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"*Centaurea* L. section *Phrygia* Pers.: Phylogeny and Biogeography"

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À minha família

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INTRODUCTION

Molecular systematics, along with other biosystematic approaches, is an essential tool when trying to decipher the natural relationships among taxa, and in complex groups, such as *Centaurea* L., this approach seems useful, if not necessary. However, depending on the working level, i. e. high taxonomic ranges, species level or infraspecific levels, we need to use different molecular approaches. When working from high taxonomic ranges down to the species level, the phylogenetic is the favourite approach, by comparing different evolving-rate introns or interspacer regions from nuclear and chloroplast DNA that are chosen according to the taxonomic rank studied. But it can also be useful to follow a phylogreographic approach, usually at specific or infraspecific level, studying the taxa's relationships within a geographical framework using cpDNA haplotypes. Finally, the study can be addressed with a population genetics approach, allowing us to understand the distribution of variability among and within populations and to infer processes and patterns which are affecting them. The approaches are multiple but the ultimate goal is the same, i.e. capturing the maximum amount of information to make the better decision about which is the best possible arrangement of such variability detecting those independent lineages deserving taxonomic recognition.

The lineage concept (Darwin, 1859; Simpson, 1961; Hull, 1980; de Queiroz, 1998 and 2005) is an interesting attempt to order biological variability, understood as a process rather than as a result or "essence" (species as taxon), which derives from the Aristotle, and subsequently Linnaean, concept of species (Hull, 1965; Wilkins, 2009). Although not free of problems and with difficulties on real implementation, lineage concept fits well with the behaviour of live organisms, which are dynamic and affected by combinations of different evolutionary processes.

Although many attempts for defining an adequate species concept were done (de Queiroz, 1998 and references therein) the inherent uncertainty of the issue, "which biologists cannot hope to avoid or eradicate" (Hey et al., 2003), have led to an amount of species definitions that usually are unable to solve the real problems of taxonomists who try to clarify their group's classification. However, when it comes down to it, most of taxonomists agree in defining the entities (lineages or taxa), the differences usually arise when assigning the taxonomical rank or the "level of lineages [which] one wants to call a species" (Wilkins, 2009). As a last resort, species should be a functional concept so the integration of different approaches, for example morphological and molecular, is necessary. If a taxon is morphologically defined, understood as the presence of constant morphological characters, geographically or ecologically recognizable and it presents some molecular features that make it a likely independent lineage, but even if one of this features is lacking, we agree that the entity deserves taxonomical recognition. If it deserves specific rank or not will depend on the researcher's knowledge of taxa variability and taxa relationships, even if evidences of weak reproductive barriers were found, since taxonomical rank can not be considered static, as organisms are not, and since taxonomical rank could change if new evidences were achieved.

Even though the "species problem" exceeds largely our purposes, the major aim of this work is the clarification of the phylogenetic relationships of *Centaurea* section *Phrygia* Pers., we are presenting evidences which suggest a reassessment of sectional classification within *Centaurea* subgenus *Centaurea* and we are proposing a new arrangement of some Iberian species from sect. *Phrygia*. Therefore we consider appropriate to point out which criteria we are following for defining the taxonomic categories, since it will clearly influence our final results, especially in a genus like

Centaurea with such weak reproductive barriers and uncertain relationships among taxa.

1.1 Systematics and Conservation Biology

The Mediterranean basin is one of the richest regions in number of animal and vegetal endemism around the world. Around a 4.3% of the total plants in the Earth are endemics to the Mediterranean region and therefore it has been considered as a Biodiversity Hotspot (Myers et al., 2000). *Centaurea* L. is a large genus of the Compositae family with around 250 species (Susanna and Garcia-Jacas, 2007) that is contributing to this extraordinary diversity with a high number of species in the Mediterranean basin and in particular in the Iberian Peninsula with approximately 100 endemic taxa (Muñoz and Devesa, 2010).

As the Mediterranean region is characterized by a large degree of endemism and also high anthropogenic footprint (Thompson, 2005 and references therein) plant conservation strategies are necessary. In order to design strong and useful conservation plans, is essential to possess a convenient knowledge of the systematic relationships among involved taxa (Mayden and Wood, 1995; Dimmick et al., 1999), but also a good comprehension of the amount of genetic diversity within populations. If the systematic relationships of the taxa are not clear, indispensable information for designing conservation strategies, like the number of populations, the number of individuals per population, its genetic diversity and so on, cannot be properly traced and therefore conservation plans become useless. For conservation purposes, it is also interesting to consider the species as evolving units (understood as lineages) rather than taxa, and researchers working on conservation issues have realized about the importance to conserve populations with different evolving paths, the Evolutionary Significant Units (ESU; Waples, 1991). Taking into account such entities, we could conserve lines with

putative different evolving directions avoiding the uncertainty of the species definition, but this approach, however, is not free of problems either, and the ESUs are also of difficult application (Hey et. al, 2003, and references therein). We have to be able to find the midpoint allowing ordering biological diversity from an evolutionary point of view without getting lost in the philosophical debate.

1.2 The Genus *Centaurea* L.

Centaurea is one of the largest genera of the Asteraceae family and also one of the most representative genera within the tribe Cardueae Cass. and the subtribe Centaureinae (Cass.) Dumort. with around 250 species (Susanna and Garcia-Jacas, 2007). It is mainly distributed in the Mediterranean region and SW Asia and it is characterized for the great degree of endemism (Colas et al., 1997) which is usually associated to restricted geographical areas (Suárez-Santiago et al., 2007b).

Centaurea is formed by annual, biennial or perennial herbs, sometimes shrubs, usually unarmed. The capitula are surrounded by scarious involucral bracts, rarely leaf-like, with an apical appendix variable in form which possesses an elevated taxonomic value. It presents two kinds of florets: sterile, which are showy and radiant although sometimes lacking, and hermaphrodite placed on the centre of capitula. Its colours range from blue to pink, orange to yellow and sometimes white. The achenes are oblong, laterally compressed, with basal elaiosome and generally presenting a short double pappus.

The genus has historically been considered a difficult taxonomic group and maybe is one of the most taxonomically complex genera in the old world. Such categorical affirmation arise as a consequence of the great degree of morphological and karyological variability which *Centaurea* presents, and to the difficulty to establish well defined limits between forms, which eventually have led to a high number of different

taxonomical arrangements with subsequently nomenclature confusion. First attempts of classification were made by Cassini (1819) who divided *Centaurea* in 40 different genera which later Bentham (1873), Hoffmann (1894) and later Hayek (1901) treated as sections. Therefore, taxonomic treatment of *Centaurea* has always been complicated.

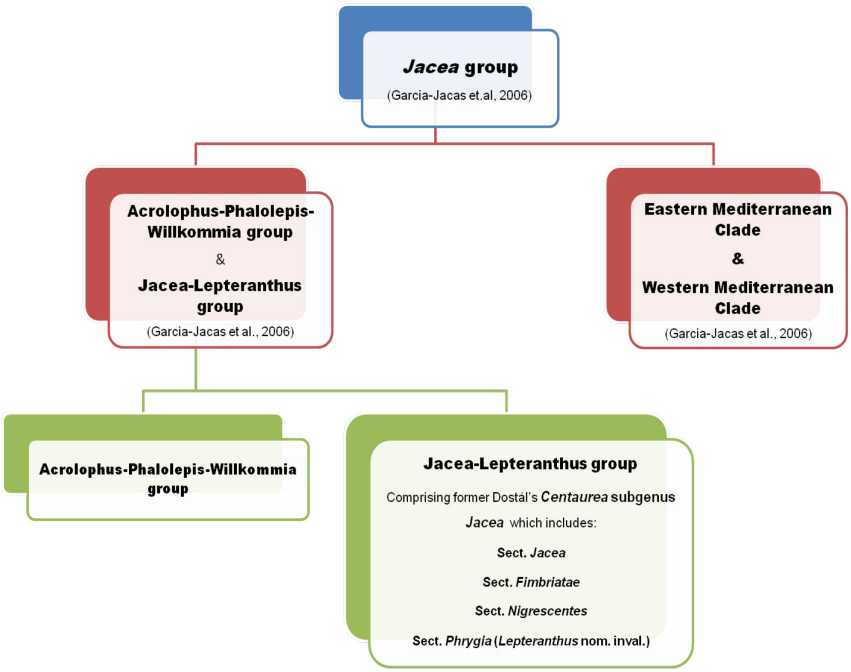

Figure 1. Informal groups content in the *Jacea* group sensu Garcia-Jacas et al. (2006).

However, more recently, the latest morphological (Wagenitz and Hellwig, 1996) and molecular studies (Garcia-Jacas et al., 2000, 2001, and 2006) have provided clues to defining some informal natural groups within the genus. *Centaurea* is now reduced to three groups (Susanna and Garcia-Jacas, 2007). The first two are subgenus *Lopholoma*, which includes section *Acrocentron*, and subgenus *Cyanus*, integrated by section *Cyanus*, both forming well-delimited subgenera. The rest of the genus, made up of a broad group of species, constitutes the subgenus *Centaurea*, known informally as the *Jacea* group. Regarding to *Jacea* group, a comprehensive molecular survey has 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

group comprising sections *Centaurea* [former *Acrolophus* (Cass.) DC.], *Phalolepis* (Cass.) DC., *Willkommia* G. Blanca, *Jacea* (Juss.) DC., and *Lepteranthus* (Neck.) DC. nom. inval., (= *Phrygia* Pers.). Within the second group, the first three sections constitute the monophyletic *Acrolophus-Phalolepis-Willkommia* group, clearly separated from the other one, the *Jacea-Lepteranthus* group (Garcia-Jacas et al., 2006; Figure 1). These two diverse groups await further classification, and although some approaches have already been attempted, it is a difficult task due to intense hybridization (Suárez-Santiago et al. 2007a, 2007b).

As a main objective, the present work tries to elucidate the relationships within the *Jacea-Lepteranthus* group since Garcia-Jacas et al. (2006) concluded that the molecular differences between the two sections were not significant enough to consider them as independent entities.

1.2.1 *Jacea-Lepterantus* group

Within current subgenus *Centaurea* (*Jacea* group), we are going to focus in the *Jacea-Lepteranthus* group. Leaving aside the recent propositions of classification, one of the latest taxonomical arrangements of the whole genus in Europe has been done by Dostál (1976), so we are going to follow his classification, i.e. the subgenus *Jacea* sensu *Flora Europaea*, as the framework for defining the *Jacea-Lepteranthus* group sensu Garcia-Jacas et al. (2006).

The origin of name *Jacea* could probably be traced to ancient, pre-Linnaean authors such as Bauhin (1623) and Tournefort (1694) who used *Jacea* as a genus name which later Linnaeus would include within his great genus *Centaurea*. More recently, at turning of the twentieth century, Hayek (1901) proposed a wide subgenus *Jacea*, although including species now considered part of section *Phalolepis* (Cass.) DC. More recently, the concept of the subgenus became more restricted in Dostál's review for

Flora Europaea. The subgenus sensu Dostál (1976) was principally defined by the presence of undivided leaves as well as by the presence of entire or fimbriate appendages. Dostál recognized the subgenus as subdivided into four different sections mainly on the basis of its appendage morphology:

1. **Sect. *Jacea*** (Mill.) DC.**:** "Appendages broadly ovate or orbicular, usually covering the bracts, with the margin entire, lacerate or denticulate. Pappus usually absent."
2. **Sect. *Fimbriatae*** (Hayek) Dostál: "Appendages triangular or ovate-triangular, lanceolate or ovate-lanceolate, covering bracts, the margin pectinate-fimbriate, the terminal fimbria longer than the lateral. Pappus present or absent."
3. **Sect. *Nigrescentes*** (Hayek) Dostál: "Appendages triangular, ovate-triangular or lanceolate to orbicular, usually not covering bracts, the margin pectinate-fimbriate, the terminal fimbriate shorter than the lateral. Pappus present or absent."
4. **Sect. *Phrygia*:** "Appendages linear to lanceolate, rarely orbicular, usually covering the bracts, the margin pectinate-fimbriate, the terminal fimbria longer than the lateral. Pappus usually present."

1.2.2 Section *Phrygia*

Firstly it has to be pointed out that the name *Lepteranthu*s should be rejected as invalid since Necker work was declared opera utiq. oppr. (Lanjouw et al.,1961). Therefore the section name *Phrygia* Pers. is the first available name.

Dostál (1976), recognized section *Lepteranthus* (= *Phrygia*) as formed by plants with long, usually reflexed, linear, pectinate-fimbriate appendages and achenes with pappus. It was composed of around 19 species, which were divided at the same time into different numbers of subspecies.

Tracing the origin of section *Phrygia* is confusing since different authors have historically considered different forms of classification for the species belonging to the section. Firstly, some authors considered a wide *Jacea* section that included species from both current sections *Jacea* and *Phrygia*. For example, Boissier (1875) proposed a *Centaurea* sect. *Jacea* in a broad sense subdivided into different subsections, which included *Phrygia*. More recently, Garcia-Jacas et al. (2006), in a molecular survey of the *Jacea* group, concluded that the differences between the two sections were not significant enough to separate *Phrygia* from *Jacea*. Other authors, however, have followed other approaches, considering the plumose morphology of appendages as a key character for separating the two sections, for example Necker (1790), who created the genus *Lepteranthus*; Persoon, who divided Linneus' genus in ten sections among which defined one section *Jacea*, including *Centaurea jacea*, and one section *Phrygia*, including *Centaurea phrygia* (Persoon, 1807; cf. Cassini, 1823); and finally, De Candolle, who included Necker's *Lepteranthus* in his treatment of *Centaurea* (cf. Cassini 1823; De Candolle 1838). In the twentieth century both Hayek (1901) and Dostál (1976) proposed *Phrygia* group as a section of *Centaurea* under the name of *Lepteranthus*.

1.3 Objectives

The main goals of present dissertation are:

1. To test the monophyly of *Centaurea* section *Phrygia* reconstructing phylogenetic relationships within subgenus *Jacea*.
2. To clarify the taxonomical arrangement of Iberian *Phrygia* species on the basis of morphological and molecular characters.
3. To study the genetic variability and conservation status of the two narrow endemic Iberian *Phrygia* species, *Centaurea emigrantis* Bubani and *Centaurea*

tripontina López-Alvarado, L. Sáez, Filigheddu, Guardiola and Susanna, using DNA microsatellite markers (SSR markers).

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THE LIMITS OF MOLECULAR MARKERS IN PHYLOGENETIC RECONSTRUCTION: THE CASE OF *CENTAUREA* SECTION *PHRYGIA* (COMPOSITAE)

The limits of molecular markers in phylogenetic reconstruction: the case of *Centaurea* section *Phrygia* (Compositae)

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ABSTRACT

The sectional delineation of sections *Jacea* and *Phrygia* (=*Lepteranthus* nom. inval.) has been controversial for long time. The present review tries to elucidate nomenclature and systematics of sect. *Phrygia*. A molecular phylogenetic approach is used, however, as a recent group, the lack of informative characters both in nuclear and chloroplast markers, the presence of hybridization and reticulate evolution, and the occurrence of shared ancestral polymorphisms emerge as crucial factors limiting resolution of the phylogenetic trees. Otherwise, the study has allowed interpreting the present European distribution of section *Phrygia* as well as its possible eastern centre of origin. The results support previous studies in *Centaurea*, suggesting that section *Phrygia* is currently entangled with sect. *Jacea* on molecular grounds, especially when coexisting with *C. nigra* s. l. and *C. jacea* s. l. Nevertheless, we propose to conserve current separation in both groups based on morphology, given the high level of introgression detected, which make molecular methods a less reliable tool.

INTRODUCTION

Centaurea L. is one of the largest genera of the Compositae with some 250 species (Susanna and Garcia-Jacas, 2007) distributed mainly in the Mediterranean region and SW Asia. Taxonomic treatment of *Centaurea* has always been complicated; however, the latest morphological (Wagenitz and Hellwig, 1996) and molecular studies (Garcia-Jacas et al., 2000, 2001, and 2006) have provided clues to defining some informal natural groups within the genus. *Centaurea* is now reduced to three groups (Susanna and Garcia-Jacas, 2007). The first two are subgenus *Lopholoma*, which includes section *Acrocentron*, and subgenus *Cyanus*, integrated by section *Cyanus*, both forming well-delimited subgenera. The rest of the genus, made up of a broad group of species, constitutes the subgenus *Centaurea*, known informally as the *Jacea* group.

A comprehensive molecular survey of the *Jacea* group revealed that the vast majority of the species were classified into 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 group with unarmed appendages comprising sections *Acrolophus* (Cass.) DC., *Phalolepis* (Cass.) DC., *Willkommia* G. Blanca, *Jacea* (Mill.) DC., and *Phrygia* Pers. [=sect. *Lepteranthus* (Neck.) DC. nom. inval., cf. Lanjouw et al., 1961]. The first three sections constitute the monophyletic *Acrolophus-Phalolepis-Willkommia* group, which appears clearly separated from the *Jacea-Lepteranthus* group (Garcia-Jacas et al., 2006). These two diverse groups await further classification, and although some approaches have already been attempted, it is a difficult task due to intense hybridization (Suárez-Santiago et al., 2007a, 2007b).

One of the latest taxonomic arrangement of the whole genus in Europe was Dostál's review for *Flora Europaea* (Dostál, 1976). This author recognized *Centaurea* section *Lepteranthus* (Neck.) DC. (= sect. *Phrygia*) as including species with long,

usually reflexed, linear, pectinate-fimbriate appendages and achenes with pappus. This section is distributed mainly in Europe, with the only exception of the narrow endemic *C. ali-beyana* Font Quer from Northern Morocco. The Iberian and the Balkan peninsulas constitute the two main diversification centres. The group is composed both of wide-range species, such as *C. phrygia* L., together with several narrow endemics as *C. pangaea* Greuter & Papan., *C. triamularia* Alden, *C. emigrantis* Bubani or the recently described *C. tripontina* López-Alvarado, Sáez, Filigheddu, Guardiola & Susanna.

Nevertheless, there are some doubts on the sectional delineation of sections *Jacea* and *Phrygia*. Historically, different authors have considered different forms of classification for species of these two sections. Firstly, some authors considered a wide section *Jacea* that includes species from *Jacea* and *Phrygia*. For example, Boissier (1875) proposed a *Centaurea* sect. *Jacea* sensu lato subdivided into different subsections, which included *Phrygiae*. More recently, Garcia-Jacas et al. (2006), in a molecular survey of the *Jacea* group, concluded that the differences between the two sections were not significant enough to separate *Phrygia* from *Jacea*. Other authors, however, have followed other approaches, considering the plumose morphology of appendages as a key character for separating the two sections. For example, Necker (1790), who created the genus *Lepteranthus,* and De Candolle, who included Necker's *Lepteranthus* in his treatment of *Centaurea* (cf. Cassini 1823; De Candolle 1838). Both Hayek (1901) and Dostál (1976) suggested that the *Lepteranthus* group was a section of *Centaurea*, and this latest author recognized 19 species, which are divided into different numbers of subspecies. However, depending on the authors, many different combinations and arrangements can be found to classify the different taxa at specific or

infraspecific level (Hayek, 1901; Wagenitz, 1987; Strid and Tan, 1991; Adler et al., 1994; Bolòs and Vigo, 1996; Bancheva et al., 2006).

In this context, a molecular phylogenetic approach could provide an accurate framework to decipher the relationships between both sections as has been done before in other groups of *Centaurea*. The nrDNA internal transcribed spacer region (ITS) and nrDNA external transcribed spacer region (ETS) have been demonstrated to be very useful for deciphering phylogenetic relationships of many closely related species in the genus (Garcia-Jacas et al., 2000, 2002, 2006; Suárez-Santiago et al., 2007a, 2007b). However, as evidenced in other sections, e.g. sect. *Acrocentron* (Font et al., 2002) and sect. *Willkommia* (Suárez-Santiago et al., 2007b), molecular phylogenetic studies on *Centaurea* could present some drawbacks, especially presence of hybridization, introgression and reticulation, which makes difficult the reconstruction task. Preliminary data indicate that these biological phenomena have been also present in the evolution of *Phrygia* and *Jacea* sections (Vanderhoeven et al., 2002; Koutecký, 2007 and references therein), which has the constant chromosome base number x =11 (Hellwig, 2004), increasing the evolution complexity and consequently also the taxonomic problems. In this context nrDNA is also useful for hybridization studies allowing the detection of different parental sequences on cloned PCR products (Baldwin et al., 1995; Sang et al., 1995; Fuertes Aguilar et al., 1999b; Fuertes Aguilar & Nieto Feliner, 2003) as well as the use of noncoding cpDNA regions which are useful to increase the phylogenetic signal and also to reveal incongruence attributable to gene flow (Garcia-Jacas et al., 2009 and references therein). However, it is also important to note that not all the molecular incongruence or all the within-species genetic variation can be explained always by hybridization, and phenomena like incomplete lineage sorting, especially in a recent evolving group such as *Centaurea,* could be involved.

The aims of the present work are to test the hypothesis of the monophyly of sect. *Phrygia* and clarify its internal phylogenetic relationships, paying special attention to the usefulness of classical molecular phylogenetic approach when dealing with complex recent evolving groups. We will also try to elucidate the biogeographic history of the group and to explain the present pattern of species distribution.

MATERIALS AND METHODS

Plant material

Sampling for molecular analysis was focused on species of section *Phrygia* following Dostál (1976). However, because this author overlooked some previously described well-defined species and because many changes in nomenclature have been made since his review, we are going to follow the latest nomenclature proposals by Greuter (2011) and other regional floras or reviews (Laínz, 1967; Soldano, 1978, 1994; Amich, 1991; Strid and Tan, 1991; Bolòs and Vigo, 1996; Bancheva et al., 2006). Even though *Phrygia* is mainly a European group, there is one species out of this range, the north African endemic *C. ali-beyana*. However, it was not available for the study despite it was searched for in the type locality but it was not found. Furthermore, available herbarium material was scarce and useless for DNA extraction since it was chemically treated. Sampling also included species from section *Jacea*: *C. exarata* Coss., *C. inexpectata* Wagenitz and individuals from *C. jacea* L and *C. nigra* L. aggregates. Individuals from these aggregates were labeled as *C. nigra* sensu lato (s. l.) or *C. jacea* s. l. when they could not be unambiguously assigned to one subspecies Also one individual of *C. nigrescens*, classified by Dostál in its own section, *Nigrescentes* but with unclear sectional affinities (Boissier, 1875) was included. Outgroup species were *Centaurea triumfetti* All. subsp. *stricta* (Waldst. & Kit.) Dostál and *C. napulifera* Rochel subsp. *thirkei* (Sch. Bip.) Dostál from section *Cyanus*, which is a sister clade to

the whole *Jacea* group (Garcia-Jacas et al., 2006). According to Hilpold et al. (2009a), two species from section *Centaurea* (*Acrolophus*), which is sister to *Jacea-Lepteranthus*, were chosen also as outgroups: *C. hieropolitana* Boiss. and *C. tossiensis* Freyn & Sint. ex Freyn. The list of studied material for molecular analysis is given in the appendix 1.1.

