# Cytogenetic insights into an oceanic island radiation: the dramatic evolution of pre-existing traits in *Cheirolophus* (Asteraceae, Cardueae-Centaureinae)

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Running head: Cytogenetic insights into an oceanic island radiation: a case study in *Cheirolophus*

**Abstract** The genus *Cheirolophus* constitutes one of the most striking cases of species radiation in Macaronesia, where it diversified into a lineage of ca. 20 endemic species at a rate that is amongst the fastest reported for oceanic islands. Whilst the cytogenetic dynamics of many of the Macaronesian *Cheirolophus* species have been comparatively well studied, an overall vision of chromosome and genome evolution has been hampered by the lack of data for the earliest-diverging species, *Ch. crassifolius*. In this study, we have completed the cytogenetic survey of *Cheirolophus* to investigate how different cytogenetic traits may have contributed to the dramatic radiation of the genus in Macaronesia. We provide new cytogenetic data (i.e. chromosome counts, genome size estimates and physical mapping of 35S rDNA loci) for several key species, including *Ch. crassifolius*, and then model trait evolution within a phylogenetic context. Our results reveal a trend of genome downsizing accompanied by a dramatic increase in number of 35S rDNA loci which started early in the evolutionary history of the genus, before its radiation in Macaronesia. It is notable that the increasing number of 35S rDNA loci has not been driven by polyploidisation, in contrast to the more typical trend observed in many angiosperms. In addition, the number of 35S rDNA loci was observed to negatively correlate with genome size, which is also very unusual in angiosperms. It is suggested that non-homologous and unequal homologous recombination are the most likely mechanisms to explain these observations and we discuss whether the unique genomic architectures of *Cheirolophus* could have predisposed the genus to its successful and rapid speciation in Macaronesia.

**KEYWORDS:** C-value, chromosome number, genomic trait evolution, oceanic island radiation, rDNA loci, speciation

## INTRODUCTION

The genus *Cheirolophus* Cass. (Asteraceae, subtribe Centaureinae) is notable for its remarkably rapid species radiation in Macaronesia (Canary Islands and Madeira). Following just one colonisation event [most likely from the Iberian Peninsula 1.7 million years ago (Mya)], its explosive diversification into c. 20 endemic species is considered to represent the fastest oceanic island species radiation so far documented (Vitales & al., 2014b). In contrast to this, only nine *Cheirolophus* species are known from the continent and continental islands (Balearic and Maltese) in the Western Mediterranean region (see Appendix).

Within subtribe Centaureinae (32 genera, c. 600 sp.; Susanna & Garcia-Jacas, 2007), *Cheirolophus* (29 sp.; see Appendix) is a successful genus in terms of species diversity. The genus is mostly made up of shrubby perennials [except the hemicryptophyte *Ch. uliginosus* (Brot.) Dostál] which show a tendency towards increased height and woodiness in Macaronesia. The enhanced arborescence and appearance of inflorescences arranged in a candelabrum-like structure probably evolved in the archipelagos as a result of secondary environmental adaptations. Shrubs –and particularly arborescent shrubs– are generally rare in the Centaureinae, and typically constitute a habit that evolved secondarily and appears phylogenetically scattered across the subtribe (e.g. *Centaurodendron* Johow, *Centaurothamnus* Wagenitz & Dittrich, *Ochrocephala* Dittrich and *Centaurea ptosimopappa* Hayek; Hidalgo & al., 2006). *Cheirolophus* therefore represents an exception within the subtribe as it combines, in Macaronesia, a treelet shrubby habit and high species diversity. Whether the pre-existing woody nature of *Cheirolophus* promoted its radiation following the colonisation of Macaronesia remains to be demonstrated. Nevertheless, it has been

68 noted that besides an increase in plant size and woodiness, the *Cheirolophus* radiation
69 has been characterised by only moderate morphological divergence (Susanna & al.,
70 1999). Such observations are certainly consistent with current theory suggesting that
71 geographic isolation and long-distance dispersal are the main forces driving
72 diversification in the genus, while ecological adaptation, usually related to adaptive
73 radiation with high levels of morphological divergence, has played only a secondary
74 role in the process (Vitales & al., 2014a).

75 Early studies considered *Cheirolophus* as part of the Tertiary circum-
76 Mediterranean stock that gave rise to the Canarian flora (Bramwell, 1976). However,
77 this assumption was challenged by an isozyme analysis that provided convincing
78 evidence for a younger age of *Cheirolophus* species (Garnatje & al., 1998), although
79 that study did not include *Ch. crassifolius* (Bertol.) Susanna. The most recent
80 phylogenetic data (Vitales & al., 2014b) have accommodated both theories by
81 establishing a Mid-late Miocene origin for *Ch. crassifolius* (ca. 10.4 Mya), while
82 demonstrating that the westward expansion of the genus towards the Mediterranean
83 basin (ca. 3.08 Mya) and its subsequent Macaronesian diversification (ca. 1.7 Mya) are
84 more recent processes (i.e. Late Pliocene – Early/Mid Pleistocene). Nevertheless, there
85 are still unresolved issues. For example, the enigmatic Iberian endemic *Ch. uliginosus*
86 appears phylogenetically isolated in an unresolved trichotomy with the Macaronesian
87 and Mediterannean clades, while the identity of the species or even the lineage, which
88 colonised Macaronesia remains unclear despite weak phylogenetic signal suggesting
89 that this took place from Iberia rather than Africa (Vitales & al., 2014a).

90 Whether certain traits belong to the so-called ‘background variables’ that
91 provide the right conditions for radiations to start, act as ‘triggers’ or ‘modulators’ in the
92 radiation process (Bouchenak-Khelladi & al., 2015), or are unrelated to the radiation,

has to be evaluated within a phylogenetic framework documenting the history of lineage diversification. Certainly such an approach has been successfully used to evaluate the processes underpinning changes in morphology in many radiating lineages (e.g. the evolution of woodiness, *Aeonium* Webb & Berthel., Mort & al., 2002; *Echium* L., García-Maroto & al., 2009; *Sonchus* L., Kim, 2012). However, other aspects of plant evolution, including the role of genomic (including cytogenetic) traits are also starting to receive increased attention, and these are contributing significant and valuable new insights into plant radiations (e.g. *Pachycladon* Hook.f., Mandakova & al., 2010; *Schiedea* Cham. & Schltdl., Kapralov & al., 2009; Kapralov & Filatov, 2011). *Cheirolophus* is the only genus of subtribe Centaureinae to have undergone a radiation in Macaronesia. It has already received considerable attention in this respect given the exceptionally high and variable number of 35S ribosomal DNA (rDNA) loci (which is unique among Centaureinae) (Garnatje & al., 2012) despite all species being considered diploid with $2n = 30(32)$. Such a conserved chromosome number is in line with the theory of chromosomal stasis during oceanic island speciation (Stuessy & Crawford, 1998). These reported chromosome numbers for the genus are common amongst early-diverging Centaureinae (Hellwig, 2004), and likely arose through ancient whole-genome duplications that predated the emergence of the subtribe itself (Huang & al., 2016). Polyploidy and to a lesser extent paleopolyploidy are seen as mechanisms that can increase the genetic diversity of colonisers and enhance lineage diversification in oceanic islands (Crawford & al., 2009). In this sense, while the Canary Islands stand out by the paucity of polyploidy, *Cheirolophus* better fits the overall trend (Crawford & al., 2009). Other genomic studies have shown that Macaronesian *Cheirolophus* possesses smaller genomes than their continental counterparts (Garnatje & al., 2007), a trend that has also been observed at the level of the whole Macaronesian flora (Suda & al., 2003;

2005). Nevertheless, despite these studies, an overall vision of island speciation in *Cheirolophus* has been hampered by the lack of data for the earliest-diverging species, *Ch. crassifolius*, which, together with the unresolved phylogenetic positioning of *Ch. uliginosus*, has impeded the reconstruction of ancestral states. Here we use the most recent phylogenetic framework available for the genus (Vitales & al., 2014b), together with an extensive survey of nuclear DNA contents and physical mapping of 35S rDNA loci distribution (including key species, such as *Ch. crassifolius*), to provide the most comprehensive analysis, to date, of genomic trait evolution in the course of the oceanic radiation of *Cheirolophus*.

