guara, Hovhannisyan, Susanna 1589, Tamanyan & Vallès* (BC)	DQ319108
Centaurea donetzica Klokov	Ukraine endemic	Ukraine, Donetzkaya: Krasny Liman, 12.08.2002, *Romashchenko s. n.* (BC)	DQ319110
Centaurea exarata Coss.	Iberian Peninsula endemic	Spain, Huelva: road A-983, Almonte to Matalascañas km 25, 09.07.1999, *Roché & Susanna 1909* (BC)	DQ319113

Table 1. (Cont.)

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

Table 1. (Cont.)

Species	Range	Voucher	ITS accession
Centaurea vankovii Klokov	Ukraine endemic	Ukraine, Crimea: Alupka, Ai-Petri mountain, 30.08.2002, *Romashchenko s. n.* (BC)	DQ319173
Centaurea virgata Lam.	Turkey endemic	Turkey, Muğla: Köyceğiz district, Sandras Dag range 13 km from Ağla, 1700 m, 29.07.2002, *Ertuğrul, Garcia-Jacas, Susanna 2252 & Uysal* (BC)	DQ319174
Centaurea wiedemanniana Fisch. & C. A. Mey.	Turkey endemic	Turkey, Bilecik: Selimiye, between Osmaneli and Bilecik, 100 m, 01.07.1962, *Davis & Coode* (E)	DQ319175
Outgroup			
Centaurea mollis Waldst. & Kit.	E Europe	Ukraine, Podolia: Lysa Hora, 2 km E of Vilshanitsa near Zolochiv, 05.06.2000, *Boratyñski & Romo 0506D* (BC)	DQ319133
Centaurea napulifera Rochel subsp. *thirkei* (Sch. Bip.) Stoj. & Acht.	E Europe	Romania, Constanta: Dobrogea, N from Cheia village, Cheia gorges of the Casimcea river, 130 m, 02.04.1996, *Badarau* (BC)	DQ319136
Vouchers used for figures 1 and 3			
Centaurea subtilis Bertol.	Italian endemic	as above	
Centaurea exarata Coss.	Iberian Peninsula endemic	as above	
Centaurea linifolia L.	Iberian Peninsula endemic	as above	
Centaurea jacea subsp. *vinyalsii* (Sennen) O. Bolòs, Vigo & J. M. Panareda	Iberian Peninsula endemic	as above	
Centaurea jacea subsp. *gaudinii* (Boiss. & Reut.) Gremli	Europe, Mediterranean	Italy, Südtirol, Sarntaler Alps, Feldthurns, 1 km WNW Garner Wetterkreuz, 1400 m, 16.08.2008, *Hilpold* (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 ≥ 95% were considered statistically significant.

Morphological survey

To prepare Fig. 3, the middle involucral 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 paralellisms with *C. exarata*. Their appendages of the involucral 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, mesophyllic, mostly glabrous or sparsely hirsute leaves, whereas *C. subtilis* and *C. exarata* have narrow, tomentose, more

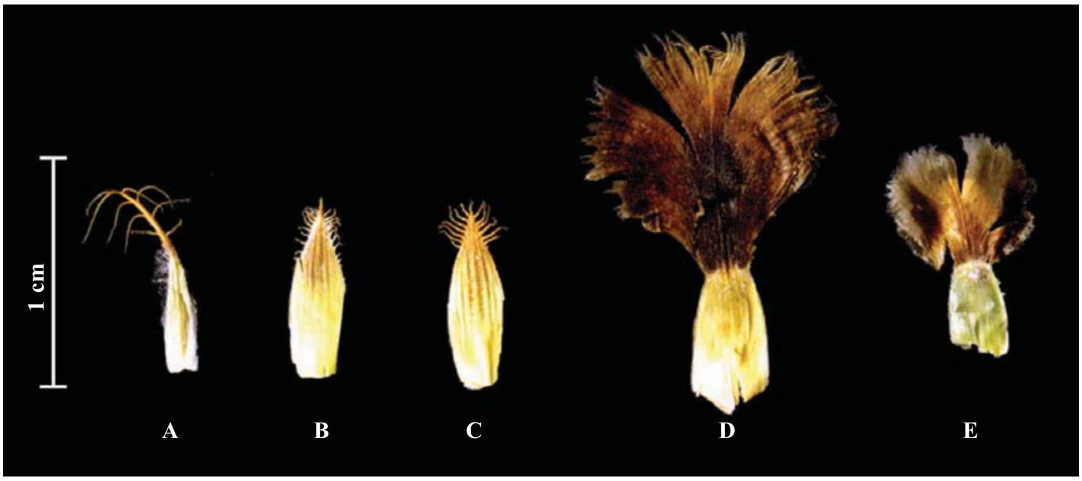

Figure 3. Middle involucral bracts of five different taxa of the Jacea Group. (A) *Centaurea linifolia* L.; (B) *C. subtilis*; (C) *C. exarata*; (D) *C. jacea* subsp. *vinyalsii* (Sennen) O. Bolòs, Vigo & J. M. Panareda ; (E) *C. jacea* subsp. *gaudinii* (Boiss. & Reut.) Gremli.

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-radiated 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).

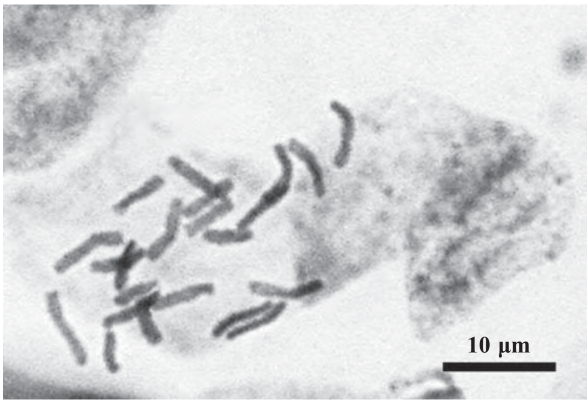

Figure 4. Orcein-stained metaphase plate of *Centaurea subtilis*. $2n = 22$.

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*.

ACKNOWLEDGEMENTS

Financial support from the Spanish Ministry of Education and Science (Project CGL2007-60781/BOS) and the Generalitat de Catalunya (*Ajuts a Grups de Recerca Consolidats* 2005/SGR/00344) is gratefully acknowledged. A. Hilpold benefited from a predoctoral grant of the JAE program (CSIC). Special thanks go to the *Parco Nazionale del Gargano* for the permission to collect plants in its territory.

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Capítol 3: s'ha publicat en *Taxon*
[*first publication, has been published in* Taxon]

Títol original [*original title*]:

Evolution of the central Mediterranean *Centaurea cineraria* group (Asteraceae): Evidence for relatively recent, allopatric diversification following transoceanic seed dispersal

[*Evolució del grup* Centaurea cineraria *(*Asteraceae*) del Mediterrani central: Evidència d'una diversificació recent i al·lopàtrica seguida d'una dispersió transoceànica de llavors*]

[*Evolución del grupo* Centaurea cineraria *(*Asteraceae*) del Mediterráneo central: Evidencia de diversificación reciente y alopátrica seguida de dispersión transoceánica de semillas*]

Autors [*authors*]:

Andreas Hilpold, Peter Schönswetter, Alfonso Susanna, Núria Garcia-Jacas & Roser Vilatersana

Resum en català [*abstract in Catalan*]:

Es va explorar la diversificació espai-temporal del grup de *Centaurea cineraria*, a partir de marcadors *AFLP* i de les seqüències de DNA cloroplàstic, en un ampli mostreig de tàxons del Mediterrani central del grup *Centaurea* (= *Acrolophus* subgroup). Malgrat la seva singularitat morfològica, no es va poder recolzar la monofília del grup de *C. cineraria*. Es defineix un llinatge diferent, sobretot restringit a Sicília (el *Sicily group*), que conté alguns membres del grup de *C. cineraria*, però també inclou *C. parlatoris*, considerada membre del grup *C. dissecta*. No s'ha pogut aclarir les relacions del *Sicily group* amb altres membres del grup *Centaurea*. La datació molecular recolza una diversificació recent, presumiblement al·lopàtrica, iniciada fa menys de 250.000 anys. Tunísia, les illes Eòliques i l'illa de Ventotene del mar Tirrè van ser probablement colonitzades des de Sicília. La diversificació recent del *Sicily group* descarta la possibilitat de vicariança a través de ponts terrestres en favor d'una dispersió transoceànica. Aquesta dispersió podria haver estat afavorida pel nivell més baix del mar durant les èpoques fredes del Pleistocè. Les dades moleculars indiquen que la taxonomia del grup de *C. cineararia* necessita una revisió.

Resumen en castellano [*abstract in Spanish*]:

Investigamos la diversificación espacio-temporal del grupo de *Centaurea cineraria*, a partir de marcadores *AFLP* y ADN plastídico, aplicados a un amplio muestreo de táxones del Mediterráneo central pertenecientes al grupo *Centaurea* (= *Acrolophus* subgroup). A pesar de su singularidad morfológica, no pudimos apoyar la monofilia del grupo de *C. cineraria*. Se define un linaje distinto, sobre todo restringido a Sicilia (el *Sicily group*), que contiene algunos miembros del grupo de *C. cineraria*, pero también incluye *C. parlatoris*, considerada miembro del grupo *C. dissecta*. No hemos podido aclarar las relaciones del *Sicily group* con otros miembros del grupo *Centaurea*. La datación molecular apoya una diversificación reciente, presumiblemente alopátrica, iniciada hace menos de 250.000 años. Túnez, las islas Eólicas y la isla Ventotene del mar Tirreno fueron probablemente colonizadas desde Sicilia. La diversificación reciente del *Sicily group* descarta la posibilidad de vicarianza a través de puentes terrestres en favor de una dispersión transoceánica. Esta dispersión podría haber estado favorecida por el nivel más bajo del mar

durante las épocas frías del Pleistoceno. Los datos moleculares indican que la taxonomía del grupo de *C. cineararia* necesita una revisión.

TAXON 60 (2) • April 2011: 528–538

SPECIATION

Evolution of the central Mediterranean *Centaurea cineraria* group (Asteraceae): Evidence for relatively recent, allopatric diversification following transoceanic seed dispersal

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Abstract We explored the spatiotemporal diversification of the *Centaurea cineraria* group based on AFLP fingerprints and plastid DNA sequences applied to a broad sampling of Central Mediterranean taxa of the Acrolophus subgroup. Despite its morphological distinctness, monophyly of the *C. cineraria* group was not supported by our data. A distinct lineage mostly restricted to Sicily (the Sicily group) comprised some members of the *C. cineraria* group but also included *C. parlatoris*, assumed to be a member of the *C. dissecta* group. The relationships of the Sicily group with other members of the Acrolophus subgroup could not be clarified. Molecular dating supported recent, presumably allopatric diversification whose onset dates back to less than 250,000 years within the Sicily group. Tunisia, the Aeolian Islands and the Island Ventotene in the central Tyrrhenian Sea were likely colonised from Sicily. The young age of diversification within the Sicily group rejects land bridges between Africa and Sicily, which were only available during significantly older periods, in favour of transoceanic seed dispersal. Dispersal might have been favoured by low sea levels during cold stages of the Pleistocene. The molecular data indicate that taxonomy of the *C. cineraria* group needs to be revised..

Keywords AFLP; biogeography; Mediterranean; phylogeography; Sicily; transoceanic dispersal

■ INTRODUCTION

The Mediterranean is one of the global centres of biodiversity (Myers & al., 2000) owing to a complex geographic and climatic history (Thompson, 2005). Moreover, the Mediterranean Basin is situated at the crossroads of three continents and has served as meeting ground and eventually melting pot for a variety of lineages (Blondel & Aronson, 1999). A plethora of diversification processes resulted in a highly "reticulate" biogeographical history (Sanmartín, 2003). The overall richness of about 25,000 plant species (Quézel, 1985) is to a large extent due to the presence of a high number of locally or regionally endemic taxa (Greuter, 1991; Médail & Diadema, 2009). Islands and mountain ranges are known for their richness (Médail & Quézel, 1997), with mountainous islands exhibiting the highest rates of species diversity and endemism. One such example is Sicily: with 2,700 plant species it is the island with the greatest species richness in the Mediterranean (Raimondo & al., 1994; Médail & Quézel, 1997; Giardina & al., 2007) and is considered one of ten "hot spots" of biodiversity within the Mediterranean (Médail & Quézel, 1997). The percentage of endemics among vascular plants ranges from 10% to 20% in the mountainous interior part of the island (Médail & Quézel, 1997; Conti & al., 2005). In addition to complex history (see below), a precondition for biotic diversity is a highly diverse landscape. Coastal regions of the island harbour evergreen, thermo-mediterranean vegetation whereas the top of Mt. Etna, Europe's highest active volcano, is snow-covered for most of the year. In between, a wide range of natural and anthropogenic habitats on both volcanic and calcareous substrate can be observed. This natural diversity offered an outstanding playground for evolutionary processes during an eventful geographical and geological history.

Sicily existed as an island through most of the Miocene (23.0–5.3 Ma [million years]; Meulenkamp & Sissingh, 2003; Goes & al., 2004), and was in tight contact with Calabria (S. Italy), Corsica, and Sardinia especially during late stages of this epoch (Speranza & al., 2002; Goes & al., 2004). Consequently, Sicily has probably had an important function as migration route and stepping stone between Africa and Europe and between the eastern and the western Mediterranean (shown for *Centaurea* subg. *Acrocentron*; Font & al., 2009). The connection with Corsica and Sardinia is evidenced, for example, in Araceae (Mansion & al., 2008). A direct connection with the African continent certainly existed during the Messinian Salinity Crisis (MSC, 5.96–5.33 Ma), when the Mediterranean desiccated due to the closure of the Strait of Gibraltar (Hsü & al., 1972; Butler & al., 1999; Krijgsman & al., 1999). With the end of the MSC, Sicily became an island again (Blanc, 2002; Garcia-Castellanos & al., 2009). The Strait of Sicily functioned as barrier between Sicily and Africa, whereas the Strait of Messina and the Isthmus of Catanzaro were barriers between Sicily and the Italian mainland (Bonfiglio & al., 2002). This fact, in combination with the subsequent onset of the Mediterranean climate around 3.2–2.8 Ma (Suc, 1984;

Thompson, 2005), may have been crucial for the development of a specific Sicilian flora. The Pleistocene climatic oscillations led to strong sea level fluctuations (Rohling & al., 1998). During warm periods, the island was much more isolated than during cold periods, when the sea level was 120–150 m below the recent level (Rohling & al., 1998; Lambeck & Bard, 2000; Lambeck & al., 2004). The distance to Africa was only 50 km during the Last Glacial Maximum (Thiede, 1978; Stöck & al., 2008), i.e., less than half of today's distance. The Strait of Messina was almost or entirely closed and various small islands around Sicily were connected to the main island (Lambeck & al., 2004). However, there is no evidence for a land bridge to Africa after the MSC.

The islands to the north of Sicily originated from volcanic eruptions during the last one million years. The larger Aeolian Islands in the Southern Tyrrhenian Sea were formed between 430,000 and 120,000 years BP (Barca & Ventura, 1991; Crisci & al., 1991; Astis & al., 1997), and the rise of Ventotene and Ponza situated in the northern Tyrrhenian Sea happened more or less at the same time (Bellucci & al., 1999). The distance of the Aeolian Islands to Sicily is small suggesting that their colonisation may have taken place mostly from Sicily. The islands in the northern Tyrrhenian Sea, however, are close to the Italian mainland rendering colonisation from there more probable.

Despite its biotic richness and the high number of endemics, Sicily has been largely neglected in phylogeographic studies. In contrast, disjunctions across the Strait of Gibraltar have been examined in detail (e.g., Martínez-Solano & al., 2004; Veith & al., 2004; Busack & Lawson, 2008; Cano-Maqueda & al., 2008; Ortiz & al., 2009). Most phylogeographic studies concerning Sicily and the adjacent areas affect a broader region, such as the entire central or western Mediterranean, and are primarily phylogenetic (e.g., Mansion & al., 2008). Whereas the paramount role of the MSC for plant migrations was emphasised for Holm Oak (*Quercus ilex* L., Lumaret & al., 2002), other studies demonstrated post-Messinian diversifications (*Matthiola* W.T. Aiton: Sánchez & al., 2005; *Jacobaea* Mill.: Passalacqua & al., 2008; *Anthemis* L.: Lo Presti & Oberprieler, 2009).

A good model to explore biogeographic relationships among Sicily, Northern Africa, the Apennine Peninsula and the islands of the Tyrrhenian Sea is provided by the relatives of *Centaurea cineraria*, perennial herbs with tomentose leaves and purple florets. They belong to the morphologically defined *C.* sect. *Acrolophus* (Candolle, 1838), which was combined with *C.* sect. *Phalolepis* and sect. *Willkommia* to create the molecularly defined, monophyletic "Acrolophus subgroup" of the "*Jacea* group" (Garcia-Jacas & al., 2006; Suárez-Santiago & al., 2007b; Hilpold & al., 2009). The *Jacea* group was suggested to have originated in the late Miocene in the eastern Mediterranean (Suárez-Santiago & al., 2007b). The classification of the Acrolophus subgroup is not sufficiently resolved as extensive hybridisation has likely played a key role in its evolution (Suárez-Santiago & al., 2007b). The *C. cineraria* group (Pignatti, 1982; Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004; Raimondo & al., 2004) comprises ten species, three of which are further divided into subspecies (*C. cineraria*, *C. aeolica*, *C. panormitana*). Four species occur in Sicily (*C. busambarensis*, *C. erycina*, *C. panormitana*, *C. saccensis*), three grow in adjacent areas (*C. aeolica* on islands of the southern and central Tyrrhenian Sea, *C. cineraria* on the southwestern coast of the Apennine Peninsula and *C. papposa* in Tunisia), two more around the northern Tyrrhenian Sea (*C. gymnocarpa* and *C. veneris*) and *C. leucadea* at the southeastern tip of Italy (Fig. 1). To date, it is unclear if the latter three species belong to the *C. cineraria* group. It is noteworthy that *C. aeolica* is a rare example of a species which inhabits the two different volcanic island archipelagos of the Tyrrhenian Sea; subsp. *aeolica* occurs on the Aeolian Islands while subsp. *pandataria* occurs on Ventotene offshore Naples. All members of the *C. cineraria* group inhabit rocky places, predominantly coastal cliffs. Sicily is the only area where species occur in mountain areas (*C. busambarensis* and *C. erycina*) and *C. saccensis* is restricted to an inland gorge. The preferred bedrock is mostly calcareous; an exception is *C. aeolica* from volcanic islands. All species except *C. gymnocarpa* possess achenes with a pappus, but the achene is glabrous, fairly heavy and the pappus is short. Consequently, gravity appears to be the only mechanism of seed dispersal (Bancheva, unpub.). In *C. corymbosa* Pourr., a close relative of the *C. cineraria* group (Hilpold & al., 2009), the average seed dispersal distance was only 32 cm (Colas & al., 1997; Hardy & al., 2004). Pollination is mainly carried out by insects (Cela-Renzoni & Viegi, 1982) and reproduction is mostly sexual, but facultative apospory was reported in *C. cineraria*, *C. gymnocarpa* and *C. panormitana* (Cela-Renzoni & Viegi, 1982). The chromosome number is $2n = 18$ (Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004), only a single tetraploid individual was ever counted (Damboldt & Matthäs, 1975).

Although several molecular studies were conducted in the Acrolophus subgroup (Ochsmann, 2000; Garcia-Jacas & al., 2006; Suárez-Santiago & al., 2007a,b), the *C. cineraria* group has been fairly neglected with only a single isoenzyme study available (Bancheva & al., 2006). Here, we apply complementary molecular markers, i.e., maternally inherited (Zhang & al., 2003), slowly evolving plastid DNA sequences and biparentally inherited, rapidly evolving (Bussell & al., 2005) AFLPs to a dense population sampling throughout the distribution area of all taxa of the *C. cineraria* group plus an exhaustive sample of potential outgroups. Our aim is to answer the following questions. (1) Does the *C. cineraria* group constitute a natural, monophyletic entity and which species of the Acrolophus subgroup in the Central Mediterranean are the next relatives? (2) If the *C. cineraria* group is monophyletic, at what time did it diversify? (3) Furthermore, we test the two hypotheses for the origin of the disjunction across the strait of Sicily, i.e., vicariance due to the fragmentation of a land-bridge established during the Messinian Salinity Crisis or transoceanic seed dispersal. (4) In addition, we aim to elucidate the phylogenetic relationships of the two subspecies of *C. aeolica* from geographically distant volcanic islands in the Tyrrhenian Sea in order to trace their colonisation history. Finally (5), we point out shortcomings of the presently used taxonomic framework.

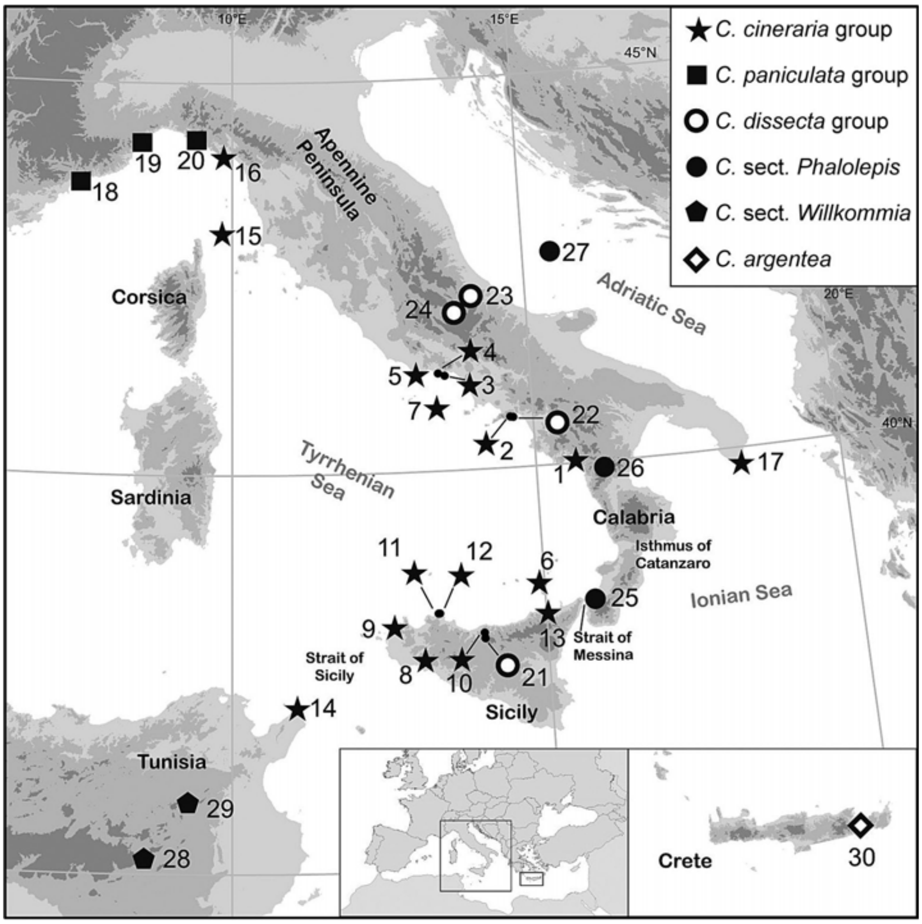
■ MATERIALS AND METHODS

In the summer of 2008, 30 sample sites (subsequently referred to as "populations") of *Centaurea* sect. *Acrolophus*, sect. *Phalolepis* and sect. *Willkommia* were visited in the field (Fig. 1; Table 1). In addition to all taxa of the *C. cineraria* group, we also included southern Italian members of the two other groups constituting sect. *Acrolophus*, i.e., the *C. paniculata* and *C. dissecta* groups (Pignatti, 1982) as well as *C. argentea*, a member of sect. *Acrolophus* from Crete. *Centaurea subtilis* Bertol. was not included, as it belongs to the *Jacea-Lepteranthus* clade (Hilpold & al., 2009) rather than to the *C. cineraria* group. Voucher specimens were determined based on Pottier-Alapetite (1981), Pignatti (1982), Breitwieser & Podlech (1986), Turland & Chilton (2000), Arrigoni (2003), Raimondo & Bancheva (2004) and Raimondo & al. (2004). Nomenclature follows Greuter (2008) and Peruzzi (2008). Leaf material was collected and immediately stored in silica gel. Voucher specimens are deposited at the herbarium of the Institut Botànic de Barcelona (BC).

Total genomic DNA was extracted from silica gel–dried tissue (ca. 10 mg) of eight to ten plants from each population (only two plants in population 13). Extraction followed the CTAB-protocol of Doyle & Doyle (1987) with the modifications of Tel-Zur & al. (1999), including three washing steps with sorbitol buffer and a few further modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed in 70% ethanol, dried at 37°C and re-suspended in TE-buffer. The quality of the extracted DNA was checked on 0.7% TBE agarose gels.

The AFLP procedure followed Vos & al. (1995) with the modifications described in Schönswetter & al. (2009). Initially, selective primers were screened using 24 selective primer combinations. The two final primer combinations for the selective PCR (fluorescent dye in brackets) were *Eco*RI (6-Fam)-ACA/*Mse*I-CAC and *Eco*RI (NED)-AGC/*Mse*I-CTG. Five microlitres of each selective PCR product were combined and purified using Sephadex G-50 Superfine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a Multi Screen-HV plate (Millipore, Molsheim, France). One microlitre of the elution product was mixed with 10 µl formamide (Applied Biosystems, Foster City, California, U.S.A.) and 0.1 µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130x automated capillary sequencer. Nineteen individuals were used as replicates to calculate the error rate and to exclude non-reproducible fragments from the analysis. Raw AFLP data were aligned with

Fig. 1. Sampled populations of *Centaurea* in the central Mediterranean and in Crete. Details of the collected populations are given in Table 1. Symbols reflect the taxonomic framework of Pignatti (1982) and Breitwieser & Podlech (1986).



the internal size standard using ABI Prism GeneScan v.3.7.1 (Applied Biosystems), and imported into Genographer v.1.6.0 (available at http://hordeum.oscs.montana.edu/genographer) for scoring. The error rate (Bonin & al., 2004) was calculated as the ratio of mismatches (scoring of 0 vs. 1) over matches (1 vs. 1) in the AFLP profiles of replicated individuals.

