6. Molecular insights into the diversification of *Cheirolophus* (Asteraceae) in Macaronesia

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Abstract. The diversification of *Cheirolophus* in Macaronesian archipelagos constitutes a paradigmatic example of radiation on oceanic islands. Phylogenetic and molecular dating analyses indicate an extraordinarily fast process, showing one of the highest speciation rates ever found on plants from oceanic islands. Such radiation has been recently studied employing phylogeographic, population genetic and molecular cytogenetic approaches. Here, the main potential patterns and processes involved in the diversification of the genus in the Canary Islands and Madeira are reviewed and discussed as a whole.

Introduction

The observations of Darwin [1] and Wallace [2] about diversification processes on island biotas meant an outstanding contribution to the origins of

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modern evolutionary biology. From that moment, island radiations have become the object of numerous studies focusing on plants and animals from archipelagos all over the world [3, 4]. This interest on islands relies in the number of characteristics they possess, which make them attractive to researchers in the field –such as their relative small size, the existence of clear boundaries, the ecological simplicity compared to the continent, and the high diversity they usually harbour– making them natural laboratories where it is easier to observe, test and interpret general evolutionary patterns [5]. In recent times, Macaronesian archipelagos (Azores, Madeira, Canaries, Selvagens and Cape Verde, which constitute, together with a small nearby fraction of the African continent, the Macaronesian biogeographic region) and particularly the Canary Islands have become one of the favorite scenarios for researchers working on plant diversification processes (e.g. [6–9]). In this sense, many of the groups that have shown higher insular endemism levels -and therefore offering better possibilities to analyse island radiation processes- have been targeted (e.g. *Argyranthemum* Webb [10]; *Aeonium* Webb & Berthel. [11]; *Bystropogon* L.Hér. [12]; *Sideritis* L., [13]; *Sonchus* L. alliance [14]; *Echium* L. [15]; *Tolpis* Adans. [16]; *Pericallis* Webb & Berthel., [17]; *Cheirolophus* Cass. [18, 19]).

Among them all, in this review we will focus in the genus *Cheirolophus* Cass., whose diversification in the Canary Islands is considered as one of the top ten explosive plant radiations in this oceanic archipelago [20]. In addition to that, the Macaronesian representatives of this genus feature several typical traits of plants that have been able to diversify on insular environments, such as e.g. increasing woodiness [3], larger inflorescences and both showier flowers and inflorescences [21], reduction in genome size [7, 22] and small population size [23], thus making *Cheirolophus* a perfect model to study radiations on oceanic islands. Until recently, however, comprehensive understanding of the diversification of the genus in Macaronesia was missing. Based in previous phylogenetic reconstructions, it had been hypothesized that the radiation of *Cheirolophus* in the Canary Islands was a considerably fast and recent process [24, 25]. Unfortunately, those early studies lacked of a solid temporal frame, so it was neither possible to estimate speciation rate nor to establish comparisons with other well-known cases of explosive radiations in Macaronesia (e.g. [15]) or in other oceanic archipelagos (e.g. [26]).

Figure 1. Morphological and ecological diversity found on the genus *Cheirolophus* a) *Ch. arbutifolius* (Svent.) G.Kunkel., b) *Ch. burchardii* Susanna, c) *Ch. canariensis* (Willd.) Holub, d) *Ch. crassifolius* (Bertol.) Susanna, e) *Ch. falcisectus* Svent. ex Montelongo & Moraleda, f) *Ch. intybaceus* (Lam.) Dostál, g) *Ch. junonianus* (Svent.) Holub, h, i) Cliffs in Madeira with *Ch. massonianus* (Lowe) A.Hansen & Sunding, j) *Ch. tagananensis* (Svent.) Holub, k) *Ch. teydis* (C.Sm.) G.López, l) *Ch. uliginosus* (Brot.) Dostál. (Images: L. Barres, T. Garnatje, D. Vitales, http://commons.wikimedia.org).

The genus *Cheirolophus* has also been pointed out to represent a paradigmatic example of non-adaptive radiation on islands [4]. Indeed, most of the Macaronesian species of the genus exploit similar ecological niches, showing at the same time mostly inconspicuous morphological

differences. Nevertheless, Macaronesian *Cheirolophus* show as well a few cases of ecological adaptations to particular habitats (e.g. *Ch. teydis* from subalpine zone of Tenerife and La Palma; or *Ch. junonianus* from the southern arid part of La Palma island; see Figure 1). These and other questions that have been found to play an important role on island diversification -such as hybridisation and among population gene flow [10, 11, 17]; colonisation and dispersal patterns among islands [9, 27]; or reproduction biology factors [28, 29]– have been the subject of some recent studies focusing on *Cheirolophus* radiation [18, 19, 25, 30]. In this chapter, we will present different methodological approaches employed to disentangle the evolutionary history of this genus, discussing the results obtained from a holistic perspective.

