Filogeografia genètica de poblacions i citogenètica molecular del gènere *Cheirolophus (Asteraceae, Cardueae)*

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SECCIÓ DE BOTÀNICA

FILOGEOGRAFIA, GENÈTICA DE POBLACIONS I CITOGENÈTICA MOLECULAR DEL GÈNERE *CHEIROLOPHUS* (*ASTERACEAE, CARDUEAE*)

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Memòria presentada per Daniel Vitales Serrano per a optar al títol de doctor per la Universitat de Barcelona

Amb el vist-i-plau dels directors de la tesi:

Prof. Joan Vallès Xirau Dr. Jaume Pellicer Moscardó Dra. Teresa Garnatje Roca

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A la meva mare

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1. Marc taxonòmic

1.1 Breu introducció a la família de les *Asteraceae* Berchtold & J.Presl

La família de les *Asteraceae*, també coneguda com a *Compositae* Giseke, a la qual pertany el gènere *Cheirolophus* Cass., és una de les famílies més grans dins de les plantes vasculars. Les revisions taxonòmiques més actuals estimen que comprèn al voltant d'unes 23.000 a 26.000 espècies repartides en un total de 1.600 gèneres (Kubitzki, 2007; Funk et al., 2009). És una família bastant diversa i cosmopolita (Figura 1), formada bàsicament per subarbusts, arbusts i herbes perennes, tot i que la gran versatilitat del grup fa que no siguin estranys els casos on trobem també herbes de comportament anual o biennal. Tenen una distribució global que abasta la gran majoria dels continents, tret de l'Antàrtida (Funk et al., 2005). De fet, part de l'èxit colonitzador d'aquesta família radica en la seva adaptabilitat a un ampli espectre d'hàbitats i ecosistemes (Figura 1), que inclou des d'aiguamolls (*Eupatorium* L.; Kirkman et al., 2000), ambients ruderals altament antropitzats (*Artemisia* L., *Onopordum* L.; Sanz Elorza, 2014), fins als cims muntanyosos i cingleres més inhòspites i inaccessibles (*Argyroxiphium* DC., *Cheirolophus* Cass.; Bramwell & Bramwell, 2001; Carlquist et al., 2003).

D'altra banda, aquesta plasticitat ecològica també és influenciada, entre d'altres, per la presència de caràcters morfològics específics, i per la capacitat per a produir un enorme ventall de metabòlits secundaris com els terpens, els flavonoides i les lactones entre molts altres (Funk et al., 2009). És per aquest fet principalment, que les asteràcies tenen una gran importància socioeconòmica com a productores de principis actius i altres productes utilitzats en diferents activitats industrials. No menys importants són altres de les aplicacions que els humans fem d'aquestes plantes, com per exemple les medicinals, les alimentàries i també les ornamentals [p. e. *Artemisia* L., *Matricaria* L. (propietats medicinals), *Lactuca* L., *Helianthus* L. (producció vegetal), *Echinops* L., *Gerbera* L. (horticultura)].

Basant-nos en dades del registre fòssil i en reconstruccions de filogènia molecular, l'origen d'aquesta família se situa a Amèrica del Sud i l'eocè es postula com a possible edat de diversificació, tot i que la hipòtesi que un estoc d'asteràcies ja formava part de la flora del sud de Gondwana abans de l'existència de barreres entre els continents sembla cada vegada més plausible (Barreda et al., 2010).

Figura 1. Diversitat morfològica i ecosistemes variats on es poden trobar asteràcies. a) Bosc humit a l'illa de Santa Cruz (Galápagos) amb *Scalesia pedunculata* Arn., b) Estepa semidesèrtica amb espècies d'*Artemisia* a Mongòlia, c) *Artemisia vulgaris* L., tàxon ruderal ocupant el marge d'una carretera, d) *Argyroxiphium sandwicense* DC. subsp. *macrocephalum* (A.Gray) Mérat, e) *Barnadesia* sp., f) *Cheirolophus webbianus* (Sch. Bip.) Holub, g) *Echinops sphaerocephalus* L., h) *Eupatorium cannabinum* L., i) *Gymnarrhena micrantha* Desf., j) *Helianthus annus* L., k) *Leontopodium alpinum* Cass., l) *Mutisia acuminata* Ruiz & Pav., m) *Wunderlichia mirabilis* Reidel ex Baker. (Imatges: J. Pellicer, J. Vallès, http://commons.wikimedia.org).

Els trets morfològics principals que caracteritzen les asteràcies dins de les angiospermes són els següents:

- Una estructura inflorescencial particular, i quasi específica de la família anomenada capítol (Figura 2a), que consisteix en un receptacle envoltat de bràctees involucrals en el qual s'insereixen les flors, que poden ser flòsculs o lígules (Figura 2b) i que prové d'una inflorescència en raïm amb els eixos curts, engruixits i fusionats, de manera que es produeix una floració centrípeta (Weberling, 1992).
- Un fruit monocarpelar indehiscent procedent de l'ovari ínfer anomenat aqueni o cípsela, coronat (de vegades) per un apèndix format per un anell de pèls, de setes o d'esquames a la seva part superior, anomenat papus (Figura 2c).
- La presència d'anteres fusionades formant un tub per l'interior del qual passa l'estigma.

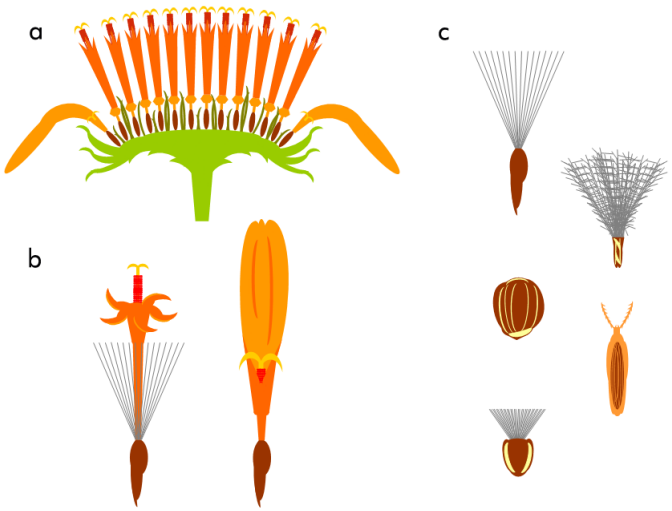

Figura 2. Representació esquemàtica de les principals característiques de la família *Asteraceae*. a) inflorescència en capítol, b) flors ligulades i tubulars on hi ha les anteres fusionades, c) fruits característics de les asteràcies [Imatge extreta de Pellicer (2009)].

1.2 La tribu de les *Cardueae* Cass.

La tribu de les *Cardueae* pertany a la subfamília de les *Carduoideae* Cass. ex Sweet. És una de les tribus més nombroses de les asteràcies, que inclou aproximadament

2.400 espècies repartides en 73 gèneres segons (Susanna & Garcia-Jacas, 2009), i es divideix taxonòmicament en cinc subtribus:

- ***Cardopatiinae*** Less.
- ***Carduinae*** Cass. ex Dumort
- ***Carlininae*** Dumort
- ***Centaureinae*** Dumort
- ***Echinopinae*** Cass. ex Dumort

La gran majoria de les *Cardueae* són herbes anuals o perennes -sovint espinoses- algunes d'elles desenvolupen un port arbustiu, i de manera més excepcional arborescent. La disposició de les seves fulles és alterna, i les basals són freqüentment agrupades en una roseta (Figura 3). Els capítols dels representants d'aquesta tribu solen presentar-se de forma solitària, o bé disposats en inflorescències corimbiformes amb algunes excepcions, on aquests es presenten en forma més complexa, com la de capítol de capítols [p. e. *Echinops* (Figura 1g, 3d), *Leontopodium* (Pers.) R. Br. (Figura 1k)]. Els aquenis de les *Cardueae* són molt variables, tot i que generalment són pilosos, amb el papus més o menys desenvolupat compost per setes o esquames.

Figura 3. Diversitat morfològica en la tribu de les *Cardueae*. a) *Berardia subacaulis* Vill., b) *Carlina corymbosa* L., c) *Centaurea napifolia* L., d) *Echinops ritro* L., e) *Mantisalca salmantica* L., f) *Olgaea leucophylla* (Turcz.) Iljin, g) *Onopordum acanthium* L., h) *Volutaria tubiflora* Sennen. (Imatges: http://commons.wikimedia.org).

1.3 El gènere *Cheirolophus* i la seva posició en la tribu de les *Cardueae*

El gènere *Cheirolophus* va ser descrit per Cassini (1817) en base a la segregació d'un grup d'espècies que pertanyien al gènere *Centaurea* L. Anys més tard, Boissier (1839-1845) va incorporar noves espècies a les ja segregades prèviament per Cassini, agrupant-les totes sota un gènere nou, *Ptosimopappus* Boiss., que comprenia les espècies de les seccions *Cheirolophus* Cass. i *Microlophus* (Cass.) DC., que havien estat descrites dintre del gènere *Centaurea*. Aquest autor proposà, a més, l'addició de *Centaurea arguta* Nees i *Centaurea uliginosa* Brotero a aquest nou gènere i va descriure'n algunes espècies noves, com per exemple *Ptosimopappus bracteatus* Boiss. i *Ptosimopappus arboreus* Boiss. (Boissier, 1875).

Aquestes reordenacions taxonòmiques no han estat exemptes de conflicte. De fet, mentre autors com Pomel (1874), Holub (1973), Dostál (1976) o Bremer (1994) van continuar considerant *Cheirolophus* com un gènere independent, alguns com Dittrich (1968) i Talavera (1987), entre altres, preferiren mantenir-lo com una secció de *Centaurea*. Tot i aquests conflictes, les revisions més recents del grup basades en dades de filogènia molecular (p. e. Susanna et al., 2011; Barres et al., 2013) donen un suport convincent a la segregació de *Cheirolophus* com una entitat taxonòmicament independent.

Actualment, aquest gènere conté aproximadament unes 27-30 espècies (Figura 4), depenent dels autors consultats, amb una distribució que inclou la Mediterrània occidental fins a Malta i els arxipèlags macaronèsics de Canàries i Madeira. Algunes d'aquestes espècies són de distribució bastant àmplia, com per exemple *Ch. intybaceus* (Lam.) Dostál i *Ch. sempervirens* Pomel. D'altres, en canvi, presenten una distribució més restringida que en molts casos es limita a unes poques poblacions, com n'és el cas de *Ch. duranii* (Burchard) Holub de l'illa d'El Hierro o *Ch. tagananensis* (Svent.) Holub de la península d'Anaga, a la part nord de Tenerife. De fet, és en aquest grup de distribució més restringida on s'inclouen la majoria d'espècies endèmiques de la regió macaronèsica. Fins ara, en aquesta regió s'han descrit unes 19 espècies, una subespècie i dues varietats en l'arxipèlag canari (Bramwell & Bramwell, 2001) i una altra espècie a les illes de Madeira i Porto Santo. A la Figura 5 es poden veure més

detalls sobre la distribució de les espècies d'aquest gènere. El nombre de poblacions reportades per a alguns dels membres del gènere -a la península Ibèrica i a les illes Canàries- així com la quantitat d'individus per població es poden trobar al *Libro Rojo de la Flora Amenazada* (Bañares et al., 2010) en el qual es pot veure que moltes d'aquestes espècies es troben dins de la categoria d'amenaçades (vulnerables) o en perill d'extinció i per tant, subjectes a regulacions mitjançant figures de protecció estatals i europees.

Figura 4. Diversitat morfològica i ecosistemes on es pot trobar el gènere *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*, g) *Ch. junonianus* (Svent.) Holub, h, i) Penya-segat a Madeira on creix *Ch. massonianus* (Lowe) A.Hansen & Sunding, j) *Ch. tagananensis,* k) *Ch. teydis* (C.Sm.) G.López, l) *Ch. uliginosus*. (Imatges: L. Barres, T. Garnatje, D. Vitales, http://commons.wikimedia.org).

Mentre que la delimitació taxonòmica del gènere és clara, les relacions amb els altres gèneres propers han resultat més complicades. Com s'ha indicat a l'apartat anterior, el gènere *Cheirolophus* pertany a la tribu de les *Cardueae* i a la subtribu de les *Centaureinae* (Dostál, 1976). Els caràcters sistemàtics que tradicionalment s'han usat per a establir diverses classificacions han estat els morfològics, especialment els que fan referència al tipus de pol·len, a la carpologia i al port de la planta (Wagenitz, 1955; Bremer, 1994). La cariologia també dóna suport a la posició filogenètica d'aquest gènere que té un nombre cromosòmic de base *x* = 15, el qual comparteix amb els altres gèneres propers (Funk et al., 2009).

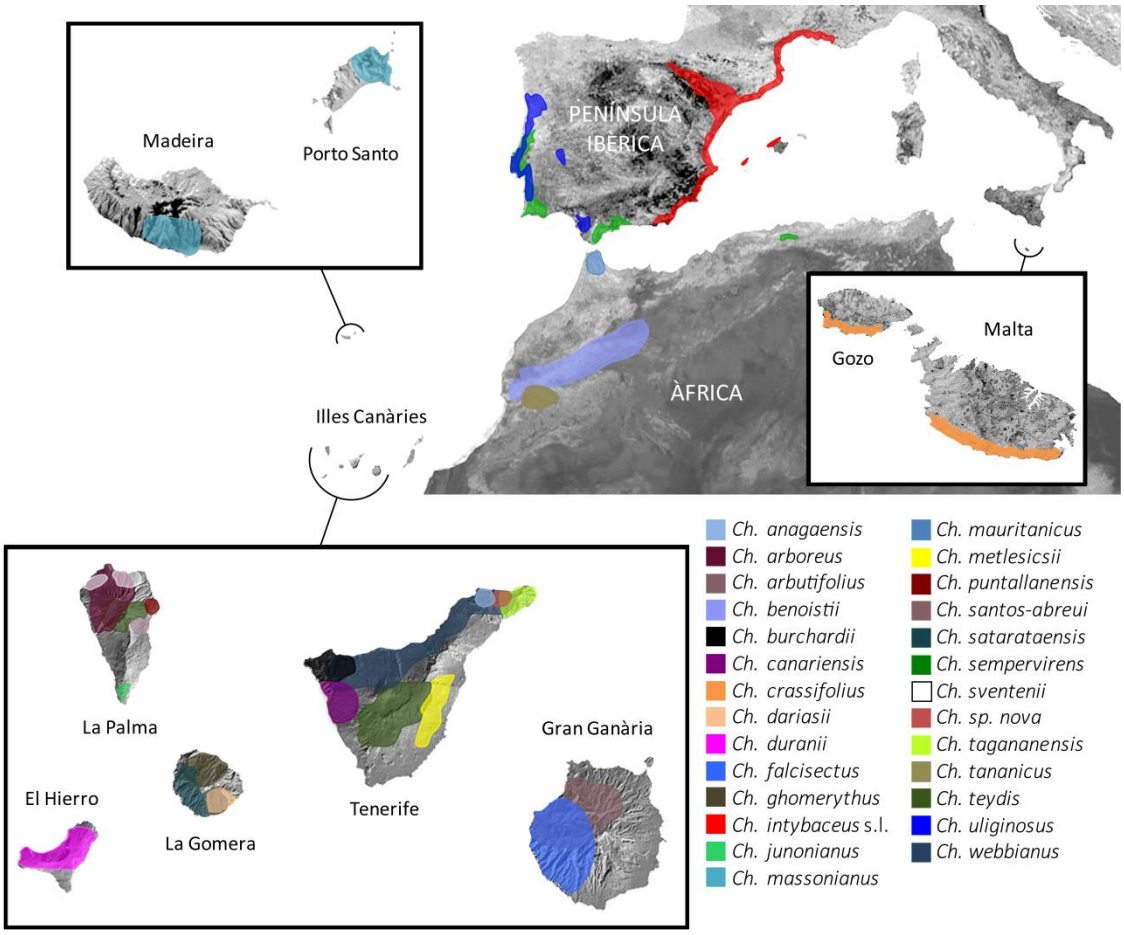

Figura 5. Distribució geogràfica aproximada del gènere *Cheirolophus* a la Mediterrània occidental, al Nord d'Àfrica i a la Macaronèsia.

Com a complement als estudis de taxonomia basats en caràcters morfològics, també s'han dut a terme un seguit d'estudis de filogènia molecular per a esclarir les relacions evolutives en la tribu (p. e. Garcia-Jacas et al., 2001, 2002; Susanna et al.,

2006; Barres et al., 2013). Aquests treballs han revelat la posició del gènere *Cheirolophus* en els clades més basals dins de la subtribu de les *Centaureinae*. Tot i això, els marcadors moleculars usats fins a l'actualitat no han aconseguit però, establir d'una forma robusta les relacions amb altres gèneres basals com *Centaurodendron* Johow, *Callicephalus* C.A.Mey, *Crupina* (Pers.) DC., *Plectocephalus* D.Don, *Psephellus* Cass., *Rhaponticoides* Vaill. i *Serratula* L. (Funk et al., 2009).

2. Antecedents

Aquesta tesi doctoral està estructurada com a compendi de publicacions, i comprèn un conjunt de treballs duts a terme en el gènere *Cheirolophus*, amb l'objectiu principal d'aportar nous punts de vista sobre la seva diversificació així com la seva empremta genètica. Usant *Cheirolophus* com a eix vertebrador, s'ha plantejat una aproximació multidisciplinària que abasta tècniques variades i, a la vegada, complementàries, com la seqüenciació de DNA, l'ús de marcadors hipervariables com els AFLP i la citogenètica clàssica i molecular. Aquestes tècniques han estat combinades amb els mètodes analítics més recents, amb la finalitat d'optimitzar al màxim l'aprofitament de les dades de què disposem i, és clar, comprendre millor la història evolutiva del gènere.

En els següents apartats es presenta una síntesi dels antecedents i dels treballs que s'han fet fins a l'actualitat, i que han estat el punt de partida per als que es presenten en aquesta dissertació.

2.1 Estudis de morfologia, distribució i descripció de noves espècies

Són nombrosos els autors que des d'anys enrere fins a l'actualitat han dut a terme estudis morfològics i de distribució de les espècies del gènere. Entre ells destaquen els treballs citats en apartats anteriors, que van servir per a establir una primera configuració taxonòmica que delimités el gènere i les espècies associades (p. e Pomel, 1874; Boissier, 1875 ; Holub, 1973; Dostál, 1976; Bremer, 1994). També han

vist la llum un seguit de treballs de caire més generalista, com ho són les flores, que han inclòs aspectes rellevants de la taxonomia i distribució de *Cheirolophus*. Entre elles destaquen *Flora Europaea* (Tutin et al., 1976), *Flora Vascular de Andalucía Occidental* (Valdés et al., 1987) o les diverses flores publicades sobre les illes Cànaries [p. e. Santos-Guerra (1983); Bramwell (1997)]. D'altra banda, estudis monogràfics on s'han descrit noves espècies basant-se en aspectes morfològics són relativament abundants, tot i que en alguns casos no han estat exempts de debat. Per exemple, el complex de tàxons de *Ch. intybaceu*s ha estat segregat en diferents espècies: *Ch. lagunae* Olivares, Peris, Stübing & J.Martín (Olivares et al., 1995), *Ch. grandifolius* (Font Quer) Stübing, Peris, Olivares & J.Martín (Stübing et al., 1997), *Ch. mansanetianus* Stübing, Peris, Olivares & J.Martín (Stübing et al., 1997). En canvi, estudis poblacionals duts a terme en aquest grup per Garnatje et al. (2013) no han trobat un suport molecular per a aquesta segregació, que més aviat respon probablement a adaptacions morfològiques locals o, si de cas, a processos incipients de diversificació. Si hi ha algun indret on els treballs de taxonomia han estat més exhaustius ha estat la Macaronèsia (vegeu p. e. Sventenius, 1960; Santos-Guerra, 1974; Montelongo Parada, 1982), on el gènere ha radiat de forma impressionant i, per tant, on se n'acumula la major diversitat d'espècies.

Aquesta radiació insular ha donat lloc a l'aparició d'un gran nombre d'endemismes que presenten en molts casos una àrea de distribució molt reduïda, qualificats en aquests casos com a microendemismes (p. e. *Ch. puntallanensis* A. Santos; *Ch. tagananensis*; *Ch. metlesicsii* Montelongo). Això, junt amb la progressiva pèrdua d'hàbitat (p. e. al parc natural de Doñana i el seu entorn) i a la falta de figures de protecció legal en molts indrets (p. e. Portugal), ha fet que en els darrers anys hagin proliferat els estudis sobre la conservació de diferents espècies del gènere, entre els quals cal destacar l'*Atlas y Libro Rojo de la Flora Vascular Amenazada de España* (Bañares et al., 2010) i el *Libro Rojo de las especies vegetales amenazadas de las islas Canarias* (Gómez-Campo et al., 1996). Actualment, 18 espècies de *Cheirolophus* es troben incloses a la *Lista Roja de la Flora Vascular Española* (Moreno, 2008; Bañares et al., 2010), i 10 d'elles figuren a la llista vermella global d'espècies amenaçades de la Unió Internacional per a la Conservació de la Natura (IUCN, 2015) (Taula 1).

Taula 1. Distribució i categories d'amenaça dels tàxons de *Cheirolophus* recollits al *Atlas y Libro Rojo de la Flora Vascular Amenazada de España* i a la IUCN *Red List of Threatened Species.* [1]EN: *endangered*; VU: *vulnerable*; CR: *critically endangered*.