A Neighbour-joining (NJ) analysis based on a Nei-Li distance matrix (Nei & Li, 1979) was conducted and bootstrapped (2000 pseudo-replicates) with TreeCon 1.3b (Van de Peer & De Wachter, 1997). Population mixture analysis implemented in BAPS v.5.2 (Corander & al., 2004) was used to infer the genetic structure of the Sicily group (see Results for a definition). This software can handle dominant markers like AFLPs under the module "clustering with linked loci" (Corander & Tang, 2007). Mixture analysis was conducted with the maximal number of groups (K) set to 2 to 10. Each run was replicated ten times and the results were averaged according to the resultant likelihood scores. Admixture coefficients were estimated using 500 iterations, and the significance of these coefficients was estimated by employing the simulation strategy described by Corander & Marttinen (2006) using 50 reference individuals and 10 iterations each. An Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree among clusters was inferred based on Nei distances.

Out of several plastid DNA markers screened, only the *rpl32-trnL* intergenic spacer (primers rpl32-F, trnL (UAG); Shaw & al., 2007) showed a sufficient degree of variation, while other markers were either entirely invariable or exhibited only very few variable sites. This result is in accordance with the survey of Shaw & al. (2007), who identified the *rpl32-trnL* intergenic spacer as the most variable plastid DNA region out several tested. It was sequenced from five to six individuals per population from the Sicily group and the geographically distant *C. argentea*. Reactions were performed in 25 µl volumes with 2.5 µl 10× AmpliTaq buffer, 2.5 µl 25 mM $MgCl_2$, 2.5 µl 2 mM dNTPs mix, 1 µl of each primer at 5 µM, 2.5 µl of 400 ng/µl BSA (bovine serum albumine; New England Biolabs, Ipswich, Massachusetts, U.S.A.), 1 U AmpliTaq DNA polymerase (Applied Biosystems) and 2 µl of template DNA of unknown concentration. PCR was conducted using the following conditions: 3 min at 95°C, followed by 35 cycles of 95°C for 40 s, 54°C for 40 s and 72°C for 1 min 40 s, followed by 10 min at 72°C. The PCR product was purified with ExoSAP-IT (USB Corp., Cleveland, Ohio, U.S.A.). Direct sequencing of the amplified DNA segments was performed using BigDye Terminator Cycle Sequencing v.3.1 (Applied Biosystems), following the manufacturer's protocol at the University of Florida ICBR Core Facility on an ABI 3730xl capillary sequencer (Applied Biosystems). Nucleotide sequences were edited using BioEdit v.7.0.5.3 (Hall, 1999) and were aligned manually.

A statistical parsimony haplotype network was constructed using TCS v.1.21 (Clement & al., 2000). For the latter analysis, insertions/deletions longer than one base pair were re-coded as single base pair mutations, and sequence gaps were treated as a fifth character state.

Molecular dating of the onset of plastid DNA diversification within the Sicily group was conducted using BEAST v.1.5.3 (Drummond & Rambaut, 2007). The best substitution model had an Akaike weight (determined with Modeltest v.3.6; Posada & Crandall, 1998) of 0.31 and 17 models (out of 56 tested) were included until the cumulative Akaike weight exceeded 0.95. The included models ranged from having only three (F81) up to eight free parameters, and for the final analysis we used HKY + Gamma which is a medium complex model with five free parameters, using six rate categories instead of the default four. Given the low level of sequence variation within the Sicily group (see Results), we used a strict clock model with a prior on the substitution rate modelled as a lognormal distribution and a model of constant population size as demographic model. Setting a rate of 4.5×10^{-3} substitutions per site and per million years and a deliberately wide standard deviation of 3×10^{-2} resulted in the mode of the prior distribution at 4.0×10^{-3} substitutions per site and per million years in accordance with published substitution rates for plastid markers (Yamane & al., 2003; Smith & al., 2008). After initial analyses, the root of the tree was constrained to be maximally 5 million years old. The Markov chain was run three times for 5×10^7 generations each, sampling every 1000th generation. After removing the first 10% of sample points as burn-in, parameter estimates and their 95% highest posterior density intervals (HPD) were obtained from 135,000 generations.

■ RESULTS

We scored 476 AFLP fragments ranging from 100 to 550 base pairs. The error rate amounted to 4.7% and was thus within the range deemed acceptable by Bonin & al. (2004). Neighbour-joining analysis (Fig. 2) linked all individuals of each population to separate clusters with bootstrap support (BS) between 94% and 100%. Exceptions were the two geographically close populations of *C. panormitana* (populations 11 and 12) as well as *C. delicatula* (population 28). One individual of *C. busambarensis* (population 10) grouped with low support (BS 51%) with *C. parlatoris* (population 21). *Centaurea argentea* (population 30) was separated along the longest branch in an unrooted NJ analysis and was therefore used to root the tree. Only a few deeper nodes had BS > 50%. A branch formed by *C. leucadea*, *C. deusta* and *C. scillae* (populations 17, 26, 25, respectively) was sister to all other populations with BS 60%. Within this latter group three members of the *C. paniculata* group s.str. and *C. veneris* constituted a branch with BS 81%. The best supported larger group within the tree consisted of nine populations of the *C. cineraria* group from Sicily, the Tyrrhenian Islands and Tunisia and *C. parlatoris* from Sicily. The group received BS 93% and is referred to as the Sicily group. The Sicily group was split into two smaller groups with low support, the first consisting of the two subspecies of *C. aeolica* from the Tyrrhenian Islands and the Tunisian *C. papposa*, the second consisting of all populations from Sicily.

Mixture analysis of the Sicily group identified eight clusters that consisted mainly of single populations or several populations of the same taxon, with the exception of a cluster joining *C. parlatoris* (population 21), *C. panormitana* subsp.

seguenzae (population 13) and one individual of *C. busambarensis* (population 10). The UPGMA tree among the clusters identified two main groups (Fig. 3A). The first was distributed from Tunisia over Sicily to the islands of the central and northern Tyrrhenian and comprised two subgroups, constituted by *C. saccensis* (population 8) plus the Tunisian *C. papposa* (population 14), and by *C. aeolica* subsp. *aeolica* (population 6) plus subsp. *pandataria* (population 7). The second group was restricted to Sicily and fell into a northwestern (populations 9, 11, 12) and an eastern subgroup (populations 10, 21, and 13). The subsequent admixture analysis revealed admixture in a single individual from population 13 and is presented on a geographical basis in Fig. 3C.

The *rpl32-trnL* sequences of members of the Sicily group were 843–867 bp long and yielded six haplotypes altogether (Tables 1–2; Fig. 3B). Haplotype IV occurred in six populations (Fig. 3C): *C. busambarensis* (population 10), *C. parlatoris* (population 21), both subspecies of *C. aeolica* (populations 6 and 7), *C. erycina* (population 9) and *C. papposa* (population 14). The haplotypes of the remaining populations differed only in a

Table 1. Population number (Pop.), taxon, collecting locality and collection number, coordinates, number of individuals investigated with AFLPs, cpDNA haplotype for members of the Sicily group (for a definition, see text) and GenBank accession numbers of 30 sampled populations of …

	Pop.	Taxon
C. cineraria group	1	*Centaurea cineraria* L. subsp. *cineraria*
	2	*Centaurea cineraria* L. subsp. *cineraria*
	3	*Centaurea cineraria* L. subsp. *cineraria*
	4	*Centaurea cineraria* L. subsp. *cineraria*
	5	*Centaurea cineraria* L. subsp. *circae* (Sommier) Cela Renz. & Viegi
	6	*Centaurea aeolica* Lojac. subsp. *aeolica*
	7	*Centaurea aeolica* Lojac. subsp. *pandataria* (Fiori & Bég.) Anzal.
	8	*Centaurea saccensis* Raimondo & al.
	9	*Centaurea erycina* Raimondo & Bancheva
	10	*Centaurea busambarensis* Guss.
	11	*Centaurea panormitana* Lojac. s.l.
	12	*Centaurea panormitana* Lojac. s.l.
	13	*Centaurea panormitana* Lojac. subsp. *seguenzae* (Lacaita) Greuter
	14	*Centaurea papposa* (Coss.) Greuter
	15	*Centaurea gymnocarpa* Moris & De Not.
	16	*Centaurea veneris* (Sommier) Bég.
	17	*Centaurea leucadea* Lacaita
C. paniculata group	18	*Centaurea leucophaea* Jord. subsp. *controversa* (Briq. & Cavill.) Kerguélen
	19	*Centaurea aplolepa* Moretti subsp. *aplolepa*
	20	*Centaurea aplolepa* Moretti subsp. *levantina* (Arrigoni) Greuter
C. dissecta group	21	*Centaurea parlatoris* Heldr.
	22	*Centaurea lacaitae* Peruzzi
	23	*Centaurea ambigua* Guss.
	24	*Centaurea scannensis* Anzal. & al.
Centaurea sect. *Phalolepis*	25	*Centaurea scillae* Brullo
	26	*Centaurea deusta* Ten.
	27	*Centaurea diomedea* Gasp.
Centaurea sect. *Willkommia*	28	*Centaurea delicatula* Breitw. & Podlech
	29	*Centaurea delicatula* Breitw. & Podlech
Centaurea sect. *Acrolophus* (Incertae sedis)	30	*Centaurea argentea* L. subsp. *argentea*

few mutational steps from haplotype IV. Haplotypes from populations 2 and 5 of the outgroup C. *cineraria* were also linked directly to this haplotype. *Centaurea argentea* (population 30) is separated from haplotype IV by five mutational steps.

Using a model of constant population size, the onset of diversification within the Sicily group was inferred to be (given as mean/median) 0.248/0.207 Ma (95% highest posterior density interval 0.576–0.018 Ma). The use of a more complex demographic model (extended Bayesian Skyline Plot) resulted in slightly younger age estimates (not shown).

■ DISCUSSION

Despite the clear morphological diagnostic features of the *Centaurea cineraria* group (Pignatti, 1982, Cela-Renzoni & Viegi, 1982; Raimondo & Bancheva, 2004; Raimondo & al., 2004), our AFLP data suggest that even though most populations are strongly reciprocally divergent, little higher-level structure is evident. Monophyly of the *C. cineraria* group in the traditional circumscription is not supported. Only a mostly geographically defined group centred on Sicily, but also including

... *Centaurea* from the central Mediterranean and Crete. All vouchers are deposited in the herbarium BC. Collections numbers are those of Vilatersana (V) or Galbany-Casals (G).

Locality and collection number	Coordinates (E, N)	N AFLP	Haplotype	GenBank accession
Italy, Potenza: Acquafredda, *V-1120*	15°39′53″, 40°02′21″	8	–	
Italy, Salerno: Minori, *V-1118*	14°37′48″, 40°38′55″	10	–	
Italy, Latina: Gaeta, *V-1112*	13°34′00″, 41°12′30″	10	–	
Italy, Latina: Torre Capovento, *V-1107*	13°27′45″, 41°14′30″	10	–	
Italy, Latina: S. Felice Circeo, *V-1104*	13°05′22″, 41°13′38″	10	–	
Italy, Messina: Lipari, *V-1181*	14°57′44″, 38°30′17″	10	IV (6)	HQ441794–HQ441799
Italy, Latina: Isola di Ventotene, *V-1113*	13°24′55″, 40°47′13″	9	IV (6)	HQ441800–HQ441805
Italy, Agrigento: Lago Arancio, *V-1159*	13°03′39″, 37°37′35″	10	I (6)	HQ441776–HQ441781
Italy, Trapani: Erice, *V-1160bis*	12°35′17″, 38°02′10″	10	IV (6)	HQ441782–HQ441787
Italy, Palermo: Isnello, *V-1144*	14°00′08″, 37°56′45″	10	IV (4), V (1), VI (1)	HQ441765–HQ441770
Italy, Palermo: Sferracavallo, *V-1166*	13°17′24″, 38°12′42″	10	II (6)	HQ441752–HQ441757
Italy, Palermo: Mondello, *V-1167*	13°18′44″, 38°12′55″	9	II (5)	HQ441758–HQ441762
Italy, Messina: Tindari, *V-1173*	15°02′27″, 38°08′40″	2	III (2)	HQ441763, HQ441764
Tunisia, Nabeul: Cap Bon, *V-1384*	11°01′41.9″, 37°04′22.3″	9	IV (6)	HQ441788–HQ441793
Italy, Livorno: Isola di Capraia, *V-1248*	09°50′35″, 43°03′00″	10	–	
Italy, La Spezia: Palmaria, *V-1255*	09°51′04″, 44°02′33″	10	–	
Italy, Lecce: S. Maria di Leuca, *V-1205*	18°22′22″, 39°47′57″	10	–	
France, Alpes-Maritimes: Eze, *V-1274*	07°21′02″, 43°42′59″	10	–	
Italy, Savona: Spotorno, *V-*1265	08°24′02″, 44°13′46″	10	–	
Italy, Genova: Sestri Levante, *V-1262*	09°23′19″, 44°16′11″	10	–	
Italy, Palermo: Piano Battaglia, *V-1150*	14°00′45″, 37°52′24″	8	IV (4), V (1)	HQ441771–HQ441775
Italy, Salerno: Capo d'Orso, *V-1116*	14°40′42″, 40°38′10″	9	–	
Italy, Pescara: Eremo S. Spirito, *V-1223*	14°04′20″, 42°10′30″	8	–	
Italy, L'Aquila: Gole del Sagittario, *V-1225*	13°47′59″, 41°59′30″	9	–	
Italy, Reggio Calabria: Bagnara, *V-1140*	15°47′07″, 38°16′28″	10	–	
Italy, Potenza: Prastio, *V-1131*	16°08′13″, 39°56′15″	10	–	
Italy, Foggia: San Domino, *V-1211*	15°29′42″, 42°42′13″	8	–	
Tunisia, Siliana: Jebel Seri, *V-1346*	09°23′27.7″, 35°51′24.4″	9	–	
Tunisia, Kasserine: Jebel ech Chambi, *V-1355*	08°39′53.9″, 35°12′09.2″	9	–	
Greece, Crete: Kavousi, *G-2008*	25°49′12.2″, 35°06′17.4″	8	–	HQ441806

C. papposa from North Africa, *C. aeolica* from the Tyrrhenian Islands and the morphologically clearly distinct *C. parlatoris* from Sicily form a supported monophyletic entity termed here the "Sicily group". The next relatives of the Sicily group cannot be determined due to insufficient resolution (Fig. 2). Our AFLP data provide no direct evidence that hybridisation, previously suggested to be a driving force in the diversification of *Centaurea*, is the main cause for the lack of coherence of the *C. cineraria* group. If at all, hybridisation had already occurred in early stages of diversification.

Highly fragmented distribution patterns as exemplified by the *C. cineraria* group were considered indicative for old, "relic" groups that failed to re-gain a wider distribution after restriction to the warmest available habitats such as coastal cliffs during cold stages of the Pleistocene (Ferrarini, 1971; Cela-Renzoni & Viegi, 1982). In contrast, plastid DNA data generated for the Sicily group strongly discourage an old age but instead suggest a fairly recent diversification taking place less than 250,000 years ago. As suggested by the AFLP data (Fig. 2) albeit without bootstrap support, the closest extant relatives of the Sicily group may be sought for in northern populations of *C. cineraria* subsp. *cineraria*. In any event, the ancestors of the Sicily group have most probably evolved on the Italian mainland and subsequently dispersed to Sicily. As southernmost Italy is predominated by granitic bedrock, an unsuitable substrate for members of the *C. cineraria* group, long-distance seed dispersal across the Tyrrhenian Sea appears more probable than the significantly shorter crossing of the Strait of Messina.

Diversification within Sicily and further dispersal into circumjacent areas (North Africa, Tyrrhenian Islands) was accompanied by genetic divergence in probably small and isolated populations as well as by rapid morphological diversification. Differentiation of populations may have been fostered by natural habitat fragmentation. Sandy beaches and river estuaries have probably acted as barriers for members of the Sicily group, which are restricted to rock faces close to the sea. Despite the fact that their achenes lack adaptations for long-distance dispersal (Bancheva, unpub.), members of the Sicily group were obviously able to expand their distribution areas. We found the same plastid DNA haplotype in populations from

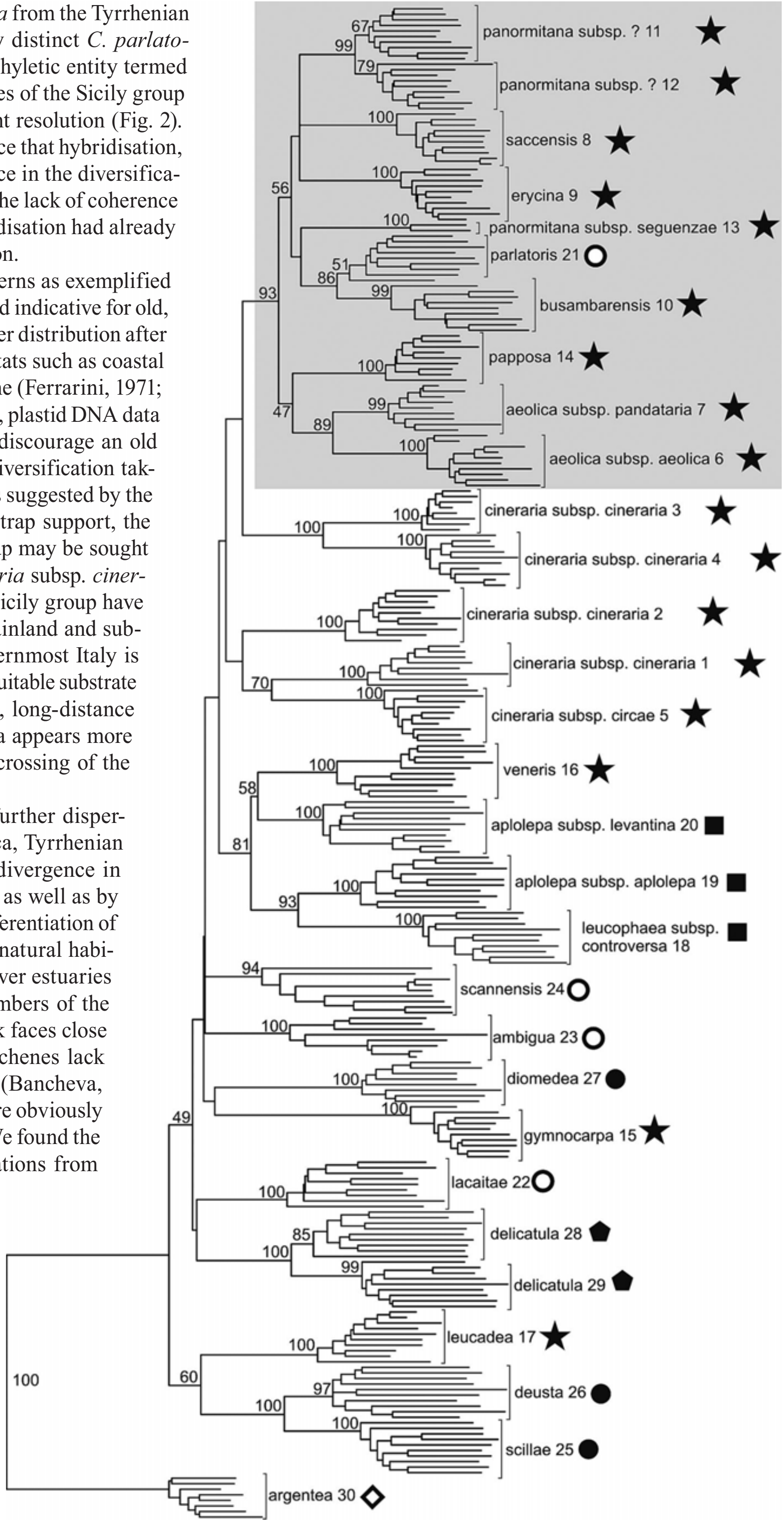


Fig. 2. Neighbour-joining tree of AFLP data of 30 populations of *Centaurea* in the central Mediterranean and in Crete. Numbers along branches are bootstrap support values above 45%. As in Fig. 1, symbols reflect the taxonomic framework of Pignatti (1982) and Breitwieser & Podlech (1986). The Sicily group (for circumscription see text) is shaded in grey.

North Africa on the one hand and Sicily and the Tyrrhenian Islands on the other hand (Fig. 3C). As the earliest diversification within the Sicily group clearly post-dates the MSC when a land bridge connected Sicily and Africa, vicariance can be outright rejected as cause for the disjunction in favour of transoceanic seed dispersal. The dispersal from Sicily to northern Africa likely occurred during cold periods of the Pleistocene when the distance between Sicily and Tunisia was much smaller (Thiede, 1978; Stöck & al., 2008), and may have been easier leaped. Similarly, the disjunction seen in *C. aeolica* between Lipari and Ventotene over a distance of about 250 km is best explained by long-distance seed dispersal. The relatively recent emergence of these islands during the last one million years fits well with our dating of the group's diversification. Colonisation likely occurred directly from the Aeolian Islands to Ventotene and Ischia, where the species was reported in the 19th century (Gussone cit. in Pignatti, 1982), but appears to be extinct now. *Centaurea aeolica* grows on volcanic soil, but the rocks along the coast south of Naples are almost exclusively calcareous or granitic, rendering dispersal across the mainland difficult. Seed dispersal probably occurred during cold periods with lower sea level and may have involved a stepping stone, the volcanic Palinuro seamount that nowadays lies below sea level, but was a large, flat island during cold periods (Kenyon & al., 2003; Siani & al., 2004). Similar disjunction patterns between the volcanic area of Naples and the nearby Tyrrhenian Island Ventotene on the one hand and the Aeolian Islands on the other hand are rarely observed; further examples are provided by *Bassia saxicola* (Guss.) A.J. Scott and *Heliotropium suaveolens* M. Bieb. subsp. *bocconei* (Guss.) Brummitt (Pignatti, 1982).

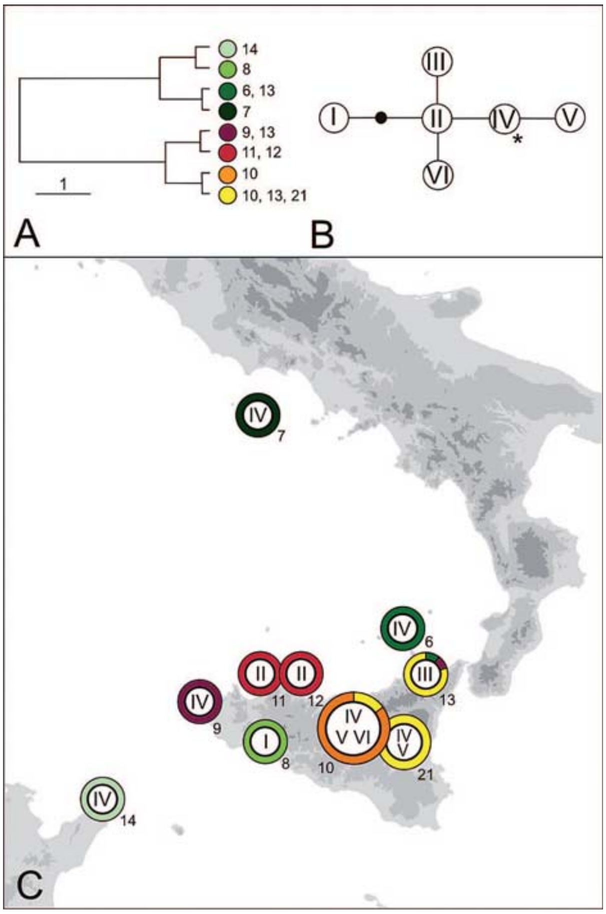


Fig. 3. AFLP and plastid DNA variation in populations of the Sicily group of *Centaurea* from Sicily, Tunisia and the Tyrrhenian (for definition, see text). **A,** Unweighted Pair Group Method with Arithmetic mean (UPGMA) tree based on Nei distances among gene pools derived by Bayesian clustering with BAPS. **B,** Statistical parsimony network of plastid DNA haplotypes. A mutational step not sampled is given as small black dot. The asterisk shows the connection with haplotypes sampled in the outgroups *Centaurea cineraria* and *C. argentea*. **C,** Geographical distribution of plastid DNA haplotypes derived from *rpl32-trnL* sequences (indicated with roman numerals) and of eight gene pools as defined by Bayesian clustering (indicated in colour).

Table 2. List of haplotypes in the studied populations of the Sicily group and in *Centaurea argentea* with indication of nucleotide site variation within the chloroplast region *rpl32-trnL*.

