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

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Summary

- Nuclear DNA amount has been assessed in 21 populations of 19 species of the genus Cheirolophus, and phylogenetic analyses have been performed in order to find the relationships between the species of the Mediterranean Basin and those of Macaronesia.
- A combined analysis of nuclear ribosomal DNA ITS and ETS has been performed based on Bayesian inference, and nuclear DNA amount has been assessed by flow cytometry.
- The monophyly of the Macaronesian group and its relationship with Ch. sempervirens indicate a unique colonization event of Macaronesia from the continent, contrarily to the various colonizations that should have occurred in the Canarian archipelago. DNA amount is significantly lower
in the insular species than in the continental ones, and a parallelism appears between the evolution of the genus *Cheirolophus* (colonization of the islands) and the loss of DNA, probably due to the selection pressure existing in the oceanic islands or to the founding effect.

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

**Introduction**

The biogeographic region of Macaronesia comprises several groups of islands: the archipelagos of the Canary Islands, the Azores, Madeira, Cape Verde and the Selvagens Islands. All of these are of volcanic origin and located in the Atlantic Ocean between the coordinates 39° 45’ N, 31° 17’ W and 14° 49’ N, 13° 20’ W. The flora of Macaronesia is highly diverse and complex, with a considerable number of endemics. In fact, oceanic islands are usually rich in endemcity, and at least 500 endemic species are found in Canary Islands (González, 1992). It is believed that there is a connection between the flora of this region and that of the Mediterranean Basin and north-west Africa (Suda *et al.*, 2003) and that a major role is played by the sea mounts surrounding the archipelagos, which acted as bridges of dispersion and colonization in the geological past, when sea levels were lower (García-Talavera, 1999). This is called the stepping-stone model (see Juan *et al.*, 2000).
The highest biodiversity of Macaronesia is found in the Canary Islands, possibly due to the great variety of habitats and the broad range of geological ages (Fernandopullé, 1976; Carracedo, 1994), among other possible reasons. The Canaries are the biggest and most developed archipelago of Macaronesia and form a volcanically active alignment of seven islands distributed in a band near the African coast. The nearest island is Fuerteventura, only 100 km from Africa. Fuerteventura is thought to have been joined to Lanzarote and to other islets 18,000 years ago, forming a single island only 60 km from the coast of Africa (García-Talavera, 1999). The fact that the Macaronesian archipelagos, and especially the Canaries, are relatively near the African continent makes them different from the other oceanic islands.

The genus *Cheirolophus* (Asteraceae, Cardueae) was originally described by Cassini (1817), who based his description on some species from *Centaurea* L. Ever since then it has been considered as an independent genus by different authors (Dostál, 1976; Bremer, 1994). *Cheirolophus* includes ca. 20 species distributed along the west Mediterranean Basin and Macaronesia; some *Cheirolophus* have a wide distribution, such as *Ch. intybaceus*, *Ch. sempervirens* and *Ch. uliginosus*, while approximately 17 are described, some of them quite recently, as endemic to the Canary Islands (Bramwell & Bramwell, 2001). Only, one species is endemic to Madeira, *Ch. massonianus* (Lowe) A. Hansen & Sunding. All *Cheirolophus* are shrubs, subshrubs or shrublets, excluding *Ch. uliginosus* (Brot.) Dostál, which is a hemichryptophyte. Preliminary studies on this genus (Susanna *et al.*, 1995, 1999) upheld its monophyly with the inclusion of *Ch. crassifolius* (Bertol.) Susanna, previously named *Paleocyanus crassifolius* (Bertol.) Dostál. *Cheirolophus* is regarded as a basal genus of the Centaureinae (García-Jacas *et al.*, 2001; Hellwig, 2004) and it would have diverged from this subtribe during the late Oligocene and Miocene (Hellwig, 2004), although its speciation is relatively recent (Garnatje *et al.*, 1998; Susanna *et al.*, 1999).
The performance of molecular phylogenetic analysis has long been useful in tracing the origin and evolution of island endemics, as has been shown in some other Asteraceae genera, such as *Robinsonia* (Sang et al., 1995), *Aeonium* (Mes, 1995) or the Macaronesian *Argyranthemum* (Francisco-Ortega et al., 1997). Sometimes, however, the resolution obtained with these methods is not as high as desired. From a phylogenetic perspective, three issues still remain unresolved in this group. The first is the delimitation of *Ch. intybaceus* (Lam.) Dostál, a species with a large distribution area from the south of France to the south of Spain, and embracing part of the Balearic Islands. Some authors (Olivares et al., 1995, Stübing et al., 1997) have conferred the status of distinct species to some populations from this area. In a previous study (Susanna et al., 1999), one of these species, *Ch. grandifolius* (Font Quer) Stübing, Peris, Olivares & Martín did not show any molecular difference with respect to *Ch. intybaceus*, on the basis of the sequences of the ITS region. In the present work, we have also included another of these species, *Ch. lagunae* Olivares, Peris, Stübing & Martín, which inhabits specific localities on the coast of Alacant (Spain). The second question is the low resolution of the phylogeny within the Macaronesian group, but Baldwin et al. (1998) outlined the difficulty of an accurate estimation of the phylogeny of insular plants due to the low variability that the DNA regions used for the analysis usually show. A combination of different data could possibly solve the phylogeny of the insular taxa (Mort et al., 2002). With this purpose, the present work combines data from the analysis of the ITS sequences (Susanna et al., 1999) with those from the ETS region of nuclear ribosomal DNA. Finally, the third question is the clarification of the phylogenetic placement of *Ch. crassifolius*.