## MATERIALS AND METHODS

**Plant materials. —** Table 1 contains the provenance of *Cheirolophus* populations from which we were able to obtain samples plus the herbaria where corresponding vouchers are deposited. Leaves and root tips were either collected in the field or obtained from individuals grown from cypselae collected in the field. The only exception was *Ch. crassifolius* that was provided by the Orto Botanico di Palermo (Università degli Studi di Palermo, Sicily, Italy), where plants from Malta are cultivated. Previously published karyological, cytogenetic and genome size (GS) data available for *Cheirolophus* and used in subsequent analyses have been collated (Appendix).

**Preparation of chromosomes for counts, fluorochrome banding and fluorescent *in situ* hybridisation (FISH). —** Root tip meristems were pretreated with 0.05% (w/v) aqueous colchicine for 2.5–3 h at room temperature, fixed in 3:1 (v/v) absolute ethanol/glacial acetic acid for 24 h, transferred to 70 % ethanol and stored at -4 °C. For chromosome counts, fixed root tips were hydrolysed in 1 M hydrochloric acid

at 60 °C for 7-9 min, rinsed in water and stained with Schiff's reagent for 30 min. Meristems were subsequently excised and squashed in a drop of 2% (w/v) aceto-orcein for microscopic observations.

Protoplasts were prepared with the air-drying technique of Geber & Hasibeder (1980) modified as follows: root tips were washed in citrate buffer (0.01 M citric acid-sodium citrate pH = 4.6) for 10 min at room temperature and further incubated at 37 °C for 30 min in an enzyme mixture [4% (w/v) cellulase Onozuka R-10 (Yakult Honsha Co. Tokyo, Japan), 1% (w/v) pectolyase Y-23 (Seishin Co. Tokyo, Japan) and 4% (w/v) hemicellulase (Sigma-Aldrich, Paris, France)] diluted to 50% in citrate buffer. Digested meristems were excised, placed on a slide, washed in citrate buffer and spread with a drop of 3:1 (v/v) absolute ethanol/glacial acetic acid. The slides were subsequently air-dried.

**Fluorochrome banding with chromomycin A3 (CMA) and fluorescent *in situ* hybridisation (FISH). —** We followed the protocols of Schweizer (1976) and Cerbah & al. (1995) for CMA banding (to preferentially stain GC-rich DNA), and Heslop-Harrison & al. (1991) and Cerbah & al. (1998) for FISH experiments (to localise the 35S rDNA), with the modifications described in Garnatje & al. (2004). The 35S rDNA probe comprised a 4 kb *Eco*RI fragment that includes the 18S-5.8S-26S rDNA sequences from *Arabidopsis thaliana* (L.) Heynh. It was directly labelled with the Cy3 fluorochrome (Amersham, Courtaboeuf, France) by nick translation, according to the manufacturer's protocol.

**DNA content assessments. —** Nuclear DNA contents were estimated by propidium iodide (PI) flow cytometry using the internal standard *Petunia ×hybrida* (Hook.) Vilm. 'PxPc6' (2C = 2.85 pg; Marie & Brown, 1993). Seeds of the standard were provided by the Institut des Sciences du Végétal, Gif-sur-Yvette (France). Leaf

tissue of *Cheirolophus* and the internal standard were co-chopped in 600 µl of LB01 isolation buffer (Doležel & al., 1989) with a razor blade and supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). Samples were filtered through a 70 µm pore size nylon mesh and subsequently stained with PI to a final concentration of 60 µg/ml (Sigma-Aldrich Química), kept on ice for 20 min and measured in an Epics XL flow cytometer (Coulter Corporation). Whenever possible, five specimens per population were processed, and two independent samples were prepared per individual. Further technical details on the procedure can be found in Garnatje & al. (2007). Measurements were carried out at the Centres Científics i Tecnològics of the Universitat de Barcelona.

**Ancestral character state reconstructions. —** From the phylogenetic inferences conducted by Vitales & al. (2014b), we used the trees resulting from the Bayesian inference of the nuclear (ITS+ETS) dataset since it provides > 10-fold more variable sites than the combined cpDNA data, and produces a more robust phylogenetic backbone for *Cheirolophus*. In contrast, the phylogenetic trees inferred using the set of cp markers segregate conspecific samples into different clades and unexpectedly place *Ch. massonianus* (a species from Macaronesia) amongst the early-diverged species of the genus. Vitales & al. (2014b) considered that such conflicting relationships were most likely due to hybridisation and chloroplast capture processes. The results presented in the text are therefore all based on the nuclear dataset. However, in our preliminary analyses of the data, we explored the use of trees resulting from the cpDNA data to reconstruct ancestral GS (see methods below and results in the Supplementary file). Two samples of 1000 post burn-in trees from nuclear and plastid datasets were generated to reconstruct the ancestral GS using BayesTraits v.2 (http://www.evolution.rdg.ac.uk/BayesTraits.html). Genome size values (2C) were

boxcox transformed with a lambda setting of -6.61 in order to achieve a normal distribution of the data prior to further analysis (Kolmogorov-Smirnov test, P = 0.654). The best fitting model for analysis of continuous characters (i.e. random walk vs. directional) was selected by running a BayesFactor test using the logarithm of the harmonic mean estimated from five independent runs under the MCMC option. The settings used were as follows: sampling every 1000 generations, iterations = 100 x $10^6$, burn-in = 10 x $10^6$ iterations, scaling parameters estimated = delta ($\delta$), kappa ($\kappa$) and lambda ($\lambda$). Parameter values were inspected with Tracer v.1.5 (Rambaut & Drummond, 2007) to ensure they had reached a stationarity. The random walk model was supported in most of the runs, and the posterior distributions of the scaling parameters generated were used as the model-settings for the second phase of the analysis where the GSs at specific nodes were estimated using the addMRCA (most recent common ancestor) command. Alternatively, ancestral GSs were also reconstructed using maximum parsimony (MP) for continuous traits in Mesquite v.3.04 software (Maddison & Maddison, 2015).

We also performed analyses to infer the ancestral number of 35S rDNA loci and of 2*n* chromosome number in Mesquite v.3.04 under MP as implemented for meristic characters, with multiple entries per cell to accommodate polymorphism of 35S rDNA loci of *Ch. uliginosus*. These analyses used the consensus phylogenetic tree obtained from the BEAST analysis of Vitales & al. (2014b), pruned with BayesTrees v.1.3 (Meade, 2011) to the same set of species used to infer the ancestral GS. Since the closest relatives of *Cheirolophus* within Centaureinae are still unknown, we attributed missing values to the outgroup species. Inferences of ancestral chromosome numbers were based on using counts verified in the present study.

**Statistical analyses. —** We conducted principal component analyses (PCA) using the *prcomp* function in the *stats* package of R v.3.2.2 (R Core Team, 2016) on log-transformed and standardised data to investigate the distribution of *Cheirolophus* species within the total karyological-cytogenetic variation of the genus, the Centaureinae, the Asteraceae and the angiosperms as a whole. Results of the PCAs were visualised with the *ggbiplot* function (https://github.com/vqv/ggbiplot). The data for GSs, chromosome and rDNA loci numbers across Angiosperms were retrieved from the databases of Garcia & al. (2012; 2014) and the present study. This dataset was also used to plot GS against 35S rDNA loci number. To address trait correlation while taking into account phylogenetic relatedness, we conducted phylogenetic generalised least squares analysis (PGLS) under a Brownian motion model of evolution with the *ape* and *nlme* packages of R (Paradis & al., 2004; Pinheiro & al., 2015). The tree used was the ultrametric consensus tree from the BEAST analysis of Vitales & al. (2014b) based on the nuclear DNA dataset but reduced to the set of species with available GS and 35S rDNA loci data. *Cheirolophus santos-abreui* A.Santos was removed from the sample after examination of the residuals using ordinary least squares with a normal QQ plot.