DNA extraction, amplification and sequencing

Genomic DNA was extracted following the 2x CTAB method of Doyle and Doyle (1987) as modified by Cullings (1992) from silicagel-dried leaves collected in the field. In some cases, herbarium material was used. The ITS region was then amplified for sequencing using primers 17SE and 26SE (Sun et al., 1994) following profiles from Garcia-Jacas et al. (2006). The ETS was amplified with primers ETS1F (Linder et al., 2001) and 18SETS (Baldwin and Markos, 1998) following profiles from Garcia-Jacas et al. (2009). In addition, double-stranded cpDNA of the *trnL*$^{(UAG)}$*-rpl32* and *ycf3-trnS* regions was amplified using rpl32F as forward primer and trnL$^{(UAG)}$ as the reverse primer for the *trnL*$^{(UAG)}$*-rpl32* region (Shaw et al., 2007), and SP43122F as the forward primer and SP44097R as the reverse primer for the *ycf3-trnS* intergenic spacer region (Hershkovitz, 2006). The profile used for cpDNA amplification included a hot start at 95ºC for 3 min. Then 30 amplification cycles were carried out under the following conditions: 95ºC for 40 s, 54ºC for 40 s and 72ºC for 1 min, with an additional extension step of 10 min at 72ºC. The PCR products was purified with ExoSAP-IT (USB Corp., Cleveland, OH, USA) and sequenced using a BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol, at the University of Florida ICBR Core Facility using an ABI 3730xl DNA analyzer (Applied Biosystems). For the nuclear markers, the ITS region was sequenced with 26SE, and the ETS with 18SETS as reverse primers. The cpDNA

trnL$^{(UAG)}$*-rpl32* with trnL$^{(UAG)}$ as reverse primer, and *ycf3-trnS* with SP43122F as forward primer to avoid a poly-A region.

To determine possible hybridization events or individual polimorphysm, ITS and ETS PCR products from some species were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Eight to sixteen positive colonies from the reaction were screened with direct PCR using T7 and M13 universal primers under the following conditions: 3 min denaturation at 95ºC followed by 35 cycles of 94ºC, denaturation for 45 s, 50ºC annealing for 45 s and 72ºC extension for 1 min, with an additional min at 72ºC. Six to twelve PCR products were selected for sequencing in both directions using the same primers.

Phylogenetic analysis

Sequences for phylogenetic analyses were edited using BioEdit (Hall, 1999) and aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990). The cloned sequences of ITS and ETS were grouped by similarity into different consensus sequences, or inserted directly in the matrix, to carry out the phylogenetic analyses.

In order to test the possible incongruence between nuclear and nuclear-chloroplastic datasets, a partition homogeneity test (Farris et al., 1994) was performed using WinClada ver. 1.00.08 (Nixon, 2002) with 10000 homogeneity replicates. Additionaly, an indel codification of the cpDNA matrix was performed with IndelCoder 1.0 (Müller, 2006) using the Modified Complex Indel Coding (MCIC) algorithm. Coded indels were included as additional characters in the final phylogenetic analyses.

Subsequent phylogenetic analyses for the combined nrDNA and the cpDNA matrix were carried out using maximum parsimony and Bayesian methods. Analyses were performed also with a simplified nuclear matrix excluding taxa suspected to be of hybrid or introgressed origin in order to eliminate phylogenetic noise and improve

resolution and statistical support (Vriesendorp and Bakker, 2005 and references therein).

Bayesian inference estimation was calculated using MrBayes 3.1.2 (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) and Bayesian information criterion (BIC) as implemented in the software jModeltest 0.1.1 (Guindon and Gascuel, 2003; Posada, 2008), which considers nucleotide substitution models that are currently implemented in MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The HKY model with variable base frequencies was assumed to follow a discrete gamma distribution (Hasegawa et al., 1985) and was selected as the best-fit model of nucleotide substitution for ETS dataset. For the ITS alignment, the symmetrical model with equal base frequencies and invariable sites (SYM+I) was selected (Zharkikh, 1994). The cpDNA matrix, with indels coded as characters were analysed under the presence-absence model F81 (Felsenstein, 1981). Bayesian inference analyses were initiated with random starting trees and were run for $1x10^6$ generations. Four Markov Chains were run using Markov Chain Monte Carlo (MCMC) principle sample trees. We saved one out of every 100 generations, which resulted in 10000 sample trees. Data from the first 2500 generations were discarded as the "burn-in" period, after we had confirmed that the likelihood values had stabilized prior to the 2500^{th} generation.

Maximum Parsimony (MP) analyses involved heuristic searches conducted with PAUP* version 4.0b10 (Swofford, 2002) using tree-bisection-reconnection (TBR) branch swapping with character states specified as unordered and unweighted. We conducted a heuristic search with 1000 replicates and random taxon addition, saving

500 trees per replicate. The strict consensus of most parsimoniousus trees were calculated (trees not shown). Bootstrap analysis (Felsenstein, 1985) was performed using a heuristic search with 1000 replicates and random taxon addition, saving a maximum of 10 trees per replicate.

RESULTS

The nuclear aligned matrix (ETS and ITS) consisted of 78 sequences of 1181 bp and 170 parsimony-informative characters. The simplified nrDNA matrix consisted of 50 sequences of 1181 bp and 151 parsimony-informative characters. For the cpDNA matrix (*trnL*$^{(UAG)}$*-rpl32* and *ycf3-trnS*), a total of 68 sequences of 1766 bp and 61 parsimony informative characters were obtained. Discordances between the number of sequences for nuclear and cpDNA matrices are due to the presence of cloned sequences or cloning consensus in the nuclear alignment. The figures 1, 2 and 3 show the trees yielded from Bayesian analyses with the addition of the Bayesian posterior probabilities (PP) and MP bootstrap values (BS) for nrDNA, simplified nrDNA and cpDNA. The MP numerical results are shown on Table 1.

A combined analysis of the ETS and ITS was not recommended by the ILD test (P value = 0.001). The combination of nuclear and cpDNA data was also not recommended (P value = 0.001). However, as suggested by Yoder et al. (2001) and Dowtow and Austin (2002), the ILD test does not have practical utility, and since the combination of different datasets is useful to improve the degree of information in phylogeny reconstruction, combination can be justified. Moreover, the results should be taken with caution considering that the possible discordances could be due to hybridization, incomplete lineage sorting, heterogeneous rates of molecular evolution, homoplasy or even stochastical error (Maddison, 1997). In the present study, besides hybridization, differences in sequence variation rates among nuclear and cpDNA

alignments, the presence of relatively high levels of homoplasy (Table 1) and the lack of sequence divergence could be acting as the main source of incongruence. Therefore both nuclear datasets (ETS and ITS) were combined since the possible incongruent relationships, in a separate analysis of both regions, are not statistically supported and since both markers yield similar topologies.

None of the phylogenetic analyses separate the sections *Jacea* and *Phrygia*. The analysed markers fail to resolve the deep relationships between taxa, giving statistical support only to outgroup nodes and terminal clades, usually with clear morphological or geographical affinities.

Nuclear markers

No good resolution is given by nuclear markers to deep nodes, only terminal branches reach statistical support, especially in Bayesian analysis (Figures 1, 2). Four major clades could be defined in the nrDNA analysis (Fig. 2): the *C. nigra clade* (PP = 1.00; BS = 98 %), the *C. phrygia clade* (PP = 0.98), the *Alpine clade* (PP = 1.00; BS = 84 %), and the *Macedonian clade* (PP = 1.00), and other small clades with few species usually with morphological affinities. Performing the analysis with the simplified nrDNA matrix, a fifth group, the *C. linifolia clade* (Fig. 1) reached statistical support (PP = 0.95). These four major clades include species from both Dostal's sections *Jacea* and *Phrygia*.

cpDNA markers

Clear incongruence was found by eye between nuclear and chloroplast markers (Figure 3). The expected basal species *C. inexpectata* (Garcia-Jacas, 2006; and also for the nuclear ETS+ITS Bayesian and MP analyses) was unexpectedly clustered with Alpine plants (PP = 1.00; BS = 80 %), whereas the Greek species *C. pangaea*, *C. triamularia* and *C. nervosa* subsp. *promota* were placed sister to the rest (PP = 0.97). The

relationship among *C. inexpectata*, *C. jordaniana* subsp. *verguinii* and *C. rhaetica*, is interesting due to the divergent ranges of distribution, Turkey and the Alps respectively. As to the rest of species, a large clade containing species from a wide range of geographical distributions and morphological affinities was supported in Bayesian analysis (PP = 0.97). Other clades usually formed by two species are supported in both Bayesian and MP analyses.

Combined nuclear and plastid alignment analysis

The combined matrix (tree not shown) does not contribute to clarify the phylogeny. Therefore, since the ILD test do not recommend the combination and no different information is achieved with this analysis, its results are not presented nor discussed.

Cloning

ITS and ETS markers were cloned for some taxa and populations when presence of hybridization were suspected, either due to the presence of morphological intermediancy, either to the presence of multiple electrophoresis bands on PCR products, especially for the ETS. Usually the presence of multiple electrophoresis bands in the ETS was due to different number of motif repetitions in the ETS 5' extreme. Most of the cloned copies usually were clustered together in the same clade and only for *C. janeri* subsp. *janeri*, *C. caballeroi*, *C. emigrantis*, *C. linifolia* and *C. montis-borlae* cloning efforts have provided evidence of intraindividual polymorphism by having two different copies belonging to different clades (Figures 1 and 2).

DISCUSSION

The molecular phylogeny of section *Phrygia* has revealed the limitations of molecular markers for resolving systematic relationships among studied taxa. There are different reasons which explain such limitations. One of them could be the recent divergence

time of the group: *Centaurea* s. str. is recent as demonstrated in previous dating studies among Cardueae (López-Vinyallonga et al., 2009; Barres et al., Botanic Institute of Barcelona, in prep.) and confirmed by the limited number of informative characters. However, other phenomena like incomplete lineage sorting or reticulation can mask the phylogenetic history of individuals, explaining some of the relationships yielded by the phylogenetic analysis. Incomplete lineage sorting could be detected when different species without apparent systematic relationship appear in the phylogeny as having the same evolving history even when distribution ranges are far away and the possibility of gene flow is scarce. Otherwise, ancestral hybridization also explains the phenomenon if some copies of the nrDNA have remained due to biased concerted evolution. Moreover, when the geographical component predominates over the systematic one, weak reproductive barriers are present and therefore gene flow and reticulate evolution is the more realistic explanation. In this context, it is interesting to remember that these evolutionary processes are not always independent, and recent evolving ages can imply all the aforementioned processes together: lack of differentiation, weak reproductive barriers and probably also higher probabilities of conserved ancestral polymorphisms (Nieto Feliner and Rosselló, 2007).

Following the exposed above, the analysis of trees yielded from nuclear data reveals a recent origin and suggests that some of the aforementioned phenomena are concurrent. Although not fully resolved, some clades of nrDNA tree are statistically supported and their compositions suggest that different evolution processes are involved. We are going to discuss the results based on tree yielded by the analysis of the simplified nrDNA matrix since it presents the maximum number of solved clades. The first clade, the *Macedonian clade* (Fig. 1 and 2) is formed by species growing in the Greek and Bulgarian mountains. The Greek accession of *C. nervosa* subsp. *nervosa*

(accesion *C. nervosa* subsp. *nervosa* C) is also clustered here. Such results indicate either the geographical component is masking systematic relationships among *C. nervosa* taxa by introgression (see Fig. 1 and 2, also for *Alpine clade*) or that Greek *C. nervosa* subsp. *nervosa*, as noted by Strid (In Sched.; *C. nervosa* subsp. *nervosa*, *Strid 24893*), is a different subspecies. This fact could be indicating some morphological differences with regard to *C. nervosa* type. This clade also includes two species which the latest proposal (Greuter, 2011) includes within *C. phrygia*: *C. indurata* and *C. stenolepis* subsp. *razgradensis* in the classification by Dostál (1976). Since this study has revealed the limitations to separate the geographic component from the systematic one, is difficult to extract definitive taxonomic conclusions, however, since these two species appears in a different clade that other taxa from *C. phrygia* aggr., the retention of Dostál (1976) names seems the more appropriate option.

The *Alpine clade* (Fig. 1 and 2), together with the *C. linifolia clade* (Fig. 1), is the only clade that presents geographical, morphological and taxonomical affinities since includes species belonging to the proposed *C. uniflora* aggregate (Greuter, 2011). The only exception is an individual attributed to *C. nigrescens*, a species suspected to be of hybrid origin (Susanna pers. comm.). *Centaurea nigrescens* was classified by Dostál (1976) within its own section, *Nigrescentes* (Hayek) Dostál, along with *C. transalpina*, which has been considered as a subspecies of *C. nigrescens* (Greuter, 2011). Keeping section *Nigrescentes* as an independent group does not seem appropriate on the basis of molecular data and morphological traits. Furthermore, as has been demonstrated before in previous studies of *Centaurea*, the inflation in number of sections in Dostál's review is manifest (Garcia-Jacas, 2006; Mameli et al., 2007; Hilpold, 2009b). In any case, further investigation specifically for this species should be done.

The *C. nigra clade* (Fig. 1 and 2) comprises mainly individuals attributable to *C. jacea* and *C. nigra* from the Iberian Peninsula. One individual of *C. janeri* subsp. *gallaecica* is also clustered in this group. It is worth noting that *C. janeri* s. l. morphologically belongs clearly to *Phrygia*, but the morphology of subsp. *gallaecica* could be in some aspects more related to the *C. nigra* aggr., as pointed out by Silva-Pando (2008). Despite this, it does not seem appropriate to make nomenclatural rearrangements, because one cloning consensus copy from an individual of *C. janeri* subsp. *janeri* is also grouped within this clade. Such evidences could indicate introgression or reticulate evolution, especially considering that *C. nigra* presents an aggressive genetic behaviour and hybridizes with species of *Centaurea* from other sections (Roché and Susanna, 2010) and even with a different subgenus (*Centaurea x valdesii-bermejoi* Fern. Casas & Susanna, cf. Fernández Casas and Susanna 1982). The ETS of *C. janeri* subsp. *gallaecica* was also cloned, but no different copies were detected (unpublished data).

The *C. phrygia clade* (Fig. 1 and 2) has strong Bayesian support, even though it is not supported by bootstrap. It also presents high geographical affinity as found in the other supported clades. The composition of this clade is similar to that of *C. nigra aggr. clade*, as it comprises taxa of wide distribution, some individuals of *C. jacea* from the Alps to Balkans, and *C. phrygia* instead of *C. nigra*. The lack of genetic differentiation is clear in this clade, surely due to lack of reproductive barriers as seems demonstrated by the hybrid accession from a locality near Kostenec (Bulgaria), with clear morphological intermediance between *C. jacea* and *C. phrygia* but having a sequence that is indistinguishable from the rest. A particular case appears in individual #2 of *C. montis-borlae*, an Italian narrow endemic, which has an ITS cloned copy falling within this clade, whereas the other one is clustered with the rest of the *C. montis-borlae*

individuals. An ancient hybridization event is the easiest explanation since the Alpine and Balkan ranges were connected as evidenced by the biogeographic affinities found at several groups of plants and animals (Schmitt, 2009 and references therein). Although *C. montis-borlae* is restricted to the Apuan Alps (Apennines), which are isolated from the Alps by the Po valley, a connection might be possible along the Ligurian mountain corridor to Maritime Alps (Ansell et al., 2008). Besides *C. montis-borlae*, two other western species, *C. jordaniana* subsp. *verguinii* and *C. pectinata*, appear nested within this clade composed mainly by eastern individuals. Since both species were cloned without finding more than one copy in its genome, neither ancestral hybridization nor incomplete lineage sorting can be discarded.

The analysis of the two previous groups reveals also that species attributable to *C. jacea* aggr. appear nested independently in both clades. It could be interpreted as a non-monophyletic species but, as evidenced in previous studies in *Centaurea* (Suárez-Santiago, 2007a), it agrees better with the "transclade species" (Fuertes Aguilar and Nieto Feliner, 2003), which follows the compilospecies model.

Finally, a *C. linifolia* clade (Fig. 1), a morphologic-geographic credible clade, is supported when the analysis is performed using the nrDNA simplified dataset. Species of this clade follow a reticulate pattern because *C. emigrantis*, *C. caballeroi*, and *C. linifolia* appear as having different affinities within the group. In particular for *C. linifolia*, cloning efforts have failed in providing evidence of intraindividual polymorphism. Instead, sequencing two individuals from separate populations has revealed these differences. Nevertheless, such behaviour does not allow differentiating between reticulation and incomplete lineage sorting. Further studies are required to clarify the relationships within this clade.

Regarding the analysis of the cpDNA (Fig. 3), it also presents the same drawback that nuclear markers, i. e. the low number of informative characters. The tree topology shows low structure without systematic information. There are some supported clades, revealing affinities among species whose distribution areas are not in contact at present suggesting ancestral polymorphism sharing or ancestral gene flow. In particular, *C. pectinata* and *C. nervosa* (PP = 1.00; BS = 85 %) or *C. montis-borlae*, *C. jacea* subsp. *weldeniana* and *C. jacea* subsp. *vinyalsii* (PP = 0.95). Others, instead, reveals geographic information, as the clade formed by *C. parilica*, *C.phrygia* subsp. *indurata* and *C. phrygia* subsp. *razgradensis* (PP = 0.95). Finally, there is one clade formed by *C. janeri* subsp. *janeri* and *C. jacea* subsp. *vinyalsii* which corroborates the gene flow existing between *C. nigra* s. l. or *C. jacea* s. l. and *C. janeri* (PP = 1.00; BS = 95 %).

It is remarkable that, even though cpDNA data do not have strong systematic signal, when confronted with nrDNA it gives information that allows making inferences about biogeography, in particular about the centre of origin and the patterns of group diversification. Regarding the centre of origin, as in other groups of *Centaurea*, an eastern origin is highly probable (Font et al., 2009 Suárez-Santiago et al. 2007b). *Centaurea inexpectata*, a Turkish endemic, appears as the sister taxon to the rest of the species in the nrDNA tree. Otherwise, the presence of related cpDNA haplotypes among *C. inexpectata* and some narrow endemic Alpine plants like *C. jordaniana* subsp. *verguinii* and *C. rhaetica* could indicate a range expansion over the continent later disaggregated in several narrow groups with retention of ancient haplotypes. Such hypothesis could be also confirmed if we consider the clade formed by the nuclear alignment of *C. pectinata* and *C. triamularia* (PP = 0.98; BS = 76%) which is also supported by a striking morphological affinity. Considering that the first species grows in southern France and north-eastern Spain and the second one in a restricted locality,

Pachtourion Mountain, in Greece, allopatric speciation becomes the most likely explanation. The pattern of cpDNA haplotypes distribution in the remaining species does not follow either systematics or geography, which also supports a wide ancestral distribution and recent isolation. Moreover, recent isolation times could explain the large degree of gene flow among species when they get in contact. Also interesting is the basal position in the cpDNA tree of Greek species, *C. pangaea* and *C. triamularia*, and the greek accesion *C. nervosa* subps. *nervosa* C, which present cpDNA haplotypes similar to the outgroup by sharing similar gap structure, indicating either shared ancestral polymorphism, either cpDNA capture (Schaal et al., 1998 and references therein). If it represents retention of ancient haplotypes instead of chloroplast capture, it could support the eastern origin of the group.

In conclusion, the study reveals that the molecular markers used in this study do not fit well with the morphology and most of the classical systematic treatment of the group. It seems clear that we need to re-examine the phylogenetic method used to study the genus *Centaurea* at species-level. The recent age of the group and the lack of differentiation appear as the main factors for low resolution of phylogenetic relationships within *Jacea-Phrygia* (sensu Garcia-Jacas et al., 2006). However, processes like incomplete lineage sorting and especially reticulate evolution are also important. Otherwise, it seems difficult to explain an intensive introgression or reticulate evolution without a real unifying effect on taxa's morphology implying that, either the used molecular markers are sensitive to gene flow and therefore differentiation forces are stronger than introgression, either the morphological characters used do not reflect the systyematic relationships, as found in other morphologically defined *Centaurea* groups (Hilpold et al., 2011).

Therefore, we propose to conserve *Jacea* and *Phrygia* as separate entities within a subgenus *Centaurea* (Susanna and Garcia-Jacas, 2007), even if not natural on the basis of our results, since the morphological traits to separate both groups are clear in most cases. Surely this is the best solution to avoid confusions and misinterpretations in a taxonomical and nomenclatural complex group such as *Centaurea* while waiting for an integrative study; morphology, at least, represents the addition of all the information kept in the genome.

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TABLES

Table 1. Numerical results of the analyses of the nrDNA, simplified nrDNA and cpdNA alignments. Tree length, CI, RI and HI were calculated for entire trees and for trees without outgroups Abbreviations: CI, consistency index; HI, homoplasy index; Informative char., phylogenetically informative characters; RI, retention index; tree, the entire tree including outgroups; ingroup, tree excluding outgroups.

	nrDNA		**Simplified nrDNA**		**cpDNA**	
Total Characters	1181		1181		1766	
	tree	**ingroup**	**tree**	**ingroup**	**tree**	**ingroup**
Informative char.	170	113	151	92	61	53
Tree length	361	238	316	191	106	88
CI	0,516	0,533	0,577	0,536	0,423	0,529
RI	0,390	0,821	0,737	0,751	0,713	0,818
HI	0,483	0,467	0,425	0,464	0,577	0,471

FIGURES

Figure 1. 50% majority-rule consensus tree obtained from the Bayesian analysis of the simplified nrDNA dataset indicating supported clades. Numbers above branches are posterior probabilities and Bootstrap values under branches. Names in dark blue correspond to *Phrygia* species, *Jacea* species are in light blue and *Nigrescentes* in green. CL = abbreviation for clones. Con = abbreviation for consensus sequence. s. l = abbreviation for *sensu lato*. Country names are given following ISO standard. Different individuals of the same population are indicated with consecutive numbers (1, 2, etc.). Individuals belonging to different populations are indicated with consecutive capital letters (A, B, etc.); population codes are shown in the appendix table.

Figure 2. 50% majority-rule consensus tree obtained from the Bayesian analysis of the nrDNA dataset indicating supported clades. Numbers above branches are posterior probabilities and Bootstrap values under branches. Names in dark blue correspond to *Phrygia* species, *Jacea* species are in light blue and *Nigrescentes* in green. CL = abbreviation for clones. Con = abbreviation for consensus sequence. s. l = abbreviation for *sensu lato*. Country names are given following ISO standard. Different individuals of the same population are indicated with consecutive numbers (1, 2, etc.). Individuals belonging to different populations are indicated with consecutive capital letters (A, B, etc.); population codes are shown in the appendix table.

