Pl. Syst. Evol. 214: 147–160 (1999)

Plant Systematics and Evolution

Molecular phylogeny of *Cheirolophus (Asteraceae: Cardueae–Centaureinae)* based on ITS sequences of nuclear ribosomal DNA

ALFONSO SUSANNA, TERESA GARNATJE, and NÚRIA GARCIA-JACAS

Received June 9, 1997; in revised version September 25, 1997

Key words: *Asteraceae*, *Cardueae–Centaureinae*, *Cheirolophus*. – Mediterranean, molecular phylogeny, ITS.

Abstract: The genus *Cheirolophus* has an interesting western Mediterranean and Macaronesian distribution. Here we investigate the delimitation of the genus and its exclusion from the large genus *Centaurea*, the systematic position of the related genus *Paleocyanus*, the delimitation of some species and the phylogeny of the group. We have carried out a phylogenetic analysis of the PCR-generated sequences of the internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA. The results suggest that the genus, including *Paleocyanus crassifolius* is monophyletic; thus, a new combination of this species under *Cheirolophus* is proposed. The Macaronesian group of species is also monophyletic, indicating a single colonization of the archipelago. The poor resolution of microspecies in the Macaronesian group reinforces the hypothesis of a very recent differentiation of the group.

The genus *Cheirolophus* CASS. (*Asteraceae*: *Cardueae–Centaureinae*) comprises 23 species from the Tethyan floristic subkingdom of TAKHTAJAN (1986): western Mediterranean region and Macaronesia (Fig. 1). The genus was described in 1817 (CASSINI 1817) on the basis of some species of the large genus *Centaurea* L., but it was not broadly accepted until very recently after its treatment as a segregate by DOSTÁL (1976) in Flora Europaea. General opinion among synantherologists favours an independent genus (BREMER 1994).

A previous analysis of the ITS sequences of the subtribe *Centaureinae* (SUSANNA & al. 1995) suggested that *Cheirolophus* could not be included in *Centaurea*. In that work, we included two species of *Cheirolophus* in the analysis; in the ITS tree they were associated to *Centaurea africana* L., the representative of *Centaurea* sect. *Centaurea*. The statistical support of the group was very low. In addition, isozyme analyses in the genus (GARNATJE & al. 1998) suggested a closer relationship with *Serratula* sect. *Klasea*.

Species of the genus *Cheirolophus* are variations on a theme; morphologically, the genus is one of the best defined in the subtribe. Differences between species

rely mostly on vegetative characters (the shape and size of the leaves); differences in the appendages of the phyllaries – the most useful character in the subtribe – are scarce.

Morphological differences between *Cheirolophus* and *Centaurea* s. l. are especially remarkable in the habit of the plants, the anatomy of the fruits, the type of pollen, and the geographical distribution.

Species of the genus *Cheirolophus* are shrubs, subshrubs or shrublets, with the exception of *Ch. uliginosus* (BROT.) DOSTÁL, an hemichryptophyte. This shrubby habit is found only in *Centaurea* sect. *Ptosimopappus* BOISS. from west Asia, a fact that lead BOISSIER (1839–1845) to group the last section with *Cheirolophus* in a single genus. However, as pointed out by WAGENITZ (1974), the coincidence in habit is one of the frequent examples of homoplasy in the subtribe. This habit should be secondary according to CARLQUIST (1976); even so, it is a good character for the generic delimitation of *Cheirolophus*.

The fruits have many plesiomorphic characters: the hilum of the seed is basal, the more archaic of the three types described by DITTRICH (1968); they do not have an elaiosome, another archaic character shared only with some species of the genus *Serratula*, and the paleae of the pappus are caducous. The detachment scar with five small teeth is unique in the whole subtribe. The parenchymatic tissue below the scar is often eaten by insects, a fact which suggests that this parenchyma plays the biological role of an elaiosome.

Cheirolophus has pollen of the most primitive type of the subtribe *Centaureinae*, *Serratula* type (WAGENITZ 1955), characterised by a double foot layer and conspicuous spines. This pollen type is frequent in the more primitive genera of the *Centaureinae*, in the subtribe *Carduinae* and even in the genus *Warionia* of the tribe *Mutisieae* (DITTRICH 1977), but it is very rare in *Centaurea*: only in sect. *Plectocephalus* D. DON from east Africa and America.

A striking difference between *Cheirolophus* and *Centaurea* s. l. is the geographical distribution. *Cheirolophus* is a genus with the main centre in the western Mediterranean (Fig. 1) and a explosive radiation in the Canaries. The more oriental species in the genus as presently circumscribed is *Ch. sempervirens* (L.) POMEL, which reaches Algeria. The distribution of many of the species is relictual (e.g. *Ch. uliginosus* and *Ch. sempervirens*) because they have extremely discontinuous areas. This suggests that *Cheirolophus* belongs to the preglacial circummediterranean stock. Nevertheless, as isozyme analyses have already established (GARNATJE & al. 1998), the genus is comparatively young; at least, its origin is more recent than the Tertiary age assigned by BRAMWELL (1976). There are no differences between widely separated populations of *Ch. sempervirens* from the Iberian Peninsula and Algeria (Fig. 1), a fact which reinforces the idea of a recent origin. Instead, *Centaurea* s. l. has a very wide, circummediterranean distribution and it did not colonize Macaronesia until very recently by anthropic action: there are no endemic *Centaurea* s. l. in the Canaries.