## RESULTS

**Chromosome numbers. —** Somatic chromosome counts made with the Feulgen squash technique (Fig. 1) and protoplast preparations (Fig. 2) from this and a previous study (Garnatje & al., 2012) were compiled with data from the literature, resulting in chromosome numbers for 22 of the 29 *Cheirolophus* species currently recognised (Appendix; Watanabe, 2002; 2004). Our data include first counts for four species [*Ch. burchardii* A.Susanna, *Ch. duranii* (Burchard) Holub, *Ch. puntallanensis* A.Santos and *Ch. santos-abreui*] and one taxon whose status as a new species is currently under

consideration [*Ch.* cf. *webbianus* (Sch.Bip.) Holub; A. Santos personal communication; Appendix]. Reassessments of several previous counts, especially records of $2n = 30$ that were corrected to $2n = 32$, suggest that $2n = 32$ is more common in *Cheirolophus* than previously thought, although a number of records still remain to be confirmed (Fig. 3, Appendix).

**GC-rich heterochromatin-rich regions and 35S rDNA loci. —** Results from the CMA fluorochrome banding, staining GC-rich heterochromatin, and FISH for physical mapping of 35S rDNA loci (10 populations of nine species analysed) are presented in Fig. 2 and the Appendix. The number of rDNA loci ranges from four in the early diverging *Ch. crassifolius* to 10, found in both continental and in several Macaronesian endemics (Figs. 2 & 3). These results, together with those obtained by Garnatje & al. (2012), are summarised in Fig. 3A which shows the number of 35S loci for each species superimposed on the branches of the phylogenetic tree.

**Genome size. —** New nuclear DNA contents data were obtained for 11 species (Table 2). Overall, available values for the genus show moderate diversity with a 1.35-fold difference between the smallest (*Cheirolophus duranii*; 2C = 1.33 pg) and largest (*Ch. crassifolius*; 2C = 1.80 pg; Appendix) genome sizes.

**Ancestral characters. —** The ancestral GSs inferred for *Cheirolophus* were very similar regardless of the method used, i.e. 2C = 1.65 pg (parsimony) or 2C = 1.59 pg for the same node (Bayesian; Table 3). While the ancestral GS values inferred for the deeper nodes (e.g. MRCA of the genus and the major Mediterranean and Macaronesia clades) were largely consistent when using the phylogenetic trees derived from nuclear vs. chloroplast DNA (see Supplementary file), the low level of resolution in the phylogenetic trees prevented us from comparing the ancestral GS inferred for the more derived clades, and hence they are not discussed further.

Insights into the evolution of GS using Bayesian approaches showed that the likelihood score for a random-walk model was significantly greater than for the directional model. Overall, the analysis supported a general trend of decreasing GS during the evolution of the genus (Fig. 3A). The lambda ($\lambda$) value of 0.87 (close to 1) indicated that phylogenetic relationships notably contributed to the observed pattern of GS variation, while the kappa ($\kappa$) value of 2.61 (> 1) suggested proportionally more GS evolution in longer branches, indicative of a gradual mode of GS evolution. The delta ($\delta$) value, which sheds light on the tempo of GS evolution, was 1.76 (> 1), revealing an accelerated evolution of GS over time. This is consistent with a model of species-specific adaptation of GS in *Cheirolophus*.

The ancestral number of 35S rDNA loci inferred for *Cheirolophus* was between 4 and 8 (Fig. 3A, node 1), which was obtained after assigning missing values for the outgroup. Given that *Ch. crassifolius* has four 35S rDNA loci, which is similar to the number found in other early diverging Centaureinae lineages such as the *Rhaponticum* group (Hidalgo & al., 2008; Fig. 3C), it is hypothesised that the outgroup is unlikely to have had more than four 35S rDNA loci. Indeed, if this value is used for ancestral reconstruction then the ancestral 35S rDNA locus number inferred for the genus is four while values at all other branches remain unchanged. Taken together, the ancestral state for *Cheirolophus* was probably a low to moderate number of 35S rDNA loci inherited from its Centaureinae ancestor, followed by a dramatic increase during the diversification of the genus.

The ancestral chromosome number inferred for *Cheirolophus* is $2n = 32$, with several independent transitions to $2n = 30$ (Fig. 3A).

**Trait correlation. —** The PCA including *Cheirolophus* species with available GS, chromosome and rDNA loci number data allows a general outline of the

karyological-cytogenetic variation within the genus (Fig. 3B). It shows that the species with higher 35S rDNA loci number tend to have smaller GSs, and reversely, suggesting a possible negative correlation between these two traits. A PGLS analysis further confirmed the negative correlation between rDNA loci and GSs ($p < 0.0005$). The distinctiveness of *Cheirolophus* species regarding karyological-cytogenetic pattern compared to Centaureinae and Asteraceae as a whole is illustrated by Figs 3C and 3D.

## DISCUSSION

Currently, few data are available on the evolution of both GS and number of 35S rDNA loci in genera that have undergone oceanic island radiations, and, to our knowledge, *Cheirolophus* is the only example where these traits have been combined in a single study (Garnatje & al., 2012; present study).

**Genome downsizing and increase in 35S rDNA loci number started early in the evolutionary history of *Cheirolophus*, even before its radiation in Macaronesia.** — There is growing evidence that oceanic island colonisations tend to involve species with smaller GSs than their continental counterparts (e.g. *Cheirolophus*, Garnatje & al., 2007; *Schiedea*, Kapralov & al., 2009; *Veronica* L., Meudt & al., 2015). Indeed, this trend is seen even at the level of whole floras (e.g. in Macaronesia, Suda & al., 2003; 2005; in Marquesas, Macaronesian and Hawaiian Islands, Kapralov & Filatov, 2011). It has also been observed that genera of oceanic island floras that undergo species radiations have significantly smaller GS than non-radiating genera (e.g. in Canarian flora, Pérez de Paz & Caujapé-Castells, 2013). However, little is known about the genomic processes underlying such trends, and the question remains whether (i) the radiation processes mainly operate on taxa with smaller GS, (ii) GS downsizing has a

316 direct impact on the rate of island speciation, hence promoting the radiation, or finally,
317 (iii) GS downsizing arises as a consequence of the island species radiation but plays no
318 direct role in driving the evolutionary processes.

319 For *Cheirolophus*, the ancestral character state reconstructions showed that the
320 trends in GS and 35S loci evolution largely preceded the island radiation, providing
321 support for the hypothesis that there was selection for a coloniser that already had a
322 small GS and high number of 35S loci. Under this scenario, having a small GS would
323 therefore be seen as facilitating the radiation process. In support of this, recent
324 developments in the field of GS research have shown (i) a link between those selective
325 evolutionary forces favouring small GS with those promoting speciation, and (ii) the
326 highest frequency and/or more stable inheritance of new genetic variants in species with
327 smaller genomes (Kraaijeveld & al., 2010). Taken together, it is possible to see how
328 these could accelerate adaptation and subsequent species divergence (reviewed in
329 Kraaijeveld & al., 2010).

330 During the diversification of *Cheirolophus*, the most substantial GS decreases
331 were coincident with the Macaronesian radiation (Table 3, Fig. 3A), regardless of the
332 conflicting phylogenetic signal reported by Vitales & al. (2014b) for *Ch. massonianus*
333 (see Supplementary file). This suggests that reduction in GS may well have played a
334 direct role by acting as a trigger for the radiation. This hypothesis is certainly consistent
335 with the growing pool of data showing that a reduction of GS itself can accelerate
336 speciation rate (Puttick & al., 2015). Indeed, this may also be facilitated by certain
337 chromosome rearrangements that generate variation in GS, as they may also cause
338 genetic incompatibilities between incipient species, creating reproductive barriers and
339 hence further promoting speciation (Kraaijeveld & al., 2010).