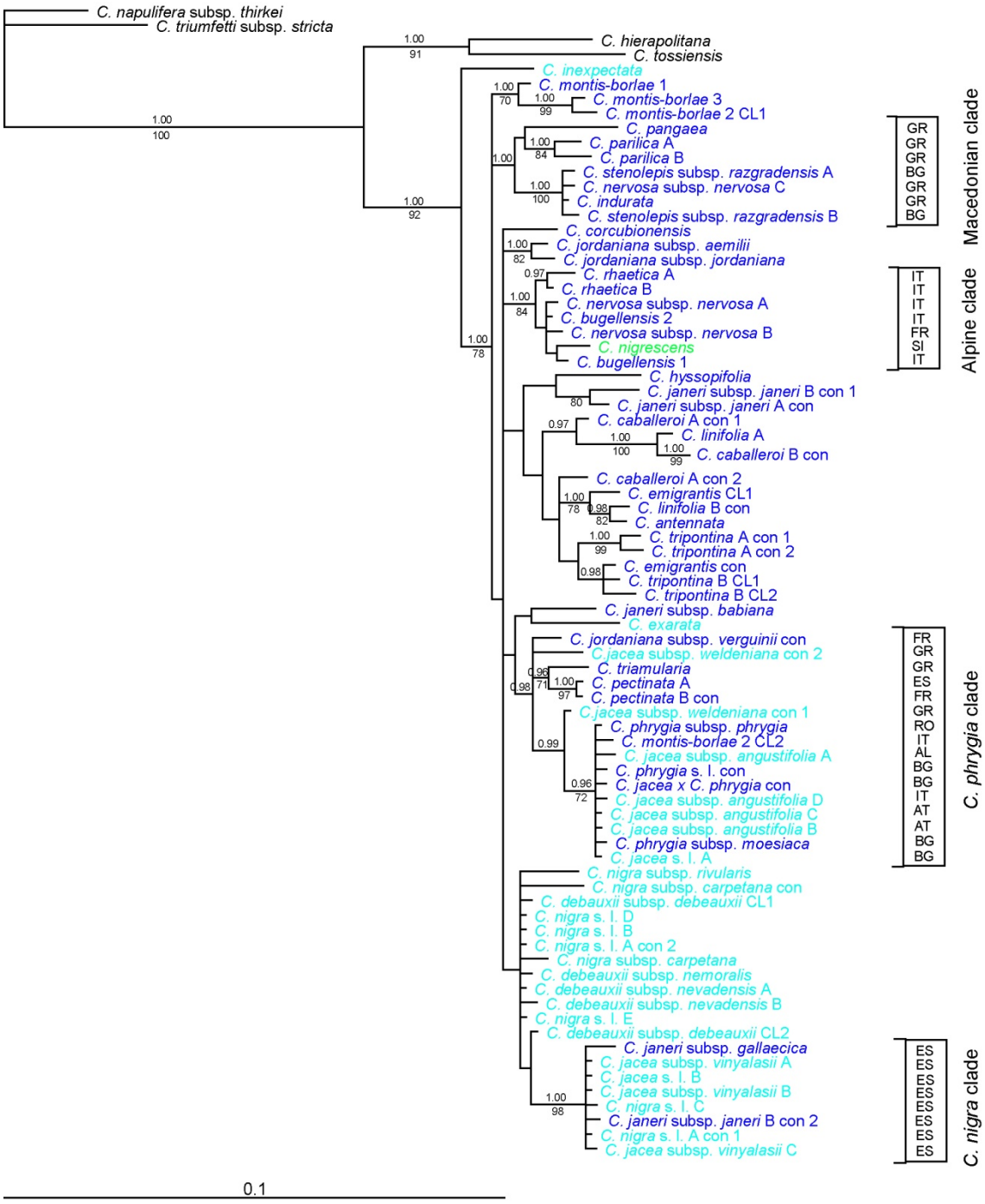
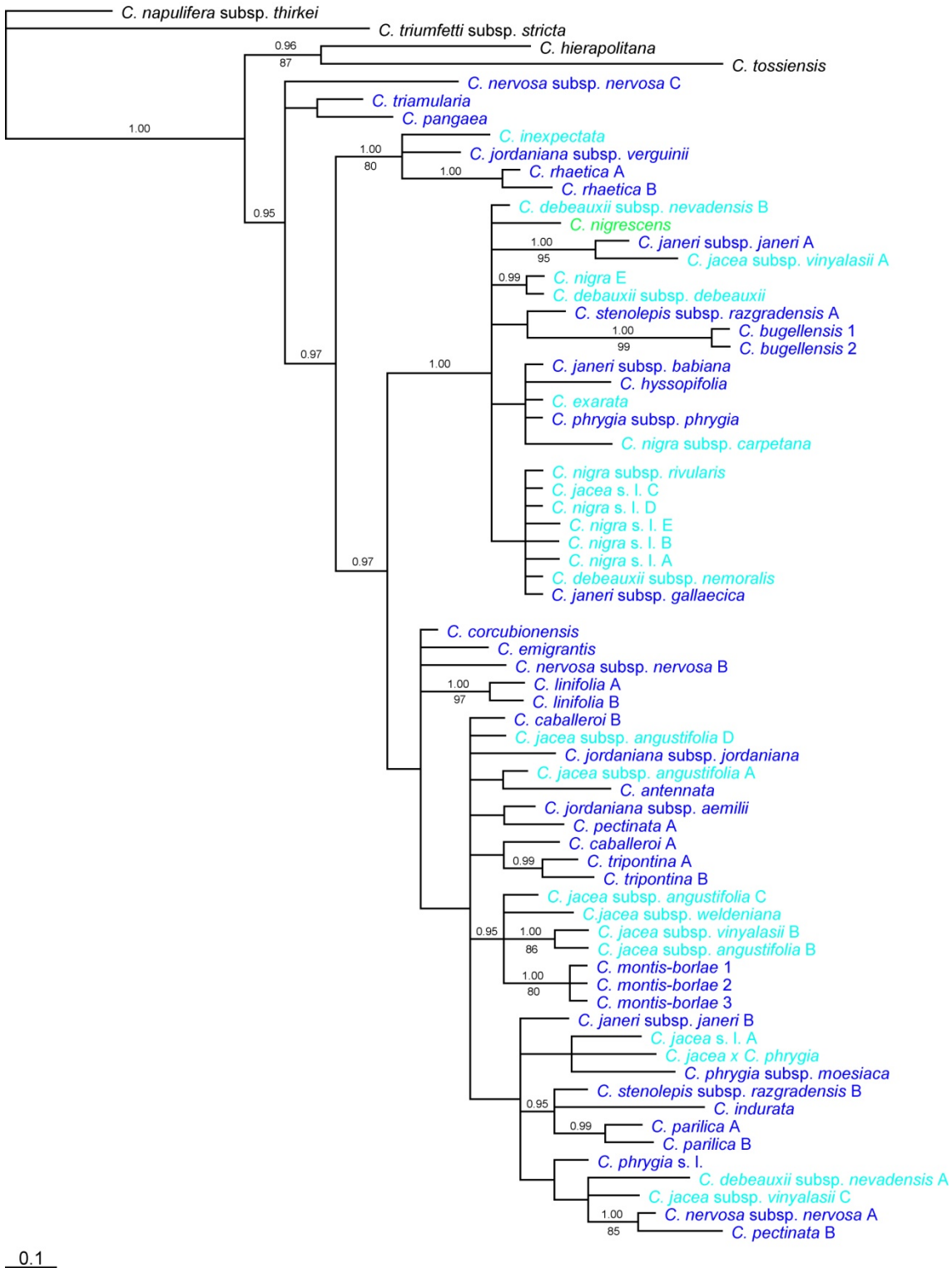


Figure 3. 50% majority-rule consensus tree obtained from the Bayesian analysis of the cpDNA alignment. Numbers above branches are posterior probabilities and Bootstrap values under branches. Names in dark blue correspond to *Phrygia* species, *Jacea* species are in light blue and *Nigrescentes* in green. s. l = abbreviation for *sensu lato*. Country names are given following ISO standard. Different individuals from the same population are indicated with consecutive numbers (1, 2, etc.). Individuals belonging to different populations are indicated with consecutive capital letters (A, B, etc.); population codes are shown in the appendix table.



APPENDIX 1.1

Species	Pop.	Range	Voucher
*Centaurea antennata*Dufour		Spain	Spain, Valencia, Serra Calderona, Font del Berro, 10-VI-2011, *Figueroa & López-Alvarado* (BC)
Centaurea bugellensis (Soldano) Soldano		Italy	Italy, Torino, Biellese-Valsesia, Biella, Strada Paniramica Zegna verso il Bielmonte, 28-VI-2011, *Figueroa & López-Alvarado* (BC)
Centaurea caballeroi Font Quer	A	Iberian Peninsula	Spain, Tarragona, Serra de Montsià, Mas de Comú, 04-VI-2009, *Barres & López-Alvarado* (BC)
Centaurea caballeroi Font Quer	B	Iberian Peninsula	Spain, Tarragona, Ports de Beceit, Portell de Caro, 02-VII-2008, *L. Sáez* (LSG pers-BCB)
*Centaurea corcubionensis*M. Laínz		Iberian Peninsula	Spain, A Coruña, Carnota, O Pindo, carretera Pindo-Carnota, 10 m, orla de matorral, granito, 09-VI-2002, *Iglesias-Louzan* (BCN20949)
Centaurea debeauxii Godr. & Gren.		Eurosiberian	Spain, Huesca, Ribereta de Arazas, Ordesa y Monte Perdido, 14-IX-2008, *Hilpold & Kosinsky* (BC)
Centaurea emigrantis Bubani		Iberian Peninsula	Spain, Huesca, Castellonroi, Congost de Santa Ana, Huesca, 26-VI-2008, *Roquet & Sáez* (LSG pers-BCB)
Centaurea exarata Coss.		Iberian Peninsula	Spain, Huelva, road A-983, Almonte to Matalascañas, km 25, 9-VIII-1999, *Roché & Susanna 1909* (BC)
Centaurea hierapolitana Boiss.		Turkey	Turkey, Afyonkarahisar: Dazkırı, Çıkışı, 870 m, 24-VI-2004, *Bağcı YBK-1523* (KNYA)
Centaurea hyssopifolia Vahl		Iberian Peninsula	Spain, Toledo, near Ontígola, 500 m, 22-VI-1996, *Garcia-Jacas, Susanna 1600 & Vilatersana* (BC)
Centaurea indurata Janka		Balkans	Greece, Dramas, Rodopi Mts., Place called Megalo Livadi ENE Dipotama, 1450 m, 06-VIII-2005, *Strid 55839* (pers. herb.)
Centaurea inexpectata Wagenitz		Turkey	Turkey, Antalya, Gevne valley, high of village Küçüklü, 1750 m, 30-VI-2004, *Uysal 598* (KNYA)
Centaurea jacea L.	A	Eurosiberian	Bulgaria, Pazardjik District, Velingrad, road margins 10 km from Yundola to Kostenec, 22-VII-2010, *Figueroa & López-Alvarado* (BC)
Centaurea jacea L.	B	Eurosiberian	Spain, Huesca, 1 Km ENE Refugio Linza, 21-VIII-2009, *Hilpold AH20095040 & Kosinsky* (BC)
Centaurea jacea subsp. *angustifolia* (DC.) Gremli	A	Eurosiberian	Albania, mountain pass between Tirana and Elbasan,17-VIII-2009, *Garnatje & Sánchez-Jiménez 17* (BC)
Centaurea jacea subsp. *angustifolia* (DC.) Gremli	B	Eurosiberian	Austria, Wien, Alpengarten im Balvedere, 11-X-2009, *Hilpold AH20096014* (BC)
Centaurea jacea subsp. *angustifolia* (DC.) Gremli	C	Eurosiberian	Austria, Marchegg , between Lange Luss and Schloss Gänsendorf, 10-X-2009, *Hilpold AH20093235*
Centaurea jacea subsp. *angustifolia* (DC.) Gremli	D	Eurosiberian	Italy, Campania, Caserta, Matese, 2,5 Km ENE Letino, 28-VII-2009, *Hilpold AH20094010* (BC)
Centaurea jacea subsp. *vinyalsii* (Sennen) O. Bolòs et al.	A	Iberian Peninsula	Spain, Lleida, Lliminana, in Barcedana riverbed, near road LV-9121, 01-VII-2010, *Hilpold AH4058 & López-Alvarado* (BC)
Centaurea jacea subsp. *vinyalsii* (Sennen) O. Bolòs et al.	B	Iberian Peninsula	Spain, Huesca, road from Sigües to Roncal, 19-VIII-2009, *Hilpold AH20095001 & Kosisnsky* (BC)
Centaurea jacea subsp. *vinyalsii* (Sennen) O. Bolòs et al.	C	Iberian Peninsula	Spain, Barcelona: La Garrotxa, Pass of Bracons, 1100 m, 04-XI-1995, *Garcia-Jacas & Susanna 1593* (BC)
Centaurea jacea subsp. *weldeniana* (Rchb.) Greuter		Eastern Mediterranean	Greece, Nomos Karditsis, Eparchia Karditsis, c. 2 km S and E of Messenikolas village, along the road to Karditsa, *Garcia-Jacas, Karamplianis & Susanna 2740* (BC)
Centaurea janeri Graells subsp. *gallaecica* M. Laínz		Iberian Peninsula	Spain, A Coruña, Toques, Serra do Careón, 700 m, na orla do matorral. Serpentinas, 04-VII-2005, *Iglesias-Louzán* (BCN38899)
Centaurea janeri Graells subsp. *janeri*	A	Iberian Peninsula	Spain, Salamanca, carretera entre el Casarito i El Cabaco, 14-VII-2009, *Figueroa & López-Alvarado* (BC)
Centaurea janeri Graells subsp. *janeri*	B	Iberian Peninsula	Spain, La Rioja, Haro, 0,3 Km W San Felices de Bilibio, near service area of AP-68, 02-VII-2009, *Hilpold AH20093007, Garcia-Jacas & Vilatersana* (BC)
Centaurea janeri Graells subsp. *babiana* M. Laínz		Iberian Peninsula	Spain, León, Sena de Luna, Ermita de Rabanal, 13-VII-2009, *Figueroa & López-Alvarado* (BC)
Centaurea jordaniana Godr. & Gren. subsp. *jordaniana*		France	France, Provence-Alpes-Côte d'Azur, Alpes-Maritimes, Duranus, Gorges de la Vésubie, 17-VI-2009, *Diadema* (CBNMED)
Centaurea jordaniana subsp. *aemilii* (Briq.) Kerguélen		France	France, Provence-Alpes-Côte d'Azur, Alpes-Maritimes, Toudon, Mont Vial, 17-VI-2009, *Diadema* (CBNMED)
Centaurea jordaniana subsp. *verguinii* (Briq. & Cavill.) Kerguélen		France	France, Provence-Alpes-Côte d'Azur, Alpes-Maritimes, Thiery, Forèt Domaniale de la Madone, 17-VI-2009, *Diadema* (CBNMED)
Centaurea linifolia L.	A	Iberian Peninsula	Spain, Barcelona, 4 Km E Balsareny, 4 Km W Avinyó, 15-V-2009, *Hilpold AH20092068* (BC)
Centaurea linifolia L.	B	Iberian Peninsula	Spain, Tarragona, Vandellós, 09-VII-2008, *L. Sáez* (LSG pers-BCB)
Centaurea montis-borlae Soldano		Italy	Italy, Toscana, Massa-Carrara, Carrara, Alpi Apuane. Campo Cecina, 25-VI-2011, *Figueroa & López-Alvarado* (BC)
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-IV-1996, *Badarau* (BC)
Centaurea nemoralis Jord.		Eurosiberian	Saint Cyr des Gats, "La Boucherie" (85) Cor. 41-5 (Index Seminum Nantensis)
Centaurea nervosa Willd. subsp. *nervosa*	A	Alpine	Italy, Trentino Alto Adige, Bozen, Aldein, Jochgrimm, 0,1 km WSW Hotel Schwarzhorn, 02-VIII-2001, *Wilhalm Thomas* (PVASC 3014)
Centaurea nervosa Willd. subsp. *nervosa*	B	Alpine	France , Rhône-Alpes, Savoie, La Thuile, Pont Serrand, 1700 m (Chanousia Botanical Gardens)

Centaurea nervosa Willd. subsp. *nervosa*	C	Greece	Greece, Florinis, Voras Mt., Kaimaksalan, 2-3 km WNW of the filakion at point 1963, 1800-1850 m, 23-VII-1985, *Strid 24893* (pers. herb.)
Centaurea nevadensis Boiss. & Reut.	A	Iberian Peninsula	Spain, Granada, road from Granada to Prado Llano, Prados del Aire, 03-VII-2008, *Garcia-Jacas & López-Pujol* (BC)
Centaurea nevadensis Boiss. & Reut.	B	Iberian Peninsula	Spain, Teruel, Guadalaviar, vora el riu, al començament del poble, 01-VII-2009, *López-Alvarado & López-López* (BC)
Centaurea nigra L.	A	Eurosiberian	Spain, Pontevedra, Caldelas de Tui, Os Baños, 04-VII-2009, *Hilpold AH20093038, Garcia-Jacas & Vilatersana* (BC)
Centaurea nigra L.	B	Eurosiberian	Spain, Lugo, Becerreá, 03-VII-2009, *Hilpold AH20093025, Garcia-Jacas & Vilatersana* (BC)
Centaurea nigra L.	C	Eurosiberian	Spain, Lleida, Val d'Aran, aigüestortes, Estanh Major de Colòmers, 12-IX-2009, *Hilpold AH20095129* (BC)
Centaurea nigra L.	D	Eurosiberian	Spain, Navarra, Isaba to Utárroz, 19-VIII-2009, *Hilpold AH20095002 & Kosisnsky* (BC)
Centaurea nigra L.	E	Eurosiberian	Spain, La Coruña: near Carballo, 03-VIII-1992, *Garcia-Jacas & Susanna 1446* (BC)
Centaurea nigra subsp. *carpetana* (Boiss. & Reut.) Nyman		Iberian Peninsula	Spain, Cáceres, Sierra de Gredos, Hervás, 09-VII-2009, *Hilpold AH20093088, Garcia-Jacas & Vilatersana* (BC)
Centaurea nigra subsp. *rivularis* (Brot.) Cout.		Iberian Peninsula	Portugal, Boticas, junto Sapiaos, 650 m, buma da estrada, 18-VII-2002, *Espirito-Santo* (BCN20446)
Centaurea nigrescens Willd.		Eurosiberian	Slovenia, next to Srednia Vas v. Bohinju, E of Bohinsko Jezero, 16-X-2009, *Hilpold AH20096025*
Centaurea pangaea Greuter & Papan.		Greece	Greece, Kavalas, Pangaion Mt., E. part. Along road from the village of Akrovounion to the ERT Station, 1250 m, 19-VII-1979, *Strid 15747* (pers. herb.)
Centaurea parilica Stoj. & Stef.	A	Greece, Bulgaria	Greece, Makedonia, Limen, Serres et Dhrama: mons Orvilos, in latere meridionali verticis principis usque ad cacumen, 1750-2212 m, 21-VIII-1978, *Greuter16673* (MA540713)
Centaurea parilica Stoj. & Stef.	B	Greece, Bulgaria	Greece, Drama, mons Orvilos, supra pagum Kataphyton, Grècia, 06-VIII-1978, *Tzanoudakis & Georgiadis* (LD1423377)
Centaurea pectinata L.	A	France, Spain	Spain, Barcelona: Montseny, Santa Fe to Sant Marçal, 1300-1400 m, 06-VII-1994, *Garcia-Jacas & Susanna 1469* (BC)
Centaurea pectinata L.	B	France, Spain	France, Languedoc-Rousillon, Pyrénées-Orientales, Canigó, 200 m SE Marialles refuge, 14-VI-2009, *Hilpold AH20092216, Roquet & Nogué* (BC)
Centaurea phrygia L. s. l.		Bulgaria	Bulgaria, Rila Mountains, road to Rila Monastery, 23-VII-2010, *Figueroa & López-Alvarado* (BC)
Centaurea phrygia L. subsp. *phrygia*		Eurosiberian	Romania, Distr. Cluj, Valea Morii (*Al Borza Botanic Garden 1897*)
Centaurea phrygia subsp. *moesiaca* (Urum. & J. Wagner) Hayek		Bulgaria	Bulgaria, Kyustendil District, Dupnitsa, Bistrica, 23-VII-2010, *Figueroa & López-Alvarado* (BC)
Centaurea phrygia x *C. jacea*			Bulgaria, Pazardjik District, Velingrad, road margins 10 km from Yundola to Kostenec, 22-VII-2010, *Figueroa & López-Alvarado* (BC)
Centaurea rhaetica Moritzi	A	Switzerland, Italy	Italy, Lombardia, Brescia, Toscolano-Maderno, Valle di Campiglio, sinistra idrografica, loc. Fiogarie, 02-VI-2005, *Galasso* (MSNM39961)
Centaurea rhaetica Moritzi	B	Switzerland, Italy	Italy, Lombardia, Bergamo, Valbondione, Baita di Mezzo, Vizna, Varza, 31-VII-1994, *Galasso* (MSNM32734)
Centaurea stenolepis Kerner subsp. *razgradensis* (Velen.) Stoj. & Acht.	A	Bulgaria	Bulgaria, Pazardjik District, Velingrad, Cherna Mesta, near Yundola village, 22-VII-2010, *Figueroa & López-Alvarado* (BC)
Centaurea stenolepis Kerner subsp. *razgradensis* (Velen.) Stoj. & Acht.	B	Bulgaria	Bulgaria, Varna, c. 48 Km NE of Burgas, c. 5 km W from Obsor along minor road, 50 m, 9-VIII-1988, *Jury & Thorton-Wood 9590* (MA452594)
Centaurea tossiensis Freyn & Sint.		Turkey	Turkey, Kastamonu: Tosya, *Bağcı* (KNYA)
Centaurea triamularia Aldén		Greece	Greece, Trikalon, Mt. Pachtouri, 5 km SSW of Athamania. Between Katafili and Soufli, 31-VII-1974, *Aldén 4792* (LD1085092)
Centaurea tripontina López-Alvarado, Sáez, Filigheddu, Guardiola & Susanna	A	Iberian Peninsula	Spain, Lleida, Organyà, Congost de Tresponts, 15-V-1972, *P. Motserrat & Villar* (JACA116472)
Centaurea tripontina López-Alvarado, Sáez, Filigheddu, Guardiola & Susanna	B	Iberian Peninsula	Spain, Lleida, Organyà, Congost de Tresponts, 21-VIII-2008, *L. Sáez* (LSG pers-BCB)
Centaurea triumfetti All. subsp. *stricta* (Waldst. & Kit.) Dostál (C. mollis in Garcia-Jacas et al., 2006)		E Europe	Ukraine, Podolia, Lysa Hora, 2 km E of Vilshanitsa near Zolochiv, 05-VI-2000, *Boratyński & Romo 0506D* (BC)

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CENTAUREA SECT. *PHRYGIA* (COMPOSITAE) IN THE IBERIAN PENINSULA: A MORPHOMETRIC AND PHYLOGEOGRAPHIC STUDY

Centaurea sect. *Phrygia* (Compositae) in the Iberian Peninsula: a morphometric and phylogeographic study

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ABSTRACT

Morphologically related Iberian species belonging to sect. *Phrygia* have historically presented many taxonomic problems as they have few good discriminant qualitative characters and their quantitative ones can vary. Furthermore, some Catalonian Pre-Pyrenees individuals, found at Tresponts gorge, which were misidentified as *C. emigrantis* and as *C. pectinata*, have been recently described as *C. tripontina*. This lack of evident morphological characters has led to a great variety of classification propositions. In order to clarify the systematics of Iberian *Phrygia* species, we used morphology, with morphometric and micromorphological approaches, as well as molecular data. Four species and one aggregate can be clearly differentiated: *C. antennata* aggr., *C. emigrantis*, *C. janeri*, *C. pectinata*, and *C. tripontina*.

Keywords—*Centaurea,* Iberian Peninsula, section *Phrygia*, morphometrics, phylogeography.

INTRODUCTION

Centaurea L. is one of the largest genera of the Asteraceae family with around 250 species (Susanna and Garcia-Jacas, 2007) distributed mainly in the Mediterranean region and SW Asia. Taxonomic treatment of *Centaurea* has always been complicated and many attempts of classification were made (Wagenitz and Hellwig, 1996; Garcia-Jacas, et al. 2000, 2001, 2006; Susanna and Garcia-Jacas, 2007). One of such groups is the section *Phrygia* Pers., which is formed by morphologically related species with entire leaves and plumose-like recurved bract's appendages.