Another interesting problem regarding *Cheirolophus* is the relationship with the monotypic genus *Paleocyanus*. DOSTÁL (1971) described *Paleocyanus* on the basis of *Centaurea crassifolia* BERTOL., an endemic from Malta. The species was usually included in sect. *Centaurea* (e.g. FIORI & PAOLETTI 1896–1909) because of the complete lack of appendages in its bracts. Nevertheless, some characters were

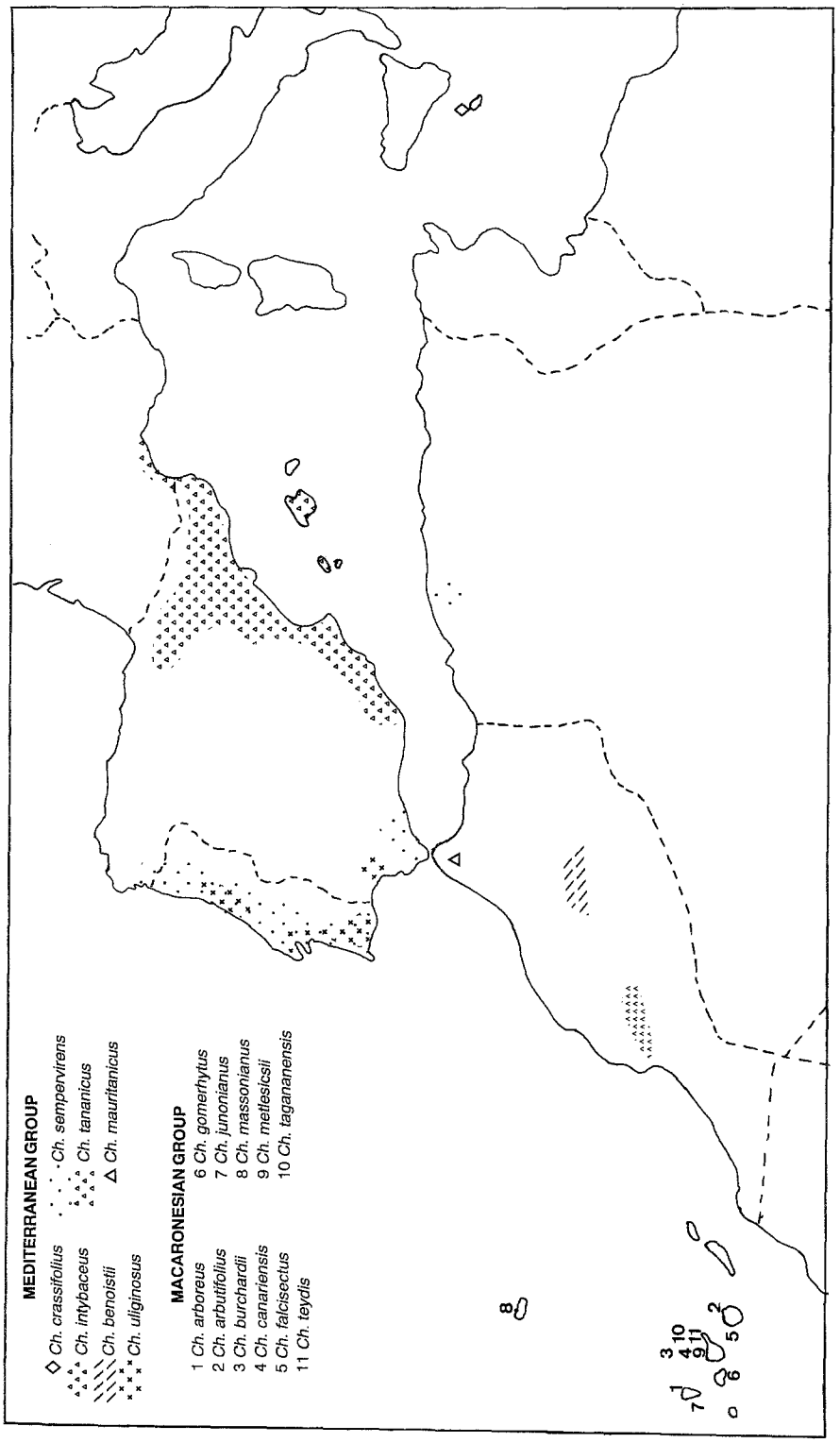

Fig. 1. Geographic distribution of the species of the genus *Cheirolophus* included in this study

very incongruent in *Centaurea* sect. *Centaurea*: the pollen type *Serratula* (WAGENITZ 1955) and the cypselas with caducous paleae, without an elaiosome and a basal hilum (DITTRICH 1968). Affinities, as suggested by DITTRICH (1968) on the basis of carpologic characters, must be in *Cheirolophus*.

Finally, we have the problem of the specific limits of *Ch. intybaceus*. This species shows the widest distribution in the genus (Fig. 1), from southern France to southern Spain, including the Baleares (excepting Menorca). In this wide area, some populations have recently been given the rank of species (OLIVARES & al. 1995, STÜBING & al. 1997) on the basis of very inadequate morphological evidence; for example, pollen size and ornamentation. Both characters are of great importance in the generic and sectional classification of the subtribe, but they are irrelevant at the species level (WAGENITZ 1955). We have included in our study one of the purportedly new species, *Cheirolophus grandifolius* (FONT QUER) OLIVARES & al. (population of *Ch. intybaceus* from Formentera, Table 1). This population was originally described as a variety of *Ch. intybaceus* because it has slightly wider leaves.

Our present research addresses three systematic problems: (i) The phylogeny of the genus and the history of its expansion in the western Mediterranean region. (ii) The systematic position of the genus *Paleocyanus*, DOSTÁL. (iii) The identity of the population of *Ch. intybaceus* from Formentera.

Materials and methods

Plants. The origin of the samples is detailed in Table 1. With the exception of *Paleocyanus crassifolius* from the Botanic Garden of La Valetta, Malta, and *Cheirolophus massonianus* (LOWE) ERIKSSON & al. from the Botanic Garden of Madeira, all the materials are of wild origin; in the case of the Canarian microspecies, all were collected in their type localities (with the exception of *Cheirolophus canariensis*, whose type locality is unknown). Voucher specimens are preserved in the herbarium BC.

We analyzed ITS sequences of 18 species of *Cheirolophus* and four outgroups. The sequences of two species of *Cheirolophus* (*Ch. arbutifolius* and *Ch. teydis*) and two species of outgroups (*Serratula nudicaulis* and *Centaurea africana*) were provided by a previous study (SUSANNA & al. 1995).