340 As stated earlier, GS values within the Macaronesian clade were shown to be

relatively stable (Figs. 3A & 4), suggesting only limited DNA gain and/or loss after the radiation. This contrasts with the GS dynamics observed in the oceanic radiation of *Schiedea*, where genome upsizing was shown to have accompanied the inter-island colonisations of Hawaii (Kapralov & al., 2009). Nevertheless, whilst GS was relatively stable amongst Macaronesian *Cheirolophus*, the number of 35S loci varied from 8 to 10 and dysploid changes in chromosome number from $2n = 32$ to 30 were also observed in some species. Given that these chromosomal changes occurred during the island radiation they may well represent examples of 'modulator variables' *sensu* Bouchenak-Khelladi & al. (2015), stimulating and/or maintaining diversification. A similar role for chromosomal lability has also been suggested for the oceanic island radiation of *Sideritis* L., where dysploidy, changes in number and size of rDNA loci and, unlike *Cheirolophus*, polyploidy were observed to accompany its diversification in the Macaronesia (reviewed in Raskina & al., 2008).

**Proliferation of 35S loci within *Cheirolophus* coincided with GS decrease. —** Across angiosperms, an increase in the number of 35S rDNA loci is usually associated with polyploidisation (see rDNA loci number database; Garcia & al., 2012; Fig. 3D). *Cheirolophus* is therefore highly unusual in this respect as the observed proliferation of 35S loci has occurred within a diploid framework. *Cheirolophus* is also unusual in that the number of 35S rDNA loci is negatively correlated with GS, a situation that contrasts with the widely documented positive association between these traits across angiosperms. Certainly among closely related taxa, GS and 35S loci number are typically seen to increase proportionally with ploidy level (e.g. Pellicer & al., 2010; 2013), and while this positive relationship is less strong when considering angiosperms as a whole (Fig. 3D) it is still clearly evident (e.g. Prokopowich & al., 2003). This makes the trend observed within *Cheirolophus* a striking exception to the general rule.

366 The only other example of a somewhat similar pattern is *Oligochaeta* K.Koch, another
367 early diverging genus of Centaureinae, relatively closely related to *Cheirolophus*. Here
368 genome downsizing was also associated with a proliferation of 35S loci (from 4 to 12;
369 Hidalgo & al., 2008). However, the positions of the 35S loci of *Oligochaeta* were
370 shown to range from terminal to intercalary, most likely arising from extensive
371 chromosome restructuring. In contrast, the GS decrease and 35S rDNA loci
372 proliferation in *Cheirolophus* took place while maintaining a (sub)-terminal position for
373 all 35S sites. Amongst the mechanisms that may lead to a proliferation of novel rDNA
374 units –e.g. locus duplication, amplification of orphaned rDNA loci previously generated
375 by transposon-mediated insertions, chromosome translocations (Matyasek & al., 2012)–
376 locus duplication through non-homologous recombination is probably the most likely
377 explanation for *Cheirolophus* since it is expected to preserve the rDNA site position. In
378 addition, given that rDNA coding sequences are highly conserved and hence likely to
379 undergo heterologous recombination, they may therefore be seen as potential powerful
380 generators of chromosomal lability (Raskina & al., 2008). Illegitimate and unequal
381 homologous recombination are indeed one of the most frequently invoked phenomena
382 for producing small deletions, and hence may well play a role in genome downsizing
383 (see Leitch & Leitch, 2013; for a review), which would explain the GS miniaturisation
384 of *Cheirolophus*.

385 While this study has highlighted how the proliferation of 35S rDNA units
386 appears tightly linked with the diversification of the genus, it is still unclear whether it
387 happened once in the common ancestor of the Macaronesian and Mediterranean
388 lineages, or independently in each of the crown clades. Only a qualitative
389 characterisation of the rDNA repeats through sequencing can provide strong evidence to
390 support either of the alternatives. Likewise, it remains to be clarified whether the

surprising increase in number of rDNA loci within the overall context of GS downsizing is just a consequence of the genomic changes taking place during radiation or if indeed the increase played an active role in the process, providing increased fitness. To date, there are little data available to explain the putative advantages of harbouring numerous rDNA loci. However, available evidence suggests that the rate of rDNA sequence homogenisation slows down as the number of rDNA loci increases, providing a possible explanation of the observed relationship between loci number and rDNA sequence diversity (Matyasek & al., 2012).

We also found some degree of loci number variation among populations. For example, we identified seven 35S loci in *Ch. uliginosus*, which is slightly higher than the former report of six loci by Garnatje & al. (2012). A recent study aiming to understand the phylogeography of this relict species (Vitales & al., 2015) revealed strong population structure probably enhanced by long-term isolation in glacial refugia. Such results suggest that habitat fragmentation and small population sizes underlie the higher levels of among-population genetic diversity, and it is plausible that these factors could also have contributed to fixing this diversity of chromosomal reorganisations in different individuals and hence giving rise to the intrapopulation heterogeneity in 35S loci number.

***Cheirolophus* has one of the highest ratios of 35S signals: nuclear DNA contents in angiosperms. —** As stated above, *Cheirolophus* is exceptional amongst angiosperms as a rare case where a negative correlation between GS and rDNA loci number has been reported. As a consequence, the density of 35S rRNA signals in the genome is particularly high in some species (e.g. *Ch. puntallanensis* with 20 35S signals for 1.36 pg/2C, corresponding to a ratio of 14.71), although far from the highest values reported; these are found in genera of Rosaceae (*Fragaria* L., e.g. *F. vesca* L. with six

35S signals for 0.20 pg/2C giving a ratio of 30.0) and Brassicaceae (*Arabidopsis* Heynh., *Brassica* L., *Neslia* Desv. and *Olimarabidopsis* Al-Shehbaz, O'Kane & R.A.Price, with e.g. *A. pumila* Busch presenting 16 35S signals for 0.67 pg/2C giving a ratio of 23.88), which are all characterised by very small GS < 1 pg/2C (Garcia & al., 2014 and references therein). Whether such a high density of 35S rDNA loci in these genomes has an impact on genomic processes such as recombination frequency remains to be determined.

*Cheirolophus* is also distinctive in being one of only 11 angiosperm genera reported to possess 20 or more 35S signals [Alstroemeriaceae: *Alstroemeria* L., Asteraceae: *Artemisia* L., *Cheirolophus* and *Dendranthema* (DC.) Des Moul., Cyperaceae: *Rynchospora* Vahl, Iridaceae: *Iris* L., Liliaceae: *Lilium* L., Malvaceae: *Gossypium* L., Poaceae: *Hordeum* L., Primulaceae: *Lysimachia* L. and Solanaceae: *Capsicum* L.; Garcia & al., 2014 and references therein], and the only Asteraceae to have reached this number without polyploidy being involved in the 35S proliferation process.

Taken together, *Cheirolophus* clearly presents a distinctive genomic architecture that has arisen during the course of its evolutionary diversification. It is also noteworthy that the karyological-cytogenetic characteristics of Centaureinae as a whole appear distinct from the remaining Asteraceae, and intermediate between *Cheirolophus* and other members of the family (Fig. 3D). Unfortunately, however, available data for other Centaureinae are too limited to address the question as to whether these distinctive karyological-cytogenetic traits of *Cheirolophus* represent the culmination of a series of cytogenetic dynamics that already existed in the remaining subtribe.

**Concluding remarks. —** The present study has contributed significantly to enhancing our understanding of cytogenetic trait evolution in *Cheirolophus* from a

phylogenetic perspective. It is now clear that the overall evolutionary trends of GS reduction accompanied by increasing numbers of 35S rDNA loci to unusually high numbers are distinctive traits of the genus. Indeed, the Macaronesian radiation has been characterised by an enhancement of these pre-existing traits, a dynamic that seems to have been triggered just after the divergence of *Ch. crassifolius*. Notwithstanding, while the reduction of GS observed here is in line with the current view that larger GS might limit speciation in island floras (Kapralov & Filatov, 2011), it remains to be demonstrated whether the increase in number of rDNA loci has been relevant for the colonisation and subsequent explosive radiation of *Cheirolophus* in the Canary and Madeira archipelagos.