Tàxon	Distribució	Categoria[1] Espanya	Categoria[1] IUCN
Cheirolophus arboreus (Webb & Berthel.) Holub	La Palma, Canàries	EN	-
Cheirolophus arbutifolius (Svent.) G. Kunkel	Gran Canària, Canàries	VU	-
Cheirolophus burchardii Susanna	Tenerife, Canàries	VU	-
Cheirolophus canariensis (Brouss. ex Willd.) Holub	Tenerife, Canàries	VU	-
Cheirolophus crassifolius Susanna	Malta i Gozo, Malta	-	CR
Cheirolophus dariasii (Svent.) Bramwell	La Gomera, Canàries	CR	-
Cheirolophus duranii (Burchard) Holub	El Hierro, Canàries	CR	CR
Cheirolophus falcisectus Svent. ex Montelongo & Moraleda	Gran Canària, Canàries	EN	EN
Cheirolophus ghomerytus (Svent.) Holub	La Gomera, Canàries	EN	EN
Cheirolophus junonianus (Svent.) Holub	La Palma, Canàries	EN	EN
Cheirolophus lagunae Olivares, Peris, Stübing & J. Martín	Alacant, País Valencià	CR	-
Cheirolophus massonianus (Lowe) A.Hansen & Sunding	Madeira, Portugal	-	EN
Cheirolophus metlesicsii Montelongo	Tenerife, Canàries	CR	CR
Cheirolophus santos-abreui A. Santos	La Palma, Canàries	CR	CR
Cheirolophus sataratensis (Svent.) Holub	La Gomera, Canàries	VU	VU
Cheirolophus sventenii subsp. *gracilis* A. Santos	La Gomera, Canàries	EN	-
Cheirolophus sventenii (A. Santos) G.Kunkel subsp. *sventenii*	La Gomera, Canàries	VU	-
Cheirolophus tagananensis (Svent.) Holub	Tenerife, Canàries	EN	VU
Cheirolophus uliginosus (Brot.) Dostál	Península Ibèrica	CR	-
Cheirolophus webbianus (Sch. Bip.) Holub	Tenerife, Canàries	VU	-

2.2 Estudis de cariologia i citogenètica

Fins fa pocs anys els estudis de cariologia en espècies d'aquest gènere es limitaven bàsicament a uns quants treballs de recomptes cromosòmics duts a terme principalment en espècies de distribució continental, entre elles *Ch. uliginosus* i *Ch. intybaceus* (vegeu Watanabe, 2002, 2004). D'acord amb aquests treballs es va establir que *Cheirolophus* és un gènere amb un nombre cromosòmic de base *x* = 15, en el qual és freqüent trobar-hi a més cromosomes B (Michaelis, 1964; Bramwell et al., 1971). Amb el temps s'han anat duent a terme recomptes de cromosomes de forma més o menys esparsa en el gènere, la majoria dels quals han confirmat 2*n* = 30 com el nombre prevalent (vegeu Watanabe, 2002, 2004 per a una recopilació de dades). No obstant, així com alguns autors consideren la presència de cromosomes B, d'altres, a causa de la mida reduïda dels cromosomes, apunten la presència d'un segon nombre 2*n* = 32. De forma més excepcional, s'ha reportat la presència de 2*n* = 45 en una població de l'espècie *Ch. taganananensis* a Canàries, i 2*n* = 24 a *Ch. uliginosus* del sud de la península Ibèrica. Aquestes dues dades però, representen una gran divergència respecte del nombre de cromosomes habitual en el gènere. El fet que cap d'aquests nombres hagi pogut ser confirmat posteriorment fa pensar que en *Ch. taganananensis* es pogués haver donat excepcionalment per un cas d'apomixi (amb un individu híbrid triploide), i el cas de *Ch. uliginosus* porta a pensar en un possible artefacte i/o una confusió taxonòmica. Finalment, Crawford et al. (2009) han proposat que el gènere *Cheirolophus* presentaria un nombre cromosòmic de base *x* = 16, que tindria un origen poliploide. Segons els mateixos autors, aquesta paleopoliploïdia podria haver comportat duplicacions gèniques i una diversitat genètica més gran, la qual cosa podria estar implicada en l'èxit radiatiu de *Cheirolophus* a la Macaronèsia.

Altres treballs de citogenètica en el gènere són bàsicament orientats a l'estudi de la quantitat de DNA nuclear. El primer treball que va incloure dades per a l'estudi de la mida del genoma en *Cheirolophus* va ser publicat per Garnatje et al. (2007). En aquest treball es va reportar com a resultat principal el fet que la colonització a la Macaronèsia havia comportat una reducció del genoma en les espècies insulars respecte de les continentals. Aquestes dades cariològiques i citogènetiques van ser

l'origen d'una de les principals preguntes que ha guiat les nostres recerques sobre aquest gènere en els darrers anys: De quina manera determinats trets genòmics estan relacionats amb els fenòmens de radiació? Alguns autors, com per exemple Suda et al. (2005, 2007) o Kapralov & Filatov (2011), ja havien apuntat a una reducció del genoma lligada a l'hàbitat insular oceànic. Altres han suggerit que certs caràcters citogenètics com ara els marcatges de DNA ribosòmic (Mandáková et al., 2010) també podrien estar relacionats amb els processos de diversificació en illes. En aquest sentit, l'estudi dels canvis que es produeixen en el genoma de les espècies durant els processos de radiació no només té un gran interès en el cas de *Cheirolophus*, sinó que també pot servir com a model per a altres grups amb històries evolutives similars.

2.3 Estudis de palinologia

Els estudis de palinologia han tingut molta rellevància en la família de les compostes (Heywood et al., 1977; Blackmore et al., 2009). El pol·len s'ha mostrat com un marcador molt usat en sistemàtica en aquesta família i així ho testifiquen els nombrosos estudis que podem trobar, des d'algun dels clàssics de Wagenitz (1955) en el qual es proposava un seguit de models pol·línics, fins a estudis més recents i de revisió com són els de Wagenitz & Hellwig (1996) o Stübing et al. (1997). Malauradament, en *Cheirolophus*, els estudis pol·línics previs que s'han dut a terme no han reportat cap tipus de variabilitat interespecífica que pogués tenir alguna implicació taxonòmica rellevant en el gènere.

2.4 Estudis de filogènia i sistemàtica molecular

L'ús d'eines moleculars i especialment de la seqüenciació de DNA per a analitzar les relacions filogenètiques entre les espècies ha estat un dels avenços més importants en l'estudi del gènere, i dels que més ha contribuït a la comprensió de la seva evolució. De fet, han estat treballs recents, que han utilitzat la seqüenciació de

regions del DNA, com els de Susanna et al. (2011) i Barres et al. (2013), que han permès confirmar l'estatus independent d'aquest gènere, així com la seva posició basal dintre de la subtribu de les *Centaureinae*. Si ens centrem, però, en els inicis, són d'obligada referència els primers treballs que van tenir el gènere *Cheirolophus* com a principal objecte d'estudi, i que van ser els treballs realitzats per Garnatje (1995) i Garnatje et al. (1998). Aquests treballs pioners consistiren en estudis de variabilitat poblacional basats en la tècnica d'anàlisi d'isoenzims. Aquesta aproximació metodològica va aportar poca resolució sobre les relacions filogenètiques entre les espècies del gènere, però va servir per a proposar per primera vegada l'origen recent de les espècies macaronèsiques del grup. Poc més tard es va dur a terme una reconstrucció filogenètica basada en l'anàlisi de seqüències de l'espaiador intern transcrit (ITS) del DNA ribosòmic nuclear (Susanna et al., 1999). Aquest treball va posar de manifest el limitat poder de resolució de l'ITS en el gènere, però va resultar útil per tal de delimitar-lo taxonòmicament -incloent-hi *Ch. crassifolius*- i va permetre inferir la monofília del grup macaronèsic de *Cheirolophus*. Uns anys més tard, es va intentar abordar aquesta qüestió amb una aproximació combinada, consistent a mesurar la grandària del genoma i analitzar les dades en un marc filogenètic aconseguit a través de l'anàlisi de les seqüències de la regió ETS (espaiador extern transcrit) junt amb les anteriorment generades per a l'ITS (Garnatje et al., 2007). En aquest darrer estudi es va poder identificar *Ch. crassifolius* com a espècie germana de la resta de congèneres i es va suggerir l'existència de dos grans llinatges d'espècies, les macaronèsiques i les continentals. Malauradament, cap d'aquests treballs no va aconseguir construir amb solidesa la filogènia de *Cheirolophus* i en particular les relacions interespecífiques dintre del grup macaronèsic van romandre fonamentalment irresoltes.

2.5 Estudis de filogeografia i genètica de poblacions

Les aproximacions poblacionals per a resoldre qüestions filogeogràfiques han experimentat un augment molt significatiu en els darrers anys, sobretot en grups de diversificació recent on els marcadors clàssics no han demostrat una capacitat suficient

de resolució (p. e. Abbott & Comes, 2003; Burnier et al., 2009; Li et al., 2013) i/o amb històries evolutives complexes (p. e. Álvarez & Wendel, 2003; Meudt et al., 2009). De manera similar, els estudis de genètica poblacional es consideren una eina fonamental per a l'anàlisi de la variabilitat i els processos de diferenciació a nivell infraespecífic, particularment interessants en l'àmbit de la biologia de la conservació (Young et al., 1996; Petit & Mousadik, 1998; Frankham et al., 2002). En canvi, malgrat que les dades prèvies -filogenètiques, de distribució i de conservació- sobre *Cheirolophus* suggereixen que aquest podria ser un grup especialment indicat per ser estudiat des d'un punt de vista filogeogràfic i de genètica poblacional, el gènere ha rebut una limitada atenció en aquest sentit. De fet, fins a dia d'avui només s'ha publicat un treball sobre la filogeografia de les espècies incloses en el complex de *Ch. intybaceus* a la Mediterrània occidental, basat en l'anàlisi de polimorfismes de llargària en fragments amplificats de DNA (coneguda per l'acrònim anglès AFLPs) (Garnatje et al., 2013). En aquest treball es va situar l'origen d'aquest complex en el sud de la península Ibèrica durant el plistocè mitjà, seguit d'una dispersió cap al nord fins a terres franceses, que inclou un únic esdeveniment de colonització de les illes Balears des de l'àrea diànica. El mateix estudi també va posar de manifest un procés de recolonització del continent des de les illes Balears, en el qual els autors van suggerir la implicació d'aus marines.

2.6 Estudis de radiació insular

Els orígens de la biologia evolutiva moderna estan marcats per les observacions que Darwin (1859) i Wallace (1878) van realitzar sobre els processos de diversificació de les espècies a illes de diferents punts del planeta. Des d'aleshores, els fenòmens de radiació insular han estat objecte de nombrosos treballs centrats en multitud de plantes i animals d'arxipèlags repartits per tot el món (Carlquist, 1974; Whittaker & Fernández-Palacios, 2007). Aquest interès per les illes s'explica per una sèrie de característiques -com ara la mida generalment reduïda, els seus límits clars, la relativa simplicitat ecològica o l'elevada diversitat que contenen- que les fan una mena de

laboratoris naturals on és fàcil observar i interpretar patrons evolutius més generals (Losos & Ricklefs, 2009). Els arxipèlags macaronèsics -i les illes Canàries molt particularment- són des de fa molts anys un dels escenaris preferits dels investigadors interessats en estudiar processos de diversificació en plantes (p. e. Juan et al., 2000; Suda et al., 2005; Kim et al., 2008; Sanmartín et al., 2008). En aquest sentit, els grups amb un major nombre d'espècies endèmiques -i que, per tant, ofereixen millors oportunitats d'analitzar els processos de radiació insular- han estat els més estudiats des d'un punt de vista evolutiu [p. e. *Argyranthemum* Webb, (Francisco-Ortega et al., 1996); *Aeonium*, (Jorgensen & Olesen, 2001); *Bystropogon* L'Hér., (Trusty et al., 2005); *Sideritis* L., (Barber et al., 2007); *Sonchus* L. i afins, (Kim et al., 1996); *Echium* L., (García-Maroto et al., 2009); *Tolpis* Adans, (Gruenstaeudl et al., 2012); *Pericallis* Webb & Berthel (Jones et al., 2014)].

La radiació del gènere *Cheirolophus* a les illes Canàries és considerada com una de les deu més importants que les plantes han experimentat en aquest arxipèlag oceànic (Fernández-Palacios, 2008). A més, les espècies macaronèsiques d'aquest grup presenten tota una sèrie de trets típics d'altres plantes que han aconseguit diversificar en ambient insulars -increment del caràcter llenyós (Carlquist, 1974); augment en les dimensions i el nombre de les inflorescències (Bölhe et al., 1996); reducció de la mida del seu genoma (Suda et al., 2005; Kapralov & Filatov, 2011); o mida reduïda de les poblacions (Caujapé-Castells et al., 2010)- que fan de *Cheirolophus* un model ideal per a l'estudi de la radiació en illes oceàniques. En canvi, fins al desenvolupament d'aquesta tesi, la gran diversificació que aquest gènere va experimentar als arxipèlags macaronèsics no ha estat objecte de cap estudi en particular. Basant-se en les reconstruccions filogenètiques prèvies, s'ha especulat que la radiació de *Cheirolophus* a la Macaronèsia hauria estat un procés significativament ràpid i recent (Susanna et al., 1999; Garnatje et al., 2007). Malauradament, aquests primers treballs mancaven d'un marc temporal sòlid i, per tant, no permetien determinar la taxa d'especiació en aquest grup ni realitzar comparacions amb altres casos ben coneguts de radiació explosiva a la Macaronèsia (p. e. García-Maroto et al., 2009) o a d'altres arxipèlags oceànics (p. e. Knope et al., 2012). Altres autors també han proposat que *Cheirolophus* representa un exemple paradigmàtic de radiació no adaptativa en illes (Whittaker &

Fernández-Palacios, 2007). Efectivament, la major part de les espècies del grup exploten nínxols ecològics força semblants i presenten diferències morfològiques poc espectaculars. No obstant això, els *Cheirolophus* macaronèsics presenten també uns quants casos d'adaptacions ecològiques concretes a uns hàbitats certament diferenciats (vegeu p. e. *Ch. teydis* de les zones subalpines de Tenerife i La Palma o *Ch. junonianus* de l'extrem sud més àrid de l'illa de la Palma, a la Figura 5). Aquestes i altres qüestions que s'han revelat com a fonamentals per tal d'entendre el procés de diversificació en illes -com ara el paper de la hibridació i del flux gènic entre les poblacions (Francisco-Ortega et al., 1996; Jorgensen & Olesen, 2001; Jones et al., 2014); els patrons de colonització i dispersió entre illes (Cowie & Holland, 2006; Sanmartín et al., 2008); o la biologia reproductiva de les espècies (Crawford et al., 2009, 2011)- formen part dels temes relacionats amb la radiació insular que es tracten en aquesta tesi.

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OBJECTIUS

OBJECTIUS DE LA TESI DOCTORAL

El projecte de tesi doctoral que aquí es presenta ha estat emmarcat dins de la línia de recerca que duu a terme el grup d'investigació en Biosistemàtica, Filogènia i Citogenètica moleculars de Plantes (BioFiC-PLANTA, www.etnobiofic.cat), format per investigadors del Laboratori de Botànica de la Facultat de Farmàcia de la Universitat de Barcelona (Unitat Associada CSIC) i l'Institut Botànic de Barcelona (IBB-CSIC-ICUB), integrat en el Grup de Recerca en Biodiversitat i Biosistemàtica Vegetals (GReB, www.webgreb.org), consolidat per l'Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR) de la Generalitat de Catalunya.

Els objectius principals d'aquesta tesi han estat els següents:

i) Construir un nou marc filogenètic robust del gènere *Cheirolophus*, amb un interès especial a resoldre acuradament les relacions filogenètiques dels representants macaronèsics (que constitueixen la major part del gènere).

ii) Establir el marc temporal corresponent, en el qual discutir tots els altres mecanismes i fenòmens de divergència que han tingut lloc en la història evolutiva del gènere *Cheirolophus*.

iii) Investigar l'origen de la colonització macaronèsica per part de *Cheirolophus*, així com estudiar els procés posterior de radiació insular que ha tingut lloc en aquest gènere.

iv) Establir un marc citogenètic en el gènere, amb dades sobre l'evolució del DNA ribosòmic 5S i 35S i del DNA repetitiu ric en bases GC.

v) Aprofundir en el coneixement sobre l'evolució de la mida del genoma amb noves dades que complementen aquelles de què disposem per tal d'elucidar quins canvis es produeixen durant els processos de radiació del gènere.

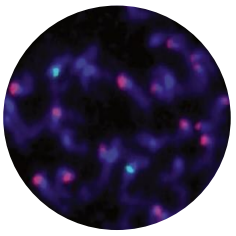
vi) Analitzar la història filogeogràfica del conjunt d'espècies macaronèsiques del gènere esmentat mitjançant marcadors hipervariables a nivell poblacional,

intentant establir els factors intrínsecs i extrínsecs que faciliten o dificulten el fenomen radiatiu.

vii) Analitzar els patrons filogeogràfics en l'evolució de dues espècies ibèriques (*Ch. sempervirens* i *Ch. uliginosus*) mitjançant seqüenciació de DNA cloroplàstic i marcadors hipervariables.

viii) Estudiar *Cheirolophus uliginosus* des d'un punt de vista de biologia de la conservació, analitzant la relació entre diversitat genètica, mida poblacional i l'eficàcia reproductiva en aquesta espècie amenaçada.

MATERIALS I MÈTODES



MATERIALS I MÈTODES

Consignem aquí els materials i les metodologies que s'han utilitzat durant els treballs d'aquesta tesi, en alguns casos d'una manera més detallada de com s'explica als articles del compendi de publicacions.

1. Materials

1.1 Tàxons estudiats

En el present projecte de tesi hem mostrejat la totalitat d'espècies reconegudes actualment en el gènere *Cheirolophus*, així com espècies d'altres gèneres estretament relacionats (*Rhaponthicum, Rhaponticoides* i *Serratula*) a l'hora de dur a terme les aproximacions filogenètiques. S'ha pogut obtenir aquenis viables de la majoria d'espècies estudiades (principalment per als treballs de citogenètica), a més de cultivar exemplars als hivernacles de l'Institut Botànic de Barcelona (IBB-CSIC-ICUB). Tot i això, d'algunes espècies solament s'han pogut estudiar plecs d'herbari atesa la impossibilitat d'obtenir material fresc del camp en les nostres expedicions. Així doncs, hem obtingut els materials a través de les següents vies:

- Campanyes de recol·lecció i expedicions realitzades pel doctorand, així com els investigadors i personal del Laboratori de Botànica de la Facultat de Farmàcia de la Universitat de Barcelona i de l'Institut Botànic de Barcelona juntament amb investigadors de diversos centres internacionals.
- Enviaments de material, principalment aquenis (tot i que també s'ha rebut plantes vives, p. e. de l'Orto botanico di Palermo), per part d'investigadors amb qui col·labora actualment aquest grup d'investigació.
- Recepció de plecs d'herbari provinents d'institucions internacionals [herbari (R) Reading], així com de l'herbari propi de l'Institut Botànic de Barcelona (BC).

Els *Cheirolophus* que s'han recol·lectat o dels quals s'ha rebut material representen el total de l'àrea de distribució del gènere (vegeu Figura 5 a la introducció), tot i que el mostreig en els treballs amb un enfocament poblacional s'ha centrat bàsicament a la regió macaronèsica i en els dos representants del sud-oest de la península Ibèrica.

2. Mètodes

2.1 Cariologia i citogenètica clàssica: recomptes de cromosomes i bandatge amb cromomicina A_3

Obtenció, preparació i visualització de cromosomes. Els aquenis s'han sembrat en plaques de Petri amb paper de filtre mullat, o bé directament en germinadors als hivernacles de l'Institut Botànic de Barcelona. Aproximadament unes 24 h després de la geminació, els extrems apicals de les arrels de plàntules germinades han estat pretractats amb una solució de colquicina aquosa al 0,05% a temperatura ambient o amb 8-hidroxiquinolina 0,002 M durant aproximadament 2,5-3,5 h. Posteriorment, el material ha estat fixat amb una solució d'etanol absolut i àcid acètic glacial (3:1) durant 12 h a temperatura ambient, per a conservar-lo finalment a 4ºC. Un cop incubades en la solució fixadora durant 24 h aproximadament, les mostres s'han transferit a etanol de 70º a 4ºC per tal d'assegurar una millor conservació a llarg termini, ja que els teixits es mantenen en un nivell d'hidratació òptima. Les mostres han estat hidrolitzades amb HCl 1N durant 5-9 min a 60ºC, posteriorment s'han rentat amb aigua destil·lada i s'han tenyit amb orceïna acètica al 2% durant 2-4 h, o bé amb reactiu de Schiff (també coneguda com tinció de Feulgen). Per a observar el material al microscopi òptic, l'àpex de l'arrel s'ha tallat amb l'ajuda d'un bisturí i s'ha muntat en un portaobjectes amb una gota d'àcid acètic al 45%. Un cop col·locat el cobreobjectes s'ha aixafat durant uns segons la mostra per tal de facilitar la dispersió cel·lular (tècnica d'*squash* o esclafament). Les millors plaques metafàsiques s'han fotografiat amb una càmera digital (SPOT RT) acoblada a un microscopi Zeiss Axioplan 2. Les imatges han estat analitzades amb el programa SPOT RT v. 4.0.4 i editades amb Adobe Photoshop CS5.

Obtenció i preparació de protoplasts. Entre 3 i 10 arrels fixades s'han rentat amb tampó citrat 0,01 M (pH = 4,6) i posteriorment s'han incubat en vidres de rellotge que contenien 1 ml d'una solució enzimàtica [cel·lulasa 4% Onozuka-RS (Yakult Honsha), pectoliasa Y-23 1% (Kikkoman) i hemicel·lulasa 4% (Sigma)] preparada amb el mateix tampó citrat durant 30-40 min, dintre d'una placa de Petri amb paper mullat, a 37ºC. Un cop digerides les arrels s'han separat els àpexs, s'han rentat amb tampó citrat durant aproximadament 20 min i s'han col·locat individualment en un portaobjectes per a homogeneïtzar-los amb una gota d'etanol absolut i àcid acètic glacial (3:1) amb l'ajuda d'unes pinces. Per a comprovar l'existència de protoplasts amb cromosomes metafàsics, les preparacions s'han observat amb un microscopi òptic de contrast de fase. Les preparacions s'han conservat a temperatura ambient amb un agent deshidratant (gel de sílice).

Bandatge amb cromomicina A_3. Les preparacions de protoplasts s'han incubat amb tampó McIlvaine (pH = 7) amb $MgCl_2 \cdot 6H_2O$ durant 15 min. Un cop retirat el tampó s'han tenyit amb 90-100 µl de cromomicina A_3 (0,02 g/100 ml) preparada amb el mateix tampó, i s'han deixat tenyir durant 90 min. Després s'han rentat les mostres amb tampó McIlvaine (pH = 7) i s'ha efectuat una contracoloració amb una dilució de verd de metil (0,1%) en tampó McIlvaine (pH = 5,5) durant 7 min en obscuritat. Posteriorment s'han retirat les restes de la tinció amb el mateix tampó McIlvaine (pH = 5,5) i s'han deixat assecar a l'aire. Les preparacions han estat muntades amb una gota de Citifluor (Agar Scientific) o Vectashield (Vector Laboratories), i s'han observat al microscopi de fluorescència. Per tal d'eliminar la tinció de cromomicina i dur a terme posteriorment la hibridació *in situ* sobre les mateixes plaques, s'han incubat les preparacions durant 30-40 min en una solució d'etanol absolut i àcid acètic glacial (3:1) a temperatura ambient i amb agitació per a facilitar la separació dels cobreobjectes. Un cop destenyides les preparacions, s'han deshidratat durant 3 min amb sèries d'etanol de 70º, 90º i 100º i, finalment, s'han deixat assecar.

