The Cardueae (Compositae) revisited: insights from a combined ITS, *trn*L-*trn*F and *mat*K nuclear and chloroplast DNA analysis

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The classification of Compositae has changed dramatically in recent years. The new outline of relationships in basal branches confirms that the sister group to the large tribe Cardueae are not Mutisieae, but only a small group of African genera of this tribe that are now classified as tribe Tarchonantheae. This change implies that our outgroup selection in previous molecular studies was not suitable, and monophyly of Cardueae must be reassessed on a molecular basis. Moreover, new collections in recent years allow us to extend our sampling to 70 of the 74 genera of the tribe. We performed a new molecular study of the tribe using one nuclear region (ITS) and two chloroplastic ones (*trn*L-*trn*F and *mat*K) and a more appropriate outgroup. Our results confirm that Cardueae are a natural group but indicate some changes in subtribal delineation: a new subtribe Cardopatiinae is recognized and some genera are moved to other subtribes (*Myopordon*, *Nikitinia*, *Syreitschikovia* and the *Xeranthemum* group). A recapitulation of a number of interesting questions that remain unresolved in the classification of some large genera is presented.

Key Words: Compositae; Cardueae; ITS; *trn*L-*trn*F; *mat*K; phylogeny; tribal delimitation; genus delineation.

INTRODUCTION

Overview *of Compositae systematics*

The systematics of Compositae is marked by three milestones, each one involving deep changes in the classification of the family. Since the history of this classification has been revised in depth in Funk *et al.* (in press), here we give only a short summary. The first attempt to classify Compositae was made by Cassini (1819), who defined twenty tribes. A more synthetic system was proposed by Bentham (1873) and soon after Hoffmann (1894). Both authors proposed a classification in two subfamilies and 16 tribes that gained general acceptation until very recently. The two latest revisions of the family towards the end of the 20th century (Dittrich, 1977; Bremer, 1994) followed Hoffmann's classification. The third large-scale changes were produced by the introduction of methods based on DNA analysis. First came the pioneering study by Jansen and Palmer (1987) using cpDNA restriction site polymorphisms, which led to the description of a third subfamily, Barnadesioideae, a proposal that was reflected in Bremer (1994). Second, the latest and more revolutionary study by Panero and Funk (2002), analysed sequences of nine chloroplast regions across the entire family and proposed a new classification with 11 subfamilies and 35 tribes, in some ways closer to Cassini's analytical views than to synthetic approaches. The dramatic differences between Bremer's (1994) classification and Panero and Funk (2002) are illustrated in Table 1 (only the basal groups are shown). The high statistical support for the latter and its sound correlation with morphology leads us to believe that the new classification of Compositae is near to being definitive final.

The tribe Cardueae

Cardueae are one of the largest tribes of Compositae with ca. 2500 species. Previous studies based on DNA sequence analyses, both nuclear (Susanna *et al.*, 1995) and combined chloroplast and nuclear (Garcia-Jacas *et al.*, 2002) confirm Cardueae as monophyletic. However, the new classification shows that our previous outgroup choice was partly inadequate. In the classic system of Compositae (e. g., Heywood *et al.*, 1977; Bremer, 1994), Cardueae were classified in subfamily Cichorioideae, close to tribes Lactuceae and Mutiseae (Table 1). Thereafter, in our first nuclear-DNA-based phylogeny (Susanna *et al.*, 1995) outgroup was composed of one Lactuceae (*Tragopogon* L.) and three Mutisieae (*Ainsliaea* DC., *Gerbera* L. and *Warionia* Benth. & Coss.). In Garcia-Jacas *et al.* (2002), we replaced *Ainsliaea*, *Tragopogon* and *Warionia* because of the increasing difficulties in aligning the ITS region and we used two Mutisieae as outgroups, *Gerbera* and *Mutisia* L. f. However, according to the new classification by Panero and Funk (2002), tribe Lactuceae (*Tragopogon*) is derived in relation to Cardueae; *Ainsliaea* and *Warionia* don't belong to Mutisieae but to Pertyeae and Gundelieae respectively, both tribes also derived with regard to Cardueae; and *Gerbera* and *Mutisia* are placed in Mutisieae sensu stricto, phylogenetically far from Cardueae (Table 1). With these outgroup species, Cardueae will always be monophyletic, and monophyly of the tribe has always been a controversial issue. The outgroup should be chosen from Tarchonantheae, the true sister group to Cardueae (Table 1). In fact, Cardueae, Dicomeae and Tarchonantheae compose a monophyletic subfamily, Carduoideae (Panero and Funk, 2002).

Tribal limits of Cardueae: In the earliest classification (Cassini, 1819), present Cardueae were divided in three tribes: Echinopeae, Carlineae, and Cardueae, the latter with two subtribes: Carduinae and Centaureinae. Bentham (1873) and Hoffmann (1894) proposed grouping the three tribes in a single tribe Cardueae that held four subtribes:

Echinopinae, Carlininae, Carduinae, and Centaureinae. This was a conservative approach that was generally accepted for a very long time. However, discussion on the status of Echinopinae restarted when Wagenitz (1976) proposed the segregation of the subtribe as a separate tribe, Echinopeae. Dittrich (1977) returned to Cassini's early views and proposed the restoration of Echinopeae and Carlineae. Finally, Bremer (1994) reintroduced the conservative approach with only one tribe, Cardueae, which, according to our molecular studies, is a better solution (Susanna *et al.,* 1995; Garcia-Jacas *et al.,* 2002).

Subtribal classification: Within Cardueae, there is general agreement in accepting four groups, regardless of the rank (tribe or subtribe) adopted. Three subtribes are natural (Carlininae, Echinopinae and Centaureinae) and the fourth (Carduinae) is a heteroclite paraphyletic assemblage (Garcia-Jacas *et al.,* 2002).

Subtribe Carlininae is sister to the rest of the tribe. A striking and probably plesiomorphic character is the presence of true ray florets in at least one genus of Carlininae, *Atractylis* L., whilst remaining subtribes have only disc florets. Capitula are usually subtended by pectinate-pinnatisect leaf-like bracts; corolla lobes are very short, only 1-3 mm long; and the pappus has long, plumose bristles, often connate at the basis into broader, robust scales (Susanna and Garcia-Jacas, in press).

Subtribe Echinopinae is easily characterized by its second-order inflorescences (uniflowered capitula clustered in a large synflorescence). Our latest molecular phylogeny indicates that Echinopinae should also include the genera of the *Xeranthemum* group and we previously proposed that the small heads of the genus *Xeranthemum* and allies could be interpreted as reduced synflorescences (Garcia-Jacas *et al.,* 2002).

Subtribe Carduinae is a paraphyletic complex of genera with some well defined groups (*Arctium* L. group, *Onopordum* L. group, *Saussurea* DC. group, or the thistles) together with genera of problematic ascription like *Berardia* Vill. or *Staehelina* L. All the genera of Carduinae have basal or basal-abaxial insertion areole of achenes and usually a simple pappus, and they are often spiny.

Finally, subtribe Centaureinae is the most derived group and is characterized by achenes with lateral-adaxial insertion areole, a double pappus and -with few exceptions- unarmed leaves. However, examining the limits between Carduinae and Centaureinae is a challenge because differences lie in microcharacters of achene and pappus that are difficult to observe in incomplete or immature herbarium materials. The examples of *Nikitinia* Iljin and *Syreitschikovia* Pavlov illustrate these difficulties (Susanna *et al.*, 2002) and the ascription of these and other genera should be checked against a molecular phylogeny.

In Garcia-Jacas *et al.* (2002) we proposed a fifth subtribe Cardopatiinae Less., with two genera: *Cardopatium* Juss. and *Cousiniopsis* Nevski. Cardopatiinae were placed in an intermediate position between Carlininae and the rest of the tribe (Garcia-Jacas *et al.*, 2002). However, we postponed the acceptation of this subtribe until more unambiguous evidence had been collected.