Outgroups were chosen from the related genera *Serratula* – with one representative of sectt. *Serratula* (*S. tinctoria* L.) and *Klasea* (*S. nudicaulis* L.); *Centaurothamnus* WAGENITZ & al., from Yemen, a shrub related to *Serratula* according to WAGENITZ & al. (1982). All these species share with *Cheirolophus* the *Serratula* pollen type. On the basis of carpologic morphology, *Serratula* is the closest relative to *Cheirolophus* in the subtribe. In a previous study based on isozyme analysis (GARNATJE & al. 1998), *Cheirolophus* was close to *Serratula*.

The fourth outgroup was *Centaurea africana* LAM., with a different pollen type (*C. centaurium* of WAGENITZ 1955). In the previous survey of the whole subtribe (SUSANNA & al. 1995), this section appeared to be the sister group of the genus *Cheirolophus* (with a low support both by the bootstrap value and the decay index). This contradicted morphological and isozyme data.

ITS amplification and sequencing strategies. Fresh or dry leaves were taken from one or more individuals (seedlings or adult plants), always from the same population; in some cases, leaves were sampled from herbarium specimens. DNA was isolated following the miniprep procedure of DOYLE & DOYLE (1987) as modified in SOLTIS & al. (1991). Another

Table 1. Origin of studied populations of *Cheirolophus* and outgroup taxa. Vouchers are deposited in herbarium BC. GenBank accession numbers are given in brackets (ITS-1 and ITS-2, respectively)

Species	Collection data, Genebank no.
Centaurothamnus maximus Wagenitz & al.	Yemen: 150 km north of San'aa, Jebel Shuharah, 2500 m, Molero, 7.II.1996 [AF021159, AF021176].
Cheirolophus arboreus (Webb) Holub	Spain, La Palma: San Andrés y Sauces, natural reserve "El Canal y Los Tiles", Susanna 1425, 29.VII.1990 [AF021147, AF021164].
Ch. benoistii (Humbert) Holub	Morocco, Ksar ES Souk: S side of Tizi n'Talrhem, 47 km from Midelt, 1100 m, Garnatje, Susanna 1787 & Vilatersana, 17.VI.1997 [AF045415, AF079945].
Ch. burchardii Susanna	Spain, Santa Cruz de Tenerife: between Buenavista and Teno, Susanna 1430, 6.VII.1990 [AF021145, AF021162].
Ch. canariensis (Brouss. ex Willd.) Holub	Spain, Tenerife: Masca ravine, Garnatje 1 & Luque, VIII.1996 [AF021151, AF021168].
Ch. falcisectus Montel. & Moral.	Spain, Gran Canaria: above San Nicolás de Tolentino, old road to Mogán, Susanna 1422, 25.VII.1990 [AF021146, AF021163].
Ch. gomerythus (Svent.) Holub	Spain, La Gomera: near Agulo, San Marcos ravine, Susanna 1426, 4.VII.1990 [AF021149, AF021166].
Ch. intybaceus (Lam.) Dostál	Spain, Alicante: between Alcoy and Pego, km 40, near Benirrama, Garcia-Jacas & Susanna 1249, 5.VIII.1988 [AF021152, AF021169].
	Spain, Formentera: mount Sa Mola, Freixenet, VIII. 1988.
Ch. junonianus (Svent.) Holub	Spain, La Palma: Fuencaliente, volcano of San Antonio, Susanna 1423, 28.VII.1990 [AF021148, AF021165].
Ch. massonianus (Lowe) O. Eriksson & al.	Botanical Garden of Madeira [AF021143, AF021160].
Ch. mauritanicus (Font Quer) Susanna	Morocco, Tetouan: mount Tissouka above Chefchaouen, Molero, Romo 4617 & Susanna, 20.VI.1988 [AF021155, AF021172].
Ch. metlesicsii Montel.	Spain, Tenerife: Arafo, Añavingo ravine, Susanna 1427, 6.VII.1990 [AF021150, AF021167].
Ch. sempervirens (L.) Pomel	Portugal, Faro: 4 km from N of Monchique, Garcia-Jacas & Susanna 1218, 28.VII.1988 [AF021156, AF021173].
Ch. tagananensis (Svent.) Holub	Spain, Tenerife: Taganana, Roque de las Ánimas, Garnatje 3 & Luque, VIII.1996 [AF021144, AF021161].
Ch. tananicus (Maire) Holub	Morocco, Agadir: 1 km S of the Tizi n'Test, Garcia-Jacas, Susanna 1395 & Vallès, 11.VII.1990 [AF021153, AF021170].
Ch. uliginosus (Brot.) Dostál	Spain, Huelva: Mazagón, El Loro, Garcia-Jacas, Julià, J. M. Montserrat 1875, Susanna & Veny, 6.VII.1988 [AF021154, AF021171].
Paleocyanus crassifolius (Bertol.) Dostál	Botanical Garden of La Valetta, Malta [AF021157, AF021174].
Serratula tinctoria L.	Spain, Segovia: Riaza, Cantó, VII.1993 [AF021158, AF021175].

modification was introduced for some *Cheirolophus*, because of the high amount of gums and resins in the leaves: both fresh and dried materials were washed with ethanol/ether 2:1 overnight to remove the gums.

The ITS-1 and ITS-2 regions were amplified separately using the polymerase chain reaction (PCR) following the protocol described in SOLTIS & KUZOFF (1993). For amplification of ITS-1, we used primer 1406F (D. NICKRENT, Southern Illinois University, Carbondale, IL, pers. comm.), located near the 3′ end of the 18S gene; the reverse primer was ITS2 (WHITE & al. 1990), located in the 5′ end of the 5.8S gene. The ITS-2 region was amplified using primer ITS3 (the reverse compliment of ITS2, WHITE & al. 1990) located in the 5.8S gene as the forward primer and 307R (D. NICKRENT, pers. comm.) as the reverse primer located in the 26S gene.

Two different protocols were followed for the purification of ITS-1 and 2 regions because of the sensitivity of the non- radioactive sequencing system. Yields of ITS-1 PCR products were lower than ITS-2 and required a more thorough purification.