2.2 Citogenètica molecular

Preparació de les sondes del DNA ribosòmic.

- **Sonda de DNA ribosòmic 5S.** La sonda utilitzada per a la detecció i localització de la regió 5S del DNA ribosòmic ha estat un clon del pTa794; un fragment de 410 kb del 5S del *Bam*HI aïllat del blat i clonat en pBR322 (Gerlach & Dyer, 1980). Aquest fragment conté el gen 5S del DNA ribosòmic (120 pb) i l'espaiador intergènic no codificant (290 pb). Aquesta sonda s'ha marcat amb fluor-roig-dUTP (Amersham) mitjançant reacció en cadena per la polimerasa (PCR).
- **Sonda de DNA ribosòmic 35S.** S'ha utilitzat un fragment de la TaqI de 0,8 kb del gen 18S de blat (Richard et al., 1995) aïllat i clonat en pUC18. La sonda ha estat marcada amb dioxigenina-11-dUTP (Boehringer Mannheim) mitjançant PCR.

Hibridació *in situ* fluorescent (FISH). Les preparacions s'han incubat en 100 µg/ml d'RNasa, lliure de DNasa, diluïda en 2xSSC durant 1h a 37ºC en una cambra humida. Posteriorment s'han rentat dos cops en 2xSSC durant 5 min amb agitació suau, un cop amb HCl 0,01N a temperatura ambient i s'han incubat afegint 80-100 µl/preparació de pepsina (0,1 mg/ml en HCl 0,01N) durant 10 min a 37ºC. Seguidament s'han rentat dos cops en 2xSSC durant 5 min, i s'han deshidratat amb sèries de 3 min amb etanol (70º, 90º i 100º). Un cop assecades, s'han dipositat 50 µl de la mescla d'hibridació* a cada preparació, s'han cobert amb un cobreobjectes de plàstic i s'han desnaturalitzat durant 10 min a 75ºC, i després, a 55ºC durant 5 min. Seguidament s'ha dut a terme la hibridació a 37ºC en cambra humida durant 15-18 h.

Transcorregut el temps d'hibridació, les preparacions s'han rentat amb agitació suau amb les següents solucions astringents: un rentat de 3 min amb 2xSSC a temperatura ambient, tres rentats de 5 min amb 2xSSC a 42ºC, dos rentats de 5 min amb formamida desionitzada 20% amb 0,1xSSC a 42ºC, un rentat de 5 min amb 0,1xSSC a 42ºC, dos rentats de 5 min amb 2xSSC a 42ºC, un rentat durant 5 min amb 4xSSCT (SSCT: 0,2% Tween 20) a 42ºC i tres rentats de 5 min amb 4xSSCT a

temperatura ambient. Per a poder detectar els senyals de la sonda 5S les preparacions han estat pretractades durant 5 min a temperatura ambient amb 100 µl/mostra d'una dissolució BSA (concentració final 5%) amb 4xSSCT, i seguidament s'han dipositat 50 µl/mostra, de la mescla de detecció preparada amb 55 µl de la dissolució anterior (BSA i 4xSSCT) a la qual s'han afegit 0,75 µl d'ADF (antidigoxigenina; Boehringer Mannheim) a una concentració de 200 µg/ml, i s'han incubat durant 1 h a 37ºC. Tot seguit s'han rentat les preparacions tres cops durant 5 min amb 4xSCCT a temperatura ambient, i s'han muntat amb Vectashield (Vector Laboratories), que conté 500 ng/ml de DAPI (4',6-diamidino-2-fenilindol). Les mostres han estat observades al microscopi d'epifluorescència (Zeiss Axiophot), utilitzant diferents filtres d'excitació (01, 07, 15). La captura d'imatges s'ha fet amb una càmera CCD (Princeton Instruments), i posteriorment s'han manipulat amb un programa d'anàlisi d'imatges (Metavue, v. 4.6, Molecular Devices Corporation).

***Mescla d'hibridació:** Ha estat preparada afegint aproximadament per a cada preparació d'1-1,5 µl de les sondes 35S i 5S a una concentració aproximada de 25-100 ng/µl, 25 µl de formamida desionitzada (concentració final 50%, pH = 7), 10 µl de sulfat de dextrà al 50% (v/v), 3 µl de dodecilsulfat sòdic 10% (p/v), 1,5 µl d'esperma de salmó 250 µg/ml i 5 µl de 20xSSC. Un cop preparada la mescla ha estat desnaturalitzada durant 10 min a 75ºC i seguidament s'ha preservat en gel.

Llistat de tampons utilitzats:

- **20xSSC (pH = 7):** clorur sòdic (NaCl) 3 M, citrat sòdic ($Na_3C_6H_5O_7 \cdot 2H_2O$) 0,3 M
- **10xPBS (pH = 7):** clorur sòdic (NaCl) 1,3 M, hidrogenofosfat sòdic ($Na_2HPO_4 \cdot 12H_2O$) 0,07 M, dihidrogenofosfat sòdic ($NaH_2PO_4 \cdot 2H_2O$) 0,03 M.

2.3 Mesura de la quantitat de DNA nuclear mitjançant citometria de flux

S'ha trossejat amb una fulleta d'afaitar alhora porcions de fulles joves de la mostra a problema a analitzar i del patró escollit per a fer les mesures (*Petunia hybrida* 'PxPc6' 2C = 2,85 pg, Marie & Brown, 1993). Generalment s'utilitza més o menys la mateixa quantitat de mostra de l'espècie problema (uns 25 mm^2 aproximadament) que del patró intern. Sempre que ha estat possible, de cada població estudiada hem analitzat cinc individus i s'han realitzat dues mesures independents per a cada individu. El procés de trossejat de les mostres s'ha dut a terme en una placa de Petri amb 600 µl de tampó de lisi LB01 (Doležel et al., 1989) per a provocar l'alliberament dels nuclis cel·lulars. La suspensió de nuclis en el tampó d'extracció s'ha filtrat amb una malla de niló d'un diàmetre de porus de 30 µm, amb la finalitat d'eliminar qualsevol traça de resta vegetal que pogués obturar el capil·lar del citòmetre. Un cop preparada i filtrada la suspensió de nuclis s'ha tenyit amb el fluorocrom d'elecció, iodur de propidi (1 mg/ml; Sigma-Aldrich Química), del qual se n'hi afegeixen 36-40 µl, amb una concentració final de 60 µg/ml. S'hi ha afegit ribonucleasa A (100µg/ml; RNasa A Boehringer) i s'han conservat en gel durant aproximadament 15-20 min abans de mesurar-les.

Els assaigs de citometria de flux s'han realitzat als Centres Científics i Tecnològics de la Universitat de Barcelona, amb el citòmetre Epics XL (Coulter Corporation). L'instrument ha estat preparat en la configuració estàndard: l'excitació de la mostra s'ha fet utilitzant com a font de llum un làser d'argó a baixa intensitat (488 nm, 15 mW) i refrigerat amb aire. S'han adquirit les dades proporcionades pel detector de FSC (*forward scatter*), que capta la llum dispersada en posició central, pel detector de SSC (*side scatter*), que capta la llum dispersada a 90º, i les de fluorescència vermella (620 nm) del iodur de propidi, el fluorocrom emprat.

Tampó de lisi LB01 (Doležel et al., 1989)**:** 15 mM Tris, 2 mM Na_2EDTA, 0,5 mM espermina 4HCl, 80 mM KCl, 20 mM NaCl, 0,1 % (v/v) Triton X-100. Ajustar el pH a 7,5. Afegir β-mercaptoetanol a una concentració final 15 mM.

2.4 Filogènia molecular i genètica poblacional

Extracció de DNA (mètode de Doyle & Doyle (1987)); amb modificacions de Soltis et al. (1991) i Cullins (1992). L'extracció de DNA s'ha dut a terme en material fresc, dessecat amb gel de sílice o de plecs d'herbari, que es trobava en bon estat de conservació. S'han pesat aproximadament 10 mg de material i posteriorment s'han homogeneïtzat mitjançant un aparell de trituració mecànica (Retsch GmbH). S'han afegit 500 μl de tampó d'extracció CTAB a la mostra que s'ha incubat d'una a dues hores a 65ºC. Posteriorment, les mostres s'han refredat en gel i s'han centrifugat durant 1 min a 13.300 rpm. S'han afegit 500 μl d'una mescla de triclorometà (cloroform) i alcohol isoamílic (24:1) i s'ha barrejat per inversió fins a obtenir una mescla homogènia. El contingut s'ha centrifugat durant 5 min a 13.300 rpm per a recollir el sobrenedant i transferir-lo a un nou tub. Aquest pas s'ha tornat a repetir afegint 500 μl més de la mescla de triclorometà i alcohol isoamílic, i s'ha tornat a centrifugar 5 min a 13.300 rpm. Un cop més, el sobrenedant s'ha recuperat i transferit a un nou tub d'1,5 ml. Amb la finalitat de precipitar el DNA, s'han afegit acetat amònic 3 M i isopropanol fred en les proporcions adequades segons el volum que s'ha recuperat en el pas anterior. Les mostres s'han centrifugat durant 3 min a 13.300 rpm i s'ha retirat el sobrenedant amb una pipeta Pasteur amb cura de no perdre el precipitat de DNA. Després s'ha afegit 1 ml d'etanol de 70º fred, s'ha mesclat per inversió amb la intenció de rentar el precipitat, i s'ha centrifugat durant 3 min a 13.300 rpm. L'etanol s'ha retirat dels tubs amb una pipeta Pasteur i les mostres s'han deshidratat durant 40-60 min al buit. El DNA s'ha tornat a suspendre afegint 50 μl de tampó TE i/o d'aigua desionitzada i incubant les mostres a 45ºC durant 20 min. En molts casos, les mostres han estat purificades usant el kit QIAquick (Qiagen), tot seguint les especificacions del proveïdor. La concentració i la qualitat del DNA han estat determinades amb un espectrofotòmetre NanoDrop ND-1000 (ThermoScientific).

Amplificació, purificació i seqüenciació de les regions escollides del DNA nuclear i cloroplàstic. Les reaccions d'amplificació del DNA mitjançant PCR s'han realitzat

utilitzant diferents termocicladors [p. e. PTC-100, PTC-200 (MJ Research Inc., Massachusetts, EUA), G-Storm (GRI Labcare, Essex, Regne Unit)], en un volum de reacció total de 25 µl [encebadors 5 µM, dNTPs 1 mM, tampó 10x, $MgCl_2$ 25 mM i 0,5 unitats de *Taq* polimerasa (Sigma), on s'han afegit aproximadament 50 ng de DNA genòmic]. Els productes de PCR han estat purificats mitjançant QIAquick PCR Purification Kit (Qiagen). Un cop purificat el DNA, la reacció de seqüenciació s'ha realitzat en un volum de reacció de 20 µl (tampó de seqüenciació 5x, tampó de seqüenciació RR-100, encebador 5 µM, 1-1,5 µl de DNA purificat). La seqüenciació directa dels fragments amplificats s'ha realitzat utilitzant Big Dye Terminator Cicle sequencing v. 3.1 (PE Biosystems), en un ABI Prism 3700 DNA analyzer (PE Biosystems) a les instal·lacions de la Unitat de Genòmica dels Centres Científics i Tecnològics de la Universitat de Barcelona. Les seqüències de DNA s'han editat mitjançant el programa Chromas Lite v. 2.01 (Technelysium Pty Ltd.) i s'han alineat manualment amb el programa Bioedit v. 7.0 (Hall, 1999).

- **Regions del DNA analitzades.** Se seqüenciaren les regions (ITS i ETS) del DNA ribosòmic nuclear i les regions (*rpl32-trnL, rpoB-trnD, rps16-trnK i trnS-trnC*) del DNA cloroplàstic, essent aquestes últimes les que mostraren els nivells més alts de polimorfisme d'entre totes les regions provades (*ndhF-rpl32, prbA-trnH, psbD-trnT, psbE-PeT, rpl32-trnL, rps16-trnK, trnD-rpoB, trnK-matK, trnL-trnF, trnQ-5'rps16, trnS-trnC, trnS-trnfM, trnT-trnG i trnV-ndhC*).

Protocol d'amplificació de DNA amb la tècnica d'AFLP. Aquest protocol està basat en la metodologia descrita per Vos et al. (1995) en concordança amb l'*AFLP Plant Mapping Protocol* (Applied Biosystems). S'han usat els enzims EcoRI i MseI per a digerir 500 ng de DNA per mostra. S'han provat 18 combinacions d'encebadors en sis individus de tres poblacions diferents per a discriminar aquelles combinacions que aportaren un major nivell d'informació. S'han seleccionat els tres parells d'encebadors que han proporcionat un patró més polimòrfic: *EcoRI*-CTT/*MseI*-AC; *EcoRI*-CTC/*MseI*-AA; and *EcoRI*-CAG/*MseI*-AT. L'èxit de cada pas ha estat verificat mitjançant una reacció PCR dels productes generats en un gel d'agarosa a l'1,5%. Els fragments han

estat posteriorment analitzats en un ABI Prism 3100 Genetic Analyzer (Applied Biosystems) amb 10 ml de High Dye i 0,2 ml de GeneScan 500 ROX Size Standard per mostra. Els fragments amplificats han estat genotipats en base a la seva presència/absència usant GenMarker AFLP/Genotyping software v. 1.9 (SoftGenetics). S'ha dut a terme un cribratge inicial, usant els al·lels compresos en un rang de 50-490 pb. Seguidament, s'ha realitzat una correcció visual per a eliminar aquells pics de baixa intensitat o amb poca reproductibilitat. La taxa d'error dels AFLP ha estat calculada seguint Bonin et al. (2004). Vint-i-cinc mostres aleatòries per combinació d'encebadors han estat replicades per tal d'assegurar la reproductibilitat de la tècnica. Aquells al·lels amb una taxa d'error del 5% han estat eliminats. Per tal de verificar l'índex d'homoplàsia per mida de fragment, s'ha calculat la correlació entre la mida del fragment d'AFLP i la seva freqüència usant AFLP-SURV v. 1.0 (Vekemans et al., 2002).

2.5 Anàlisis filogenètiques, filogeogràfiques i de datació molecular

Les anàlisis filogenètiques de les matrius de dades nuclears i cloroplàstiques s'han dut a terme mitjançant inferència bayesiana amb MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003). Abans de concatenar les diferents regions nuclears i cloroplàstiques, s'ha comprovat la seva congruència mitjançant el test ILD (Farris et al., 1994) implementat a PAUP. En cada cas, la selecció del millor model evolutiu de les seqüències s'ha basat en el criteri d'informació d'Akaike implementat a jModelTest (Posada, 2008). Els arbres consens que han resultat de les anàlisis d'inferència bayesiana s'han visualitzat mitjançant el programa FigTree v. 1.3.1 (Rambaut & Drummond, 2009).

Els temps de divergència de les espècies de *Cheirolophus* s'han obtingut utilitzant inferència bayesiana amb rellotge relaxat implementada a BEAST v. 1.7.1 (Drummond et al., 2012). L'anàlisi s'ha dut a terme només amb el conjunt de dades de DNA nuclear, per la falta de variabilitat i per la baixa resolució que havia mostrat el conjunt de dades del DNA cloroplàstic a nivell filogenètic d'espècie. L'elecció dels paràmetres s'ha basat en els mètodes de *Path Sampling* (PS) i *Stepping Stone* (SS)

sampling de BEAST, que han demostrat que superen altres estimadors de probabilitat en termes de consistència (Baele et al., 2012).

Per a l'anàlisi de datació, el mostreig s'ha limitat a només un individu per tàxon (incloent subespècies). Tracer v. 1.4 (Rambaut & Drummond, 2007) s'ha utilitzat primer per a comprovar la convergència i la barreja de cada paràmetre i, a continuació, per a confirmar una grandària efectiva de la mostra (ESS) raonable de cada paràmetre (és a dir, valors d'ESS > 200, després d'excloure una fracció de *burn-in* del 10%). Els arbres s'han consensuat en un arbre de màxima credibilitat (MCC) obtingut amb TreeAnnotator v. 1.6.2 (Drummond & Rambaut, 2007) i han estat visualitzats amb el programa FigTree v. 1.3.1 (Rambaut & Drummond, 2009). Com que no es disposa de registre fòssil conegut per a *Cheirolophus,* l'estimació del temps de divergència absolut dels llinatges s'ha obtingut a partir de punts de calibratge secundaris de la filogènia molecular de Barres et al. (2013). Aquest estudi es basà en cinc punts de calibratge diferents, incloent fòssils recentment descoberts d'*Asteraceae*, i constitueix l'anàlisi de datació més completa de la tribu *Cardueae* actualment.

S'ha utilitzat una àmplia gamma de programes estadístics implementats en el llenguatge de programació R (http://www.R-project.org, R Development Core Team 2012) per a analitzar el temps i el mode de diversificació d'espècies en el gènere *Cheirolophus.* El paquet d'R TreePar v. 2.1 (Stadler, 2011) s'ha usat per a detectar els canvis temporals en les taxes de diversificació. El TreePar pot detectar canvis temporals en les taxes de diversificació, però no permet detectar la variació de la taxa de diversificació entre els llinatges. Per aquest motiu, alternativament s'ha usat MEDUSA (Alfaro et al., 2009), implementat al paquet GEIGER (Harmon et al., 2008) d'R per a localitzar la posició d'aquests tipus de variacions a la filogènia.

Per a inferir la història filogeogràfica de *Cheirolophus* s'han construït xarxes d'haplotips per a explorar visualment la diversitat genètica dins de cada espècie amb el programari TCS v. 1.21 (Clement et al., 2000). Les insercions/delecions de més d'un parell de bases han estat recodificades com a mutacions de parells de bases i aquestes insercions/delecions (indels) han estat tractades com un cinquè estat. Les anàlisis filogeogràfiques per a establir l'origen i el moment de les diferents colonitzacions a

l'arxipèlag han estat realitzades mitjançant el model *discrete-state continuous-time Markov chain* (CTMC) (Lemey et al., 2009) implementat a BEAST v. 1.6.2.

2.6 Anàlisis de diversitat genètica i estructura poblacional basades en els AFLPs

Per a estimar la diversitat genètica de cada població amb més d'un individu s'han calculat els següents paràmetres: a) al·lels privats; b) al·lels rars, presents en <10% de les mostres; i c) heterozigosi no esbiaixada (Hj), tots ells calculats utilitzant TFPGA v. 1.3 (Miller, 1997). Altres mesures de diversitat genètica s'han estimat a través de: (i) l'índex DW de Schönswetter & Tribsch (2005) utilitzant AFLPDAT (Ehrich, 2006); (ii) la riquesa de bandes (Br), que és el nombre de fenotips esperats en cada *locus*, i pot ser interpretat com un anàleg de la riquesa al·lèlica, que oscil·la entre 1 i 2 (Coart et al., 2005); i (iii) el percentatge de *loci* polimòrfics (PLP) amb una significació de l'1% (P = 0,99). Els índexs Br i PLP s'han calculat d'acord amb el mètode de rarefacció de Hurlbert (Petit & El Mousadik, 1998) i van ser condicionats a la mida de la població més petita (N = 3) mitjançant v. 1.0 AFLPDIV (Coart et al., 2005). Com a conseqüència, Br i PLP no s'han calculat per a les poblacions que tenien menys de tres individus. La incidència del possible mostreig de clons en aquestes anàlisis es va estudiar mitjançant la funció Clones del programa AFLPDAT (Ehrich, 2006). Els valors de FST per a cada parell de poblacions incloses s'han estimat utilitzant AFLP SURV v. 1.0 (Vekemans et al., 2002). La significació d'aquests s'ha avaluat amb 10.000 permutacions. El coeficient d'endogàmia es va estimar mitjançant el programa I4A (Chybicki et al., 2011). El test de Mantel s'ha utilitzat per a avaluar l'efecte de la distància espacial en les diferències genètiques entre les poblacions. Aquest test s'ha calculat mitjançant el paquet Vegan (Oksanen et al., 2008) implementat al programa R, fent servir els valors de distància FST entre poblacions. L'autocorrelació de l'estructura genètica espacial també es va estimar mitjançant el preograma SPAGeDi (Hardy & Vekemans, 2002).

L'estructura genètica de les poblacions s'ha analitzat mitjançant mètodes d'agrupament com STRUCTURE (Hubisz et al., 2009) o BAPS (Corander & Marttinen,

2006). A més, s'han usat mètodes d'ordenació com l'anàlisi de coordenades principals (PCoA), implementada a R, i mètodes d'anàlisi filogenètica en xarxes com el *Neighbor-Net* (Bryant & Moulton, 2004) executat al programa SplitsTree v. 4.12.08. L'anàlisi molecular de la variància (AMOVA) s'ha calculat mitjançant el programa ARLEQUIN v. 3.5. (Excoffier et al., 2005). Finalment, la correlació entre les diferències morfològiques i les diferències genètiques de les espècies macaronèsiques s'ha estudiat mitjançant una anàlisi de components principals (PCA) i mitjançant una anàlisi generalitzada de la variància molecular (GAMOVA, Nievergelt et al., 2007), totes dues implementades a R.