1. Brief overview of the genus *Cheirolophus*

First of all, we must circumscribe *Cheirolophus* (Asteraceae, Cardueae, Centureineae) within a taxonomic and phylogenetic context. The genus was first described by Cassini [31] based on the segregation of a group of species formerly included in the genus *Centaurea* L. Afterwards, Boissier [32] included additional species to those previously segregated by Cassini, grouping them all in a new genus, *Ptosimopappus* Boiss., which comprised species from sections *Cheirolophus* Cass. and *Microlophus* (Cass.) DC., that had been described within the genus *Centaurea*. Moreover, this author proposed the inclusion of *Centaurea arguta* Nees and *Centaurea uliginosa* Brot. to this new genus, describing as well some new species such as *Ptosimopappus bracteatus* Boiss. and *Ptosimopappus arboreus* Boiss. [33].

These taxonomic reorganisations have been in some cases conflicting. In fact, while authors like Pomel [34], Holub [35], Dostál [36], or Bremer [37] continued considering *Cheirolophus* as an independent genus, Dittrich [38] or Talavera [39], among others, preferred maintaining it as a section of *Centaurea*. Nonetheless, the most recent revisions of the Cardueae tribe based on molecular phylogenetic data (e.g.[40, 41]), clearly supported the segregation of *Cheirolophus* as an independent taxonomic entity, revealing the position of the genus as a basal lineage within subtribe Centaureinae.

This genus contains approximately 27-30 species, depending on the authors consulted, distributed along the western Mediterranean basin as far as Malta, and the Macaronesian archipelagos of Madeira and Canary Islands (Figure 2). Some of the species have a wide distribution, such as *Ch. intybaceus* (Lam.) Dostál or and *Ch. sempervirens* Pomel (Fig. 2).
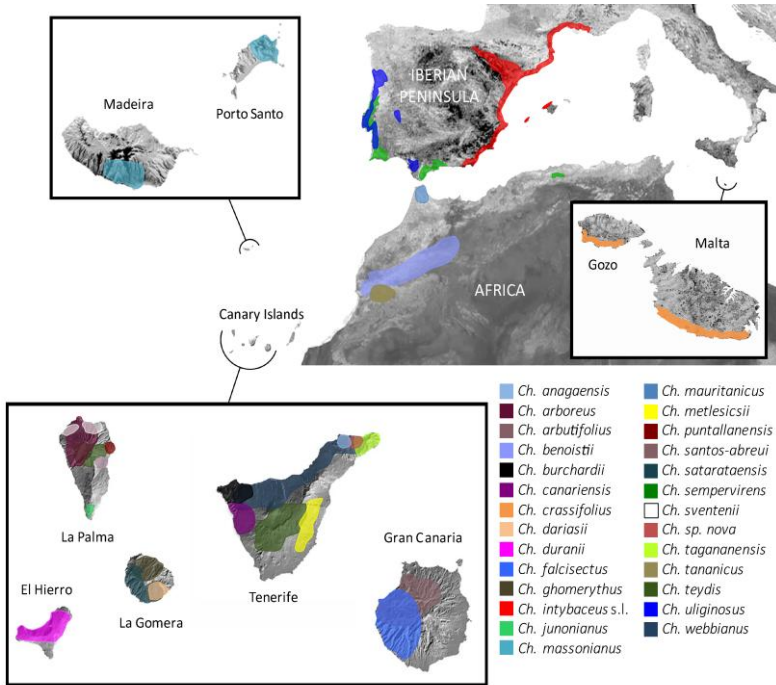
Figure 2. Approximate geographical distribution of the genus *Cheirolophus* on Western Mediterranean, North Africa and Macaronesia.

Others, however, are more restricted geographically, and limited in many cases to very few populations, such as *Ch. duranii* (Burchard) Holub from El Hierro island or *Ch. tagananensis* (Svent.) Holub from Anaga peninsula in the northern part of Tenerife (Fig. 2). Indeed, most of the endemic *Cheirolophus* species from Macaronesia present extremely restricted distributions. To date, 20 species, one subspecies and one variety have been described in the Canarian archipelago [42], plus another species endemic to the islands of Madeira and Porto Santo. The low number of populations as well as the small population size reported for some of these species was a key factor for many of them to be included in different national and international red lists of endangered flora (e.g. Libro Rojo de la Flora Vascular Amenazada de España [43]; or the IUCN Red List [44]).