Double-stranded ITS-1 DNA was purified by electrophoresis through 1% low melting point agarose. After staining with ethidium bromide, the band was excised and the agarose fragment was digested using the Gelase Agarose Gel-Digesting Preparation, Epicentre Technologies. DNA was then precipitated with 5M ammonium acetate and ethanol, and vacuum dried.

Double-stranded ITS-2 DNA was purified using the following procedure: DNA was precipitated with 3M sodium acetate and absolute ethanol. After 15 min of centrifugation, the supernatant was discarded and the pellet was vacuum dried. In both cases, dried DNA was resuspended in water.

The sequencing primers used for the ITS-1 and ITS-2 regions were ITS1 and ITS4 (WHITE & al. 1990), respectively. Both were labelled with digoxigenine, Boehringer Mannheim. After direct sequencing of the double stranded DNA using the cycle sequencing Fmol kit, Promega Corp., sequences were resolved in 6% acrylamide gels. After the transferring of the DNA fragments to a positively charged nylon membrane, sequences were stained with the DIG detection kit, Boehringer Mannheim.

Data analysis. DNA sequences were aligned visually by sequential pairwise comparison (SWOFFORD & OLSEN 1990). The alignment of ITS-1 sequences for all taxa required interpretation of small (1–2 bp) insertions/deletions (indels) at 1.18% of the sites, whereas alignment of ITS-2 sequences required insertion of small (1 bp) gaps at 2.31% of all sites. These indels were coded as "missing data" and were omitted from phylogenetic analyses, following the recommendations of WOJCIECHOWSKI & al. (1993). All ITS-1 and ITS- 2 sequences have been submitted to GenBank (the accession numbers are detailed in Table 1).

A phylogenetic analysis was conducted to establish the molecular phylogeny. Parsimony analysis involved heuristic searches conducted with PAUP version 3.1.1 (SWOFFORD, 1991) using TBR branch swapping with character states specified as unordered and unweighted. All most-parsimonious trees were saved. To locate other potential "islands" of most-parsimonious trees (MADDISON 1991), we performed 100 replications with random taxon addition, also with TBR branch swapping. Bootstrap analyses (FELSENSTEIN 1985) with 100 replicates and decay analyses (BREMER 1988, DONOGHUE & al. 1992) were performed to obtain estimates of reliability for each monophyletic group. The decay index is the number of steps longer than the shortest trees at which a node collapses (decays). Decay analyses were continued for six steps more than the most-parsimonious trees. While swapping on trees three steps longer than the shortest trees, the memory limit of PAUP (32,767 trees) was reached. Therefore, decay analyses of trees three, four, five and six steps longer than the shortest trees were conducted following MORGAN & al. (1994), a procedure revised by MORGAN (1997). The strict consensus of all trees up to two steps

longer was used as a constraint tree in searches of trees three steps longer than the shortest trees. Trees three steps longer that were incompatible with the constraint tree were saved, and a strict consensus of the constraint tree and the incompatible trees was used to find those branches with a decay index of three. To prevent the decay analysis from being marooned on an island of suboptimal trees, the search was conducted using 1000 replicates with random taxon addition. These steps were followed for obtaining the decay indices of four, five and six.

Results

Size and composition of ITS. The length of the ITS-1 region in the species of *Cheirolophus* varied from 250 to 253 bp and the ITS-2 region varied between 214 and 216 bp.

Sequence divergence was calculated separately for the ITS-1 and ITS-2 regions using the Distance Matrix option available in PAUP. ITS-1 sequence divergence varied within species of *Cheirolophus* from 0% between different species of the Macaronesian group (ten combinations) to 7.6% (pairwise distance between *Ch. mauritanicus* and *Ch. arbutifolius* and also between *Ch. crassifolius* and *Ch. arbutifolius*). Divergence between ingroup and outgroup genera varied from 5.2% between *Centaurea africana* and *Ch. uliginosus*, to 12.3% between *Serratula tinctoria* and *Ch. arbutifolius*.

ITS-2 sequence divergence values are lower than those for ITS-1, which is consistent with the results in other *Asteraceae* (BALDWIN 1992, 1993; SUSANNA & al. 1995; BAYER & al. 1996). We have found a maximum of divergence within genera (12.4% between *Ch. uliginosus* and *Ch. benoistii*), but ITS-2 sequence divergence was 0% in 17 combinations of species in the Macaronesian complex and one in the Mediterranean complex (*Ch. mauritanicus* and *Ch. sempervirens*). Divergence between ingroup and outgroup genera varied from 5.7% between *Ch. taganananensis* and *Serratula nudicaulis* and between *Ch. junonianus* and *Serratula nudicaulis* to 12.1% between *Ch. benoistii* and *Centaurea africana*.

Phylogenetic analysis. The analysis resulted in eight minimal length trees of 160 steps, all in one island. The strict consensus of all eight trees is shown in Fig. 2. The clade that includes all the species of *Cheirolophus* and *Paleocyanus* has very strong statistical support (a bootstrap value of 97% and a decay index of 5). *Cheirolophus uliginosus* is the sister group to the whole genus (including *Paleocyanus*!) with a bootstrap support of 82% and decay index of 3. Next, we have two equal branches: one is the Mediterranean clade, with a bootstrap value of 80% and decay index of 2; the other one is the Macaronesian clade, which has the highest support in the whole analysis: 100% bootstrap value and a decay index of 6 steps (the monophyly of the Macaronesian species has more support than the monophyly of the genus).