Together with all the data coming from phylogenetic studies, data on genome size variation can contribute to a better understanding in the interpretation of the systematics and the evolutionary relationships within a narrow group of plants (Ohri,
Genome size is an important biodiversity character, linked to many aspects in the biology of a species (Bennett, 1998). Although the meaning of the variation in the C-value (i.e. genome size) is still not completely well understood (Gregory, 2001, 2005), and that it is still too soon to establish any model or to see if this parameter could have remarkable systematic implications (only approximately 3% of angiosperm species have a known C-value), its study can certainly provide valuable information in the clarification of the relationships between close species. Thus, for example, Zonneveld & Iren (2001) found that when many species in a genus had the same chromosome numbers, differences in nuclear DNA contents proved to be effective in delimiting infrageneric divisions in a number of taxa. Thus, the study of genome size variation could reveal divergence within a genus (like in Hypochaeris, Cerbah et al., 1999, or in Musa, Lysák et al., 1999).

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

**Materials and Methods**

**Plant material**

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

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

DNA amplification and sequencing strategies

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

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

PCR products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, California, U.S.A.). Both strands were sequenced with the sequencing primers ETS 1f as forward primer and 18S 2L as reverse primer. Direct sequencing of the amplified DNA segment was performed using the Big Dye Terminator Cycle sequencing v3.1 (PE Biosystems, Foster City, California, U.S.A.). Nucleotide
sequencing was carried out at the Serveis Científicotècnics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, California, U.S.A.).

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

Data analysis

Bayesian Inference Analysis

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

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

Likelihood calculations

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

Statistical analyses

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

Results and Discussion

Genome size

Nuclear DNA amounts of all the species studied are shown in Table 2. The differences between the mean DNA amount of continental and insular Macaronesian islands species of *Cheirolophus* were statistically significant (P<0.001) - *Ch. intybaeus* (including *Ch. grandifolius* and *Ch. laguna*) is regarded as a continental species although some populations are present in the Balearic Islands- (Fig. 1). The mean value for the species of continental distribution is 1.58 pg, ahead the 1.38 pg for those of
This is the first extensive genome size investigation in the genus *Cheirolophus*, with nuclear content reports for 19 species (21 populations), which represent almost the whole genus. Before the present study only one species had been investigated from this point of view (Suda et al., 2003). Our values are rather low (from 1.35 to 1.69 pg) and basically agree with the slightly larger reports by Suda et al. (2003).