Generic limits in tribe Cardueae: Other points of interest are genus affinities and limits in Cardueae, a tribe with some of the largest genera of the family. Regarding genus affinities, on the basis of morphology and partial molecular studies, the two largest subtribes (Carduinae and Centaureinae) were subdivided into informal groups (Susanna and Garcia-Jacas, in press), which should be checked against a more comprehensive molecular phylogeny. As to genus limits, in our latest revision of Cardueae (Susanna and Garcia-Jacas, in press) we adopted a broad generic concept for

Cousinia Cass. (600 species), *Jurinea* Cass. (200) and *Saussurea* (400), because of the lack of recent systematics revisions for all the three. Recently, on the basis of a partial study of DNA sequences and achene morphology, Raab-Staube (2003) proposed the restoration of two small genera, *Frolovia* (DC.) Lipsch. and *Lipschitziella* Kamelin, and described a new genus, *Himalaiella* Raab-Staube, all of these within the *Saussurea* group.

Our study

With the addition of new materials, our DNA sampling covers 70 of the 74 accepted genera of Cardueae: only *Ancathia* DC. (Carduinae, Central Asia), *Centaurodendron* Johow (Centaureinae, Juan Fernández archipelago), *Goniocaulon* Cass. (Centaureinae, India and East Tropical Africa) and *Takeikadzuchia* Kitag. & Kitam. (Carduinae, Mongolia) are absent, and the position of these within the tribe and their subtribal ascription is clear. To test our broad generic concept, we included the genera *Frolovia*, *Lipschitziella* and *Modestia* Kharadze & Tamamsch., which we introduced in *Jurinea*; and *Anura* (Kult.) Tscherneva and *Tiarocarpus* Rech. f., which we had previously placed in *Cousinia* (Susanna and Garcia-Jacas, in press). We also included the published sequence of the recently described genus *Himalaiella*. On this wide representation of Cardueae, we completed the ITS and *mat*K regions and, in view of the low resolution of basal groups in previous analyses, we added a new marker. Low definition in many molecular phylogenies can be solved by adding more data to DNA sequence matrices. We used a chloroplast marker, the *trn*L-*trn*F region, which is widely credited in Compositae (Bayer and Starr, 1998; Liu *et al.*, 2002; Oberprieler, 2002; Panero and Funk, 2002). Our goals were:

a) To verify monophyly of Cardueae using species from their sister tribe Tarchonantheae as outgroup.

b) To clarify subtribal classification and define the position of Cardopatiinae, which could constitute a fifth subtribe.

c) To examine whether the informal groups defined in subtribes Carduinae and Centaureinae are natural, and check the systematic position within these groups of many genera not included in our previous studies, and

d) To verify the suitability of a broad generic concept in some large genera of Cardueae by analyzing species from genera that we had previously rejected on the basis of morphological characters.

MATERIAL AND METHODS

Plant Material

Sampling was defined on the basis on Garcia-Jacas *et al.* (2001), Garcia-Jacas *et al.* (2002), Susanna *et al.* (2003) and Susanna and Garcia-Jacas (in press), in order to represent most of the genera of tribe Cardueae. Thirteen accepted genera (*Amphoricarpos* Vis., *Karvandarina* Rech. f., *Lamyropappus* Knorring & Tamamsch., *Lamyropsis* (Kharadze) Dittrich, *Myopordon* Boiss., *Nikitinia*, *Olgaea* Iljin, *Plagiobasis* Schrenk, *Polytaxis* Bunge, *Russowia* C. Winkl., *Syreitschikovia* Pavlov, *Siebera* J. Gay, *Tricholepis* DC. and *Tugarinovia* Iljin) are sequenced here for the first time. Six other genera that were not accepted in our latest revision are *Aegopordon* Boiss., *Anura* Tscherneva, *Frolovia*, *Lipschitziella*, *Modestia* and *Tiarocarpus* Rech. f. Two more genera, *Dolomiaea* DC. and *Himalaiella*, were obtained from published sequences. Three outgroup species were chosen among Tarchonantheae, which are sister to Cardueae (Panero and Funk, 2002). Many of our ITS1 and ITS2 sequences from

previous studies (Garcia-Jacas *et al.*, 2001, 2002) have been completed with the sequence of the 5.8S gene, and some of our old manual ITS sequences (Susanna *et al.*, 1995) have been sequenced again by automatic sequencing. Both previously published (Garcia-Jacas *et al.*, 2002) and new sequences of the *mat*K gene were used in the analysis. All the *trn*L-*trn*F sequences analysed are new, with the exception of *Dolomiaea* (from Liu, unpub.) and some species of *Saussurea* (from Liu, unpub. and Raab-Staube, 2003). The number of new sequences is 283. The origin of the samples and their GenBank accession numbers are given in Table 2.

DNA Extraction, Amplification, and Sequencing

Total genomic DNA was extracted following the miniprep procedure of Doyle and Doyle (1987) as modified by Soltis *et al.* (1991) and Cullings (1992), from silica-gel-dried leaves collected in the field or from fresh leaves of plants cultivated in the Botanic Institute of Barcelona. In some cases, herbarium material was used.

cpDNA *trn*L-*trn*F region strategies: The *trn*L-*trn*F region includes the *trn*L intron, the 3' *trn*L (UAA) exon, and the intergenic spacer between *trn*L (UAA) and *trn*F (GAA), that were amplified and sequenced together. Universal primers *trn*L-c, forward, and *trn*L-f, reverse (Taberlet *et al.*, 1991) were used for amplifying the *trn*L-*trn*F region. In some cases, *trn*L-d, reverse, and *trn*L-e, forward, were used. Polymerase chain reaction (PCR) was conducted in a termocycler (MJ Research PTC 100). The PCR procedure include a warm start at 95°C for 1 minute 35 seconds, followed by 80°C during which the polymerase (Ecotaq, Ecogen S.R.L., Barcelona, Spain) is added, and

34 cycles of 1 minute denaturation at 93°C, 1 minute annealing at 58°C, 1 minute extension at 72°C, and a final 10 minute extension at 72°C.

PCR products were cleaned with a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sequenced with the *trn*L-c and *trn*L-f primers. Direct sequencing of the amplified DNA segments was performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA), following the protocol recommended by the manufacturer. Nucleotide sequencing was carried out at the Serveis Científico-Tècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, CA).

cpDNA *mat*K gene strategies: We have sequenced the first 1000 base pairs at the 5' end because this region includes most of the variability in *mat*K (Khidir and Hongping, 1997). Partial *mat*K was amplified by PCR with the primers *trn*K-710F (Johnson and Soltis, 1995) and AST-1R (Garcia-Jacas *et al.*, 2002). The PCR procedure included a warm start at 94°C for 1 minute 20 seconds, followed by 80°C during which the polymerase (Ecotaq, Ecogen S.R.L., Barcelona, Spain) was added, and 40 cycles of 45 seconds denaturation at 94°C, 1 minute annealing at 58°C, 2 minutes extension at 72°C, and a final 10 minutes extension at 72°C. PCR products were cleaned with QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and sequenced with *trn*K-710F and AST-1R primers. Direct sequencing of the amplified DNA segments was performed as for the *trn*L-*trn*F region.

nrDNA ITS region strategies: Both ITS1, 5.8S gene, and ITS2 (the ITS region) were amplified and sequenced together. The ITS region was amplified by PCR with 1406F (Nickrent *et al.*, 1994) and ITS1 (White *et al.*, 1990) as forward primers, and ITS4

(White *et al.*, 1990) as reverse primer, referring to the protocol described in Soltis and Kuzoff (1993). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA). Sequencing primers 1406F and ITS4 were used. Direct sequencing of the amplified DNA segments was performed as for the *trn*L-*trn*F region.