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COMPENDI DE PUBLICACIONS

• **Genome 2012, 55, 529–535**

Eixam de 35S terminals en *Cheirolophus* (*Asteraceae*, *Centaureinae*)

Teresa Garnatje, Oriane Hidalgo, Daniel Vitales, Jaume Pellicer, Joan Vallès, Odile Robin, Sònia Garcia i Sonja Siljak-Yakovlev

Les radiacions insulars constitueixen una plataforma de treball per a estudiar la diversificació de les espècies, fet que durant molt temps, i també avui en dia, ha fascinat investigadors en la matèria. El fet que sols una petita fracció dels representants dintre del grup de colonitzadors insulars estigui involucrat en aquest procés, la qüestió que es planteja és si alguns factors poden contribuir a la capacitat d'un tàxon per a radiar. *Cheirolophus* es l'únic gènere de la tribu de les *Centaureinae* que ha experimentat una radiació a les illes Canàries. La caracterització citogenètica a través de FISH dels gens de l'RNA ribosòmic 5S i 35S en vuit espècies de *Cheirolophus* continentals i de les illes Canàries revelaren un nombre alt i inusual de 35S majoritàriament en posició terminal, amb un únic *locus* 5S de DNAr en posició intersticial en tots els tàxons estudiats. Aquesta abundància de senyals de DNAr 35S és únic entre les *Centaureineae* i precedeix l'arribada de *Cheirolophus* a les illes Canàries. Discutim doncs, el possible lligam entre els perfils de DNAr amb el procés de radiació, tot comparant els resultats amb dos altres casos relacionats, el grup *Rhaponticum* i el gènere *Centaurea.*

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Swarm of terminal 35S in *Cheirolophus* (Asteraceae, Centaureinae)

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Abstract: Island radiation constitutes a playground for species diversification, which has long fascinated researchers and still does today. Because only a small subset of taxa within the pool of island colonizers is concerned by this process, the question is raised on whether some factors could make a taxon prone to radiate. *Cheirolophus* is the only genus of Centaureinae subtribe to have experienced a radiation in the Canary Islands. Cytogenetic characterization through FISH of 5S and 35S ribosomal RNA genes in eight *Cheirolophus* species from continent and Canary Islands revealed an unusually high number of 35S predominantly at terminal position, together with a single interstitial 5S rDNA locus in all the studied taxa. Such an abundance of 35S rDNA signals is unique among Centaureinae and predates *Cheirolophus* arrival in Canary Islands. The possible link of the rDNA profile with radiation process is discussed through a comparison with two other case studies, the closely related *Rhaponticum* group and the genus *Centaurea*.

Key words: Asteraceae, Canary Islands, fluorescent in situ hybridization (FISH), fluorochrome banding, radiation.

Résumé : La radiation adaptative en milieu insulaire constitue un matériel d'exception pour étudier la diversification des espèces, qui fascine depuis toujours les chercheurs. Ce phénomène concernant seulement une infime partie des taxons parmi l'ensemble des espèces atteignant les îles, la question se pose de savoir s'il existerait des facteurs qui pourraient rendre un taxon plus enclin à entreprendre une radiation. *Cheirolophus* est le seul genre des Centaureinae à présenter une radiation dans les îles Canaries. La caractérisation cytogénétique des gènes ribosomiques 5S et 35S par la technique d'hybridation in situ fluorescente (HISF), chez huit espèces de *Cheirolophus* du continent et des îles Canaries, a mis en évidence que toutes les espèces étudiées présentent un nombre de 35S particulièrement élevé, presque exclusivement en position terminale, ainsi qu'un unique locus 5S, en position interstitielle. Une telle abondance de signaux 35S est unique parmi les Centaureinae, et a précédé l'arrivée de *Cheirolophus* dans les îles Canaries. La possibilité d'une relation entre le profil d'ADNr et le processus de radiation est discutée par comparaison avec deux autres cas d'étude, le groupe *Rhaponticum*, phylogénétiquement proche de *Cheirolophus*, et le genre *Centaurea*.

Mots-clés : Asteraceae, Iles Canaries, hybridation in situ fluorescente (HISF), coloration différentielle aux fluorochromes, radiation.

Introduction

Because they constitute a privileged material for studying species diversification, island radiations have allowed crucial advances in evolutionary understanding, starting by Galapagos finches that inspired Darwin's theory of evolution (Darwin 1859). Phylogenetic studies have evidenced a great number of radiations in plants, most of them from islands (Baldwin 2007; Guzmán and Vargas 2010). The mechanisms taking place during radiation at genome level remain, however, largely unknown. The same applies for the characteristics that make a given taxon more prone to radiate or, in contrast, prevent it from undergoing this process. Being by definition a fast process, radiation usually combines high morphological diversity and low molecular divergence, hampering to trace the temporal sequence of morphological evolution. In this context, determining if a trait is possibly a cause, a consequence, or has no relationship with radiation is often challenging. Radiations have been poorly studied from the cytogenetic perspective, although this approach would certainly contribute to a better understanding of this process (Mandáková et al. 2010).

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Table 1. Provenance of the populations of *Cheirolophus* studied.

Species	Origin
Ch. benoistii (Humbert) Holub	Morocco, Ksar es Souk: south side of Tizi n'Talrhem, Garnatje, Susanna 1787 and Vilatersana, 17 June 1997
Ch. burchardii Susanna	Spain, Canary Islands, Tenerife: between Buenavista and Teno, Susanna 1430, 6 July 1990
Ch. canariensis (Brouss. ex Willd.) Holub	Spain, Canary Islands, Tenerife: Masca ravine, Garnatje 1 and Luque, August 1996
Ch. gomerythus (Svent.) Holub	Spain, Canary Islands, La Gomera: near Agulo, San Marcos ravine, Susanna 1426, 4 July 1990
Ch. junonianus (Svent.) Holub	Spain, Canary Islands, La Palma: Fuencaliente, volcano of San Antonio, Susanna 1423, 28 July 1990
Ch. massonianus (Lowe) A.Hansen & Sunding	Portugal, Botanical Garden of Madeira
Ch. uliginosus (Brot.) Dostál	Spain, Huelva: Mazagón, El Loro, Garcia-Jacas, Molero, JM Montserrat 1875, Susanna and Veny, 6 July 1998
Ch. cf. *webbianus* (Sch.Bip.) Holub	Spain, Canary Islands, Tenerife: Taganana, roque de las Ánimas, Garnatje 3 and Luque, August 1996

Note: Herbarium vouchers are deposited at the Institut Botànic de Barcelona (BC).

We have selected for a physical genome study the genus *Cheirolophus* Cass. of the Centaureinae subtribe (Cardueae, Asteraceae), which has profusely radiated towards the Canary Islands (Bramwell and Bramwell 2001). The genus comprises 25 species occurring in the western Mediterranean and Macaronesia (Susanna and Garcia-Jacas 2007), with 17 species that inhabit Canary Islands (Bramwell and Bramwell 2001), all of them endemic (Hansen and Sunding 1993). *Cheirolophus* is related to other basal genera of the subtribe (Garcia-Jacas et al. 2001; Hellwig 2004), with which it shares a number of morphological, palynological, and karyological features (Wagenitz and Hellwig 1996), such as the absence of radiant peripheral florets, cypselae with basal hilum, pollens of the *Serratula* type, and a base chromosome number > 12. One of those close relatives is the genus *Rhaponticum* Vaill., which, interestingly, has only a single representative in Tenerife. *Cheirolophus* and *Rhaponticum* are both diploid (Garnatje et al. 2007; Hidalgo et al. 2007). Despite their close phylogenetic relationship, ploidy level, and morphological similarities, the latter did not radiate across the Canary Islands, being the unique representative in the archipelago *R. canariense* DC. (= *Stemmancantha cynaroides* (C.Sm.) Dittrich), which is in addition considered endangered (Bañares et al. 2004). Another point of comparison is constituted by the largest genus of the Centaureinae, *Centaurea* L., which is extensively spread in the Mediterranean region (~200 species; Hellwig 2004), but not in the Canary Islands where it is, however, present (with seven species and two subspecies, although only *C. conocephala* Bolle is endemic; Hansen and Sunding 1993).

The fluorochromes chromomycin A_3 (CMA) and 4′,6-diamidino-2-phenylindole (DAPI) exhibit preferential staining for GC- and AT-rich DNA sequences, respectively, allowing the identification of different types of heterochromatin (Las Peñas et al. 2009). They have also been largely used for systematic and evolutionary purposes, especially in Asteraceae, with data available for some *Centaurea* (Dydak et al. 2009) and *Rhaponticum* group species (Hidalgo 2006; Hidalgo et al. 2008). The development of fluorescent in situ hybridization (FISH) allowed the detection of labelled DNA probes to homologous chromosomal targets (Schwarzacher and Heslop-Harrison 2000; Schwarzacher 2003). The probes for the 5S and 18S-5.8S-26S (hereafter 35S) rDNAs, tandemly arranged DNA repeats highly conserved among plants, have been and continue to be extensively used as tools for karyotyping, as well as markers to detect chromosome rearrangements in an evolutionary context (Garcia et al. 2012, and references therein). The ribosomal genes are among the most highly expressed genes (Abirached-Darmency et al. 2005), and particularly, some 35S rDNA loci form the nucleolar organizer regions (NORs).

In this context, the main goals of the present work are (*i*) to describe the physical pattern of distribution of both rDNA and heterochromatin in some species of *Cheirolophus* from Macaronesia, (*ii*) to analyse possible changes in the number and distribution of rDNA loci confronting continental and island representatives of the genus, and (*iii*) to compare the results obtained in *Cheirolophus* with the pattern previously reported in representatives of the *Rhaponticum* group and the genus *Centaurea*.

Materials and methods

The plants studied come from cypselae, collected in the field. Table 1 shows the provenance of all species investigated. We chose six island species and two continental species of *Cheirolophus*. Protoplast preparation, CMA banding, and FISH were carried out following the procedures described in Garnatje et al. (2004). The probe used for 35S rDNA detection was a 0.8 kb TaqI fragment of 18S gene of wheat (Richard et al. 1995) isolated from pTa71 and cloned in pUC18. This probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) using the polymerase chain reaction (PCR). The probe used for 5S rDNA location was a clone of a pTa794, a 410 bp BamHI fragment of 5S rDNA isolated also from wheat and cloned into pBR322 (Gerlach and Dyer 1980). It contains the 5S rRNA gene (120 bp) and the noncoding intergenic spacer (290 bp). This probe was labelled with Fluoro-Red-dUTP (Amersham) by PCR.

Results and discussion

Pattern of rDNA in island vs. continental species of *Cheirolophus*

All *Cheirolophus* species studied present the 35S and 5S genes located in separated chromosome loci (Fig. 1), which is the most common rDNA organization in flowering plants, i.e., they do not belong to the nearly 25% of the Asteraceae species that might have a linked arrangement of rRNA genes (Garcia et al. 2010), as first described in the genus *Artemisia* (Garcia et al. 2009*a*, 2009*b*). This result is consistent with the ribosomal gene configuration found so far in other Centaureinae (genera *Callicephalus*, *Myopordon*, *Oligochaeta*, and *Rhaponticum*; Hidalgo 2006; Hidalgo et al. 2008; and *Centaurea*; Dydak et al. 2009), which extends to other more distant genera outside Centaureinae but within tribe Cardueae, such as *Amphoricarpos*, *Chardinia*, *Siebera*, *Xeranthemum* (Garnatje et al. 2004), *Arctium*, *Atractylodes*, *Berardia*, and *Cnicus* (Garcia et al. 2010).

Results from the rDNA FISH revealed that both island and continental *Cheirolophus* representatives display a surprisingly high number of usually coincident (compare Figs. 1*f* and 1*m*) CMA+ bands and 35S sites, predominantly located at terminal position (Table 2; Fig. 1). It is tempting to relate this 35S pattern of *Cheirolophus*, unique among Centaureinae (Hidalgo 2006; Hidalgo et al. 2008; Dydak et al. 2009), to the island radiation, which is also unique among the subtribe. However, this high number of 35S loci was not generated during the course of the radiation, since the continental (e.g., *Ch. benoistii* (Humbert) Holub, *Ch. uliginosus* (Brot.) Dostál) and the island *Cheirolophus* species have comparable numbers of 35S sites (Table 2; Fig. 1), in all cases higher than those of the genera in its subtribe. Therefore, the abundance of terminal 35S predated the radiation in Canary Islands. If a relationship between 35S pattern and radiation existed, it might consist in a positive effect of the 35S locus pattern promoting the radiation of *Cheirolophus*. This connection should be taken most carefully, given the scarcity (or absence) of available data linking radiation processes to cytogenetic patterns, but in any case it constitutes the first evidence of rDNA arrangement in a group of plants exhibiting island radiation and might indicate a starting point in this sense.

Consistent with previous results accounting for chromosomal stasis in island radiation (Mandáková et al. 2010), *Cheirolophus* radiation did not proceed through changes in chromosome number or ploidy level. However, species-specific rDNA patterns (Table 2; Fig. 1) and genome size decrease (Garnatje et al. 2007) evidence a certain degree of genomic reshuffling during *Cheirolophus* radiation. All island species of *Cheirolophus* share a common life form, and their ecological conditions are very similar as well. Differences mainly concentrate in the diversity of habitats colonized in the islands, with habitat adaptation being a clue in the *Cheirolophus* radiation process. Is the increased number of 35S sites somehow related to the ability to colonize these new habitats? Is this capability related to an overall increase of NORs activity? The single species of the genus *Cheirolophus* showing special features regarding the life form is *Ch. uliginosus*, a hemicryptophyte occurring along the Atlantic shore of the Iberian Peninsula, whereas the remaining species are phanerophytes or nanophanerophytes. In addition, this species is closely related to freshwater habitats (D. Vitales, J. Pellicer, J. Vallès, and T. Garnatje, unpublished data). The cytogenetic pattern of this species shows that the number of 35S sites is considerably lower (12) than in the other studied species. Two main reasons might be evoked. The first one relates to the fact that *Ch. uliginosus* is, along with *Ch. crassifolius* (Bertol.) Susanna, the possible earliest-diverging species within the genus (Garnatje et al. 2007). Therefore, its number of 35S sites would be representative of the ancestral state of the genus. Again, there is no literature available about any relationship between rDNA locus number and ancestry or late divergence of a given species, and patterns may be different in different plant groups. The second reason has to do with the threatened status of the species because of the loss of its habitat (D. Vitales, J. Pellicer, J. Vallès, and T. Garnatje, unpublished data), which may somehow be related to a 35S rDNA loci loss. However, before drawing conclusions it should be investigated whether the low number of 35S sites is restricted to the population under study, whether it took place only in impoverished populations, or whether it constitutes a general pattern in the species. Interspecific and intraspecific variability in number and location of the ribosomal genes have been observed in many plant species and also between and within cultivars (e.g., in grain legumes; Abirached-Darmency et al. 2005, and references therein). Furthermore, it has been shown that populations under ecological stress may show a rDNA FISH pattern divergent from remaining ones (e.g., on peridotite–serpentine soils, populations of *Lilium* L. present an increased number of rDNA loci with respect to those growing in regular substrates; Muratović 2007; in populations of *Narcissus* L., changes have also been detected in this regard; F. Pustahija and S. Siljak-Yakovlev, unpublished data). Further cytogenetic studies involving *Ch. crassifolius*, previously mentioned as one of the first species to have diverged within the genus (Garnatje et al. 2007), might help to shed light on this question, since it would permit to confirm the primitive state for the number of 35S sites in *Cheirolophus*.

Patterns of rDNA in *Cheirolophus* vs. *Rhaponticum* and related genera

With only four or six 35S marks (Hidalgo 2006; Hidalgo et al. 2008), the genus *Rhaponticum* shows much fewer 35S sites than *Cheirolophus* (12–20; Table 2). Within *Rhaponticum*, the third 35S locus is restricted to species that have experienced hybridization (*R. heleniifolium* Gren. & Godr. and *R. scariosum* Lam.; Hidalgo 2006) or dysploidy (*R. carthamoides* (Willd.) Iljin; Hidalgo et al. 2008), in all cases related to recent chromosome restructurings. Strong genome reorganization leading to the multiplication and scattering of 35S sites (from 4 to 12) has been evidenced in the genus *Oligochaeta* K.Koch (Fig. 1*v*) of the *Rhaponticum* group, as triggered by a drastic genome size reduction (Hidalgo et al. 2008). Beside shifts in genome size, dysploidy, and increase of 35S loci, the chromosome restructurings in the *Rhaponticum* group were also associated with more 35S loci at intercalary positions (Hidalgo et al. 2008). Although the most common position of 35S rDNA loci is terminal, the location of all 35S sites in *Oligochaeta* was always intercalary (Hi-

Fig. 1. (*a*–*f*) Fluorescent in situ hybridization metaphase plates of both 5S (bluish-green) and 35S (red) rDNA. (*g*–*n*) Chromomycin A_3 banding. (*o*–*v*) Schematic representation of the number and location (terminal versus interstitial position) of chromomycin marks (yellow), 5S (green), and 35S (red) rDNAs on the haploid set of chromosomes. (*a*, *g*, *o*) *Cheirolophus benoistii*, (*b*, *i*, *p*) *Ch. canariensis*, (*c*, *j*, *q*) *Ch. gomerytus*, (*d*, *k*, *r*) *Ch. junonianus*, (*e*, *l*, *s*) *Ch. massonianus*, (*f*, *m*, *n*, *u*) *Ch.* cf. *webbianus*, (*h*) *Ch. burchardii*, (*t*) *Ch. uliginosus* (dotted line chromosome indicates the 16th pair of the ~32 chromosome count), (*v*) *Oligochaeta divaricata* (from Hidalgo et al. 2008) is included here for comparative purposes (asterisks indicate either entire chromosomes or possible chromosome fragments, as the species is $2n = 24$). Scale bars = 10 µm.

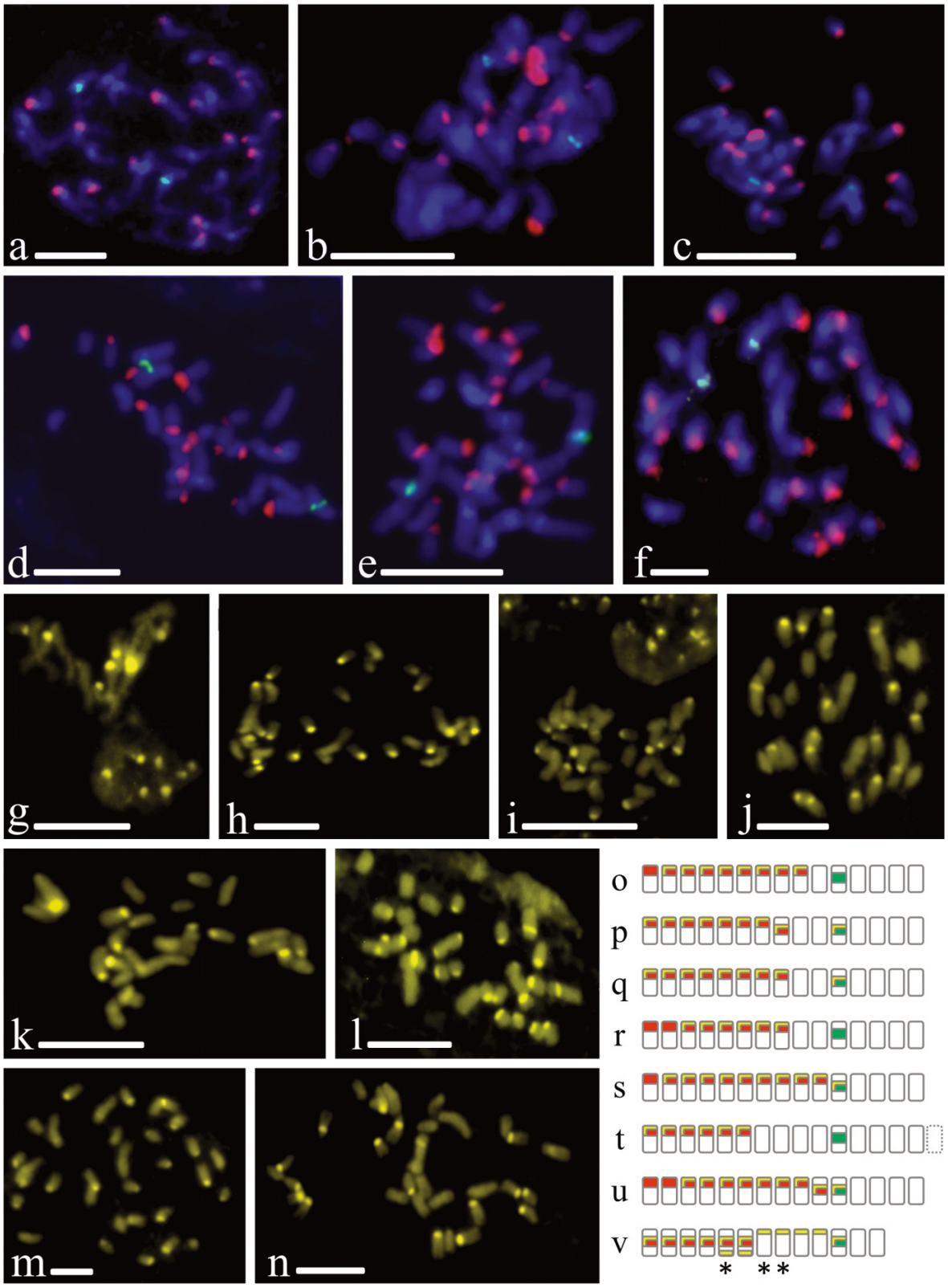

Table 2. Number of chromosomes ($2n$), genome size (2C value), number of chromomycin A_3-positive bands (CMA), and 35S and 5S rDNA sites for the species studied.

Species	$2n$	2C (pg)*	CMA	35S sites	5S sites
Ch. benoistii	30	1.55	16	18	2 (2i)
Ch. burchardii	30	1.42	16	—	—
Ch. canariensis	30	1.38	18 (4i)	16 (2i)	2 (2i)
Ch. gomerythus	30	1.41	18 (2i)	16	2 (2i)
Ch. junonianus	—	1.37	12	16	2 (2i)
Ch. massonianus	30	1.44	20 (2i)	20	2 (2i)
Ch. uliginosus	~32	1.69	12	12	2 (2i)
Ch. cf. *webbianus*	30	1.38	18 (4i)	20 (2i)	2 (2i)

Note: Indication of positive signals with interstitial position (i) is provided.
*Garnatje et al. (2007).

dalgo et al. 2008; Fig. 1), seeming randomly scattered by chromosome restructurings.

The increased number of 35S sites in *Cheirolophus* with respect to close relatives was not coupled with changes either in the chromosome number, all species being $x = 15$-based, or with recent polyploidization events. Multiplication of 35S loci neither took place at random position (Fig. 1). On the contrary, *Cheirolophus* 35S signals are almost exclusively terminal, which may suggest some kind of constraint or preference for this 35S positioning. In plants, terminally located 35S rDNA are predominant (Sousa et al. 2011), probably reflecting the prevalence of NORs in terminal and subterminal regions (Lima-De-Faria 1976). It is thought that preferential terminal location of 35S rDNA helps maintaining linkage groups during the frequent non-allelic recombinations affecting these genes, then preserving the genetic balance of the organisms (Sousa et al. 2011, and references therein).

