

1 Genome size variation and molecular phylogeny in the genus *Cheirolophus*  
2 Cass. : biogeographic implications.

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## 15 **Summary**

- 16
- 17 • Nuclear DNA amount has been assessed in 21 populations of 19 species  
18 of the genus *Cheirolophus*, and phylogenetic analyses have been  
19 performed in order to find the relationships between the species of the  
Mediterranean Basin and those of Macaronesia.
  - 20 • A combined analysis of nuclear ribosomal DNA ITS and ETS has been  
21 performed based on Bayesian inference, and nuclear DNA amount has  
22 been assessed by flow cytometry.
  - 23 • The monophyly of the Macaronesian group and its relationship with *Ch.*  
24 *sempervirens* indicate a unique colonization event of Macaronesia from  
25 the continent, contraryly to the various colonizations that should have  
26 occurred in the Canarian archipelago. DNA amount is significantly lower

1 in the insular species than in the continental ones, and a parallelism  
2 appears between the evolution of the genus *Cheirolophus* (colonization  
3 of the islands) and the loss of DNA, probably due to the selection  
4 pressure existing in the oceanic islands or to the founding effect.

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6  
7 **Keywords:** Asteraceae, biogeography, *Cheirolophus*, C-value, DNA amount, genome  
8 size, islands, molecular phylogeny.

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13 **Introduction**

14  
15 The biogeographic region of Macaronesia comprises several groups of islands: the  
16 archipelagos of the Canary Islands, the Azores, Madeira, Cape Verde and the Selvagens  
17 Islands. All of these are of volcanic origin and located in the Atlantic Ocean between  
18 the coordinates 39° 45' N, 31° 17' W and 14° 49' N, 13° 20' W. The flora of  
19 Macaronesia is highly diverse and complex, with a considerable number of endemics. In  
20 fact, oceanic islands are usually rich in endemism, and at least 500 endemic species are  
21 found in Canary Islands (González, 1992). It is believed that there is a connection  
22 between the flora of this region and that of the Mediterranean Basin and north-west  
23 Africa (Suda *et al.*, 2003) and that a major role is played by the sea mounts surrounding  
24 the archipelagos, which acted as bridges of dispersion and colonization in the geological  
25 past, when sea levels were lower (García-Talavera, 1999). This is called the stepping-  
26 stone model (see Juan *et al.*, 2000).

1           The highest biodiversity of Macaronesia is found in the Canary Islands, possibly  
2 due to the great variety of habitats and the broad range of geological ages  
3 (Fernandopullé, 1976; Carracedo, 1994), among other possible reasons. The Canaries  
4 are the biggest and most developed archipelago of Macaronesia and form a volcanically  
5 active alignment of seven islands distributed in a band near the African coast. The  
6 nearest island is Fuerteventura, only 100 km from Africa. Fuerteventura is thought to  
7 have been joined to Lanzarote and to other islets 18,000 years ago, forming a single  
8 island only 60 km from the coast of Africa (García-Talavera, 1999). The fact that the  
9 Macaronesian archipelagos, and especially the Canaries, are relatively near the African  
10 continent makes them different from the other oceanic islands.

11           The genus *Cheirolophus* (Asteraceae, Cardueae) was originally described by  
12 Cassini (1817), who based his description on some species from *Centaurea* L. Ever  
13 since then it has been considered as an independent genus by different authors (Dostál,  
14 1976; Bremer, 1994). *Cheirolophus* includes *ca.* 20 species distributed along the west  
15 Mediterranean Basin and Macaronesia; some *Cheirolophus* have a wide distribution,  
16 such as *Ch. intybaceus*, *Ch. sempervirens* and *Ch. uliginosus*, while approximately 17  
17 are described, some of them quite recently, as endemic to the Canary Islands (Bramwell  
18 & Bramwell, 2001). Only, one species is endemic to Madeira, *Ch. massonianus* (Lowe)  
19 A. Hansen & Sunding. All *Cheirolophus* are shrubs, subshrubs or shrublets, excluding  
20 *Ch. uliginosus* (Brot.) Dostál, which is a hemichryptophyte. Preliminary studies on this  
21 genus (Susanna *et al.*, 1995, 1999) uphelded its monophyly with the inclusion of *Ch.*  
22 *crassifolius* (Bertol.) Susanna, previoulsy named *Paleocyanus crassifolius* (Bertol.)  
23 Dostál. *Cheirolophus* is regarded as a basal genus of the Centaureinae (Garcia-Jacas *et*  
24 *al.*, 2001; Hellwig, 2004) and it would have diverged from this subtribe during the late  
25 Oligocene and Miocene (Hellwig, 2004), although its speciation is relatively recent  
26 (Garnatje *et al.*, 1998; Susanna *et al.*, 1999).

1           The performance of molecular phylogenetic analysis has long been useful in  
2 tracing the origin and evolution of island endemics, as has been shown in some other  
3 Asteraceae genera, such as *Robinsonia* (Sang *et al.*, 1995), *Aeonium* (Mes, 1995) or the  
4 Macaronesian *Argyranthemum* (Francisco-Ortega *et al.*, 1997). Sometimes, however,  
5 the resolution obtained with these methods is not as high as desired. From a  
6 phylogenetic perspective, three issues still remain unresolved in this group. The first is  
7 the delimitation of *Ch. intybaceus* (Lam.) Dostál, a species with a large distribution area  
8 from the south of France to the south of Spain, and embracing part of the Balearic  
9 Islands. Some authors (Olivares *et al.*, 1995, Stübing *et al.*, 1997) have conferred the  
10 status of distinct species to some populations from this area. In a previous study  
11 (Susanna *et al.*, 1999), one of these species, *Ch. grandifolius* (Font Quer) Stübing,  
12 Peris, Olivares & Martín did not show any molecular difference with respect to *Ch.*  
13 *intybaceus*, on the basis of the sequences of the ITS region. In the present work, we  
14 have also included another of these species, *Ch. lagunae* Olivares, Peris, Stübing &  
15 Martín, which inhabits specific localities on the coast of Alacant (Spain). The second  
16 question is the low resolution of the phylogeny within the Macaronesian group, but  
17 Baldwin *et al.* (1998) outlined the difficulty of an accurate estimation of the phylogeny  
18 of insular plants due to the low variability that the DNA regions used for the analysis  
19 usually show. A combination of different data could possibly solve the phylogeny of the  
20 insular taxa (Mort *et al.*, 2002). With this purpose, the present work combines data from  
21 the analysis of the ITS sequences (Susanna *et al.*, 1999) with those from the ETS region  
22 of nuclear ribosomal DNA. Finally, the third question is the clarification of the  
23 phylogenetic placement of *Ch. crassifolius*.