In *Flora Europaea* section *Phrygia* (reported as sect. *Lepteranthus* nom. inval.) is recognized to be composed of around 19 species (Dostál, 1976), divided at the same time into different numbers of subspecies. In the Iberian Peninsula, sect. *Phrygia*, is represented by nine taxa, some of which were not recognized by Dostál (1976): *Centaurea antennata* Dufour, *C. caballeroi* Font Quer, *C. corcubionensis* M. Laínz, *C. emigrantis* Bubani, *C. hyssopifolia* Vahl, *C. janeri* Graells, *C. linifolia* L., *C. pectinata* L. and *C. tripontina* López-Alvarado, L. Sáez, Filigheddu, Guardiola and Susanna. In particular, seven of these Iberian taxa present a controversial taxonomic status due to the different treatments they have received by different authors: *C. antennata*, *C. caballeroi*, *C. emigrantis*, *C. janeri*, *C. linifolia*, *C. pectinata* and *C. tripontina.* The morphological similarity and the different specific criteria applied by different botanist have led to confusion in its taxonomic treatment. For example, individuals occurring in the western Catalonian mountains of Montsec (Pre-Pyrenees), have been classified as either *C. emigrantis*, a taxon of uncertain status related to *C. janeri* (Dostál, 1976), as a subspecies of *C. uniflora* Turra (Bolòs & Vigo, 1996) or as a well defined species (Romo, 1989; Sáez et al., 2010). However, *C. janeri*, recognized at species level by Dostál (1976), was considered synonymous of *C. uniflora* subsp. *emigrantis* by Bolòs

and Vigo (1996). Individuals usually identified as *C. janeri* occur in three different geographical areas corresponding to three different subspecies: *C. janeri* subsp. *janeri* mainly in the Sierra de Ávila in the center of Iberian Peninsula, *C. janeri* subsp. *gallaecica* on serpentines in Galicia (North-western Spain), and *C. janeri* subsp. *babiana* in Picos de Europa (León and Asturias provinces, Northern Spain). On another front, some Catalonian Pre-Pyrenees individuals found in Tresponts gorge which were miscidentified as *C. emigrantis* and as *C. pectinata*, have been recently described as a new species, *C. tripontina* (López-Alvarado et al., 2011). Another taxon with controversial status is *C. caballeroi,* which was described with species rank (Font Quer, 1918), brought down to subspecies level as *C. linifolia* subsp. *caballeroi* by Bolòs and Vigo (1996) and overlooked by Dostál (1976). This taxon occurs in the north-east of the Iberian Peninsula, southwards of *C. linifolia*.

These taxonomic problems may be attributed to polyploidy, reticulate evolution or shared ancestry as demonstrated in a recent phylogenetic survey of sect. *Phrygia* (López-Alvarado, in prep.). Furthermore, other studies have confirmed the great importance of these phenomena in the evolution of the group (Vanderhoeven et al., 2002; Koutecký, 2007 and references therein), which are favoured by the constant chromosome base number $x = 11$ (Hellwig, 2004). However, such complexity in evolutionary processes could be partially overcome by the study of cpDNA, which is free of recombination and is only maternally inherited (Schaal, 1998). These markers can be useful to study the reticulate evolution above the species level avoiding the problems of recombination and also can indicate gene flow between species if haplotypes of different origin are found in a single population. Furthermore it is a good source of systematic information without the prejudices of taxonomic criteria (Schaal et al., 1998) by revealing the ancestor-descendant relationships.

Otherwise, an accurate study of the morphology with a morphometric approach gives complementary information which can help to discern if morphological discontinuities are related to ancestor-descendant relationships. Although it has been argued that some nucleotide changes in a few genes can lead to great changes in morphology (Schaal et al., 1998 and references therein), thus discrediting the use of morphology in systematic study, if the study of morphological traits is integrated with molecular one and it is based in as many characters as possible, morphology can be a great source of valuable systematic information.

Therefore, in order to clarify the systematics of conflicting Iberian *Phrygia* species an integrative study with preliminary data was made. We have used morphological, phytodermological and molecular data.

MATERIALS AND METHODS

Plant material

Samples for morphometric and phytodermological studies were chosen from conflicting Iberian species of sect. *Phrygia*. The studied species were *C. antennata*, *C. caballeroi*, *C. emigrantis*, *C. janeri*, *C. linifolia*, *C. pectinata*, *C. tripontina* and *C. uniflora*. Due to the morphological cohesiveness of *C. janeri* subspecies, they were considered as *C. janeri* in broad sense for morphometric and phytodermology analysis. Morphological characters were measured on herbarium specimens. The list of studied material is given in Appendix 2.1 and Appendix 2.2.

Sampling for molecular analysis was focused on the following species of section *Phrygia* : *C. antennata*, *C. caballeroi*, *C. corcubionensis*, *C. emigrantis*, *C. janeri*, *C. linifolia*, *C. pectinata* and *C. tripontina*. Unlike morphological analysis, the three subspecies of *C. janeri* have been considered separatedly in molecular analysis in order to decipher the ongoing evolutionary processes. *Centaurea corcubionensis* was chosen

as outgroup species since has been considered a relict on the basis of ecology and distribution features (López-Alvarado, pers. observ.). Ten individuals per population were sampled, except for population ANT1 (Table 4) of *C. antennata* for which seven individuals were sampled, each one corresponding to a herbarium sheet. Not all herbarium sheets of ANT1 belong to the same population. Nevertheless, since it is not the aim of the work to study population genetic diversity, and since all the accessions are collected from close geographic areas, it has been considered as one population in phylogeographic analyses. The list of studied material for molecular analysis is given in Table 4.

Morphometric analysis

A total number of 87 herbarium individuals were reviewed and used for morphometric multivariate analysis. Twenty characters were chosen for their taxonomic discriminatory value (Table 1). Ten of them are quantitative continuous (leaf length [Leaf Lgth], leaf width [Leaf Wdth], capitulum length [Cap Lgth], capitulum width [Cap Wdth], bract length [Bract Lgth], bract width [Bract Wdth], appendage length [Apdg Lgth], appendage width [Apdg Wdth], fimbriae length [Fimb Lgth] and stem length [Stem Lgth]) and one is quantitative discrete (fimbriae number [Fimb Num]), five are ratios calculated between these and other not included quantitative characters, and four are qualitative (leaf shape [Leaf Shp], leaf indumentum [Leaf Pub], appendage color [Apdg Col] and leaf margin [Margin]). The normality of each character was tested using Shapiro-Wilks statistics. Leaf Lght, Leaf Wdth, Cap Lght, Cap Wdth, Apdg Lgth, Leaf Ratio, and AL/NF Ratio were log-transformed and Apdg Wdth was square root transformed in order to improve their distribution.

Phytodermology

Leaf samples were removed from herbarium specimens. Sometimes it was possible to study more than one specimen per sheet. The list of the studied material is detailed in Appendix 2. Leaves were boiled in water for 10 minutes to rehydrate them, followed by maceration in a 50% commercial sodium hypochlorite solution (40g/l) for about 24 hours (depending on the hardness of the material). After maceration, the adaxial and abaxial epidermises were removed from the mesophyll using a scalpel. Both epidermises were washed with distilled water, stained in 1% methyl green solution and mounted in glycerine gel. Slides for light microscope observation (OLYMPUS BX51) were prepared for each species from two leaves of five different individuals from different locations. Stomatal and cell frequency, Stomatal Index, and stomatal and trichome type were determined by counting on five different microscope fields. We have also measured the trichome length, considering only the stalk of trichoma, not the terminal cell which usually appears broken.

Statistical analysis

All morphometric analyses were computed using SPSS 15.0 (SPSS, 2006). Principal Component Analysis based on a correlation matrix (PCA; Sneath & Sokal, 1973) and Discriminant Analysis (DA) were performed using individuals as OTUs (operational taxonomic units). PCA was used to reduce the overall variation of the 20 examined characters into three new uncorrelated components, which were used to depict morphological relationships among individual specimens. DA, a statistical analysis that maximizes differences between groups and makes linear relationships among quantitative variables, was conducted over rescaled variables derived from PCA to reveal the robustness of predetermined groups. Micromorphological data were not included in the multivariate analysis as it was the impossible to obtain

micromorphological data for all of the 87 specimens reviewed in the morphometric study.

DNA extraction, amplification and sequencing

Genomic DNA was extracted following the 2x CTAB method of Doyle and Doyle (1987) as modified by Cullings (1992) and Tel-Zur et al. (1999) from silicagel-dried leaves collected in the field. For *C. antennata* and *C. caballeroi*, herbarium material was used. The double-stranded cpDNA *trnL*$^{(UAG)}$*-rpl32* region was amplified using rpl32F as forward primer and trnL$^{(UAG)}$ as the reverse primer (Shaw et al., 2007). The profile used for cpDNA amplification included a hot start at 95 ºC for 3 min. Then 30 amplification cycles were carried out under the following conditions: 95 ºC for 40 s, 54 ºC for 40 s and 72 ºC for 1 min, with an additional extension step of 10 min at 72 ºC. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) and ExoSAP-IT (USB Corp., Cleveland, OH, USA). The *trnL*$^{(UAG)}$*-rpl32* region was sequenced with trnL$^{(UAG)}$ as the reverse primer. Direct sequencing of the amplified DNA segments was performed using a BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol at the University of Florida ICBR Core Facility using an ABI 3730xl DNA analyzer (Applied Biosystems).

cpDNA analysis

Sequences for phylogenetic analyses were edited using BioEdit (Hall, 1999) and aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990). A phylogenetic network of cpDNA haplotypes was constructed under a Statistical Parsimony approach with software TCS 1.21 (Clement et al., 2000).

RESULTS

Morphometric multivariate analysis

For the PCA, the first three components were selected using slope change on a components scree plot. All three components explain 52.41% of the total variance (PC1 = 26.85 %; PC2 = 15.15 % and PC3 = 10.41 %). The factors that contribute most to the first PC axis are appendage characters (Fimb Num, Apdg Col, Apdg Lgth and Apdg Wdth), the Cap Lgth, the Stem Lgth and also leaf variables (Leaf Wdth and Leaf Shp) as positive values and the bract lenght/appendage length ratio (BL/AL ratio) and the appendage length and fimbriae number ratio (AL/NF ratio) as negative values. Variables contributing to the second component are the appendage length (Apdg Lgth) and AL/NF Ratio as positive values and BL/AL ratio and capitulum width (Cap Wdth) as negative values. The Stem Lgth and leaf characters (Leaf pub, Leaf Wdth and Margin) are the variables that contribute most to the third component as positive values, and the capitulum lenght (Cap Lgth) as a negative value (Table 2). We have represented the scatterplot of 87 specimens against the first three principal component axes (Figure 1). The scatterplot (Fig. 1) shows two clear separated groups, one formed by *C. uniflora* individuals and other by Iberian species. Nevertheless a grouping of specimens into relatively identifiable discrete groups can be done although represented very closely. Moreover, most of observed overlap in the figure it is due to the limitations to represent 3D graphics in a paper support.

The first two canonical discriminant functions explain the 77.7 % of the variance. The first function is mostly correlated with the second PC while the second function is mostly correlated with first PC. The cross-validation procedure, which tests the consistency of discriminant functions to separate cases in previously formed groups, classifies 92.0 % of cases correctly. One individual of *C. emigrantis* was misclassified as *C. caballeroi*, one individual of *C. caballeroi* as *C. linifolia*, three individuals of *C.*

linifolia as *C. caballeroi* and finally one individual of *C. antennata* as *C. linifolia*. No *C. pectinata*, *C. tripontina* or *C. uniflora* individuals were misclassified. The DA scatterplot represents the 87 individuals against the first two discriminant functions (Fig. 2).

Phytodermology

Micromorphological characters, like macromorphological or molecular, are of systematic importance as has been demonstrated in many plant groups (Metcalfe and Chalk, 1950, 1979) as well as in the Compositae family (Ogundipe and Adegbite, 1991). Despite this, the micromorphological quantitative characters studied have a large degree of variance within the same species, surely due to small size, and therefore do not allow the differentiation of taxa easily (Table 3). Nevertheless, the trichomas provide valuable qualitative information for taxonomic classification.

We have classified the different kinds of trichomas found into two main groups. The first group is composed of pluricellular uniseriate flaccid trichomas with a basal cell followed by some walled cells (usually one) and a long terminal whip-like cell (Fig. 3; A, B and D). This type of trichome is present in *C. emigrantis*, *C. janeri* and *C. pectinata* (*C. pectinata* sometimes has thicker walled cells between the basal and whip-like terminal cell, but never as thick as in the second trichome type). Macroscopically, this kind of trichome gives these species their typical pubescent appearance. The second group is formed by pluricellular uniseriate rigid conical trichomas with thicker walled cells followed by a shorter terminal whip-like cell (usually deciduous) (Fig. 3; C, E, F and G). This second type is present in *C. antennata*, *C. caballeroi*, *C. linifolia*, *C. tripontina* and *C. uniflora*. Unlike the first kind of trichome, this one gives the leaf a glabrescent or even hispid aspect, except in *C. uniflora*, which has a pubescent appearance, as it has a high density of trichomas that are less rigid, and it does not tend

to lose the terminal whip-like cell. We have provided the length of the stalk of the trichomas excluding the terminal whip-like cell; however, we have not given the trichome length for *C. emigrantis* and *C. janeri* because, due to their fragility, it was difficult to find whole trichomas that had not collapsed.

cpDNA analysis

Preliminar statistical parsimony analysis of cpDNA has revealed 26 haplotypes for the *trnL*$^{(UAG)}$*-rpl32*. Among the 26 haplotypes (Fig. 4), the haplotype A, which is found among different taxa distributed far away from each other, was chosen as the central haplotypes in the network. In particular this haplotype is present in *C. corcubionensis*, which has been considered the outgroup species. This is suggesting that haplotype A represents the ancestral cpDNA gene pool of Iberian species from sect. *Phrygia* included in present study. The parsimony network also shows three clear lineages derived from haplotype A. The first one (Fig. 4, green colors), contains haplotypes belonging all to *C. janeri* subspecies forming a western Iberian lineage. The second one (Fig. 4, violet colors), is formed by species with clear Pyrenean affinities, with the exception of one population which belongs to *C. caballeroi* expected to be placed with Levantine haplotypes. The third lineage (Fig. 4, blue colors) contains populations of the Levantine species: *C. linifolia*, *C. antennata* and *C. caballeroi*. A fourth line of haplotypes was only found in one population of *C. janeri* subsp. *janeri* from La Rioja can be also defined (Fig. 4, yellow colors). Should to be note that La Rioja population present haplotypes which seems to have different evolutionary histories. The haplotype network has revealed also two private haplotypes (Fig. 4, grey colors), one found exclusively in *C. corcubionensis* (haplotypes C) and derived by one nucleotide change from haplotype A, and the haplotype B, only present in one french population of *C. pectinata* and also derived from haplotype A by one nucleotide change.

DISCUSSION

Morphology

The morphometric approach, for both multivariate analysis and micromorphology, indicates that an analytic point of view is a suitable solution for the classification for most taxa included in the study except for *C. antennata*, *C. caballeroi* and *C. linifolia*. The data presented show that most of studied taxa have good defining characteristics to be considered as separate entities. Multivariate analysis, PCA and DA, show discontinuities among sets of individuals and cluster individuals into five well defined groups which broadly agree with the proposed species except in the case of *C. antennata*, *C. caballeroi* and *C. linifolia* which appears on both analysis as not fully separated. Although morphological discontinuities revealed by morphometric analysis for *C. antennata*, *C. caballeroi* and *C. linifolia* are not as clear-cut as in other cases, there are some qualitative and quantitative differences, like leaf shape and leaf size, as well as other characters that are difficult to code for the morphometric analysis, like growth patterns or the kind of leaf layout on the stem, which allows to discriminate them, nevertheless further research involving these three species is needed to achieve the best possible classification. Micromorphology does not improve the separation among three taxa, *C. antennata*, *C. linifolia* and *C. caballeroi*; only differences in trichome size, longer for *C. antennata*, and not conclusive stomatal length differences, which could point to different ploidy levels, are noteworthy. Moreover, the trichome type is very similar and is not useful for taxonomic discrimination. In addition to the above, the cpDNA analysis has revealed that they form a clear lineage, indicating that there is an ongoing speciation process (Fig. 4). Probably the best solution for these species is the treatement as aggregate until more individuals and populations are studied.

The morphometric study (Fig. 1, Fig. 2) allows *C. emigrantis* to be accurately differentiated from *C. uniflora*, contrary to Bolòs and Vigo (1996), and also from *C. janeri* as suggested by Dostál (1976) and Romo (1989); and contrary to the proposal by Bolòs and Vigo (1996). Only one individual of *C. emigrantis* was misclassified as *C. caballeroi* in DA analysis. Such classification surely is due to similar quantitative traits with regard to *C. caballeroi* at this particular individual, nevertheless, quantitative characters and also trichome type allows a positive identification. In addition to the above, micromorphology provides some interesting information. The indumentum differences, as shown in Fig. 3, greatly support the separation of *C. emigrantis* and *C. uniflora.* Micromorphological data of *C. emigrantis* and *C. janeri* do not provide good characters to improve their separation; however, in this case morphometric and molecular data are very useful for separating these taxa (Fig. 1, 2 and 4). Therefore, the morphological and molecular data provide enough differences regarding to the rest of the taxa to support the recognition of *C. emigrantis* as independent species, a status doubtfully accepted by Dostál (1976).

Morphological data of *C. tripontina* individuals suggest that they show enough differences to be considered at specific level. Principal Component Analysis and Discriminant Analysis (Fig. 1, Fig. 2) show a good definition of *C. tripontina* as a separate taxon, which is located in the middle of the PCA scatterplot. This central position on the PCA representation could explain why J. Prudhouse and P. Montserrat & L. Villar (in sched.) identified the plant as *C. emigrantis* and *C. pectinata* respectively. This morphological evidence could suggest hybridization, but it is interesting to note that the intermediacy in the PCA analysis does not necessarily indicate hybridization, but rather it is the intermediacy in each of the characters studied that indicates a possible hybrid origin (Wilson 1992), and only if the hybrid is not the

result of introgreded lines (Nieto Feliner et al. 2001). Analyzing quantitative characters individually using box-plots (data not shown) did not reveal clear intermediacy between the characters of the new species and any other of the studied species. Furthermore, there are also useful qualitative characters to discriminate *C. tripontina* from C. *emigrantis* and *C. pectinata* (López-Alvarado et al., 2011). Finally, as revealed in cpDNA analysis, *C. tripontina* does not share haplotypes either with surveyed populations of *C. emigrantis* or *C. pectinata*. Moreover, *C. tripontina*, appears as having ancestral haplotypes regarding to *C. emigrantis*.

Chloroplast data

Considering molecular data only, cpDNA has revealed three main lineages in the Iberian Peninsula. The first one, the western Iberian lineage, has confirmed the natural relationships of the three *C. janeri* subspecies which in a molecular survey of sect. *Phrygia* using nrDNA molecular markers was hidden by low phylogenetic resolution and introgression phenomena with taxa belonging to *C. nigra* aggr. (López-Alvarado et al., in prep.). Furthermore, chloroplast data confirms the importance of gene flow, pointed by the aforementioned phylogenetic survey, by the presence of haplotypes of suitable different origin in one population of *C. janeri* subsp. *janeri* from La Rioja (Fig. 4). Such data fit also with the particular morphology found at individuals of this population which suggest hybridization, surely with some taxa of *C. jacea* aggr. (López-Alvarado, pers. observ.). Finally, molecular data confirms definitively that, unlike proposed by Dostál (1976) ans Bolós and Vigo (1996), *C. janeri* form and independent evolving lineage with regard to *C. emigrantis*.

A second lineage can be defined as containing *C. tripontina* and *C. emigrantis*, as has been exposed above, they show an ancestor-descendant relationship. Nevertheless, the molecular survey of sect. *Phrygia* (López-Alvarado et al., in prep.)

has suggested gene flow between both species by the presence of two different nrDNA ETS copies within the genome of *C. emigrantis*, one clustered in phylogenetic analysis with *C. tripontina* and the other with *C. linifolia*. However, *C. emigrantis* does not present also haplotypes related to *C. linifolia*. Therefore, caution is needed with regard to a possible hybrid origin for *C. emigrantis*. Firstly, there is the possibility that ETS copies found in the phylogenetic survey are due to introgression rather than to hybridization. Secondly, as some populations of *C. linifolia* has the ancestral haplotype and it is one of the most widespread taxa of the group, it could be the possibility that *C. linifolia* is most ancient lineage of the group. Therefore, different copies found at *C. emigrantis* would be related with ancestral polymorphysm sharing and therefore present also at the rest of the taxa of this group. However these copies have not been found in cloning efforts, surely due to multiple array nature of nrDNA (Álvarez and Wendel, 2003), and the low intensity of cloning in this particular group in López-Alvarado et al. (in prep.) survey. This last argument could be confirmed by the grouping of one haplotype of *C. caballeroi*, a taxon clearly related to *C. linifolia*, withint the *C. emigrantis*-*C. tripontina* lineage, since not evidences of gene flow with *C. tripontina* were found (López-Alvarado et al., in prep.). In any case, caution is needed until more evidences are achieved.

It is also interesting to comment that the two studied populations of *C. pectinata* have yielded two different lineages. The first one, corresponding to the french population, derives directly from haplotype A. However, population from Montseny Mountain, NE Spain, is placed as a particular lineage derived from the same hypotetical ancestor than *C. janeri* and *C. emigrantis*-*C. tripontina* lineages. As *C. pectinata* is a widespread and morphologically variable species, more population sampling is needed

also including other species with which it can be in contact due to its eurosiberian distribution such as *C. jordaniana* Godr. & Gren., *C. nigra* L. aggr. or *C. jacea* L. aggr.

Finally, there is a third lineage including haplotypes belonging to *C. antennata* and *C. linifolia*. It should to be note that populations of *C. antennata*, *C. caballeroi* and *C. linifolia* present a great diversity of haplotypes: some populations present haplotypes belonging to this third lineage, other popuations has the haplotype A, suspected to be the ancestral one, and finally one population of *C. caballeroi* has an haplotype related to *C. tripontina* and *C. emigrantis*. Furthermore, if we consider the three species as one taxon, the *C. antennata* aggr., it presents the largest area of distribution among studied species and some morphological continuity. All this evidences could suggest that *C. antennata* aggr. harvest the oldest genetic pool among the iberian studied species. At any rate, an increase in population sampling and in number of markers surveyed is necessary to improve the degree of phylogeographic information.

As a conclusion, the best taxonomical arrangement for Iberian species from section *Phrygia* should take in consideration the evolutionary processes reveald by molecular data but also the morphological identity of every taxon. Therefore, we propose to recognize four species clearly differentiated: *C. emigrantis*, *C. janeri*, *C. pectinata*, and *C. tripontina*. Otherwise, the arragement of three related taxa *C. antennata*, *C. caballeroi* and *C. linifolia* should be a compromise between differences and similarities revealed in the present study, therefore we propose to clasify three taxa within a *C. antennata* aggr. until a deepest systematic review.

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TABLES

Table 1. Acronyms used for morphometric multivariate analysis characters.

ACRONYM	*Character description*
Leaf Lgth	Leaf lenght
Leaf Wdth	Leaf width
Leaf Shp	Leaf shape: cuneate = 1, liniar = 2, liniar-lanceolate = 3, lanceolate = 4, ovate = 5
Leaf Pub	Leaf hair: no hair = 0, rigid thick walled trichome = 1, flaccid thin walled trichome = 2
Margin	Leaf margin type: entire = 1, entire convolute = 2, dentate/lobulate = 3, entire/dentate = 4
Cap Wdth	Capitulum width
Cap Lgth	Capitulum lenght
Stem Lgth	Stem length
Bract Wdth	Bract width
Bract Pub	Bract Pubescence
Apdg Lgth	Appendage length
Apdg Wdth	Appendage width
Apdg Col	Appendage colour: pale brown = 1, brown = 2, dark brown = 3, blackish = 4, dark base = 5
Fimb Num	Fimbriae number
Fimb Lgth	Frimbriae lenght
Cap Ratio	Capitulum Length/Cap Wdth
Bract Ratio	Bract length/ Bract Width
BL/AL Ratio	Bract length/ Apdg Lgth
Leaf Ratio	Leaf Length/ Leaf Wdth
AL/NF Ratio	Apdg Lgth/Fimb Num

Table 2. PCA-eigenvectors showing the correlation of characters used and the three principal components extracted.