As for the 5S rRNA genes, all studied species present a single locus located interstitially (although sometimes it is subterminal). The 5S rDNA sites usually have a more stable position among related taxa (Berjano et al. 2009, and references therein) and might be more frequently located at proximal region as well (Sousa et al. 2011). It is remarkable that in all the Cardueae studied up to now by FISH (Garnatje et al. 2004; Hidalgo 2006; Hidalgo et al. 2008; Dydak et al. 2009), the pattern is very similar, with much more 35S than 5S rDNA signals, in a constant or near constant number for a given genus and ploidy level, and mostly located interstitially. Also, in most of the studied cases, in this tribe as in *Cheirolophus*, the number of 5S rDNA loci of a genome is a reflection of their ploidy level.

A 35S site increase in *Cheirolophus*, associated with a genome size contraction, such as in *Oligochaeta*, cannot be established because of the lack of phylogenetic and genome size data. However, since *Cheirolophus* falls among the lowest C values in the Centaureinae (Garnatje et al. 2007, 2010, 2011; O. Hidalgo, M.À. Canela, T. Garnatje, J. Pellicer, S. Garcia, S. Siljak-Yakovlev, and J. Vallès, unpublished data) and even in the Cardueae (Garnatje et al. 2011), the hypothesis of a genome size decrease at the base of the genus is the more likely. Interestingly, island *Cheirolophus* species comprise the lowest genome sizes and the highest numbers of 35S sites (Table 2).

Patterns of rDNA in *Cheirolophus* vs. *Centaurea*

Unfortunately, except for the members of the *Rhaponticum* group, none of the other *Cheirolophus* relatives have been studied from the cytogenetic point of view. Only few data are available for some *Centaurea* species, a genus that has radiated in the continents of the Northern Hemisphere but not in the Macaronesian archipelagos. In the study of species belonging to the *Centaurea* genus (Dydak et al. 2009), there was a polymorphism in the number of 35S rDNA loci in tetraploids, with five to six pairs observed, one of them smaller than the remaining ones, and this phenomenon is also visible in *Cheirolophus* (Fig. 1). Ribosomal DNA sites may not be detected by FISH if their gene copy number is very low, owing to the limited sensitivity of the technique. Also, the number of copies can evolve very fast (Dydak et al. 2009), and some of them could be lost quickly. Transpositional events, chromosome reorganization, unequal crossing-over, or gene conversion are possible mechanisms explaining this process (Thomas et al. 2001; Raskina et al. 2004; Dydak et al. 2009). Siljak-Yakovlev et al. (2002) have demonstrated the relationship of rDNA loci with heterochromatic regions. The NOR regions probably favour rDNA mobility and affect both the evolution and expression of rDNA loci, although analysis of repetitive DNA in some species from Fabaceae have shown the absence of retroelement sequences in secondary constrictions (Abirached-Darmency et al. 2005).

The terminal position for the 35S rDNA is a common trait in *Cheirolophus* and *Centaurea* species. There are six sites in diploid species of *Centaurea* and 10–12 in the tetraploid species; four pairs are transcriptionally active. These data are not available for *Cheirolophus*, as it presents very small chromosomes that make secondary constrictions difficult to visualize. Usually the active NORs are in the secondary constriction and they are GC-rich, but cannot be revealed by CMA if they possess a low number of GC base pairs. This could explain the bigger number of 35S sites than CMA+ bands in *C. benoistii*, *C. junonianus*, and *C.* cf. *webbianus.* Regarding the 5S rDNA in *Cheirolophus* compared with *Centaurea*, again there are fewer 5S than 35S sites in both genera, and this number is, also in both genera, much more constant throughout the species studied, as in the other Cardueae previously studied.

Concluding remarks

This work reports the first data on rDNA physical mapping in *Cheirolophus* and reveals that in continental as well as island species there is a very high number of 35S marks,

almost exclusively at terminal position. Additionally, the number of 5S signals is constant, much lower than the number of 35S, and typically located interstitially. Because this genus is the only one within the Centaureinae to have radiated in the Canary Islands, a possible relationship between the two trends — island radiation and swarm of 35S — could be envisaged. This result, undoubtedly exciting, calls for more in-depth research, which would start by extending rDNA physical survey to more Centaureinae and also to more taxa having undergone island radiation. In this sense, this study constitutes the first step to a deeper understanding of island radiation processes from the perspective of molecular cytogenetics.

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Estructura genètica i germinació de llavors en poblacions portugueses de *Cheirolophus uliginosus* (*Asteraceae*): Implicacions per a la seva conservació

Daniel Vitales, Jaume Pellicer, Joan Vallès i Teresa Garnatje

Cheirolophus uliginosus és una espècie endèmica amenaçada de la costa atlàntica de la península Ibèrica, on ocupa unes poques localitats reduïdes. En el nostre estudi, analitzem els patrons de variació dels haplotips de DNA cloroplàstic i l'èxit reproductiu -capacitat de germinació- en set poblacions portugueses de mida diferent. L'èxit reproductiu de *Ch. uliginosus* s'ha examinat en relació amb l'estructura genètica i la mida de les seves poblacions. Els resultats indiquen una variabilitat intrapoblacional molt baixa per als marcadors cloroplàstics emprats. El nostre estudi mosta una taxa de germinació significativament reduïda en les poblacions petites (< 50 individus) respecte a aquelles de mida mitjana (50-250 individus) o gran (> 250 individus). Per a explicar aquest fenomen, s'han de tenir en compte les limitacions ecològiques i l'aïllament genètic. D'altra banda, en les poblacions de *Ch. uliginosus* més grans (> 250 individus) s'ha observat una incidència més acusada de la depredació de llavors abans de la seva dispersió, fet que podria estar afectant la seva resposta reproductiva. Finalment, les poblacions més petites -que presenten un èxit reproductiu reduït- contenen els haplotips més distants evolutivament i la seva conservació hauria de ser, per tant, prioritària.

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Genetic structure and seed germination in Portuguese populations of *Cheirolophus uliginosus* (Asteraceae): Implications for conservation strategies

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Abstract

Genetic structure and seed germination in Portuguese populations of *Cheirolophus uliginosus* (Asteraceae): Implications for conservation strategies.— *Cheirolophus uliginosus* is a threatened species, endemic to the Atlantic coast of the Iberian Peninsula, where it occupies a few restricted localities. In our study we analysed the patterns of cpDNA haplotypes variation and reproductive success—germinability—among seven Portuguese populations of varying size. The aim was to examine the reproductive performance of *Ch. uliginosus* related to genetic structure and population size. The results showed very low within-population variability of cpDNA markers. Our study indicates that the germination rate is significantly reduced in small populations (< 50 plants) whereas medium (50–250 individuals) and large-sized (> 250 individuals) do not show any constraint. In the search for plausible causes explaining the lower germination success in the smallest populations, ecological concerns and genetic isolation must be taken into account. Besides, in large-sized populations of *Ch. uliginosus* (> 250 plants) a higher incidence of predispersal seed predation was observed, maybe affecting their sexual reproductive response. Finally, smaller populations—presenting a reduced reproductive success—contain also the most evolutionary distant haplotypes, so their conservation should be a priority.

Key words: germination rate; habitat loss; haplotype structure; seed predation; small populations.

Resumen

Estructura genética y germinación de semillas en poblaciones portuguesas de *Cheirolophus uliginosus* (*Asteraceae*): Implicaciones para su conservación.— *Cheirolophus uliginosus* es una especie amenazada endémica de la costa atlántica de la península ibérica, donde ocupa unas pocas y reducidas localidades. En nuestro estudio, analizamos los patrones de variación de los haplotipos de ADN cloroplástico y el éxito reproductivo —capacidad germinativa— en siete poblaciones portuguesas de diferente tamaño. El éxito reproductivo de *Ch. uliginosus* se ha examinado en relación con la estructura genética y el tamaño de sus poblaciones. Los resultados indican una variabilidad intrapoblacional muy baja para los marcadores cloroplásticos utilizados. Nuestro estudio muestra una tasa de germinación significativamente reducida en las poblaciones pequeñas (< 50 individuos) respecto a aquellas de tamaño mediano (50-250 individuos) o grande (> 250 individuos). Para explicar este fenómeno, se deben tomar en consideración las limitaciones ecológicas y el aislamiento genético. Por otro lado, en las poblaciones de *Ch. uliginosus* de mayor tamaño (> 250 individuos) se ha observado una incidencia más acusada de la depredación de semillas antes de su dispersión, lo cual podría estar afectando a su respuesta reproductiva. Finalmente, las poblaciones más pequeñas —que presentan un reducido éxito reproductivo— contienen los haplotipos más distantes evolutivamente y su conservación debería ser, por tanto, prioritaria.

Palabras clave: depredación de semillas; estructura haplotípica; pérdida de hábitat; poblaciones pequeñas; tasa de germinación.

INTRODUCTION

Habitat loss is one of the main forces causing plant population decline and extinction (Brooks *et al.*, 2002). This phenomenon has a strong impact on small and isolated plant populations, leading to genetic deterioration and greater sensitivity to non-genetic forces such as environmental and demographic stochasticity (Gilpin & Soulé, 1986). These dynamics may result in an Allee effect, predicting that the primary outcome of a reduction in population size is a potential erosion in the fitness (Allee, 1931; Groom, 1998; Bataillon & Kirkpatrick, 2000; Higgins & Lynch, 2001). Many studies (e.g. Menges, 1991; Lamont *et al*., 1993; Fischer & Matthies, 1998; Paschke *et al.*, 2002; Hensen & Oberprieler, 2005) have compared certain fitness parameters, mainly seed production and germinability, between plants from small and large populations, finding a clear relationship between reproductive success and population size. Alternatively, other authors (Ouborg & van Treuren, 1995; Morgan, 1999; Costin *et al.*, 2001; Rabasa *et al.*, 2009) point to the existence of several factors that may mask or limit the impact of population size on reproduction capacity. Identifying factors involved in fitness response is a prerequisite for designing management plans to increase the size and persistence of endangered populations, thereby decreasing the probability of extinction (Pavlik, 1994).

Cheirolophus uliginosus (Brot.) Dostál is a hemicryptophyte with a habitat preference for the wetlands of the southwestern and western Iberian Peninsula (Fig. 1A), differing from the remaining representatives of the genus as it is the only herbaceous one. As a species closely associated with either permanent or intermittent water streams, it is very vulnerable to fluctuations in the water table. In relation to this water dependence, extensive plantations of eucalypts (*Eucalyptus globulus* Labill.) and crops such as corn (*Zea mays* L.), especially in Portugal, and strawberry [*Fragaria* ×*ananassa* (Weston) Duchesne ex Rozier] fields, nearby Doñana National Park (Andalusia, Spain), represent excessive water consumption leading to a decrease in the availability of this resource (Casermeiro *et al.*, 2002) for wild species. Watercourses are scarce and this, together with an increase in grazing and subsequent land nitrification, allows wetlands to be colonized by brambles (*Rubus ulmifolius* Schott) and ferns [*Pteridium aquilinum* (L.) Kuhn], which represent an excessive competition for *Ch. uliginosus* (Bañares *et al.*, 2009). Furthermore, flower heads of this species, as well as those from other congeners, are usually infested by insects, destroying achenes and capitula (Bañares *et al.*, 2009).

Presumably as a result of the above-mentioned threats, numerous localities cited during the early and middle 20th century by Rivas-Martínez *et al.* (1980) or Susanna (1993) have subsequently been revisited and have either not been rediscovered or have recently undergone a significant reduction in the number of individuals and/or extension. In fact, this species is included in the Spanish red book of threatened vascular flora (Bañares *et al.*, 2009) and classified as CR ("Critically Endangered") according to IUCN criteria. In contrast, since there does not exist any red list of threatened vascular flora in Portugal, there is a lack of knowledge about the conservation status of the species in this country. Given that Spanish populations of *Ch. uliginosus*—occurring in Andalusia and Extremadura regions—have already been the subject of specific conservation studies (Palacios-González *et al.*, 2008; Bañares *et al.*, 2009), we decided to focus our investigation on the lesser known Portuguese populations.

DNA-sequence analysis has a long-standing history in population and conservation biology research (for reviews, see Schlötterer, 2004; Höglund, 2009). Particularly, chloroplast DNA (cpDNA) haplotyping has been extensively used to infer genetic structure and diversity in endangered plant populations (Soltis *et al.*, 1992; El Mousadik & Petit, 1996; Ueno *et al.*, 2005; Saeki & Murakami, 2009; Zhao *et al.*, 2012). In our work, the molecular approach arises as a preliminary survey of genetic structure in *Ch. uliginosus* that may help us to interpret the results derived from reproduction components.

The present study investigates (1) the population genetic structure of *Ch. uliginosus* by using chloroplast haplotypes, (2) the actual population size, (3) the germinability of the seeds, and (4) the predispersal predation of the seeds. These data may help to find out the causes of the observed demographic decline and propose conservation strategies for this endangered species.

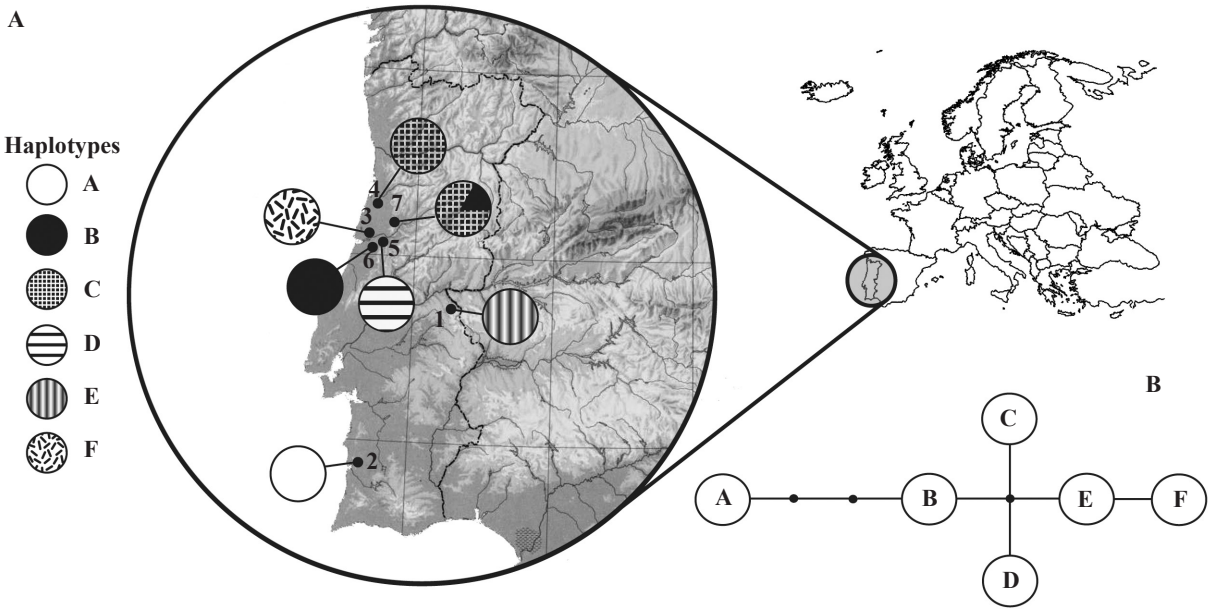

Figure 1. (A), geographical distribution of cpDNA haplotypes and studied populations of *Ch. uliginosus*. The population numbers correspond to those in Table 1, and the pie charts represent the percentage of each haplotype in each population; (B), statistical parsimony network showing relationships of the six plastid haplotypes. Each line between haplotypes indicates a mutational step, and black dots represent extinct or unsampled haplotypes.

MATERIALS AND METHODS

Sampling strategy

The sampling strategy was designed to cover a large range of the distribution of *Ch. uliginosus* to detect intra- and inter-population variability. Two field collections were carried out during the flowering season in 2009. Seven populations of *Ch. uliginosus* were sampled in Portugal (Fig. 1A), located within an altitudinal range from 0 to 705 m a.s.l. Detailed information of each locality and herbarium voucher is summarized in Table 1.

The number of individuals per population was not always precisely counted, because in dense populations it was difficult to distinguish between different individuals in dense clusters of rosettes. As *Ch. uliginosus* also reproduces vegetatively, the accurate estimation of population size at the genet level (i.e. group of genetically identical individuals) may be very difficult (e.g. Luijten *et al.*, 1996). Alternatively, population size was categorised adapting the IUCN criteria for scarce and declining populations (IUCN, 2011): small populations < 50 individuals, medium-sized populations with 50–250 individuals, and large populations > 250 individuals. The number of individuals of small-sized populations was visually counted one by one (Table 1). For larger populations, poor accessibility and/or leafy vegetation did not allow visual contact with all individuals so the approximate size was estimated according to apparent density and extension of populations.

For genetic analyses, we collected healthy leaves from each population studied in this work. We sampled one leaf per plant covering the entire population area—up to 13 individuals per population separated by more than one meter where possible— resulting in a total of 57 samples (see Table 1). Leaf material was immediately dried in silica gel and stored at room temperature (20–25ºC) until DNA extraction.

Achenes were taken from three to 20 plants per population depending on population size and taking into account the distribution of individuals. Seeds were dried in silica gel and stored at room temperature. The sampling of capitula was conducted during the autumn in populations 3–7 from Centro Region (Portugal). Approximately 40 flower heads were randomly collected from 20 individuals for each population. Capitula were also dried in silica

gel and stored at room temperature. Unfortunately, we were unable to sample capitula from the smallest populations [Reguengo (1) and Almograve (2)].

Germination tests

Full-sized, healthy-looking achenes were incubated at 20°C with a 12 h day-night photoperiod in Petri dishes with filter paper soaked with distilled water to saturation, following the recommendations of Garnatje (1995). Viable seeds are easily distinguished from unviable seeds (aborted seeds) by their colour and shape. The number of tested achenes per population ranged between 14 and 40 according to the available achene stock (with a maximum of 20 achenes per Petri dish). Germination was considered to be successful when a 1 mm-long radicle emerged from the achene; each achene that germinated was removed from the Petri dish. The number of achenes germinating was recorded daily during one month. In order to compare possible differences of germination vigour due to seasonality of achene field collections, germination tests of achenes from population 4 collected in August and November were carried out.

State of capitula

Randomly sampled flower heads from populations 3 to 7 of *Ch. uliginosus* were dissected and classified in three different categories according to their status: (1) good condition, (2) capitula abortion and (3) predated capitula. Flower heads were recorded as predated when they had direct evidence of damage caused by insects such as wounds, holes or occurrence of insect larvae inside the seeds. Incompletely developed capitula were recorded as aborted only when there was no evidence of insect damage; otherwise they were recorded as predated. The occurrence of predatory insects was also reported and specimens were subsequently preserved in 70% ethanol and determined taxonomically.

Genetic analyses

Total genomic DNA was extracted from silica gel-dried tissue (*ca*. 10 mg) following the CTAB protocol of Doyle & Doyle (1987) with the modifications of Soltis *et al.* (1991) and Cullings (1992).

We conducted a previous screening test involving nuclear (ITS and ETS) and chloroplast (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*) markers that were sequenced for a few individuals of different populations. Subsequently, we selected the regions that yielded the highest level of polymorphism: *rpl32-trnL* and *trnS-trnC*. The *rpl32-trnL* region was amplified and sequenced with rpl32f as forward primer and $trnL^{UAG}$ as reverse primer (Shaw *et al.*, 2007) referring to the PCR procedure described in the same publication. The *trnS-trnC* region was amplified and sequenced with $trnS^{GCU}$ as forward primer and $trnC^{GCAR}$ for reverse (Shaw *et al.*, 2005) with the following PCR conditions: 95ºC, 5 min; 30x (94ºC, 30 s; 62ºC, 1 min; 72ºC, 2 min); 72ºC, 5 min, and storage at 10ºC. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany) or DNA Clean and Concentrator™-5 D4004 (Zymo Research, Orange, CA, USA) depending on the quality of the amplification. Direct sequencing of the amplified DNA segments was performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA, USA) following the protocol recommended by the manufacturer. Nucleotide sequencing was carried out at the Centres Científics i Tecnològics of the University of Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems, Foster City, CA, USA).

The number of individuals finally analysed per population range between three and 13 due to the plant material availability and the uneven success of extraction, amplification and sequencing procedures (Table 1). Locality 1 (Reguengo) presents only three plants, so all the individuals were sampled in this case. Regarding other sparsely sampled populations (i.e. Almograve and Mata da Foja), the difficult accessibility prevented getting more material. Nucleotide sequences were edited using Chromas LITE v2.01 (Technelysium Pty, Tewantin, Australia) and subsequently aligned manually with BioEdit v7.0.5.3 (Hall, 1999). Chloroplast DNA haplotypes were determined using the number and position of nucleotide substitutions and indels from the aligned sequences. A statistical parsimony haplotype network was also constructed using TCS v1.21 (Clement *et al.*, 2000). For this latter analysis, insertions/deletions longer than one base pair were re-coded as single base pair mutations, and sequence gaps were treated as a fifth character state.

Data analyses

Statistical analyses of fitness-related parameters

Table 1. Studied populations of *Cheirolophus uliginosus* including localities, collection data, geographical coordinates and estimated population size. Herbarium vouchers are deposited at the Institut Botànic de Barcelona (BC). Localities 4 and 4′ correspond to the same population sampled in August and November, respectively.