Phylogenetic Analysis

Nucleotide sequences were edited with Chromas 1.56 (Technelysium Pty., Tewantin, Australia). The *trn*L-*trn*F and *mat*K sequences were aligned visually by sequential pairwise comparison (Swofford and Olsen, 1990). *Mat*K sequences were translated into proteins with GeneJockey (Biosoft, Cambridge, U. K.) to verify the absence of internal stop codons. Due to the high level of variability of the ITS sequences, our alignment was checked with the ITS alignment for the whole Compositae by Goertzen *et al.* (2003) and adjusted manually. In order to conserve the phylogenetic information of insertions-deletions which constitute the main part of the variation of the *trn*L-*trn*F region and at the same time avoiding over-estimation of lengthy indels, they where coded as presence-absence characters and added to the end of matrices in all the cases. Data matrices are available on request from the corresponding author.

The ITS matrix was analyzed by Neighbor-Joining method, using parsimony and the default options as specified in PAUP4b10, because heuristic search was impossible due to the size of the matrix (190 species). For the combined data sets, parsimony analysis involved heuristic searches conducted with PAUP version 4.0b10 (Swofford, 1999) using Tree Bisection Recognition (TBR) branch swapping with character states specified as unordered and unweighted. All most-parsimonious trees (MPT) were saved.

To locate islands of most-parsimonious trees (Maddison, 1991), we performed 100 replicates with random taxon addition, also with TBR branch swapping. Tree lengths, consistency index (CI) and retention index (RI) are always given excluding uninformative characters. Two combined analyses were performed, with different data sets: the ITS + *trn*L-*trn*F data, and the ITS + *trn*L-*trn*F + *mat*K data.

Bootstrap (BS) and Bremer support (Bremer, 1988; Donoghue *et al.*, 1992) or "decay index" (DI) were carried out to obtain support estimates of the nodes of the consensus trees. Bootstrap analysis was performed (Felsenstein, 1985) using 1000 replicates and heuristic search with the default options. In the nrDNA ITS data matrix, we used the approach by Lidén *et al.* (1997) using 1000 replicates, random taxon addition with 20 replicates per replicate, and no branch swapping. Regarding decay index, it was technically impossible for the ITS matrix. For the two combined analyses, DI was calculated for each node by successive analysis using the clade constraint approach as discussed in Morgan (1997) with 10 replicates. ACCTRAN (accelerated transformation) character-state optimization was used for all illustrated trees.

RESULTS AND DISCUSSION

Since we were unable to obtain the sequences of the three regions for all our samples, we performed three distinct analyses: the ITS alone, to examine the position of some genera not included in previous analyses and for which we were not able to amplify any chloroplast region; a combined ITS and *trn*L-*trn*F regions, to study the generic limits in the *Saussurea* group; and a combined analysis of the three regions, to elucidate subtribal delineation and confirming the natural limits of the informal groups

in Carduinae. The numeric results of the three analyses are summarized in Table 3. The resulting trees are shown in Figure 1a and 1b (NJ phylogram for the ITS alone), Figure 2 (strict consensus of the combined ITS and *trn*L-*trn*F sequences) and Figure 3 (strict consensus of the combined ITS, *trn*L-*trn*F and *mat*K sequences).

Delineation of Cardueae

The monophyly of Cardueae was confirmed with the new outgroup in all the analyses with the highest statistical support: BS = 99, 100% (Fig. 1a, 2 and 3) and DI= 11 (Fig. 2 and 3). Thus, the most adequate status for Echinopinae and Carlininae is subtribal. Indeed, Cardueae could be divided in five tribes, but we consider it inpractical to fragment a natural group that can be so easily recognized on the basis of macromorphology.

Subtribal classification

The four subtribes recognized by the latest report on the tribe (Garcia-Jacas *et al.*, 2002), Carlininae, Echinopinae, Carduinae and Centaureinae, were confirmed. However, subtribe Cardopatiinae must be restored and some changes made to correlate molecular phylogeny and subtribal delineation.

Carlininae and *Tugarinovia*: Our *results* do not modify the circumscription of Carlininae in our latest surveys of Cardueae (Garcia-Jacas *et al.*, 2002; Susanna and Garcia-Jacas, in press). The subtribe is monophyletic with high support (BS = 75, 95%, 100%; DI = 7, 9, Fig. 1a, 2 and 3) and includes *Atractylodes* DC., *Atractylis*, *Carlina* L. and *Thevenotia* DC. (not included in present analyses, but confirmed in our previous work). The rest of the genera that were classified by other authors in Carlininae belong either to Carduinae (*Staehelina* and the *Xeranthemum* group) or Cardopatiinae

(*Cardopatium* and *Cousiniopsis*). The classic definition of Carlininae was based mainly on achene characters (Dittrich, 1977, 1996b): parenchymatic pericarp usually hirsute, and pappus setae very long and plumose directly attached to the pericarp. However, these characters must be interpreted as plesiomorphic because they appear across all basal subtribes (Carlininae, Cardopatiinae and Echinopinae) and even in Carduinae. If we rely only on achene characters for classification, the resulting definition of Carlininae (Dittrich, 1977, 1996b) differs greatly from the delineation on the basis of DNA sequence analyses and macromorphology (Susanna and Garcia-Jacas, in press).

Curiously, after confirmtion on molecular grounds that *Tugarinovia* belongs to Cardueae, the inclusion of this puzzling monotypic genus from Mongolia in Carlininae, as first proposed by Dittrich *et al.* (1987) is unsupported, even though the only subtribe where it can be placed on the basis of morphological affinities (leaves, involucral bracts and pappus) is Carlininae. However, our analyses showed no connection of *Tugarinovia* with the only other East Asian representative of Carlininae, *Atractylodes*, or with any other genus of the subtribe.

Cardopatiinae: This subtribe had moderate support in our analyses (BS = 62%, 65%, 81% and DI = 4, 6, Fig. 1a, 2 and 3), probably because of the different evolving rates of annual *Cousiniopsis* and perennial *Cardopatium*. Regarding the position of Cardopatiinae near the basis of the tree, in view of the moderate support for these basal branches, subtribes Carlininae, Echinopinae and Cardopatiinae should be considered a polytomy basal to Carduinae-Centaureinae.

Cardopatiinae, as first defined, included only the east Mediterranean genus *Cardopatium*. Later, Nevski (1937) described a monotypic genus from central Asia, *Cousiniopsis*, closely related to *Cardopatium* (it was first described as *Cardopatium atractyloides* C. Winkler). Classic monographers of Compositae (Bentham, 1873;

Hoffmann, 1894; Dittrich, 1977; Bremer, 1994) consistently placed both genera among Carlininae, but the only characters that connect these two groups are achenes, which could equally relate *Cardopatium* and *Cousiniopsis* to Echinopinae. It is tempting to interpret the corymbose inflorescence of *Cardopatium*, formed by very small, few-flowered capitula, as a first step towards syncephaly. On this basis, Petit (1997) considered *Cardopatium* sister to *Echinops* L. and placed *Cardopatium* and *Cousiniopsis* in Echinopinae. On the basis of our results, we prefer interpret these similarities as convergence, especially since the same trend towards syncephaly appears in all subtribes of Cardueae (Garcia-Jacas *et al.*, 2002).

Echinopinae: Our results demonstrate, contrary to our previous studies (Garcia-Jacas *et al.*, 2002), that Echinopinae include only *Echinops sensu lato* (*Echinops* and *Acantholepis* Less.) with strong support (BS = 100% and DI = 26, 30; Fig. 1b, 2 and 3). In fact, a recent molecular study indicates that *Acantholepis* is a reduced, unarmed species of *Echinops* (Garnatje, pers. comm.), as originally described (*Echinops acantholepis* Jaub. & Spach). Our combined analyses reveal that the *Xeranthemum* group doesn't belong to Echinopinae (Fig. 1a, 1b, 2 and 3).

The origin of the compound inflorescence of *Echinops* cannot be tracked on molecular grounds because the subtribe does not show affinity to any other group. Cardopatiinae and Carlininae are the best candidates for being sister of subtribe Echinopinae (the structure of the achenes is very similar, cf. Dittrich, 1977). However, syncephalies at various states of development involving small, few-flowered heads occur in all the subtribes across Cardueae.