24           Together with all the data coming from phylogenetic studies, data on genome  
25 size variation can contribute to a better understanding in the interpretation of the  
26 systematics and the evolutionary relationships within a narrow group of plants (Ohri,

1 1998). Genome size is an important biodiversity character, linked to many aspects in the  
2 biology of a species (Bennett, 1998). Although the meaning of the variation in the C-  
3 value (i.e. genome size) is still not completely well understood (Gregory, 2001, 2005),  
4 and that it is still too soon to establish any model or to see if this parameter could have  
5 remarkable systematic implications (only approximately 3% of angiosperm species have  
6 a known C-value), its study can certainly provide valuable information in the  
7 clarification of the relationships between close species. Thus, for example, Zonneveld &  
8 Iren (2001) found that when many species in a genus had the same chromosome  
9 numbers, differences in nuclear DNA contents proved to be effective in delimiting  
10 infrageneric divisions in a number of taxa. Thus, the study of genome size variation  
11 could reveal divergence within a genus (like in *Hypochaeris*, Cerbah *et al.*, 1999, or in  
12 *Musa*, Lysák *et al.*, 1999).

13 Hence, the objectives of the present work are: a) to obtain a molecular  
14 phylogeny from the combined data sets of the different DNA regions, b) to relate the  
15 phylogeny with the biogeographic framework of these species, and c) to use the  
16 information provided by genome size in the analysis of the phylogeny.

17

## 18 **Materials and Methods**

19

### 20 **Plant material**

21

22 Table 1 shows the provenance of all the species investigated. *Petunia hybrida* cv. PxPc6  
23 (2C=2.85pg) was used as internal standard for flow cytometric measurements. It was  
24 provided by the Institut des Sciences du Végétal, Gif-sur-Yvette (France). Voucher  
25 specimens are preserved in the herbarium BC.

26

1 DNA content assessment

2

3 Although this is the first genome size study on *Cheirolophus*, the measurements were  
4 carried out following the protocols described in a previous study on *Echinops* L. and  
5 related genera (Garnatje *et al.*, 2004).

6

7

8 DNA amplification and sequencing strategies

9

10 The sequences of the ETS (external transcribed spacers) of the nuclear ribosomal DNA  
11 were examined and an attempt was made to analyze the *psbA-trnH* region of the  
12 chloroplastic DNA, but this did not contribute any information for the species of our  
13 study.

14 Double-stranded DNA ETS region was amplified with the ETS 1f and 18S 2L  
15 primers (Linder *et al.*, 2000). The profile used for amplification is the following: initial  
16 denaturation at 95° C for 5 min followed by 30 cycles of 94° C for 45 s (denaturation),  
17 50° C for 45 s (primers annealing) and 72° for 40 s (primers extension), and conclusion  
18 with 72° C for 7 min (for completion of primer extension). Some ITS sequences for  
19 several species which were not included in the previous work (Susanna *et al.*, 1999)  
20 have been added in the present study following the methods described on the above-  
21 cited paper.

22 PCR products were purified with the QIAquick PCR purification kit (Qiagen,  
23 Valencia, California, U.S.A.). Both strands were sequenced with the sequencing primers  
24 ETS 1f as forward primer and 18S 2L as reverse primer. Direct sequencing of the  
25 amplified DNA segment was performed using the Big Dye Terminator Cycle  
26 sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.). Nucleotide

1 sequencing was carried out at the Serveis Científicotècnics of the University of  
2 Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City,  
3 California, U.S.A.).

4 DNA sequences were edited by Chromas 1.56 (Technelysium PTy, Tewantin,  
5 Queensland, Australia) and aligned visually. This matrix is available from the  
6 corresponding author.

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9 Data analysis

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11 *Bayesian Inference Analysis*

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13 Markov Chain Monte Carlo (MCMC) simulation, as implemented in MrBayes 3.0  
14 (Huelsenbeck & Ronquist, 2001), was used for generating trees for the combined ITS  
15 and ETS data set. After some exploratory analysis, we selected the HKY (Hasegawa *et*  
16 *al.*, 1985) and TN (Tamura & Nei, 1993) evolutionary models, comparing them through  
17 a likelihood ratio test. The HKY model involves, besides the branch lengths, 4 base  
18 frequencies ( $\pi_A$ ,  $\pi_C$ ,  $\pi_G$  and  $\pi_T$ ) and the ratio transitions/transversions ( $\kappa$ ). In the TN  
19 model, the substitution rates can be different, so that  $\kappa$  is replaced by five rates ( $r_{CT}$ ,  $r_{CG}$ ,  
20  $r_{AT}$ ,  $r_{AG}$  and  $r_{AC}$ ), the other one having been fixed at 1. This leaves us with four degrees  
21 of freedom in the likelihood ratio test.

22 In both models, we also allowed a proportion of constant sites and a random  
23 distribution of rates, using a gamma distribution. This gives two additional parameters,  
24 the proportion of invariant sites and the shape ( $\alpha$ ) of the gamma distributions. We ran  
25 500,000 generations, with four chains, sampling one of each 100 generations, and  
26 discarding the first 50 trees sampled (burn-in). Then, a consensus tree was selected by

1 the majority rule. The differences between the consensus trees obtained with the two  
2 models were minimal.