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627 **Table 1.** Taxa and collection data of the *Cheirolophus* populations studied.

| Species | Locality |
|---|---|
| *Ch. anagensis* A.Santos | Spain, Canary Islands: Tenerife, Anaga, Roque de los Pinos, Santos-Guerra 14.V.08 (ORT) |
| *Ch. arbutifolius* (Svent.) G.Kunkel | Spain, Canary Islands: Gran Canaria, Agaete, Santos-Guerra 17.II.09 (ORT) |
| *Ch. canariensis* (Willd.) Holub | Spain, Canary Islands: Tenerife, Los Carrizales, Santos-Guerra 17.VIII.08 (ORT) |
| *Ch. crassifolius* (Bertol.) Susanna | Italy, Sicily: Palermo, Orto Botanico di Palermo (cultivated from Malta), Vitales (BC) |
| *Ch. duranii* (Burchard) Holub | Spain, Canary Islands: El Hierro, Temijiraque, Barranco Balcón, Santos-Guerra 23.VII.09 (ORT) |
| *Ch. intybaceus* (Lam.) Dostál | Spain: Pedralba, Garnatje & Pellicer 01.XI.2006 (BC) |
| *Ch. junonianus* (Svent.) Holub var. *junonianus* | Spain, Canary Islands: La Palma, Fuencaliente, Teneguía, Santos-Guerra 26.VI.09 (ORT) |
| *Ch. puntallanensis* A.Santos | Spain, Canary Islands: La Palma, Puntallana, Barranco Nogales, Santos-Guerra 17.II.08 (ORT) |
| *Ch. santos-abreui* A.Santos | Spain, Canary Islands: La Palma, Barranco Madera, Santos-Guerra 15.II.08 (ORT) |
| *Ch. sempervirens* (L.) Pomel | [1] Portugal, Faro: 4 km from N of Monchique, Garcia-Jacas & Susanna 1218 (BC) |
| | [2] Portugal, Alentejo: Odemira, Vila Nova de Milfontes, Furnas, Garnatje 267 & Pellicer (BC) |
| *Ch. tagananensis* (Svent.) Holub | Spain, Canary Islands: Tenerife, Taganana, Roque de las Ánimas, Santos-Guerra 07.IX.09 (ORT) |
| *Ch. uliginosus* (Brot.) Dostál | Portugal, Beira Litoral: Pateira de Fermentelos, Vitales 13, Pellicer & Garnatje (BC) |
| *Ch. webbianus* (Sch.Bip.) Holub | Spain: Tenerife, Anaga, Chinamada, Santos-Guerra 14.V.08 (ORT) |
| *Ch.* cf. *webbianus* (Sch.Bip.) Holub | Spain: Canary Islands, Tenerife: Taganana, at the base of Roque de las Ánimas, Garnatje 3 & Luque (BC) |

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633 **Table 2.** Nuclear DNA contents estimated in the present study.

| **Species** | **2C (SD) [pg]** | **1C*x*[1] [pg]** | **2C [Mbp][2]** |
|---|---|---|---|
| *Ch. anagensis* A.Santos | 1.42 (0.03) | 0.71 | 1389 |
| *Ch. canariensis* (Willd.) Holub | 1.36 (0.01) | 0.68 | 1330 |
| *Ch. crassifolius* (Bertol.) Susanna | 1.80 (0.04) | 0.90 | 1760 |
| *Ch. duranii* (Burchard) Holub | 1.33 (0.05) | 0.67 | 1301 |
| *Ch. junonianus* (Svent.) Holub var. *junonianus* | 1.48 (0.05) | 0.74 | 1447 |
| *Ch. puntallanensis* A.Santos | 1.36 (0.02) | 0.68 | 1330 |
| *Ch. santos-abreui* A.Santos | 1.46 (0.05) | 0.73 | 1428 |
| *Ch. sempervirens* (L.) Pomel [2] | 1.53 (0.04) | 0.77 | 1496 |
| *Ch. taganannensis* (Svent.) Holub | 1.41 (0.01) | 0.71 | 1379 |
| *Ch. uliginosus* (Brot.) Dostál | 1.55 (0.04) | 0.78 | 1516 |
| *Ch. webbianus* (Sch.Bip.) Holub | 1.44 (0.00) | 0.72 | 1408 |

[1]1C*x*: monoploid genome size (DNA content per basic chromosome set).
[2]2C [Mbp]: 1 pg = 978 Mbp (Doležel & al., 2003).

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636 **Table 3.** Ancestral genome size values (2C, in pg) for the MRCAs of selected nodes
637 inferred using either parsimony or Bayesian (MCMC) approaches (node numbers are
638 shown in Fig. 3).
639

| Node | Parsimony | MCMC (95% CI) |
|---|---|---|
| 1 | 1.654 | 1.5902 (1.5897–1.5906) |
| 2 | 1.615 | 1.6087 (1.6084–1.6091) |
| 3 | 1.412 | 1.3996 (1.3994–1.3999) |
| 4 | 1.418 | 1.3999 (1.3995–1.4001) |
| 5 | 1.592 | 1.5778 (1.5776–1.5781) |
| 6 | 1.598 | 1.5809 (1.5807–1.5812) |
| 7 | 1.560 | 1.5582 (1.5584–1.5585) |
| 8 | 1.586 | 1.5806 (1.5803–1.5810) |
| 9 | 1.510 | 1.5096 (1.5095–1.5098) |

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643 **FIGURE LEGENDS**

644 **Figure 1.** Somatic metaphase plates of *Cheirolophus* species. (A) *Ch. crassifolius*, (B)
645 *Ch. intybaceus*, (C) *Ch. sempervirens*, (D) *Ch. uliginosus*. Scale bar = 10 μm.

646

647 **Figure 2.** Fluorochrome banding and fluorescent *in situ* hybridisation (FISH) of somatic
648 metaphase plates of *Cheirolophus* species. A–J: Chromomycin $A_3$ banding. K–T: FISH
649 showing location of 35S rDNA loci (= red/pink fluorescent signal). Number of 35S loci
650 given in brackets after the species name. (A,K) *Ch. arbutifolius* (10), (B,L) *Ch.*
651 *crassifolius* (4), (C,M) *Ch. duranii* (9), (D,N) *Ch. intybaceus* (10), (E,O) *Ch.*
652 *puntallanensis* (10), (F,P) *Ch. santos-abreui* (9), (G,Q) *Ch. sempervirens* [1] (8), (H,R)
653 *Ch. sempervirens* [2] (8), (I,S) *Ch. uliginosus* (10), (J,T) *Ch.* cf. *webbianus* (7). Scale
654 bar = 10 μm.

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656 **Figure 3.** A: Phylogenetic tree of *Cheirolophus* showing genome size, 35S rDNA loci
657 number and chromosome number of the extant species, and their reconstructed ancestral
658 values. Note that 35S loci (red) are depicted in chromosomes (blue) for illustration
659 purposes only, they do not represent idiograms. Inferences done with Mesquite v.3.04
660 are depicted as coloured branches for GS, with diamonds for chromosome numbers and
661 as pie charts for 35S rDNA loci number. Chromosome numbers which were not
662 reassessed/confirmed in this study are shown as "?". The numbers along branches
663 indicate posterior probabilities ≥ 0.95. Numbers in squares on the branches refer to the
664 nodes for which ancestral GS values are given in Table 3. The black star corresponds to
665 the Macaronesian radiation. B: Principal component analysis of the log-transformed
666 karyological and cytogenetic *Cheirolophus* data, with black and grey shades indicating

667 phylogenetic groups. The ellipses represent the 68 % confidence interval of the
668 respective points in the same colour. C-D: Analyses showing the karyological-
669 cytogenetic profiles of *Cheirolophus* species (N = 15, in green) in comparison to those
670 of Centaureinae (N = 31, blue), Asteraceae (N = 149, red) and angiosperms as a whole
671 (N = 1161, grey). Data were extracted from the databases of Garcia & al. (2012; 2014)
672 and the present study. Graph showing GS plotted against 35S rDNA loci number (C).
673 Principal component analysis of log-transformed karyological and cytogenetic data (D).

674

675 **Figure 4.** Box-plots showing the distribution of genome size values in *Cheirolophus*
676 species occupying different geographical areas.