## 2. Molecular phylogenetic studies in *Cheirolophus*

The pioneering molecular systematic studies in *Cheirolophus* employed isoenzyme electrophoresis technique to analyse the genetic variability among populations of different species [45, 46]. This methodological approach provided little resolution on the evolutionary relationships among species, but served to propose for the first time a recent origin for the diversification of the Macaronesian members of the genus. Sometime later, a phylogenetic reconstruction based on the internal transcript spacer (ITS) of the nrDNA allowed a taxonomic delimitation of *Cheirolophus*, including the species *Ch. crassifolius* (Bertol.) Susanna and confirming the monophyly of the Macaronesian grade [24]. This question was also readdressed afterwards with a combined approach consisting on genome size data and sequencing of both the ITS and external transcript spacer (ETS) regions [25]. As mentioned above, this study indentified *Ch. crassifolius* as the sister species to the rest of the members and evidenced the existence of two main lineages, the Macaronesian and the Mediterranean clades. Unfortunately, none of these works were able to accurately reconstruct the evolutionary history of *Cheirolophus*, and the interspecific relationships within the Macaronesian group remained particularly unresolved.

A recent reconstruction of the evolutionary history of the genus [18] has, however, provided a relevant contribution to former phylogenetic works. This new study has been based on the analysis of two nrDNA regions (ITS and ETS) as well as four cpDNA regions (*rpl*32-*trn*L, *rpo*B-*trn*D, *rps*16-*trn*K and *trn*S-*trn*C), to analyse the whole specific and virtually all infraspecific diversity within the genus.

The results showed a significant phylogenetic incongruence among nuclear and plastid markers, a pattern usually explained by phenomena like incomplete lineage sorting (ILS), hybridisation or polyploidy, among others [47]. Incongruent position of species within the Mediterranean clade was explained by the slow evolutionary rate of plastid markers, preventing us from tracking accurately the speciation events in this group. A different hypothesis was proposed for the conflicting position of *Ch. massonianus* (Lowe) A.Hansen & Sunding between the plastid and nuclear trees. In this case, the placement of this Madeiran endemic in each phylogenetic reconstruction is so discordant that phenomena related to sharing alleles were discarded. Moreover, *Ch. massonianus* shows an intermediate genome size (though not exclusive) half way between that of the continental and the remaining insular species [25], hence constituting another evidence of potential hybridisation in this species.

Regardless of this incongruence, both markers resulted useful to reconstruct the phylogeny when analysed independently (see Vitales *et al*. [18]). As it had been reported in former studies (e.g. [48]), whereas nuclear DNA provided resolution to the backbone of the phylogeny as well as for generating a temporal frame for the evolutionary history, plastid markers resulted more helpful unravelling the phylogeographic relationships among closely related species. Both datasets supported the monophyly of the genus and the existence of a well-differentiated insular clade. However, as a result of the potential hybridisation involving *Ch. massonianus*, the nuclear tree included the endemic species from Madeira within the insular clade, while the sampling on the plastid tree was restricted to Canarian species. The reconstruction based on ITS and ETS markers also resolved the Mediterranean clade, reflecting morphological and geographical affinities among the species of this group. *Cheirolophus crassifolius*, endemic to Malta and Gozo Islands, appeared in both analyses as an early-diverged lineage, and sister to the rest of the members of the genus. In relation to *Ch. uliginosus*, the only hemicryptophyte representative, this species is also placed in a basal position relative to the diversification of the species within the Mediterranean and the Macaronesian clades - although not entirely resolved- according to both nuclear and plastid datasets.