3

#### 4 *Likelihood calculations*

5

6 Maximum loglikelihood values, used in the likelihood ratio test in order to compare the  
7 evolutionary models examined, were calculated with PAML 3.14 (Yang, 2004).

8

#### 9 *Statistical analyses*

10

11 The analysis of the differences between the mean DNA amount of continental and  
12 insular habitat species was performed using the phylogenetically based generalized least  
13 squares (PGLS) algorithm, as implemented in the PHYLOGR R package (R Project,  
14 2005).

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16

## 17 **Results and Discussion**

18

### 19 **Genome size**

20

21 Nuclear DNA amounts of all the species studied are shown in Table 2. The  
22 differences between the mean DNA amount of continental and insular Macaronesian  
23 islands species of *Cheirolophus* were statistically significant ( $P < 0.001$ ) - *Ch. intybaceus*  
24 (including *Ch. grandifolius* and *Ch. lagunae*) is regarded as a continental species  
25 although some populations are present in the Balearic Islands- (Fig. 1). The mean value  
26 for the species of continental distribution is 1.58 pg, ahead the 1.38 pg for those of



1 insular distribution (without considering *Ch. crassifolius*, on which material has been  
2 impossible to acquire).

3 This is the first extensive genome size investigation in the genus *Cheirolophus*,  
4 with nuclear content reports for 19 species (21 populations), which represent almost the  
5 whole genus. Before the present study only one species had been investigated from this  
6 point of view (Suda *et al.*, 2003). Our values are rather low ( from 1.35 to 1.69 pg) and  
7 basically agree with the slightly larger reports by Suda *et al.* (2003).

8 The majority of authors agree with the hypothesis that ancestral species possess  
9 low genome sizes (Leitch *et al.*, 1998; Soltis *et al.*, 2003), and others also support the  
10 idea that evolutionary trend is toward increasing nuclear DNA amounts, because  
11 molecular mechanisms such as amplification of retrotransposons can account for it  
12 (Bennetzen & Kellogg, 1997). However, both increments and decrements have been  
13 found during evolution in different plant genera (Bennett & Leitch, 2005). In  
14 *Cheirolophus*, the presumed ancestors of each group, i.e. *Ch. uliginosus* in the  
15 Mediterranean and *Ch. massonianus* or *Ch. sempervirens* in the Macaronesian, show  
16 nuclear DNA amounts slightly higher than the derived species of each of the clades.  
17 Indeed, these are low C-values if compared with those of other groups of the Cardueae  
18 (in *Echinops*, Garnatje *et al.*, 2004; in *Carthamus* Garnatje *et al.*, unpublished; in  
19 *Rhaponticum*, Hidalgo, pers. comm.) and also if they are compared with most of the  
20 known angiosperm C-values (Bennett & Leitch, 2005). Besides, *Cheirolophus* is  
21 regarded as a basal genus of the Centaureinae (Garcia-Jacas *et al.*, 2001; Hellwig,  
22 2004). The lower genome sizes detected in the derived species with respect to their  
23 ancestors could be due to evolutionary constraints such as, in the case of *Cheirolophus*,  
24 the effect of insular selection pressures.

25 Some authors (Suda *et al.*, 2003) have already described this phenomenon for  
26 other colonizer genera of oceanic islands and the fact that *Ch. massonianus* (endemic to

1 Madeira) shows 1.44 pg of DNA (the highest value of all the insular species) could  
2 suggest that this colonization was previous to that of the Canary Islands (with a mean  
3 nuclear DNA content of 1.38 pg). This decrease in genome size would be a consequence  
4 of the founding effect, because when a species colonizes a new territory (and  
5 particularly an island), it only carries a part of all the genetic information of the  
6 population from which it has split (García-Talavera, 1999). Another possibility could be  
7 the loss of selfish or junk DNA, which constitutes the greatest part of many plant  
8 genomes and would be an unnecessary load in the stressful process of colonization.  
9 These species do not own the same genetic resources of their ancestors and must rapidly  
10 adapt to the new insular environments. Speciation and adaptive radiation are faster in  
11 the islands than on the continent (Carlquist, 1974; García-Talavera, 1999) and a smaller  
12 amount of nuclear DNA, i. e. a smaller genome size, can favour these processes.

13 Plant populations in general and particularly those of *Cheirolophus* show quite a  
14 reduced size and also a reduced nuclear DNA amount as compared with continental  
15 relatives. On the contrary, no decrease in genome size has been detected in the bigger  
16 populations of *Ch. intybaceus* from the Mediterranean islands in comparison with their  
17 continental counterparts. However, it is still unclear whether this is due to the  
18 population size or to the kind and formation of the islands. The oceanic islands  
19 analysed in this study are of volcanic origin, unlike the Mediterranean islands, and  
20 species inhabiting the former are subject to higher selection pressures which can  
21 provoke changes in their growing strategy (this point will be discussed later). Suda *et*  
22 *al.* (2003) postulated in a recent work that insular selection pressures acting on the  
23 Macaronesian archipelagos favoured small C-values (most of the species analysed in  
24 that study, from diverse angiosperm families, show less than 1.6 pg of nuclear DNA  
25 amount). As claimed by these authors, the smaller C-values of Macaronesian taxa  
26 would be explained by two main hypotheses: a loss of DNA since those islands were

1 colonized, and the fact that ancestral species possessed small genomes and only  
2 negligible changes took place during subsequent speciation processes; the  
3 diversification of ancestral plant types into several related taxa being favored by  
4 mechanisms such as adaptive radiation (in response to different ecological pressures)  
5 and vicariance (divergence due to geographic isolation) (Crawford *et al.*, 1987).