Carduinae: If monophyletic Centaureinae are moved to a distinct subtribe, the Carduinae constitute a paraphyletic assemblage (Fig. 1a, 2 and 3). However, alternate solutions are unpractical: either subtribe level is adscribed to all the monophyletic

groups recognized in present Carduinae and a fragmented classification made, or a single large subtribe Carduinae is maintained, which includes Centaureinae, thereby encompassing almost ninety per cent of the species of the tribe (Garcia-Jacas *et al.*, 2002).

Even in this heteroclite assemblage, some well-defined groups emerge, together with genera without known affinities like *Berardia* or *Staehelina*.

Berardia and *Staehelina*: Our analyses show that these two genera present no affinities. They are clustered in an isolate position within Carduinae in both combined analyses, without statistical support (Fig. 2 and 3). *Berardia* was ranked among Mutisieae on the basis of achene characters (Dittrich, 1977 and 1996a) and we agree in that the pericarp wall is very similar to the type found in Gochnatiinae, a subtribe of Mutiseae. An fact that could support this faint relationship between *Berardia* and *Staehelina* is that the pericarp of *Staehelina* is also "gochnatioid" (Dittrich, 1996a). However, we cannot state whether this similarity is convergence or a very old character conserved in these two strange genera.

Staehelina was usually placed among Carlininae (Bentham, 1873; Hoffmann, 1894; Dittrich, 1977; Bremer, 1994), but Petit (1997) proposed moving it to Carduinae. For Dittrich (1996b), the two species of *Staehelina* with hirsute pericarp (*S. fruticosa* L. and *S. lobelii* DC.) should be classified in a distinct genus, *Hirtellina* Cass. All our analyses grouped the included species of *Staehelina* (five out of eight) in a robust clade with very high support (BS = 100% and DI = 34, 47, Fig. 1a, 2 and 3). However, they all divided the genus in two highly supported clades that coincide with *Staehelina* s. stir. and *Hirtellina* (Fig. 1a, 2 and 3), which is compatible with the division of the genus. Nevertheless, morphological differences other than achene pilosity are virtually non-

existent and we prefer to keep a single genus with *Staehelina* and *Hirtellina* recognized with sectional rank (Susanna and Garcia-Jacas, in press).

The *Xeranthemum* group: In a previous study, this group (genera *Amphoricarpos*, *Chardinia* Desf., *Siebera* and *Xeranthemum* L.) was placed among subtribe Echinopinae. This unexpected result led us to propose that the very small and peculiar heads of the genera of the group, with very large receptacular bracts, could constitute a syncephaly (Garcia-Jacas *et al.*, 2002). Our new analyses show that this view was erroneous and, in fact, Harris (1995) had already demonstrated that the inflorescence of *Xeranthemum* was not a syncephaly. The *Xeranthemum* group appears in the combined analyses as part of the Carduinae, sister to the rest of the subtribe with low support but indeed in an isolate position. The monophyly of the group has very high support (BS = 100% and DI = 23, 28, Fig. 1a, 2 and 3). Traditional classification (Dittrich, 1977; Bremer, 1994) placed it in Carlininae, and Petit (1997) was the first to suggest Carduinae. Species of the annual genera of the group (*Chardinia*, *Siebera* and *Xeranthemum*) are colonizers of arid and waste-land thoroughout the Mediterranean region. In contrast, species of the dwarf shrubby genus *Amphoricarpos* are narrow mountain endemics and are sister to the rest of the genera in all the analyses (Fig. 1a, 2 and 3).

The *Onopordum* group: The usual definition of this group is founded on the absence of receptacular bracts. A pitted, naked receptacle is rare in the tribe. However, not all the species of at least one genus (*Alfredia* Cass.) show epaleate receptacle. In addition to this character, achenes are also peculiar with pericarp diversely pitted, wrinkled or rugulose (Susanna and Garcia-Jacas, in press), seldom smooth (*Olgaea* and *Syreitschikovia*). The group has negative importance because species of *Onopordum*

include some highly noxious weeds widespread in the Mediterranean region like *O. acanthium* L. and *O. nervosum* Boiss., giant thistles that can reach up to 3 m high.

Three genera not included in previous studies, *Lamyropappus*, *Olgaea* and *Syreitschikovia*, are classified in the *Onopordum* clade in all the analyses, which confirm the group as a natural one with high support (BS = 77%, 91%, 97% and DI = 6, 4; Fig. 1a, 2 and 3). *Syreitschikovia* was placed by Dittrich (1977) and Bremer (1994) in Centaureinae. Its classification in Carduinae and its relationship to the *Onopordum* group was reported out by Susanna *et al.* (2002) on the basis of morphology.

Generic definitions in the group are unclear. *Synurus* Iljin forms a polytomy with *Olgaea* and *Syreitschikovia* (Fig. 1a, 2 and 3). The inclusion of more species of *Olgaea* (which comprises some 15 taxa from the Tien Shan mountains) and its strange relative *Takeikadzuchia* from Mongolia may contribute to a better definition of the genera in the group.

The *Carduus* group: This is the large complex of very spiny plants which are usually called "thistles". All of these have medium or large-sized heads, spiny leaves and a long pappus detachable as a single piece. Our results indicate that at least the largest part (*Carduus* L. , *Cirsium* Mill., *Notobasis* Cass., *Picnomon* Adans., *Silybum* Adans. and *Tyrimnus* Cass.) are a natural group with good support (BS = 78%, 91%, 100% and DI = 10, 14, Fig. 1a, 2 and 3). The rest of the genera, *Cynara* L., *Galactites* Moench, *Lamyropsis* (Kharadze) Dittrich and *Ptilostemon* Cass., are also placed in the group in all the analyses, but only the combined analysis of the three regions support this branch, albeit weakly (BS = 60% and DI = 2, Fig. 3).

As pointed out by Häffner and Hellwig (1999) and Garcia-Jacas *et al.* (2002), phylogenetic relationships and generic boundaries within the clade are obscure (Fig. 1a, 2, and 3). One of the reasons for this is that the co-existence of annual or biennial

species (most of *Carduus*, *Galactites*, *Picnomon* Adans., *Silybum* or *Tyrimnus*) together with perennials (many *Cirsium*, *Cynara*, *Lamyropsis* and *Ptilostemon*) hinders the assessment of the two aspects from a molecular standpoint. Differences in mutation rates between annuals and perennials (Gaut *et al.*, 1997; Laroche *et al.*, 1997; Andreasen and Baldwin, 2001) make comparison of sequences a less reliable tool. In fact, some unexpected results like the strange position of the annual genus *Galactites* could be caused by this character of annual: *Galactites* is placed close to the basis of the thistles grouped to *Ptilostemon* (Fig. 1a, 2 and 3), thereby contradicting morphological evidence (*Galactites* is very similar to *Carduus* or *Cirsium*). Regarding *Lamyropsis*, the only genus of the thistles that was missing in our previous studies and is sequenced here for the first time, appears related to *Ptilostemon*, without support (Fig. 1a, 2 and 3). Species of *Lamyropsis* have dentate-spiny leaves with very prominent veins beneath like many species of *Ptilostemon*, and a relationship between the two genera was already pointed out by Dittrich (1971). The *Carduus* group, together with the two following ones, requires a more comprehensive molecular analysis.

The *Arctium* group: this group has been subject of a recent preliminary molecular survey, using ITS and *mat*K sequences (Susanna *et al.*, 2003). The results of the new analyses including the *trn*L-*trn*F region (Fig. 2 and 3) do not change our main previous conclusions: the limits of *Arctium* L. and *Cousinia* are unclear. Our study (Susanna *et al.*, 2003) demonstrated two main lines in the *Arctium* group: the Arctioid clade (supported only by the combined analyses with BS = 85%, 100% and DI = 5, 7; Fig. 2 and 3) and the Cousinioid clade (support by all the analyses with BS = 84%, 92%, 94% and DI = 4, 3; Fig. 1a, 2 and 3). The two groups can be segregated by molecular, chromosome and pollen characters, but this grouping is not consistent with morphology: two genera of the group, *Schmalhausenia* C. Winkl. and *Hypacanthium* Juz., are part of

Arctium on the basis of pollen, chromosomes and DNA sequences (Fig. 1a and 2), but are morphologically much closer to *Cousinia*. In addition to an "Arctioid" group of *Cousinia*, there is also a "Cousinioid" group of *Arctium*. More sampling of the obscure *Cousinia* subgenus *Hypacanthodes* from Central Asia is required, but it is highly probable that all four genera will have to be grouped in *Arctium*.