	2	2	3	4			6	6	6		
	4	9	9	5			3	3	3		
Haplotype	7	6	2	8	502–514	523–534	1	2	4	770–773	Taxon (population number)
Haplotype I	A	T	G	C	–	CTATAAGGATTT	G	T	A	GAAA	*Centaurea saccensis* (8)
Haplotype II	A	T	G	A	–	CTATAAGGATTT	G	G	A	GAAA	*Centaurea panormitana* (11, 12)
Haplotype III	A	G	G	A	–	CTATAAGGATTT	G	G	A	GAAA	*Centaurea panormitana* subsp. *seguenzae* (13)
Haplotype IV	A	T	G	A	CTTCTTAATTATC	CTATAAGGATTT	G	G	A	GAAA	*Centaurea aeolica* subsp. *aeolica* (6), *C. aeolica* subsp. *pandataria* (7), *C. busambarensis* (10), *C. papposa* (14), *C. parlatoris* (21)
Haplotype V	A	T	G	A	CTTCTTAATTATC	CTATAAGGATTT	G	G	A	–	*Centaurea parlatoris* (21)
Haplotype VI	A	T	G	A	–	CTATAAGGATTT	G	G	A	GAAA	*Centaurea busambarensis* (10)
Haplotype *C. argentea*	G	T	A	A	CTTCTTAATTATC	–	T	G	C	GAAA	*Centaurea argentea* (30)

It is known that AFLPs are no marker of choice for resolving relationships at deep nodes as the frequency of non-homologous fragments being scored as one AFLP band increases in parallel with the phylogenetic distance. The very limited plastid DNA variation, however, suggests that diversification within the group is recent and we therefore do not expect that our AFLP dataset contains much homoplasy. The molecular data suggest that the current taxonomic framework needs to be significantly revised. As taxonomic ranks should be applied in a comparable way throughout the entire genus once phylogenetic relationships are better understood and because a detailed morphometric analysis of the *C. cineraria* group is underway (Guarino, in prep.), we here refrain from taking taxonomic decisions but rather point out shortcomings of the presently used classification. (1) First of all, the lack of supported hierarchical structure in the AFLP dataset does neither support the division of the Acrolophus subgroup into sects. *Acrolophus*, *Phalolepis* and *Willkommia* (Candolle, 1838; Blanca, 1981) nor the division into informal groups as proposed by Pignatti (1982). It has been suggested that hybridisation and introgression within this group confound reconstruction of sectionial boundaries (Suárez-Santiago & al., 2007b) but our data provide no evidence for this hypothesis. (2) It was previously shown (Schönswetter & al., 2009) that in groups that exhibit geographically isolated, fairly small populations and were suggested to have undergone relatively recent, synchronous, allopatric differentiation, geographically close populations tend to be related independent of their taxonomic assignment, the latter often being based on a single or very few characters only. As our data strongly suggest recent diversification in the *C. cineraria* group and because populations are often small, allowing for rapid fixation of probably parallely evolving characters, we expect that entirely morphology-based classifications such as those by Dostál (1976) and Pignatti (1982) are suited for practical purposes but do not reflect a natural system. This is well illustrated by the morphologically divergent *C. parlatoris* that was previously assigned to the *C. dissecta* group. It is not only deeply nested in the Sicily group but is also genetically only weakly differentiated from its next relative, *C. busambarensis* (Fig. 2), growing in geographic proximity. (3) Taxonomic ranks used for members of the Sicily group and for *C. cineraria* do not reflect observed patterns of genetic divergence. For example, the four investigated populations of *C. cineraria* subsp. *cineraria* are strongly reciprocally divergent and do not even form a monophyletic group; their genetic differentiation is at least as strong as that among the constituents of the Sicily group classified at specific level (Fig. 2). It is unclear if this pattern is exclusively caused by genetic drift in small, geographically isolated populations or if facultative apospory previously reported in *C. cineraria* (Cela-Renzoni & Viegi, 1982) also plays a role. (4) Of the remaining three species previously included in the *C. cineraria* group, *C. veneris* from the northern Tyrrhenian is more closely related to the *C. paniculata* group (Fig. 2). *Centaurea gymnocarpa* and *C. leucadea*, in contrast, clearly fall outside both groups. *Centaurea leucadea* groups with weak support with two members of sect. *Phalolepis* (*C. deusta*, *C. scillae*). In consequence, similarities of *C. gymnocarpa*, *C. leucadea* and *C. veneris* to *C. cineraria* might be caused by convergence or by gene flow between these groups. The later hypothesis is supported by the presence of morphologically intermediate individuals in some populations not included in this study (A. Hilpold, pers. obs.). (5) As a minor point, the subspecies of *C. panormitana* are highly doubtful. On Mt. Pellegrino, where all three subspecies should occur, neither morphological differentiation was possible (A. Hilpold, pers. obs.) nor did the AFLP data suggest any differentiation. In contrast, subsp. *seguenzae* is morphologically separated by prolonged bract appendages and is clearly not the next relative of the other subspecies (Fig. 2).

In conclusion, our study shows that diversification in groups that – based on their highly disjunct distribution – were assumed to be old relics may be surprisingly recent. A further example for a recent, homoploid diversification in the Mediterranean is provided by the genus *Nigella* L. in the Aegean (Bittkau & Comes, 2008). Moreover, it becomes more and more apparent that disregarding the “flat tail of the dispersal curve” might lead to erroneous conclusions regarding the evolutionary significance of long-distance seed dispersal. As illustrated by the *C. cineraria* group, this concerns not only species with diaspores that are morphologically adapted to dispersal, but also those which are lacking such adaptations. As a consequence, we could reconstruct a large number of biogeographic processes, i.e., various allopatric-vicariant divergences in Sicily as well as transoceanic dispersal across the Strait of Sicily and between the geographically distant but edaphically similar volcanic islands Lipari and Ventotene in the Tyrrhenian Sea.

■ ACKNOWLEDGEMENTS

Financial support from the Spanish Ministry of Education and Science (Project CGL2007-60781/BOS) and the Generalitat de Catalunya (Ajuts a Grups de Recerca Consolidats 2009/SGR/00439) is gratefully acknowledged. A. Hilpold benefited from a predoctoral grant of the JAE program (CSIC). Special thanks go to the Parco Nazionale della Majella (prot. n. 1763), to the Parco Naturale Regionale di Porto Venere (prot. n. 2804), to the Parco Nazionale Arcipelago Toscano (prot. n. 3363), to the Parco della Madonie (prot. n. 2195), to the Parco Nazionale delle Cinque Terre (prot. n. 2137), to the Parco Nazionale del Circeo (prot. n. PNC/2008/196) and to the Riserva Naturale delle Gole del Sagittario (prot. n. 1081) for the permission to collect plants in their territory. M. Galbany-Casals and A. Romo are acknowledged for their help in collecting specimens, K. Bardy for her help in preparing Fig. 1, and G.M. Schneeweiss for helping with the software BEAST. We are grateful to three anonymous reviewers, who provided insightful comments on a previous version of the manuscript.

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Capítol 4: s'enviarà a *Systematic Biology*

[*forth publication. The here presented manuscript is a first draft, which may undergo major revisions before submitting to the journal. The journal aimed at is* Systematic Biology]

Títol provisional [*preliminary title*]:

Current taxonomy in light of a species coalescence approach in the *Centaurea alba* complex.

[*La taxonomia de plantes actual des d'una perspectiva de coalèscencia d'espècies en el complex de* Centaurea alba]

[*La taxonomía de plantas actual desde una perspectiva de coalescencia de especies en el complejo de* Centaurea alba]

Autors [*authors*]:

Andreas Hilpold, Núria Garcia-Jacas, Alfonso Susanna, Carsten Löser, Roser Vilatersana & Bengt Oxelman.

Resum en catalá [*abstract in Catalan*]:

La delimitació d'espècies en plantes segueix tradicionalment sobretot criteris morfològics. No obstant, no existeix cap certesa que els taxons basats en la morfologia siguin veritablement espècies en el sentit d'un llinatge metapoplacional evolucionat separadament. Recentment s'han desenvolupat models que descriuen l'arbre d'espècies sota la teoria de la coalescència de multiespècies i s'ha usat també per a la delimitació d'espècies. En aquest treball s'usa aquest enfocament dins d'un grup que presenta grans problemes de delimitació d'espècies, ben reflectit en tractaments taxonòmics molt divergents. *Centaurea alba* és una espècie de la família de les Compostes que creix a la península Ibèrica. Forma part del grup *Centaurea*, present amb centenars d'espècies en el Mediterrani. Hem usat un mostreig ampli de *C. alba* i espècies pròximes de tot el Mediterrani per esbrinar si el tractament taxonòmic actual és congruent amb un enfocament de coalescència. Per arribar a aquest objectiu, s'ha seqüenciat una regió cloroplàstica, la regió nuclear ITS i cinc regions nuclears de còpia única. S'ha usat el programa *BEAST basat en coalescència per deduir l'arbre d'espècies i s'ha examinat l'arbre buscant esdeveniments d'especiació amb el programa BPP. S'han testat diverses classificacions mitjançant el Factor de Bayes. Les classificacions que unien diverses espècies en una única són les que van donar un resultat significativament millor que les que usaren la delimitació actual. Es discuteix el paper de la hibridació. Finalment, s'expliquen les possibles conseqüències pràctiques del nostre enfocament.

Resumen en castellano [*abstract in Spanish*]

La delimitación de especies en plantas sigue tradicionalmente sobre todo criterios morfológicos. Sin embargo, no existe ninguna certidumbre de que estos táxones basados en la morfología sean de verdad especies en el sentido de un linaje metapoblacional evolucionado separadamente. Recientemente se han desarrollado modelos

que describen el árbol de especies bajo la teoría de la coalescencia de multispecies y se ha usado también para la delimitación de especies. En nuestro trabajo, usamos este enfoque dentro de un grupo que sufre de grandes problemas de delimitación de especies, bien reflejado en tratamientos taxonómicos muy divergentes. *Centaurea alba* es una especie de la familia de las Compuestas que crece en la Península Ibérica. Forma parte del grupo *Centaurea*, presente con cientos de especies en el Mediterráneo. Hemos usado un muestreo amplio de *C. alba* y especies próximas de todo el Mediterráneo para averiguar si el tratamiento taxonómico actual es congruente con un enfoque de coalescencia. Para lograr este objetivo, hemos secuenciado una región cloroplástica, la región nuclear ITS y cinco regiones nucleares de copia única. Hemos usado el programa *BEAST basado en coalescencia para deducir el árbol de especies y hemos examinado el árbol buscando eventos de especiación con el programa BPP. Se han testado distintas clasificaciones mediante el Factor de Bayes. Las clasificaciones que unían varias especies en una única son las que nos dieron resultados significativamente mejores que las que usaron la delimitación actual. Se discute el papel de la hibridación. Finalmente, explicamos las posibles consecuencias prácticas de nuestro enfoque.

Current plant taxonomy in light of a species coalescence approach in the *Centaurea alba* complex

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Abstract.— Species delimitation in plants follows traditionally mainly morphological criteria. There is, however, no certainty that these morphology based taxa display indeed a species in sense of a separately evolving metapopulation lineage. Recently, models that adequately describe species trees under the multispecies coalescent have been developed and have also been used for species delimitation. In our work, we used such an approach in a group of plants which suffer from large problems in species delimitation reflected in very divergent taxonomic treatments. *Centaurea alba* is a species of the Compositae family occurring on the Iberian Peninsula. It is nested within the *Centaurea* group, present with some 200 species around the Mediterranean Sea. We used a broad sampling of *C. alba* and closely related species from all over the Mediterranean to figure out if the actual taxonomical treatment is in agreement with the results of a multispecies coalescence approach. To reach this aim we sequenced one cpDNA region, the nuclear ITS region, and five more nuclear single copy regions. We used the coalescence based program *BEAST to deduce a species tree and screened the resulting species tree for speciation events applying the program BPP. Several species classifications were tested via Bayes factor calculation. Classifications that unite several species into one, gave significantly better results, than those that use current species delimitation. The role of hybridization is discussed. Finally, we illustrate possible, practical consequences of our approach.
[*BEAST; BPP; Cardueae; Mediterranean; species delimitation; species concepts.]

INTRODUCTION

Species are considered basic, often also fundamental, units of biology (Linnaeus 1753, 1758; Darwin 1859; Dobzhansky 1937; Mayr 1942, 1970, 1982; Ereshefsky 1992; Claridge et al. 1997; de Queiroz 2005). The definition of what a species is, is despite decades of intensive disputes and controversies, still not settled. The most important and widely accepted species concepts, for example the biological, ecological and phylogenetic ones (for summary see Mayden 1997; de Queiroz 1998), have one attribute in common: they all treat species as separately evolving metapopulation lineage (de Queiroz 2007). They differ by how the separation of two lineages is assessed.

In botany, the traditional way of describing species taxonomically is to group those plants that share diagnostic morphological similarities (Davis and Heywood 1963; Cronquist 1978; McDade 1995; Zapata and Jiménez 2012). It corresponds therefore to a phenetic (=typological) species concept (Sokal and Crovello 1970), which may be based on the hope that such morphological similarities are the product of a single speciation event, which forms one single, well separated lineage (e.g., Andersson 1985; Wanntorp 1988). The question "how much morphological difference of one clade is needed to recognize that a speciation process is completed in order to be considered as a separate species?" is unresolved. In addition, the fact that morphological differences can arise from processes beyond speciation, needs special attention. Especially, ancient polymorphisms and convergent evolution may lead to taxon-descriptions, which may not correspond to a single lineage. The use of BLAST scores and similar approaches that have been proposed in conjunction with DNA barcoding (Hebert et al. 2004a, b) suffer from corresponding problems (Kress et al. 2005; Brower 2006; Vogler and Monaghan 2006; Markolf et al. 2011).

All these approaches assess single characters or combinations of them to deduce the complex speciation process. These characters may or may not be product of such a process, which itself can not be observed directly, only its marks, since speciation usually takes place in time units far off from human perception. The development of models for phylogenetic inference based on molecular sequence data has reached a considerable sophistication, and recently models that adequately describe species trees under the multispecies coalescent have been developed (*BEAST, Heled and Drummond 2010; BEST, Liu and Pearl 2007; Liu et al. 2008) – Naturally, it is critical for such methods that alleles are assigned correctly to species. A few attempts to use coalescent approaches for delimiting species have been made (e.g., Pons et al. 2006; Kubatko et al. 2009; Leache and Fujita 2010; O'Meara 2010; Yang and Rannala 2010; Barret and Freudenstein 2011).

Actual species delimitation is controversial for many plant groups, and species numbers may vary considerably among taxonomic treatments (Isaak et al. 2004; Knapp et al. 2005). One of such groups is the plant genus *Centaurea* L., and therein depicted especially well by the *Centaurea* group (*Acrolophus* subgroup in Wagenitz and Hellwig 1996). This group, shown to be monophyletic (Garcia-Jacas et al. 2006; Hilpold et al. in prep.) has its center of distribution around the Mediterranean and Black Sea (Hilpold et al. in prep.). It is characterized by usually perennial life form, and showy purple or more seldom yellow inflorescences. Systematics within this group is exceptionally difficult, a fact which is well reflected by extremely unequal taxonomical treatments. While Dostál (1976) distinguished in his treatment for Flora Europaea 83 species and 155 subspecies, Greuter (2008) in the Euro+Med Plantbase accepts more than 200 species with about 70 subspecies for the same area belonging to the *Centaurea* group. This increase is only partly due to the description of new species, but in large part it is due to an upgrading of many subspecies or varieties to separate species.

Classical taxonomy of the group uses the distinctive bract appendages to distinguish two main sections within the subgroup: section *Centaurea* (formerly *Acrolophus* (Cass.) DC.) has ciliate to fimbriate bract appendages whereas section *Phalolepis* (Cass.) DC. has membranaceous, lacerate bract appendages. A third section, section *Willkommia* Blanca, was elevated to sectional rank later (Willkomm and Lange 1870; Blanca López 1981) and is defined by a continuous distribution area, ranging from Tunisia over Morocco to the north of the Iberian Peninsula, and ciliate to fimbriate bract appendages with spiny tip and a subshrubby life-form.

Centaurea alba L. is a member of section *Phalolepis*, abundant on the Iberian Peninsula where a number of subspecies have been recognized (recently a group of subspecies was upgraded to species rank: *C. costae*

Willk.; López and Devesa 2011). Some dozens of further described species of section *Phalolepis* can be found on the Apennine and Balkan Peninsula, as well as around the Black Sea. In northern Africa only two species of this section have been described so far. Morphologically, the Italian *Centaurea deusta* Ten. is particularly similar to the Iberian *C. alba*, which led even to the merging of the two into one single species (Dostál 1976). However, the clear separation between the three sections has been questioned since there frequently exist intermediary forms (Wagenitz 1989) – a fact that has been explained in some cases by ongoing hybridization between the sections, mainly between section *Phalolepis* and *Centaurea* (López and Devesa 2008). Recent molecular studies (Garcia-Jacas et al. 2006; Suárez-Santiago et al. 2007; Hilpold et al. 2011) have contradicted morphology-based classification and suggested that hybridization may be one explanation. An ITS-based study showed *C. alba* as being closely related to geographically co-occuring species of the sections *Centaurea* and *Willkommia* and did not give any evidence for a close relationship to the Italian *C. deusta*-complex or to other members of section *Phalolepis* (Suárez-Santiago et al. 2007). Preliminary results from chloroplast DNA sequences are in line with the ITS study (Hilpold et al. in prep.). In addition, preliminary studies using these two sequence markers did not show any evidence that different populations of *C. alba* are closer related to each other than to other species of the *Centaurea* group within the Iberian Peninsula, casting doubt on the monophyly of the Iberian species in particular and on the species concept in general.

The main aim of this study is, therefore, to test whether the current morphology based taxonomic treatment into sections and species is supported by a multispecies coalescence approach using several putatively unlinked loci. To address this question, a representative sampling of members of all three sections with special emphasis on the Iberian *C. alba* was collected and is subjected to a comparison of 7 gene regions, six of them fast evolving nuclear genes and one chloroplast region (*rpl32-trnL* intergenic spacer).

MATERIAL AND METHODS

Sampling

We selected 27 populations (Table 1; Fig. 1) with one or maximally two (cpDNA) individuals per population of the *Centaurea* group (i.e. the *Acrolophus* subgroup sensu Garcia-Jacas et al. 2006). Eight of them belong to the *C. alba* species complex from the Iberian Peninsula, i.e. the only members of section *Phalolepis* in the Peninsula. Four further Iberian populations belong to sections *Centaurea* and *Willkommia*. Four N African species were included, three of them of sect. *Willkommia* and one (*C. tougourensis* Boiss. & Reut.) member of *Phalolepis.* From NW Italy, *Centaurea aplolepa* Moretti member of the *C. paniculata* L. complex (section *Centaurea*) was included. As outgroups ten populations belonging to nine different species of section *Phalolepis* from the central and eastern Mediterranean were included. Diploid populations were favored against tetraploid ones to avoid excessive allele numbers for sequencing. There are only two exceptions: *C. acarnanica* (Matthäs) Greuter is tetraploid (Matthäs 1976), and for *C. pawlowskii* Phitos & Damboldt both diploid (Damboldt and Matthäs 1975) and tetraploid (Ochsmann 2000) counts were published. We follow Greuter (2008) as taxonomic treatment.

TABLE 1 Population number (Pop.), taxon, collecting locality and collection number, coordinates and in analysis included sequences (signed with a +*). Vouchers are deposited in the herbaria BC, GB, BOZ, ACA and KNYA

Pop.	Species name, internal number	Locality, collection number	Coordinates (X,Y)	ITS	*rpl32*	A04	A26	B27	C20	D28
Iberian Peninsula										
1	*Centaurea alba* L.	Spain, Castilla y León: Puerta de Guadarrama, S of the road, *Hilpold* 20093100	40° 42' 32'', -4° 8' 26''	+	+	+	-	+	+	+
2	*Centaurea alba* L.	Spain, Castilla y León: Hoz de Arriba, Peña del Santo, *Romaschenko* 641	-	+	+	-	+	+	+	+
3	*Centaurea alba* L.	Spain, Catalunya: Pallars Sobirá, along road from Sellui to Ancs, 1.4 km NNW Sellui, *Hilpold* 20104074	42° 41' 47'', 1° 0' 48''	+	+	+	-	+	+	+
4	Centaurea alba L.	Spain, Aragón: between castle Viacamp and road Lleida-Vielha (N230), 0.2–0.5 km NW castle, *A. Hilpold* s.n.	42° 7' 52'', 0° 36' 43''	+	+	+	+	+	+	+
5	*Centaurea alba* L.	Spain, Aragón: Sobrarbe, 0.8 km N Fiscal, along road Torla-Ainsa, *Hilpold* 20095109	42° 30' 16'', -0° 7' 13''	+	+	+	+	+	+	+
6	*Centaurea alba* L. subsp. *macrocephala* (Pau) Talavera	Spain, Andalucía: between Arcos de la Frontera and Bornos, on km 7.5, *Susanna* 2631	-	+	+	+	+	+	+	+
7	*Centaurea alba* L. subsp. *strepens* (Hoffmanns. & Link) Rocha Afonso	Portugal, Região Centro: by coast of Tejo, N of Tramagal, W Abrantes, *Hilpold* 20093068	39° 28' 16'', -8° 15' 7''	+	+	+	+	+	+	-
8	*Centaurea alba* L. subsp. *tartesiana* Talavera	Spain, Andalucía: between Marines and Fuenteheridos, before Fuenteheridos, *L. Barres* & *J. Molero* s.n.	37° 54' 4,7'', 6° 39' 16,2''	+	+	+	+	+	+	+
9	*Centaurea gadorensis* Blanca	Spain, Andalucía: road from Puerto de la Ragua to Berja, 1.4 km NW Bayárcal, *Hilpold* 1023	37° 2' 20'', -3° 0' 28''	+	+	+	+	+	+.	+
10	*Centaurea cordubensis* Font Quer	Spain, Extremadura: NW Almendralejo, urbanización San Marcos, *Hilpold* 20093076	38° 42' 5'', -6° 27' 10''	+	+	+	+	+	+	+
11	*Centaurea limbata* Hoffmanns. & Link subsp. *limbata*	Spain, Galicia: A Coruña, about 0.1 km SSW Torre de Hércules, *Hilpold* 20093029	43° 23' 6'', -8° 24' 26''	+	+	+	-	+	+	+
12	*Centaurea hanrii* Jord. subsp. *spinabadia* (Timb.-Lagr.) Smythies	Spain, Catalunya: Alt Empordà, about 1.2 km NNE of Espolla, by the road towards french border, *A. Hilpold* s.n.	42° 24' 23'', 3° 0' 36''	+	+	+	+	+	+	+
NW Africa										
13	*Centaurea boissieri* Coss. subsp. *atlantica* (Font Quer) Blanca	Morocco, Meknès-Tafilalt: Middle Atlas, along road from Ifrane to Dayet Achlef, 4.6 km SSW Ifrane, *Hilpold* 20103093	33° 30' 29'', -5° 1' 1''	+	+	+	-	+	+	+
14	*Centaurea debdouensis* Breitw. & Podlech	Morocco, Souss-Massa-Daraâ: Djebel Sarhro, 0.7 km NE Ilknoun market square, *Hilpold* 20103061	31° 10' 32'', -5° 40' 10''	+	+	-	+	+	+	+
15	*Centaurea resupinata* subsp. *rifana* (Emb. & Maire) Breitw. & Podlech	Morocco, Taza-Al-Hoceima-Taounate: Rif, between Tarquist and Jebel Timouzai, about 2 km NE Tarquist, *Hilpold* 20103106	34° 57' 11'', -4° 18' 35''	+	+	+	-	+	+	+
16	*Centaurea tougourensis* Boiss. & Reut.	Algeria, Batna: Aurés Tighanimine, *Lidén* 660	-	+	+	+	-	+	+	+
Italy										
17	*Centaurea aplolepa* Moretti subsp. *bertolonii* (Arrigoni) Greuter	Italy, Liguria: Chiavari, sidestreet of the Via Aurelia (SP1), about 1.3 km NNW of the port, *Vilatersana* 1263	44° 19' 31'', 9° 19' 2''	+	+	+	+	+	+	+
18	*Centaurea deusta* Ten.	Italy, Puglia: road from Madonne delle Grazie to Monte Sant'Angelo, 1 km SSE Monte Sant'Angelo,	41° 41' 49'', 15° 58' 8''	+	+	-	+	+	+	+

		Vilatersana 1210								
19	*Centaurea deusta* Ten.	Italy, Veneto: Monte Pastello, dry slope above Adige river, N of La Quara, *F. Prosser* s.n.	-	+	+	+	+	+	+	+
20	*Centaurea pentadactyli* Brullo & al.	Italy, Calabria: Pentidattilo, on the eastern side of the rocks, *Vilatersana* 1183	37° 57' 16", 15° 45' 48"	+	+	+	+	+	+	+
21	*Centaurea tenacissima* (H. Groves) Brullo	Italy, Puglia: San Foca, Porto, *B. Medagli s.n.*	40° 18' 3", 18° 24' 23"	+	+	-	+	+	+	+
Greece										
22	*Centaurea acarnanica* (Matthäs) Greuter	Greece, Aetolia-Acarnania: Mt. Akarnanika Ori, ca. 0.5 km SW of the Romvou monastery, *Karamplianis* 1513	38° 47', 20° 59'	+	+	+	+	-	+	+
23	*Centaurea messenicolasiana* T. Georgiadis & al.	Greece, Karditsa: 1.9 km S and E of Messenikolas village, along the road to Karditsa, *T. Constantinidis* & *T. Karamplianis* s.n.	39° 20', 21° 45'	+	+	+	-	+	+	+
24	*Centaurea pawlowskii* Phitos & Damboldt	Greece, Epirus: Mt. Timfi. Vikos gorge, c. 0.8-1.2 km NE of the village of Monodendri, *T. Constantinidis* & *T. Karamplianis* s.n.	39° 53', 20° 46'	+	+	+	-	+	+	+
Turkey										
25	*Centaurea amaena* Boiss. & Balansa	Turkey, Kayseri: Kayseri, *Bağcı* 1529	-	+	+	+	+	+	+	+
26	*Centaurea lycia* Boiss.	Turkey, Antalya: Kazdaği, *Y. Bağcı* s.n.	-	+	+	+	-	+	+	+
27	*Centaurea wagenitzii* Hub.-Mor.	Turkey, Antalya: Adrasan, way to Sazak, *Bağcı* 1520	-	+	+	+	+	+	+	+

* the plus symbols will be substituted by the GenBank accession numbers in the published article.