6 As Leitch *et al.* (1998) have stated it seems that there is a phylogenetic  
7 component to quantitative genome size variation which must be evaluated before the  
8 evolutionary significance of C-value diversity can be fully explained (Pagel &  
9 Johnstone, 1992; Bharathan, 1996). In many cases, the lack of a clear phylogenetic  
10 framework has limited the ability to resolve the directionality of evolutionary changes  
11 in DNA content, but recent progress in molecular biology studies has allowed the  
12 analysis of genome size variation in a phylogenetic context (Bennetzen & Kellogg, 1997;  
13 Wendel *et al.*, 2002; Soltis *et al.*, 2003; Price *et al.*, 2005). For instance, an explicit  
14 phylogenetic view on grass genomes resulted in clear genome size differences among  
15 the monophyletic grass subfamilies (Kellogg, 1998; Gaut, 2002).

16

#### 17 Phylogenetic analyses

18 The aligned sequences data matrix, including ETS and ITS regions, of  
19 *Cheirolophus* and the outgroups was 1616 bp long with 129 potentially informative  
20 characters. Among evolutionary models, HKY and TN models are usually favored,  
21 since the implicit assumptions in simpler models like the Jukes-Cantor (Jukes & Cantor,  
22 1969), Kimura (Kimura, 1980) or Felsenstein (Felsenstein, 1984) are too simplistic.  
23 Indeed, in this study, the base frequencies, as estimated by MrBayes under the HKY  
24 model (the results were quite similar under the TN model) range from  
25  $\pi_A=0.2017\pm 0.0175$  (95% posterior probability confidence limits) to  $\pi_T=0.3018\pm 0.0216$ .

1 Also, the estimate for the transitions/transversions ratio was  $\kappa=4.5572\pm 1.3094$ , leaving  
2 the Felsentein model out.

3 Thus, the analysis was restricted to HKY and TN models. A proportion of  
4 invariant sites was allowed, giving an estimate of about an 50% invariance in both  
5 models. Maximum loglikelihood values were -2578.57 and -2571.04, respectively. The  
6 likelihood ratio test yields a significance level of  $P=0.0047$ , suggesting the use of the  
7 TN model. Moreover, the rates of substitution vary by a factor of 2, both among  
8 transitions and transversions. Nevertheless, the phylogeny trees were almost identical,  
9 the only changes being found in branch lengths and posterior probabilities. Only the TN  
10 tree, with posterior probabilities included, is presented here (Fig. 2).

11

#### 12 Monophyly of the genus *Cheirolophus*

13 The analyses based on Bayesian inference (Fig. 2) support the monophyly of this  
14 genus, *Ch. crassifolius* included (PP=1.00), which had already been evidenced in a  
15 previous study based on the analysis of the ITS region (Susanna *et al.*, 1999). The tree  
16 shows a great distance between the genus *Cheirolophus* and the outgroup (*Centaurea*  
17 *iconiensis* and *C. hajastana*). It is obvious, then, that these are not species close enough  
18 to *Cheirolophus*, although the goal is not the delimitation of the genus, which is clear,  
19 as previously stated, but the clarification of the relationships at the infrageneric level.

20

#### 21 Position of *Ch. uliginosus* and *Ch. crassifolius*

22

23 All the topologies obtained show that within *Cheirolophus* there are two well-  
24 defined clades and also two species, *Ch. crassifolius* and *Ch. uliginosus*, which are not  
25 included in any supported clade. Although these species do not show a clear position,  
26 all the topologies based on BI point out that *Ch. uliginosus* would be the sister group of

1 the remaining species (PP=1.00). It is likely to be an ancestral species within the genus.  
2 These findings support the most recent hypotheses, which state that woodiness is a  
3 secondary character (Barber *et al.*, 2002 in *Sideritis*) and that small, herbaceous and  
4 least woody species occupy a basal position in their genera (Mes & Hart, 1996). In this  
5 sense, this species is the only one of the whole genus which is a hemichryptophyte, the  
6 remaining species being woody and some of them in fact being shrubs of considerable  
7 height, such as *Ch. arboreus* or *Ch. arbutifolius*. *Cheirolophus uliginosus* shows the  
8 highest nuclear DNA amount of the genus (1.69 pg). As previously stated, ancestral  
9 species are believed to possess small genomes (Leitch *et al.*, 1998; Soltis *et al.*, 2003).  
10 This species seems to be the ancestor of this group, and the fact that it has the highest  
11 genome size does not contradict this hypothesis, because the nuclear DNA content in  
12 question is still quite a decreased amount.

13       Concerning *Ch. crassifolius*, the other species with an unclear phylogenetic  
14 position, the Bayesian analysis points out that it could be a sister group of the clade  
15 constituted by the Macaronesian and Mediterranean species, but only with a PP=0.54.  
16 This species is endemic to the islands of Malta and Gozo (Mediterranean Basin) and  
17 both its geographical distribution and position in the tree suggest that it could be a  
18 relictual species, as proposed in a previous study (Susanna *et al.*, 1999). Moreover,  
19 nucleotidic evidence could also support the hypothesis of a relictual nature of this  
20 species: the ETS region of the genus *Cheirolophus* shows a deletion of 478 bp with  
21 respect to the outgroups, while *Ch. crassifolius* shows a deletion of 464 bp. This could  
22 possibly be as a result of two subsequent deletions, and the second evolutionary event  
23 (the second deletion) could have taken place when the remaining *Cheirolophus* had still  
24 not separated from *Ch. crassifolius*. Assuming this hypothesis to be correct, this species  
25 should be the subject of further research.

26

1 Mediterranean clade

2         Within this clade (PP=1.00) two groups can be observed (Fig. 2). On the one  
3 hand, the assembling of *Ch. mauritanicus* (a species from Morocco, only known from  
4 one location) with *Ch. intybaceus* complex (PP=1.00) and, on the other hand, that of *Ch.*  
5 *tananicus* with *Ch. benoistii* (PP=1.00, both species growing in Morocco, with a more  
6 restricted distribution than the former). These two later species had already been related  
7 in previous studies (Garnatje *et al.*, 1998, Susanna *et al.*, 1999), while the link between  
8 *Ch. mauritanicus* and *Ch. intybaceus* had not yet been found before in those analyses.  
9 Morphological characteristics do not agree with this grouping. Vargas *et al.* (1999)  
10 observed in their study on *Saxifraga* that some species from North Africa had been  
11 isolated and evolved independently from those of the Iberian Peninsula. These authors  
12 believed that the Mediterranean sea acted as a barrier for some groups of plants of  
13 Europe and North Africa. These *Cheirolophus* species could have undergone a similar  
14 process.