Finally, our ITS analysis (Fig. 1a) confirms that the genera *Anura* and *Tiarocarpus* (*Cousinia pallidivirens* Kult. and *C. neubaueri* Rech. f. respectively in Fig. 1a) cannot be segregated from *Cousinia*, as previously proposed by Susanna and Garcia-Jacas (in press).

The *Saussurea* group: The only genus placed in the *Saussurea* group by Susanna and Garcia-Jacas (in press) that was not included in our previous studies is *Polytaxis*. The ITS and the combined ITS+*trn*L-*trn*F analyses place it basal to *Saussurea* with moderate support (BS = 75%, 87% and DI = 6, Fig. 1a and 2). Because species of *Polytaxis* are the only annuals in this clade, its basal position could originate in the faster evolution of annuals compared to perennials (Gaut *et al.*, 1997; Laroche *et al.*, 1997; Andreasen and Baldwin, 2001), in the same way as annual *Acantholepis* always appears basal to perennial *Echinops* (Fig. 1b, 2 and 3).

Another taxon that was not included in our previous study is the purported genus *Aegopordon* (*Jurinea berardioides* in Fig. 1a and 2), which, according to Susanna and Garcia-Jacas (in press), should be considered a synonym of *Jurinea*. The combined ITS+*trn*L-*trn*F analysis (Fig. 2) place it in a robust clade (BS = 91% and DI = 4) with *Jurinea carduiformis* Boiss., formerly also considered a distinct genus (*Outreya* Jaub. & Spach) that we merged in *Jurinea* Cass. (Garcia-Jacas *et al.*, 2002).

Our results confirm that the limits between *Jurinea* and *Saussurea* are not well established (as pointd out recently by Kita *et al.*, 2004) because some species formerly

included in *Saussurea* are grouped in the genus *Jurinea* (Fig. 1a and 2): they are *Saussurea carduicephala* (Iljin) Iljin and *S. deltoidea* (DC.) Sch. Bip., considered by Raab-Staube (2003) a new genus *Himalaiella* (Fig. 1a and 2), and *Saussurea ceratocarpa* Decne, for Raab-Staube (2003) a restored genus *Lipschitziella* (Fig. 1a and 2). *Himalaiella* and *Lipschitziella* form a monophyletic clade with *Jurinea sensu stricto*, with very high support (BS = 100% and DI = 15, Fig. 1a and 2). A third genus restored by Raab-Staube (2003), *Frolovia* Lipsch. (*Saussurea asbukinii* Iljin and *S. frolovii* Ledeb. in the ITS and the combined ITS and *trn*L-*trn*F analyses, Fig. 1a and 2), on the basis of our well-supported results (BS = 95%, 94% and DI = 6, Fig. 1a and 2), must be considered a synonym of *Dolomiaea* DC.

No final conclusions can be drawn from this entanglement of genera because our sampling of *Jurinea* was very limited. However, a redefinition of the boundaries between *Jurinea* and *Saussurea* is clearly required. A good example of these troublesome limits is illustrated from the GenBank: one sequence is retrieved either as *Saussurea asbukinii* (data from Raab-Staube) or *Jurinea asbukinii* (data from Liu, a better approach according to our results). However, *Jurinea–Saussurea asbukinii* is the type of a third genus, *Frolovia*, which in turn is most probably a synonym of a *fourth* genus, *Dolomiaea*...

The clarification of these limits and indeed the description of new genera in a complex in which no less than 15 have been already described (Susanna and Garcia-Jacas, in press) calls for a much more comprehensive sampling than any performed to date.

Centaureinae: Our results confirm the general outline of Centaureinae proposed by Garcia-Jacas *et al.*, 2001, this time on the basis of three regions of the genome (Fig. 1b,

2 and 3). Here we describe only the most important results, namely the inclusion of two genera formerly classified in Carduinae, *Myopordon* and *Nikitinia*, in the subtribe.

Myopordon was considered related to *Onopordum* (hence the name) and placed in subtribe Carduinae because of the absence of receptacular setae (Wagenitz, 1958; Dittrich, 1977). In contrast, the ITS and the combined ITS + *trn*L-*trn*F analyses places *Myopordon* deeply nested in the *Leuzea* group of subtribe Centaureinae with moderate (BS = 75, Fig. 1b) or high support (BS= 85%, DI = 5, Fig. 2). Difficulties of interpretation of even apparently unambiguous characters are constant in tribe Cardueae: as we have seen above, the naked receptacle is a sound character of the *Onopordum* group (Susanna and Garcia-Jacas, in press), but they are too many exceptions. Epaleate genera are present in almost all subtribes: *Tugarinovia* in Carlininae, *Dolomiaea* and part of the *Onopordum* group in Carduinae, *Myopordon* and *Russowia* C. Winkl. in Centaureinae. To verify the position of *Myopordon* within Centaureinae on morphological grounds, the characters of the achenes are critical, but we were unable to find herbarium material with mature fruits. Mouterde (1983) described the insertion areole of the achenes as oblique, a character of Centaureinae. This observation contrasts with that of Wagenitz (1958), who reported the insertion as straight, which therefore points towards Carduinae.

Nikitinia was described in Carduinae and in all recent reviews of the tribe it was maintained in that subtribe (Dittrich, 1977; Bremer, 1994). However, achene characters are undoubtedly centauroid (especially, the double pappus, illustrated in Susanna *et al.*, 2002) as confirmed by molecular analyses (Fig. 1b).

CONCLUDING REMARKS

With a more suitable outgroup and with the addition of the *trn*L-*trn*F region, systematics of Cardueae now appears to be mature. However, there are some points that remain unclear and their clarification require better sampling and more morphological and molecular data. In addition to only moderate support for basal branches in the combined analysis of the three regions, the remaining doubts relate to the typical problems of delimitation of very large genera that are so frequent in Compositae (classic examples are *Aster* L., *Erigeron* L. or *Senecio* L.). In Cardueae, generic boundaries are difficult to establish in *Carduus*, *Cirsium*, *Cousinia*, *Jurinea* and *Saussurea*. In the case of *Carduus* and *Cirsium*, extensive sampling in Africa and North America is required. For *Cousinia*, *Jurinea* and *Saussurea*, which are the easternmost representatives of the tribe in Eurasia, intense collections are called for in Central and East Asia.