No.	Locality	Date	Coordinates	Population size[1]	Haplotypes	*N*[2]	Number of tested achenes
1	Portugal, Alentejo: Reguengo, *Garnatje*, *Pellicer & Vitales* (BC)	2.08.2009	39°17′ N, 7°23′ W	(3 ind.) small	E	3	39
2	Portugal, Alentejo: Almograve, *Garnatje*, *Pellicer & Vitales* (BC)	31.07.2009	37°39′ N, 8°47′ W	(25 ind.) small	A	5	34
3	Portugal, Centro: Mata da Foja, *Paiva & Vitales* (BC)	2.11.2009	40°13′ N, 8°43′ W	(40 ind.) small	F	7	14
4	Portugal, Centro: Fermentelos, *Garnatje*, *Pellicer & Vitales* (BC)	4.08.2009	40°34′ N, 8°32′ W	medium	C	13	40
4′	Portugal, Centro: Fermentelos, *Paiva & Vitales* (BC)	2.11.2009					40
5	Portugal, Centro: Figueiró do Campo, *Paiva & Vitales* (BC)	3.11.2009	40°09′ N, 8°35′ W	medium	D	9	40
6	Portugal, Centro: Paul da Madriz, *Paiva & Vitales* (BC)	2.11.2009	40°07′ N, 8°37′ W	large	B	8	29
7	Portugal, Centro: Pampilhosa, Quinta do Valdoeiro, *Paiva & Vitales* (BC)	2.11.2009	40°21′ N, 8°25′ W	large	C (10 ind.) B (2 ind.)	12	20

[1] Size categories: small (< 50 individuals), medium (50–250 individuals) and large (> 250 individuals).
[2] No. of individuals studied for haplotypes.

were performed with the R software v2.7.0 (R Development Core Team, 2008). Prior to the analysis, data were tested for normality and homocedasticity and nonparametric statistics were applied when the assumptions of parametric tests were not met. The results of germination capacity tests were analysed within a generalised linear model (GLM) framework, assuming a binomial distribution and logit link function. Population size—i.e. small, medium and large size—and populations—nested to size categories—were used as independent variables of the GLM. In relation to the effect of seasonality on achene collection, data distribution did not fit a normal distribution, so a non-parametrical test (Mann-Whitney's U) was used to check whether data from summer and autumn field collections could be considered as the same statistical population.

In order to compare the frequencies of the three considered capitula variables among the different populations, a contingency table was created. The significance of the differences observed was analysed applying a chi-square test to the data. When the value of the expected frequency in any of the cases of contingence table was less than 5, Yates correction was applied in the chi-square calculation.

RESULTS

Germination tests

The percentages of seed germination in the populations studied are detailed in Fig. 2. Differences in germination vigour between the two batches from population 3 collected in August and November 2009 have not been found significant (U = 326.5,

$P > 0.05$), and therefore, both samples have been treated as the same statistic pool of data. The GLM analysis detected significant differences in the germination rates related to population size ($\chi^2 = 23.18$, d.f. = 11, $P < 0.05$). However, not all the levels of "population size" factor were statistically significant. Partial Wald Z-test checked the significance of each coefficient (i.e. levels of "size" factor) in the presence of the others. The significant differences in seed germination among size levels were largely due to small populations (Z-value = −3.388, $P < 0.05$) whereas the rest of coefficients did not have a significant effect on the model.

Evaluation of capitula predation

The results obtained from the classification of capitula according to their condition are summarized in percentages in Table 2. The chi-square test with Yates correction revealed significant differences between populations related to the capitula conditions ($\chi^2 = 25.26$, d.f. = 8, $P < 0.05$).

Among the insects found inside the capitula some were only determined as unidentified adults of Alydidae heteropterans, but in-depth taxonomical classification made it possible to determine adults and larvae of the curculionid coleopterans *Larinus leuzeae* H. Fabre (P. Gurrea, pers. comm.).

Haplotypic structure

Out of several plastid and nuclear DNA markers screened, only the *rpl32-trnL* intergenic cpDNA spacer (988 base pairs; bp) and the *trnS-trnC* intergenic cpDNA spacer (849 bp) showed a sufficient degree of variation, while other markers (*rpoB-trnD*, *rps16-trnK*, ITS, ETS) were either entirely invariable or exhibited only very few variable sites. The two chloroplast markers were finally sequenced for 57 samples from seven different populations resulting in an alignment of 1816 bp. We detected eight polymorphic sites—including a 22 bp indel—representing six different haplotypes (Fig. 1A–B and Table 3). All populations except one (population 7) contained one single haplotype. Haplotypes B and C were shared by more than one population while the rest of haplotypes (A, D, E and F) were each one restricted to a single population (private haplotypes). Phylogenetic relationships between haplotypes inferred by the parsimony network is shown in Fig. 1B. Different haplotypes of *Ch. uliginosus* distinguish from adjacent haplotypes by one, two or three evolutionary events (substitutions or indels) and extinct or unsampled haplotypes are represented as black dots in the parsimony network.

DISCUSSION

Genetic structure, population size, and fitness

The low variability of employed DNA markers and the limited extension of sampling prevent us from inferring accurate statements about the genetic diversity and structure of *Ch. uliginosus* populations. However, because this species was more common at the beginning of the twentieth century, and because its habitat is declining rapidly, we suggest that genetic drift and bottlenecks are likely to be the main causes for the loss of genetic variation in the small populations. Indeed, the sole population presenting two haplotypes—instead of only one—is precisely the population with a larger size (Pampilhosa; > 1000 individuals). Assuming that additional analyses are required to confirm this preliminary result, a positive association between genetic diversity and population size can be envisaged.

Our results also suggest that geographic distance is not shaping the genetic structure of populations in *Ch. uliginosus*. Two medium and large populations separated by only 5 km [(i.e. Figueiró do Campo (5) and Paul da Madriz (6)] do not share any cpDNA haplotype whereas Pampilhosa (7)—the only population showing two haplotypes that are shared—is separated from its nearest neighbouring populations by more than 25 km (Fig. 1A and Table 1). Furthermore, haplotypes evolutionarily distant, as in the case of B and F (Fig. 1B) are only 12 km away. Large cpDNA differentiation among spatially close populations may indicate ancient isolation and—at least for seeds—restricted gene flow. In any case, further analyses involving other molecular markers more sensitive for population genetics studies (i.e. AFLP) may help to confirm the accuracy of these preliminary hypotheses (Vitales *et al.*, unpubl. data).

The results of the germination tests suggest a significant heterogeneity in germination behaviour between *Ch. uliginosus* populations (Fig. 2). Similar results have been previously described for other species (see review in Baskin & Baskin, 1973), some of

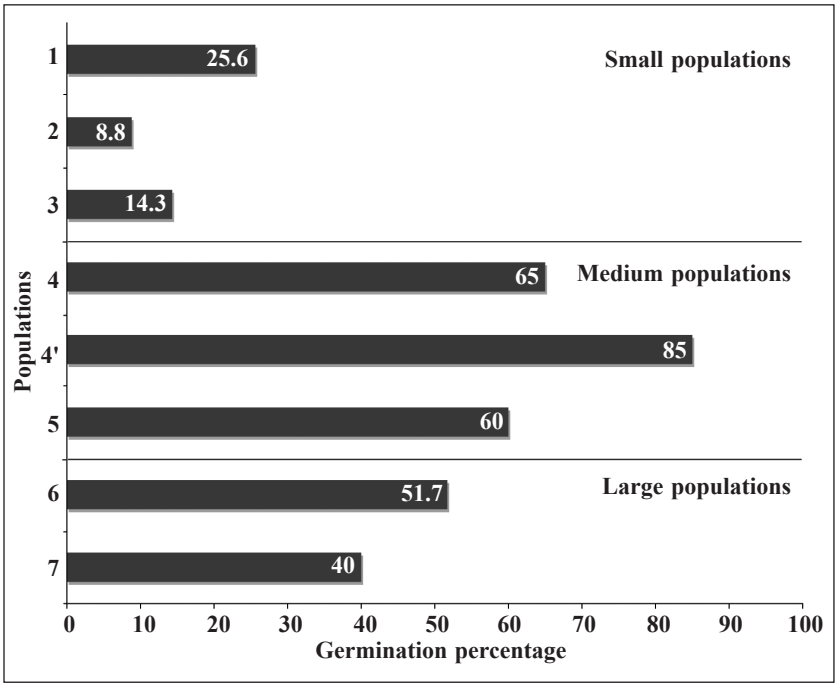

Figure 2. Germination rate of achenes (%) in *Ch. uliginosus* populations. Populations 4 and 4′ correspond to seeds from the same location (Fermentelos) collected in August and November respectively. The horizontal lines delimitate populations according to their size category [small (< 50 individuals), medium (50–250 individuals) and large (> 250 individuals)].

them attributing the inter-population differences to genetic factors and others proposing environmental factors as being of importance.

In *Ch. uliginosus*, reproductive fitness—measured by means of the percentage of germinated achenes—seems to follow a nonlinear pattern related to the number of individuals per population. Certainly, achenes from small-sized populations (1, 2 and 3), all of them displaying fewer than 50 individuals, showed the lowest germination percentages. Nonetheless, the largest germination rates were not found in large populations (> 250 individuals) but in medium-sized ones (50–250 individuals). The lack of a plain Allee effect (Stephens & Sutherland, 1999) on germination capacity has been severally reported in different species (see for instance Hauser & Loeschcke, 1994; Lammi *et al.*, 1999; Costin *et al.*, 2001; Le Cadre *et al.*, 2008) and factors causing unequal response to population size or density are multiple and difficult to disentangle (Vergeer *et al.*, 2003).

Table 2. Percentages of capitula condition in the studied populations**.** The estimated size per population (see Table 1) is indicated beside population number (in parentheses).

		Capitula condition		
Population number (estimated size)	**Number of evaluated capitula**	**Good condition (%)**	**Capitula abortion (%)**	**Predated capitula (%)**
3 (small)	20	55.00	10.00	35.00
4 (medium)	38	50.00	18.42	31.58
5 (medium)	33	51.51	0.00	48.48
6 (large)	31	32.26	0.00	67.74
7 (large)	40	25.00	7.50	67.50

Table 3. List of haplotypes found in the studied populations of *Cheirolophus uliginosus* with indication of nucleotide site variation within the chloroplast regions *rpl32-trnL* and *trnS-trnC*.

Regions	***rpl32-trnL***						***trnS-trnC***	
Positions / Haplotypes	318	382	590–611[1]	644	653	951	1497	1794
A	A	T	+	G	A	C	C	T
B	A	T	–	G	A	A	A	T
C	A	C	–	G	C	A	A	T
D	A	T	–	G	C	A	A	C
E	A	T	–	A	C	A	A	T
F	T	T	–	A	C	A	A	T

[1] At the indel position, "+" means presence and "–" means absence of the fragment.

Our study indicates that reproductive success is severely reduced in small populations (< 50 plants) whereas medium and large-size ones (50–250 and > 250 individuals) do not show any fitness constraint. Reed (2005) pointed out that clearest effects of genetic erosion on fitness should start at some limited population size and it has been suggested by different authors that 50 individuals are required to retain reproductive fitness (Frankel & Soulé, 1981). Apart from this, other non-genetic mechanisms such as pollen scarcity (Berec *et al.*, 2007) may also be responsible for component Allee effects in reproduction. Furthermore, the three smallest populations of *Ch. uliginosus* grow under disadvantageous environmental conditions (D. Vitales, pers. obs.) that might increase stress on reproductive plants resulting in a reduced investment of maternal energy in the offspring (Oostermeijer *et al.*, 1995). In conclusion, either or both ecological and genetic deterioration could explain the lower germination success in small populations of this species.

Predispersal predation of seeds was expected to influence sexual reproduction, since it is a determinant factor in many Asteraceae, especially in the Cardueae tribe (Colas *et al*., 2001; Kolb *et al*., 2007). Interestingly, the extent of predation was not equally distributed among different populations: populations 6 and 7, both with more than 250 plants each, showed a significantly higher proportion of damaged seed and capitula than the small and medium-sized ones. Large host plant populations are more frequently colonized and seed predators may be more likely to persist when the food resource is more concentrated (Östergard & Ehrlén, 2005), this resulting in a major damage in reproductive output (Cunningham, 2000; Arvanitis *et al.*, 2007). Insects found inside *Ch. uliginosus* flower heads belong to taxonomic groups specialised in achene and capitulum consumption (Metcalf *et al.*, 2009) and a relationship between the presence of curculionid and hemiptera larvae and the damage to developing seeds has also been reported in other species (Louda, 1983; Fernández *et al.*, 2008). Therefore, the observed increase in seed predation in larger populations could explain the lower but not statistically significant decrease in germination capacity.

Implications for conservation and concluding remarks

Habitat loss and deterioration have resulted in a rapid decline of Portuguese *Ch. uliginosus* populations, probably also limiting the gene flow between them. Reproductive success in the small populations is clearly reduced and, therefore, recruitment in these populations is virtually absent at present (D. Vitales, pers. obs.). Thus, taking also into account the anthropogenic pressures in its habitat, the viability of small populations of *Ch. uliginosus* may be limited in the short term. The smallest populations contain the most evolutionarily-distant haplotypes and are, therefore, of especially high conservation value. In

order to preserve their distinctive genetic diversity, we recommend ex situ conservation strategies for these small populations (e.g. collection and storage of seeds in germplasm banks). Most of the studied populations are located within protected natural areas included in Nature 2000 network (ICNF, 2013) hence they benefit from some in situ protection. Unfortunately, populations of Mata da Foja (3), Figueiró do Campo (5) and Quinta do Valdoeiro (7) do not show any statutory protection so they should be considered as targets for in situ conservation measures.

Our study reinforces the modern idea on demography and conservation biology proposing that plant species respond somewhat individually to the effects of population decline and fragmentation (Costin *et al.*, 2001). Large populations of *Ch. uliginosus* were not reproductively advantaged in relation to medium-sized populations, at least when seed germination is used as an indicator of plant performance. As a moderate size may favour escape from seed predation (Janzen, 1971; Kéry *et al.*, 2000), medium-sized populations may achieve a better sexual reproduction performance than large ones. Besides, *Ch. uliginosus* could also be showing reproductive erosion signs, but only in populations below 50 individuals, this suggesting the existence of an asymptote in the relationship between population size and germination capacity in this species. Further studies including more populations varying in size as well as alternative molecular approaches such AFLPs (Vitales *et al.*, unpubl. data), may help to further elucidate the relationship between population size and reproductive success in *Ch. uliginosus*.

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La radiació explosiva de *Cheirolophus* (*Asteraceae*, *Cardueae*) a la Macaronèsia

Daniel Vitales, Teresa Garnatje, Jaume Pellicer, Joan Vallès, Arnoldo Santos-Guerra i Isabel Sanmartín

Antecedents: Les illes Canàries, considerades com un punt calent de diversitat, han estat objecte de nombrosos estudis evolutius centrats en una gran varietat d'organismes. El gènere *Cheirolophus* (*Asteraceae*) representa una de les majors radiacions que han tingut lloc a l'arxipèlag canari. Per contra, només unes poques espècies habiten a la conca mediterrània, que representa l'àrea de distribució ancestral del gènere. Aquí, el nostre objectiu principal fou recontruir la història filogenètica i biogeogràfica de *Cheirolophus*, amb l'interès especial d'explicar l'origen de la radiació canària.

Resultats: Hem trobat una incongrüència significativa en les relacions filogenètiques inferides entre marcadors nuclears i cloroplàstics. Cadascun dels grups de dades aporta resolució a diferents nivells en la filogènia del gènere *Cheirolophus*: els marcadors nuclears resolgueren l'esquelet de la filogènia, mentre que els marcadors cloroplàstics aportaren una major resolució dintre de la clada canària. L'origen de *Cheirolophus* fou estimat durant el període miocè mitjà-tardà, seguit d'una ràpida diversificació en tres llinatges mediterranis i la clada macaronèsica. S'ha inferit una reducció en la tasa de diversificació a finals del miocè, seguida d'un increment durant el pliocè tardà de forma contemporània a l'establiment del clima mediterrani. La diversificació en la clada macaronèsica començà cap a l'inici-meitat del plistocè, amb nivells d'especiació inusuals que donaren lloc la gran diversitat insular actual.

Conclusions: La diversificació del gènere ha estat influenciada per factors climàtics, tot explicant els inicis de la seva història evolutiva a la regió mediterrània. Sembla que l'excepcional taxa de diversificació observada en la clada canària ha estat conduïda principalment per fenòmens d'especiació al·lopàtrica (incloent diversificacions intra-illes i inter-illes). Diversos factors intrínsecs (p.e. sistema reproductiu, poliploïdia,

síndrome de dispersió de llavors) i extrínsecs (p.e. fragmentació d'hàbitats, aïllament, clima i orografia) contribuïren probablement a la diferenciació progressiva de les poblacions, donant lloc a l'aparició de nombrosos microendemismes. Finalment, esdeveniments d'hibridació i l'adaptació ecològica emergent poden haver reforçat també aquest procés de diversificació.

Revista: El factor d'impacte d'aquesta revista (corresponent a l'anys 2013, segons el JCR de la ISI web of Knowledge) és 3,407, i es troba en la posició 18 de 46 a *Evolutionary Biology* (Q2, segon quartil) i 59 de 165 a *Genetics & Heredity* (Q2, segon quartil). Article *Highly Accessed*.

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RESEARCH ARTICLE Open Access

The explosive radiation of *Cheirolophus* (Asteraceae, Cardueae) in Macaronesia

Daniel Vitales[1*], Teresa Garnatje[2], Jaume Pellicer[3], Joan Vallès[1], Arnoldo Santos-Guerra[4] and Isabel Sanmartín[5*]

Abstract

Background: Considered a biodiversity hotspot, the Canary Islands have been the key subjects of numerous evolutionary studies concerning a large variety of organisms. The genus *Cheirolophus* (Asteraceae) represents one of the largest plant radiations in the Canarian archipelago. In contrast, only a few species occur in the Mediterranean region, the putative ancestral area of the genus. Here, our main aim was to reconstruct the phylogenetic and biogeographic history of *Cheirolophus* with special focus on explaining the origin of the large Canarian radiation.

Results: We found significant incongruence in phylogenetic relationships between nuclear and plastid markers. Each dataset provided resolution at different levels in *Cheirolophus*: the nuclear markers resolved the backbone of the phylogeny while the plastid data provided better resolution within the Canarian clade. The origin of *Cheirolophus* was dated in the Mid-Late Miocene, followed by rapid diversification into the three main Mediterranean lineages and the Macaronesian clade. A decrease in diversification rates was inferred at the end of the Miocene, with a new increase in the Late Pliocene concurrent with the onset of the Mediterranean climate. Diversification within the Macaronesian clade started in the Early-Mid Pleistocene, with unusually high speciation rates giving rise to the extant insular diversity.

Conclusions: Climate-driven diversification likely explains the early evolutionary history of *Cheirolophus* in the Mediterranean region. It appears that the exceptionally high diversification rate in the Canarian clade was mainly driven by allopatric speciation (including intra- and interisland diversification). Several intrinsic (e.g. breeding system, polyploid origin, seed dispersal syndrome) and extrinsic (e.g. fragmented landscape, isolated habitats, climatic and geological changes) factors probably contributed to the progressive differentiation of populations resulting in numerous microendemisms. Finally, hybridization events and emerging ecological adaptation may have also reinforced the diversification process.

Keywords: Allopatric speciation, Canary Islands, Diversification, Island radiation, Mediterranean Basin, Phylogeography

Background

In recent decades, the Macaronesian archipelagos of Azores, Cape Verde, Madeira, Savages and Canary Islands have been the subject of numerous studies concerning patterns of colonization and speciation of different plant lineages [1-4]. In particular, the Canary Islands have drawn special attention from biogeographers because of their high degree of endemism, wide geological age ranges, variety of ecological conditions and unusual short distance to the mainland [5]. This has made the archipelago an ideal natural laboratory to test general hypotheses on island biogeography and evolution [5-9]. Recently, phylogenetic studies in Macaronesian plants have started incorporating information on lineage divergence times [3,10,11], a key factor when addressing evolutionary questions on the processes underlying lineage diversification [12] and their role in community assembly [13].

With approximately 20 endemic species, the genus *Cheirolophus* Cass. (1817) (Asteraceae, Cardueae) is considered one of the ten largest plant radiations in the Canary Islands [6]. In fact, ongoing taxonomical investigation points towards the existence of an even larger number of species (A. Santos-Guerra, unpubl. data). With the exception of *Ch. teydis* (C.Sm.) G.López from La Palma and Tenerife, all species are endemic to one of the central or western islands (Gran Canaria, Tenerife, La Gomera, La Palma and El Hierro). Most species present very narrow geographical

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ranges, but exhibit notable differences in their ecological preferences and morphological characteristics. Canarian *Cheirolophus* typically occur as small populations isolated on humid basalt cliffs. However, some species are adapted to live in remarkably different habitats, such as xeric environments (e.g. *Ch. junonianus* (Svent.) Holub), the subalpine zone (e.g. *Ch. teydis*) or coastal environments (*Ch. webbianus* (Sch.Bip.) Holub) [14]. This ecological diversity, coupled with a large species richness distributed in a clearly geographical pattern, makes *Cheirolophus* an ideal group to explore patterns and processes behind island diversification.

In addition to the large Canarian radiation, the genus occurs in Madeira [*Ch. massonianus* (Lowe) A.Hansen & Sunding] and the Western Mediterranean Basin, including the Mediterranean climate Atlantic coasts of the Iberian Peninsula (Figure 1). The species with the widest geographical distribution are the Mediterranean *Cheirolophus intybaceus* (Lam.) Dostál and the two Atlantic *Ch. sempervirens* (L.) Pomel and *Ch. uliginosus* (Brot.) Dostál [15]. There is considerable intraspecific variability within these species, especially in the *Ch. intybaceus* complex, which groups a set of morphologically similar taxa (e.g., *Ch. mansanetianus* Stübing, J.B.Peris, Olivares & J.Martín, *Ch. lagunae* Olivares & al., *Ch. grandifolius* (Font Quer) Stübing & al., *Ch. intybaceus* var. *microcephala* Rouy). This has led to an unstable taxonomy, with no clear estimate of the number of species in the genus, which ranges from 25 to 30 depending on the author. All *Cheirolophus* species are perennial plants characterized by a thickened capitulum peduncle, a cypsela with deciduous pappus, *Serratula* pollen type [16], and a shrubby habit (except *Ch. uliginosus*, which is a perennial hemicryptophyte). They usually present an outcrossing mating system, although certain degree of self-compatibility has been reported [17]. From the conservation viewpoint, 22 species and subspecies of *Cheirolophus* are officially listed as vulnerable, endangered or critically endangered taxa, of which 17 are Macaronesian [18,19].