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

Some authors (Suda et al., 2003) have already described this phenomenon for other colonizer genera of oceanic islands and the fact that *Ch. massonianus* (endemic to
Madeira) shows 1.44 pg of DNA (the highest value of all the insular species) could suggest that this colonization was previous to that of the Canary Islands (with a mean nuclear DNA content of 1.38 pg). This decrease in genome size would be a consequence of the founding effect, because when a species colonizes a new territory (and particularly an island), it only carries a part of all the genetic information of the population from which it has split (García-Talavera, 1999). Another possibility could be the loss of selfish or junk DNA, which constitutes the greatest part of many plant genomes and would be an unnecessary load in the stressful process of colonization. These species do not own the same genetic resources of their ancestors and must rapidly adapt to the new insular environments. Speciation and adaptive radiation are faster in the islands than on the continent (Carlquist, 1974; García-Talavera, 1999) and a smaller amount of nuclear DNA, i.e. a smaller genome size, can favour these processes.

Plant populations in general and particularly those of Cheirolophus show quite a reduced size and also a reduced nuclear DNA amount as compared with continental relatives. On the contrary, no decrease in genome size has been detected in the bigger populations of Ch. intybaceus from the Mediterranean islands in comparison with their continental counterparts. However, it is still unclear whether this is due to the population size or to the kind and formation of the islands. The oceanic islands analysed in this study are of volcanic origin, unlike the Mediterranean islands, and species inhabiting the former are subject to higher selection pressures which can provoke changes in their growing strategy (this point will be discussed later). Suda et al. (2003) postulated in a recent work that insular selection pressures acting on the Macaronesian archipelagos favoured small C-values (most of the species analysed in that study, from diverse angiosperm families, show less than 1.6 pg of nuclear DNA amount). As claimed by these authors, the smaller C-values of Macaronesian taxa would be explained by two main hypotheses: a loss of DNA since those islands were
colonized, and the fact that ancestral species possessed small genomes and only negligible changes took place during subsequent speciation processes; the diversification of ancestral plant types into several related taxa being favored by mechanisms such as adaptive radiation (in response to different ecological pressures) and vicariance (divergence due to geographic isolation) (Crawford et al., 1987).

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

Phylogenetic analyses

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

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

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

Monophyly of the genus Cheirolphus

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

Position of Ch. uliginosus and Ch. crassifolius

All the topologies obtained show that within Cheirolphus there are two well-defined clades and also two species, Ch. crassifolius and Ch. uliginosus, which are not included in any supported clade. Although these species do not show a clear position, all the topologies based on BI point out that Ch. uliginosus would be the sister group of
the remaining species (PP=1.00). It is likely to be an ancestral species within the genus. These findings support the most recent hypotheses, which state that woodiness is a secondary character (Barber et al., 2002 in Sideritis) and that small, herbaceous and least woody species occupy a basal position in their genera (Mes & Hart, 1996). In this sense, this species is the only one of the whole genus which is a hemichryptophyte, the remaining species being woody and some of them in fact being shrubs of considerable height, such as Ch. arboreus or Ch. arbutifolius. Cheirolophus uliginosus shows the highest nuclear DNA amount of the genus (1.69 pg). As previously stated, ancestral species are believed to possess small genomes (Leitch et al., 1998; Soltis et al., 2003). This species seems to be the ancestor of this group, and the fact that it has the highest genome size does not contradict this hypothesis, because the nuclear DNA content in question is still quite a decreased amount.

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

Within this clade (PP=1.00) two groups can be observed (Fig. 2). On the one hand, the assembling of Ch. mauritanicus (a species from Morocco, only known from one location) with Ch. intybaceus complex (PP=1.00) and, on the other hand, that of Ch. tananicus with Ch. benoistii (PP=1.00, both species growing in Morocco, with a more restricted distribution than the former). These two later species had already been related in previous studies (Garnatje et al., 1998, Susanna et al., 1999), while the link between Ch. mauritanicus and Ch. intybaceus had not yet been found before in those analyses.

Morphological characteristics do not agree with this grouping. Vargas et al. (1999) observed in their study on Saxifraga that some species from North Africa had been isolated and evolved independently from those of the Iberian Peninsula. These authors believed that the Mediterranean sea acted as a barrier for some groups of plants of Europe and North Africa. These Cheirolophus species could have undergone a similar process.

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

Another interesting result observed within the Mediterranean clade is the assembling of three species previously included in Ch. intybaceus. The population of Formentera (segregated as Ch. grandifolius), appears in the trees as a sister group of the two remaining species (Fig. 2), with a PP value of 1.00. If this is a true species, distinct from Ch. intybaceus, then it is probably undergoing a differentiation process, because although some morphological differences can be observed, nuclear DNA amount is
exactly the same as that of the continental *Ch. intybaceus* and there is no nucleotidic
variability in the studied regions. Speciation and differentiation processes, however, can
take place without any detectable change in genome size (Bennett & Leitch, 2005).