Components Matrix			
	Principal Components		
	1	2	3
Leaf Lgth	0,451	0,301	0,379
Leaf Wdth	0,662	0,155	0,509
Cap Wdth	0,452	-0,478	-0,239
Apdg Lgth	0,642	0,669	-0,183
Cap Lgth	0,681	-0,213	-0,499
Apdg Wdth	0,665	-0,465	-0,113
Stem Lgth	0,628	-0,098	0,533
Fimb Num	0,947	-0,014	-0,115
Fimb Lgth	0,525	0,554	-0,166
Leaf Shp	0,627	0,31	-0,141
Leaf Pub	0,327	0,006	0,556
Bract Pub	0,06	0,093	-0,01
Apdg Col	0,684	-0,449	0,081
Bract Wdth	-0,204	0,065	0,223
Cap Ratio	-0,254	0,544	0,146
Bract Ratio	0,466	0,068	-0,283
BL/AL Ratio	-0,401	-0,629	0,317
Leaf Ratio	-0,24	0,09	-0,259
AL/NF Ratio	-0,354	0,661	-0,308
Margin	0,034	0,456	0,502

Table 3. Median values and standard deviation (S.D.) of: stomatal frequency (StoFr, mm^2), cell frequency (CellFr, mm^2), stomatal length (StoL, µm) and trichome length (not for *C. emigrantis* and *C. janeri*, see Results; µm) and Stomatal Index (Sto In = StoFr/StoFr+CellFr).

Species	Leaf Face	Sto Fr (mm^2)		Cell Fr (mm^2)		Sto Lgth (µm)		Tric Lgth (µm)		Sto In
		Mean	S. D.	Mean	S. D.	Mean	S. D.	Mean	S. D.	
C. antennata	adaxial	48,77	36,21	569,03	152,19	34,46	3,07	431,56	82,29	0,079
	abaxial	72,12	37,67	681,44	290,47	34,17	2,61			0,096
C. caballeroi	adaxial	46,72	11,17	574,22	176,53	34,25	1,4	228,92	89,63	0,075
	abaxial	47,04	22,42	472,06	135,49	37,67	2,36			0,091
C. emigrantis	adaxial	79,31	30,84	1083,47	346,67	28,69	3,5			0,068
	abaxial	63,57	25,8	913,2	371,86	30,24	4,77			0,065
C. janeri s. l.	adaxial	53,51	22,47	580,45	183	40,31	8,7			0,084
	abaxial	43,2	14,51	544	164,87	40,45	8,4			0,074
C. linifolia	adaxial	47,21	6,72	542,61	21,12	36,32	0,43	242,3	59,98	0,080
	abaxial	42,32	12,59	492,85	58,47	40,69	3,36			0,079
C. pectinata	adaxial	45,44	27,35	651,43	94,94	32,66	3,3	77,71	17,12	0,065
	abaxial	56,39	25,82	671,16	112,54	33,59	4,16			0,078
C. tripontina	adaxial	73,44	37,01	741,01	280,76	31,93	3,04	75,5	31,36	0,090
	abaxial	59,03	15,32	627,09	201,46	34,22	4,44			0,086
C. uniflora	adaxial	78,96	33,65	1048,63	280,4	30,27	4,6	478,15	186,59	0,070
	abaxial	72,56	40,78	870,44	208,77	30,44	3,16			0,077

Table 4. Origin of material used on cpDNA parsimony network.

Species	Haplotype	Pop Code	Voucher
C. antennata Dufour	G, J	ANT 1	Spain, Alicante, Villena, Sierra de Salinas, 950 m, 04.VII.1993, *Rigual, Crespo De la Torre et al.* (ABH6477)
	H		Spain, Alicante, Villena, El Castellar, 520 m, 30.V.1994, *Alonso & Vargas* (ABH10678)
	G		Spain, Alicante, Salinas, Barranco de Salaines, 700 m, 29.V.2004, *Monerris & Monleón* (ABH48513)
	K, L		Spain, Alicante, Pinoso, Sierra del Reclot, Alto del Algarejo, 800 m, 30.V.1997, *Navarro & Crespo.* (ABH35278)
	G		Spain, Alicante, Monóvar, La Herrada, 760 m, 04.V.1997, *Ortega* (ABH34877)
	A	ANT 2	Spain, Valencia, Serra, Porta Coeli Monastery, to Font del Berro, 10.VI.2011, *Figueroa & López-Alvarado* (BC)
C. caballeroi Font Quer	A	CAB 1	Spain, Tarragona, Ulldecona, Serra del Montsià, Mas de Comú, 630 m, 06.IX.2000, *Arán & Tohá* (ABH44553)
	A		Spain, Tarragona, Ulldecona, Serra del Montsià, Mas de Comú, 630 m, 07.VI.2002, *Arán 5180* (ABH46686)
	W	CAB 2	Spain, Tarragona, Ports de Beceit, Portell de Caro, 31TBF7722, 1050 m, 02.VII.2008, *Guardiola & L. Sáez* LS-6884 (L. Sáez herb. pers.-BCB)
C. corcubionensis M. Laínz	A, B	COR 1	Spain, A Coruña, Carnota, O Pindo, path margins, 08.V.2011, *López-Alvarado & López-López* (BC)
C. emigrantis Bubani	Z	EMI 1	Spain, Lleida, Àger, near Corçà, road margins, 21.VI.2009, *Figueroa & López-Alvarado* (BC).
	Y	EMI 2	Spain, Lleida, Llimiana, along the road LV-9121 to Llimiana, 21.VI.2009, *Figueroa & López-Alvarado* (BC).
C. janeri subsp. *babiana* M. Laínz	Q	BAB	Spain, León, Sena de Luna, near Rabanal chapel, 13.VII.2009, *Figueroa & López-Alvarado* (BC)
C. janeri subsp. *gallaecica* M. Laínz	O, P	GAL 1	Spain, A Coruña, Melide, Furelos, path margins, 15.VII.2009, *Figueroa & López-Alvarado* (BC)
	T, U	GAL 2	Spain, Pontevedra, Agolada, Basadre, scrubs at road margin, 16.VII.2011, *Figueroa & López-Alvarado* (BC)
C. janeri Graells subsp. *janeri*	R	JAN 1	Spain, Salamanca, between El Casarito and el Cabaco, road margins, 14.VII.2009, *Figueroa & López-Alvarado* (BC)
	D, E, F, S	JAN 2	Spain, La Rioja, Haro, San Felices de Bilibio, path margin, 14.VII.2011, *Figueroa & López-Alvarado* (BC)
C. linifolia L.	I	LIN 1	Spain, Barcelona, Sitges, Les Botigues, road margins, 22.VI.2010, *López-Alvarado* (BC)
	G	LIN 2	Spain, Lleida, Agramunt, Serra de l'Almenara, 05.VI.2011, *Figueroa & López-Alvarado* (BC)
	A	LIN 3	Spain, Zaragoza, Leciñena, Sierra de Alcubierre, road margins, 05.VI.2011, *Figueroa & López-Alvarado* (BC)
C. pectinata L.	N	PEC 1	Spain, Barcelona, Montseny, 2 Km E Figaró, 0.4 Km S of San Cristófol de Montugues, 04.IV.2010, *Hilpold AH20090061 & Kosinski* (BC)
	C	PEC2	France, Languedoc-Roussillon, Gard, Le Vigan, road to Col du Minier, 23.VI.2011, *Figueroa & López-Alvarado* (BC)
C. tripontina López-Alvarado et al.	V, X	TRI 1	Spain, Lleida, Figols i Alinyà, Congost de Tresponts, calcareous cliffs, 630-670 m, 06.VI.2009, *L. Sáez LS-7065* (L. Sáez, herb. pers.-BCB)
	M	TRI 2	Spain, Lleida, Figols i Alinyà, Congost de Tresponts, calcareous cliffs, 630 m, 01.VIII.2008, *Guardiola* (BCB)

FIGURES

Figure 1. The PCA-scatterplot for the 87 individuals studied. Abbreviations in figure: C. emi = *C. emigrantis*, C. jan = *C. janeri*, C. cab = *C. caballeroi*, C. lin = *C. linifolia*, C. pec = *C. pectinata*, C. trip = *C. tripontina*, C. uni = *C. uniflora* and C. ant = *C. antennata*.

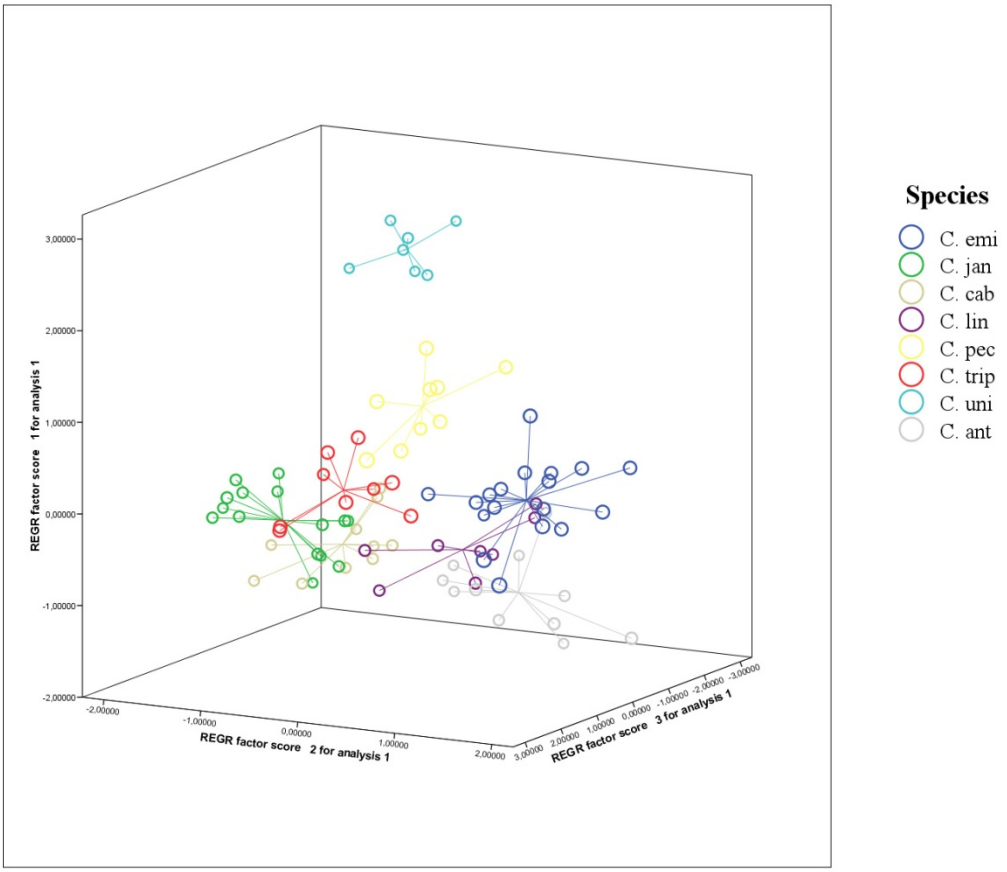


Figure 2. Scatterplot showing the distribution of individuals regarding to the first two discriminant functions. Abbreviations in figure:: C. emi = *C. emigrantis*, C. jan = *C. janeri*, C. cab = *C. caballeroi*, C. lin = *C. linifolia*, C. pec = *C. pectinata*, C. trip = *C. tripontina*, C. uni = *C. uniflora* and C. ant = *C. antennata*.

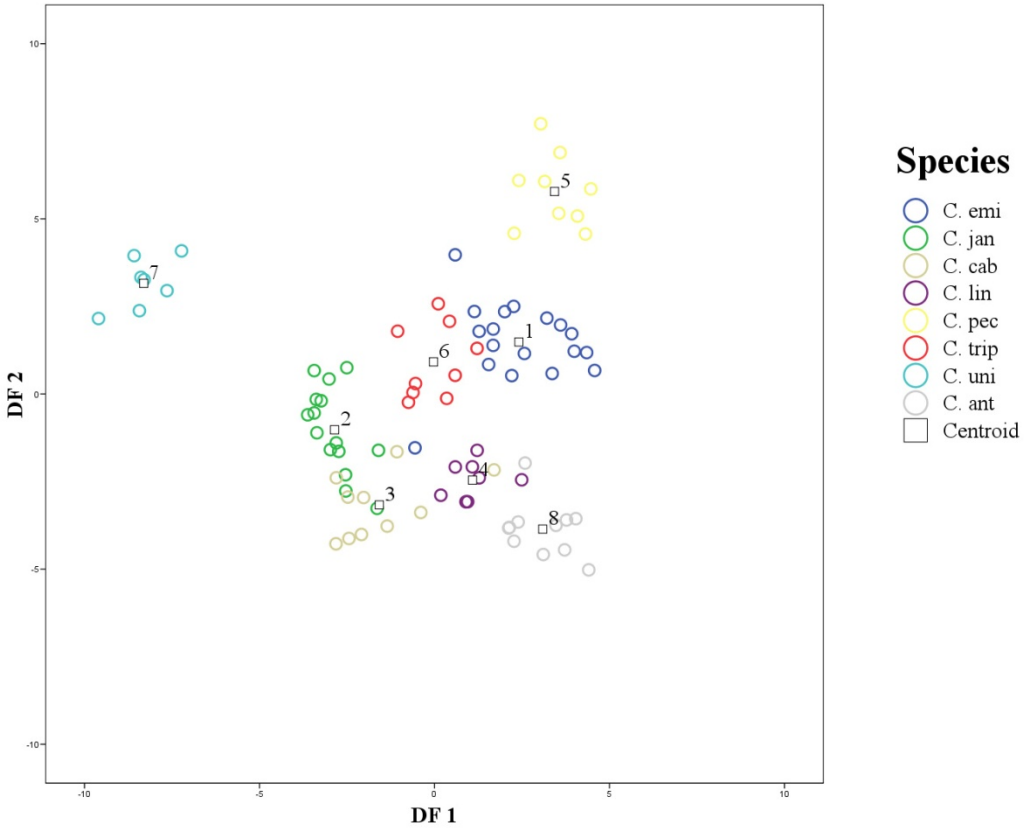


Figure 3. Representation, not in scale, of trichomas for each species. Species: A = *C. emigrantis*, B = *C. janeri*, C = *C. pectinata*, D = *C. unflora*, E = *C. caballeroi*, F = *C.antennata*, G = *C. tripontina* and H = *C. linifolia*.

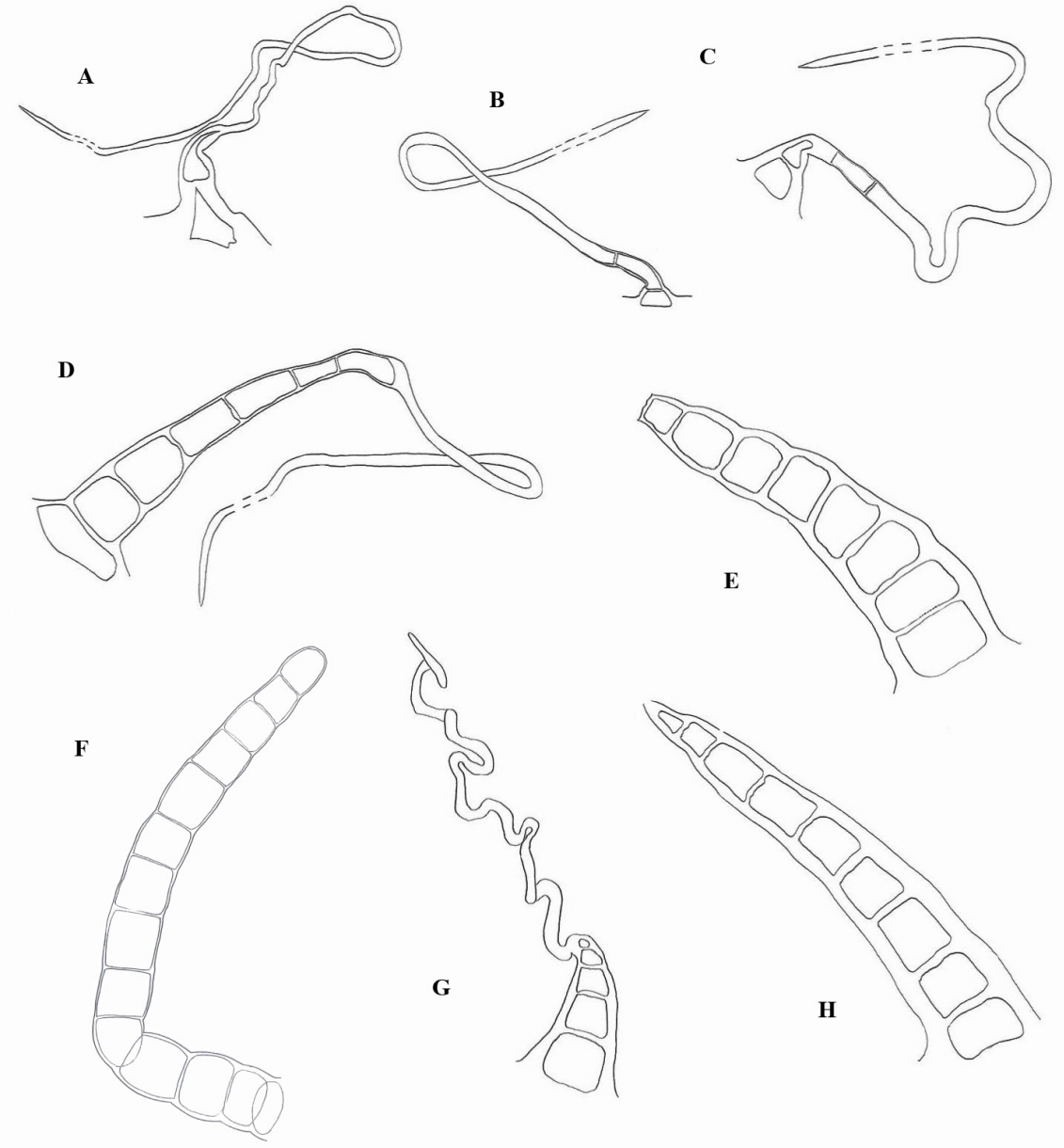
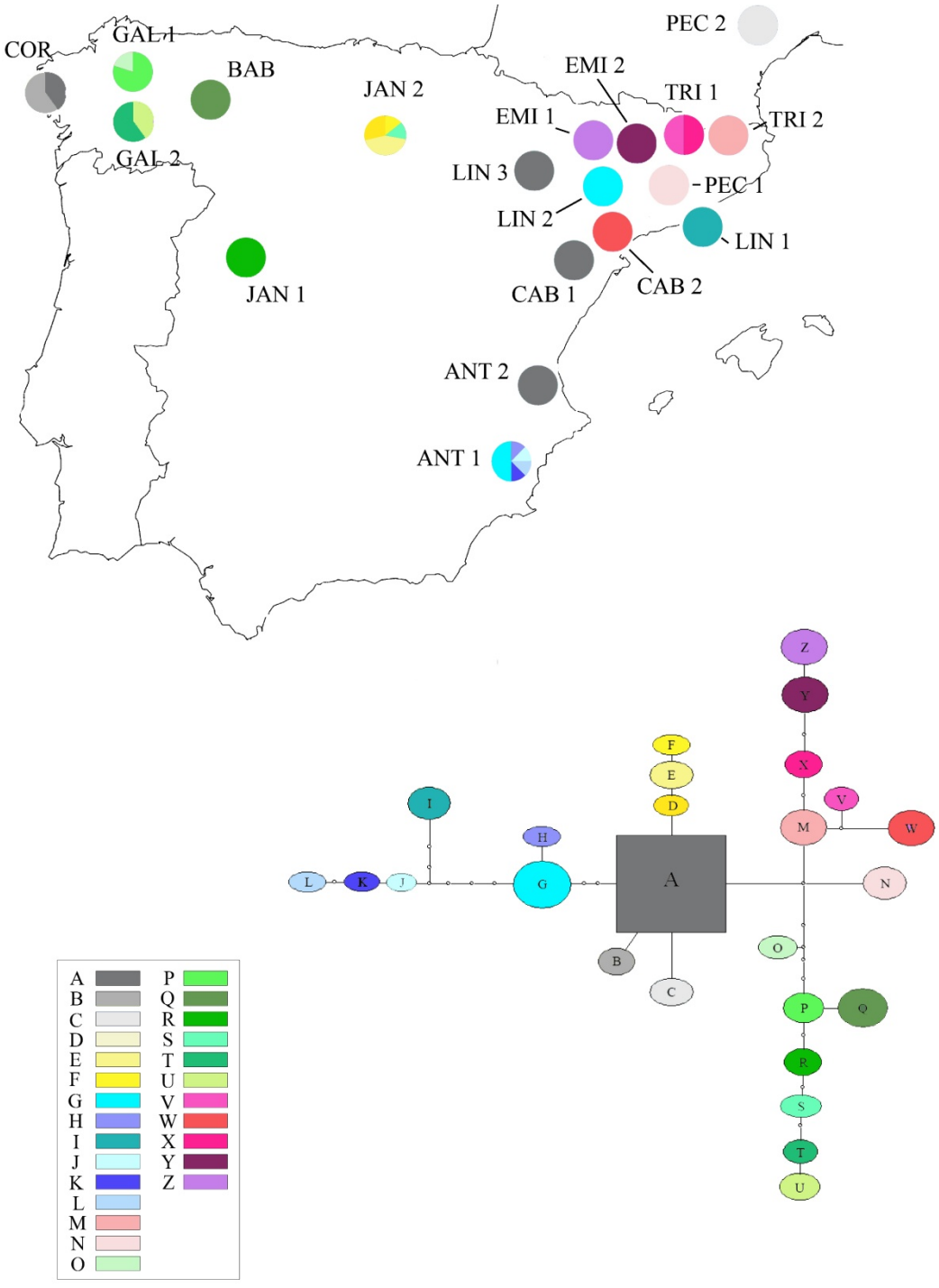


Figure 4. Haplotype network obtained from the analysis of the *trnL*$^{(UAG)}$*-rpl32* cpDNA marker. Haplotypes are represented by frequency in each population in an Iberian Peninsula map. Haplotypes are codified by color as indicated in the legend. Abbreviations for populations are shown in Table 4.



APPENDIX 2.1

List of specimens used for the morphometric study. It was possible to study more than one specimen per sheet.