Earlier attempts to address phylogenetic relationships among *Cheirolophus* species or between the genus and its closest relatives have been based on allozymes [20] or DNA sequences from the nuclear ribosomal ITS and ETS regions [21,22]. These studies supported the existence of two well-defined major lineages: a Macaronesian clade, including all Macaronesian endemics, and a Mediterranean clade, grouping the North African *Ch. benoistii* (Humbert) Holub and *Ch. tananicus* (Maire) Holub with the *Ch. intybaceus* complex, distributed along the eastern shores of the Iberian Peninsula and southern France [22]. In contrast, lack of phylogenetic resolution within the Macaronesian clade - probably due to a recent history of colonization and diversification - prevented an in-depth study of phylogenetic relationships among the Macaronesian endemics [22]. More recently, a tribal phylogenetic reconstruction based on both nuclear and chloroplast markers [23] placed *Cheirolophus* in a basally branching position within the subtribe Centaureinae, as sister-group to the *Myopordon-Rhaponticum* lineage. Using new fossil evidence for Asteraceae, these authors estimated the divergence of *Cheirolophus* from its sister genera around the Early Miocene [23].

Here, we used nuclear and chloroplast DNA sequence data and the most comprehensive sampling of the genus conducted so far - including the entire species

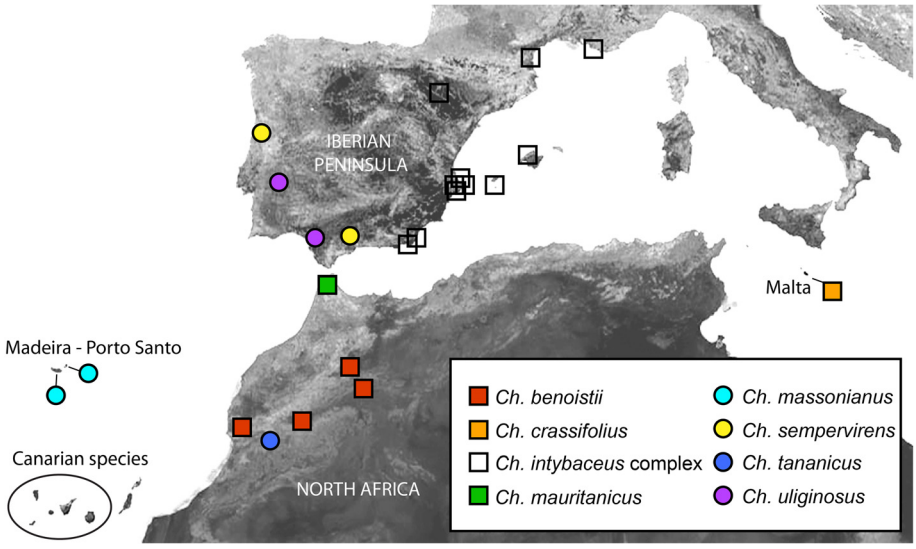

Figure 1 Map with sampled localities. Geographical distribution of the populations of *Cheirolophus* species included in this study. Further details on Canarian populations are given in Figure 4.

and infraspecific diversity registered in the International Plant Name Index - in conjunction with Bayesian phylogenetic analysis, divergence time estimation, macroevolutionary modelling, and biogeographical reconstruction to: (1) disentangle phylogenetic relationships within *Cheirolophus*, with special focus on the Canarian radiation, (2) infer the tempo and mode of lineage diversification within the genus, and (3) reconstruct the origin and colonization events in Canarian *Cheirolophus* in order to understand the factors underlying its large species richness.

Methods

Taxon sampling and DNA sequencing

DNA sequences were obtained from 57 populations representing 32 different taxa of *Cheirolophus* (Additional file 1: Table S1). *Serratula coronata* L., *Rhaponticoides hajastana* (Tzvelev) M.V.Agab. & Greuter, and *Rhaponticum pulchrum* Fisch. & C.A.Meyer were chosen as outgroup taxa based on previous phylogenetic studies of tribe Cardueae [20,22,23]. Total genomic DNA was extracted from silica gel-dried leaves and from herbarium specimens (ca. 10 mg) following the CTAB-protocol of Doyle and Doyle [24] with the modifications of Soltis et al. [25] and Cullins [26]. Nuclear rDNA regions (ITS and ETS) were newly sequenced for 11 taxa of *Cheirolophus* (eight species, three infraspecific taxa). This represents a 64% increase in the number of *Cheirolophus* species sequenced compared to previous studies [22]. For chloroplast DNA markers, a preliminary screening test involving 14 rapidly evolving cpDNA regions [27] was conducted (*ndhF-rpl32; prbA-trnH; psbD-trnT; psbE-petT; rpl32-trnL; rps16-trnK; trnD-rpoB; trnK-matK; trnL-trnF; trnQ-5′rps16; trnS-trnC; trnS-trnfM; trnT-trnG;* and *trnV-ndhC*). The regions that yielded the highest level of polymorphism were selected for further sequencing (*rpl32-trnL, rpoB-trnD, rps16-trnK* and *trnS-trnC*). All cpDNA sequences were newly generated for this study. In addition, we conducted a pilot study for the Macaronesian taxa to evaluate the within-population level of genetic diversity in the cpDNA markers. We analysed three individuals per population (when available), ensuring that these represented the entire area occupied by the population. Macaronesian populations showed no genetic variability for any of the plastid markers, so only one individual per population was included in further analyses.

DNA amplification procedures were performed as outlined by Pellicer et al. [28]. Details on primers used and polymerase chain reaction (PCR) conditions are given in the Additional file 2: Table S2. Depending on the quality of the amplification, products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA, USA) or DNA Clean and Concentrator™-5 D4004 (Zymo Research, Orange, CA, USA) following the manufacturer's protocol. Direct cycle sequencing of the purified DNA segments was performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA, USA) following the protocol recommended by the manufacturer. Nucleotide sequencing was carried out at the Centres Científics i Tecnològics of the University of Barcelona on an ABI PRISM 3700 DNA analyzer (PE Biosystems, Foster City, CA, USA). Details on species authorities, geographical localities for samples, and GenBank accession numbers are given in the Additional file 1: Table S1 of the supporting information.

Sequences were edited with Chromas LITE v2.01 (Technelysium Pty, Tewantin, Australia), and aligned manually with BioEdit version 7.0.5.3 [29].

Phylogenetic analysis

Bayesian inference, implemented in MrBayes 3.2 [30], was used to estimate phylogenetic relationships among species of *Cheirolophus* based on individual analyses of the concatenate ITS + ETS (nrDNA) dataset and the concatenate four-plastid marker (cpDNA) dataset (each with 60 sequences: 57 *Cheirolophus* samples plus three outgroup taxa). Before concatenating the different plastid and nuclear regions, we checked for conflict among them. Incongruence was assessed (i) with the ILD test implemented in PAUP v. 4.0b10 [31], using a P-value of 0.01, and 1000 replications with heuristic search and random addition of sequences and excluding uninformative characters and (ii) by looking for nodes that were strongly supported (PP ≥ 0.95) in the Bayesian 50% majority rule consensus tree of one region/dataset but were not present in the consensus tree of the other region/dataset. No incongruence was observed among the cpDNA regions and between the two nuclear (ITS/ETS) markers, so they were concatenated in two independent datasets (cpDNA and nrDNA), which were analyzed separately. The General Time Reversible model (GTR) was selected as the most appropriate nucleotide substitution model for the cpDNA dataset, and the same model with among-site rate variation (GTR + G) for the nrDNA dataset based on the Akaike information criterion implemented in jModelTest 0.1 [32]. We did not partition the plastid and nuclear datasets by gene region in the Bayesian analyses because of the observed low genetic variation among sequences and to avoid overparameterization. Gaps inferred during the alignment of the nrDNA and cpDNA regions were manually coded and modelled as different, binary partitions, using the F81-like restriction site model in MrBayes [33]. Two independent Markov chain Monte Carlo (MCMC) analyses with four Metropolis-coupled chains each were run for 5,000,000, sampling every 100 generation. The first 5,000 trees were discarded as the 'burn-in' period, after confirming that the average standard deviation of the

split frequencies was < 0.01, and the potential scale reduction factor approached 1.0 for all parameters. The remaining samples were pooled to construct a majority rule consensus tree that approximates the posterior distribution of the phylogeny – visualized in FigTree 1.3.1 [34] – and to obtain clade posterior probabilities.

Both the ILD test and the node-comparison approach revealed the existence of significant incongruence between the cpDNA and nrDNA genomes, so we decided not to concatenate these two datasets in further analyses. The cpDNA tree was in general less resolved than the nrDNA tree and most cases of incongruence concerned poorly resolved relationships at the backbone of the tree, which may be explained by the low information content (variability) at the phylogenetic species level in this dataset in comparison with nuclear markers (see Additional file 3: Table S3). One exception was the incongruent position of the Madeiran endemic *Ch. massonianus*, which showed high support and significantly distant phylogenetic positions in both the cpDNA and nrDNA datasets (see Results below). Incongruence among gene trees can be attributed to different causes, being incomplete lineage sorting (ILS) and hybridization the most commonly reported in plant groups experiencing rapid radiations (e.g. [35-37]). To explore whether the incongruent position of *Ch. massonianus* could be the result of either reticulate evolution or ILS, we conducted an additional analysis under *BEAST [38]. This multilocus coalescent method is known to address ILS phenomena, whereas it is not able to resolve incongruence derived from hybridization. *BEAST uses a multispecies coalescent approach to estimate the most probable species tree given the unlinked multi-locus sequence data (i.e., the nrDNA and cpDNA datasets) and assumes no gene flow between after population/species divergence [37]. We constructed two partitioned, concatenate nuclear-plastid dataset: the first one including all *Cheirolophus* species and a second one excluding the taxon suspected of causing the major incongruence among gene trees (i.e. *Ch. massonianus*). Theoretically, a hybrid taxon included into a multi-locus phylogeny introduces homoplasy with clades that contain the hybrid parents, because hybrid taxa are supposed to be overall intermediate to the parental taxa since they contain a mosaic of parental characters [39]. Therefore, the removal of the hybrid taxon should increase the branch support for the clades that include the parental taxa –or their most closely related species– by decreasing the amount of homoplasy in the dataset [40]. For both analyses, the same model priors employed for the MrBayes phylogenetic analysis and the BEAST divergence time analysis (see below) were selected. The Markov chain was run for 5×10^7 generations, sampling every 1000th generation. Tracer 1.4 [41] was used to check the convergence of the analyses and to confirm that the effective sample size (ESS) of each parameter are sufficiently large. Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnnotator 1.6.2 [42] and visualized in FigTree [34].

Divergence time estimation and diversification analysis

We estimated species divergence times in *Cheirolophus* using a Bayesian-relaxed clock approach implemented in BEAST 1.7.1 [43]. The analysis was carried out only on the nrDNA dataset because of lack of variability and poor resolution in the cpDNA dataset at the species phylogenetic level (see above). Choice of model priors was based on the Path Sampling (PS) and Stepping Stone (SS) sampling methods in BEAST, which have been shown to outperform other marginal likelihood estimators in terms of consistency [44]. The birth-death model [45] was selected as the tree prior and the uncorrelated lognormal rate variation among branches as the clock prior, with a broad uniform distribution (10^{-1}-10^{-6}) for the mean rate and a default exponential prior for the standard deviation parameter. Speciation birth-death models can be problematic when multiple individuals per taxon/species are included in an analysis, since they assume that tips represent extant species completely sampled from the clade of interest [38]. Consequently, for the dating analysis, we included only one individual/sample per taxon (including subspecies), resulting in a 35-sequence data matrix. The GTR + G model was used as substitution model with a separate gap partition. The Markov chain was run for 5×10^7 generations, sampling every 1000th generation. Tracer 1.4 [41] was used first to check the convergence and mixing of each parameter, and then to confirm that the effective sample size (ESS) of each parameter was sufficient to provide reasonable estimates of the variance in model parameters (i.e. ESS values > 200, after excluding a burn-in fraction of 10%). Trees were summarized in a maximum clade credibility (MCC) tree obtained in TreeAnnotator 1.6.2 [42] and visualized in FigTree [34]. Since there is no known fossil record of *Cheirolophus*, estimation of absolute lineage divergence times relied on secondary age constraints obtained from the molecular dated phylogeny of Barres et al. [23]. This study was based on five different fossil calibration points, including newly discovered fossils of Asteraceae [23], and constitutes the most complete dating analysis of tribe Cardueae to date. Barres et al. [23]'s estimate for the most recent common ancestor of *Serratula*, *Rhaponticum*, *Rhaponticoides*, and *Cheirolophus* was used to calibrate the root node in our phylogeny. To reflect the uncertainty in deriving age estimates from a more inclusive dated phylogeny, itself calibrated with the fossil record, we used a normal distribution prior [46] for the root node age parameter, with a median of 24.51 Ma

and a standard deviation (SD) of 2.7 Ma to span the entire confidence interval (95% high probability density (HPD): 20.17–29.62 Ma) obtained by Barres et al. [23].

We used a diverse array of diversification statistics implemented in the programming language R (http://www.R-project.org, R Development Core Team 2012) to analyse the tempo and mode of species diversification in genus *Cheirolophus*. The package APE 2.7-3 [47] was used to construct a lineage-through-time (LTT) plot from the nrDNA BEAST chronogram, after pruning the outgroup taxa. We used the gamma statistic [48] implemented in the R package GEIGER [49] to test whether rates of diversification have been constant through time; since taxon sampling is complete in *Cheirolophus,* there was no need to use the MCCR test to correct this statistic [48]. The R package TreePar v.2.1 [50] was used to detect temporal changes in diversification rates. In particular, we make use of episodic birth-death models in which diversification rates are allowed to change at certain points in time (rate-shifts). Maximum likelihood optimization was used to simultaneously estimate diversification parameters – the net diversification rate (r = speciation minus extinction) and the extinction fraction (ε = the extinction to speciation ratio) – for each time interval together with the rate-shift times [50]. We used likelihood ratio tests (LRT) to compare nested models of increasing complexity with one, two, three, or four rate shifts (an arbitrary high value based on the size of our phylogeny), using a grid on shift times of 0.2 Myr steps.

TreePar can detect temporal changes in diversification rates, but does not allow the rate of diversification to vary among lineages. Instead, we used MEDUSA [51] implemented in GEIGER to locate the position of these rate shifts on the phylogeny. MEDUSA uses an AIC-based stepwise approach that compares the likelihood of piecewise models, in which r and ε are estimated at various points in the phylogeny. We also used the method-of-moments estimator [52], implemented in GEIGER, to estimate the rate of diversification in Canarian *Cheirolophus* under two extreme values of the extinction fraction (ε = 0, no extinction, and ε = 0.9 high rate of extinction). This method does not require a resolved time-calibrated phylogeny, and can thus be used to direct estimation of speciation rates in groups that underwent diversification recently. In order to obtain reliable confidence limits, we used the 95% HPD interval for the age of the crown node of the Canarian clade based on the BEAST analysis of the nrDNA dataset. Additionally, we estimated the probability of obtaining a clade with the same size and age as the Canarian clade given the global diversification rate inferred for the entire genus and at increasing extinction fractions (ε = 0, 0.5, 0.8, 0.9).

Phylogeographic analysis

To infer the phylogeographical history of Canarian *Cheirolophus*, we constructed two additional datasets based on the original nrDNA and cpDNA matrices but subsampling only the Canarian taxa; in all 32 populations from 20 species were included in these further datasets. Two separate haplotype networks were constructed from each dataset to visually explore genetic diversity within each species using the software TCS v.1.21 [53]; insertions/deletions longer than one base pair were re-coded as single base pair mutations and these indels were treated as a fifth character state. The origin and timing of dispersal events in the colonization of the archipelago were inferred –independently for the nrDNA and for the cpDNA datasets – with the discrete-state continuous-time Markov chain (CTMC) model [54] implemented in BEAST 1.6.2 [42]. This composite CTMC phylogenetic-biogeographic model allows simultaneous estimation of phylogenetic relationships, lineage divergence times, ancestral ranges, and migration rates between geographic locations using Bayesian MCMC inference [55] and is similar to the Bayesian island biogeographic model described in Sanmartín et al. [8]. We used five geographical states corresponding to the islands where *Cheirolophus* is present: Gran Canaria, Tenerife, Gomera, La Palma, and El Hierro. Migration rates were modelled under both uninformative (mean = 1; SD = 0) and geographically informed priors, i.e., SD = 0 and mean equal to the normalized inverse distance between the centroids of two geographic locations [54]. To calibrate the phylogeographic analysis of Canarian populations based on cpDNA data, we carried out a new BEAST analysis using a dataset of 35 cpDNA sequences including one representative each of all *Cheirolophus* species, with identical settings as the BEAST nrDNA analysis above, except that substitution rates were modelled with GTR. The age of the crown-node of Canarian *Cheirolophus* estimated in this analysis was used to calibrate the root node height in the phylogeographic analysis of the 32-populations Canarian dataset (log normal distribution, mean = 1.22 Ma, SD = 0.4). The root node of the phylogeographic analysis based on the ITS + ETS data was calibrated with the age of crown Canarian *Cheirolophus* obtained in the BEAST analysis of the nrDNA 35-taxa dataset (log normal distribution, mean = 1.73 Ma, SD = 0.4). These phylogeographic analyses were run under a constant-size coalescent model and the uncorrelated log-normal molecular clock, based on the PS and SS selection, for 20×10^6 generations, with all other settings identical to those used in the dating analyses. Finally, Bayesian Stochastic Search Variable Selection (BSSVS) was used to identify those rates (colonization routes) that were frequently invoked to explain the diffusion process [54]; these were saved as a KML file for visualization in Google

Earth 6.2.2.6613. Given the small size of the phylogeny, we used a threshold value of Bayes Factors (BF) > 2 to consider a rate as well supported in the BSSVS analysis.

Results

Phylogenetic relationships and congruence among cpDNA loci

The main characteristics for all markers analysed are summarized in the Additional file 3: Table S3. The nrDNA dataset (ITS + ETS) for the ingroup taxa included 1138 aligned nucleotide positions, of which 140 (12.3%) were variable. The cpDNA dataset (*trnS-trnC* + *rpl32-trnL* + *trnD-rpoB* + *rps16-trnK*) included 3764 aligned positions, 58 (1.54%) of them variable. A total of 330 new sequences were generated and deposited in GenBank, of which 90 were from ITS + ETS and 240 from cpDNA markers (see Additional file 1: Table S1).

Figure 2 shows the consensus trees obtained from the separate Bayesian MCMC analysis of the nrDNA and cpDNA datasets. Phylogenetic relationships were generally better resolved in the nrDNA than in the cpDNA trees, especially for the backbone nodes. The opposite pattern was observed for the Canarian clade, which showed several well-supported subclades in the plastid phylogeny –even though some of these subclades are constituted by just one haplotype shared by several species (see below)– but poor resolution in the nrDNA tree. Both nuclear and plastid genomes support the monophyly of the genus (posterior probability, PP = 1.00) and recover a monophyletic "Macaronesian clade" (with or without *Ch. massonianus;* PP = 1.00, Figures 2A and B). In addition, the Mediterranean species appear grouped into three well-supported clades. These three clades form a well-supported "Mediterranean clade" in the nuclear dataset, but their relationships appear unresolved in the chloroplast phylogeny. *Cheirolophus crassifolius* (Bertol.) Susanna is recovered as the most basal lineage in the two trees (Figure 2). Significant incongruence between the nuclear and chloroplast phylogeny, as evidenced by node comparison and the ILD test ($p < 0.001$), concerned mainly conspecific samples grouped in the nuclear tree that appeared segregated into different clades in the plastid phylogeny, such as, the Mediterranean species *Ch. benoistii* or the Canarian *Ch. teydis* and *Ch. canariensis* (Brouss. ex Willd.) Holub (Figure 2). The only example of incongruence at the inter-species level concerned the position of the Madeiran endemic *Ch. massonianus*, which appears embedded within the Canarian clade in the nrDNA tree (Figure 2A), but occupies a position at the base of the genus together with *Ch. uliginosus* and *Ch. crassifolius* in the cpDNA phylogeny (Figure 2B). The multilocus coalescent *BEAST analysis including this species (see Additional file 4: Figure S1a) resulted in a tree topology in which the Madeiran taxon was placed at the base of the Canarian clade, in a position that was intermediate between the one it occupied in the cpDNA and the nrDNA trees (Figures 2A,B). In addition, the *BEAST analysis without *Ch. massonianus* (Additional file 4: Figure S1b) revealed increased clade support for those clades that include the putative parental taxa compared to the analysis including *Ch. massonianus* (i.e. the Canarian group, PP = 1.00 vs PP = 0.82; or *Ch. uliginosus*, PP = 0.55 vs PP = 0.47).

Divergence time estimation and diversification analyses

Figure 3 shows the BEAST maximum clade credibility tree for the nrDNA dataset, whose topology is overall congruent and slightly better resolved than the one obtained from MrBayes, i.e., *Ch. uliginosus* is resolved as sister-group to the Macaronesian clade. Mean rates of evolution were estimated as 3.13×10^{-9} substitutions per site per year for nuclear regions, which is in agreement with average absolute rate of substitution for nrDNA estimated in other perennial angiosperms with relatively long generation times [56] like *Cheirophus* species. The mean age for crown-group *Cheirolophus* was 10.37 Ma (95% HPD confidence intervals: 5.98–15.35 Ma), while the divergence time between the Macaronesian clade from the Mediterranean lineages (Figure 3) was dated at 8.50 Ma (95% HPD: 4.68–12.45 Ma), and the crown-age of the Canarian clade at 1.74 Ma (0.82–2.93).