Extractions

Total genomic DNA was extracted from silica-gel dried leaves collected in the field. Only for *Centaurea tougourensis* herbarium material was used. Extraction followed the CTAB-protocol of Doyle and Dickson (1987) with the modifications of Tel-Zur et al. (1999), including three washing steps with sorbitol buffer and a few further modifications: after precipitation with isopropanol and subsequent centrifugation, the DNA pellet was washed in 70% ethanol, dried at 37°C and re-suspended in TE-buffer. The quality of the extracted DNA was checked on 0.7% TBE agarose gels.

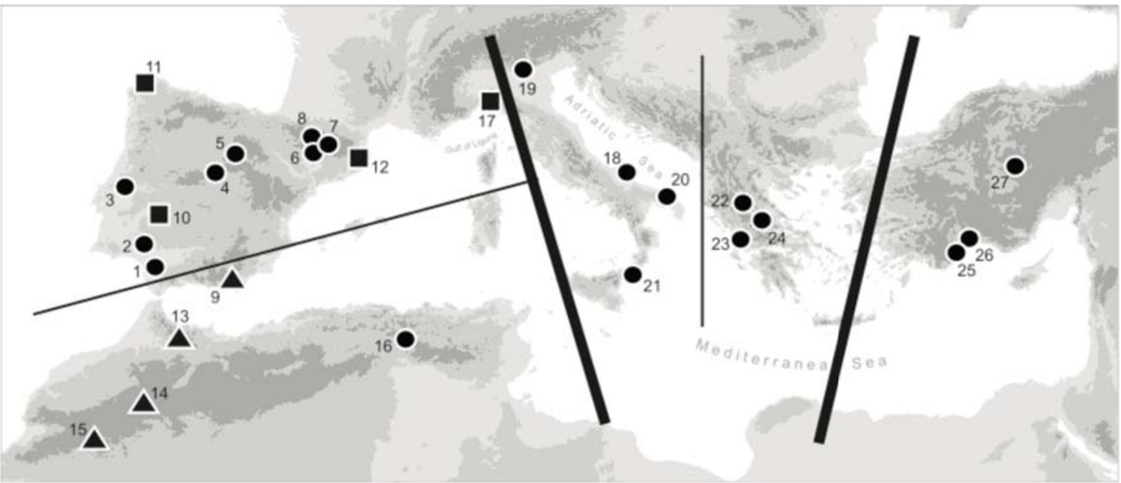

FIGURE 1 Geographical distribution of the 27 populations studied. Form or the symbols shows membership to one of the three sections: bowls = *Phalolepis*; squares = *Centaurea*; triangles = *Willkommia*. The lines show the geographical separation of populations used for the *BEAST-species-assignation. Bold lines separate the three main areas (E, C and W Mediterranean), thin lines give further separations between Greece and Italy respectively Iberian Peninsula and NW Africa (including SE Spain).

Selection of gene regions

The selection of the used gene-region followed previous experience in our own work or given in the literature. The internal transcribed spacer (ITS) has been proved to give good results also in shallow phylogenies. Its abundant gene-copies undergo rapid concerted evolution (Hillis et al. 1991; Kovarik et al. 2005; Ganley and Kobayashi 2007). In some cases, though, this concerted evolution is not yet concluded, especially postdating hybridization events (Popp and Oxelman 2001; Soltis et al 2008). The maternally inherited *rpl32-trnL* (shortly *rpl32*) region is considered to be one of the most variable in the angiosperm chloroplast genome (Shaw et al. 2007). The selection of the single copy regions followed the consultation of an accurate work on universal primers for Asteraceae (Chapman et al. 2007). The published primers were generated from conserved orthologue sets of *Helianthus* and *Lactuca*. As the result of a preliminary examination, most primers worked poorly in *Centaurea* sect. *Cyanus* (C. Löser pers. obs.). That is why a subset of primers was used as probes for BLAST searches to retrieve matching expressed sequence tag (EST) data of *Centaura solstitialis* L., *Centaurea stoebe* L., *Carthamus tinctorius* L., *Cynara scolymus* L. and further representatives of Compositae from Genbank (http://www.ncbi.nlm.nih.gov/genbank/). Those loci that showed signs of gene duplication, e.g. large number of matching sequences or duplicate clades in cursory phylogenetic trees, were discarded. For the remaining loci, new specific primers were manually designed based on the consensus of the *Cardueae* sequences. Priming sites were chosen close to inferred exon boundaries to amplify mostly non-coding intron portions of expressed genes. In this study, we chose a subset of five loci that gave high-quality amplification products in *Centaurea* sect. *Cyanus* used in the study of Löser et al. (in prep.). The used single copy regions are called (in concordance with Chapman et al. 2007): A04, A26, B27, C20 and D28.

PCR

We used AmpliTaq® (Applied Biosystems) for ITS and *rpl32* and Phusion® polymerase (Finnzymes, Espoo, Finnland) for the low-copy-regions. We conducted the PCR for the ITS with the following conditions: 4 min denaturing at 95°C, followed by 30 cycles of 94°C denaturing for 1.5 min, 55°C annealing for 55 s and 72°C extension for 3 min, with additional 15 min at 72°C. The PCR for the *rpl32* followed the following conditions: 3 min of denaturing at 95°C, followed by 35 cycles of 95°C denaturing for 40 s, 54°C annealing for 40 s and 72°C extension for 1 min 40 s with additional 10 min at 72°C. The PCRs of the low-copy regions were conducted with the following conditions: 2 min denaturing at 98°C, followed by 40 cycles of 98°C denaturing for 10 s, 67°C annealing for 20 s and 72°C extension for 7 min, with additional 7 min at 72°C. We sequenced at least six gene regions per included population (see Table 1).

Separation of alleles in single copy genes

When two alleles of a single individual differed by a maximum of three substitutions they were coded as ambiguous. However, two alleles were resolved when short insertions/deletions allowed subtraction of phase-shifted peaks in one direction and cross-validation by comparison with backward reads.

Alignments

The alignment was partly made by hand (ITS, *rpl32*), partly by ClustalW 2.0.12 (low copy genes; Larkin et al. 2007), implemented in Geneious Pro 4.8.3 (Drummond et al. 2010). In the second case an adjustment by hand followed the automatic alignment. Gaps were treated as missing data in all analyses.

Recombination tests

We used the software RDP 3.44 (Martin et al. 2010) to screen the data for possible recombinants with the default settings of the package with a highest acceptable P value of 0.05 and using the Bonferoni correction. We used following methods implemented in the program: (a) the RDP method (Martin and Rybicki 2000), with internal and external reference, (b) the Sister Scanning method implemented in SiScan (Gibbs et al. 2000), (c) the gene conversion method in GENECONV (Padidam et al. 1999), (d) the bootscanning method in BOOTSCAN (Martin et al. 2005), (e) the maximum v2 method in MAXCHI (Maynard Smith 1992), and (f) a modification of this method implemented in CHIMAERA (Posada and Crandall 2001).

Network analyses

To screen the nuclear genes for hybridizations with subsequent recombination we made network analyses using the Neighbor-Net (NN) algorithm (Bryant and Moulton 2004) as implemented in SplitsTree4 4.10 software (Huson and Bryant 2006). The adjustments used included uncorrected pair-wise (p) distances, excluding non-informative characters.

Bayesian phylogenetic analyses with MrBayes

All data-sets were subjected to Bayesian phylogenetic inference using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best available model of molecular evolution required for Bayesian estimations of phylogeny for all datasets was selected using Akaike information criteria (AIC) as implemented in MrModeltest 2.2 (Nylander 2004). The Bayesian inference analyses were initiated with random starting trees and were run for 2 x 10^6 generations. Four Markov chains run using Markov Chain Monte Carlo (MCMC) principles to sample trees. Every 1000th generation, a tree was saved, resulting in 2,000 sample trees. Using the software Tracer v1.5 (Rambaut and Drummond 2003–2009) convergence and mixing was explored. After discarding burn-in samples (10%), a majority rule consensus tree was made that was displayed on FigTree v1.3.1 (Rambaut 2006–2009). Internodes, which had a posterior probability of at least 0.95, were considered as statistically significant.

**BEAST*

We used the software *BEAST (Heled and Drummond 2010) as implemented in BEAST v1.6.1. (Drummond and Rambaut 2007). We used BEAUti v1.6.1 (Drummond et al. 2002–2010) for generation of the BEAST input file. After importing all seven matrices into BEAUti, substitution models, clock models and trees were unlinked. In various analyses the species circumscription for *BEAST was changed (see below). For the clock model priors all regions were given a strict clock. As prior for the substitution rate the posterior for this parameter in a BEAST dating analysis with ITS based on a fossil calibration (Hilpold et al. in prep., Barres et al. in press) was used. For the species tree prior a Yule-process was assumed. For the population size the default prior was used (piecewise linear and constant root). The ploidy type was changed to mitochondrial for the *rpl32* and afterwards the value was changed from 0.5 to 1 in the xml-file. The starting trees were generated by UPGMA. The alpha-parameter was changed towards a lognormal distribution. The analysis was run for 2 x 10^8 generations, every 10,000st tree was saved, resulting in 20,000 trees. The resulting log-files were checked for convergence. Ten percent of the species trees were discarded as burn-in.

Of the entire data set, consisting of all 7 regions, a total of 8 analyses were run, changing only the *BEAST-species circumscription: (1) every population, as given in Table 1, as single species, resulting in a total number of 27 "species"; (2) every taxonomic species, following Euro+Med Plantbase, as species (= 19 species); (3) every section, the species belong to (see Table 1) as different species (= three "species"); (4) populations of the same geographic area as different species, distinguishing three groups (Fig. 1, bold lines): W Mediterranean (including *C. aplolepa* Moretti from NW Italy), C Mediterranean and Turkey (hereafter referred to as GEO3); (5) populations of the same geographic area as different species, distinguishing four groups. Separation equally as in 4 but with C Mediterranean split into a group of Italian and a group of Greek populations (Fig. 1, bold lines and right thin line; hereafter referred to as GEO4); (6) populations of the same geographic area as different species, as in 5 but additionally separating the W Mediterranean group into a group including NW Africa and SE Spain (*C. gadorensis* Blanca) and a group of the remaining Iberian specimens, including *C. aplolepa* (Fig. 1, bold and thin lines; hereafter referred to as GEO5); (7) the species given by BPP (i.e. those clades shown in Fig. 4, description of BPP see below) with a division into eight species; (8) following the BPP species division, but merging clade 3 and 4 (see Fig. 4) into one large Iberian group.

Bayes factor calculations

To compare these species classifications, Bayes factors of the *BEAST log files in were calculated using the program Tracer v1.5 (Rambaut and Drummond 2003–2009) with 10,000 bootstrap replicates. This program uses the harmonic mean (HM) method (Newton and Raftery 1994), which is a computationally easy method to estimate marginal likelihoods from the output of the MCMC analysis. The HM method, however, has been shown to be less accurate than another recently developed method, the thermodynamic integration, since HM is prone to overestimate marginal likelihoods (Lartillot and Philippe 2006). This latter one, though, has to be integrated directly into the MCMC chain (Xie et al. 2011) and is therefore hardly feasible in *BEAST. For this reason, we use results of Tracer with caution, making sure that *BEAST runs have converged and running *BEAST at least twice.

BPP

We used the program BP&P v2.1 (shortly BPP; Rannala and Yang 2003; Yang and Rannala 2010) which accommodates the species phylogeny as well as lineage sorting due to ancestral polymorphism. We used the maximum posterior topology from *BEAST (classification of every population as separate species) as backbone. BPP uses reversible model jumps in the MCMC to evaluate the possible nested species classifications of that tree. A gamma prior *G* (2, 1000) is used on the population size parameters (θ_s). The age of the root in the species tree (τ_0) is assigned the gamma prior *G* (2, 1000), while the other divergence time parameters are assigned the Dirichlet prior (Yang and Rannala 2010: equation 2). Each analysis is run at least twice to confirm consistency between runs.

RESULTS

Sequencing

We succeded to sequence at least six gene regions in all 27 populations (Table 1). In 15 populations one out of seven gene regions could not be amplified. All these missing sequences are among the low copy regions, with the region A26 with the fewest populations successfully amplified (see Fig. 2). About half of the sequences per single copy region were heterozygous. Single copy markers had frequently two different copies. These copies differed in most cases only in a single or a few bases. In a few cases, however, one single individual showed very different gene copies. In the putative tetraploids *C. acarnanica* and *C. pawlowski* in all single copy genes only maximally two different gene copies were found. The presence of diploid and tetraploid individuals within one species is rather common in the *Centaurea* group (IPCN Chromosome Reports) and it is possible that the individuals included in our study are diploids.

Results from recombination tests, NeighborNet and substitution model selection

The recombination tests didn't show evidence for recombination. Even in individuals where recombination was suspected due to their intermediate allele combination between two divergent genotypes (namely in *C. aplolepa* in the B27 region; Fig. 2) no significant evidence for recombination was given. The NeighborNet (Supplementary material S1–S3) gave some contradicting splits, which, however, were mainly restricted to the three main groups given by the *BEAST analysis (see below), suggesting recombination mainly within these groups but not among them. In some cases, though, contradicting splits between these groups could indeed be observed. Examples are *C. deusta* (Verona) in A26 and *C. alba* (Soria and Cádiz) in C20. Contradictory splits can also be caused by homoplasy and/or positive selection.

The chosen models used as priors in Bayesian analyses were the following: GTR+I for C20, GTR+G+I for ITS, A04 and B27; GTR+G for *rpl32*, A26 and D28.

Gene trees given by Bayesian analysis

All seven markers showed good resolution with mostly, well delimited and well supported clades (Fig. 2). The seven gene trees are clearly different between the different markers, if compared visually (Fig. 2). There is, however a clear relation with geography, which can be seen in the fact that most clades present only species of maximally two geographic regions (see colors in Fig. 2). Populations from the Iberian Peninsula group together, often including some individuals from NW Africa and *C. aplolepa* from the Ligurian Coast. Sequences from plants from Italy (except *C. aplolepa*) group with those from Greek populations. Turkish populations share the same alleles in several gene trees. None of the seven gene trees reflects the sectional delimitation or support monophyly of those species that are present with more than one population in the sampling (i.e. *Centaurea alba* and *C. deusta*).

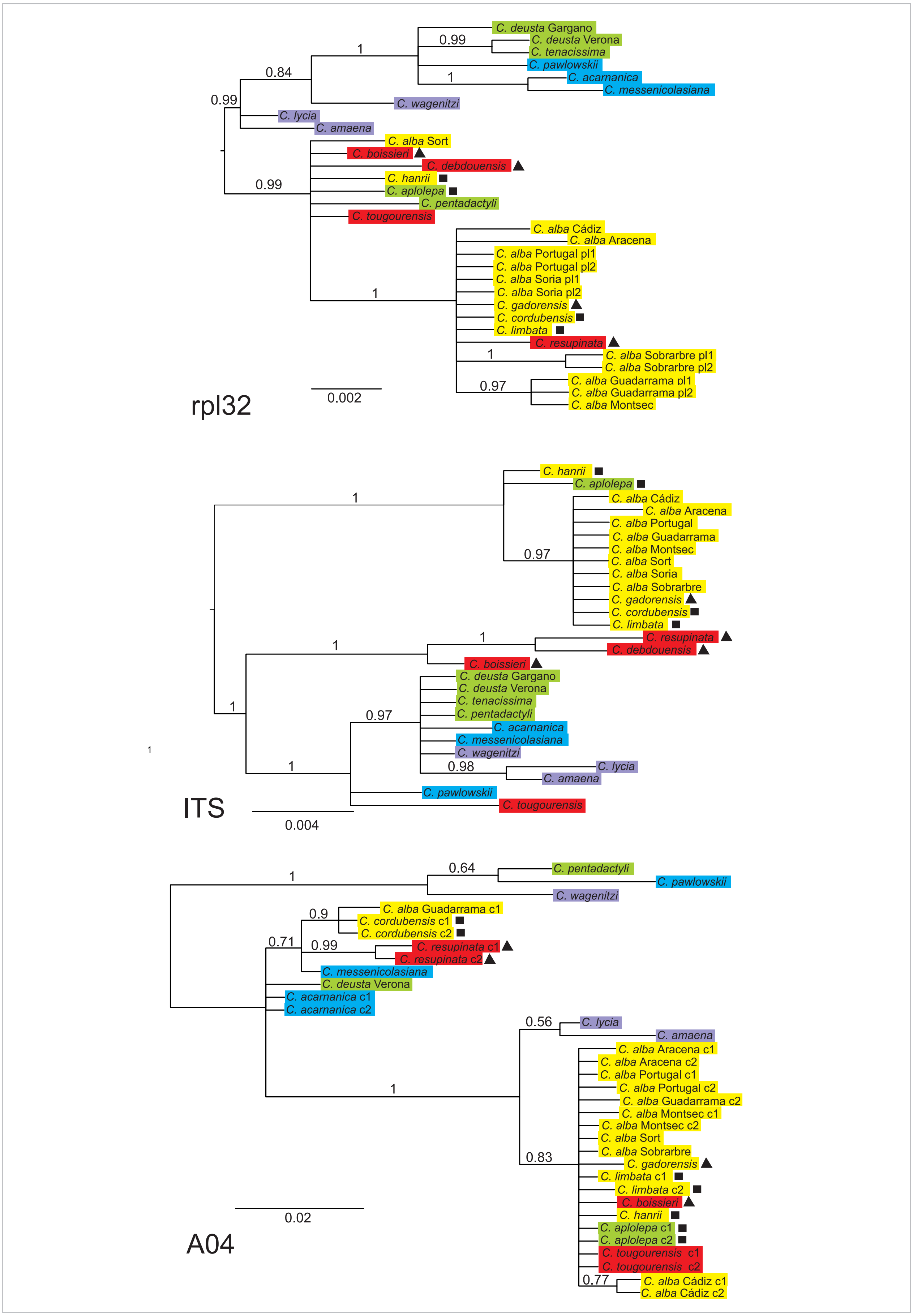

FIGURE 2 Midpoint rooted majority consensus gene trees resulting from Bayesian analysis of six nuclear and on cpDNA region. Values on branches give posterior probabilities. Underlain colors show geographical distribution of the species: yellow = Iberian Peninsula; red = NW Africa; green = Italy; blue = Greece; violet = Turkey. Symbols after the species names show sectional assignation: triangle = sect. *Willkommia*; square = sect. *Centaurea*; all others belong to sect. *Phalolepis.*

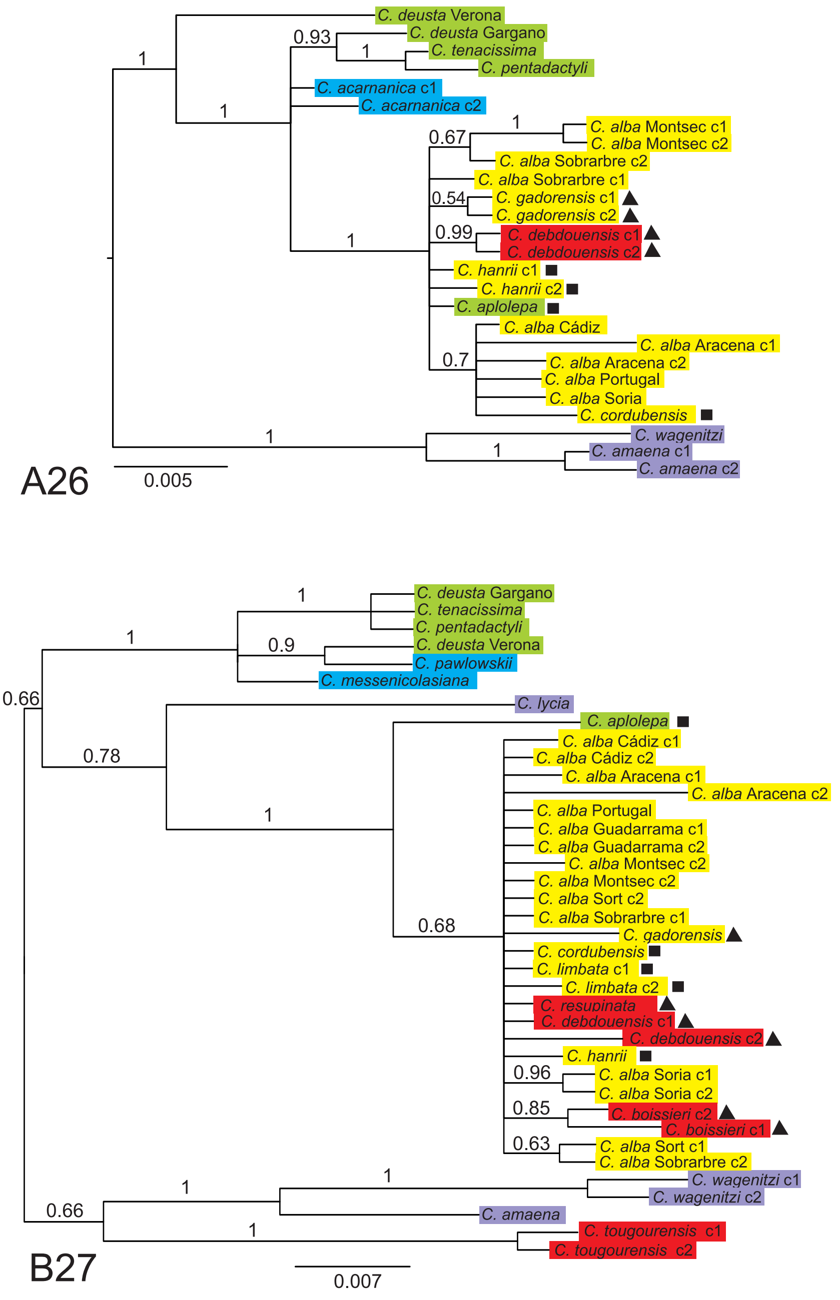

FIGURE 2 Continuation from preceding page.

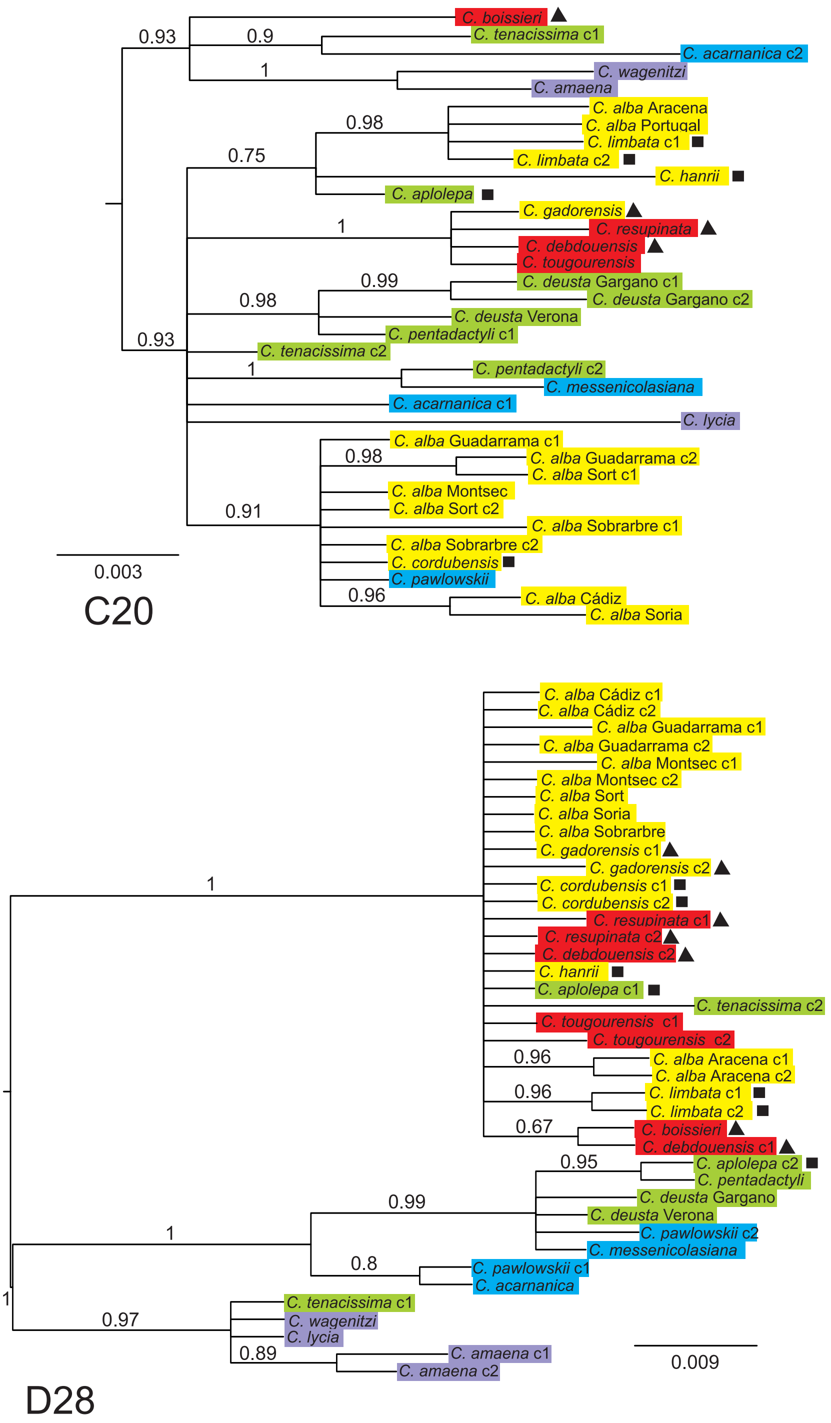

FIGURE 2 continuation from preceding two pages.