677

**Appendix. List of *Cheirolophus* species, their geographical distribution, and, where available, their chromosome number (2*n*), mean genome size (2C-value, pg), number of GC-rich heterochromatin bands (identified using CMA fluorochrome banding) and 35S rDNA sites.** Distribution codes follow Euro+Med (2006-): Ag: Algeria. Bl: Balearic Islands (I: Eivissa and Formentera; M: Mallorca). Ca: Canary Islands (C: Gran Canaria; G: La Gomera; H: El Hierro; P: La Palma; T: Tenerife). F: France. S: Spain. Lu: Portugal. Ma: Morocco. Md: Madeira. M: Maltese Islands. [1] The two varieties of *Ch. junonianus* are likely to be recognised as constituting two different species (Vitales & al., 2014a, b). [2] This population was first attributed to *Ch. taganannensis* (Susanna & al., 1999; Garnatje & al., 2007), but morphological and genetic divergence indicate that it may be a separate taxon (A. Santos personal communication). Chromosome numbers in bold are those that are new or reassessed/confirmed in this study. In addition to the data generated here, the table includes chromosome count and genome size data obtained from the Index to Chromosome Numbers in Asteraceae (Watanabe, 2002; 2004) and Genome size in Asteraceae (GSAD; Garcia & al., 2014) databases, respectively.

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| Taxon | Distribution | 2n | 2C (mean) [pg] | CMA | 35S |
|---|---|---|---|---|---|
| **1. *Ch. anagensis* A.Santos** | Ca(T) | - | 1.42 | - | - |
| **2. *Ch. arboreus* (Sch.Bip.) Holub** | Ca(P) | 30 | 1.40 | - | - |
| **3. *Ch. arbutifolius* (Svent.) G.Kunkel** | Ca(C) | 30, **32** | 1.39 | - | 20 |
| **4. *Ch. benoistii* (Humbert) Holub** | Ma | 30, **32** | 1.55 | 16 | 18 |
| **5. *Ch. burchardii* A.Susanna** [= *Ch. canariensis* var. *subexpinnatus* (Burchard) G.Kunkel] | Ca(T) | **30** | 1.38-1.42(1.439) | 16 | - |
| **6. *Ch. canariensis* (Willd.) Holub** | Ca(T) | c. 30 | 1.36-1.38 (1.37) | 18(4i) | 16(2i) |
| **7. *Ch. crassifolius* (Bertol.) Susanna** | Si(M) | 30, **32** | 1.8 | - | 8 |
| **8. *Ch. dariasii* (Svent.) Bramwell** | Ca(G) | - | - | - | |
| **9. *Ch. duranii* (Burchard) Holub** | Ca(H) | **32** | 1.33 | - | 18 |
| **10. *Ch. falcisectus* Montelongo & Moraleda** | Ca(C) | 30 | 1.35 | - | - |
| **11. *Ch. ghomerythus* (Svent.) Holub** | Ca(G) | 30 | 1.41 | 18(2i) | 16 |
| ● *Ch. ghomerythus* var. *integrifolius* (Svent.) Holub | | - | - | - | - |
| **12. *Ch. grandifolius* (Font Quer) Stübing & al.** | Bl(I, M) | | 1.47-1.61 (1.52) | | |
| **13. *Ch. intybaceus* (Lam.) Dostál** (= *Ch. mansanetianus* Stübing & al., = *Ch. cavanillesianus* Ferrer-Gallego & al.) | Ga(F), Hs(S) | 30, **32**, 32+0-2B | 1.40-1.56 (1.51) | | 20 |
| ● *Ch. intybaceus* var. *capillifolius* (Sandwith) J.R. Nebot & al. [= *Ch. cavanillesianus* subsp. *capillifolius* (Sandwith) Ferrer-Gallego & al.)] | Hs(S) | - | 1.49-1.51 | | - |
| ● *Ch. intybaceus* var. *microcephala* Rouy | Ga(F) | - | 1.47 | | - |
| **14. *Ch. junonianus* (Svent.) Holub** | Ca(P) | **30**, 32 | 1.37-1.48 (1.43) | 12 | 16 |
| ● *Ch. junonianus* var. *junonianus*[1] | Ca(P) | **30**, 32 | 1.37-1.48 (1.43) | 12 | 16 |
| ● *Ch. junonianus* var. *isoplexiphyllus* (Svent.) Kunkel[1] | | - | - | - | - |
| **15. *Ch. lagunae* A.Olivares & al.** | Hs(S) | 30, 32 | 1.51 | - | - |
| **16. *Ch. massonianus* (Lowe) A.Hansen & Sunding** | Md(M, P) | 30, **32** | 1.44 | 20(2i) | 20 |
| **17. *Ch. mauritanicus* (Font Quer) Susanna** | Ag, Ma | 30 | 1.57 | - | - |
| **18. *Ch. metlesicsii* Montelongo** | Ca(T) | 30 | 1.36 | - | - |
| **19. *Ch. puntallanensis* A.Santos** | Ca(P) | **c.30** | 1.36 | - | 20 |
| **20. *Ch. santos-abreui* A.Santos** | Ca(P) | **32** | 1.46 | - | 18 |
| **21. *Ch. sataraetaensis* (Svent.) Holub** | Ca(G) | - | - | - | - |
| **22. *Ch. sempervirens* (L.) Pomel** | Hs(S), Lu | 30, **32** | 1.53-1.59 (1.56) | - | 16 |
| **23. *Ch. sventenii* (A.Santos) G.Kunkel** | Ca(P) | - | - | - | - |
| ● *Ch. sventenii* subsp. *gracilis* A.Santos | Ca(P) | - | - | - | - |
| **24. *Ch. tagananensis* (Svent.) Holub** | Ca(T) | - | 1.41 | - | - |
| **25. *Ch. tananicus* (Maire) Holub** | Ma | 30 | 1.65 | - | - |
| **26. *Ch. teydis* (Buch) G.López** [= *Ch. argutus* (Nees) Holub] | Ca(P,T) | 30, 30+1B | 1.38-1.43(1.4) | - | - |
| **27. *Ch. uliginosus* (Brot.) Dostál** | Hs(S), Lu | 32, **32** | 1.55-1.69 (1.62) | 12 | 12-14 |
| **28. *Ch. webbianus* (Sch.Bip.) Holub.** Population of the *Ch. webbianus* complex that could constitute new species: | Ca(T) | 32 | 1.44 | - | - |
| ● *Ch.* cf. ***webbianus*** [Spain, Tenerife: Taganana, Roque de las Ánimas, Garnatje 3 and Luque (BC)][2] | Ca(P) | 30 | 1.38 | 18(4i) | 20(2i) |
| **29. *Ch.* cf. *sp. nova*** (Spain: Tenerife, near Taganana, Afur) | Ca(T) | - | - | - | - |

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**Supplementary file.** Ancestral genome sizes (2C, in pg) for the MRCAs of selected nodes of the nrDNA and cpDNA Bayesian analyses, and inferred using either parsimony (first value on branches) or Bayesian (second value) approaches.