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

*Cheirolophus sempervirens* and the Macaronesian group

The tree based on the TN model (Fig. 2) shows a clade formed by the
Macaronesian group and *Ch. sempervirens* (PP=0.99). As previously explained this
species is probably at the origin of the Macaronesian group. It is distributed along the
centre and the south of Portugal, some localities from the south of the Iberian Peninsula,
and the south of Italy (Dostál, 1976), and there are also some populations in Algeria,
probably a relict of a past wider North African distribution from which this species could have colonized the Macaronesian archipelagos. However, it is not discardable that such colonization could have taken place from the westernmost part of the Iberian Peninsula, although it is farther from the islands than the African continent. In this sense, Percy et al. (2002) observed that within some genera, the species from Madeira were more closely related to those of Mediterranean origin than to those from Morocco.

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

Our results do not prove whether Madeira or the Canary Islands were first colonized, but a trend in all the trees indicates that *Ch. massonianus* is the sister group of the clade formed by the species from the Canary Islands. Nuclear DNA amount of this species (1.44 pg) is somewhere between that of *Ch. sempervirens* (1.59 pg) and the mean of the Canarian group (1.38 pg), which agrees with the appreciable genome size loss detected in the species from oceanic islands with respect to their continental counterparts. Considering a probable African origin for the colonization, it is difficult to
believe that Madeira was colonized before the Canary Islands. On the other hand, the possibility of an Iberian origin it is not so surprising, although there is a considerable distance between both territories. In this sense, Muñoz et al. (2004) found more floristic similarities between places connected by wind highways than between geographically closer places, supporting the idea that wind can act as a powerful vehicle of dispersion.

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

Canarian Cheirolophus species do not constitute a monophyletic group (PP=0.70), so that a single colonization event by this genus in these islands can be discarded. There is a low resolution within the Canarian group, as other authors have also detected in some plant genera (Sang et al., 1994 in Dendroseris; Mort et al., 2003 in Tolpis), a circumstance that could support the theory of a fast adaptive radiation taking place in the insular environments. The species from the island of Tenerife (Ch. tagananensis excluded) form a well-supported clade in the BI analysis (PP=0.99, Fig. 2), and the species from the remaining Canarian Islands form another clade, but weakly supported (PP=0.74). Therefore it is possible that two introductions, or even three (Fig. 2),
2), took place in the colonization of the Canary Islands. Likewise, Fuertes-Aguilar et al. (2002) also observed in *Lavatera* that two introductions took place in the Canary Islands, one probably being very recent, because the species is located in a derived clade together with the Mediterranean species. Francisco-Ortega et al. (2001, 2002) did not find any clade restricted to a single island, and therefore it appears that inter-island colonization was the main avenue for speciation in these two archipelagos. According to those authors, Tenerife is likely the ancestral island for species endemic to the five westernmost islands, this one and Gran Canaria, La Gomera, El Hierro and La Palma, and this would also seem to be the case of the genus *Cheirolophus*. This last-named genus inhabits locations of similar ecology in the different islands of the archipelago, i.e., usually rocky and slightly humid places, with the presence of small water trickles. Francisco-Ortega et al. (1996) found that interisland colonization between similar ecological zones may be one of the primary factors involved in the evolution of the endemic flora of oceanic archipelagos.

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

Aside from this, the clade including all the species from the other islands (PP=0.74), namely *Ch. junonianus* and *Ch. arboreus* from the island of La Palma, *Ch. arbutifolius* and *Ch. falcisectus* from Gran Canaria and *Ch. gomerhytus* from La Gomera, shows that the length of the branches agrees with the geological ages of the
different islands, so longer branches correspond to those species inhabiting the older islands and therefore they have experienced a greater differentiation process.