As already mentioned above, the analysis of the nuclear regions was particularly useful for studying the early stages of the evolutionary history of *Cheirolophus*, as well as to establish a time frame for the phylogeny (Figure 3). The origin of the diversification of the genus was dated to the mid-Miocene period. At that time, the Mediterranean basin still featured tropical climate characteristics, but a progressive aridification starting in the east around 11-9 Ma [49] might had pushed *Cheirolophus* westwards, explaining its current Western Mediterranean and Macaronesian distribution. Concerning the radiation of the genus in Macaronesia, the time-calibrated phylogeny indicated that *Cheirolophus* diversified rapidly, with c. 20 species arising in less than 1.8 Ma, at a rate of 0.34–2.84 species per Ma. Such high speciation rate is only comparable to those exhibited by other island radiations such as Hawaiian *Bidens* L. (0.3–2.3 species Myr^{-1}) or Macaronesian *Echium* (0.4–1.5 species Myr^{-1}), considered as the fastest plant radiations on volcanic islands to date [26]. Indeed, taking into account the area covered by both the Canary Islands and Madeira (8,321 km^2), Macaronesian *Cheirolophus* may well represent the highest per-unit-area rate of diversification (4.09×10^{-5} to 3.41×10^{-4} species $Myr^{-1} km^{-2}$) observed so far in plants [26, 50, 51].
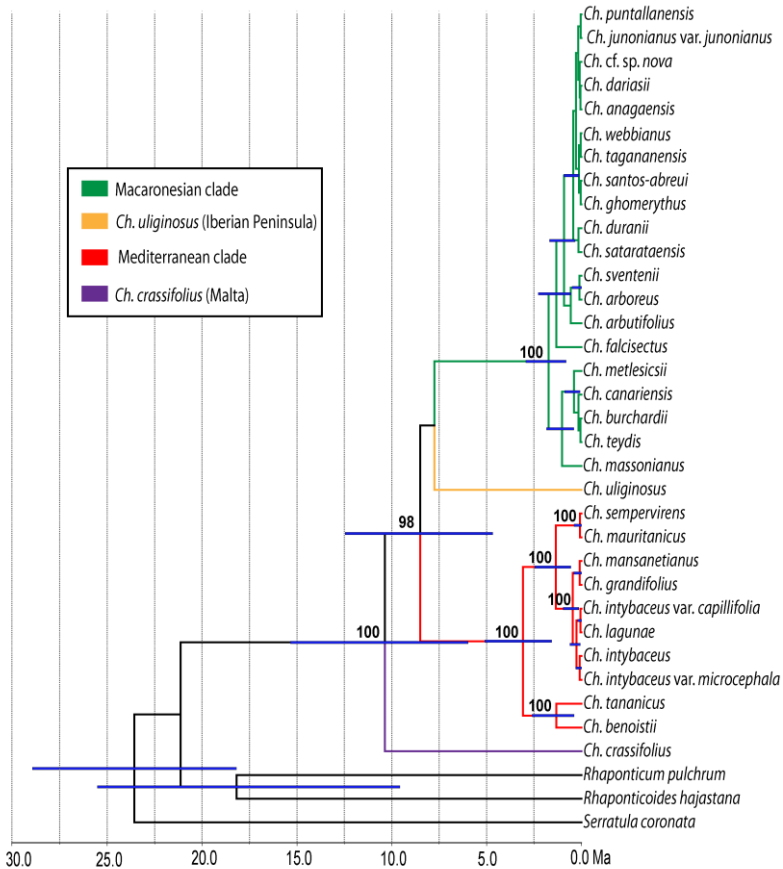
Figure 3. Time-calibrated Bayesian phylogenetic tree inferred from nuclear (ITS and ETS) DNA sequences. Bayesian posterior probabilities (PP) above 95% are indicated above main interesting branches. Blue bars indicate 95% high posterior density intervals (for nodes with posterior probability > 50%) of node ages.

3. Phylogeography of Macaronesian *Cheirolophus*

Having provided strong evidence explaining the radiation process, the question arose as to which mechanisms were underlying such rapid diversification. Another point to further investigate was the role played by the phylogeographic history of Macaronesian *Cheirolophus* in such rapid

diversification. The phylogeographic analyses performed by Vitales *et al*. [18] highlighted Tenerife island as the most likely source area of inter-island dispersal, with a pivoting role in successive colonisations towards the East (from Tenerife to Gran Canaria) and towards the West (from Tenerife to La Gomera and la Palma, on the one hand and towards La Gomera and El Hierro, on the other hand). During this process, the analyses suggested that La Gomera could have been colonised twice. These results are consistent with other phylogeographic studies focusing on the Canary Islands (e.g. [52–54]), which propose that this central island could had served as a major hub for the colonisation of the archipelago. The ancient geological history of this island [55], together with its central position in the archipelago and the high diversity of habitats, probably explains this central phylogeographic role of Tenerife. Moreover, Tenerife harbours the highest genetic diversity for *Cheirolophus* in the archipelago, as observed also in other genera in the Canarian area such as *Bystropogon* [12], *Sideritis* [13] or *Aeonium* [11]. The higher genetic and taxonomic diversity levels found in Tenerife have also been attributed to the complex palaeogeographic history of this island [55]. Successive fragmentation and connexion process among the habitats due to major climatic and geologic events affecting Tenerife during this period may have contributed to the allopatric differentiation among populations.