In the Mediterranean clade, *Paleocyanus crassifolius* is sister to the Ibero-North African group of species (*Ch. benoistii*, *Ch. tananicus*, *Ch. intybaceus*, *Ch. mauritanicus* and *Ch. sempervirens*) with a bootstrap value of 99% and a decay index of 4. In this Ibero-North African clade, there are two groups. The first one is formed by the African *Ch. benoistii* and *Ch. tananicus*, with a bootstrap value of 90% and a decay index of 3. This clade is sister to the second group of species,

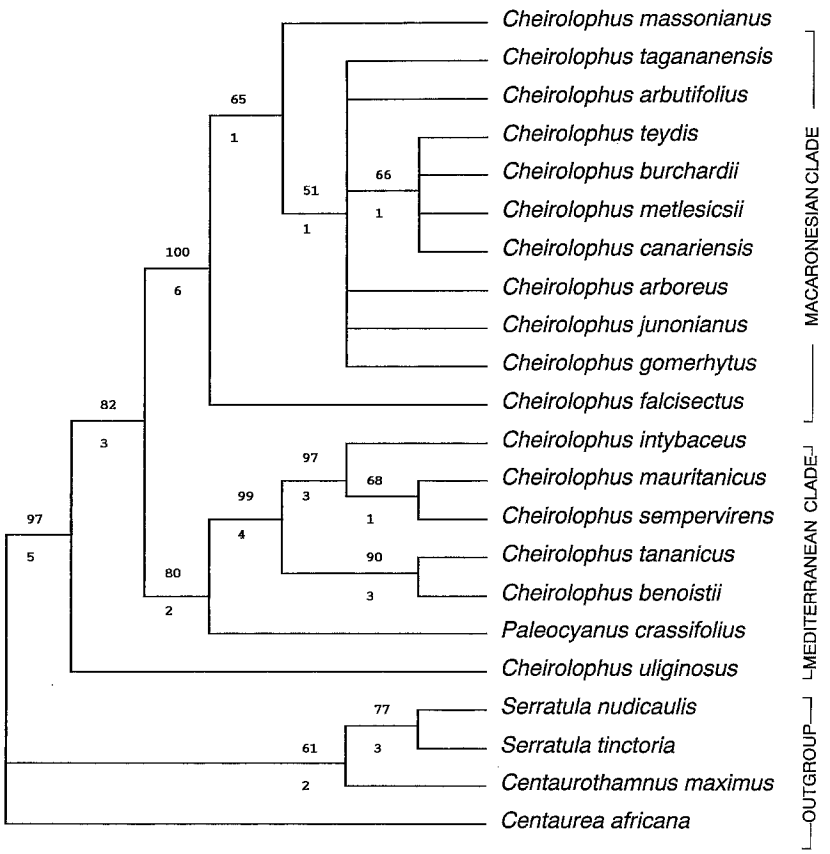

Fig. 2. Strict consensus tree of the 8 most parsimonius trees showing the hypothetical ITS phylogeny of *Cheirolophus* (160 steps; consistency index excluding uninformative characters = 0.720; retention index = 0.887). Two clades are indicated: the Macaronesian and the Mediterranean. Numbers above branches are bootstrap percentages; numbers below branches are decay values

with a bootstrap value of 97% and a decay index of 3. This second clade is formed by *Ch. intybaceus* and a clade with *Ch. mauritanicus* and *Ch. sempervirens* (with a bootstrap support of 68% and a low decay value of 1).

In the Macaronesian clade, relationships are not well solved: the groups suggested by the analysis have weak statistical support (bootstrap values of 66% or lower and decay indices of 1 in all cases).

Discussion and conclusions

As could be expected from the exact morphological definition of the genus and the result of previous analysis (SUSANNA & al. 1995), the phylogeny suggested by the comparison of ITS sequences indicates that *Cheirolophus*, with the inclusion of *Paleocyanus*, is monophyletic with a bootstrap value of 98% and a decay index of 6 (Fig. 2). Obviously, nothing can be deduced from this analysis on the position of *Cheirolophus* in the basal assemblage of the subtribe *Centaureinae*, to which it belongs (SUSANNA & al. 1995).

The systematic position of *Ch. uliginosus* as sister to the rest of the genus supports the hypothesis by CARLQUIST (1976) that the woody habit is secondary in insular *Asteraceae*. *Cheirolophus uliginosus* is the only hemicryptophyte in the whole genus. Actually, CARLQUIST (1976) used the example of the shrubby "*Centaurea*" (*Cheirolophus*) species from the Canary Islands. Similarly, TAKHTAJAN (1986) used the example of another genus of the *Centaureinae* endemic of an oceanic island: *Centaurodendron* from Juan Fernández. According to TAKHTAJAN (1986), the woody genus *Centaurodendron* is an extreme derivative of herbs of *Centaurea* sect. *Plectocephalus* (D. DON) DC. (sometimes considered as a different genus, *Plectocephalus* D. DON) from the mainland of Chile. The present distribution of *Ch. uliginosus* – reduced to the oceanic shores of Portugal and South Spain (Fig. 1) – suggests that the change to a woody habit took place on the mainland, prior to the colonization of the Canaries. The mild climatic conditions that made this change possible must have been present in the whole area of *Cheirolophus*, as all the extant species have this shrubby habit.

Regarding *Paleocyanus*, ITS sequences suggest that *P. crassifolius* is sister to the Ibero-North-African clade. As studies on pollen and cypselas had already suggested, it should be placed in *Cheirolophus*; in the Appendix we have proposed a new combination under this last genus. *Paleocyanus crassifolius* is known only from two localities in Malta and Gozo (Fig. 1); this narrow, isolated distribution and its status – basal to the Mediterranean clade – suggests that it is an old relic in the genus.

In the same Mediterranean clade, *Cheirolophus tananicus* and *Ch. benoistii* from the West and the East High Atlas respectively (Fig. 1) appear as the sister group to the rest of the species. Isozyme analyses (GARNATJE & al. 1998) supports the close relationship between *Ch. tananicus* and its vicariant species *Ch. benoistii*. Both *Ch. tananicus* and *Ch. benoistii* are morphologically similar to *Ch. intybaceus*, but all of them grow in xeric habitats; it could be a convergence.