15         Hitherto, the ITS analysis (Susanna *et al.*, 1999) had grouped *Ch. mauritanicus*  
16 with *Ch. sempervirens*, but with quite a weak support (the first species had previously  
17 been described as a variety of the second, with which it presented high morphologic  
18 affinity). However, with the addition of the ETS sequence data, this grouping varies  
19 (Fig. 2) and it seems now clear that *Ch. sempervirens* is in the origin of the  
20 Macaronesian group (PP=0.99).

21         Another interesting result observed within the Mediterranean clade is the  
22 assembling of three species previously included in *Ch. intybaceus*. The population of  
23 Formentera (segregated as *Ch. grandifolius*), appears in the trees as a sister group of the  
24 two remaining species (Fig. 2), with a PP value of 1.00. If this is a true species, distinct  
25 from *Ch. intybaceus*, then it is probably undergoing a differentiation process, because  
26 although some morphological differences can be observed, nuclear DNA amount is

1 exactly the same as that of the continental *Ch. intybaceus* and there is no nucleotidic  
2 variability in the studied regions. Speciation and differentiation processes, however, can  
3 take place without any detectable change in genome size (Bennett & Leitch, 2005).

4 On the other hand, the population from Alacant (*Ch. lagunae*) is present in all  
5 the trees constituting a clade with *Ch. intybaceus* (PP=0.92), a morphologically quite  
6 similar species, although their ETS sequences show some divergences. The main  
7 difference observed between them is the plant habit, quite procumbent in *Ch. lagunae*.  
8 This plant also grows this way when cultivated, hence, the idea that this form would be  
9 a consequence of the kind of habitat should be rejected. It would be interesting to know  
10 whether this trait disappeared after some generations. Olivares *et al.* (1995) refer to  
11 many quantitative characters in the description of this species (which inhabits coastal  
12 loamy cliffs, on rocky, weakly consolidated substrata), but some of these traits, such as  
13 leaf size, are subject to variation. The sequence analysis does not allow a definite  
14 conclusion as to whether these are truly different species or not. Thus, for the sake of  
15 clarification, a more extensive study of these taxa is required, embracing their whole  
16 distribution and also including *Ch. mansanetianus* Stübing, Peris, Olivares & Martín,  
17 also segregated from *Ch. intybaceus* (which is endemic to the Cabo de Gata, Andalusia,  
18 Spain, Stübing *et al.*, 1997).

19

20 *Cheirolophus sempervirens* and the Macaronesian group

21

22 The tree based on the TN model (Fig. 2) shows a clade formed by the  
23 Macaronesian group and *Ch. sempervirens* (PP=0.99). As previously explained this  
24 species is probably at the origin of the Macaronesian group. It is distributed along the  
25 centre and the south of Portugal, some localities from the south of the Iberian Peninsula,  
26 and the south of Italy (Dostál, 1976), and there are also some populations in Algeria,

1 probably a relict of a past wider North African distribution from which this species  
2 could have colonized the Macaronesian archipelagos. However, it is not discardable that  
3 such colonization could have taken place from the westernmost part of the Iberian  
4 Peninsula, although it is farther from the islands than the African continent. In this  
5 sense, Percy *et al.* (2002) observed that within some genera, the species from Madeira  
6 were more closely related to those of Mediterranean origin than to those from Morocco.

7         The Macaronesian group, *sensu stricto*, appears as monophyletic (PP=1.00), as  
8 is found for other plant groups (Barber *et al.*, 2000, Francisco-Ortega *et al.*, 2002), and  
9 this would point to a single colonization of Macaronesia by this genus. Similarly,  
10 Vargas *et al.* (1999) also found a single colonization event of Madeira by *Saxifraga*  
11 species from the Eurosiberian region. Moreover, Silvertown (2004) postulated that large  
12 endemic plant taxa found on oceanic archipelagos were frequently monophyletic,  
13 indicating that they originated from a single colonization event. It seems that this could  
14 be a consequence of the process of colonization, because the success of the first  
15 colonizers avoids the establishment of others arriving later through niche pre-emption  
16 and interspecific competition. Carine *et al.* (2004) found that niche pre-emption is  
17 responsible for the limited number of colonisations into the region but, conversely, their  
18 results suggest that there have been multiple colonizations of the Macaronesia and a  
19 back-colonization of the continent.

20         Our results do not prove whether Madeira or the Canary Islands were first  
21 colonized, but a trend in all the trees indicates that *Ch. massonianus* is the sister group  
22 of the clade formed by the species from the Canary Islands. Nuclear DNA amount of  
23 this species (1.44 pg) is somewhere between that of *Ch. sempervirens* (1.59 pg) and the  
24 mean of the Canarian group (1.38 pg), which agrees with the appreciable genome size  
25 loss detected in the species from oceanic islands with respect to their continental  
26 counterparts. Considering a probable African origin for the colonization, it is difficult to



1 believe that Madeira was colonized before the Canary Islands. On the other hand, the  
2 possibility of an Iberian origin it is not so surprising, although there is a considerable  
3 distance between both territories. In this sense, Muñoz *et al.* (2004) found more floristic  
4 similarities between places connected by wind highways than between geographically  
5 closer places, supporting the idea that wind can act as a powerful vehicle of dispersion.