The largest islands -particularly Tenerife and La Palma- experienced several cases of intra-island diversification, probably driven by genetic isolation, but also due to different processes such as incipient ecological adaptation or introgression events. This model of radiation has been proposed to be common in other plants and animals that diversified -more or less- in the Canary Islands (see Sanmartín *et al*. [9] for a review). Certainly, these typical patterns of colonization, dispersal and differentiation experienced by *Cheirolophus* cannot explain on their own the extraordinarily rapid radiation occurred in Macaronesia, so other intrinsic or extrinsic factors must have contributed to this spectacular process of island diversification.

4. Genomic insights in *Cheirolophus*

Several authors have recently proposed that certain genomic factors such as the genome size [7, 22, 56] or the number of nrDNA loci [57] could be related to the process of diversification on islands. In *Cheirolophus*, this topic has been the subject of different studies [25, 30], addressing genome size, karyological and molecular cytogenetic aspects of some Macaronesian and continental species of the genus.

Cheirolophus is the only genus within the Centaureinae that has radiated in the Canary Islands. Similarly to other closely related genera (*Callicephalus* C.A.Mey, *Myopordon* Boiss., *Oligochaeta* K.Koch, *Rhaponticum* Ludw. and *Centaurea*, see Hidalgo *et al.* [58, 59] for further details), the genus displays the 35S and the 5S nrDNA loci physically separated in the chromosomes. By contrast, while those phylogenetically related genera contain a relatively low number of 35S loci [60], fluorescent *in situ* hybridisation (FISH) analyses in *Cheirolophus* revealed a strikingly high number of chromomycin bands and 35S loci, predominantly located at terminal position. Likewise, a certain trend towards an increasing number of 35S loci in Macaronesian species was observed, preliminarily suggesting that this unusual number of loci appeared during island radiation process. However, other continental species of the genus such as *Ch. benoistii* (Humbert) Holub or *Ch. intybaceus* showed as well a high number of 35S signals, indicating that the abundance of terminal 35S predated the radiation in Macaronesia. Finally, Garnatje *et al.* [30] hypothesised a positive effect of the 35S loci pattern promoting the radiation of *Cheirolophus*.

In a previous study focusing on genome size variation within the genus *Cheirolophus,* Garnatje *et al.* [25] evidenced a significant progressive genome downsizing since early stages of its evolutionary history, and particularly noticeable within the Macaronesian clade. Some evolutionary mechanisms such as homologous recombination and illegitimate recombination (see Leitch and Leitch [61], for a review) have been proposed to be able to affect both genome size and rDNA loci distribution. In *Oligochaeta divaricata* K.Koch (another species included within basal Centaureinae and closely related to *Cheirolophus*), a deep chromosomal restructuring process resulted on a significant loss of DNA associated to an increase of 35S loci and the reorganisation of their position in the chromosomes. It should be noted, however, that terminal 35S positions in *Cheirolophus* were not affected by genome size reduction. In summary, despite that *Cheirolophus* radiation was not associated to changes in chromosome number or ploidy level, the patterns of rDNA loci distribution and the reduction of the DNA content evidence certain capacity for genomic dynamism in the group. Indeed, the association among genomic size changes, nrDNA organisation and cladogenesis has been recently discussed by several authors (e.g. [62, 63]), but the precise putative role played by these mechanisms on *Cheirolophus* radiation will require further investigation.

5. Population genetics study in Macaronesian *Cheirolophus*

As stated earlier, the phylogenetic study of *Cheirolophus* based on nrDNA and cpDNA sequences [18] provided valuable information about the early evolutionary history of the genus. However, the phylogenetic resolution within the Macaronesian lineage was poor due to the rapidity of the diversification process and the limited variability of the employed markers. For that reason, the radiation of *Cheirolophus* in the Canary Islands and Madeira was subsequently investigated using a population genetics approach with AFLP markers [19].