**BEAST*

The *BEAST analysis both based on taxonomic species and populations treated as species gave good support for three groups (Fig. 3, with the classification of populations as species), which are: (1) a western Mediterranean group, including all Iberian and African populations and additionally *C. aplolepa* from NW Italy; (2) a central Mediterranean group, including all Greek and Italian populations except *C. aplolepa*; (3) a eastern Mediterranean group, including the three Turkish populations. The division into these three clades is very robust: it is shown also if only nuclear markers are included and if only low copy markers are included. Below this tripartition no well supported clades are present. None of the single gene trees is fully congruent with the *BEAST tree (Fig. 3). There is some evidence that the individual of *Centaurea aplolepa* is product of hybridization between the western Mediterranean clade and the central Mediterranean clade since it shows two copies in the D28 marker and possible recombination was detected in B27 (Fig. 3). Hybridization may also be responsible for the position of one copy of *C. tenacissima* (H. Groves) Brullo, usually placed among E Mediterranean populations, ranged among W Mediterranean populations in D28. Quite surprising is also the position of *C. pawlowskii* within a purely Iberian clade in C20, which could be also a sign of hybridization or an error in the sequencing process.

BPP

The BPP analysis (Fig. 4) recognizes less species than given in the different taxonomic treatments. One clear speciation event happened between Turkish members (clade 8) and central to western Mediterranean populations. The latter lineage split with high support into two separate lineages, a western and a central Mediterranean one (Figs. 4–5). There is support for *C. pawlowski* being a separate species and all other Italian (except *C. aplolepa*) and Greek species a separate one (clade 2). Further species supported by BPP are, a Ligurian one (*C. aplolepa*) and *C. hanrii* Jord. (clade 7), a N African one (except *C. resupinata* Coss.; clade 6) one including *C. resupinata* from Rif Atlas and *C. gadorensis* from SE Spain (clade 5), and maximally two further Iberian species, including all the remaining species from the Iberian Peninsula (clades 3-4).

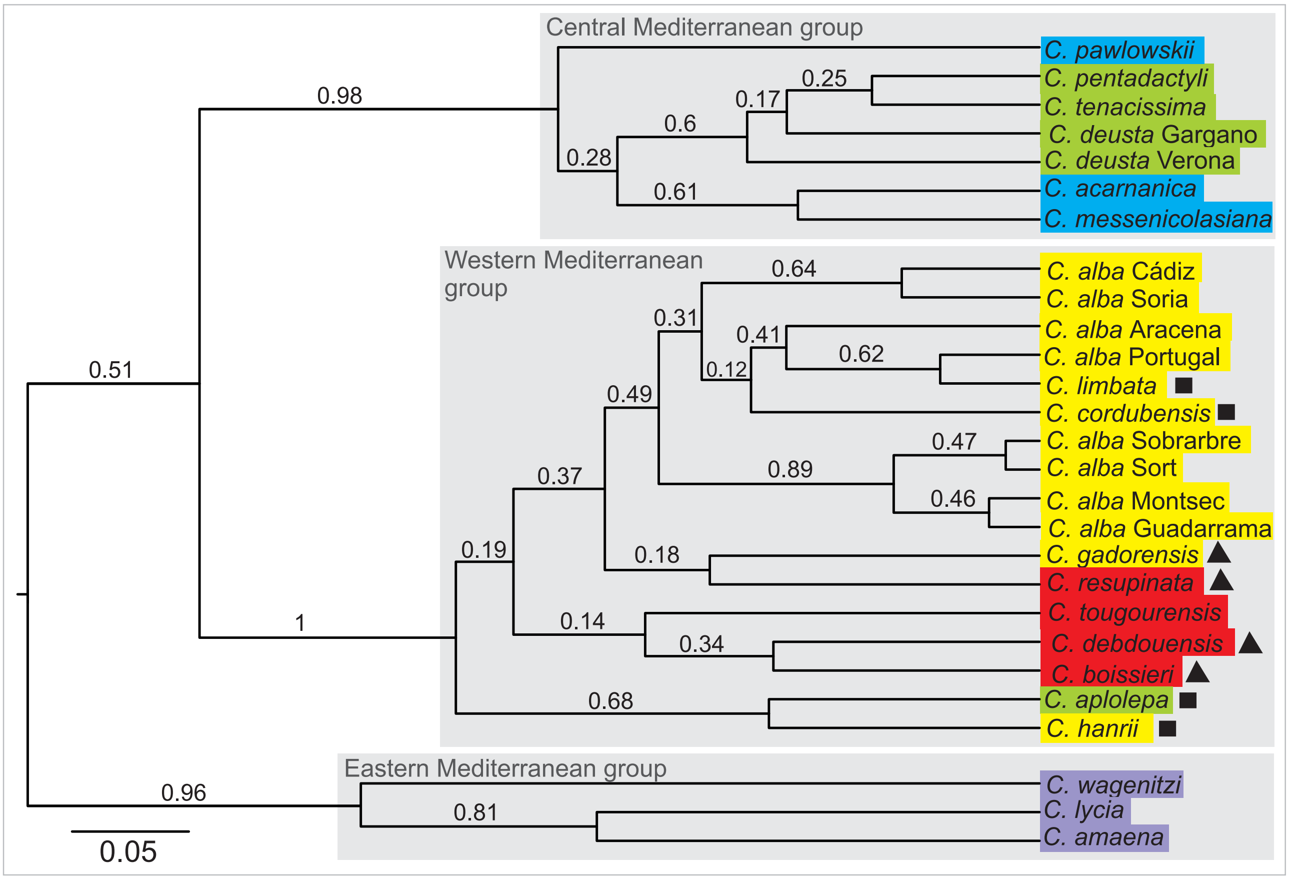

FIGURE 3 Species tree resulting from the *BEAST analysis of seven gene loci. Numbers on branches give posterior probabilities. Colors and symbols as in Figure 3.

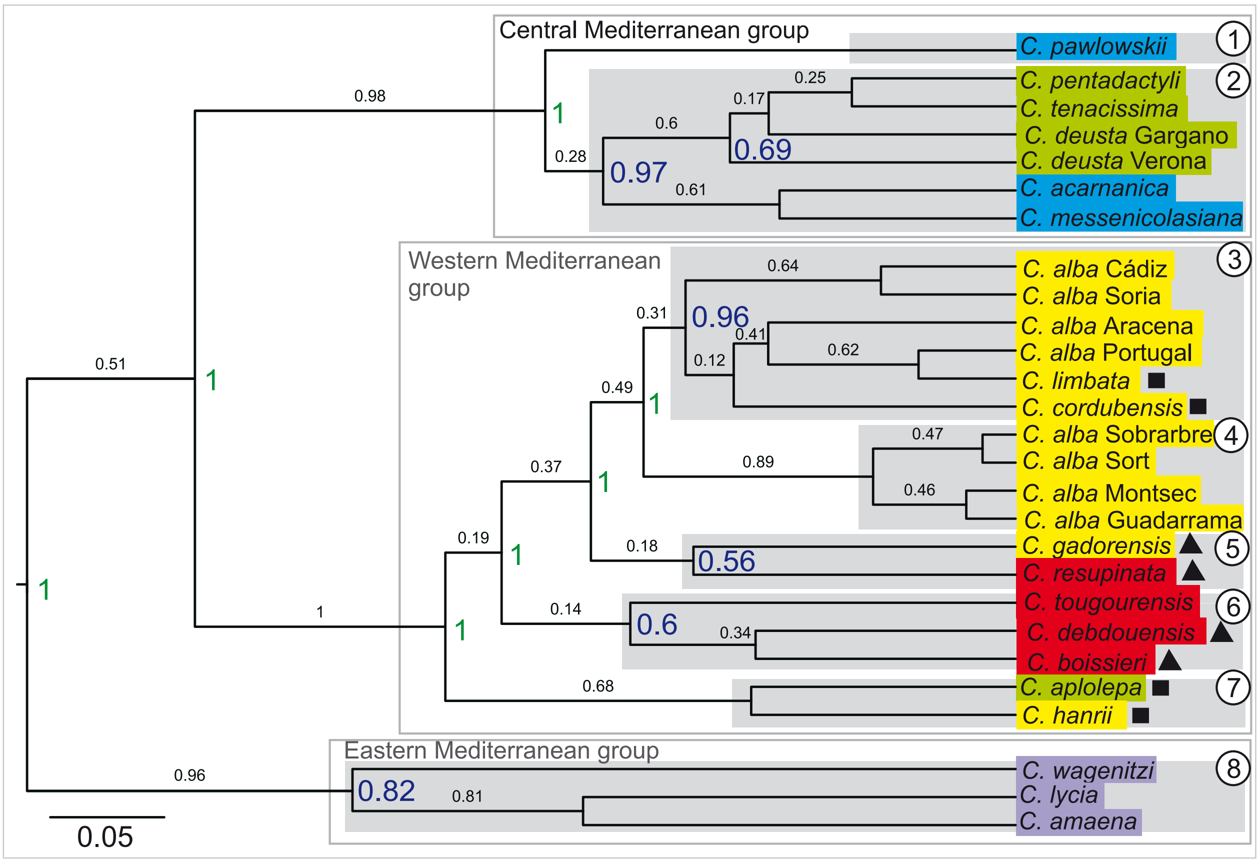

FIGURE 4 *BEAST species tree with results from BPP species delimitation. Values on the right side of the nodes show support for these notes to have split into two species: 1 (in green) means high support; blue means low support.

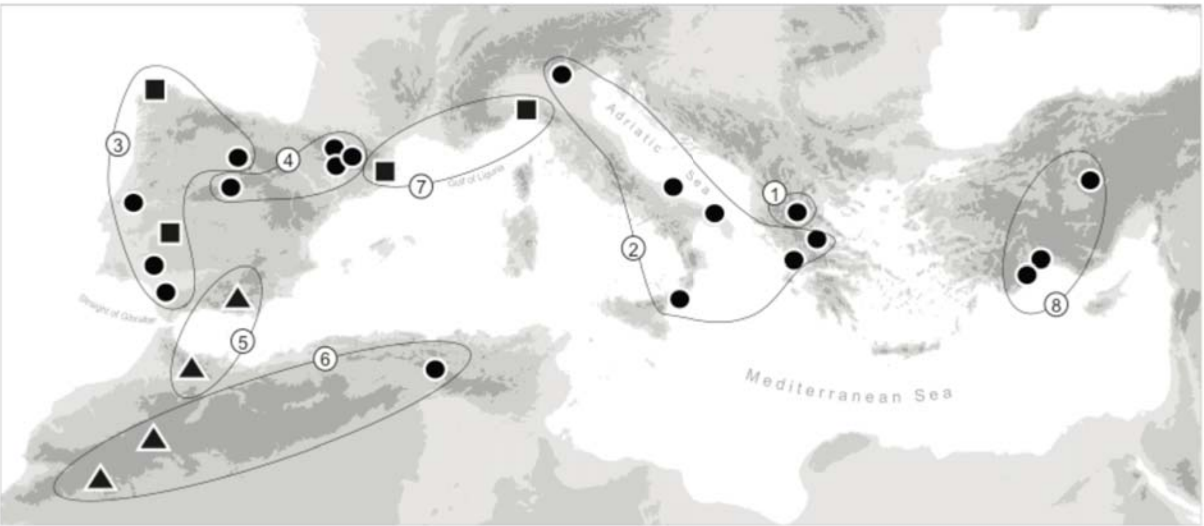

FIGURE 5 Geographical distribution of the species resulting from BPP.

Bayes factors

The variation among the estimated marginal likelihoods for the different species classifications spans almost 15 units, and could therefore be considered as conclusive (Kass and Raftery 1995; Table 2). The lowest value is obtained when every population is treated as a separate species (BF= –8594.2). Significantly better (delta 4.5) is the treatment taxonomic species as species, which is, however, clearly outperformed by the treatments section as species (delta 6.5) and especially by geographical groups as species (delta 5.2–10.5). Similar high Bayes factors are given if the species resulting from the BPP analysis are treated as separate species.

TABLE 2 Bayes factors for various species classification (see text).

*BEAST species classification	Bayes factor
Population as species	-8594.19 +/- 0.34
Species as species	-8589.74 +/- 0.38
Section as species	-8583.27 +/- 0.38
GEO3	-8580.62 +/- 0.69
GEO4	-8579.28 +/- 0.42
GEO5	-8584.52 +/- 0.38
BPP-IB2 (two Iberian groups)	-8582.64 +/- 0.36
BPP-IB1(one Iberian group only)	-8582.13 +/- 0.38

DISCUSSION

The results of our study are clear in one sense: traditional taxonomy, i.e. the breakdown of members of *Centaurea* group from the western Mediterranean, especially the Iberian Peninsula, into a multitude of species and into three sections is not congruent to the results of a multispecies coalescent approach. Our analyses gave three, well supported clades (Fig. 3) strongly different from the sections recognized by traditional taxonomy. The division of the sampled populations into 19 species showed the second-worst results in the Bayes factor comparisons (Table 2), only undermatched by every single population as a single species. The treatment of the three sections as a single species gave higher marginal likelihood estimates, but was outperformed by treating geographic groups as species. A fact that may have improved the performance of the sectional division in the Bayes factor calculation is the unbalanced sampling scheme, including only members of section *Phalolepis* from the central and the western Mediterranean. It is remarkable that the models with many species seem to work worse than those with few. Almost equally high Bayes factors were given if the species delimitation provided by BPP (Fig. 4) was used. According to the results of BPP, there is support for only three species in our sampling from the Iberian Peninsula (Fig. 5): 1. *Centaurea gadorensis* together with Moroccan *C. resupinata* (Figs. 5 and 6) 2. *C. hanrii* together with NW Italian *C. aplolepa*, 3. four out of eight included populations of the *C. alba* complex, including *C. limbata* Hoffmanns. & Link and *C. cordubensis* Font Quer and 4. four populations of the *C. alba* complex from NW and central Spain. One more species would occur in NW Africa, one in the rest of Italy and Greece, one in Greece and one in Turkey. Compared to the species classification of Greuter (2008), this would be a decrease from 19 to 8 species.

A clear reduction of species numbers when using multispecies coalescent approaches was also the result of the study of Harrington and Near (2011) on bats using the approach of Carstens and Dewey (2010), in which likelihood scores are compared between species trees generated under alternative species delimitation scenarios, whilst Salicini et al. (2011), working with the same animal group, observed a small increase in species numbers, using *BEAST. Kubatko et al. (2011) observed an increase of one species in their study about rattlesnakes. Zhou et al. (2012) found a slight increase in a group of closely related frog species. Pons et al. (2011) observed a strong increase in species numbers by using a coalescence approach in a group of beetles. The only study published about plants (Barret and Freudenstein 2011) shows an increase of maximally one species by using a species coalescent. In summary, changes in species number in these studies are in most cases moderately positive. Our study depicts from theses studies for its strong reduction in species numbers.

Hybridization

Zhang et al. (2011) show that BPP is relatively robust against hybridization (i.e. migration between different populations). A migration rate less then 0.1 migrants per generation still gives correct species limits in their simulations. The migration rate between distribution areas, which are far apart can be expected to lay clearly below that rate. Even for different populations from different regions within the Iberian Peninsula the migration rate could not be so high. If, at all, this rate should be exceeded, it might be the case for populations that grow in close proximity. Most populations of our sampling, however, are separated by at least 50 km (Fig. 1). To accomodate migration, a multispecies coalescent model must either set a very recent time for the speciation, or infer inflated population sizes. Problems with these parameters could indeed be observed, i.e. huge difference between *BEAST posterior and BPP posterior values: the *BEAST-posterior estimate of the root height was about 0.5 (not dependend on the chosen classification) whilst the BPP posterior was at about 0.002. The models in BPP and *BEAST are not identical (e.g. JC69 vs. GTR as substitution models), this could be one reason for the largely divergent posterior for the root height, probably indeed provoked by connections between the main groups that might have happened after the main divergence of species.

Morphological groups vs. metapopulation lineages

The fact that a high morphological variability exists does not imply that all these morphological groups are good species in sense of a separately evolving metapopulation lineage. But here it comes to a dilemma: What is taxonomy for, if not to describe species as a single lineage? But what happens if these lineages, which actually exist, do not constitute morphologically separable groups, or if they contradict morphological groupings? The first case is what is often referred to as cryptic species. Morphological variability within a species defined as lineage could be handled on a subspecific level, downscaling former species to the rank of subspecies. In case of the *Centaurea alba* complex, morphologically well distinguishable taxa like. *C. cordubensis* and *C. limbata* would have to be merged into one single species, probably with three different subspecies. Such a solution would probably cause more problems than it may solve, from the complication in generating determination keys to the impossibility of species determination. In addition, information about morphological variability within these traditional species would likely get lost if it is not even assigned a subspecific level (that would probably be the case for the subspecies of *C. alba*). If a high morphological variability is pressed into a system of multiple subspecies, another question arises: how many subspecies can be handled in regional and supraregional floras without losing any practicability?

A further important question is what to do in cases where molecular division is also unclear (additionally to the morphological one). A good example in our data is given by members of the morphologically relatively clearly delimited section *Willkommia* from Southern Spain and NW Africa. The molecular data are not conclusive if they really belong together, or if Iberian members are closer related to members of the *C. alba* complex than to those from Morocco. Different methodological approaches e.g. in molecular markers and tools may get to different answers about this question. Any taxonomical decision would be overhasty as long as the molecular results are so inconclusive. Although the use of a multispecies coalescence approach is very promising, it is still too tedious and expensive to be used on a big scale including hundreds of traditionally described species with several populations. One hope for the solution of these problems may lay in the use of next generation sequencing techniques, allowing for sequencing information in the plant genome on a large scale, coupled with a simultaneous enhancement of the methods of analysis.

CONCLUSIVE REMARKS

Finally, an important question is whether such a discrepancy between traditional taxonomy and species delimitations following molecular methods is limited to a few genera or species groups, or if it can constitute a common problem in recently diverged species complexes. There are several examples where this might be the case, for example in other clades of *Centaurea* s. l., like the *Jacea-Phrygia* group (J. López-Alvarado, pers. comm.), subgen. *Cyanus* (Juss.) Hayek (Boršić et al. 2011; C. Löser pers. obs.), but also in numerous not related genera like *Silene* L. (Aydın et al. in prep.), *Haworthia* Duval (Ramdhani et al. 2011). On the other hand genera or species groups may exist which have diversified millions of years ago, where both a morphological and a molecular approach might come to the same result.

SUPLEMENTARY MATERIAL

Networks resulting from the Neigbort-Net are given in the supplementary material.

FUNDING

This project was funded by the Spanish Ministry of Science and Innovation (Projects CGL2007-60781/BOS and CGL2010-18631) and the Generalitat de Catalunya (Ajuts a Grups de Recerca Consolidats 2009/SGR/00439). A. Hilpold benefited from a predoctoral grant of the JAE program of the CSIC. The CSIC financed also a research stay of A. Hilpold in Gothenburg (Sweden) in autumn of 2010.

ACKNOWLEDGEMENTS

T. Marcussen is acknowledged for the help in the lab. We thank Y. Bağcı, L. Barres, T. Constantinidis, T. Karamplianis, B. Medagli, J. Molero, F. Prosser, K. Romaschenko for their help in collecting specimens. Special thanks go also to the Junta de Andalucía (Ref. SGYB/FAO/CRH Re-519-08) for allowing recollection in their territory.

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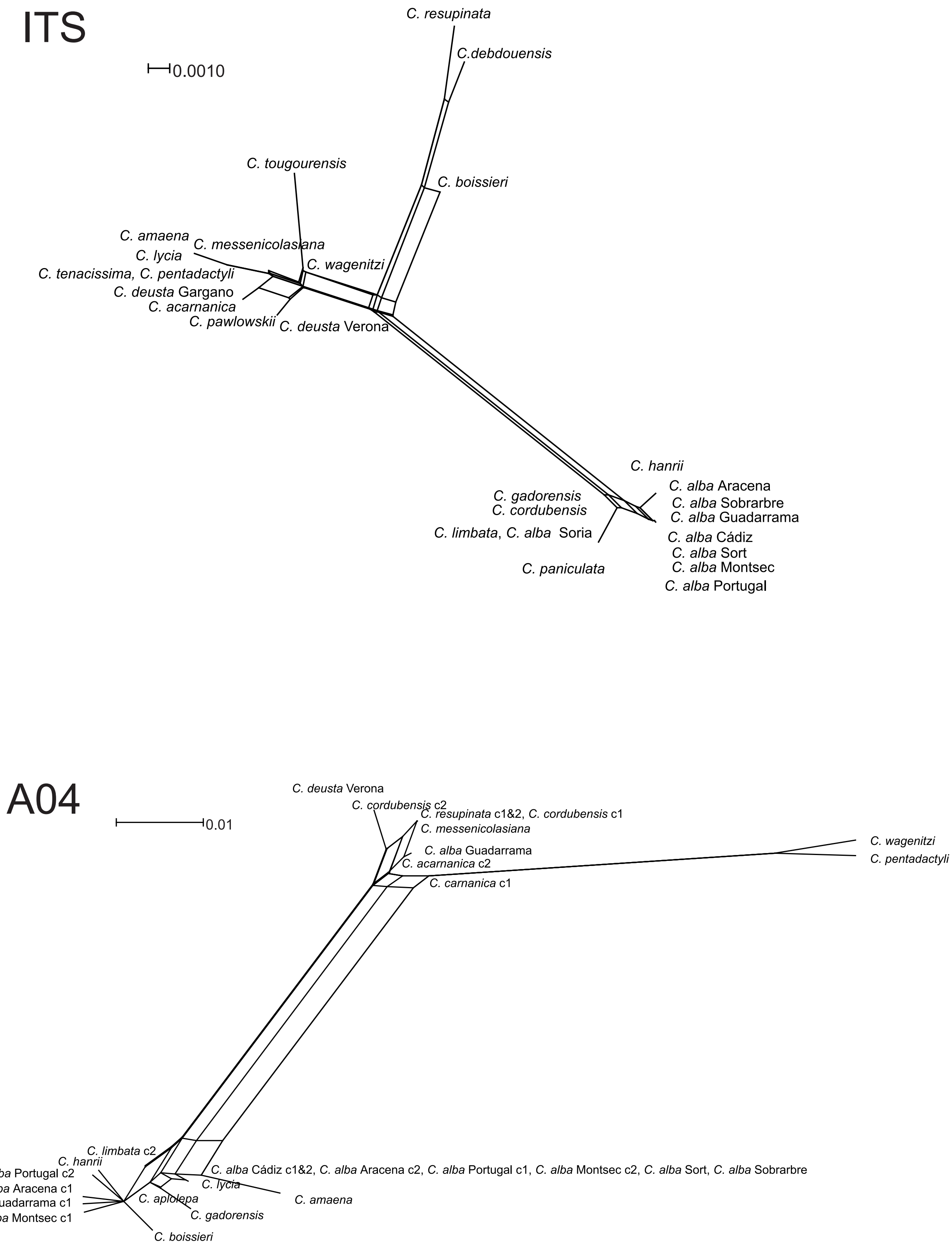
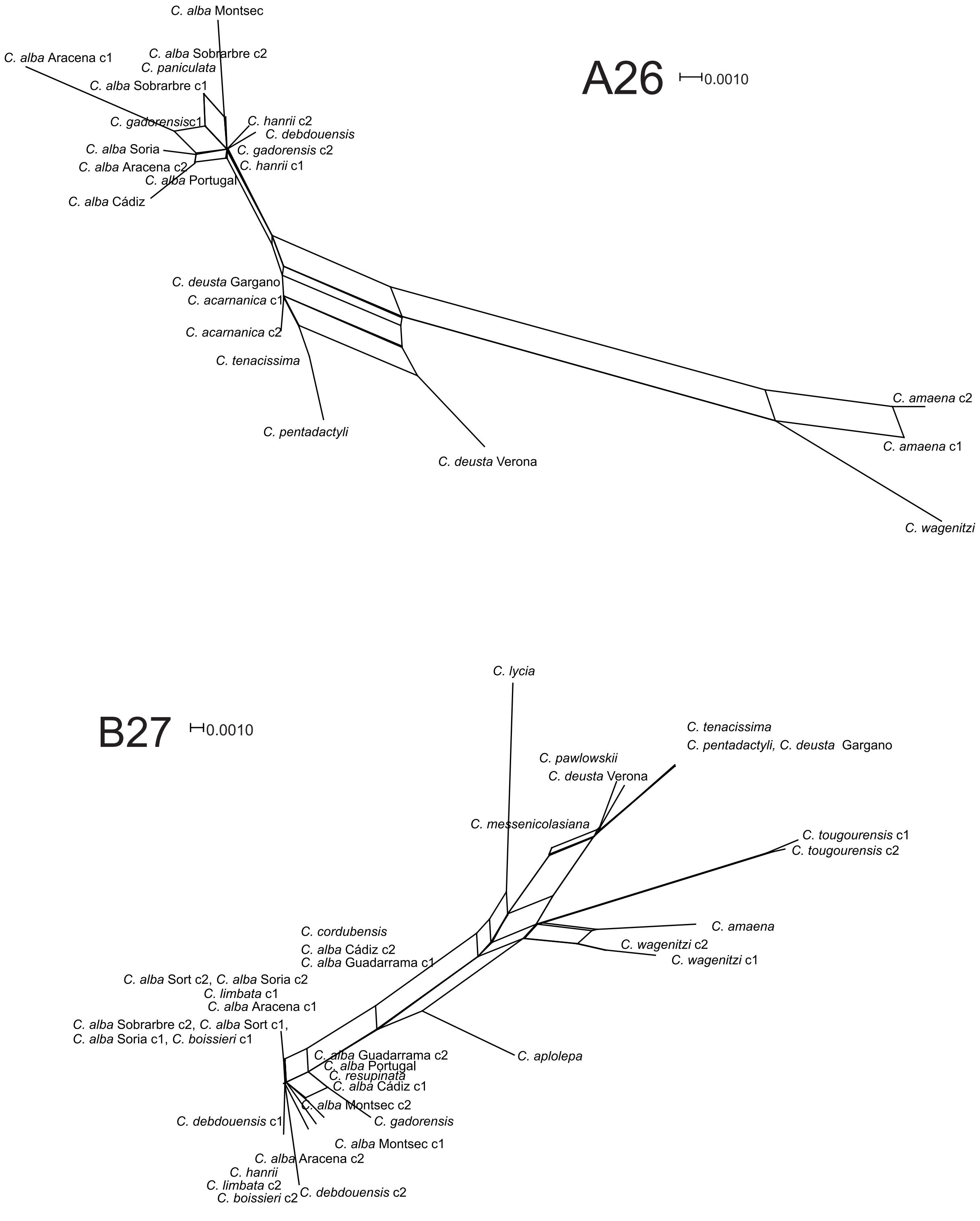
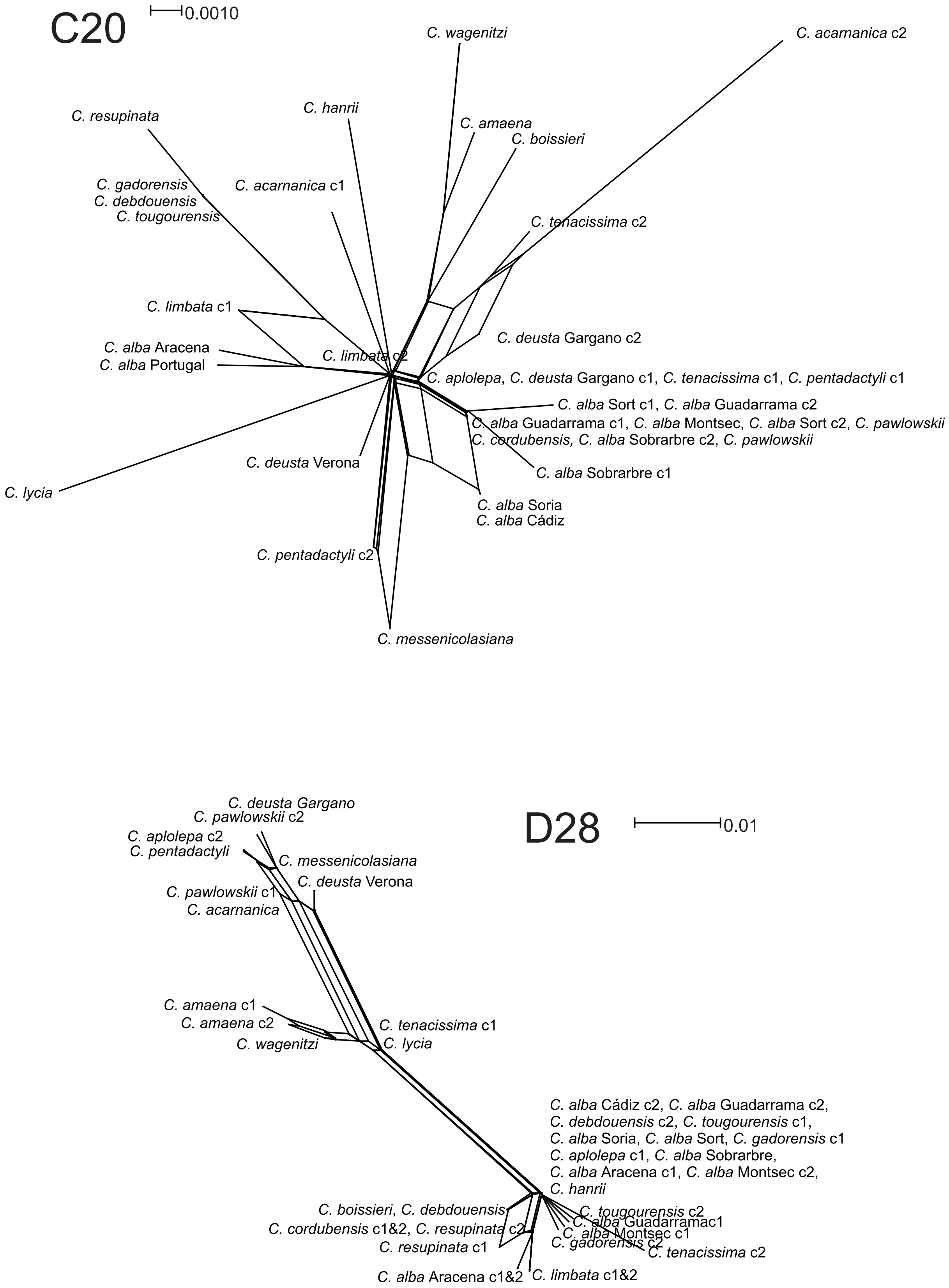