735 Figure 1

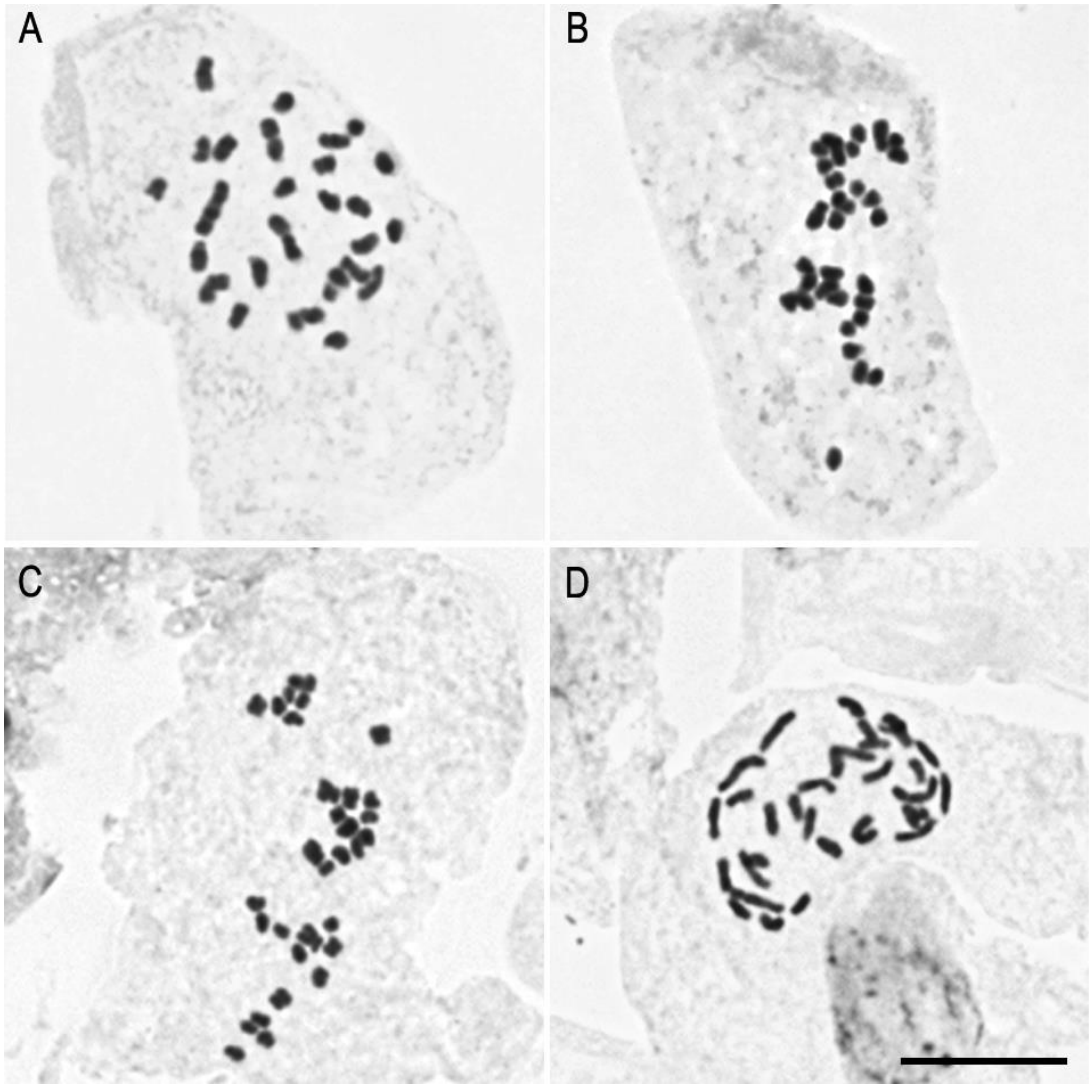


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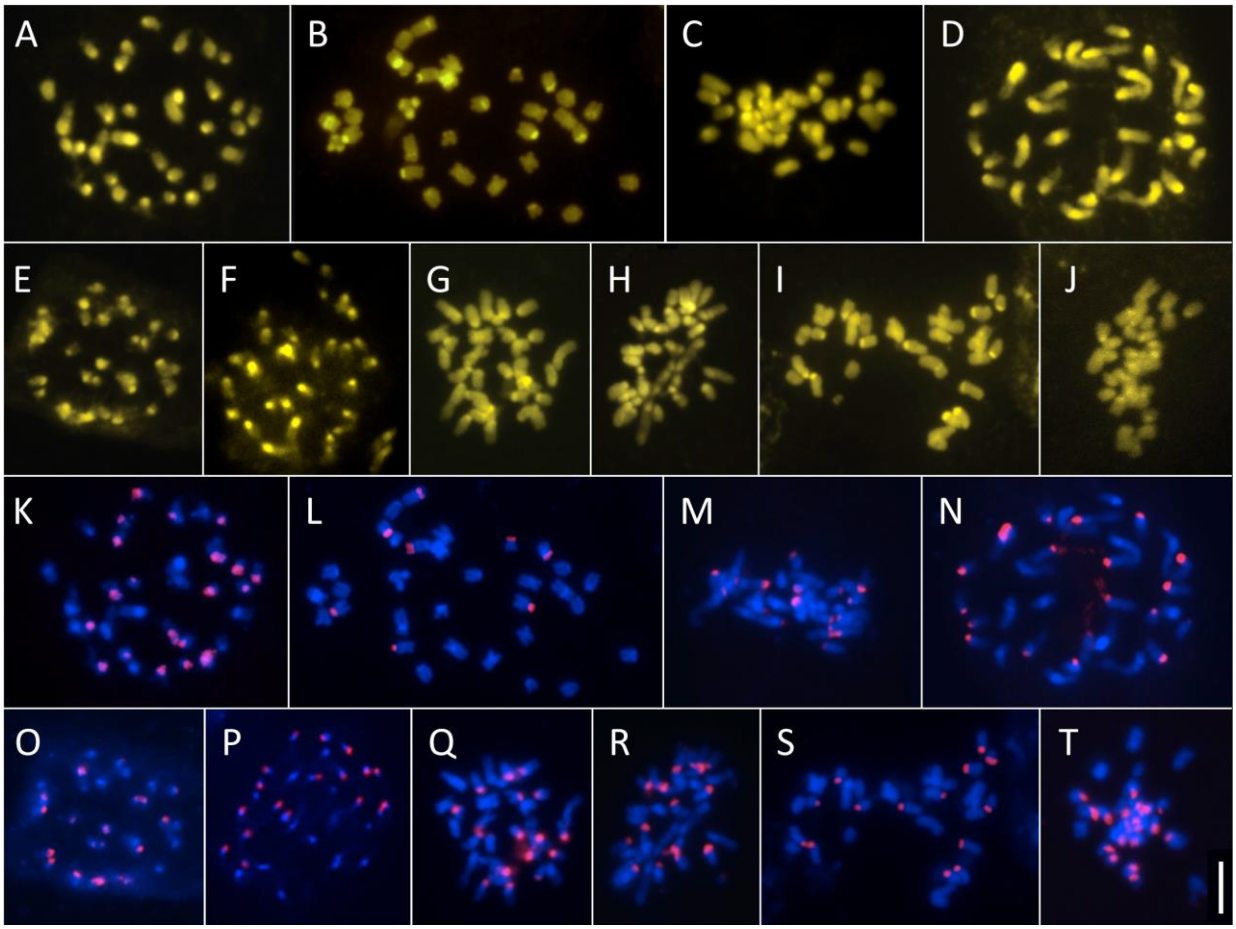
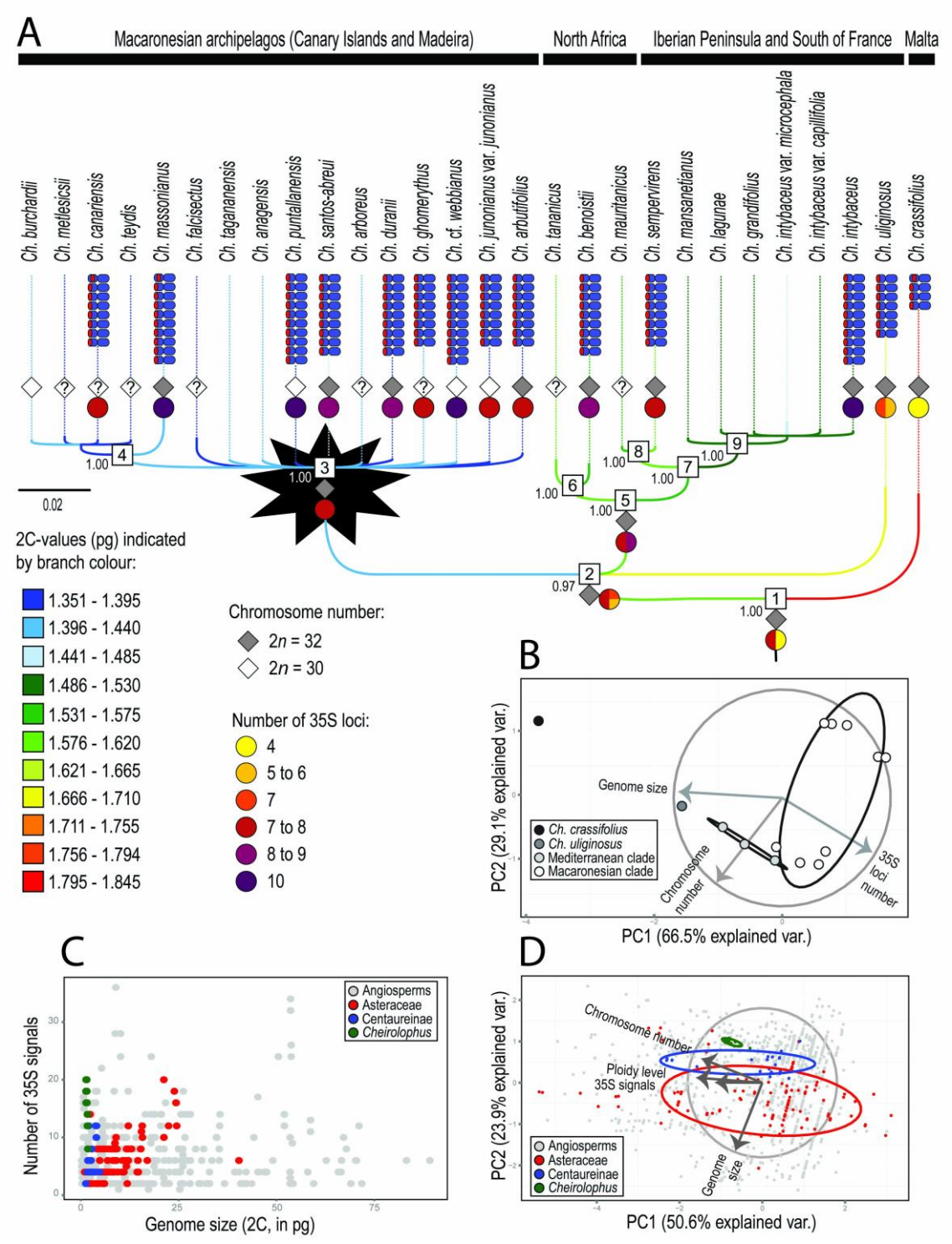
Figure 2