6 Barber *et al.* (2000) observed that the insular taxa of *Sideritis* formed a  
7 monophyletic group, and this would mean that only one colonization event of the  
8 islands took place. Moreover, the same authors found that the genus was most likely  
9 introduced in Madeira from the Canarian archipelago, probably due to the presence of a  
10 great biological/floristic diversity in these islands. Francisco-Ortega *et al.*, 1999 found  
11 in the *Asteriscus* alliance at least two colonizing events of Macaronesia from North  
12 Africa, and postulated that the derived position of these species suggested a recent  
13 colonization process of Macaronesia, contrary to the hypothesis upholding the idea of  
14 an ancient origin of Macaronesian flora (Bramwell, 1972, 1976, 1985). Similarly, the  
15 study of Helfgott *et al.* (2000) on the *Bencomia* alliance (Rosaceae) contradicts the  
16 premise that Macaronesian flora would be a relictual fragment of a widespread Tertiary  
17 subtropical European flora.

18 Canarian *Cheirolophus* species do not constitute a monophyletic group  
19 (PP=0.70), so that a single colonization event by this genus in these islands can be  
20 discarded. There is a low resolution within the Canarian group, as other authors have  
21 also detected in some plant genera (Sang *et al.*, 1994 in *Dendroseris*; Mort *et al.*, 2003  
22 in *Tolpis*), a circumstance that could support the theory of a fast adaptive radiation  
23 taking place in the insular environments. The species from the island of Tenerife (*Ch.*  
24 *tagananensis* excluded) form a well-supported clade in the BI analysis (PP=0.99, Fig.  
25 2), and the species from the remaining Canarian Islands form another clade, but weakly  
26 supported (PP=0.74). Therefore it is possible that two introductions, or even three (Fig.

1 2), took place in the colonization of the Canary Islands. Likewise, Fuertes-Aguilar *et al.*  
2 (2002) also observed in *Lavatera* that two introductions took place in the Canary  
3 Islands, one probably being very recent, because the species is located in a derived clade  
4 together with the Mediterranean species. Francisco-Ortega *et al.* (2001, 2002) did not  
5 find any clade restricted to a single island, and therefore it appears that inter-island  
6 colonization was the main avenue for speciation in these two archipelagos. According to  
7 those authors, Tenerife is likely the ancestral island for species endemic to the five  
8 westernmost islands, this one and Gran Canaria, La Gomera, El Hierro and La Palma,  
9 and this would also seem to be the case of the genus *Cheirolophus*. This last-named  
10 genus inhabits locations of similar ecology in the different islands of the archipelago, i.  
11 e., usually rocky and slightly humid places, with the presence of small water trickles.  
12 Francisco-Ortega *et al.* (1996) found that interisland colonization between similar  
13 ecological zones may be one of the primary factors involved in the evolution of the  
14 endemic flora of oceanic archipelagos.

15 We detected some groupings within the Canarian clade in our study, but with no  
16 support. As maintained by Barret (1996), for most plant groups on remote oceanic  
17 islands extreme genetic bottlenecks must have been involved in their evolutionary  
18 history. However, the occurrence of such events and their evolutionary significance is  
19 poorly understood. Likewise, Francisco-Ortega *et al.* (1997), based on the low levels of  
20 nucleotide sequence divergence found in *Argyranthemum*, suggested that this genus  
21 originated and radiated recently, and this could also be the case of *Cheirolophus*.

22 Aside from this, the clade including all the species from the other islands  
23 (PP=0.74), namely *Ch. junonianus* and *Ch. arboreus* from the island of La Palma, *Ch.*  
24 *arbutifolius* and *Ch. falcisectus* from Gran Canaria and *Ch. gomerhytus* from La  
25 Gomera, shows that the length of the branches agrees with the geological ages of the

1 different islands, so longer branches correspond to those species inhabiting the older  
2 islands and therefore they have experienced a greater differentiation process.

3

#### 4 Habit of insular plants

5

6 The insular woody habit occurs in several angiosperm families, among them the  
7 Asteraceae, as an adaptation to climatic and other factors (Böhle *et al.*, 1996). In their  
8 study on *Echium*, these authors hypothesized that selection for successful pollination  
9 could account for the woodiness (longevity) as well as for the candelabrum-like habit,  
10 and observed large inflorescences in the insular, insect-pollinated species, in an  
11 environment where insects would be expected to be rare, noting that the resulting  
12 increase in size may have provided additional advantage in niche competition among  
13 initial colonizers. Likewise, Fuertes-Aguilar *et al.* (2002) postulated that woodiness in  
14 *Lavatera* was a secondary character originated as an adaptation to the insular  
15 conditions. According to Hellwig (2004), *Cheirolophus* may be an old group within  
16 Centaureinae, but speciation may have occurred not too long ago. While only a few  
17 relictic species grow in the western Mediterranean region, *Cheirolophus* radiated on the  
18 Canary Islands, where some species have developed the candelabrum-like habit, and  
19 also some, such as *Ch. arboreus* and *Ch. arbutifolius*, have acquired an arborescent,  
20 woodier habit, than those from the continent, as can be deduced from their specific  
21 names. Moreover, they produce large amounts of quite big capitula with gorgeous  
22 flowers in Macaronesia, while the continental *Cheirolophus* (*Ch. benoistii*, *Ch.*  
23 *intybaceus*, *Ch. uliginosus*) present smaller flowers. Similar findings have been reported  
24 by Mort *et al.* (2002) in their study centred on the Crassulaceae. Helfgott *et al.* (2000) in  
25 *Bencomia* alliance (Rosaceae) considered that plant size and woodiness increased in the  
26 insular taxa in comparison with their continental relatives, contrary to what other

1 authors had stated. It is common, for example, for herbaceous colonists to speciate into  
2 a wide variety of growth forms, including shrubs and trees, and for species to radiate  
3 into new habitats (Carlquist, 1974; Givnish, 1998). To add to this, Panero *et al.* (1999)  
4 found that 65% of endemic species of the genus *Pericallis* that were originary from  
5 some Atlantic archipelagos were also woody. Additionally, according to Kim *et al.*  
6 (1996) woodiness increased in the islands in the genus *Sonchus*.