SUPPLEMENTARY MATERIAL S1 Neighbor-Net of the ITS and the A04 regions.



Supplementary Material S2 Neighbor-Net of the A26 and the B27 regions.



SUPPLEMENTARY MATERIAL S3 Neighbor-Net of the C20 and the D28 regions.

Capítol 5: s’ha enviat a *Plant Systematics and Evolution*
[*fifth publication, has been sent to* Plant Systematics and Evolution]

Títol original [*original title*]:

Tackling taxonomic ambiguity with an integrative approach: the case of *Centaurea corensis*
[*Resoldre l'ambigüitat taxonòmica amb un enfocament integratiu: el cas de Centaurea corensis*]
[*Resolver la ambigüedad taxonómica con un enfoque integrativo: el caso de* Centaurea corensis]

Autors [*authors*]:

Andreas Hilpold, Javier López-Alvarado, Núria Garcia-Jacas, Emmanuele Farris

Resum en catalá [*abstract in Catalan*]:

Centaurea corensis, espècie recenment descrita de Sardenya i fins ara només coneguda d'una única localitat, va ser trobada a l'illa de Procida, en el golf de Nàpols. L'afiliació a la mateixa espècie de les dues poblacions es confirma a partir d'una comparació morfològica, recomptes cromosòmics i seqüenciació de DNA. Es proposa la hipòtesi d'una poliploidització recent i es discuteix la curiosa disjunció, subratllant la possibilitat d'una dispersió humana recent.

Resumen en castellano [*abstract in Spanish*]:

Centaurea corensis, especie de Cerdeña recientemente descrita y hasta ahora sólo conocida de una única localidad, fue encontrada en la isla de Procida, en el golfo de Nápoles. La afiliación a la misma especie de las dos poblaciones se confirma a través de una comparación morfológica, recuentos cromosómicos y secuenciación de ADN. Se propone la hipótesis de una poliploidización reciente y se discute la curiosa disyunción, subrayando la posibilidad de una dispersión humana reciente.

[Abstract in English see below]

Tackling taxonomic ambiguity with an integrative approach: the case of *Centaurea corensis* (Compositae)

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Abstract *Centaurea corensis*, a recently described Sardinian species hitherto known only from one single locality in Sardinia, has been found on the Island Procida, at the Gulf of Naples off the Italian west coast. The identification of the two populations as *C. corensis* is confirmed by morphological comparison, chromosome counts and DNA sequence data. A recent origin of the species through polyploidization is hypothesized. Finally, this rare disjunction is discussed, focussing on the possibility of recent human dispersal.

Keywords Anthropogenic dispersal · Integrative taxonomy · Mediterranean · Polyploidization · Sardinia · Species delimitation.

Introduction

Taxonomic ambiguity is a common problem not only in plant systematics (Chang et al. 2007), but also in several applied fields, ranging from agriculture (Costea et al. 2006) to conservation biology (Rossetto 2005). In particular, when dealing with species conservation, it is of utmost importance to take into account taxonomic uncertainty (Hey et al. 2003) and try to discriminate between closely related taxa (Zhang et al. 2006; Romeiras et al. 2007).

Problems in species delimitation are frequent when species are delimited by few morphological characters. In groups where species share the same or similar morphological traits, many cryptic species have been described over the last decade, greatly aided by molecular methods (e.g. Hebert et al. 2004; Molina et al. 2011). In contrast, it has been claimed that some species, which had originally been described based on few characters, may be heterogeneous assemblages of more than one independently evolving metapopulation lineage (sensu de Queiroz 1998). A remedy to resolve this tricky situation comes from new taxonomic approaches, which seek to complement morphology, cytology or karyology with other sources of information like molecular evidence for deducing the speciation process. These approaches can be summarized under the term "integrative taxonomy" (Schlick-Steiner et al. 2010; Padial et al. 2010; Barret and Freudenstein 2011).

A group of plants full of taxonomic ambiguities is the genus *Centaurea* L. (subtribe *Centaureinae*, tribe *Cardueae*, Compositae). It includes roughly 250 species (Susanna and Garcia-Jacas 2009), with many narrow endemics (e.g. Colas et al. 1997; Pisanu et al. 2009) and new species are continuously being described (e.g. Köse et al. 2010; Kultur 2010; López-Alvarado et al. 2011). What in one flora may be treated as a widespread species, may in another treatment be considered as a local endemic. A good example is the treatment of the Italian species of the *Centaurea* group (i.e., section *Phalolepis* and section *Centaurea*, formerly section *Acrolophus*): Fiori (1927) reported seven species and 55 subspecies; Dostál (1976) 16 species and 30 subspecies; Pignatti (1982) 25 species and 12 subspecies; Conti et al. (2005) 38 species and 30 subspecies, and finally Greuter (2008) numbered 47 species and 20 subspecies. The main reasons for these problems lay on the one hand in a fairly recent diversification (Hilpold et al. 2011) with species still being prone to undergo hybridization (Pisanu et al. 2011) and introgression (Ochsmann 2000; Hellwig 2004; Suárez-Santiago et al. 2007; Hilpold unpubl. data), and high morphological variability on a small geographical scale on the other hand.

In summer 2009 we discovered a population of *Centaurea* on the island of Procida (Fig. 1b), offshore of Naples, which did not fit to any *Centaurea* species described in the literature for the Italian mainland. Although the plants were quite similar to *Centaurea deusta*, which was also collected on the same island, there were two clear differences: the perennial, almost sub-shrubby habit and the white, sometimes yellowish or purplish, flowers. Initially, we suspected it to be a new species and designated it provisionally as "*Centaurea prochytae* spec. nov.". However, after a more detailed study of the literature, we noted a high morphological similarity to *Centaurea corensis* from Sardinia (Fig. 1b), located more than 450 km away. Thus, it is unclear whether the two populations are really conspecific, which implies a remarkable and unlikely disjunction, or whether the morphological similarity of the two populations is merely a product of convergence.

In this study, we therefore aim to resolve the identity of the Procida population using an integrative taxonomic approach involving (1) a morphometric comparison of material from Sardinia and Procida Island, (2) a karyological study, and (3) a comparison of the nuclear internal transcribed spacer region (ITS).

Materials and Methods

Species and area of study

Centaurea corensis Vals. & Filigheddu (Fig. 2b) is a perennial herb, woody at base (chamaephyte), 80–100 cm tall. Leaves, mainly disposed in a basal rosette, are hairy and glandular. Capitula are numerous in a single plant, on average 10–12 mm in length and 5–6 mm in width. Flowers are white to pale pink, sterile ones 30 mm in length and fertile ones 20 mm in length. Achenes are oblong, 3.5–4 mm in length. The pappus is short and scarious. It flowers from May to September-October.

It is present at only one site (40°41'49"N-8°35'11"E) in the municipality of Ossi (province of Sassari), Sardinia, where it covers an area of 0.5 hectares with roughly 5,000 adult individuals (Valsecchi and

Filigheddu 1991; Filigheddu et al. 2010). On the small island of Procida also only one population (40°45'42''N-14°2'7''E) occurs. It covers large parts of the easternmost tip of the island with some hundred individuals. The habitat is a disturbed coastal garrigue and roadsides on tuff.

Chromosome counts

We used the squash technique on somatic metaphases of root meristems from germinating seeds collected in the wild. After pretreating them with 0.002 M 8-hydroxyquinoline at 4°C for 8 h, the material was fixed with Carnoy at low temperatures for 24 h. Afterwards it was hydrolysed with 5N HCl at room temperature for 1 h. We stained the material with 1% acetic orcein at room temperature for more than 2 hours and mounted the root tips in 45% acetic acid. Five metaphase plates from different individuals were examined on an Olympus microscope U-TV1-X.

DNA isolation, amplification and sequencing

In order to prove the identity of the new population discovered at Procida, we carried out a comparison with DNA sequences of the *Centaurea* of the surrounding Central Italian mainland and Sardinia. We used plant material of 23 populations belonging to 17 described species (Table 1) and dried and stored it in silica. DNA Extraction, amplification and sequencing followed the procedures detailed in Garcia-Jacas et al. (2006), cloning procedures followed Vilatersana et al. (2007).

Phylogenetic analysis

The ITS sequences were joined in a data matrix. We aligned the sequences visually by Sequential pairwise comparison (Swofford and Olsen 1990) using BioEdit v.7.0.5.3 (Hall 1999). We calculated the Bayesian inference estimation using MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) under the GTR + G + I model of substitution as proposed by the software MrModeltest 2.2 (Nylander 2004). Indels were treated as missing data. Bayesian inference analyses were run with four MCMC chains and random starting trees for 5×10^6 generations, with trees sampled every 1,000 generations. The first 10^6 generations were identified and discarded as burnin. We considered internodes with posterior probabilities ≥ 95% as statistically significant.

Morphological comparison

Morphological analyses were conducted on individuals collected in the field in spring-summer 2010 at the Sardinian locality, and in 2009 at Procida. Vouchers of the specimens collected in Procida are preserved in herbaria BC, BOZ and SS. Furthermore, in order to observe the life cycle of the new population, we cultivated 15 plants at the Botanical Garden of Barcelona from wild-collected seeds.

Table 1 List of included species in the Bayesian analysis. The newly detected *Centaurea* population is given in bold

Taxon	Locality, date and collection number	Accession
Centaurea aeolica subsp. pandataria (Fiori & Bég.) Anzal.	Italy, Latina: Ventotene Island, *Hilpold V-1113* (BC).	
Centaurea aplolepa Moretti subsp. *aplolepa*	Italy, Savona: Spotorno, *Vilatersana 1265 et al.* (BC).	
Centaurea cineraria L. subsp. *cineraria* Gaeta	Italy, Latina: Gaeta, Torre Capovento, *Vilatersana 1107 et al.* (BC).	
Centaurea cineraria L. subsp. *cineraria* Amalfi	Italy, Salerno: Sorrentine peninsula, Minori, *Vilatersana 1118 et al.* (BC).	
Centaurea cineraria subsp. *circae* (Sommier) Cela Renz. & Viegi 38	Italy, Latina: S. Felice Circeo, *Vilatersana 1104 et al.* (BC).	
***Centaurea corensis* Vals. & Filigh.**	**Italy, Napoli: Isola di Procida, Terra Murata, 0.1 km N Church San Michele. 60 m. 40°45'42" N, 14°2'7" E, disturbed coastal garrigue and waysides on tuff, *Hilpold 20094006 & Granitto*, 26.7.2009 (BC, BOZ).**	
Centaurea corensis Vals. & Filigh.	Italy, Sardinia: Scala di Giocca, *Filigheddu & al. s.n.,* 26.11.2008 (BC).	
Centaurea crithmifolia Vis.	Croatia, Split-Dalmatia: Jabuka Island, *Boršić 33* (ZA).	
Centaurea deusta Ten.	Italy, Napoli: Procida Island, *Hilpold 20094007 et al.* (BC).	
Centaurea deusta Ten. Caserta	Italy, Caserta: San Gregorio Matese, *Hilpold 20094008 et al.* (BC).	
Centaurea deusta Ten. Verona	Italy, Verona: Monte Pastello, *Bertolli & Prosser s.n.*, 20.5.2010 (BC).	
Centaurea ferulacea Martelli	Italy, Sardinia: Baunei, *Mameli & Pisanu s.n.*, 12.10.2007 (SS).	
Centaurea filiformis Viv.	Italy, Sardinia: Monte Maccione, Oliena, *Pisanu s.n.*, 28.5.2007 (SS).	
Centaurea horrida Badarò st	Italy, Sardinia: Valle della Luna, Stintino Peninsula, *Pisanu s.n.*, 8.5.2007 (SS).	
Centaurea ionica Brullo	Italy, Reggio Calabria: Pazzano, *Vilatersana 1191 et al.* (BC).	
Centaurea magistrorum Arrigoni & Camarda	Italy, Sardinia: Villagrande, Monte Luas, *Camarda et al. s.n.*, 28.7.1995 (Herbarium Camarda).	
Centaurea pentadactyli Brullo & al.	Italy, Reggio Calabria: Pentidattilo, *Vilatersana 1183 et al.* (BC).	
Centaurea poeltiana Puntillo	Italy, Reggio Calabria: Montalto, *Vilatersana 1184 et al.* (BC).	
Centaurea scillae Brullo	Italy, Reggio Calabria: Bagnara, *Vilatersana 1140 et al.* (BC).	
Centaurea tenoreana Willk.	Italy, Chieti: Majella, Rifugio Puntilio, *Garcia-Jacas et al. V-1221* (BC).	
Centaurea tenorei Lacaita	Italy, Salerno: Capo d'Orso, *Vilatersana 1116 et al.* (BC).	

We measured the traits that are considered diagnostic at the species level (Ertuğrul et al. 2004): capitulum length (CL), capitulum width (CW), medium appendage length (AL), medium appendages width (AW), fimbrium width (FW), and spine length (SL). We analysed 40 capitula from 10 Sardinian individuals (hereafter called: S), 23 capitula from 6 specimens collected at Procida (hereafter: P), and 22 capitula from 6 individuals cultivated at the Botanical Garden of Barcelona from seeds collected in the wild at Procida (Pc). Thus a total of 85 capitula were measured for this study.

Morphometric data (CL, CW, AL, AW, FW and SL) were analysed using multivariate techniques with the PRIMER software package (Plymouth Marine Laboratory, UK; Clarke and Warwick 1994). Data were not transformed. A Bray-Curtis similarity matrix was used to generate a two-dimensional ordination plot applying non-metric multidimensional scaling (nMDS) (Clarke 1993). The similarity percentages procedure (SIMPER; Clarke 1993) was employed to determine similarities within populations and dissimilarities among populations, and to identify the major morphological traits contributing to the differences among the populations.

Results

Karyological results

All the examined achenes showed a tetraploid chromosome number of $2n = 36$, based on $x = 9$. This differs from the chromosome number of most of the other Italian members of the *Centaurea* group which are diploid with 2*n* = 18 (Cela Renzoni and Viegi 1982; Tornadore and Marcucci 1988; Arrigoni and Mori 1972; Arrigoni et al. 1980; Pisanu et al. 2011).

Molecular results

Several ribotypes per individual were found after cloning individuals from Procida and Sardinia. Of these clones three clearly different copies of both populations were included into Bayesian analysis. Figure 1A shows an unrooted tree resulting from the Bayesian analysis of the ITS sequence data.

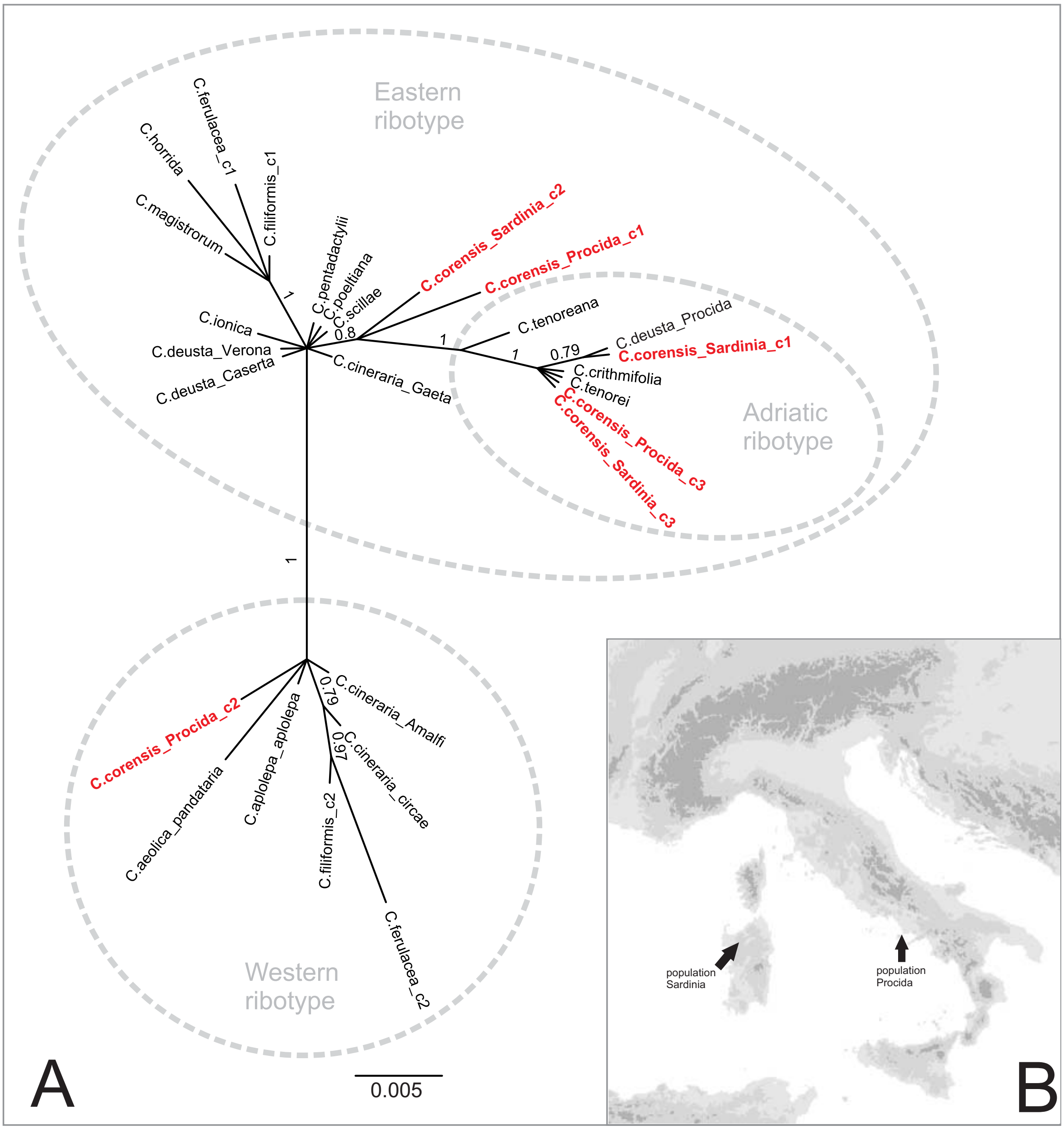

Fig. 1 Unrooted consensus tree of the ITS from Bayesian analysis. Support values on branches are Bayesian analysis posterior probabilities. Taxon names of *C. corensis* are shown in red. B, Geographic distribution of the two *C. corensis* populations

Two of the three copies of the two populations are closely related. One of these ribotypes is otherwise also found in *C. deusta* from Procida, *C. tenorei* from the Sorrentine peninsula south of Naples, in *C. tenoreana* from the central Italian Apennines and in several members of the *Centaurea* group from the Adriatic (only *C. crithmifolia* is shown in the tree). This ribotype is named the Adriatic ribotype in Hilpold (unpubl. data.) It has never been detected so far in any other member of the *Centaurea* group from Sardinia.

The second ribotype found in both populations is closer related to a ribotype frequently found in central Italy, which is named as *Acrolophus-Phalolepis* ribotype in Suárez-Santiago et al. (2007), hereafter called Eastern ribotype. A third ribotype, only found in the Procida population, belongs to a group of ribotypes distributed in the Western Mediterranean, called as *Willkommia*-ribotype in Suárez-Santiago et al. (2007), hereafter simply called Western ribotype.

Morphological comparison

The average values of the considered traits are shown in Table 2: the two populations S and P differed primarily in CW (24.2% higher in P) and SL (38.5% higher in P), whereas the other measures were quite similar (CL 4.1% higher in S). The cultivated plants (Pc) had higher morphometric scores than the wild plants (both S and P): in particular the wild individuals from P showed intermediate measures between S and Pc, except for SL which was intermediate in Pc.

Table 2 Morphometric characters used in multivariate analyses and their values expressed in mm (± SE)

	CL	CW	AL	AW	FW	SL
SAR (n=40)	13.92±0.16	9.59±0.13	4.94±0.05	4.94±0.06	1.67±0.04	0.59±0.02
PRO (n=23)	13.35±0.30	12.65±0.34	4.25±0.12	5.37±0.18	1.75±0.45	0.96±0.07
PRO_CU (n=22)	15.16±0.38	14.29±0.47	5.24±0.13	7.01±0.18	2.42±0.09	0.76±0.10

The nMDS plot of morphological traits did not show a clear-cut separation of the different populations (Fig. 2b). The SIMPER procedure showed similarity within P at 92.32%, similarity within Pc at 91.98%, and similarity within S at 95.33%. SIMPER also identified certain morphological traits as major contributors to the dissimilarities observed between different populations (Table 3): the trait mostly contributing to the dissimilarity among the different populations was CW in all cases. Surprisingly, dissimilarity between P and Pc was higher (10.47%) than dissimilarity between P and S (9.36%); the highest dissimilarity was between Pc and S (12.78%).

The plants grown at the Botanical Garden of Barcelona were all perennial. They grew very fast from the beginning and flowered abundantly in the second year. After flowering they developed sterile basal rosettes, which allowed persistence into the third year.