With respect to the clade formed by *Ch. sempervirens* and *Ch. mauritanicus* resulting from the analysis, our results suggest a close relationship between both species, but they also support the classification of *Ch. mauritanicus* as a different species (*Ch. mauritanicus* was originally described as a variety of *Ch. sempervirens*): there are slight changes in the sequence of both taxa (Fig. 3) and the decay value that supports the association is very low. Present distribution of *Ch. mauritanicus* is reduced to a single locality in Morocco (Fig. 1). On the other hand, *Ch. sempervirens* has a extreme discontinuity between the Iberian Peninsula and the mountains of northern Algeria (Fig. 1). This discontinuity is correlated to another remarkable discontinuity in its area in west Iberia, a pattern also shown by the rest of the Iberian species (SUSANNA 1989, 1991, 1993). This pattern is not reflected in the Fig. 1 because of the small scale of the map. This distribution in scattered, small, often discontinuous populations reinforces the hypothesis of a preglacial origin of the genus; also, as previously pointed out, the fact that the populations of *Ch. sempervirens* from the extremes of the area are identical suggests a relatively recent origin of the disjunction.

With respect to the problem of the population of *Ch. intybaceus* from Formentera described as a new species, the difference between ITS sequences of Iberian and insular populations was zero; for this reason, we have excluded it from

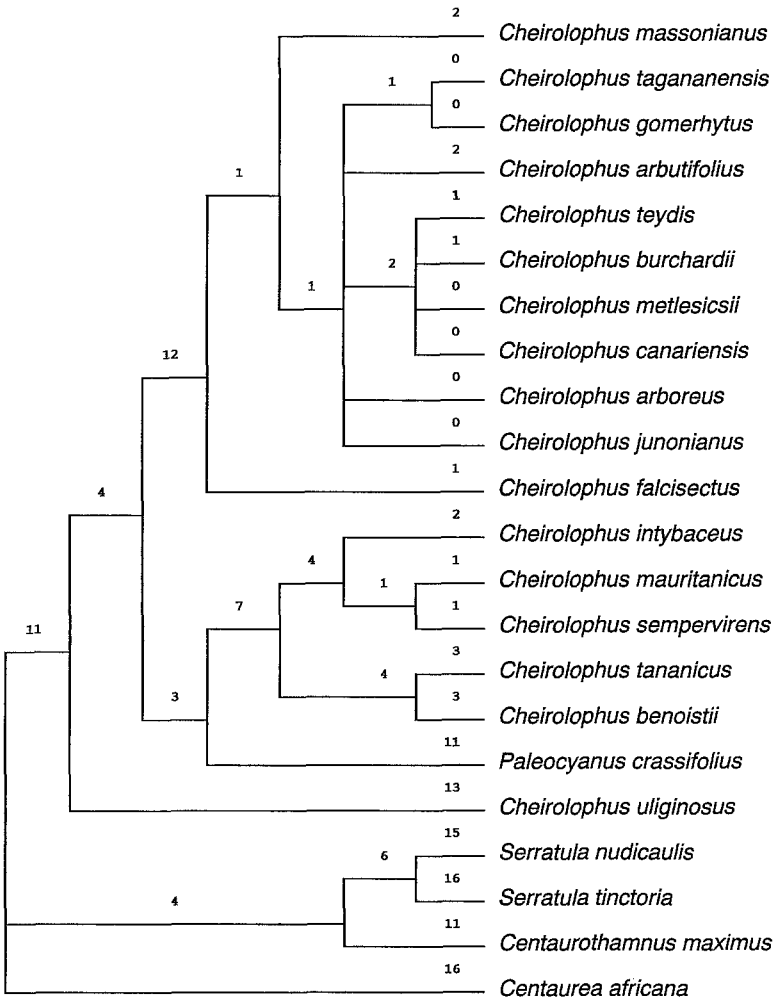

Fig. 3. One of the eight most parsimonious trees resulting from the heuristic search (160 steps; consistency index excluding uninformative characters = 0.728; retention index = 0.892). Numbers above branches indicate the number of apomorphies for each branch

the final analysis. This result supports our previous idea (SUSANNA 1989) on the uniformity of *Ch. intybaceus*, a species with a very wide distribution and many local variants of no taxonomic weight. We have found the same lack of variation in the ITS sequences between some Macaronesian species (Fig. 3), but in all these cases morphological differences were obvious. Moreover, the pattern of speciation in the Canary islands is very different from the pattern of the Baleares: the Canaries are isolated from ancient times, whereas the Pityuses (Ibiza and Formentera) share almost all their flora with the adjacent Dianic territory of the Iberian Peninsula (FONT QUER 1927, BOLÒS 1958).

Small differences in the ITS sequences (zero in most cases) within the Macaronesian clade show that we have reached the limit of resolution of the ITS for phylogenetic reconstruction in *Cheirolophus*. Groups suggested by the analysis of the ITS sequences have weak statistical support. However, there are two

suggesting results. First, one of the species from the oldest island – Gran Canaria –, *Ch. falcisectus*, is sister to the rest of the Macaronesian species. Second, the only group defined within the Macaronesian clade is formed by four of the five species of the island of Tenerife (Fig. 1). However, a weak statistical support make these suggestions somewhat vague.

The low resolution of our analysis is consistent with some studies of ITS sequences in oceanic islands: in the genus *Psychotria* from Hawaii (NEPOKROEFF & SYTSMA 1996) and in the genus *Aeonium* from the Canaries (MES & al. 1996). Instead, SANG & al. (1994, 1995) obtained well resolved phylogenies with ITS sequences in Juan Fernández islands. In the Canarian taxa, other regions of DNA should be evaluated. FRANCISCO-ORTEGA & al. (1996) obtained a well detailed phylogeny of *Argyranthemum* based on cp-DNA restriction site analysis.

In the same line, relationships suggested by isozyme analyses (GARNATJE & al. 1998) are very confusing – Iberian and Macaronesian species are intermixed in the tree based on allelic frequencies – underlining the extreme youth of the speciation processes in *Cheirolophus*. The Macaronesian complex has always been a good example of the explosive radiation that occurs in oceanic archipelagos; even BRAMWELL (1979) and GARNATJE (1995) suggested that some Canarian narrow endemics of *Cheirolophus* should be treated as a single species.

Our results are not conclusive on the origin of the Macaronesian group of species, but it is clear that it is a secondary centre of speciation of *Cheirolophus*: the Macaronesian group is strongly monophyletic, which implies a single colonization event; and differences between species are smaller than in the Mediterranean group (the number of apomorphies is lower, see Fig. 3), in which clear phylogenetic inferences can be done. This pattern contrasts with the model of *Aeonium*, a genus primarily originated in the Canaries and secondarily escaped to Africa (cf. MES & al. 1996).