7

8 Appearance of the genus in the islands

9

10 As stated before, the results from our analyses bring ambiguous data on the  
11 order of colonization of the Canary Islands. The fact that there are no *Cheirolophus*  
12 representatives in the islands of Lanzarote and Fuerteventura, the two nearest to the  
13 continent and also among the most ancient (between 15 and 21 M.y.), does not  
14 necessarily mean that they had never been there, because they could have been  
15 extinguished due to volcanic activity or to the special climatic conditions of these  
16 islands. Mean annual precipitation in these islands, which were united 18,000 years ago,  
17 seldom exceeds 150 mm, and the presence of winds from the Sahara is quite usual there,  
18 frequently carrying dust particles, another weather incident to consider (García-  
19 Talavera, 1999). Plants from these islands could be recent groups because of their  
20 proximity to the continent and they could also function as the link between Macaronesia  
21 and Africa, an entry or departure point for their dispersion.

22 As claimed by Carracedo (1994) the eastern Canary Islands volcanoes may have been  
23 active since the late cretaceous. According to their eruptive activity, Tenerife, La Palma,  
24 Lanzarote and Hierro, have had eruptions in historic time (<500 year) and are  
25 volcanically active; Fuerteventura and Gran Canaria had Quaternary volcanism, while  
26 no evidence of Quaternary volcanism has been found in La Gomera. This would

1 probably explain why *Cheirolophus* habitually colonizes the most ancient places of the  
2 islands, and this is fairly noticeable in the island of Tenerife, where some species are  
3 found over basaltic mountains of the Tertiary, such as *Ch. tagananensis* at the Anaga  
4 coasts and *Ch. canariensis* in the southern sector of the Teno Mountains (Bramwell &  
5 Bramwell, 2001).

6 Finally, from the information obtained with the phylogenetic trees, no  
7 recolonization from the islands to the continent can be deduced. The same results were  
8 found by Böhle *et al.* (1996), who also considered it curious that no continental *Echium*  
9 species studied was a descendant of any of the island inhabitants. It would seem, then,  
10 that there was no fruitful recolonization of the continent.

11

## 12 **Conclusions**

13

14 Differences between mean nuclear DNA amounts of insular (1.38 pg) and  
15 continental (1.58 pg) species are statistically significant and this has been related to the  
16 adaptation leading to the colonization process of oceanic islands.

17 The Macaronesian group is monophyletic, and this could mean a single  
18 colonization event of Macaronesia by this genus. *Ch. massonianus* shows an  
19 intermediate C-value, between continental and insular species, suggesting a former  
20 colonization of Madeira from the continent. Additionally, *Ch. sempervirens* appears  
21 related to the Macaronesian group; therefore, this species could be at the origin of this  
22 colonization process. Conversely, the Canarian group is not monophyletic; hence, we  
23 suppose more than one colonization event of the archipelago. Finally, the species from  
24 the island of Tenerife, with the exception of *Ch. tagananensis*, constitute a single clade,  
25 while all the representatives of this genus from all the other islands are grouped in

1 another clade; thus, there has been more speciation in Tenerife (probably because of a  
2 higher diversity of habitats in this island).

3

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5

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20

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1 Figure captions

2

3 Figure 1. Box and whisker plot comparing nuclear DNA amount of continental vs.

4 insular species.

5

6 Figure 2. Consensus tree produced by Bayesian inference. Posterior probabilities equal

7 or above 0.5 are indicate at the branches.

8

1 Table 1. Origin, herbarium vouchers, and GenBank accession numbers of the species  
 2 studied. The accessions marked with an asterisk (\*) were only used to assess the nuclear  
 3 DNA amount.