Habit of insular plants

The insular woody habit occurs in several angiosperm families, among them the Asteraceae, as an adaptation to climatic and other factors (Böhle et al., 1996). In their study on *Echium*, these authors hypothesized that selection for successful pollination could account for the woodiness (longevity) as well as for the candelabrum-like habit, and observed large inflorescences in the insular, insect-pollinated species, in an environment where insects would be expected to be rare, noting that the resulting increase in size may have provided additional advantage in niche competition among initial colonizers. Likewise, Fuertes-Aguilar et al. (2002) postulated that woodiness in *Lavatera* was a secondary character originated as an adaptation to the insular conditions. According to Hellwig (2004), *Cheirolophus* may be an old group within Centaureinae, but speciation may have occurred not too long ago. While only a few relictic species grow in the western Mediterranean region, *Cheirolophus* radiated on the Canary Islands, where some species have developed the candelabrum-like habit, and also some, such as *Ch. arboreus* and *Ch. arbutifolius*, have acquired an arborescent, woodier habit, than those from the continent, as can be deduced from their specific names. Moreover, they produce large amounts of quite big capitula with gorgeous flowers in Macaronesia, while the continental *Cheirolophus* (*Ch. benoistii, Ch. intybacæus, Ch. uliginosus*) present smaller flowers. Similar findings have been reported by Mort et al. (2002) in their study centred on the Crassulaceae. Helfgott *et al.* (2000) in *Bencomia* alliance (Rosaceae) considered that plant size and woodiness increased in the insular taxa in comparison with their continental relatives, contrary to what other
authors had stated. It is common, for example, for herbaceous colonists to speciate into a wide variety of growth forms, including shrubs and trees, and for species to radiate into new habitats (Carlquist, 1974; Givnish, 1998). To add to this, Panero et al. (1999) found that 65% of endemic species of the genus *Pericallis* that were originary from some Atlantic archipelagos were also woody. Additionally, according to Kim et al. (1996) woodiness increased in the islands in the genus *Sonchus*.

Appearance of the genus in the islands

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

As claimed by Carracedo (1994) the eastern Canary Islands volcanoes may have been active since the late cretaceous. According to their eruptive activity, Tenerife, La Palma, Lanzarote and Hierro, have had eruptions in historic time (<500 year) and are volcanically active; Fuerteventura and Gran Canaria had Quaternary volcanism, while no evidence of Quaternary volcanism has been found in La Gomera. This would
probably explain why *Cheirolophus* habitually colonizes the most ancient places of the islands, and this is fairly noticeable in the island of Tenerife, where some species are found over basaltic mountains of the Tertiary, such as *Ch. tagananensis* at the Anaga coasts and *Ch. canariensis* in the southern sector of the Teno Mountains (Bramwell & Bramwell, 2001).

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

**Conclusions**

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

The Macaronesian group is monophyletic, and this could mean a single colonization event of Macaronesia by this genus. *Ch. massonianus* shows an intermediate C-value, between continental and insular species, suggesting a former colonization of Madeira from the continent. Additionally, *Ch. sempervirens* appears related to the Macaronesian group; therefore, this species could be at the origin of this colonization process. Conversely, the Canarian group is not monophyletic; hence, we suppose more than one colonization event of the archipelago. Finally, the species from the island of Tenerife, with the exception of *Ch. tagananensis*, constitute a single clade, while all the representatives of this genus from all the other islands are grouped in
another clade; thus, there has been more speciation in Tenerife (probably because of a higher diversity of habitats in this island).

Acknowledgements

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Macaronesian flora. Proceedings of the National Academy of Sciences USA 93: 4085-4090.


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Figure captions

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

Figure 2. Consensus tree produced by Bayesian inference. Posterior probabilities equal or above 0.5 are indicated at the branches.
Table 1. Origin, herbarium vouchers, and GenBank accession numbers of the species studied. The accessions marked with an asterisk (*) were only used to assess the nuclear DNA amount.