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Figure Captions

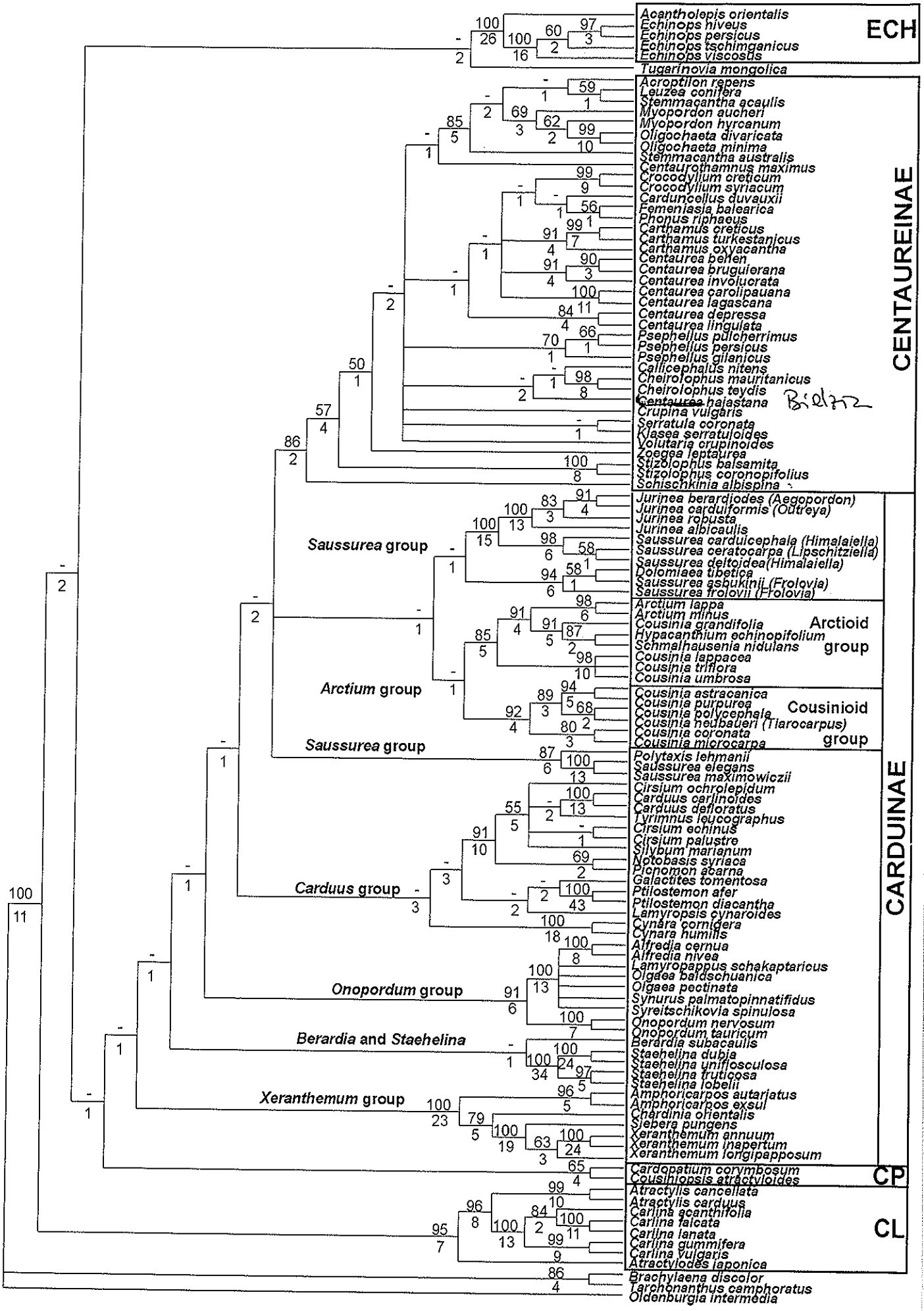
1a. Neighbor-Joining tree of the ITS sequence data matrix (basal part of the tree only). Numbers above branches are bootstrap percentages.

1b. Neighbor-Joining tree of the ITS sequence data matrix (upper part of the tree only). Numbers above branches are bootstrap percentages.

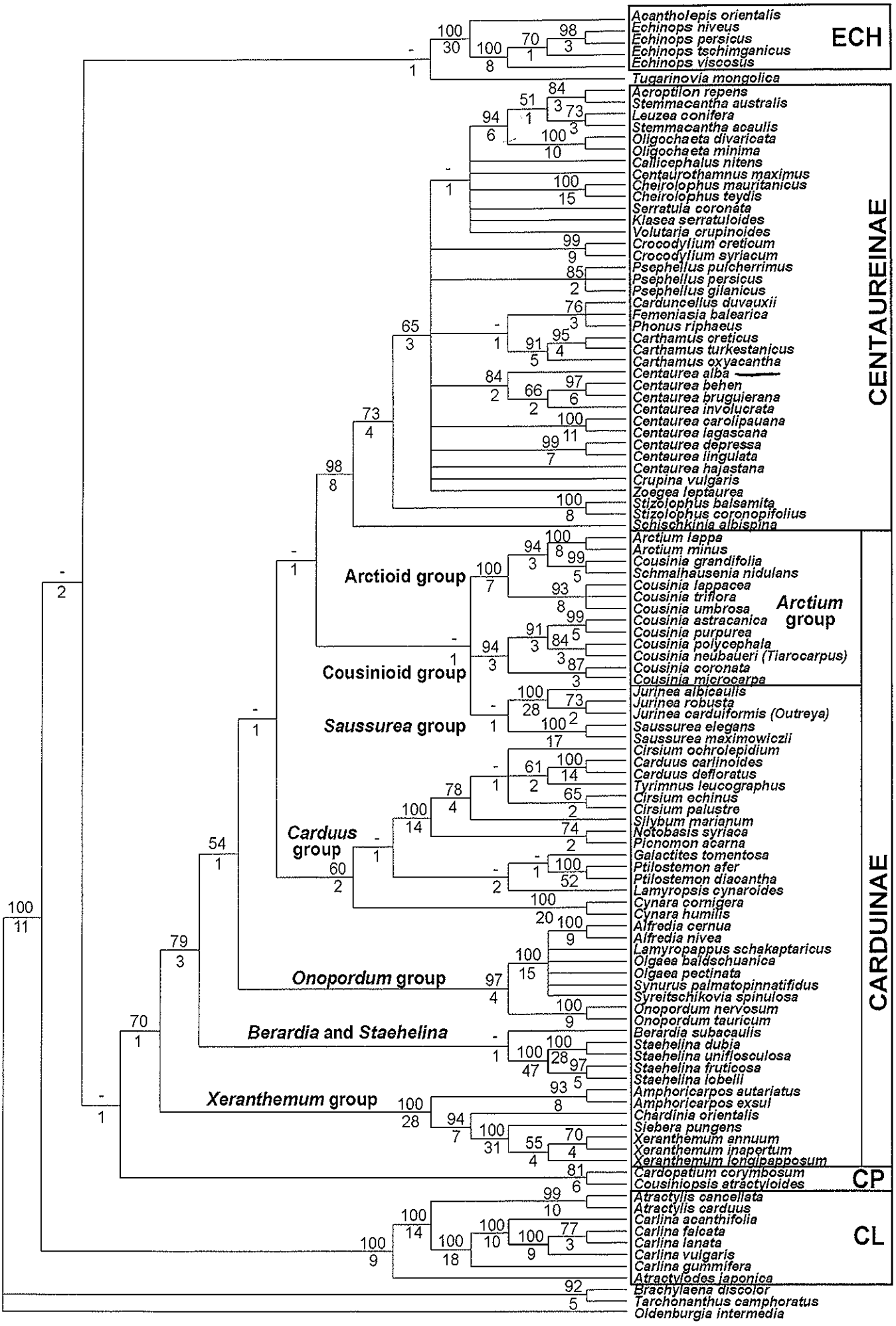
2. Strict consensus tree of the most parsimonious trees resulting from the ITS and *trn*L-*trn*F combined data matrix. Numbers above branches are bootstrap percentages; below branches, decay indices. CL = Carlininae; CP = Cardopatiinae; ECH = Echinopinae.