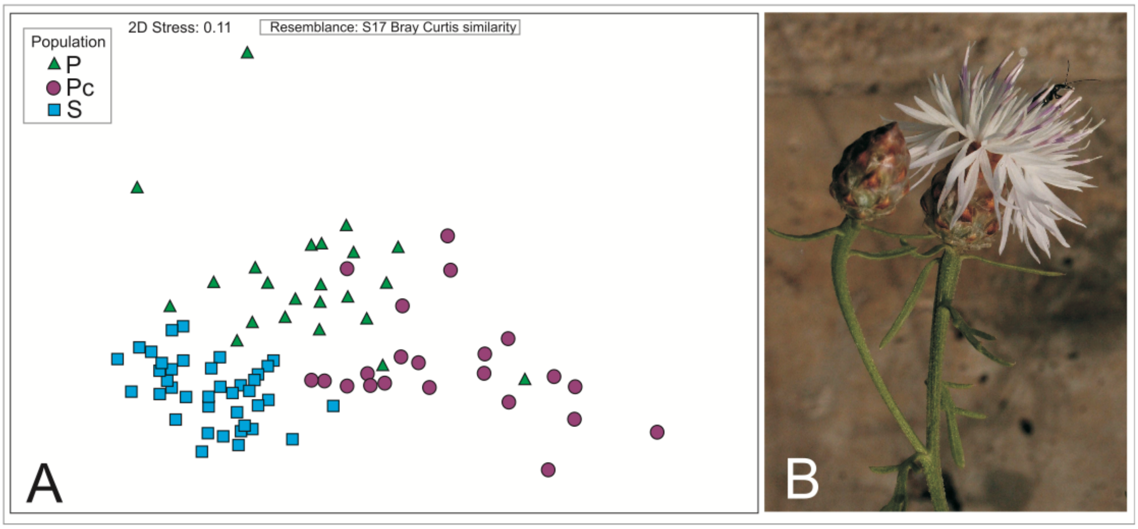

Fig. 2 Two-dimensional non-metric multidimensional scaling ordination (nMDS) of individual replicates (capitula) comparing morphological characters among three populations (P: specimens collected at Procida; Pc: specimens cultivated at the Botanical Garden of Barcelona from seeds collected at Procida; S: specimens collected in Sardinia). B, Photo of *Centaurea corensis*, Botanical Garden of Barcelona, plants grown from seed material collected in Procida (photo: A. Hilpold)

Table 3 Major morphological characters contributing (%) to dissimilarity between populations (P: Procida; Pc: specimens cultivated at the Botanical Garden of Barcelona from seeds collected at Procida; S: Sardinia) according to SIMPER analysis. Cut off for low contributions: 90.00%

Groups P & Pc
Average dissimilarity = 10.47%

Character	Av. Abund. P	Av. Abund. Pc	Av. Diss.	Contrib. %
CW	12.65	14.29	2.99	28.58
CL	13.35	15.16	2.58	24.69
AW	5.37	7.01	2.09	19.93
AL	4.25	5.25	1.33	12.71
FW	1.75	2.42	0.88	8.43

Groups P & S
Average dissimilarity = 9.36%

Species	Av. Abund. P	Av. Abund. S	Av. Diss.	Contrib. %
CW	12.65	9.59	4.15	44.34
CL	13.35	13.92	1.84	19.61
AW	5.37	4.94	1.13	12.10
AL	4.25	4.95	1.08	11.54
SL	0.96	0.59	0.61	6.48

Groups Pc & S
Average dissimilarity = 12.78%

Species	Av. Abund. Pc	Av. Abund. S	Av. Diss.	Contrib. %
CW	14.29	9.59	5.78	45.21
AW	7.01	4.94	2.57	20.11
CL	15.16	13.92	2.17	16.99
FW	2.42	1.67	0.93	7.27
AL	5.25	4.95	0.76	5.91

Results in summary

High morphological similarity, identical tetraploid chromosome numbers and similarity in ITS sequences suggest that the population from Procida should be considered conspecific with *C. corensis*, as described by Valsecchi and Filigheddu (1991).

Discussion

New descriptions in the genus *Centaurea* from Italy are not rare. Several new species have been described during the last 20 years (e.g. Puntillo 1996; Brullo et al. 2001; Arrigoni and Camarda 2003; Raimondo and Bancheva 2004; Raimondo et al. 2004; Guarino and Rampone 2006; Raimondo and Spadaro 2006, 2008). In most of these cases morphologically somewhat distinct populations or groups of populations were upgraded to species rank, following a strict phenetic species concept (Michener 1970). However, the distinction of these narrow endemic species to neighboring populations is usually not sharp, and the evidence that these species belong to an independently evolving metapopulation lineage was rather poor.

The main difference of the description of *Centaurea corensis* from Sardinia was, that its morphologically most similar populations are far away. The morphological traits and the base chromosome number $x = 9$ place *C. corensis* in sect. *Centaurea* s.l. This section is represented in Sardinia by four more endemic species (*C. horrida*, *C. filiformis*, *C. ferulacea,* and *C. magistrorum*), but these are morphologically different from *C. corensis*. From a morphological point of view, *C. corensis* is reminescent of the *C. deusta*-group from mainland Italy. Taxa from this group differ however from *C. corensis* in their biennial life cycle (perennial in *C. corensis*), the purple flowers (dirty white in *C. corensis*), and the diploid chromosome number $2n = 18$ (*C. corensis* is tetraploid, $2n = 36$). Presumably the *C. deusta* complex contributed at least one of the two genomes to the tetraploid *C. corensis*.

Hybridization has been recognized to play a significant role in *Centaurea*, with several taxa of putatively hybrid origin (Garcia-Jacas 1998; Pisanu et al. 2011). The fact that *C. corensis* shows distinct ribotypes within the same individual (Fig. 1a) strongly points towards allopolyploidization for explaining the origin of the species.

From a biogeographic perspective, it is surprising that *C. corensis* occurs extremely disjunctly on Sardinia and on the Island Procida next to Naples, more than 450 km from each other (Fig. 1b). The degree of floristic research in Italy over the last centuries is fairly high, and it is a good question why the populations were not described earlier. A possible explanation might be that *C. corensis* could be a species of recent origin (cf. Soltis and Soltis 2009), which is expanding its range. Closest relatives are found in Central Italy as suggested by molecular data and morphology. The site of origin of the species may therefore be indeed the area around Naples. The dispersal to Sardinia could have happened with human help as the rather disturbed habitat of the Sardinian population suggests: it grows at a cement dump near a roadside. Also in Procida, the habitat where the population occurs is a disturbed wasteground in connection with an old military complex. The success of the species in such disturbed places is best explained by its ecology. The seedlings brought up in the greenhouse showed an extremely fast growth compared to closely related *Centaurea* species, especially in the first months. They became quickly robust, highly competitive perennial herbs. These characters are shared with many plant species of ruderal places and weeds, many of them also originated by polyploidization. By example, diploid *Centaurea stoebe* is a non-colonizing biennial, while the allotetraploid cytotype is a highly invasive perennial (Mráz et al. 2012). It is believed that polyploidy gives these species the robustness and competitiveness to settle in such difficult conditions (Stebbins 1985). Another explanation for the observed, strong performance would be hybrid vigor (Shull 1914). In this sense, *C. corensis* might be a valuable study object for future investigation.

The findings shown in this investigation have important implications not only for the comprehension of the polyploid speciation in plants, but also for the field of biodiversity conservation. The discrimination between closely related species, and the clarification of a species' range, are crucial to evaluate the extinction risk of a given species and to plan conservation measures. *Centaurea corensis* is a good example on how the finding of new populations can have consequences on the species' conservation status. This plant was believed until now to be an exclusive Sardinian narrow endemic, and was indeed classified among the first 10 endangered Sardinian plants (Bacchetta et al. 2012), with a CR IUCN global status (Filigheddu et al. 2010). This new discovery on Procida not only expands its Extent of Occurrence (EOO) and doubles its Area of Occupancy (AOO), but will also change the local responsibility (sensu Gauthier et al. 2010) between two different Italian municipalities (Ossi and Procida) and two different administrative Italian regions (Sardinia and Campania). These data will probably lower the extinction risk of *C. corensis*, allowing us to invoke a new assessment of its conservation status. Most importantly, this case of study suggests caution before describing new plant species with extremely narrow ranges, and encourages an integrated morphological-molecular approach to detect species relationships and resolve cases of taxonomic ambiguity.

Acknowledgements

Thanks go to the Spanish Ministry of Science and Innovation (Projects CGL2007-60781/BOS and CGL2010-18631) and the Generalitat de Catalunya (Ajuts a Grups de Recerca Consolidats 2009/SGR/00439) for financial support. A. Hilpold benefited from a predoctoral grant of the JAE program of the CSIC. This study was supported also by the Regione Autonoma della Sardegna, LR 7/2007 – PO Sardegna FSE 2007–2013, with the grant nr. CRP2_474 for EF.

Special thanks go to R. Granitto, Caserta, for introducing the first author to the Island of Procida, rendering possible the discovery of the new *Centaurea* population and subsequently the entire work here presented. The authors are grateful to G. Becca for chromosome counts from Sardinian material and to S. Pisanu for helping in multivariate analysis. R. Filigheddu, T. Marcussen, A. Sánchez-Meseguer and A. Susanna made useful comments on the manuscript. I. Boršić, G. Mameli, S. Pisanu, F. Prosser, and R. Vilatersana are acknowledged for their help in collecting specimens.

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Appendix 1

Species list of the *Centaurea* group with chromosome numbers, molecular evidence, literature, number of subspecies and geographic distribution.

This list will be part of a taxonomic article about the *Centaurea* group, that will be sent to the journal Anales del Jardín Botánico de Madrid.

[*Llista d'espècies del grup* Centaurea*, amb informacions geogràfiques, nombres cromosòmics, evidència molecular i literatura.*
Aquesta llista s' inclourà en un article taxonòmic del grup Centaurea, *que s'enviarà a la revista Anales del Jardín Botánico de Madrid*.]

[*Lista de especies del grupo* Centaurea *con informaciones geográficas, números cromosómicos, evidencia molecular i literatura.*
Esta lista se incluirá en un artículo taxonómico del grupo Centaurea *que se enviará a la revista Anales del Jardín Botánico de Madrid*.]

Species list of the *Centaurea* group of the genus *Centaurea*

Working title for the planned article:

Nomenclatural notes on *Centaurea*: A proposal of classification and a species list of the Central Mediterranean Clade (CMC) of the genus Centaurea

Our current list centers in the CMC, including al species of the *Centaurea* group, the *Ammocyanus* group, the *C. hierapolitana* group and the two isolated species *C. akamantis* and *C. benedicta*, formerly *Cnicus benedictus*. Only recent literature is incorporated. Taxonomy follows Euro+Med Plantbase (in the following document only given as Euro+Med; Greuter, 2008), which is the most complete listing of the genus *Centaurea*. For areas not included in Euro+Med, local floras were used. Cases where our treatment diverges from that of Euro+Med are specially mentioned. Synonyms are only given if the name in Euro+Med diverges from the name used in this list. The second column gives information about the chromosome number. In this list we give maximally two citations per chromosome number, further information can frequently be found in the Index to plant chromosome numbers (Goldblatt & Johnson, 1979–). The third column of the tables shows the assignation to any of the three traditional sections *Willkommia* (W), *Phalolepis* (P) and *Centaurea* (C, formerly sect. *Acrolophus*). If two sections are mentioned, it means that there is uncertainty about the sectional membership. The fourth column shows if there exists evidence from the ITS that the taxon belongs to the respective systematic group. The information derives, if not otherwise mentioned, from the studies of Ochsmann (2000), Garcia-Jacas et al. (2006), Suárez-Santiago et al. (2007) and Hilpold et al. (in prep.). The last two columns give information about the existence of subspecies (only those accepted in Euro+Med) and the approximate distribution of the taxon.

1.

Centaurea group

The species list for the *Centaurea* group (i.e., sections *Centaurea*, *Phalolepis* and *Willkommia*) is split into eight smaller tables divided after their geographic distribution (Fig. 1). Species, that occur in two of these areas are treated only in one table with a reference in the other tables.

Figure 1: Map with the geographic division used for the eight species tables of the *Centaurea* group. 1. Iberian Peninsula; 2. N Africa; 3. Italy and C Europe; 4. Balkans, with subdivision into Greece, NW and NE Balkans; 5. N Black Sea; 6. Anatolian Peninsula; 7. Caucasus; 8. Near-East and Iran.

1.1. – Iberian Peninsula

For *Flora Iberica* (Castroviejo 1986–) a complete revision of the genus *Centaurea* is under way. Systematic treatment followed for the *C. paniculata* and the *C. alba* groups preparatory work for *Flora Iberica*: López & Devesa (2008abc, 2010, 2011) and López et al. (2011), the one used for the taxa traditionally subsumed as sect. *Willkommia* is from Euro+Med. Identification keys can be found in López & Devesa (2010; *C. paniculata* complex & *C. diffusa*), López & Devesa (2011; *C. alba* complex) and Blanca López (1981a) for members of sect. *Willkommia*. Whether *C. paniculata* and *C. leucophaea* from the Iberian Peninsula are really next-related or even homonymous to populations from SE France and NE Italy is an open question. The same holds true for *Centaurea boissieri*, *C. resupinata* and *C. monticola*, all listed for the Iberian Peninsula and NW Africa.

Table 1: Species list of the *Centaurea* group from the Iberian Peninsula.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. alba L.	18 (López & Devesa, 2008d; López & Devesa, 2011)	P	+	López & Devesa, 2011	3	Iberian Peninsula
C. aristata Hoffmanns. & Link	36 (López & Devesa, 2008d)	C	+	López & Devesa, 2008a		Iberian Peninsula
C. avilae Pau	18 (Blanca López, 1980a)	W(P)	+			Iberian Peninsula
C. bethurica E. López & Devesa	18 (López & Devesa, 2008d)	C	+	López & Devesa, 2008c		Iberian Peninsula
C. boissieri DC.	18 (Blanca López, 1980a, 1981b)	W	+		7	Iberian Peninsula, NW-Africa
C. bombycina DC.	18 + 2B (Blanca López, 1981b)	W	+		2	Iberian Peninsula
C. carratracensis Lange	18+ 0–1B (Blanca López, 1980a)	W	+			Iberian Peninsula
C. castellanoides Talavera	18 (López & Devesa, 2008d)	C	+	López & Devesa, 2008a	3	Iberian Peninsula
C. citricolor Font Quer	36 + 1B (Blanca López, 1980a)	W	+			Iberian Peninsula
C. cordubensis Font Quer	36 (López & Devesa, 2008d)	C	+	López & Devesa 2008c		Iberian Peninsula
C. costae Willk. (*C. alba* p.p.)	18 (López & Devesa, 2008d)	P	+	López & Devesa, 2011		Iberian Peninsula
C. diffusa Lam.	See Balkans					
C. gadorensis Blanca	18 (Blanca López, 1981b under *C. sagredoi* subsp. *tenuiloba*))	W	+			Iberian Peninsula
C. hanrii Jord.	18 (López & Devesa, 2008d)	C	+	López & Devesa, 2010		NW-Mediterranean (Spain, France)
C. jaennensis Degen & Debeaux	18 (Blanca López, 1980a)	W	+			Iberian Peninsula
C. langei Nyman	18 (López & Devesa, 2008d; López et al., 2011)	C	+	López et al., 2011	6	Iberian Peninsula
C. leucophaea Jord.	See Italy					
C. limbata Hoffmanns. & Link	18 (López & Devesa, 2008d)	C	+	López & Devesa, 2008b	2	Iberian Peninsula
C. monticola DC.	18, 19, 20, 18+1B (Blanca López, 1980a; Blanca, 1983)	W(C)	+			Iberian Peninsula, NW Africa
C. paniculata L.	See Italy					
C. paui Willk.	18 (Blanca López, 1980a)	W	+			Iberian Peninsula

C. pauneroi Talavera & J. M. Muñoz	-	W	+	Talavera & Muñoz, 1984		Iberian Peninsula
C. pinae Pau	18 (Blanca López, 1981b)	W	+			Iberian Peninsula
C. pinnata Vicioso	18 (Blanca López, 1980a)	W	+			Iberian Peninsula
C. pulvinata (Blanca) Blanca	18 (Blanca López, 1980a)	W	+	Blanca López, 1980b		Iberian Peninsula
C. resupinata Coss.	18 (Blanca López, 1981b under *C. dufourii*)	W	+		9	Iberian Peninsula, NW-Africa
C. rouyi Coincy	18 (Blanca López, 1981b)	W	+	Blanca López, 1981a; Figuerola et al., 1991		Iberian Peninsula
C. sagredoi Blanca	18 (Blanca López, 1980a)	W	+	Blanca López, 1980b		Iberian Peninsula
C. schousboei Lange	36 (López & Deves, 2008d)	C/P	+	López & Devesa 2008c		Iberian Peninsula
C. segariensis Figuerola et al.	18 (Boscaiu et al., 1997)	W	+ *			Iberian Peninsula

*** in Suárez-Santiago et al. (2007) under *C. rouyi* var. *suffrutescens*.**

1.2 – N-Africa

Most members of *Centaurea* group occur in the NW of the continent, namely in various ranges of the Atlas. Only *C. cyrenaica* occurs in the NE of the continent. No recent complete treatments of the group are available, only the revision of section *Willkommia* from NW Africa by Breitwieser & Podlech (1986).

Table 2: Species list of the *Centaurea* group from Northern Africa.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. boissieri	See Iberian Peninsula					
C. cyrenaica Bég. & A. Vacc.	18 (Brullo & Pavone, 1977)	P/W	-	Alavi, 1983 (with illustration)		Libya
C. debdouensis Breitw. & Podlech	-	W(P)	+			NW Africa
C. delicatula Breitw. & Podlech	-	W	+	Hilpold et al., 2011		NW Africa
C. djebel-amouri Greuter	-	P	-	Quézel & Santa, 1963 (under *C. alba* var. *mauritanica*)		NW Africa
C. monticola	See Iberian Peninsula					
C. olivieri Pomel (*C. resupinata* s.l.)	-	W	-			NW Africa
C. papposa (Coss.) Greuter	-	C	+	Quézel & Santa, 1963 and Pottier-Alapetite, 1981 (under *C. cineraria*); Hilpold et al., 2011		NW Africa
C. pomeliana Batt.	-	W(P)	+		2	NW Africa
C. resupinata	See Iberian Peninsula					
C. tougourensis Boiss. & Reut.	*x* = 9 (Benamara et al., 2010)	P	+			NW Africa
C. vesceritensis Boiss. (*C. resupinata* s.l.)	-	W	.			NW Africa

1.3 – Italy, France and central Europe

All Italian *Centaurea* species are listed in Conti et al. (2005) and in Network Nazionale della Biodiversità (2012 ongoing) but are not treated in detail. Complete treatments are given in Pignatti (1982) and in Fiori (1927). Treatments for single groups: Arrigoni (2003; *C. paniculata* complex), Cela Renzoni & Viegi (1982; *C. cineraria* complex); Guarino & Rampone (2006; *C. dissectae* group). Many new descriptions have been published over the last 30 years. In the meantime *C. subtilis* has been removed (Hilpold et al., 2009). Here we follow the very tight treatment of Euro+Med.

Table 2: Species list of the *Centaurea* group from Italy, France and Central Europe.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. aeolica Lojac.	18 (Viegi et al., 1972; Cela Renzoni & Viegi, 1982)	C	+	Cela-Renzoni & Viegi, 1982; Anzalone, 1995; Hilpold et al., 2011	2	Italy
C. aetaliae (Sommier) Bég.	18 (Viegi & Renzoni, 1976)	C	+			Italy
C. ambigua Guss.	-	C	+	Guarino & Rampone, 2006; Hilpold et al., 2011		Italy
C. aplolepa Moretti	18 (Viegi & Renzoni, 1976; Arrigoni et al., 1980)	C	+	Arrigoni, 2003; Hilpold et al., 2011	10	Italy
C. arrigonii Greuter	18 (Signorini et al., 2001, under *C. dissecta* var. *intermedia*)	C	+			Italy
C. aspromontana Brullo & al. (*C. deusta* s.l.)	-	P	+	Brullo et al., 2001		Italy
C. brulla Greuter (*C. deusta* s.l.)	-	P	+	Brullo, 1988		Italy
C. busambarensis Guss.	18 (Viegi et al., 1972, Cela-Renzoni & Viegi, 1982;)	C	+	Cela-Renzoni & Viegi, 1982; Hilpold et al., 2011		Italy
C. cineraria L.	18 (Cela-Renzoni & Viegi, 1982); 36 (Damboldt & Matthäs, 1975)	C	+	Cela-Renzoni & Viegi, 1982; Hilpold et al., 2011		Italy
C. corensis Vals. & Filigh.	36 (Hilpold et al., in prep.)	P	+	Valsecchi & Filigheddu, 1991		Italy
C. corymbosa Pourr.	-	C	+			France
C. cristata Bartl. (*C. spinosociliata* aggr.)	36 (Lausi, 1966; Ochsmann, 1999; pers. obs. A. Hilpold)	C	+			Italy, W Balkan
C. delucae C. Guarino & Rampone	18 (Baltisberger, 1990; under *C. ambigua* subsp. *nigra*)	C	+	Guarino & Rampone, 2006		Italy
C. deusta Ten.	18 + 0–1B (Matthäs, 1976; Brullo et al., 1991)	P	+	Hilpold et al., 2011		Italy
C. diffusa Lam.	See Balkans					
C. diomedea Gasp.	18 (D'Amato & Pavesi, 1990; pers. obs. A. Hilpold)	P	+	Hilpold et al., 2011		Italy
C. erycina Raimondo & Bancheva	18 (Raimondo & Bancheva, 2004)	C	+	Raimondo & Bancheva, 2004; Hilpold et al., 2011		Italy
C. filiformis Viv.	18 (both subsp.; Arrigoni & Mori, 1971)	P/C	+	Arrigoni et al., 1972	2	Italy
C. giardinae Raimondo & Spadaro	18 (Raimondo & Spadaro, 2006)	C	+	Raimondo & Spadaro, 2006; Bancheva et al., 2011		Italy
C. gymnocarpa Moris & De Not.	18 (Cela Renzoni & Viegi, 1972)	C	+	Cela-Renzoni & Viegi, 1982; Hilpold et al., 2011		Italy
C. horrida Badarò	18 (Desole, 1954)	C	+			Italy
C. ilvensis (Sommier) Arrigoni (*C. aetaliae* s.l.)	18 (Viegi & Cela Renzoni, 1976)	C	+			Italy
C. ionica Brullo (*C. deusta* s.l.)	-	P	+	Brullo et al., 2001		Italy
	18 (Tornadore et al., 2000)	P	+	Francini, 1951; Tornadore et al., 2000		Italy
C. japygica (Lacaita) Brullo						
C. kartschiana Scop.	18 (Lausi, 1966; Siljak-Yakovlev, 1982)	C	-	Lovrić, 1971; Marcucci et al., 1999	6	Italy, W Balkan

C. leucadea Lacaita	18 (Tornadore et al., 2000)	C	+	Francini, 1951; Tornadore et al., 2000; Hilpold et al., 2011		Italy
C. leucophaea Jord.	18 (Ochsmann, 1999; López & Devesa, 2008d)	C	+	Arrigoni, 2003; López & Devesa, 2010; Hilpold et al., 2011	6	NW Mediterranean (Spain, France, Italy)
C. litigiosa (Fiori) Arrigoni (*C. aetaliae* s.l.)	-	C	+			Italy
C. magistrorum Arrigoni & Camarda	-	C	+	Arrigoni & Camarda, 2003		Italy
C. nobilis (H. Groves) Brullo	18 (Brullo et al., 1991; Tornadore et al., 2000)	P	+	Tornadore et al., 2000		Italy
C. paniculata L.	18 (López & Devesa, 2008d)	C	+	Arrigoni, 2003; López & Devesa, 2010	5	NW Mediterranean (Spain, France, Italy)
C. panormitana Lojac.	18 (Viegi et al., 1972; Cela Renzoni & Viegi, 1982)	C	+	Hilpold et al., 2011		Italy
C. parlatoris Heldr.	18 (Colombo & Trapani, 1990)	C	+	Guarino & Rampone, 2006; Bancheva et al., 2011; Hilpold et al., 2011		Italy
C. pentadactyli Brullo & al. (*C. deusta* s.l.)	-	P	+	Brullo et al., 2001		Italy
C. pestalotii Ces.	18 (Ochsmann, 1999); 36 (Lovrić, 1982, under *C. brachtii*)	C/P	+			Italy, W Balkans
C. poeltiana Puntillo (*C. deusta* s.l.)	-	P	+	Puntillo, 1996		Italy
C. saccensis Raimondo & al.	18 (Bancheva et al., 2006, secondary)	C	+	Raimondo et al., 2004		Italy
C. sarfattiana Brullo & al. (*C. deusta* s.l.)	-	P	-	Brullo et al., 2004		Italy
C. scannensis Anzal. & al.	18 (Anzalone, 1961)	C	+	Hilpold et al., 2011		Italy
C. scillae Brullo (*C. deusta* s.l.)	-	P	+	Brullo et al., 2001; Hilpold et al., 2011		Italy
C. sicana Raimondo & Spadaro	18 (Raimondo & Spadaro, 2008)	C	-	Raimondo & Spadaro, 2008; Bancheva et al., 2011		Italy
C. stoebe L.	18, 36 (Španiel et al., 2008)	C	+	Manifold, partly published under synonym *C. maculosa*	2	Europe, except Ib. Pen., introduced to N-America and parts of S America.
C. tenacissima (H. Groves) Brullo	18 (Brullo et al., 1991)	P	+			Italy
C. tenoreana Willk.	-	P	+			Italy
C. tenorei Lacaita	18, 36 (Peruzzi & Cesca, 2002)	C	+	Guarino & Rampone, 2006		Italy
C. tommasinii A. Kern. (*C. spinosociliata* aggr.)	36 (Lovrić, 1982a; Marcucci et al., 1999)	C	+	Marcucci et al., 1999		Italy, NW Balkans
C. valesiaca (DC.) Jord.	18 (Ochsmann, 1999)	C/P	+	Ochsmann, 2000		Switzerland, Italy
C. veneris (Sommier) Bég.	18 (Viegi et al., 1972; Cela Renzoni & Viegi, 1982)	C	+	Cela-Renzoni, 1970; Cela-Renzoni & Viegi, 1982; Hilpold et al., 2011		Italy

1.4 – Balkans (including Romania, Aegean Sea and Crete)

The most complete taxonomic treatments concentrating on the Balkan area are those of Boissier (1875) and Hayek (1931). Newer treatments of the entire area, except Flora Europaea, do not exist. In the NW Balkans, besides some widely distributed species, there are two main species groups: the *C. spinoso-ciliata* group and the *C. cuspidata* group. The latter one, though only distributed in a relatively small area, was split into several microspecies of doubtful rank. These two species groups are treated in Lovrić (1968). A treatment for Rumania is given in Ciocarlan (2000), for Greece in Halácsy, (1902), for the Greek Mountains in Gamal-Eldin & Wagenitz (1991), for the Aegean Sea in Rechinger (1943), for N Greece in Rechinger (1939). Listings of sect *Phalolepis* from Greece are given in Wagenitz (1971) and Georgiadis et al. (1996), of the Bulgarian members of genus *Centaurea* in Assyov et al., (2002) of the Croatian members in Flora Croatica Database (Nicolić, 2010).