Species	Collection data and herbarium voucher	GenBank accession number ITS1, ITS2	GenBank accession number ETS
<i>Rhaponticoides hajastana</i> (Tzvelev) M. V. Agab. & Greuter	Armenia, Talin: between villages Pokr Artik and Bagravan, <i>Susanna</i> 1587 et al. 26.VIII.1995 (BC).	AF041281	AF041263
<i>Rhaponticoides iconiensis</i> (Hub.-Mor.) M. V. Agab. & Greuter	Turkey, Ala Dag: track above the of Demir Kazk. Ertugrul 1761, 7.VIII.2003 (BC).	AF041280	AF041256
<i>Cheirolophus arboreus</i> (Webb & Berth.) Holub	Spain, Canary Islands, La Palma: San Andrés y Sauces, <i>Susanna</i> 1425, 29.VII.1990 (BC).	AF021147, AF021164	AF041257
<i>Ch. arbutifolius</i> (Svent.) Kunkel	Spain, Canary Islands, Gran Canaria: Agaete, Los Berrazales, <i>Susanna</i> 1420, 25.VII.1990 (BC).	AF041279	AF041258
<i>Ch. benoistii</i> (Humbert) Holub	Morocco, Ksar es Souk: S side of Tizi n'Talrhem, <i>Garnatje, Susanna</i> 1787 & <i>Vilatersana</i> , 17.VI.1997 (BC). Morocco, Ksar es Souk: S side of Tizi n'Talrhem, <i>Hidalgo &amp; Romo</i> 12699, 19.VII.2004 (BC)*.	AF045415, AF079945	AF041249
<i>Ch. burchardii</i> Susanna	Spain, Canary Islands, Tenerife: between Buenavista and Teno, <i>Susanna</i> 1430, 6.VII.1990 (BC).	AF021145, AF021162	AF041250
<i>Ch. canariensis</i> (Brouss. ex Willd.) Holub	Spain, Canary Islands, Tenerife: Masca ravine, <i>Garnatje 1 &amp; Luque</i> , VIII, 1996 (BC).	AF021151, AF021168	AF041253
<i>Ch. crassifolius</i> (Bertoloni) Susanna	Malta: Botanical Garden of la Valetta	AF021157, AF021174	AF041252
<i>Ch. falcisectus</i> Montel. & Moral.	Spain, Canary Islands, Gran Canaria: above san Nicolás de Tolentino, old road to Mogán, <i>Susanna</i> 1422, 25.VII.1990 (BC).	AF021146, AF021163	AF041255
<i>Ch. gomerythus</i> (Svent.) Holub	Spain, Canary Islands, La Gomera: near Agulo, San Marcos ravine, <i>Susanna</i> 1426, 4.VII.1990 (BC).	AF021149, AF021166	AF041259
<i>Ch. grandifolius</i>	Spain, Balearic Islands, Formentera: mount Sa Mola, <i>Freixenet</i> , VIII.1988 (BC). Balearic Islands, Formentera: La Mola <i>Garnatje &amp; Vilatersana V-411</i> , 18.IV.2005 (BC).*	AF041282	AF041261
<i>Ch. intybaceus</i> (Lam.) Dostál	Spain, Alacant: between Alcoy and Pego, km 40, near Benirrama, <i>Garcia-Jacas &amp; Susanna</i> 1249, 5.VIII.1988 (BC). Spain, Balearic Islands, Eivissa: Cala d'Albarca, <i>Garnatje &amp; Vilatersana V-400</i> , 14.IV.2005 (BC).*	AF021152, AF021169	AF041276
<i>Ch. junonianus</i> (Svent.) Holub	Spain, Canary Islands, La Palma: Fuencaliente, volcano of San Antonio, <i>Susanna</i> 1423, 28.VII.1990 (BC).	AF021148, AF021165	AF041284
<i>Ch. lagunae</i> A.Olivares, J.B.Peris, G.Stübing & J.Martín	Spain, Alacant, Xàvia: Cap Prim, 31SBC5894 <i>Garnatje &amp; Vilatersana</i> 442, 20.V.2005 (BC).	AF041283	AF041289
<i>Ch. massonianus</i> (Lowe) O. Eriksson & al.	Portugal, Botanical Garden of Madeira (BC).	AF021143, AF021160	AF041287
<i>Ch. mauritanicus</i> (Font Quer) Susanna	Morocco, Tetouan : mount Tissouka above Chefchaouen, <i>Molero, Romo</i> 4617 & <i>Susanna</i> , 20.VI.1988 (BC).	AF021155, AF021172	AF041291
<i>Ch. metlesicsii</i> Montel.	Spain, Canary Islands, Tenerife: Arafo, Afiavingo ravine, <i>Susanna</i> 1427, 6.VII.1990 (BC).	AF021150, AF021167	AF041293
<i>Ch. sempervirens</i> (L.) Pomel	Portugal, Faro : 4 km from N of Monchique, <i>Garcia-Jacas &amp; Susanna</i> 1218, 28.VII.1988 (BC).	AF021156, AF021173	AF041294
<i>Ch. taganensis</i> (Svent.)	Spain, Canary Islands, Tenerife:	AF021144, AF021161	AF041295



Holub	Taganana, roque de las Animas, <i>Garnatje 3 &amp; Luque</i> , VIII.1996 (BC).		
<i>Ch. tananicus</i> (Maire) Holub	Morocco, Agadir: 1 km S of the Tizi n'Test, <i>Garcia-Jacas, Susanna 1395 &amp; Vallès</i> , 11.VII.1990 (BC).	AF021153, AF021170	AF041296
<i>Ch. teydis</i> (Ch. Smith) G. López	Spain, Canary Islands, Tenerife: El Teide, boca de tauce, <i>Susanna 1429</i> , 6.VII.1990 (BC).	AF041283	AF041297
<i>Ch. uliginosus</i> (Brot.) Dostál	Spain, Huelva: Mazagón, El Loro, <i>Garcia-Jacas, Julià, J. M. Montserrat 1875, Susanna &amp; Veny</i> , 6.VII.1988 (BC).	AF021154, AF021171	AF041298

1

1 Table 2. Nuclear DNA content and other characters of the populations studied.  
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 3

Species	2C ± SD (pg)	2C (Mbp)	Distribution
<i>Cheirolophus arboreus</i>	1.40 ± 0.02	1369.20	Insular
<i>Ch. arbutifolius</i>	1.39 ± 0.02	1359.42	Insular
<i>Ch. benoistii</i>	1.55 ± 0.02	1515.90	Continental
<i>Ch. burchardii</i>	1.42 ± 0.03	1388.76	Insular
<i>Ch. canariensis</i>	1.38 ± 0.04	1349.64	Insular
<i>Ch. falcisectus</i>	1.35 ± 0.03	1320.30	Insular
<i>Ch. gomerythus</i>	1.41 ± 0.04	1378.98	Insular
<i>Ch. grandifolius</i>	1.51 ± 0.03	1476.78	Cont/Insu
<i>Ch. intybaceus</i> Alacant	1.50 ± 0.02	1467.00	Cont/Insu
<i>Ch. intybaceus</i> Eivissa	1.51 ± 0.02	1476.78	Cont/Insu
<i>Ch. junoniamus</i>	1.37 ± 0.02	1339.86	Insular
<i>Ch. lagunae</i>	1.51 ± 0.01	1476.78	Continental
<i>Ch. massonianus</i>	1.44 ± 0.02	1408.32	Insular
<i>Ch. mauritanicus</i>	1.57 ± 0.03	1535.46	Continental
<i>Ch. metlesicsii</i>	1.36 ± 0.01	1330.08	Insular
<i>Ch. sempervirens</i>	1.59 ± 0.02	1555.02	Continental
<i>Ch. taganensis</i>	1.38 ± 0.02	1349.64	Insular
<i>Ch. tananicus</i>	1.65 ± 0.01	1613.70	Continental
<i>Ch. teydis</i>	1.38 ± 0.03	1349.64	Insular
<i>Ch. uliginosus</i>	1.69 ± 0.01	1652.82	Continental

4