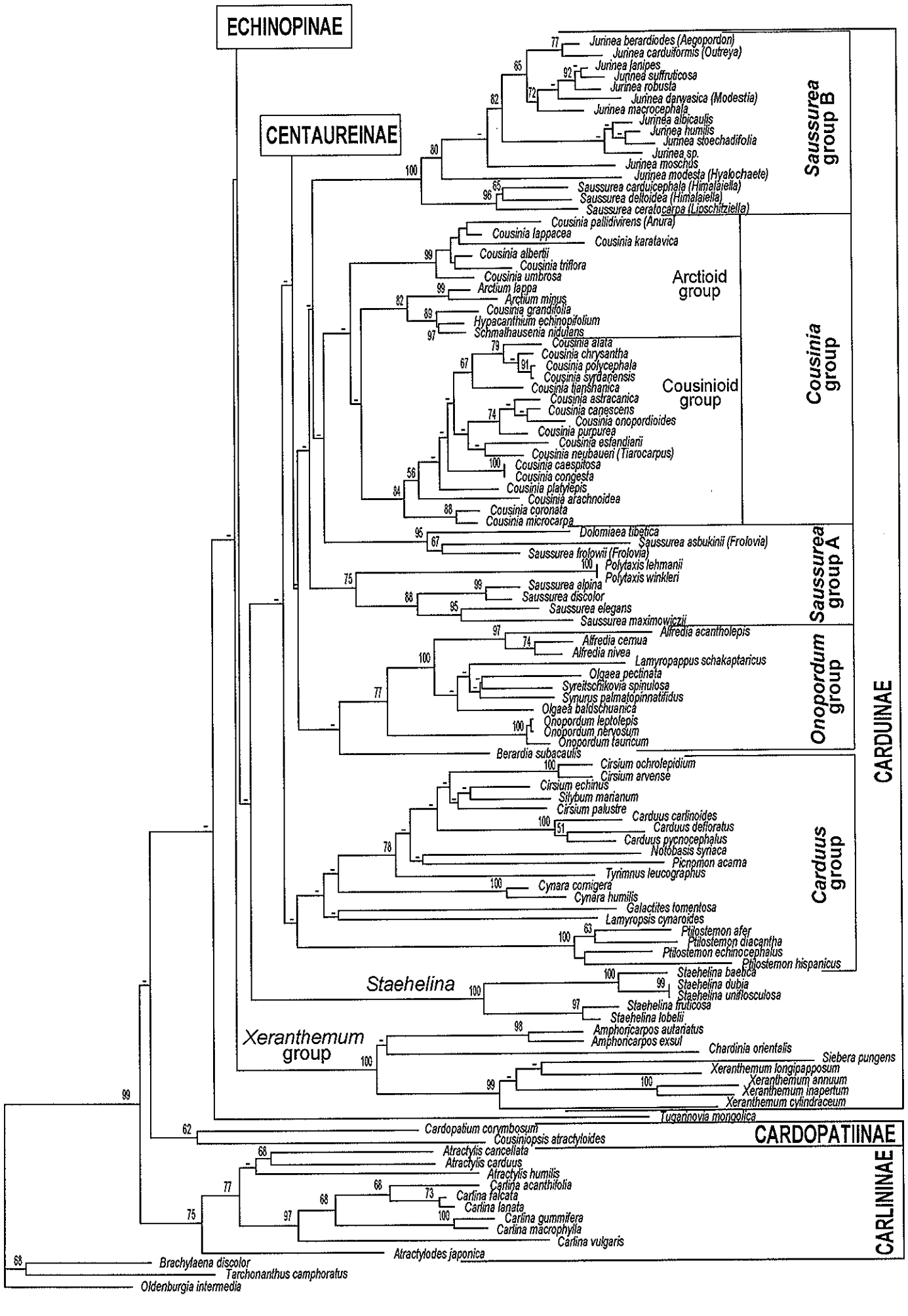
3. Strict consensus tree of the most parsimonious trees resulting from the ITS, *trn*L-*trn*F and *mat*K combined data matrix. Numbers above branches are bootstrap percentages; below branches, decay indices. CL = Carlininae; CP = Cardopatiinae; ECH = Echinopinae.