Table 4: List of members of the *Centaurea* group from the Balkan and the Aegean Area. Distribution areas apply to: W Balkans = former Yugoslavia and Albania, NE Balkans = Bulgaria, Romania and European part of Turkey.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. acarnanica (Matthäs) Greuter	36 (Matthäs, 1976, under *C. subciliaris* subsp. *acarnanica*)	P	+	Matthäs, 1976		Greece
C. affinis Friv.	18, 36 (Georgiadis, 1983)	C	+		6	Balkans
C. albanica Halácsy	-	P	-	Phitos & Damboldt, 1971		Greece
C. arenaria Willd.	See N Black Sea region					
C. argentea L.	18 (Georgiadis, 1983, Montmollin, 1986)	C	+	Runemark, 1967, Turland & Chilton, 2000		Greece
C. attica Nyman	18 (Georgiadis & Phitos, 1976; Georgiadis, 1983); 36 (Georgiadis & Phitos, 1976)	C	-		3	Greece, W Balkans
C. besseriana DC. (*C. ovina* aggr.)	See N Black See region					
C. biokovensis Teyber (*C. cuspidata* aggr.)	36 (Lovrić, 1982a)	C	-			W Balkans
C. borysthenica Gruner (*C. arenaria* aggr.)	See N Black Sea					
C. bovina Velen.	18 (Georgiadis, 1983)	C	-			Greece, NE Balkans
C. brunnea (Halácsy) Halácsy	18 (Geogiadis & Phitos, 1977)	P	+			Greece, W Balkans
C. carystea Trigas & Constantin.	54 (Trigas et al., 2009)	C	-	Trigas et al., 2009		Greece
C. caliacrae Prodan	18 (Ciocarlan, 2000, secondary citation!?)	P	-			NE Balkans
C. chalcidicaea Hayek	18 (Damboldt & Melzheimer, 1974, Georgiadis, 1983)	C	-			Greece
C. chrysocephala Phitos & T. Georgiadis	18 (Geogiadis & Phitos, 1977)	P	+	Geogiadis & Phitos, 1977		Greece
C. cithaeronea Phitos & Constantin.	54 (Phitos & Constantinidis 1993)	P	+	Phitos & Constantinidis 1993		Greece
C. codruensis Prodan (*C. kanitziana* s.l.)	-	C	-			NE Balkans
C. coziensis Nyár. (*C. stoebe* s.l.)	-	C	-			NE Balkans
C. crithmifolia Vis.	36 (Siljak-Yakovlev, 1980)	C	+			W Balkans
C. cuneifolia Sm.	18 (Kuzmanov et al., 1979), 36 (Georgiadis & Phitos, 1976; Georgiadis, 1983)	C	+		2	Balkans, Anatolia
C. cuspidata Vis. (*C. cuspidata* aggr.)	18 (Lovrić, 1982a)	C	-			W Balkans

C. cylindrocephala Bornm. (*C. stoebe* s.l.)	36 (Georgiadis, 1983 under *C. biebersteinii* subsp. *cylindrocephala*)	C	-			Greece, W Balkans
C. derventana Vis. & Pančić (*C. cuspidata* aggr.)	18 (Strid & Franzen, 1981, under *C. incompta* subsp. *derventana*)	C	-			W Balkans
C. deustiformis Adamović	16 (Strid, 1983)	P	+	Phitos & Damboldt, 1971		Greece, W Balkans
C. diffusa Lam.	18 (Georgiadis & Phitos, 1976; Taylor & Taylor, 1977; Kuzmanov et al., 1979); 16 (Bancheva & Greilhuber, 2006)	C	+	López & Devesa 2010		Originally NE Mediterranean and Black Sea, introduced to C and W Europe, N America, Australia
C. edith-mariae Radić (*C. cuspidata* aggr.)	36 (Papes & Radić, 1982)	C	-			W Balkans
C. epapposa Velen.	-	P	-			N Balkans
C. euxina Velen.	-	P	-			NE Balkans
C. formanekii Halácsy	-	P	-			W Balkans
C. friderici Vis.	36 (Siljak-Yakovlev, 1980)	C	+		2 subsp.	W Balkans
C. galicicae Micevski	-	C	-	Micevski, 1985		W Balkans
C. glaberrima Tausch	36 (Siljak-Yakovlev, 1980; (Siljak-Yakovlev et al., 2005)	C	+		2	W Balkans
C. gloriosa Radić (*C. cuspidata* aggr.)	18 (Papes & Radić, 1982; Siljak-Yakovlev et al., 2005)	C	-			W Balkans
C. gracilenta Velen.	-	C	-			NE Balkans
C. greuteri E. Gamal-Eldin & Wagenitz	-	P	-	Gamal-Eldin & Wagenitz, 1983		Greece
C. grisebachii (Nyman) Heldr.	36 (Strid & Franzen, 1981; Georgiadis, 1983)	C	+		4	Greece, W Balkans
C. heldreichii Halácsy	18 (Phitos & Damboldt, 1971)	P	+	Kalpoutzakis & Constantinidis, 2004		Greece
C. huljakii J. Wagner	18, 36 (Damboldt & Melzheimer 1974, Matthäs 1976)	P	-			Greece
C. incompleta Halácsy	18 (Strid & Franzen, 1981)	C	+			Greece
C. incompta Vis. (*C. cuspidata* aggr.)	36 (Lovrić, 1982b)	C	-			W Balkans
C. inermis Velen.	See Anatolia					
C. ipecensis Rech. f.	-	P	-			W Balkans
C. jankana Simonk. (*C. arenaria* aggr.)	-	C	-			N Balkans
C. johnseniana Strid & Kit Tan	-	C	-	Strid & Tan (2003)		Greece
C. jurineifolia Boiss.	-	C	-			NE Balkans
C. kalambakensis Freyn & Sint.	18 (Georgiadis & Phitos, 1976)	C	-			Greece
C. kanitziana D. Brândză	-	C	-			NE Balkans
C. kilaea Boiss.	See Anatolia					
C. kusanii Radić (*C. cuspidata* aggr.)	36 + 1–5B (Papes & Radić, 1982; Siljak-Yakovlev et al., 2005)	C	-			W Balkans

C. lacerata (Hausskn.) Halácsy	36 (Georgiadis, 1983)	C	-		Greece
C. lactiflora Halácsy	18 (Georgiadis & Phitos, 1976)	C	-		Greece
C. laureotica Halácsy	36 (Georgiadis & Phitos, 1976; Georgiadis, 1981)	C	-		Greece
C. leonidia Kalpoutz. & Constantin.	18 (Kalpoutzakis & Constantinidis, 2004)	P	+	Kalpoutzakis & Constantinidis, 2004	Greece
C. leucomalla Bornm.	-	P	-		W Balkans
C. leucomelaena Hayek	-	C	-		W Balkans
C. litochorea T. Georgiadis & Phitos	16 (Strid & Franzen, 1981)	P	+	Georgiadis & Phitos, 1978	Greece
C. marmorea Bornm. & Soška	-	P	-		W Balkans
C. mayeri Radić (C. cuspidata aggr.)	36 (Papes & Radić, 1982; Siljak-Yakovlev et al., 2005)	C	-		W Balkans
C. messenicolasiana T. Georgiadis & al.	18 (Georgiadis et al., 1996)	P	+	Georgiadis et al., 1996	Greece
C. mucurensis Teyber (*C. cuspidata* aggr.)	36 (Lovrić, 1982a, under *C. biokovensis* subsp. *mucuriensis)*	C	-		W Balkans
C. musakii T. Georgiadis	18 (Georgiadis, 1979)	C	+	Georgiadis, 1979	Greece
C. musarum Boiss. & Orph.	18 (Constantinidis & Vassiliades, 1996)	P	+	Constantinidis & Vassiliades, 1996	Greece
C. niederi Heldr.	18 (Georgiadis & Phitos, 1976)	C	+	Kalpoutzakis & Constantinidis, 2004	Greece
C. orphanidea Boiss.	18 (Georgiadis & Phitos, 1976)	C	-		Greece
C. ossaea Halácsy	18 (Georgiadis, 1983)	C	-		Greece
C. pawlowskii Phitos & Damboldt	18 + 1B (Damboldt & Matthäs, 1975), 36 +1B (Ochsmann, 2000)	P	+	Damboldt & Matthäs 1979, Phitos & Damboldt, 1983	Greece, W Balkans
C. paxorum Phitos & T. Georgiadis	18 (Geogiadis & Phitos, 1977)	P	-	Geogiadis & Phitos, 1977	Greece
C. pelia DC.	18 (Georgiadis & Phitos, 1976; Georgiadis, 1983)	C	+		Greece
C. peucedanifolia Boiss. & Orph.	18 (Georgiadis & Phitos, 1976)	C	-		Greece
C. poculatoris Greuter	18 (Montmollin, 1986; Routsi & Georgiadis, 1988)	C	+	Greuter, 1967	Greece
C. polyclada DC	18 (Garcia-Jacas et al., 1997; Martın et al., 2009)	C	+	Uysal et al., 2010	Aegean Sea
C. prespana Rech. f.	-	C	-	Rechinger, 1974, Tan et al., 2007	Greece
C. princeps Boiss. & Heldr.	18 (Geogiadis & Phitos, 1977)	P	+		Greece
C. pseudobovina Hayek (*C. jurineifolia* s.l.)	-	C	-		NE Balkans
C. pseudocadmea Wagenitz	-	P	+	Wagenitz, 1971	Greece
C. ptarmicoides Halácsy	-	P	-	Phitos & Damboldt, 1983	Greece
C. radichii Plazibat (*C. cuspidata* aggr.)	27+1B, 36 (Papes & Radić, 1982; Siljak-Yakovlev et al., 2005, under *C. elegantissima*)	C	-		W Balkans
C. reichenbachii DC.	18 (Jasievicz & Mizienty, 1975, under *C. calvescens* DC.)	C	-		N Balkans
C. rufidula Bornm.	-	C	-		W Balkans
C. rutifolia SM.	18+0-1B (Damboldt & Melzheimer, 1974; Georgiadis, 1983; Kuzmanov et al., 1983)	C	-		S and NE Balkans
C. soskae Košanin	-	C	-		W Balkans
C. spinosa L.	36 (Runemark, 1967;	C	+	Runemark, 1967	Aegean Sea

	Georgiadis & Phitos, 1976)					
C. spinosociliata Seenus	36 (Lovrić, 1982a)	C	+			W Balkans
C. subciliaris Boiss. & Heldr.	18+ 2B, 36 + 4B (Phitos & Damboldt, 1971; Matthäs, 1976)	P	+	Matthäs, 1976		Greece, NE Balkans
C. subsericans Halácsy	18 (Constantinidis et al., 1997)	C	-			Greece
C. tauscheri A. Kern. (*C. arenaria* aggr.)	18 (Sz.-Borsos, 1971 under *C. arenaria* subsp. *pseudo-rhenana*)	C	-			N Balkans
C. thasia Hayek	18 (Georgiadis, 1981, under *C. ipsaria*)	C	-			Greece
C. thessala Hausskn.	36 (Georgiadis & Phitos, 1976; Georgiadis, 1983)	C	-		2	Greece
C. tomorosii Micevski	-	C	-	Micevski, 1985		W Balkans
C. triniifolia Heuff.	-	C	+			Balkans
C. tymphaea Hausskn.	18 (Georgiadis, 1983)	C	+		2	Balkans
C. vandasii Velen.	-	P	-			NE Balkans
C. varnensis Velen.	18 (Ochsmann 1999, under *Centaurea* × *psammogena*)	C	-			N Balkans, introduced elsewhere
C. vatevii Degen & al. (*C. stoebe* s.l.)	-	C	-			NE Balkans
C. vermia Rech. f.	-	C	-			Greece
C. visianii Radić (*C. cuspidata* aggr.)	18 (Siljak-Yakovlev et al., 2005); 36 + 6B (Papes & Radić, 1982)	C	-			W Balkans
C. wettsteinii Degen & Dörfl.	-	C	-			Greece
C. zuccariniana DC.	18 (Georgiadis & Phitos, 1976; Georgiadis, 1983)	C	+			Greece, W Balkans

1.5 – Region N of Black Sea (Russia p.p., Ukraine, Moldavia)

Most species in this area belong to five species groups: the *C. stoebe* complex, the *C. margaritacea* aggr., the *C. sterilis* aggr., the *C. ovina* aggr. and the *C. arenaria* aggr. Monophyly of these groups is not clear. The most complete treatment for Russia, Ukraine and Moldavia is given in Klokov et al. (1963).

Table 5: List of members of the *Centaurea* group from the area north of the Black Sea (Russia p.p., Ukraine, Moldavia).

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. aemulans Klokov (*C. diffusa* s.l.)	-	C	-			N Black Sea
C. appendicata Klokov (*C. margaritacea* aggr.)	-	P	-			N Black Sea
C. arenaria Willd.	32 (Bancheva & Greilhuber, 2006), 36 (Kuzmanov et al., 1979)	C	+			NE Balkans, N and E of Black Sea
C. besseriana DC. (*C. ovina* aggr.)	18 (Bancheva & Greilhuber, 2006, under C. *ovina* subsp. *besserana*)	C	-			NE Balkans, N Black Sea
C. borysthenica Gruner (*C. arenaria* aggr.)	-	C	-			N Balkans, N Black Sea
C. breviceps Iljin (*C. margaritacea* aggr.)	-	P	+			N Black Sea
C. caprina Steven (*C. ovina* aggr.)	-	C	-			N Black Sea
C. demetrii Dumbadze (*C. ovina* aggr.)	-	C	-			N Black Sea
C. diffusa	See Balkans					

C. donetzica Klokov (*C. margaritacea* aggr.)	36 (Romaschenko et al., 2004)	P	+	N Black Sea
C. dubjanskyi Iljin (*C. margaritacea* aggr.)	-	P	-	N Black Sea
C. gerberi Steven (*C. margaritacea* aggr.)	-	P	-	N Black Sea
C. konkae Klokov (*C. margaritacea* aggr.)	-	P	-	N Black Sea
C. kubanica Klokov (*C. stoebe* s.l.)	-	C	-	N Black Sea
C. lavrenkoana Klokov (*C. ovina* aggr.)	-	C	-	N Black Sea
C. *majorovii* Dumbadze (*C. arenaria* aggr.)	-	C	-	N Black Sea
C. margaritacea Ten. (*C. margaritacea* aggr.)	-	P	+	N Black Sea
C. margaritalba Klokov (*C. margaritacea* aggr.)	-	P	+	N Black Sea
C. *odessana* Prodan (*C. arenaria* aggr.)	-	C	-	N Black Sea
C. paczoskii Klokov (*C. margaritacea* aggr.)	-	P	+	N Black Sea
C. pineticola Iljin (*C. margaritacea* aggr.)	-	P	-	N Black Sea
C. protogerberi Klokov (*C. margaritacea* aggr.)	18 (Romaschenko et al., 2004)	P	+	N Black Sea
C. protomargaritacea Klokov (*C. margaritacea* aggr.)	-	P	+	N Black Sea
C. pseudoleucolepis Kleopow (*C. margaritacea* aggr.)	18 (Romaschenko et al., 2004)	P	+	N Black Sea
C. pseudomaculosa Dobrocz. (*C. stoebe* s.l.)	18 (Probatova et al., 1996)	C	+	N Black Sea
C. savranica Klokov (*C. stoebe* s.l.)	-	C	-	N Black Sea
C. sophiae Klokov (*C. arenaria* aggr.)	-	C	-	N Black Sea
C. steveniana Klokov (*C. ovina* aggr.)	-	C	-	N Black Sea
C. wolgensis DC. (*C. arenaria* aggr.)	-	C	-	N Black Sea
C. sarandinakiae N. B. Illar. (*C. sterilis* aggr.)	36 (Romaschenko et al., 2004)	P	+	N Black Sea
C. semijusta Juz. (*C. sterilis* aggr.)	36 (Romaschenko et al., 2004)	P	+	N Black Sea
C. sterilis Steven (*C. sterilis* aggr.)	18 (Romaschenko et al., 2004)	P	+	N Black Sea
C. vankovii Klokov (*C. sterilis* aggr.)	36 (Romaschenko et al., 2004)	P	+	N Black Sea

1.6 – Anatolia and Cyprus

Flora of Turkey (Wagenitz, 1975) gives nine species for section *Phalolepis* and 21 species for section *Centaurea* (=*Acrolophus*). In recent years, several new species have been described. Two species of *Phalolepis* (*C. hierapolitana* and *C. tossiensis*) do not belong to the *Centaurea* group. The same is true for *C. akamantis* from Cyprus – where only one species remains (*C. cyprensis* (Holub) T. Georgiadis cf. Meikle, 1985).

Table 6: List of members of the *Centaurea* group from Anatolia and Cyprus.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. aggregata DC.	18 (Bakhshi Khaniki, 1996)	C	+		2	Anatolia, Caucasus
C. amaena Boiss. & Balansa	18 (Uysal et al., 2009)	P	+			Anatolia
C. antalyensis H. Duman & A. Duran	$x = 9$ (Köse, 2006)	P	+	Duran & Duman, 2002		Anatolia
C. anthemifolia Hub.-Mor.	-	C	+			Anatolia
C. aphrodisea Boiss.	36 (Uysal et al., 2009)	P	+			Anatolia
C. austroanatolica Hub.-Mor.	-	C	+			Anatolia
C. aziziana Rech. f. (*C. ovina* aggr.)	See Caucasus					
C. cadmea Boiss.	18 (Uysal et al., 2009)	P	+		2	Anatolia
C. calolepis Boiss.	18 (Romaschenko et al., 2004)	C	+	Wagenitz, 1972		Anatolia
C. cariensiformis Hub.-Mor.		C	+	Davis et al., 1988		Anatolia
C. cariensis Boiss.	18 (Martın et al., 2009); 36 (Georgiadis & Christodoulakis, 1984; Martın et al., 2009)	C	+	Wagenitz, 1972	5	Anatolia
C. consanguinea DC.	-	C	+			Anatolia
C. cuneifolia Sm.	See Balkans					
C. cyprensis (Holub) T. Georgiadis	-	C	+	Meikle, 1985 (under *C. veneris*)		Cyprus
C. dichroa Boiss. & Heldr.	-	C	+			Anatolia
C. diffusa	See Balkans					
C. dursunbeyensis	36 (Uysal & Köse, 2009)	P	-	Uysal & Köse, 2009		Anatolia
C. ertugruliana Uysal	18 (Uysal, 2008)	C	+	Uysal, 2008		Anatolia
C. gulissashvilii Dumbadze (*C. ovina* aggr.)	See Caucasus					
C. inermis Velen.	-	C	+			Anatolia, NE Balkans
C. kilaea Boiss.	36 (Meriç et al., 2010)	C	+			Anatolia, NE Balkans
C. luschaniana Heimerl	18 (Uysal et al., 2009)	P	+			Anatolia
C. lycaonica Boiss. & Heldr.	18 (Uysal et al., 2009; Martın et al., 2009)	P	+	Uysal et al., 2010		Anatolia
C. lycia Boiss.	18 (Uysal et al., 2009)	P	+			Anatolia
C. nydeggeri Hub.-Mor.	-	C	-	Davis et al., 1988		Anatolia
C. olympica (DC.) K. Koch (*C. cuneifolia* s.l.)	-	C	+	Wagenitz 1972		Anatolia
C. pinetorum	-	C	+	Uysal et al., 2010		Anatolia
C. polyclada DC	See Balkans					
C. sipylea Wagenitz	-	C	+			Anatolia
C. sivasica Wagenitz	18 (Bal et al., 1999)	C	+	Wagenitz, 1974		Anatolia
C. spinosa L.	See Balkans					
C. tuzgoluensis Aytaç & H. Duman	54 (Martın et al., 2009)	C	+	Vural et al., 2006		Anatolia
C. ulrichiorum Wagenitz & al.	-	?*	+	Wagenitz et al., 2006		Anatolia
C. virgata Lam.	18, 36 (Ghaffari, 1989; Martın et al., 2009)	C	+	Wagenitz 1972	2	E Europe and W Asia, casual alien in rest of Europe, introduced to N America
C. wagenitzii Hub.-Mor.	18 (Uysal et al., 2009)	P	+			Anatolia
C. werneri Wagenitz & al.	-	C	+**	Wagenitz et al., 2006		Anatolia
C. wiedemanniana Fisch. & C. A. Mey.	18 (Özaydyn, 2007)	C	+	Sozen & Ozaydin, 2010		Anatolia
C. yozgatensis Wagenitz	-	C	+	Wagenitz & Hellwig, 1996; Uysal et		Anatolia

				al., 2010		
C. zeybekii Wagenitz	-	C	+	Wagenitz, 1974		Anatolia

*** Not assignable to any section (Wagenitz et al., 2006); ** published in Wagenitz et al. (2006)**

1.7 – Caucasus Area

The Caucasus Area, including parts of Russia, Georgia, Armenia and Azerbaijan, is relatively poor in endemic taxa of the *Centaurea* group. The most complete treatment for the area is given in Iokov et al., (1963).

Table 7: List of members of the *Centaurea* group from the Caucasus region.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. aggregata DC.	See Anatolia					
C. aziziana Rech. f. (*C. ovina* aggr.)	18 (Garcia-Jacas et al., 1998)	P(C)	+			Anatolia, Caucasus, Iran
C. caspia Grossh.	-	C	-			Caucasus
C. diffusa	See Balkans					
C. gulissashvilii Dumbadze (*C. ovina* aggr.)	18 (Tonian, 1980)	C	-			Anatolia, Caucasus
C. hohenackeri Steven (*C. ovina* aggr.)	-	C	-			Caucasus
C. ovina Willd. (*C. ovina* aggr.)	18 (Bakhshi Khaniki, 1996; Tonian, 1980)	C	-			Caucasus
C. transcaucasica Grossh.	-	P	-		3	Caucasus
C. virgata	See Anatolia					

1.8 – Near-East, Middle-East

No complete listings or treatments are available postdating Boissier (1875), since Euro+Med considers only the western part of this area. The following list derives mainly from regional floras; i.e., Post & Dinsmore (1932), Feinbrun-Dothan (1978) and Wagenitz (1980),

Table 8: List of members of the *Centaurea* group from Near-East (except Turkey) and Middle-East.

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. aggregata	See Anatolia					
C. aziziana	See Caucasus					
C. damascena Boiss.	-	C	-			Near-East
C. dumulosa Boiss.	-	C	-			
C. foveolata Blakelock	-	P	-	Wagenitz 1980		Iraq
C. fusiformis Blakelock	-	C	-	Wagenitz 1980		Iraq
C. intricata Boiss.	18 (Bakhshi Khaniki, 1996)	C	-	Wagenitz 1980	2	Iran, Iraq
C. ovina	See Caucasus					
C. reducta Wagenitz		C	-	Wagenitz 1981		Syria
C. virgata	See Caucasus					

2.

Species list of the *Ammocyanus* group, the *C. hierapolitana* group and the isolated taxa within the CMC

Species	Chromosome number (2n)	Sect.	ITS	Literature	Subsp.	distribution
C. benedicta (L.) L. (= *Cnicus benedictus* L.)	22 (Ubera, 1979; Vogt & Aparicio, 1999)	Genus *Cnicus*	+			Mediterranean, introduced as weed elsewhere
C. akamantis T. Georgiadis & Hadjik.	18 (Georgiadis & Chatzikyriakou, 1993)	C	+	Georgiadis & Chatzikyriakou, 1993		Cyprus
***Ammocyanus* group**						
C. ammocyanus Boiss.	*x* = 8 (Ghaffari & Chariat-Panahi, 1985; Ghaffari, 1989)	Ammocyanus	+			Anatolia to Sinai
C. halophila Hub.-Mor.	-	Ammocyanus	-			Anatolia
C. laxa Boiss. & Hausskn. (*C. ammocyanus* s.l.)	-	Ammocyanus	-			Anatolia, Jordania, Syria
C. patula DC.	14 (Garcia-Jacas et al., 1996)	Ammocyanus	+			Anatolia
***C. hierapolitana* group**						
C. hierapolitana Boiss.	16 (Uysal et al., 2009)	P	+	Uysal et al., 2010		Anatolia
C. tossiensis Freyn & Sint.	18 (Uysal et al., 2009)	P	+			Anatolia

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