Centaurea antennata*: Spain, Alicante:** Biar, Solana del Fraile, 30SXH9374, 750 m, 03-VII-1987, *De la Torre* (ABH3401); Moratalla, near road to Calasparra, 30SXH0535, 450 m, 04-V-1985, *Selma* (ABH3403); Arenal de la Virgen, Cerro de la Virgen, 30SXH7878, 530 m, 22-V-2008, *Aragoneses & Aragoneses* (ABH53015); Pinoso, 30SXH7261, 750 m, 6-VI-2004, *Monerris* (ABH47742); Pinoso, Sierra del Reclot, 30SXH7449, 800 m, 15-VI-1996, *Navarro* (ABH34239)/// ***Centaurea caballeroi*: Spain, Tarragona:** Cardó, 20-VII-1917, *Pericot* (BC34490); Ports de Tortosa, La Vall near La Galera south from Mola del Fonollar, 500 m, 19-VI-1956, *A. & O. de Bolòs* (BC150934); Serra de Montsià, 13-VI-1916, *sine col.* (BC34506); Ports de Beceit, Portell de Caro, 31TBF7722, 1050 m, 2-VII-2008, *Guardiola & Sáez LS-6884* (L. Sáez herb. pers.-BCB)/// ***Centaurea emigrantis*: Spain, Lleida:** Montsec d'Ares, 500 m, 29-VI-1948, *Font i Quer* (BC116420); Àger, 29-V-1924, *Riofrío* (BC35535); Montsec d'Ares, 26-VI-1916, *Font i Quer* (BC34466); Montsec d'Ares, 26-VI-1916, *Font i Quer* (BC34465); *sine loc*., 26-VI-1916, *Font i Quer* (BC34467); Embanat de Sant Antoni, Tremp, 16-VI-1969, *Barrau i Andreu* (BC612144); Montsec de Rúbies, 1200 m, 12-VIII-1978, *Romo* (BC633801); **Huesca:** Congost de Santa Ana, 26-VI-2008, *Roquet & Sáez LS-6880,* (L. Sáez herb. pers.-BCB)/// ***Centaurea janeri (including three subspecies, *C. janeri* subsp. *janeri*, *C. janeri* subsp. *gallaecica* and *C. janeri* subsp. *babiana*)**: Spain, Álava:** Comunión, VI-1910, *Elías* (BC34505); **A Coruña:** Melide, Furelos, 450 m, 28-VI-1982, *Amich, Rico & Sánchez* (BC637758); Toques, Serra do Careón, 4-VII-2005, *Iglesias Louzán* (BCN38899); **Lugo:** Palas de Rei, near Ramil, near Pambre river, 30-VII-1997, *Rodríguez Oubiña & Iglesias Louzán,* (BCN21909); **Salamanca:** between El Casarito and El Cabaco, 4-VII-1985, *Amich & Elías* (BCN21910); between El Casarito and El Cabaco, 1000 m, 9-VII-1988, *Amich* (BCB)/// ***Centaurea linifolia*: Spain, Tarragona:** Tivissa, La Mola, 8-V-1944, *Font i Quer & A. de Bolòs* (BC95573); Catllar, 70 m, 8-V-1919, *Font i Quer* (BC34493); La Gritella, 9-VII-2008, *Roquet & Sáez LS-6887* (BCB); Serres de Vandellós, Portella del Xato, 9-VII-2008, *Roquet & Sáez LS-6886* (L. Sáez herb. pers.-BCB). **Teruel:** Casterlserás, 18-VII-1875, *Loscos* (BC97515)/// ***Centaurea pectinata*: Spain, Girona:** La Cellera i Sant Julià de Llor, Bosc de Can Palet, 18-VI-1920, *sine col.* (BC34452); Pas de la Selva, 27-IV-1945, *Font i Quer* (BC631868); Sant Llorenç de la Muga, 30-VI-1972, *sine col.* (BCB); vers Farners, La

Selva, 23-VI-1945, *Font i Quer* (BC631895); Agullana, 23-VI-1908, *Sennen* (BC34461); **Barcelona:** La Miranda versus Montnegre, 730 m, 18-VII-1946, *P. Montserrat* (BC617418); Serra de Montnegre, Can Vives de la Cortada versus Sant Iscle de Vallalta, 3-XI-1948, *P. Montserrat* (BC617423)/// ***C. tripontina*: Spain, Lleida:** Organyà, Congost de Tresponts, 10-VI-1950, *Le Brun* (BC145156); Congost de Tresponts, 15-V-1972, *P. Montserrat* & *Villar* (JACA116472); Congost de Tresponts, *P. Montserrat & Villar* (JACA146883); Figols i Alinyà, Congost de Tresponts, 01-VIII-2008, *Guardiola* (BCB); Organyà, Congost de Tresponts, 31TCG6376, 580 m, 21-VI-2008, *Sáez LS-6870* (BC 871572); Organyà, Congost de Tresponts, 31TCG6376, 580 m, 21-VI-2008, *Sáez LS-6870* (L. Sáez, herb. pers.-BCB); Fígols i Alinyà, Congost de Tresponts, 31TCG6376, 580 m, 6-VI-2009, *Sáez LS-7065* (L. Sáez, herb. pers.-BCB)/// ***C. uniflora*: Italy:** Vinadio, Bagni di Vinadio, Cascata delle' Schiattare, 31-VII-1912, *Zola* (BC34418); Torino, Val Perosa, VII-1990, *Rostan* (BC34421); Cuneo, Tenda, Val Casterino, 1913, *Bicknell* (BC34423); **France:** Basses Alpes, Larche, forêt de Boisset, 1800 m, VIII-1916, *sine col.* (BC34417); Hautes Alpes, Col de Lautaret, 2200 m, 1921, *Gandoger* (BC34422); Col du Mont-Cenis, 1900 m, 27-VII- 1875, *Jacob* (BC659984); **Switzerland:** La Salette, 1800 m, 28-VII-1911, *Lung* (BC34420).

List of other specimens studied (not included in morphometric analysis)

***Centaurea caballeroi*: Spain, Tarragona:** Ulldecona, Serra de Montsià, pr. Mas del Comú, 31TBF9100, 630 m, 7-VI-2002, *Arán* (JACA R273778)/// ***Centaurea emigrantis*: Spain, Lleida:** Font de les Bagasses, S of Terradets, CG2555-2556, 350 m, 6-VII-1986, *P. Montserrat* & *G. Montserrat* (JACA 474986); Camarassa, 31TCG2347, 675 m, 2-VII-1997, *P. Montserrat* & *Soriano* (JACA 145397); reservoir of Camarassa, 31TCG2441, 350 m, 5-VI-1987, *P. Montserrat* & *G. Montserrat* (JACA 501187); La Isla, Camarassa, 31TCG2441, 300 m, 4-VI-1987, *P. Montserrat* & *G. Montserrat* (JACA 497487); Montsec de l'Estall, 31TCG0761, 1000 m, 16-VIII-1996, *Ferrández* (JACA 370496); Monte Santa Ana, Castillonroy, 31TCG0138, 500-550 m, 5-VI-1987, *P. Montserrat* & *G. Montserrat* (JACA 517387); **Huesca:** Canelles, near reservoir of Canelles, 31TCG0149, 500 m, 27-V-1987, *G. Montserrat* (JACA 424687); Chapel of San Marcos, Estopiñán, CG0253, 510 m, 12-V-1996, *Ferrández* (JACA 332696); Baldellou, near Santa Ana reservoir, under Peña Roja, CG0042, 410-420 m, 11-V-1997, *Ferrández* (JACA 321297); Estopiñán, BG9951, 735 m, 14-VIII-1996, *Ferrández* (JACA 366196); Picot, Camporrells, BG9746, 840 m, 11-VIII-1997, *Ferrández* (JACA 357297); La Encalada, Camporrells,

31TBG9645, 740 m, 26-VI-1988, *G. Montserrat & E. Gil* (JACA 625888); tunnel under Santa Ana reservoir, 31TBG9939, 330 m, 9-X-1986, *P. Montserrat* & *G. Montserrat* (JACA 597386)/// ***Centaurea janeri*** (including three subspecies, *C. janeri* subsp. *janeri*, *C. janeri* subsp. *gallaecica* and *C. janeri* subsp. *babiana*): **Spain, León:** Sena de Luna, 30TTN5758, 1120 m, near chapel of Rabanal, 9-VI-1990, *P. Montserrat & Laínz* (JACA 132390); Camposagrado, 1150 m, 18-VI-1973, *P. Montserrat* (JACA 123178); Quintanilla de Flórez, 29TQG38, 19-VI-1983, *T.E. Díaz & al.* (JACA 381491); **A Coruña:** Melide, Furelos, 29TNH8352, 450 m, 28.VI-1982, *Amich & al.* 2885 (JACA 342582); Melide, Furelos, 29TNH85, ad 400 m, in dumosis raris, 19-VI-1980, *Fernández Casas 3437* exs. IV ºn 430 (JACA s.n); **Lugo:** Palas de Rei, 29TMH8545, 325 m, near Ramil, pr. Pambre river, 325 m, 30-VII-1997, *Rodríguez Oubiña & R. Iglesias* 183 (JACA 448597); **Salamanca:** between El Casarito amd El Cabaco, 29TQE4391, 1000 m, 4-VII-1985, *Amich & Elías 179* (JACA 448197); El Cabaco, 29TQE4293, 980 m, 8-VII-1993, *Ladero & G. Iglesias 17499* (JACA s.n.); **Ávila:** between Cepeda la Mora and S. Martín de la Vega de Alberche, 30TUK28, 1150 m, 19-VII-1995, *Sánchez-Mata s.n*. (JACA s.n.)/// ***Centaurea linifolia*****: Spain, Zaragoza:** Castillo de Mequinenza, 240 m, 31TBF7382, 28-V-1988, *P. Montserrat & G. Montserrat* (JACA 97188); Caspe-Bujaraloz, Cabezo del Ciervo, 30TYL48, 300 m, 29-V-1988, *J. M. Montserrat & G. Montserrat* (JACA 119288); Castejón de Valdejasa, Alto de la Sierra de Baró, 30TXM6953, 710 m, 2-V-2001, *D. Gómez* (JACA R264247); Sádaba, between Pinsoro and Sádaba, 30TXM3782, 400-410 m, 20-VI-2001, *D. Gómez* (JACA R264768); Tauste, Caída de La Negra, 30TXM3956, 600-610 m, 27-IV-1998, *Vivant & P. Montserrat* (JACA 18898); **Huesca:** Peratilla, 31TBG5259, 430 m, *P. Montserrat & Cernoch* (JACA 46393); Ballobar, SW from El Basal, 31TBF6010, 280 m, 20-VII-1995, *Ferrández* 4698 (JACA 600695); Monzón, Conchel, Terreu, 31TBG5338, 360-400 m, 8-VI-2002, *Ferrández* s.n. (JACA R270523); Alcolea de Cinca, La Codera, 31TBF6119, 230 m, 12-IV-1995, *Ferrández* 3825 (JACA 513495); San Quílez next to a chapel, Binéfar, 31TBG7236, 400 m, 20-VI-2002, *Ferrández* 3825 (JACA R270544); Civiacas, Alfántega, BG6434, 250 m, 23-V-2004, *Ferrández* s.n. (JACA R278043); Peralta de Alcofea, 30TYM4041, 370 m, 4-V-1996, *Ferrández* s.n. (JACA 326796); Castelflorite, Barbastro, 30TYM4531, 350-370 m, 13-VI-2004, *Ferrández* s.n. (JACA R276674); Las Solanas, near Torrente de Cinca, 31TBF7693, 150-180 m, 31-V-1985, *G. Montserrat* (JACA 157285); Alcubierre, San Caprasio, 30TYM1022, 800 m, 8-V-2001, *P. Montserrat & al.* (JACA R263950); Candasnos, Valcuerna gorge, 31TBF5294, 340 m, 27-V-1996, *P. Montserrat* & *D. Gómez* (JACA 26996); **Tarragona:** Benifallet, Balneario de Cardó, 31TBF93, 300 m, 7-VII-1981, *Dorda,*

Granzow & Susanna (JACA 536491); à 2 km au-dessus d'Alcover sur la route de Montreal, 400 m, 2-VI-1977, *B. De Retz* 75839 (JACA s.n.)/// ***Centaurea pectinata* subsp. *pectinata*: Spain, Huesca:** Tolva, 31TBG9864, 640 m, 30-IX-1995, *Ferrández* (JACA 644695)/// ***Centaurea pectinata* subsp. *acutifolia*: France:** Sur les rochers de la rive droite de la Loire, Saint-Just-sur-Loire, Loire, 21-VI-1876, *Fr. Faustinien* 1264 (FABR08963); St Just-sur-Loire, 18-VII-1878, *Fr. Faustinien* 2129, (FABR); Ardèche, 20-VI-1857, *ex horto Alexis Jordan* (LY)/// ***Centaurea pectinata* subsp. *supina*: France:** *sine loc.*, 23-VI-1857, *Jordan* (LY); Jonquières de Gards, 23-VI-1857, *sine col.* (LY).

APPENDIX 2.2

List of specimens for phytodermologic study

***Centaurea antennata*: Spain, Alicante:** Biar, Solana del Fraile, 30SXH9374, 750 m, 03-VII-1987, *De la Torre* (ABH3401); Moratalla, near road to Calasparra, 30SXH0535, 450 m, 04-V-1985, *Selma* (ABH3403). **Valencia:** Serra, Porta Coeli Monastery, to Font del Berro, 10.VI.2011, *Figueroa & López-Alvarado* (BC) /// ***Centaurea caballeroi:* Spain, Tarragona:** Ports de Beceit, Portell de Caro, 31TBF7722, 1050 m, 2-VII-2008, *Guardiola & Sáez LS-6884* (L. Sáez herb. pers.-BCB); Serra de Montsià, 13-VI-1916 *Font i Quer* (BC34506); Vall de Carreretes, Portell de Caro, 25-VI-1917, *Font i Quer* (BC34496)/// ***Centaurea emigrantis:* Spain, Huesca:** Congost de Santa Ana, 26-VI-2008, *Sáez & Roquet LS-6880* (L. Sáez pers. herb.-BCB). **Lleida:** Corçà, 21-VI-2009, *Figueroa & López-Alvarado* (López-Alvarado pers. herb.-BC); Ametlla de Montsec, 21-VI-2009, *Figueroa & López-Alvarado* (López-Alvarado pers. herb.-BC); Llimiana, 25-VI-2009, *Figueroa & López-Alvarado* (López-Alvarado pers. herb.-BC) /// ***Centaurea janeri*: Spain, A Coruña:** Melide, Furelos, 400 m, 19-VI-1980, *Amich, Rico & Sánchez* (BC645530); Melide, Furelos, 15-VII-2009, *Figueroa & López-Alvarado* (López-Alvarado pers. herb.-BC) . **Álava:** Comunión, VI-1910, *Elías* (BC34505). **Salamanca:** Between El Casarito and El Cabaco, 1000 m, 9-VII-1988, *Amich* (BCB); Between El Casarito and El Cabaco, 14-VII-2009, *Figueroa & López-Alvarado* (López-Alvarado pers. herb.-BC) /// ***C. linifolia*: Spain, Tarragona:** La Gritella, 9-VII-2008, *Roquet & Sáez LS-6887* (BCB); Serres de Vandellós, Portella del Xato, 9-VII-2008, *Roquet & Sáez LS-6886* (BCB). **Barcelona:** Between Balsareny and Avinyó, 420 m, 15-V-2009, *Hilpold AH20092068* (BC)/// ***Centaurea pectinata*: Spain, Girona:** La Cellera i Sant Julià de Llor, Bosc de Can Palet, 18-VI-1920, *sine col.* (BC3445); St. Llorenç de la Muga, 30-VI-1972, *sine col.* (BCB); road

GI-682, between Sant Feliu de Guíxols and Tossa de Mar, 30 m, 14-VI-2009, *Barres LB46* (BC); road GI-682, between Sant Feliu de Guíxols and Tossa de Mar, 30 m, 14-VI-2009, *Barres LB47* (BC). **France, Pyrénées-Orientales:** Canigó, 1720 m, 14-VI-2009 *Hilpold, Roquet & Nogué AH20092216* (BC)/// ***Centaurea tripontina*: Spain, Lleida:**; Organyà, Congost de Tresponts, 15-V-1972, *P. Montserrat & Villar* (JACA116472); Figols i Alinyà, Congost de Tresponts, 01-VIII-2008, *Guardiola* (BCB); Figols i Alinyà, Congost de Tresponts, 01-VIII-2008, *Guardiola* (BCB); Fígols i Alinyà, Congost de Tresponts, 31TCG6376, 580 m, 6-VI-2009, *Sáez LS-7065* (L. Sáez, pers. herb-BCB); Fígols i Alinyà, Congost de Tresponts, 31TCG6376, 580 m, 6-VI-2009, *Sáez LS-7065* (ex horto botanico)/// ***Centaurea uniflora*: Italy, Cuneo:** Tenda, Val Casterino, 1913, *Bicknell* (BC34423). **France, Hautes Alpes:** Col du Mont Cenis, 2150 m, 9-VIII-1990, *E. del Castillo* (BC808104); Col du Lautaret, 2000 m, 7-VIII-1990, *sine col.* (BC808551); Chantemerle, Serre du Chevalier, 23-VII-1980, *B. De Retz* (BC647781).

3

DECIPHERING EVOLUTIONARY RELATIONSHIPS OF TWO *CENTAUREA* NARROW ENDEMICS (COMPOSITAE): SYSTEMATICS AND CONSERVATION BIOLOGY

Deciphering evolutionary relationships of two *Centaurea* narrow endemics (Compositae): systematics and conservation biology

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ABSTRACT

The existence of cryptic species is a well known phenomenon in both plants and animals for a long time. The recently described narrow endemic *C. tripontina* is one of such unnoticed evolving lineages. In spite of that, its systematic relationships with *C. emigrantis*, another Iberian narrow endemic, remains unclear. The use of microsatellites, fast-evolving nuclear regions, and cpDNA intergenic spacers are adequate to provide information about its systematic affinities. Furthermore such markers are suitable to assess the genetic variability of both species offering the possibility to propose appropriate management plans. Data reveals that *C. emigrantis* and *C. tripontina* are isolated evolving lineages with medium-high levels of genetic diversity. The possibility of reticulation among both species is discussed.

INTRODUCTION

The existence of cryptic species, understood as two or more distinct lineages classified as a single species, is a well known phenomenon in both plants and animals for a long time (Bickford et al., 2007). Moreover, new molecular approaches have been demonstrated as a powerful tool in order to decipher such hidden variability. However, the indiscriminate use of DNA data without an integrative systematic vision could led to an overestimation of the number of species (Tan et al., 2009), and indeed to a fail of conservation measures if we are treating single evolving lineage as different taxa. Alternatively the underestimation of the number of species could lead to the same problem. In this context the use of multiple sources of information should be the chosen option. According to Rieseberg et al. (2006), 70% of plant species defined by taxonomists fits well with reproductively isolated lineages. Therefore around 30% of currently recognized plant species include more than one reproductive lineage with subsequent bad performing of species-based conservation programs. Otherwise, as has been pointed out by French et al. (2008), in taxonomically complex groups such as *Euphrasia*, with recent evolution times, divergences may exist between named taxa and the evolving lineages.

Another example of a recent evolved group is *Centaurea* L., a large genus of the Compositae with circa 250 species (Susanna and Garcia-Jacas, 2007) distributed along the Mediterranean and Irano-Turanian regions. It is supposed that the recent evolution time is the main cause of the complex taxonomy of the genus, due to weak reproduction barriers and little morphological differentiation. The Iberian Peninsula is one of the centres of diversity of the genus with approximately 100 endemic taxa (Muñoz and Devesa, 2010).

The narrow endemic *C. tripontina* López-Alvarado, Sáez, Guardiola, Filigheddu & Susanna, recently described on the basis of morphological traits (López-Alvarado et al., 2011), could be one of such unnoticed evolving lineages. The evolutionary relationships between this taxon and *C. emigrantis* Bubani, both from section *Phrygia* Pers. (= *Lepteranthus* nom. inv.), remain uncertain. A molecular survey of *Centaurea* section *Phrygia* carried by López-Alvarado et al. (in prep.) using the nrDNA regions ITS and ETS, has revealed that reticulate evolution might have been important among the Iberian species, and in particular between the two aforementioned species. Cloning of nrDNA region ETS have revealed that *C. emigrantis* presents copies related to those of *C. tripontina*. However, the presence of such copies in the rest of Iberian *Phrygia* can not be discarded, since no intensive cloning efforts were done with the rest of species. Furthermore, the nature of nrDNA, which appears forming multiple copy arrays and is subjected to concerted evolution and presence of pseudogenes, could be masking the real evolutionary events underlying in the group (Álvarez and Wendel, 2003; Garcia-Jacas et al., 2009).

The aim of present work is to test the hypothesis of the lineage differentiation between *C. emigrantis* and *C. tripontina* using fast-evolving nuclear regions, microsatellites (SSR) with co-dominant inheritance, and cpDNA intergenic spacers with maternal information. Furthermore, microsatellite markers will provide information about genetic variability of both species of usefulness on conservation strategies.

MATERIALS AND METHODS

Plant Material

The studied species are two morphologically related narrow endemics from section *Phrygia* growing on the Spanish Pre-Pyrenean Mountains.

Centaurea emigrantis is a perennial plant restricted to Serra del Montsec mountain range and its surroundings in north-eastern Spain, where it grows on shrubs and open habitats up to 1400 m. Montsec mountain range is placed between the Spanish regions of Catalonia and Aragon and it is subdivided in three well defined parts, Montsec d'Estall, Montsec d'Ares and Montsec de Rúbies. Despite its narrow range of distribution, *C. emigrantis* can be locally abundant and has been considered as LC (Least Concern) following the IUCN (2001) criteria (Sáez et al., 2010). It is a diploid plant with $2n = 22$ (Arnelas & Devesa, 2010). Although there is no data about its population dynamics and biology, we were able to observe in the field that it grows forming small and isolated patches of not many individuals and that its populations presents both flowering and vegetative individuals. Furthermore seeds have presented good levels of germination in laboratory (unpublished data).

The second species, *Centaurea tripontina*, is also a perennial narrow endemic growing on shady calcareous cliffs in a single locality near village of Organyà in Lleida province, the Tresponts gorge. Due to its extremely restricted range of distribution and decrease in habitat quality it has been considered EN (Endangered) following the IUCN (2001) criteria (López-Alvarado et al., 2011). It is suspected to be a diploid species with $2n=22$ also confirmed by stomata and pollen data (López-Alvarado, unpublished data). As well as for *C. emigrantis*, no studies in demography or biology for *C. tripontina* have been published. Our field observations have revealed low density of mature individuals and we were not able to find seedlings. Furthermore, seeds have shown low rates of germination in laboratory (López-Alvarado, unpublished data).

The study was focused on representative populations of both species. For *C. emigrantis*, with a wider range of distribution, four populations were chosen: two populations from Montsec d'Ares (COR and AME), the greatest part of Montsec range;

one population from Montsec de Rúbies (LIM), and one population from the southernmost part of the range, Serra del Mont-Roig (MTR). For *C. tripontina*, with a more restricted distribution, sampling was carried out by dividing the individuals in two different populations, one on the right side of river Segre (DRE) and other on the left margin (ESQ). Sampling for SSR analysis was conducted on 30 individuals per population in the case of *C. emigrantis* and 20 individuals per population for *C. tripontina*. Althoug recommended sample when working on SSR markers is 30 individuals, the scarcity of individuals of *C. tripontina*, and the difficult access to the plants did not allow larger sampling. Therefore, a total of 20 individuals from two populations were included in the study. The number of individuals sampled for cpDNA analysis were five per population.

DNA isolation, microsatellite loci and cpDNA markers

Genomic DNA was extracted from dried leaf tissue using the CTAB method by Doyle and Doyle (1987) as modified by Cullings (1992) and Tel-Zur et al. (1999). Simple Sequence Repeats markers (SSR) described for other *Centaurea* species (Fréville et al., 2000; Marrs et al. 2006) were tested for their ability to amplify the same SSR regions in *C. emigrantis* and *C. tripontina*. Only six of out sixteen SSR markers were selected for their capacity to amplify and to give unambiguous peak patterns (Table 2). PCR was performed under conditions shown in Marrs et al. (2006). All SSR loci were amplified using FAM, NED, PET and VIC fluorescently labeled forward primers as detailed in López-Vinyallonga et al. (2010). Genotyping was performed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the Interdisciplinary Center for Biotechnology Research (ICBR) facility at the University of Florida. Fragment analysis was performed with GeneMarker® v. 1.85 (SoftGenetics, LLC, State College, PA) software using LIZ600 size standard, and data were scored manually.