759 Figure 3

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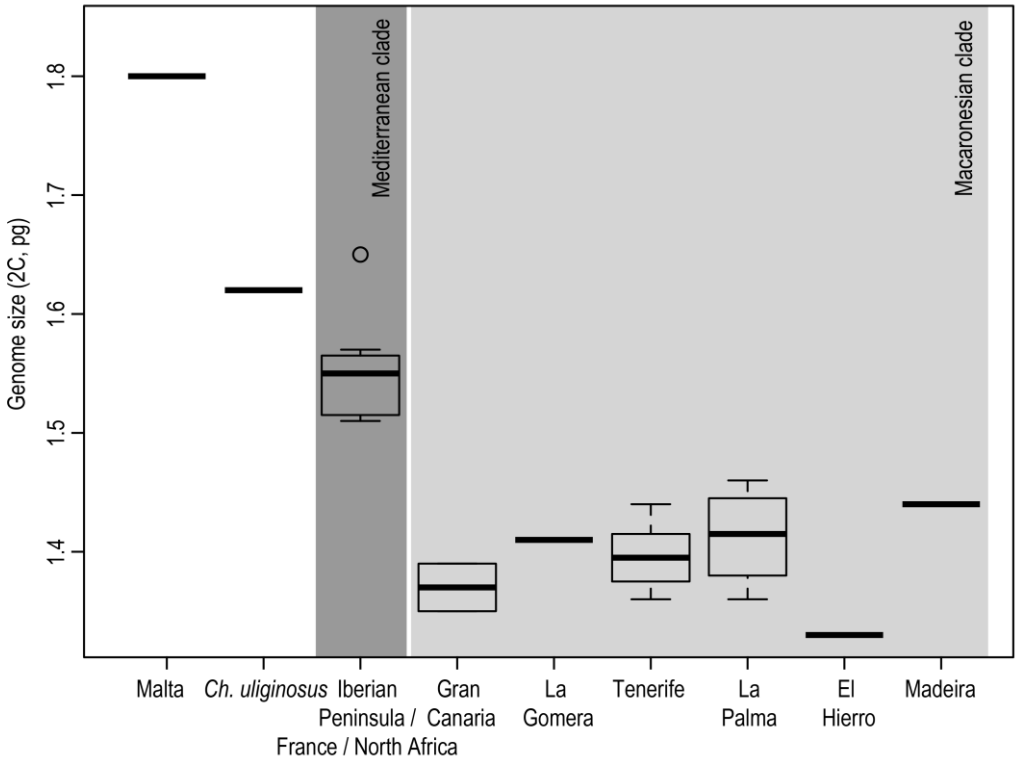
764 Figure 4

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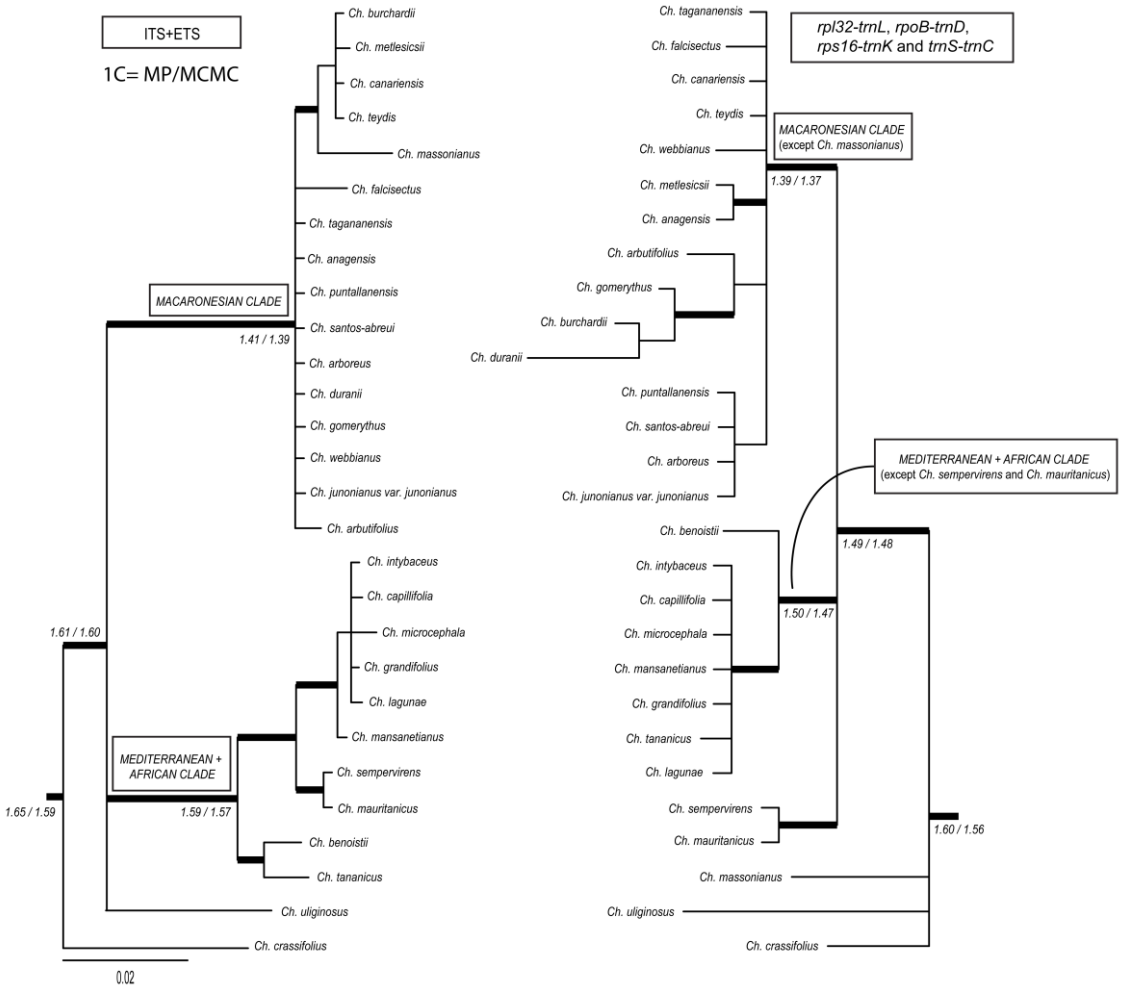
778 Figure S1

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