According to the present geographical distribution of the genus and the floristic history of Macaronesia, the most plausible hypothesis is the African origin of *Cheirolophus*. In fact, *Ch. tananicus* reaches the western end of the High Atlas, a little above the Canaries (Fig. 1). But relationships of the Macaronesian clade are not with *Ch. tananicus* or *Ch. benoistii*, not even with the morphologically closer *Ch. mauritanicus* (Fig. 1) from north Morocco: they are with the Iberian species *Ch. uliginosus*, also basal to the Macaronesian clade. The present distribution of *Ch. uliginosus* (Fig. 1) coincides with the area of some elements of the flora of Macaronesia which are also present in southwest Iberia, a refuge for the old preglacial flora (BRAMWELL 1976, TAKHTAJAN 1986). This close relationship must also be the case for *Cheirolophus*.

Appendix

***Cheirolophus crassifolius* (BERTOLONI) SUSANNA, comb. nova.**
Centaurea crassifolia BERTOLONI, Ann. Stor. Nat. **2**: 359 (1829).
Paleocyanus crassifolius (BERTOLONI) DOSTÁL, Bot. J. Linn. Soc. 71: 192 (1971).

Financial support by the CICyT within projects PB88-0033 and PB93-0032, and by the Consejo Superior de Investigaciones Científicas, is gratefully acknowledged. Technical

support by Mr M. VENY of the Botanic Institute of Barcelona has been invaluable in the conservation of the living specimens of *Cheirolophus*. Seeds of *Ch. massonianus* were provided by Dr S. SA FONTINHA from the Botanic Garden of Madeira; leaves of *Ch. crassifolius* were sent by Dr E. LANFRANCO from the University of Malta; our grateful thanks to them. We want to thank all our Canarian friends who made a gratifying experience of all the ravine-climbing in the Canaries: Dr A. MARRERO and Dr R. FEBLES from the Botanic Garden Viera y Clavijo, Gran Canaria; Mr I. NEGRÍN from Agulo and Mr A. MENDOZA from Sataratá in Gomera; Mr and Mrs JAVIER and SUSANA CARRILLO, from La Palma; and Mr A. FARIÑAS from Arafo, Tenerife.

References

BALDWIN, B. G., 1992: Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the *Compositae*. – Molec. Phylogenet. Evol. **1**: 3–16.

– 1993: Molecular phylogenetics of *Calycadenia* (*Compositae*) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. – Amer. J. Bot. **80**: 222–238.

BAYER, R. J., SOLTIS, D. E., SOLTIS, P. S., 1996: Phylogenetic inferences in *Antennaria* (*Asteraceae*: *Gnaphalieae*: *Cassiniinae*) based on sequences from nuclear ribosomal DNA internal transcribed spacers (ITS). – Amer. J. Bot. **83**: 516–527.

BOISSIER, E., 1839–1845: Voyage botanique dans le midi de l'Espagne pendant l'année 1837. – Paris.

BOLÒS, O., 1958: Grupos corológicos de la flora balear. – Publ. Inst. Biol. Apl. Barcelona **27**: 49–71.

BRAMWELL, D., 1976: The endemic flora of the Canary Islands. Distribution, relationships and phytogeography. – In KUNKEL, G., (Ed.): Biogeography and ecology in the Canary Islands, pp. 207–240. – The Hague: Junk.

– (Ed.), 1979: Plants and islands. – London: Academic Press.

BREMER, K., 1988: The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. – Evolution **42**: 795–803.

– 1994: *Asteraceae*. Cladistics and classification. – Portland: Timber Press.

CARLQUIST, S., 1976: Tribal interrelationships and phylogeny of the *Asteraceae*. – Aliso **8**: 465–492.

CASSINI, H., 1817: *Cheirolophus*. – Dictionnaire de Sciences Naturelles **1**, pp. 250. – Paris.

DITTRICH, M., 1968: Karpologische Untersuchungen zur Systematik von *Centaurea* und verwandten Gattungen. – Bot. Jahrb. Syst. **88**: 70–162.

– 1977: *Cynareae* – systematic review. – In HEWYWOOD, H. W., HARBORNE, J. B., TURNER, B. L., (Eds): The biology and chemistry of *Compositae*. – London, New York: Oriole.

DONOGHUE, M. J., OLMSTEAD, R. G., SMITH, J. F., PALMER, J. D., 1992: Phylogenetic relationships of *Dipsacales* based on *rbc*L sequences. – Ann. Missouri Bot. Gard. **79**: 333–345.

DOSTÁL, J., 1971: Preliminary notes on the subtribe *Centaureinae*. – Acta Bot. Acad. Sci. Hung. **19**: 73–79.

– 1976: *Centaurea*. – In TUTIN, T. G., HEYWOOD, V. H., BURGES, N. A., VALENTINE, D. H., WALTERS, S. M., WEBB, D. A., (Eds): Flora Europaea, **4**, pp. 254–301. – Cambridge: Cambridge University Press.

DOYLE, J. J., DOYLE, J. L., 1987: A rapid DNA isolation procedure for small quantities of fresh leaf tissue. – Phytochem. Bull. **19**: 11–15.

FELSENSTEIN, J., 1985: Confidence limits on phylogenies: an approach using the bootstrap. – Evolution **39**: 783–791.

FIORI, A., PAOLETTI, G., 1896–1909: Flora Analitica d'Italia. – Padova.

FONT QUER, P., 1927: La flora de las Pitiusas y sus afinidades con la de la Península Ibérica. – Mem. Real Acad. Ci. Barcelona **20**: 109–154.

FRANCISCO-ORTEGA, J., JANSEN, R. K., SANTOS-GUERRA, A., 1996: Chloroplast DNA evidence of colonization, adaptive radiation, and hybridization in the evolution of the Macaronesian flora. – Proc. Natl. Acad. Sci. USA **93**: 4085–4090.