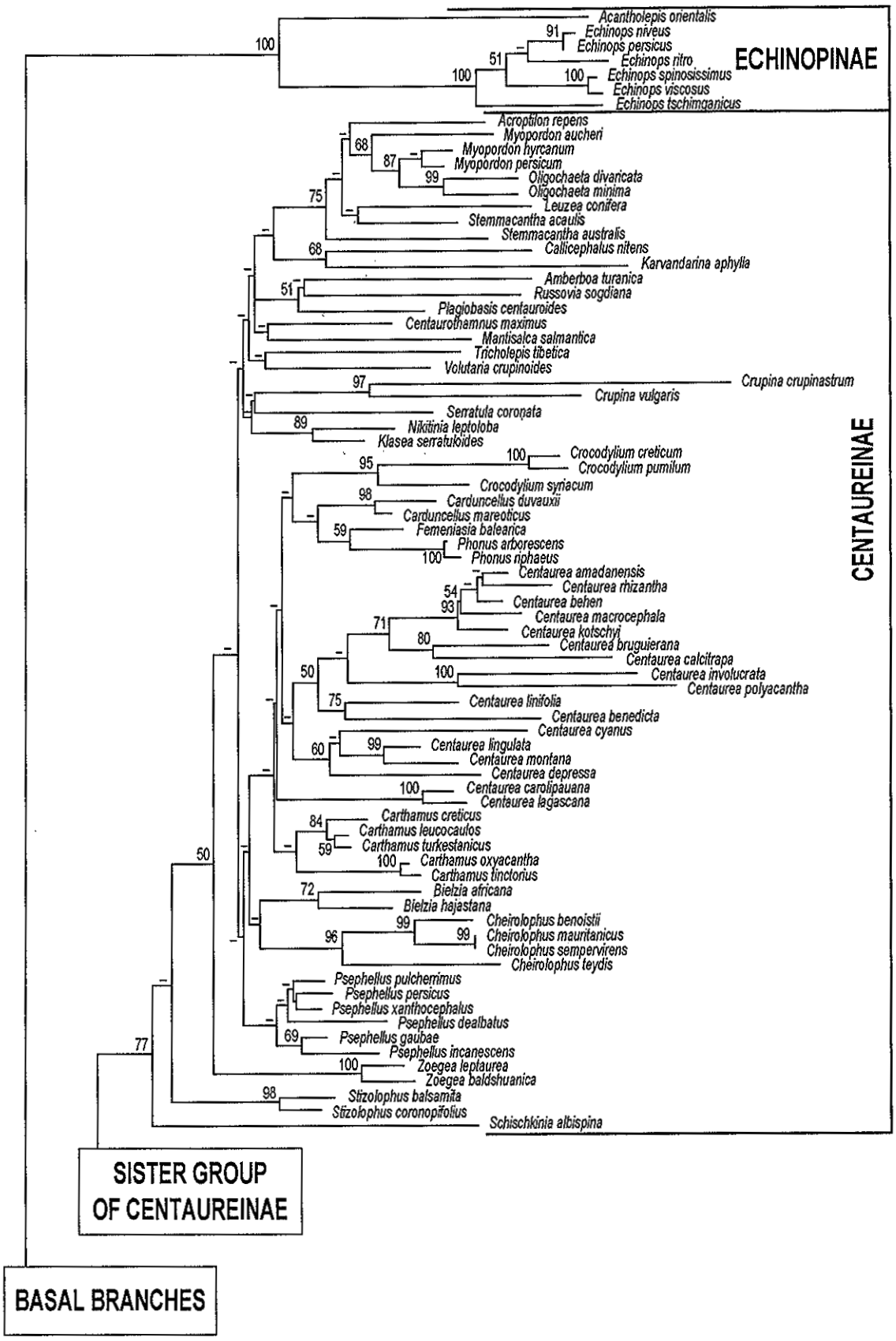
trnL + ITS


Acantholepis orientalis
Echinops hiveus
Echinops persicus
Echinops tschimganicus
Echinops viscosus
ECH
Tugarinovia mongolica
Acroptilon repens
Leuzea conifera
Stemmacantha acaulis
Myopordon aucheri
Myopordon hyrcanum
Oligochaeta divaricata
Oligochaeta minima
Stemmacantha australis
Centaurothamnus maximus
Crocodylium creticum
Crocodylium syriacum
Carduncellus duvauxii
Femeniasia balearica
Phonus riphaeus
Carthamus creticus
Carthamus turkestanicus
Carthamus oxyacantha
Centaurea behen
Centaurea bruguierana
Centaurea involucrata
Centaurea carolipauana
Centaurea lagascana
Centaurea depressa
Centaurea linguiata
Psephellus pulcherrimus
Psephellus persicus
Psephellus gilanicus
Callicephalus nitens
Cheirolophus mauritanicus
Cheirolophus teydis
Centaurea hajastana
Crupina vulgaris
Serratula coronata
Klasea serratuloides
Volutaria crupinoides
Zoegea leptaurea
Stizolophus balsamita
Stizolophus coronopifolius
Schischkinia albispina
CENTAUREINAE
Jurinea berardioides (Aegopordon)
Jurinea carduiformis (Outreya)
Jurinea robusta
Jurinea albicaulis
Saussurea carduicephala (Himalaiella)
Saussurea ceratocarpa (Lipschitziella)
Saussurea deltoidea(Himalaiella)
Dolomiaea tibetica
Saussurea asbukinii (Frolovia)
Saussurea frolovii (Frolovia)
Saussurea group
Arctium lappa
Arctium minus
Cousinia grandifolia
Hypacanthium echinopifolium
Schmalhausenia nidulans
Cousinia lappacea
Cousinia triflora
Cousinia umbrosa
Arctioid group
Arctium group
Cousinia astracanica
Cousinia purpurea
Cousinia polycephala
Cousinia neubaueri (Tiarocarpus)
Cousinia coronata
Cousinia microcarpa
Cousinioid group
Saussurea group
Polytaxis lehmanii
Saussurea elegans
Saussurea maximowiczii
Cirsium ochrolepidum
Carduus carlinoides
Carduus defloratus
Tyrimnus leucographus
Cirsium echinus
Cirsium palustre
Silybum marianum
Notobasis syriaca
Picnomon acarna
Galactites tomentosa
Ptilostemon afer
Ptilostemon diacantha
Lamyropsis cynaroides
Cynara cornigera
Cynara humilis
Carduus group
Alfredia cernua
Alfredia nivea
Lamyropappus schakaptaricus
Olgaea baldschuanica
Olgaea pectinata
Synurus palmatopinnatifidus
Syreitschikovia spinulosa
Onopordum nervosum
Onopordum tauricum
Onopordum group
Berardia subacaulis
Staehelina dubia
Staehelina uniflosculosa
Staehelina fruticosa
Staehelina lobelii
Berardia and Staehelina
Amphoricarpos autariatus
Amphoricarpos exsul
Chardinia orientalis
Siebera pungens
Xeranthemum annuum
Xeranthemum inapertum
Xeranthemum longipapposum
Xeranthemum group
CARDUINAE
Cardopatium corymbosum
Cousiniopsis atractyloides
CP
Atractylis cancellata
Atractylis carduus
Carlina acanthifolia
Carlina falcata
Carlina lanata
Carlina gummifera
Carlina vulgaris
Atractylodes japonica
CL
Brachylaena discolor
Tarchonanthus camphoratus
Oldenburgia intermedia

ITS + trnL + matK




ECHINOPINAE
CENTAUREINAE
Jurinea berardioides (Aegopordon)
Jurinea carduiformis (Outreya)
Jurinea lanipes
Jurinea suffruticosa
Jurinea robusta
Jurinea daryasica (Modestia)
Jurinea macrocephala
Jurinea albicaulis
Jurinea humilis
Jurinea stoechadifolia
Jurinea sp.
Jurinea moschus
Jurinea modesta (Hyalochaete)
Saussurea carduicephala (Himalaiella)
Saussurea deltoidea (Himalaiella)
Saussurea ceratocarpa (Lipschitziella)
Saussurea group B
Cousinia pallidivirens (Anura)
Cousinia lappacea
Cousinia karatavica
Cousinia albertii
Cousinia triflora
Cousinia umbrosa
Arctioid group
Arctium lappa
Arctium minus
Cousinia grandifolia
Hypacanthium echinopifolium
Schmalhausenia nidulans
Cousinia alata
Cousinia chrysantha
Cousinia polycephala
Cousinia syrdariensis
Cousinia tianshanica
Cousinioid group
Cousinia astracanica
Cousinia canescens
Cousinia onopordioides
Cousinia purpurea
Cousinia esfandiarii
Cousinia neubaueri (Tiarocarpus)
Cousinia caespitosa
Cousinia congesta
Cousinia platylepis
Cousinia arachnoidea
Cousinia coronata
Cousinia microcarpa
Cousinia group
Dolomiaea tibetica
Saussurea asbukinii (Frolovia)
Saussurea frolowii (Frolovia)
Polytaxis lehmanii
Polytaxis winkleri
Saussurea alpina
Saussurea discolor
Saussurea elegans
Saussurea maximowiczii
Saussurea group A
Alfredia acantholepis
Alfredia cernua
Alfredia nivea
Lamyropappus schakaptaricus
Olgaea pectinata
Syreitschikovia spinulosa
Synurus palmatopinnatifidus
Olgaea baldschuanica
Onopordum leptolepis
Onopordum nervosum
Onopordum tauricum
Onopordum group
Berardia subacaulis
Cirsium ochrolepidium
Cirsium arvense
Cirsium echinus
Silybum marianum
Cirsium palustre
Carduus carlinoides
Carduus defloratus
Carduus pycnocephalus
Notobasis syriaca
Picnomon acarna
Tyrimnus leucographus
Cynara cornigera
Cynara humilis
Galactites tomentosa
Lamyropsis cynaroides
Ptilostemon afer
Ptilostemon diacantha
Ptilostemon echinocephalus
Ptilostemon hispanicus
Carduus group
CARDUINAE
Staehelina
Staehelina baetica
Staehelina dubia
Staehelina unifloscula
Staehelina fruticosa
Staehelina lobelii
Xeranthemum group
Amphoricarpos autariatus
Amphoricarpos exsul
Chardinia orientalis
Siebera pungens
Xeranthemum longipapposum
Xeranthemum annuum
Xeranthemum inapertum
Xeranthemum cylindraceum
Tugarinovia mongolica
Cardopatium corymbosum
Cousiniopsis atractyloides
CARDOPATIINAE
Atractylis cancellata
Atractylis carduus
Atractylis humilis
Carlina acanthifolia
Carlina falcata
Carlina lanata
Carlina gummifera
Carlina macrophylla
Carlina vulgaris
CARLININAE
Atractylodes japonica
Brachylaena discolor
Tarchonanthus camphoratus
Oldenburgia intermedia
0.01 substitutions/site


Acantholepis orientalis
Echinops niveus
Echinops persicus
Echinops ritro
Echinops spinosissimus
Echinops viscosus
Echinops tschimganicus
ECHINOPINAE
Acroptilon repens
Myopordon aucheri
Myopordon hyrcanum
Myopordon persicum
Oligochaeta divaricata
Oligochaeta minima
Leuzea conifera
Stemmacantha acaulis
Stemmacantha australis
Callicephalus nitens
Karvandarina aphylla
Amberboa turanica
Russovia sogdiana
Plagiobasis centauroides
Centaurothamnus maximus
Mantisalca salmantica
Tricholepis tibetica
Volutaria crupinoides
Crupina crupinastrum
Crupina vulgaris
Serratula coronata
Nikitinia leptoloba
Klasea serratuloides
Crocodylium creticum
Crocodylium pumilum
Crocodylium syriacum
Carduncellus duvauxii
Carduncellus mareoticus
Femeniasia balearica
Phonus arborescens
Phonus riphaeus
Centaurea amadanensis
Centaurea rhizantha
Centaurea behen
Centaurea macrocephala
Centaurea kotschyi
Centaurea bruguierana
Centaurea calcitrapa
Centaurea involucrata
Centaurea polyacantha
Centaurea linifolia
Centaurea benedicta
Centaurea cyanus
Centaurea lingulata
Centaurea montana
Centaurea depressa
Centaurea carolipauana
Centaurea lagascana
Carthamus creticus
Carthamus leucocaulos
Carthamus turkestanicus
Carthamus oxyacantha
Carthamus tinctorius
Bielzia africana
Bielzia hajastana
Cheirolophus benoistii
Cheirolophus mauritanicus
Cheirolophus sempervirens
Cheirolophus teydis
Psephellus pulcherrimus
Psephellus persicus
Psephellus xanthocephalus
Psephellus dealbatus
Psephellus gaubae
Psephellus incanescens
Zoegea leptaurea
Zoegea baldshuanica
Stizolophus balsamita
Stizolophus coronopifolius
Schischkinia albispina
CENTAUREINAE
SISTER GROUP OF CENTAUREINAE
BASAL BRANCHES