Double-stranded cpDNA of the *trnL*$^{(UAG)}$*-rpl32*, *ycf3-trnS* and *rpL16* regions was amplified using rpl32F as forward primer and trnL$^{(UAG)}$ as the reverse primer for the *trnL*$^{(UAG)}$*-rpl32* region (Shaw et al., 2007); SP43122F as the forward primer and SP44097R as the reverse primer for the *ycf3-trnS* intergenic spacer region (Hershkovitz, 2006); and rpL16F71 (Jordan et al. 1996) and RexC (R. Vilatersana, Botanic Institute of Barcelona, pers. comm.) for *rpL16* intron. The profile used for cpDNA amplification included a hot start at 95 ºC for 3 min. Then 30 amplification cycles were carried out under the following conditions: 95 ºC for 40 s, 54 ºC for 40 s and 72 ºC for 1 min, with an additional extension step of 10 min at 72 ºC. The PCR products were purified with ExoSAP-IT (USB Corp., Cleveland, OH, USA) and sequenced using a BigDye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), following the manufacturer's protocol, at the University of Florida ICBR Core Facility using an ABI 3730xl DNA analyzer (Applied Biosystems). Primers used for sequencing were trnL$^{(UAG)}$ as reverse primer for *trnL*$^{(UAG)}$*-rpl32*, SP43122F as the forward primer for *ycf3-trnS* to avoid a poly-A region, and RexC for *rplF71-rpl16*.

Data analysis

The SSR data were analyzed using different software. Genetic diversity was computed using GeneAlex v. 6 (Peakall and Smouse, 2006). The statistical parameters calculated were: the number of alleles; the number of effective alleles (*NE*); the number of private alleles (*PA*); the Shannon's Information Index (*I*); the observed heterozygosity (H_O) which represents the percentage of heterozygous individuals in a given sample; and the unbiased expected heterozygosity (H_E; Nei, 1978) which measures the proportion of heterozygosity expected under random mating. All these parameters were calculated for both species and for each polymorphic locus. The population genetic structure was checked with the Bayesian clustering method as implemented in the software

STRUCTURE 2.3.3 (Pritchard et al., 2000) which allows de detection of genetic clusters (K) present in the nuclear dataset. This analysis was performed with *C. emigrantis* and *C. tripontina* together. The number of recommended clusters was chosen following the ΔK method as proposed by Evanno et al. (2005). Furthermore, genetic differentiation among populations of both species was tested by pairs with the F_{ST} statistic (Weir and Cockerham, 1984) using GeneAlex v. 6 and with the Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) using ARLEQUIN v. 3.1 (Excoffier et al., 2005) and considering the two species as a single group and each species separately. Regarding cpDNA, sequences were aligned visually using Bioedit (Hall, 1999). A phylogenetic network of cpDNA haplotypes was constructed under a Statistical Parsimony approach with software TCS 1.21 (Clement et al., 2000) codifying gaps as fifth state. Deletions of more than one bp were condensed in a single gap.

RESULTS

Genetic variability

The six SSR markers used in this study yielded 57 alleles for the 160 surveyed individuals, *C. emigrantis* presenting 51 alleles and *C. tripontina* 29 alleles. All the markers were polymorphic except 13D10 for *C. tripontina*. The number of alleles *per locus* ranged from 1 for locus 13D10 to 7 for locus 12B1 in *C. tripontina* and from 4 for locus 13D10 to 12 for locus CM17 in *C. emigrantis*. A number of 23 alleles were found to be shared by the two species while 28 private alleles were found for *C. emigrantis* and 6 for *C. tripontina*.

Regarding genetic diversity (Table 3), *C. emigrantis* presented a H_E mean value across populations of 0.641 and values ranging from 0.276 for locus 13D10 to 0.837 for locus 21CM36. Moreover, *C. tripontina* presented a mean H_E of 0.483 across populations and values ranging from 0 for locus 13D10 to 0.646 for locus 12B1.

Considering *C. emigrantis*, the H_E at population level ranged from 0.429 for population COR to 0.654 for population AME. For *C. tripontina*, DRE population presented a H_E = 0.388 and ESQ an H_E = 0.491.

The first three components of Principal Coordinate Analysis (PCoA) explained the 77.25% of the total variance of the data, and the scatterplot of individuals against the two first coordinates (64.47% of the total variance) revealed two discrete groups corresponding to *C. emigrantis* and *C. tripontina* (Fig. 1). Nevertheless, there are some individuals of *C. emigrantis*, from AME, LIM and MTR populations, which were placed in an intermediate position with regard to the two cores of maximum individual density.

Population genetic structure

The analysis of SSR with STRUCTURE software revealed that data could be divided in two different groups (K = 2). Clusters fitted highly with the two taxa *C. emigrantis* and *C. tripontina*. Some individuals belonging to *C. emigrantis*, in particular from populations AME and LIM, showed affinities with *C. tripontina* specimens (Fig. 2). Individuals 14 and 21 from population AME were excluded from analyses since they present missing data.

AMOVA analyses pointed out that the maximum degree of genetic diversity appeared always within populations and that the variance among populations was low for both species. However, variance among populations doubled when considering both taxa as a single species indicating differentiation among species (Table 4). Regarding genetic differentiation among popuations, F_{ST} value showed a maximum value of 0.24 between the populations of *C. emigrantis* COR and AME, whereas for *C. tripontina* differentiation between both studied populations had a F_{ST} value of 0.18. Maximum

differentiation among populations of the same species was found to be always lower than among populations of different species (Table 5).

cpDNA genetic structure

The cpDNA network revealed differentiation among both species (Fig. 3). Seven step-changes were found among the two species for the closest populations in the parsimony network. Furthermore, no shared or directly derived haplotypes were found between *C. emigrantis* and *C. tripontina*. *Centaurea emigrantis* presented eight of the total haplotypes whereas *C. tripontina* accounted for five of them. Regarding populations, MTR for *C. emigrantis* and DRE for *C. tripontina* presented the maximum number of haplotypes, four and three respectively. AME and COR for *C. emigrantis* and ESQ for *C. tripontina* have the lowest number of haplotypes.

DISCUSSION

The results reveal the complexity of evolutionary relationships when dealing with recent evolving groups and the difficulty to establish limits among different evolving lineages. This becomes evident when analyzing the number of alleles detected for *C. tripontina* and *C. emigrantis*. The number of total alleles is 57 where 23 alleles are shared between both species. Since the number of alleles detected in *C. tripontina* is 29, the degree of shared alleles is significantly high and may indicate either close relatedness due to common ancestry or gene flow.

Nevertheless, and despite of the high rates of allele sharing, all the statistic analyses performed on SSR markers show genetic differentiation among both taxa especially for F_{ST} (Table 5) and STRUCTURE analysis (Fig. 2). F_{ST} is always lower among populations of the same species than among those of different species, indicating low genetic interchange and genetic differentiation. In addition, STRUCTURE

corroborates genetic differentiation by dividing data in two clusters (K=2) coinciding almost perfectly with *C. emigrantis* and *C. tripontina*. However, some individuals of *C. emigrantis* seem to have more affinities with *C. tripontina* cluster than with individuals of its own population. It can be explained by the presence of at least 8 alleles, distributed among four of the six SSR markers analyzed, which are present only in some individuals of *C. tripontina* and some individuals of *C. emigrantis*. If these shared alleles were acquired by gene flow or are due to common ancestry can not be discerned. Furthermore, if shared alleles are a consequence of gene flow events, the source of such alleles is difficult to find since they are scarce in both species.

However, as revealed in the phylogenetic survey of *Centaurea* sect. *Phrygia* by López-Alvarado et al. (in prep.), *C. emigrantis* present ETS copies related to *C. tripontina* and *C. linifolia* L. In spite of that, the hybrid origin of *C. emigrantis* does not seem likely since *C. linifolia* also present different copies of ETS. This issue should be adressed in future studies but, as has been pointed out in a systematic study of Iberian species of sect. *Phrygia* (López-Alvarado, in prep.), presence of ancestral polymorphysms it is a likely explanation to nrDNA sharing among *C. emigrantis*, *C. tripontina* and *C. linifolia*. Moreover, *C. tripontina* could not be the maternal species since there is not sharing any cpDNA haplotypes with *C. emigrantis*. On the other hand, a cpDNA haplotype study of Iberian sect. *Phrygia*, including three populations of *C. linifolia*, has not revealed also any shared haplotype among *C. emigrantis* and *C. linifolia*. Therefore, although the origin of *C. emigrantis* and *C. tripontina* can not be fully solved, the presence of gene flow between *C. emigrantis* and *C. tripontina* seems to be confirmed also by SSR markers.

AMOVA, as F_{ST}, points as the same direction than STRUCTURE analysis, since percentage of variance explained by differences among populations is twice when

considering both species as a unique group unlike if considered as two different groups. This reveals a high degree of heterogeneity among *C. emigrantis* and *C. tripontina*. However, the great diversity of genotypes, both at population and species level, can be masking differences among groups.

The cpDNA, which gives complementary information to that of nuclear markers, since is only maternally inherited, supports the evidences pointed out by microsatellite markers, and a long isolation between both lineages is corroborated (Fig. 3). There are not haplotypes shared between the two species, and closer haplotypes are separated by seven nucleotide changes. Furthermore, haplotypes form two good recognizable clusters which present only internal relationships.

If the results are discussed independently for each species, analysis of SSR markers reveals that *C. emigrantis* presents a good level of genetic diversity and low degree of differentiation among populations (Tables 3 and 5) if compared with those from other studies conducted on similar *Centaurea* narrow endemics. The comparision can be done because SSR data from the two narrow endemics *C. corymbosa* Pourr. and *C. horrida* Badarò is available for the same markers (Fréville et al., 2001; Mameli et al., 2008). For *C. corymbosa*, H_E values ranged from 0.36 to 0.62 whereas for *C. horrida* values ranged from 0.603 to 0.854. These results suggest that despite its narrow range of distribution and its expected low rate of seed dispersion, all the populations are acting as a unique evolving unit. The cohesiveness of *C. emigrantis* populations is interesting due to the relatively high distances between populations (10-30 Km), which, moreover grow forming isolated patches, and the low rates of seed dispersal expected due to the lack of long dispersion accessories (Hilpold et al., 2011). Despite this, human and cattle transport should be taken into account since could be acting as a main dispersion element. However, the possibility of a recent isolation of populations due to the increase

of forest and shrubs can not be discarded, especially in herbs like *Centaurea* usually growing in open habitats and path margins. The F_{ST} pairwise comparison, which is higher between closest populations, does not help to solve that point, since both hypotheses could explain such behaviour. The population AME, which presents slightly allelic frequency differences, appears as the main distinct population. Not surprisingly it is the population that has presented more difficulties for amplifying some loci and maybe its high degree of missing data is affecting the final result. Nevertheless, the presence of two private alleles for marker 13D10, which presents only two for the rest of individuals of both species, for AME population makes it the most different population regarding to the rest. Since half of alleles found for 13D10 are present in only two individuals of population AME, it could be considered that such alleles could be derived from an introgression with a third species. The cpDNA haplotype distribution of *C. emigrantis* is intriguing due to its assimetry. The westernmost populations of the range (COR and AME) presents low variability, one haplotype each, while LIM and, specially, MTR present more than one haplotype (Fig. 3). Such behavior could be explained if an underestimation of COR and AME population size was ocurred, and therefore biased results of genetic varability were achieved. Otherwise, a population bottleneck due to a population decrease and a consequent lost of haplotype diversity could be possible, since cpDNA has a half of effective population size and therefore is more sentive to population size changes (Birky et al., 1989; Mitton, 1993). The last explanation is likely due to instability of habitat where *C. emigrantis* grows, road and path margins. However, cannot be ruled out that the presence of such high number of haplotypes at MTR population is due to introgression with other species of *Centaurea*.

Relating conservation strategies, genetic information together with the local abundance of *C. emigrantis* suggests, according to Sáez et al. (2010) who considered it as LC under IUCN critera (IUCN, 2001), that not exceptional conservation plans should be taken. Nevertheless, since its abundance could decrease as consequence of habitat changes, periodical monitoring is recommended.

Regarding *Centaurea tripontina*, it also presents a good level of genetic diversity despite its narrow range of distribution, since is known only from two populations with few individuals. However, the number of alleles is lower with regard to *C. emigrantis*. It could be argued that the smaller population size and the narrower range of distribution of *C. tripontina* implies a higher probability of genetic drift or other genetic constraints which could have led to a loss in genetic diversity, whereas in *C. emigrantis*, with larger populations, these phenomena have not been as important. Otherwise, the analysis of cpDNA data from *C. tripontina*, which are more sensitive to population bottelnecks due to smaller effective population size, do not reveal such decrease in population size since the number of haplotypes is high. Another possible explanation to the high number of alleles of *C. emigrantis* has to do, as commented above, with a possible genetic introgression of a third species. In any case, the good levels of genetic diversity presented by *C. tripontina* can be explained if species presents a long life span or high levels of vegetative reproduction (Young et al. 1996; Honnay and Bossuyt, 2005; Honnay and Jacquemyn, 2007). Nevertheless, the vegetative reproduction does not seem a probable strategy for *C. tripontina* since it grows in rocks' fissures (Sáez, pers. com.). Data also reveals that the river Segre could be acting as an effective barrier to seed dispersal, since differences between cpDNA haplotypes exists between both river margins. Nevertheless, the F_{ST} value (Table 5), which can be considered low, indicates

that pollen exchange occurs and therefore seems that both populations are not fully isolated.

The presence of three different haplotypes found only in two small populations may be indicative of the relict status of this plant. Furthermore, its interior position within a *Centaurea* sect. *Phrygia* cpDNA haplotype network regarding to *C. emigrantis* (López-Alvarado et al., in prep.), indicates that *C. tripontina* could match the 'stable rear edge' model of Hampe and Petit (2005). This model proposes that some populations of a particular species survived climatic oscillations of Quaternary in suitable areas. The habitat where *C. tripontina* grows, North-South orientated limestone gorges, fits with such description since represents an important quaternary shelter area postulated for many other species inhabiting this particular Pyrenean habitat (Montserrat and Montserrat, 1988; Mayol and Rosselló, 1999; Bosch et al., 2002; Castro et al., 2008). Such gorges have represented stable microenvironments conserving great niches' diversity and the possibility of vertical migrations which has avoided large displacements (Médail and Diadema, 2009).

Taking into account its possible relict condition and the possibility of genetic diversity conservation driven by long life span, the scarcity of individuals, the low levels of recruitment observed, the low rates of seed production and the effects of herbivory could be acting against survival of this species. Furthermore, the increasing of climbing routes in the locality where *C. tripontina* grows, which is clearly affecting natural rupicolous habitats, claims for urgent protection measures.

As a conclusion, the analysis of SSR and cpDNA data for *C. emigrantis* and *C. tripontina* suggest that both species can be considered independent evolving lineages, since they present genetic differentiation, which added to morphological differences and habitat preferences supports its divergent evolutionary histories. Nevertheless, the

genetic contact between both species can not be ruled out. Moreover, the importance of such contacts should be studied in depth by adding other Iberian species that could be involved. Finally, provided evidences recommend the treatment of both species as Evolutionary Significant Units (ESU) in conservation efforts (Moritz, 1994) paying a special attention to intraspecific population diversity both in *C. emgrantis* and *C. tripontina*.

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TABLES

Table 1. Voucher information and sources of material of the two species included in this work.

Species	Population	Locality	UTM	Habitat	Altitude	Voucher
C. emigrantis Bubani	COR	Spain, Lleida, Àger, Corçà, road to Congost de Mont Rebei	42º 02' N 0º 41' E	Road margins	700 m	21.VI.2009, *Figueroa & López-Alvarado* (BC)
	LIM	Spain, Lleida, Llimiana, along the road LV-9121 to Llimiana	42º 03' N 0º 53' E	Road margins	400 m	21.VI.2009, *Figueroa & López-Alvarado* (BC)
	AME	Spain, Lleida, Camarasa, road LV-9311 to L'Ametlla de Montsec	42º 00' N 0º 49' E	Road margins	500 m	21.VI.2009, *Figueroa & López-Alvarado* (BC)
	MTR	Spain, Lleida, Les Avellanes, Vilanova de la Sal, road to the sanctuary of Mare de Déu de Montalegre	41º 52' N 0º 47' E	Road margins	600 m	25.VI.2009, *Figueroa & López-Alvarado* (BC)
C. tripontina López-Alvarado et al.	DRE	Spain, Lleida, Figols i Alinyà, Congost de Tresponts	46º 13' N 1º 20' E	Calcareous cliffs	630-670 m	06.VI.2009, *L. Sáez LS-7065* (L. Sáez, herb. pers.-BCB)
	ESQ	Spain, Lleida, Figols i Alinyà, Congost de Tresponts	46º 13' N 1º 20' E	Calcareous cliffs	630 m	01. VIII. 2008, *Guardiola* (BCB)

Table 2. Marker information: locus names, bibliographic references, repeat motifs, size ranges of PCR products and number of alleles observed in the original publications.

Loci	Repeat	Size (bp)	No. of alleles	Reference
21CM36	$(CA)_6(TA)_5(TG)_{16}$	187–244	11	Marrs et al. (2006)
CM17	$(AC)_9$	379–430	12	
12B1	$(TA)_{27}(GA)_{22}$	168	10	Fréville et al. (2000)
13B7	$(AC)_{12}(AT)_5$	163	10	
13D10	$(AC)_7ATAC(AT)_{10}$	178	4	
28A7	$(CA)_{16}$	112	10	

Table 3. Number of effective alleles (*NE*), number of private alleles (*PA*), Shannon's Information Index (*I*) and heterozygosity values (H_O and H_E) for each species computed after the 11 polymorphic nuclear loci. *s. d.*: standard deviation.

Species	*NE*	*s. d*	*PA*	*I*	*s. d*	H_O	*s. d*	H_E	*s. d*
C. emigrantis	**3.42**	**± 1.59**	**—**	**1.39**	**± 0.49**	**0.46**	**± 0.22**	**0.64**	**± 0.19**
COR	2.17	± 0.44	2	0.82	± 0.21	0.39	± 0.28	0.43	± 0.11
LIM	2.8	± 0.53	2	1.10	± 0.20	0.48	± 0.26	0.56	± 0.08
MTR	3.05	± 0.48	5	1.23	± 0.23	0.49	± 0.31	0.60	± 0.10
AME	3.29	± 0.51	5	1.28	± 0.13	0.48	± 0.30	0.65	± 0.06
C. tripontina	**2.24**	**± 0.76**	**—**	**0.93**	**± 0.53**	**0.44**	**± 0.30**	**0.48**	**± 0.26**
DRE	1.92	± 0.32	1	0.69	± 0.21	0.39	± 0.30	0.39	± 0.11
ESQ	2.52	± 0.56	2	0.93	± 0.24	0.49	± 0.32	0.49	± 0.12

Table 4. Analysis of Molecular Variance (AMOVA) of SSR data for *C. emigrantis* and *C. tripontina* considering the two species as a single unit and each species separately.

Source of variation	d.f.	Sum of squared deviations	Variance components	% variation	Signif.
C. emigrantis and *C. tripontina*					
Among species	5	129,809	0.46	28,34	*
Among populations within species	154	195.875	0,09	5,70	0.003
Within populations	160	173.500	1,08	65,96	*
C. emigrantis					
Among populations	3	29,99	0,15	13,49	*
Within populations	236	227,78	0,96	86,51	*
C. tripontina					
Among populations	1	10,89	0,24	15,9	*
Within populations	78	99,17	1,27	84,1	*

Table 5. F_{ST} values for pairs of populations. *C. emigrantis* population abbreviations: COR = Corçà population; LIM = Llimiana population; MTR = Mont Roig population; AME = Ametlla population. *C. tripontina* population acronyms: DRE = Right side of river Segre population; ESQ = Left side of river Segre population.

	COR	LIM	MTR	AME	DRE	ESQ
COR	0.00					
LIM	0.14	0.00				
MTR	0.13	0.12	0.00			
AME	0.24	0.13	0.10	0.00		
DRE	0.50	0.32	0.45	0.38	0.00	
ESQ	0.50	0.36	0.39	0.31	0.18	0.00

FIGURES

Figure 1. Scatterplot showing individuals of *C. emigrantis* (squares) and *C. tripontina* (circles) against the two first Principal Coordinates of PCoA analysis. Coord. 1 accounts for the 47.98 % of total variance and Coord. 2 for the 16.48 %. *C. emigrantis* population abbreviations: COR = Corçà population; LIM = Llimiana population; MTR = Mont Roig population; AME = Ametlla population. *C. tripontina* population acronyms: DRE = Right side of river Segre population; ESQ = Left side of river Segre population.

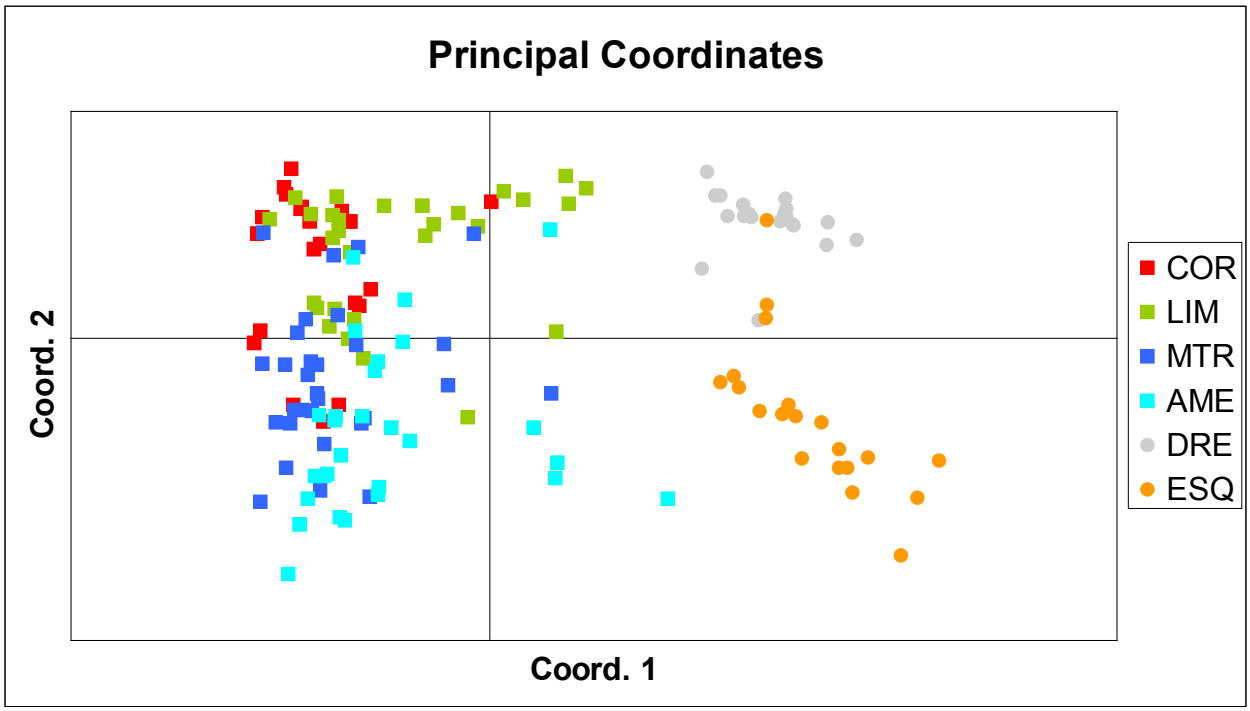


Figure 2. STRUCTURE Bar Plot for *C. emigrantis* and *C. tripontina* shown by individuals. Red corresponds to *C. emigrantis* and green to *C. tripontina*. Some individuals of *C. emigrantis* were represented as intermediate. COR = Corçà population; LIM = Llimiana population; MTR = Mont Roig population; AME = Ametlla population; DRE = Right side of river Segre population; ESQ = Left side of river Segre population.