GARNATJE, M. T., 1995: Relació entre el polimorfisme enzimàtic i alguns aspectes de l'especiació i de l'evolució en el gènere *Cheirolophus* CASS. – Doctoral Dissertation, Universitat Autònoma de Barcelona.

– SUSANNA, A., MESSEGUER, R., 1998: Isozyme studies in the genus *Cheirolophus* CASS. (*Asteraceae*: *Cardueae-Centaureinae*) in the Iberian Peninsula, North Africa and the Canary Islands. – Pl. Syst. Evol. **213**: 57–70.

MADDISON, D. R., 1991: The discovery and importance of multiple islands of most parsimonious trees. – Syst. Zool. **40**: 315–328.

MES, T. H. M., VAN BREDERODE, J., 'T HART, H. T., 1996: Origin of the woody Macaronesian *Sempervivoideae* and the phylogenetic position of the east African species of *Aeonium*. – Bot. Acta **109**: 477–491.

MORGAN, D. R., 1997: Decay analysis of large sets of phylogenetic data. – Taxon **46**: 509–517.

– SOLTIS, D. E., ROBERTSON, K. R., 1994: Systematic and evolutionary implications of *rbc*L sequence variation in *Rosaceae*. – Amer. J. Bot. **81**: 890–903.

NEPOKROEFF, M., SYTSMA, K. J., 1996: Systematics and patterns of speciation and colonization in Hawaiian *Psychotria* and relatives based on phylogenetic analysis of ITS sequence data. – Amer. J. Bot. **83**, Suppl.: 181–182.

OLIVARES, A., STÜBING, G., PERIS, J. B., MARTÍN, J., 1995: *Cheirolophus lagunae* sp. nov. (*Asteraceae*), endemismo iberolevantino. – Anales Jard. Bot. Madrid **53**: 262–265.

SANG, T., CRAWFORD, D. J., KIM, S.-C., STUESSY, T. F., 1994: Radiation of the endemic genus *Dendroseris* (*Asteraceae*) on the Juan Fernández islands: evidence from sequences of the ITS regions of nuclear ribosomal DNA. – Amer. J. Bot. **81**: 1494–1501.

– – STUESSY, T. F., SILVA, M., 1995: ITS sequences and phylogeny of the genus *Robinsonia* (*Asteraceae*). – Syst. Bot. **20**: 55–64.

SOLTIS, D. E., KUZOFF, R. K., 1993: ITS sequence homogeneity within and among populations of *Lomatium grayi* and *L. laevigatum* (*Umbelliferae*). – Molec. Phylogenet. Evol. **2**: 166–170.

– SOLTIS, P. S., COLLIER, T. G., EDGERTON, M. L., 1991: The *Heuchera* group (*Saxifragaceae*): evidence for chloroplast transfer and paraphyly. – Amer. J. Bot. **78**: 1091–1112.

STÜBING, G., PERIS, J. B., OLIVARES, A., MARTÍN, J., 1997: *Cheirolophus mansanetianus* Stübing, Peris, Olivares & Martín, sp. nov. and *Ch. grandifolius* (FONT QUER) comb. & stat. nov. (*Asteraceae*), two endemics from Spain. – Anales Jard. Bot. Madrid **55**: 170–173.

SUSANNA, A., 1989: Mapa 114. *Cheirolophus intybaceus* (LAM.) DOSTÁL. – In FERNÁNDEZ CASAS, J., (Ed.): Asientos para un atlas corológico de la flora occidental, 11. – Fontqueria **22**: 16–18.

– 1991: Mapa 478. *Cheirolophus sempervirens* (L.) POMEL. – In FERNÁNDEZ CASAS, J., GAMARRA, R., (Eds): Asientos para un atlas corológico de la flora occidental, 18. Fontqueria **31**: 267–269.

– 1993: Mapa 511. *Cheirolophus uliginosus* (BROT.) DOSTÁL. – In FERNÁNDEZ CASAS, J., MORALES, M. J., (Eds): Asientos para un atlas corológico de la flora occidental. – Fontqueria **36**: 208–210.

– GARCIA JACAS, N., SOLTIS, D. E., SOLTIS, P. S., 1995: Phylogenetic relationships in tribe *Cardueae* (*Asteraceae*) based on ITS sequences. – Amer. J. Bot. **82**: 271–294.

SWOFFORD, D. L., 1991: PAUP: phylogenetic analysis using parsimony, version 3.1. – Champaign: Illinois Natural History Survey.

– OLSEN, G. J., 1990: Phylogeny reconstruction. – In HILLIS, D., MORITZ, C., (Eds): Molecular systematics, pp. 411–501. – Sunderland: Sinauer.

TAKHTAJAN, A., 1986: Floristic regions of the world. – Berkeley: California University Press.

WAGENITZ, G., 1955: Pollenmorphologie und Systematik in der Gattung *Centaurea* L. s. l. – Flora **142**: 213–279.

– 1974: Parallele Evolution von Merkmalen in der Gattung *Centaurea*. – Phyton (Austria) **16**: 301–312.

– DITTRICH, M., DAMBOLDT, J., 1982: *Centaurothamnus*, eine neue Gattung der *Compositae-Cardueae* aus Arabien. – Candollea **37**: 101–115.

WHITE, T. J., BRUNS, T., LEE, S., TAYLOR, J., 1990: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. – In INNIS, M., GELFAND, D., SNINSKY, J., WHITE, T., (Eds): PCR protocols: a guide to methods and applications, pp. 315–322. – San Diego: Academic Press.

WOJCIECHOWSKI, M. F., SANDERSON, M. J., BALDWIN, B. G., DONOGHUE, M. J., 1993: Monophyly of aneuploid *Astragalus* (*Fabaceae*): evidence from nuclear ribosomal DNA Internal transcribed sequences. – Amer. J. Bot. **80**: 711–722.

Address of the authors: A. SUSANNA, T. GARNATJE, N. GARCIA-JACAS, Institut Botànic de Barcelona (C.S.I.C.), Av. Muntanyans s. n., E-08038 Barcelona, Spain.

Accepted September 25, 1997 by I. KRISAI-GREILHUBER