First, this methodology was employed to study the taxonomic delimitation within the Macaronesian species of the genus, a subject that had been already under discussion in earlier studies focusing on *Cheirolophus* evolution [45]. The phylogenetic results obtained from AFLP data provided full support to the current taxonomic species' circumscription. Thus, our results corroborate the distinctiveness of these extraordinarily recently diverged species and support the suitability of classical diagnostic characters employed in the taxonomical delimitation of Macaronesian *Cheirolophus*.

The phylogenetic relationships among the Macaronesian species were not entirely reconstructed with the AFLP data, but the diverse analytical approaches proved useful to better understand the evolutionary history of these insular lineages. The genetic structure of populations showed a significant segregation pattern among western islands (La Palma and El Hierro) and central/eastern islands (La Gomera, Tenerife, Gran Canaria). The important role played by allopatric differentiation became even more evident when considering additional genetic clusters: most of the populations grouped geographically, either among the islands or within the islands. Here again, the genetic structure found in Tenerife populations was particularly interesting, already reported in other plant groups [12], and suggesting a lineage disjunction related to the ancient palaeoislands of Anaga and Teno [64].

Despite the limited population sampling, the study carried out by Vitales *et al*. [19] also suggested a reduced gene flow among the Macaronesian populations. The strong signal of isolation-by-distance, the low within-population heterozygosity and the high values of the fragmentation indexes indicated a limited genetic flow among the populations. These results agreed with the low dispersal capacity of *Cheirolophus* seeds, the geographic isolation of populations and their small size, possibly contributing to their progressive genetic differentiation. In contrast, the phylogeographic pattern observed in this group -including numerous colonisation and recolonisation events, both intra- and inter-island

suggest that Macaronesian *Cheirolophus* showed a considerable ability for sporadic long distance dispersal (see Crawford *et al*. [28] for some hypotheses). In this way, authors such as Ellis *et al*. [65] or Knope *et al*. [26] have proposed that the combination of reduced gene flow and certain ability for long distance dispersal may play an important role on radiation processes experienced by plants.

As mentioned in the introduction section, the Macaronesian *Cheirolophus* have been proposed as an example of non-adaptive radiation on islands [4]. In this regard, the correlation analyses among morphological and genetic distances performed by Vitales *et al*. [19] indicated that there is not a straightforward association among genetic lineages and the ecomorphological traits studied in these species. Clearly, the data and the methods employed in this study are preliminary and somewhat insufficient to discard an essential role of adaptive selection in the radiation of this group. However, these results suggested that ecological adaptation did not drive the initial stages of Macaronesian *Cheirolophus* diversification. The examples of adaptations to specific ecological conditions found in some species (e.g. *Ch. junonianus* from the southern extreme of La Palma, or *Ch teydis* from the subalpine zone of Tenerife and La Palma) seem to correspond with relatively recent and independent processes of ecomorphological differentiation.

Another mechanism potentially playing an important role in the evolutionary history of Macaronesian *Cheirolophus* is introgression [18]. We have already discussed the case of *Ch. massonianus*, putatively experiencing a chloroplast capture process from a continental taxon. In addition, our analyses also suggested some cases of genetic introgression between several species from the Canary Islands. Particularly, some evidences of genetic admixture were found in *Ch. teydis* and *Ch. arboreus* from La Palma, and *Ch. duranii* from El Hierro. In some cases these genetic traces seemed supported by morphological data and/or heteromorphic positions found in the nrDNA regions of these species. That said, one should bear in mind that some of these signals could also be explained by retention of ancestral polymorphisms or incomplete lineage sorting (ILS) phenomena, especially considering the speed of the radiation. Therefore, the relative importance of genetic introgression in the radiation of Macaronesian *Cheirolophus* should be further studied more in detail.

6. Conclusion

Recent molecular phylogenetic studies have shown that the radiation of *Cheirolophus* in Macaronesia was an extraordinarily recent and rapid process.

Phylogeographic analyses indicated that Tenerife Island played an important role in this explosive diversification that, according to our data, could have been driven by allopatric differentiation, incipient ecological adaptation and introgression events. Molecular cytogenetic studies have revealed that *Cheirolophus* has undergone a significant increase in the number of 35S rDNA loci, which started just after the diversification of the genus in the Mediterranean region. This pattern contrasts with the gradual genome downsizing observed during the evolution of the genus, and evidences a certain genomic dynamism in the genus, probably related with the ability to radiate on islands. Finally, a population genetic approach suggested that the combination of poor gene flow capacity and a certain ability for sporadic long-distance colonization could have also played an important role enhancing the explosive diversification of this genus in Macaronesia.

Acknowledgements

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