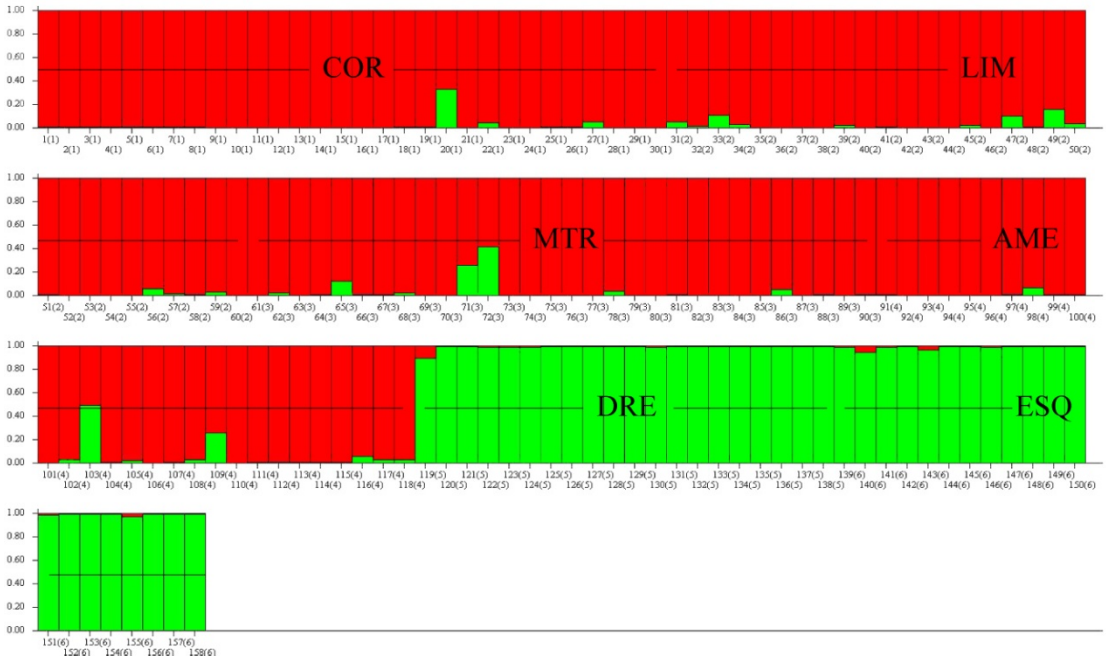
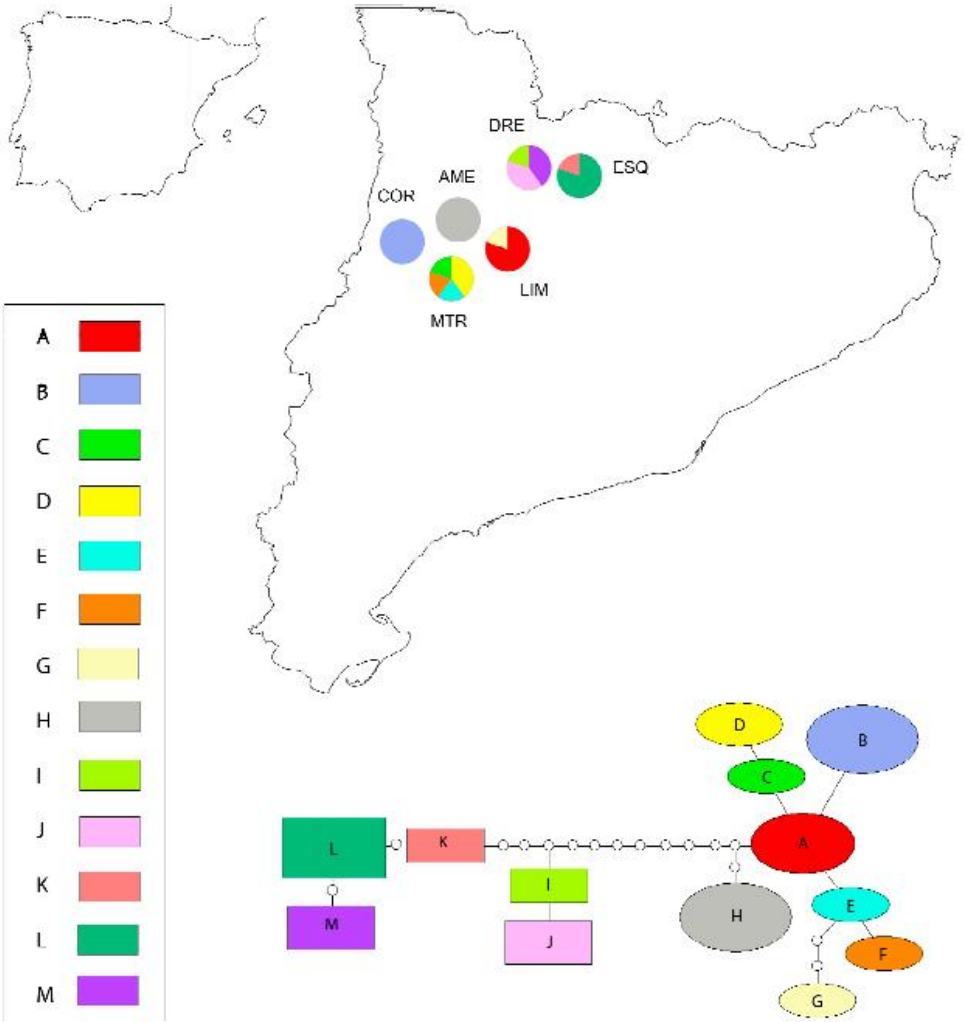


Figure 3. Frequency and geographic distribution of the 13 cpDNA haplotypes found. Haplotype network is also shown. Ellipses in the network represent haplotypes found only in *C. emigrantis*. Rectangles represent haplotypes exclusive of *C. tripontina*. Population names of *C. emigrantis*: COR, AME, LIM and MTR. Population names of *C. tripontina*: DRE and ESQ. Each letter and color is indicating one different haplotype.



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CENTAUREA TRIPONTINA (COMPOSITAE), A NEW SPECIES FROM THE PRE-PYRENEAN MOUNTAINS, SPAIN

Centaurea tripontina (compositae), a New Species from the Pre-Pyrenean mountains, Spain

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ABSTRACT

The new species *Centaurea tripontina* (Compositae) grows in Pre-Pyrenean mountains (northeast Iberian Peninsula) and belongs to *Centaurea* section *Lepteranthus*, which includes several narrow endemic species distributed in mountainous regions of Centre and South Europe and North Africa. This Catalonian Pre-Pyrenees populations, found at Tresponts gorge, which have been reported twice until the date and which were identified as once as *C. emigrantis* and once as *C. pectinata*, have gone unnoticed for years. Morphological and micromorphological data reveals that Tresponts gorge individuals should be described as a new species separated from those two other species.

Key words: Compositae, *Centaurea,* section *Lepteranthus*, Pre-Pyrenees Mountains, Iberian Peninsula.

INTRODUCTION

Centaurea L. is one of the largest genera of the Asteraceae family with around 250 species (Susanna and Garcia-Jacas 2007) distributed mainly in the Mediterranean region and SW Asia. The last taxonomic arrangement of the whole genus in Europe was Dostál's review for *Flora Europaea* (Dostál 1976). In his classification, Dostál recognized the *Centaurea* section *Lepteranthus* (Neck.) DC. based on two characters, the long, usually reflexed, linear, pectinate-fimbriate appendages and the presence of pappus in the achenes. Section *Lepteranthu*s is composed of around 19 species (Dostál 1976), which are divided into different numbers of subspecies, mainly distributed in Europe. In the Iberian Peninsula, it is represented by nine species: *Centaurea antennata* Dufour*, C. corcubionensis* M. Laínz*, C. debeauxii* Gren. & Godr.*, C. emigrantis* Bubani*, C. hyssopifolia* Vahl*, C. janeri* Graells, *C. linifolia* L.*, C. nigra* L. and *C. pectinata* L.

Some Catalonian Pre-Pyrenees populations found in Tresponts gorge were identified as *Centaurea emigrantis* by J. Prudhouse (herbarium BC 145156) and as *Centaurea pectinata* subsp. *acutifolia* (Jordan) Dostál by P. Montserrat and L. Villar (herbarium JACA 116472 & 146883). For years this herbarium sheets were unnoticed, until year 2008 when were reviewed, questioning doubts concerning the taxonomic attribution of herbarium specimens. In summer 2008 new specimens were collected and new locations were found. To clarify the identity of these populations we used morphological and phytodermological characters.

Centaurea tripontina López-Alvarado, L. Sáez, Filigheddu, Guardiola & Susanna sp. nov. TYPE: Spain. Lleida Province: Organyà, Congost de Tresponts, 31TCG6376, 580 m, calcareous cliffs, 21-VI-2008, *L. Sáez* 6870 (holotype, BC 871572;

Isotype: Ibidem, *L. Sáez*, herb. pers.-BCB). Figure 1.

Speciei *Centaurea emigrante* Bubani similis sed planta viridis, foliis latioribus, non tomentosis, pilis unicellularibus septatis brevibus (53--106 μm) munitis (in illa nullis) et corolla roseoalbida (albida in *C. emigrante*). Etiam *C. pectinata* L. affinis, a qua caulibus brevioribus, non erectis, foliis pubescentibus, superioribus sessilibus, et bractearum appendicibus brevioribus (2.25--4.5 mm), linearibus ad triangularibus, nigricantibus vel atrocastaneis differt.

Perennial herb, stems 13--36 cm long, numerous, simple or ramified, decumbent. Leaves 5--10 cm long, cuneate to lanceolate, mucronate, entire, dentate or sometimes lobed to lyrate, glabrous, with short (53--106 μm) septate trichomes on margins (also on the rest of the leaf, less densely); lower leaves cuneate, petiolate; upper lanceolate, sessile. Capitula sessile, solitary. Involucre 12.5--17 mm x 10--16 mm; bracts glabrous or slightly hairy, appendages short, linear to triangular, 2.25--4.5 x 0.8--1.5 mm, dark brown to black, somewhat recurved, pectinate-fimbriate, fimbriae 9--12, of 2.5--3.75 mm long. Florets pale pink, the outer not radiate. Stamens 6-8 mm long, anthers 2--3 mm long, violet. Style 1--1.5 mm long. Achenes 3--3.5 x 1.50--1.70 mm, lanceolate, laterally compressed, sparsely hairy, whitish to grey; pappus 1--2 mm, yellow-white.

Habitat and distribution

Centaurea tripontina is restricted to a small area of the Pre-Pyrenees (Tresponts gorge, Lleida province, northeast Spain). It grows in a narrow altitudinal zone between 570 and 780 m above sea level on calcareous cliffs, together with several endemic taxa, such as *Antirrhinum molle* L., *Asplenium seelosii* Leybold subsp. *catalaunicum* (O. Bolòs & Vigo) P. Monts., *Hieracium candidum* Scheele, *H. hastile* Arv.-Touv. & Gaut., *Ramonda myconi* (L.) Rchb., *Saxifraga longifolia* Lapeyr. as well as more widely distributed taxa such as *Asplenium fontanum* (L.) Bernh., *A. ruta-muraria* L. subsp.

ruta-muraria, *Coronilla lotoides* W.D.J. Koch, *Erinus alpinus L., Globularia repens* Lam., *Lonicera pyrenaica* L. subsp. *pyrenaica*, *Narcissus assoanus* Dufour, *Potentilla caulescens* L., *Sarcocapnos enneaphylla* (L.) DC., *Sedum dasyphyllum* L., *Silene saxifraga* L. and *Teucrium aureum* Schreb. No other species of *Centaurea* were found coexisting with the new taxon.

IUCN Red List category

Since June 2008 we have explored a number of sites (16 UTM 1x1 km^2 squares) in Tresponts gorge where suitable habitats for *C. tripontina* are known to exist. Applying the IUCN (2001) methodology to evaluate the vulnerability of *C. tripontina* produced the following results: the distribution of the species is highly restricted with extent of occurrence (6.8 km^2) and area of occupancy (0.53 km^2). At present it is known to exist at 3 locations, which are located in 3 1-km^2 UTM cells. A decline in the population currently seems imminent as a consequence of the decrease in the habitat quality and we have also registered fluctuations in the number of mature individuals. Therefore, *C. tripontina* should be included in the IUCN (2001) "endangered" category according to the following criteria and subcriteria:

EN B1ab(ii,iii)c(iv)+B2ab(ii,iii)c(iv).

The climbing routes have become the main source of direct anthropogenic disturbance in the area. Disturbance of the vegetation due to rock-climbing on limestone cliffs has been confirmed in mountain areas of central Europe (Müller et al. 2004). Management plans and conservation actions are needed to minimize the impact due to direct anthropogenic disturbance in areas with cliffs that are climbed frequently. In addition, overgrazing by herbivores constitutes a biotic threat.

Phenology

The new species was collected in flower in June--July and in fruit in July--August.

Etymology

The name refers to the locality where *C. tripontina* was found, Tresponts gorge, which is the only known locality of the new species.

DISCUSSION

Centaurea tripontina presents good qualitative and quantitative characters to discriminate the new species from *C. emigrantis* and *C. pectinata*, such as, the glabrous character of green *C. tripontina* leaves, unlike *C. emigrantis*, which has greyish lanate leaves, and *C. pectinata*, which has green pubescent leaves. This indument is also important at micromorphological level, even though *C. emigrantis* and *C. pectinata* have flaccid trichomas formed by a small basal cell followed by some thin walled cells and a long terminal whip-like cell, unlike *C. tripontina* which has a low density of conical, short, thick-walled trichomas with decidous, shorter, thin whip-like terminal cells (Figure 2). Another important trait is the appendage morphology, with long linear pale brown appendages in *C. emigrantis*, triangular in *C. pectinata* and *C. tripontina*, but shorter, wider, darker and not so recurved in *C. tripontina*. The floret configuration is also important, *C. pectinata* has capitula with pink-violet florets and without sterile radiant florets while *C. tripontina* has capitula with white-pink and non sterile radiant florets, and finally *C. emigrantis* has sterile radiant and white florets. The habitat is another important trait to differentiate these species, *C. pectinata* grow on acid soils whereas *C. emigrantis* and *C. tripontina* grows on calcareous substrates, although *C. emigrantis* appears in fields and road margins and *C. tripontina* grow in shaded calcareous cliffs. Linking with the habitat type, the grow habit presents differences, *C. emigrantis* presents a procumbent habit, erect to ascending in *C. pectinata* and decumbent in *C. tripontina*. In the new species the decumbent habit has been checked as

genetically fixed character in plants cultivated at the Botanical Institute of Barcelona (Figure 3).

Centaurea tripontina shows some morphological characters that could suggest an hybridogenic origin. However, no other taxa of the genus have been found in the vicinity and isolation and new habitat seem to have stabilized *C. tripontina* as a new species. An ongoing study using molecular markers will confirm or reject our hypothesis on its origin and affinities.

KEY TO SPECIES

1a. Involucre 6–12 mm in diameter, narrowly conical; stems procumbent sometimes ascending, simple; leaves greyish-white, lanate; appendages pale brown, strongly recurved; florets white ……. ***C. emigrantis***

1b. Involucre 10–18 mm in diameter, ovoid or subglobose; stems and leaves not as above; florets pink, pink-violet ….……...…..…….. 2

2a. Stems usually up to 40 cm, erect to ascending; leaves greyish-green, pubescent; involucre 13–18 mm in diameter; appendages black below, strongly recurved ; pappus 0.5 mm ……...…… ***C. pectinata***

2b. Stems 12 to 40 cm, pendant; leaves green, glabrescent; involucres 10–16 mm in diameter; appendages reddish-brown to black, not so recurved; pappus 1–2 mm ……..…… ***C. tripontina***

ACKNOWLEDGEMENTS

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FIGURES

Figure 1. Holotypus of *Centaurea tripontina* sp. nov. (B), with details of achene (A) and bracts (C). BC871572. Scale bars: A: 1 mm; B: 2 cm; C: 3 mm.

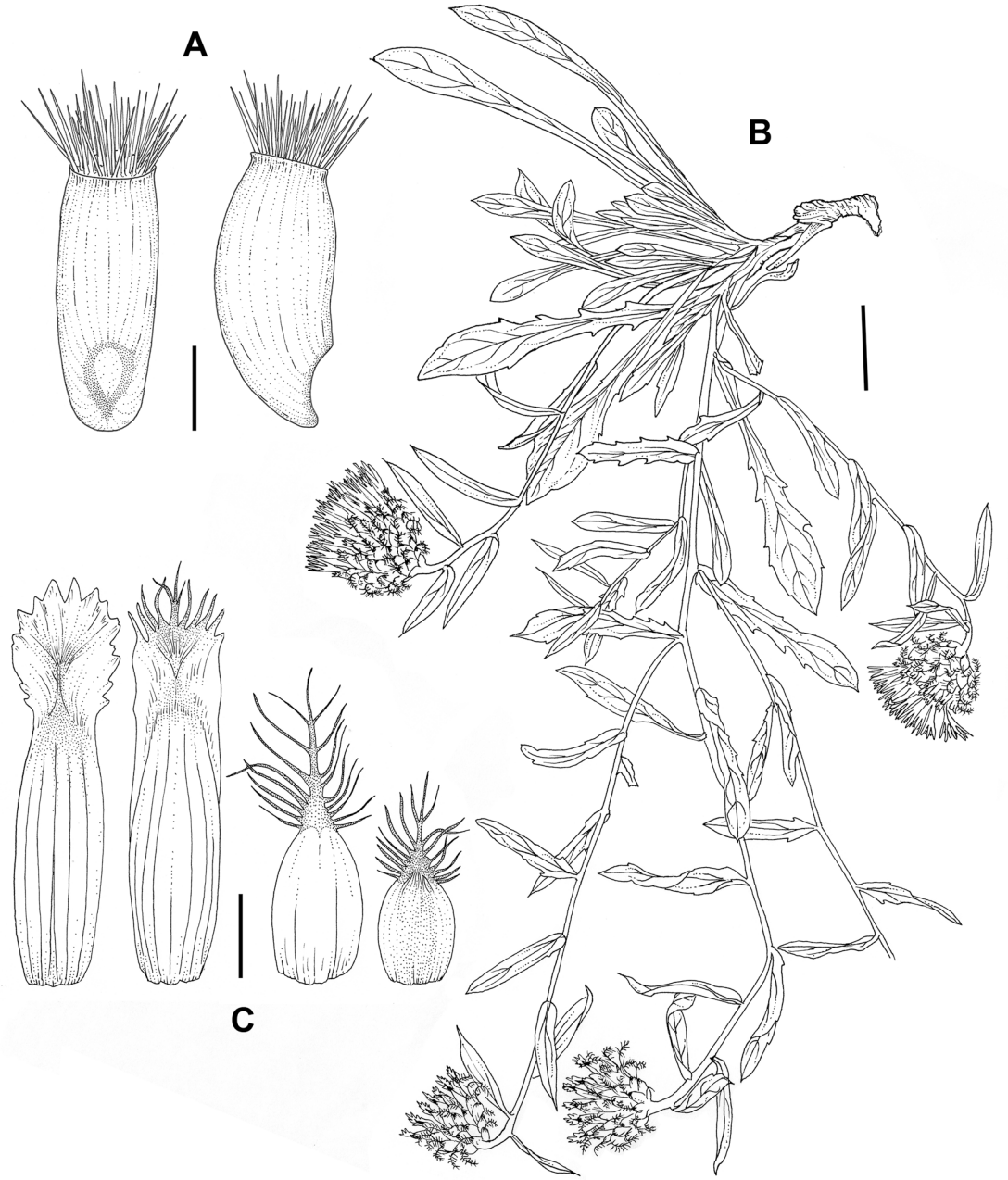

Figure 2. Scale representation of trichomas for each species. Species: A = *C. emigrantis*, B = *C. tripontina*, C = *C. pectinata*. Scale bar: 55 μm.

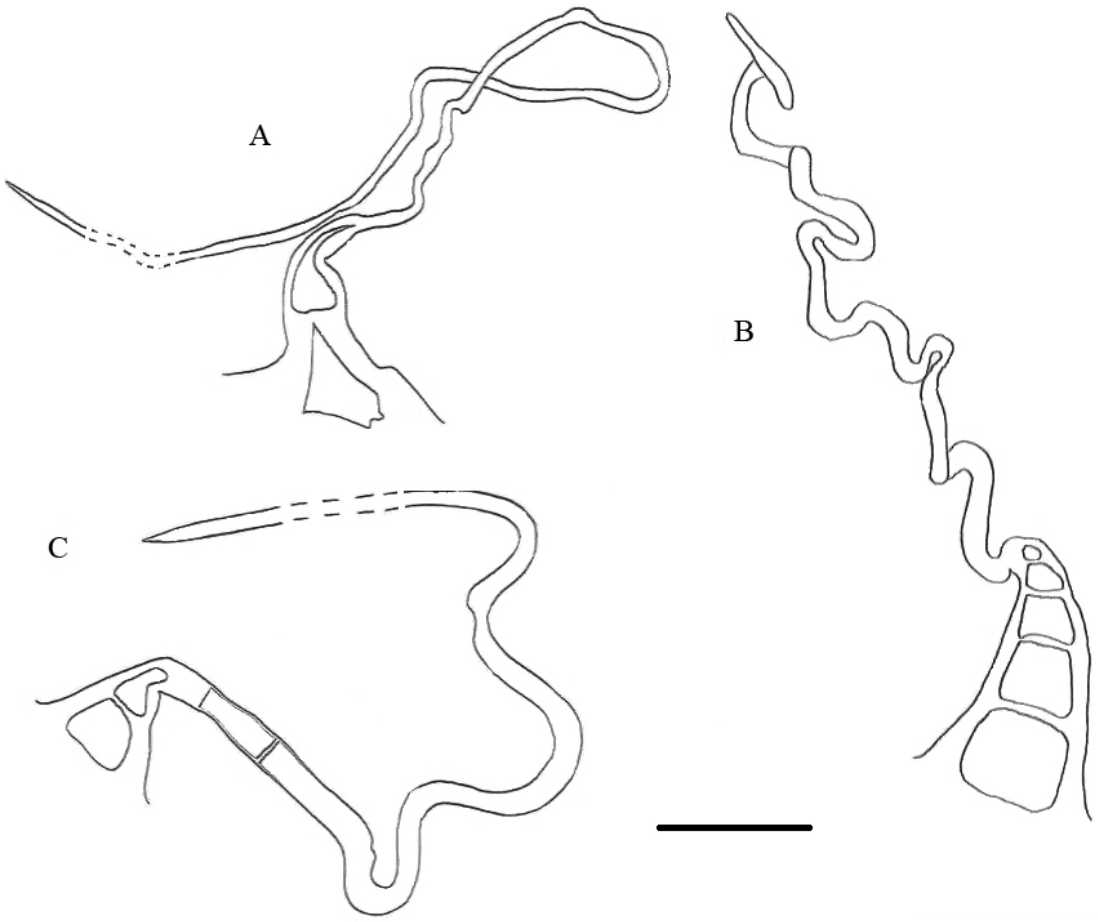

Figure 3. Specimen of *Centaurea tripontina* sp. nov. on a calcareous cliff showing the decumbent habit.