<table>
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<tr>
<th>Species</th>
<th>Collection data and herbarium voucher</th>
<th>GenBank accession number ITSI, ITS2</th>
<th>GenBank accession number ETS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rhaphonticoides hogianus</strong> (Tzvelev) M. V. Ageb. &amp; Greuter</td>
<td>Armenia, Talin: between villages Pshirik and Bregavan, Susana 1587 et al. 26.VIII.1993 (BC).</td>
<td>AF041281</td>
<td>AF041263</td>
</tr>
<tr>
<td><strong>Rhaphonticoides iconomus</strong> (Hult.-Mor.) M. V. Ageb. &amp; Greuter</td>
<td>Turkey, Als Dog: track above the of Dermit Kakik. Ertugral 1761, 7.VIII. 2003 (BC).</td>
<td>AF041280</td>
<td>AF041256</td>
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<tr>
<td><strong>Chirolopus arboresus</strong> (Webb &amp; Berth.) Holub</td>
<td>Spain, Canary Islands, La Palma: San Andrés y Sauces, Susana 1425, 29.VII.1990 (BC).</td>
<td>AF021147, AF021164</td>
<td>AF041257</td>
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<tr>
<td><strong>Ch. arbutifolius</strong> (Svant.) Kurckel</td>
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<td><strong>Ch. burchardi</strong> Susanna</td>
<td>Spain, Canary Islands, Tenerife: between Buenavista and Teno, Susana 1420, 6.VII.1990 (BC).</td>
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<td>AF041250</td>
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<td><strong>Ch. canariensis</strong> (Brouss. ex Willd.) Holub</td>
<td>Spain, Canary Islands, Tenerife: Masca ravine, Garnatje 1 &amp; Luque, VIII. 1996 (BC).</td>
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<tr>
<td><strong>Ch. crassifolius</strong> (Bertoloni) Susanna</td>
<td>Malta: Botanical Garden of La Valetta</td>
<td>AF021157, AF021174</td>
<td>AF041252</td>
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<td><strong>Ch. falcatus</strong> Monel. &amp; Morol.</td>
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<td><strong>Ch. gomerythus</strong> (Svant.) Holub</td>
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<td>AF041259</td>
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<td><strong>Ch. gravisfolius</strong></td>
<td>Spain, Balearic Islands, Formentera: mount Sa Mola, Prexaven, VIII.1988 (BC). Balearic Islands, Formentera: La Mola Garnatje &amp; Vilatoversa V-411, 18.VI.2005 (BC)*</td>
<td>AF041282</td>
<td>AF041261</td>
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<tr>
<td><strong>Ch. humilis</strong> (Lam.) Dostál</td>
<td>Spain, Alacant: between Alcoy and Pego, km 40, near Benitarrux, Garcia-Jacas &amp; Susana 1249, 5.VIII.1988 (BC). Spain, Balearic Islands, Elvissa: Cala d’Alborca, Garnatje &amp; Vilatoversa V-409, 14.IV.2005 (BC)*</td>
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<td><strong>Ch. pannonicus</strong> (Svant.) Holub</td>
<td>Spain, Canary Islands, La Palma: Fuenclairente, volcano of San Antonio, Susana 1423, 28.VII.1990 (BC).</td>
<td>AF021148, AF021165</td>
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<td><strong>Ch. massonianus</strong> (Lowe) O. Eriksson &amp; al.</td>
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<td><strong>Ch. monticiculus</strong> (Font Quer) Susanna</td>
<td>Morocco, Tetouan: mount Tissolska above Chelifouhoun, Medoro, Rome 4617 &amp; Susana, 20.VI.1988 (BC).</td>
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<tr>
<td><strong>Ch. sempervirens</strong> (L.) Pomel</td>
<td>Portugal, Faro: 4 km from N of Monchique, Garcia-Jacas &amp; Susana 1218, 28.VII.1988 (BC).</td>
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<td>Holub</td>
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<td>Morocco, Agadir: 1 km S of the Tizi n'Test, Garcia-Jacas, Susanna 1399 &amp; Vallés, 11.VII.1990 (BC).</td>
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<td>Ch. tegidis (Ch. Smith)</td>
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<td>Spain, Canary Islands, Tenerife: El Teide, boca de tauce, Susanna 1429, 6.VII.1990 (BC).</td>
<td>AF021154, AF021171</td>
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<tr>
<td>Ch. tiliгиноsum (Brot.)</td>
<td>Dostál</td>
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Table 2. Nuclear DNA content and other characters of the populations studied.

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<tr>
<th>Species</th>
<th>2C ± SD (pg)</th>
<th>2C (Mbp)</th>
<th>Distribution</th>
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<tr>
<td>Cheetrologus arboresus</td>
<td>1.40 ± 0.02</td>
<td>1369.20</td>
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<td>Ch. semenivorec</td>
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<td>Ch. uligimius</td>
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