The LTT plot of the nrDNA chronogram showed an initial short phase of diversification (10–8 Ma) followed by a plateau between 8 and 3 Ma, with a final pronounced upturn in the rate of diversification c. 2 Ma (Figure 3, inset). This increase in diversification rates is supported by the gamma test, which rejects a constant-rate diversification model (5.730, $p > 0.999$). Although this test was designed to detect decreases in diversification rates compared to the constant-rate model [48], high positive values are usually interpreted as indicating an increase in the rate of speciation [12]. On the other hand, TreePar detected a decrease in diversification rates at 7.8 Ma, and an increase at 3.2 Ma for the model allowing two shifts (Figure 3). None of these rate shifts were, however, significant (LRT, $p > 0.1$), probably due to lack of statistical power when the phylogeny is small (n < 50 taxa). MEDUSA indicated a significant increase in diversification rates along the branch leading to a Canarian subclade ($r_1 = 0.00022$; $r_2 = 6.55$, c. 0.44 Ma, Figure 3). The method-of-moments estimator indicated significantly higher diversification rates in the Canarian clade than expected, given their age and the global diversification rate for the entire genus ($r_G = 0.2673$; $p < 0.05$). This held under varying levels of the extinction fraction (ε = 0, 0.5, 0.8), except for a very high relative extinction rate of ε = 0.9 ($p < 0.1$), which is otherwise unrealistic for such a

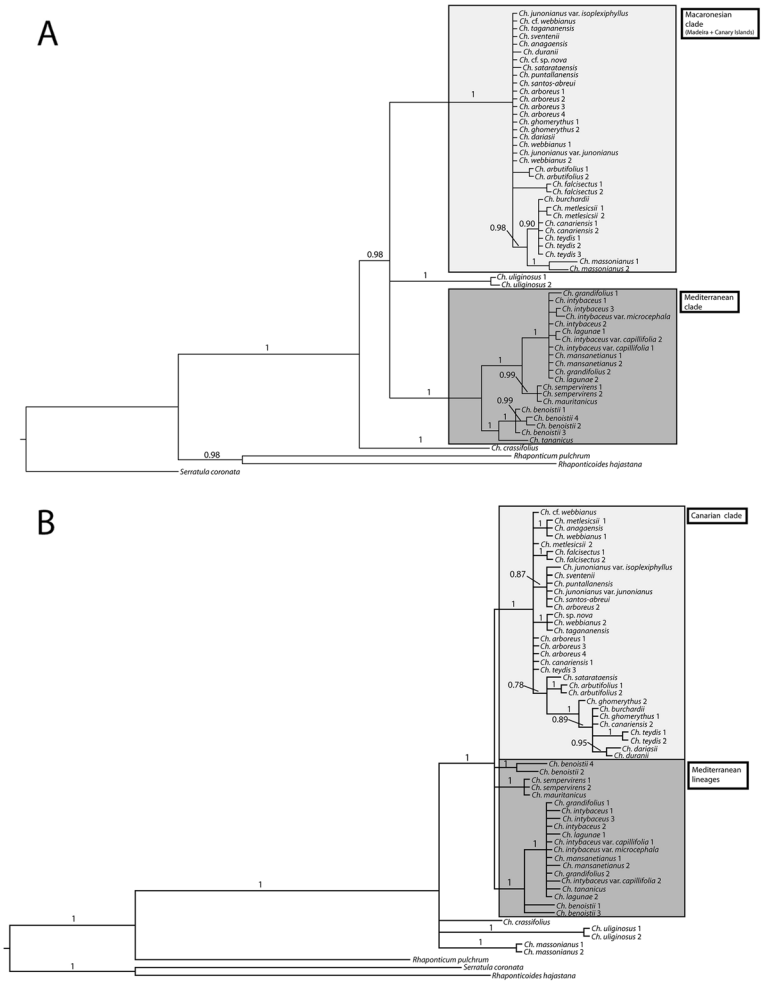

Figure 2 Bayesian phylogenetic trees of *Cheirolophus* inferred from nuclear and plastid DNA datasets. Majority-rule consensus tree resulting from a Bayesian analysis of **A)** the nuclear (ITS + ETS) dataset and the **B)** 4-marker chloroplast data set. Numbers above branches are Bayesian posterior probabilities (PP).

young group as estimated here for the Canarian clade. Diversification rates varied between r = 2.84–0.78 species Myr^{-1} for ε = 0) and 1.25–0.34 species Myr^{-1} for ε = 0.9.

Phylogeography of Canarian *Cheirolophus*

Plastid DNA regions showed higher variability within the Canarian clade than the nrDNA (ITS + ETS) regions. Twenty-one variable characters (see Additional file 3: Table S3) were found within the cpDNA regions, defining 15 haplotypes distributed among the sampled populations of Canarian *Cheirolophus* (Figure 4A). In contrast, nrDNA yielded 18 variable characters –most of them autapomorphic of one single species– that generated only seven different haplotypes (Additional file 3: Table S3; Figure 4b). Although population sampling was not dense enough to assess the entire genetic variability in each species, the cpDNA haplotype network (Figure 4A) indicated a clear geographical pattern, with all haplotypes confined to a single island, with the exception of haplotype A found in both Tenerife and La Palma. Haplotype B is the most widespread, occurring in five different species endemic to La Palma. No intra-population diversity was found, but several morphologically described species contained more than one haplotype distributed across the different sampled populations (Figure 4A). The species with the greatest haplotype diversity was *Ch. webbianus* (haplotypes A, F, and G), with a wide distributional range in Tenerife (Figure 4A). Tenerife is the island with the highest number of haplotypes (7), followed by La Palma and La Gomera (3 in each), Gran Canaria (2), and El Hierro (1). The haplotype network constructed from nrDNA data (Figure 4B) showed a less complex structure compared to the cpDNA network. Haplotype III is the most widely distributed, occurring in three different

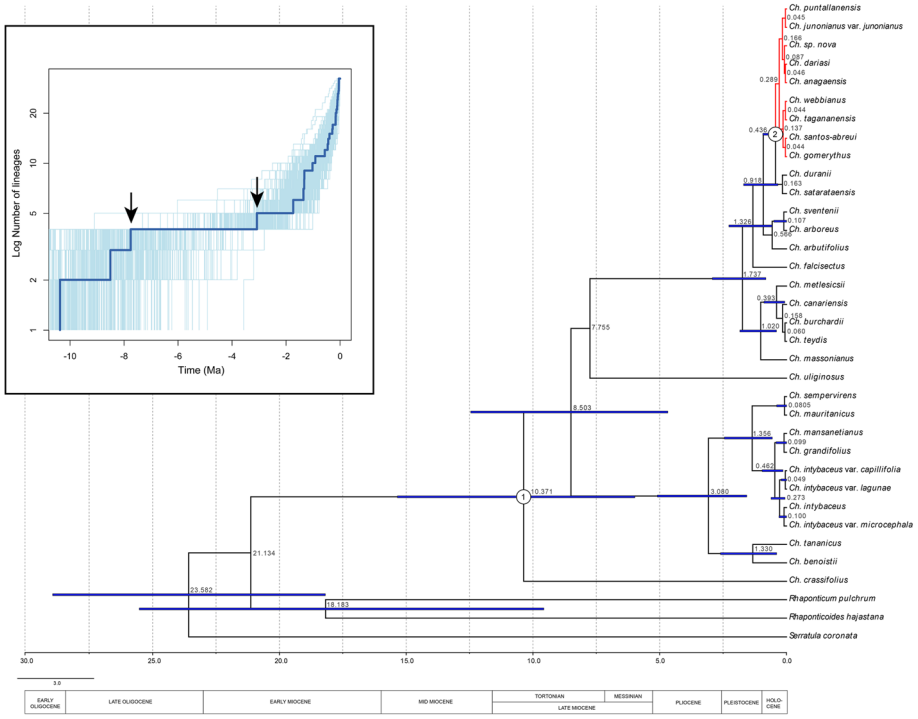

Figure 3 Divergence time chronogram of *Cheirolophus* based on the nuclear DNA dataset. BEAST maximum clade credibility tree, showing mean age estimates and 95% high posterior density intervals (for nodes with posterior probability > 0.5), obtained from the nuclear dataset. Significant changes in rates of diversification detected by MEDUSA are indicated at the appropriate node. The inset represents the lineage-through-time-plots (LTT) derived from the maximum clade credibility trees of the nuclear and chloroplast datasets. Faded lines represent the 95% confidence interval, as estimated from 1000 dated trees randomly sampled from the post burn-in BEAST distribution. Arrows indicate temporal shifts detected by TreePar.

islands (Tenerife, La Gomera and La Palma) and eleven Canarian species. Haplotype IV is present also in several species (*Ch. canariensis*, *Ch. burchardii* Susanna and *Ch. teydis*) and in different islands (Tenerife and La Palma), whereas the rest of haplotypes are restricted to one single species. Contrary to the pattern observed in some taxa for the cpDNA data, all populations within a species showed the same nrDNA haplotype.

Figure 5 shows the ancestral ranges and the history of migration events across space and time in the Canarian lineage, as reconstructed in BEAST based on the plastid DNA dataset and using uninformative rate priors (geographically informed priors gave very similar results; data not shown). This analysis recovered several well supported clades within the Canarian lineage, even if some of them seem to be constituted by just one haplotype that is shared by several species (Figure 5). The first diversification event is dated around the Late Pleistocene: 1.02 Ma (0.42–1.96 Ma), with the majority of species diverging within the last 0.5 Ma (0.12–1.25 Ma). Some species were recovered as polyphyletic: *Ch. teydis*, *Ch. canariensis*, *Ch. webbianus*, *Ch. metlesicsii* Parada and *Ch. arboreus* (Webb & Berthel.) Holub. Tenerife is reconstructed as the most likely ancestral range of the Canarian radiation, from which several dispersal events took place eastward (from Tenerife to Gran Canaria) and westward (from Tenerife to La Gomera and La Palma, and from La Gomera to El Hierro). La Gomera was colonized twice, from Tenerife and from Gran Canaria, although the dispersal events concerning Gran Canaria are not recovered by BSSVS, probably due to low clade support and the small size of the phylogeny (Figure 5). Again, the phylogeographical analysis based on the nrDNA dataset was less informative than the one based on plastid markers: very few nodes received significant support while only one migration event (from Tenerife to La Palma) was recovered by the BSSVS analysis (Additional file 5: Figure S2).

Discussion

Hybridization and incongruence among plastid and nuclear genomes

The conflicting relationships found here between the nuclear and plastid phylogenies might be attributed to different coalescence-based and biological phenomena, including ILS, duplication/gene loss, chloroplast capture (introgression), polyploidy and hybridization. The nuclear ribosomal region (ITS + ETS) is by far the most

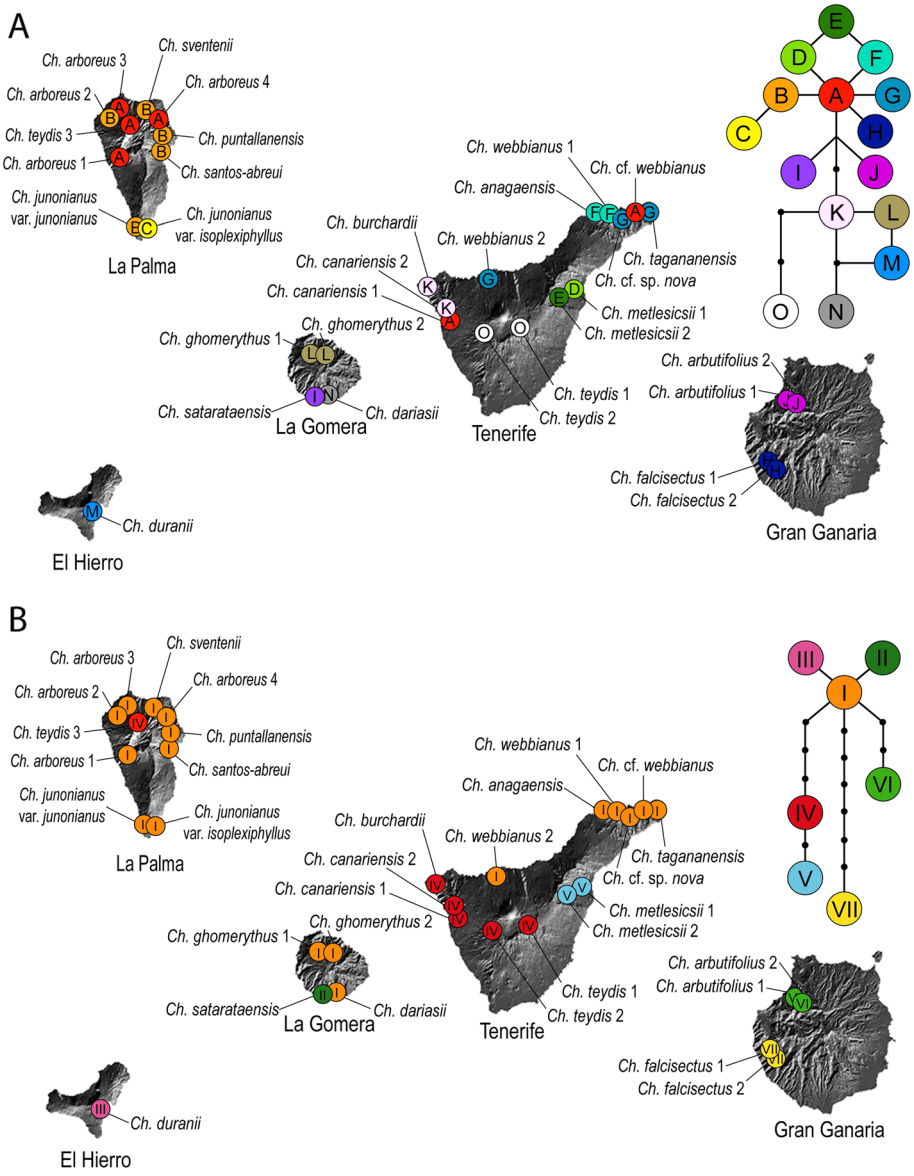

Figure 4 Geographical distribution of populations and haplotypes of Canarian *Cheirolophus* sampled in this study. The upper figure **(A)** shows plastid DNA haplotypes while the lower one **(B)** shows nuclear DNA haplotypes. The insets represent the haplotype networks estimated by TCS.

widely used marker in plant systematics, and has been the preferred marker to disentangle phylogenetic relationships in Asteraceae (e.g., [52]), but phenomena like potentially non-functional pseudogene copy types and incomplete concerted evolution across the large multicopy tandem arrays in which the nrDNA is arranged, are known to cause problems in phylogenetic reconstruction, especially among closely related species [57,58]. Although this could be the case here, there is some evidence suggesting that the incongruence between nuclear and plastid genomes is more related to differences in genetic variability than to ILS in the nrDNA markers. On the one hand, we found no evidence of double bands in the PCR amplification, while polymorphic sites (double peaks in the electrophoretogram in which the weakest signal reached 25% of the strength of the strongest signal) represented less than 1% of sites in the ITS/ETS DNA sequences (these sites were not included in the phylogenetic and phylogeographic analysis). On the other, the topology and grouping of taxa in the nrDNA tree agrees well with the current species circumscription, reflecting geographical and morphological affinities,

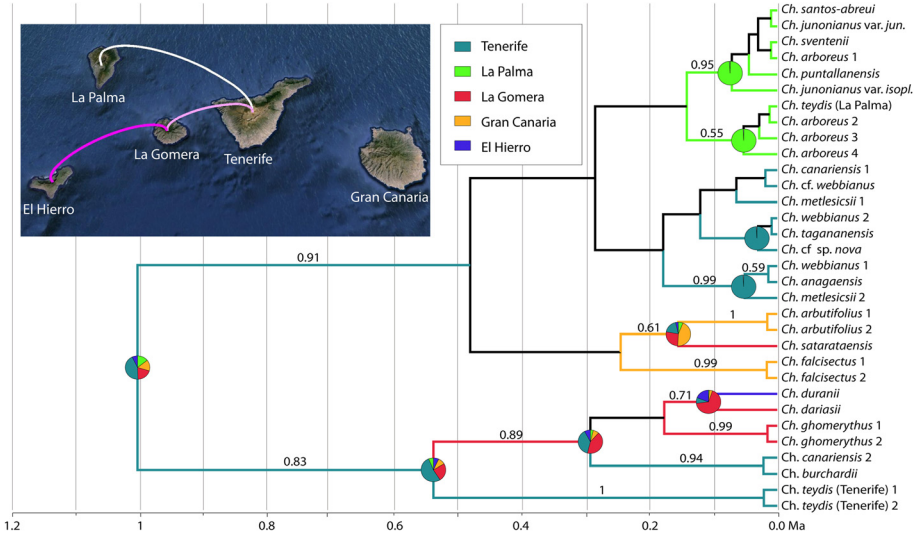

Figure 5 Bayesian ancestral range reconstruction and colonization history of Canarian *Cheirolophus* based on plastid DNA markers. Numbers above branches are Bayesian posterior probabilities (PP). The colored branch lengths represent the ancestral range with highest marginal probability for each lineage as inferred in BEAST (only branches with PP > 0.5). Node pie charts represent marginal probabilities for alternative ancestral ranges. Colonization routes identified by BSSVS are shown on the map with lines (see text for more details).

whereas some conspecific sequences fell into different clades in the cpDNA tree. Moreover, the nrDNA dataset contained at least ten-fold more variable sites than the full cpDNA dataset (see Additional file 3: Table S3), suggesting that the little variability in the latter (<2.0%) and associated homoplasy might be responsible for the artifactual positions of some species and lack of resolution at the base of the tree. Conversely, because of the their haploid nature, chloroplast markers generally require less time to fix novel mutations and present shorter coalescence times and higher polymorphism at lower taxonomical or population levels than nuclear ribosomal markers, despite their generally slower substitution rate [59]. Thus, they have been the marker of choice in species-diagnostic and phylogeographical studies on islands, outperforming the nuclear markers [60,61]. In our study, the cpDNA tree showed considerably higher levels of variability and phylogenetic resolution within the Canarian clade than the ITS + ETS tree, recovering several well-supported clades.

Finally, there is some evidence that at least part of the incongruence observed here can be caused by hybridization. Interspecific hybridization within groups that have recently radiated has been reported in many Macaronesian taxa [1,60,62,63] and might be behind the incongruent position of the Madeiran endemic *Ch. massonianus* in the nrDNA and cpDNA trees. This is supported by the position of this species in the multispecies coalescent analysis (*BEAST) intermediate between those occupied in the individual gene trees, and by the increased branch support for those clades including the putative parental taxa when this species was removed (Additional file 4: Figure S1), which are two evidences usually associated to horizontal gene transfer or hybridization (e.g. [39,40]). In addition, a genome size survey [22] revealed that the nuclear DNA content in the *Cheirolophus massonianus* (2C = 1.44 pg) was intermediate between those found in continental (mean 2C = 1.58 pg) and Canarian species (mean 2C = 1.38 pg). One potential explanation is hybridization or chloroplast capture (introgression) between a Canarian ancestor and the Atlantic Iberian endemic *Ch. uliginosus*, which occupies a basal position in the cpDNA tree together with *Ch. massonianus*. A close evolutionary relationship between species from Madeira and the western Iberian Peninsula has been documented in other studies [2], and is supported by the finding that submerged seamounts between Madeira and the continent might have acted as stepping stones during the Pleistocene glaciations [64]. Besides, preliminary amplified fragment length polymorphism (AFLP) analyses (Vitales et al., submitted) cluster *Ch. massonianus* within other Canarian *Cheirolophus* species, supporting the relationship depicted by the nrDNA tree.

Hybridization and introgression might also explain the polyphyletic nature of several Canarian species in the cpDNA tree (Figures 2 and 5). The only accession of *Ch. teydis* from La Palma exhibits haplotype A, which is very different from haplotype O of *Ch. teydis* populations occurring in Tenerife, but it is widely distributed over neighbouring populations of *Ch. arboreus*. Besides, the population of *Ch. arboreus* from north-western La Palma (Barranco Briestas) presents haplotype B, characteristic of other species from the island (Figure 4B); this

latter population also exhibits slight morphological differences with respect to conspecific populations in the same island [19]. Furthermore, both *Ch. teydis* form La Palma and *Ch. arboreus* from Barranco Briestas show considerable levels of genetic admixture according to a preliminary AFLP analyses (Vitales et al., submitted), supporting the hypothesis of ongoing gene flow. In Tenerife, discordant accessions of *Ch. canariensis* or *Ch. webbianus* presented haplotypes found in other geographically close species (*Ch. burchardii* and *Ch. anagaensis*, respectively) (Figure 4B). However, the relatively low sample size at the population level does not provide enough information to discern whether these latter cases of haplotype sharing are due to retention of ancestral polymorphism or actual gene flow among species, especially given the young age of the Canarian radiation (Figure 3). For example, the occurrence of haplotype A, ancestral according to its central position in the parsimony network (Figure 4), in accessions of three of the species recovered as polyphyletic (*Ch. teydis*, *Ch. canariensis*, and *Ch. webbianus*), might be explained by retention of ancestral polymorphisms due to insufficient time for coalescence. Further population-level studies are needed with intra-population sampling to discriminate among these explanations.

Early evolutionary history of *Cheirolophus*

Given the different level of genetic variation and potential hybridization between markers mentioned above, the evolution of the genus is based here on the nrDNA tree, whereas the divergence and biogeographic history of the Canarian clade is discussed based mainly on evidence from the cpDNA population-level analysis, albeit considering the potential of hybridization.

In reconstructing the biogeographic history of tribe Cardueae, Barres et al. [23] placed the origin of the derived subtribe Centaureinae in West Asia, followed by repeated dispersal events across the Mediterranean region during the Miocene that gave rise to most extant genera. Our nrDNA phylogeny supports this scenario and dates the first diversification event in *Cheirolophus* during the Mid-Late Miocene (Figure 3). At that time, the Mediterranean Basin still featured tropical climate characteristics, but a progressive aridification starting in the east around 11–9 Ma [65] might have pushed *Cheirolophus* westward, explaining its current Western Mediterranean distribution. The basal position within the genus of *Ch. crassifolius*, endemic to Malta in the Central Mediterranean, agrees well with this hypothesis of an early east-to-west migration.

By the late Miocene, three additional extant lineages in the genus had diverged (Figure 3): the Western Mediterranean and Macaronesian clades, and the lineage formed by the single species *Ch. uliginosus*, a rare herbaceous member of the genus. This initial period of diversification was followed by a transition period of 5 Myr characterized by no apparent diversification ending in a sharp increase in the rate of diversification (Figure 3). Either a period of stasis followed by a recent radiation or a scenario of high extinction rates – constant or punctual – removing the early lineages, might explain the phylogenetic pattern found here (Figure 3; [12]). Although these two scenarios are difficult to distinguish on the basis of extant data alone [50], several lines of evidence support the high extinction hypothesis. TreePar detected a decrease in diversification rates at 7.8 Ma (Figure 3), and MEDUSA estimated high relative extinction rates in *Cheirolophus*, prior to the rate shift within the Canarian radiation (Figure 3). This slowdown in diversification could be explained by the effects of extinction associated with the extreme drought trend that culminated with the Messinian salinity crisis [65], which led to the replacement across the Mediterranean Basin of an ancestral "tropical-like" flora by new sclerophyllous plant communities [66]. Extant *Cheirolophus* lineages might have survived this hostile environment by migrating westwards, as exemplified by the Macaronesian clade or its putative sister-group, *Ch. uliginosus*, endemic to the humid Atlantic coast of the Iberian Peninsula. Others seem have developed ecological adaptations to drought environments, i.e. severe leaf reduction is observed in *Ch. benoistii* from the western Mediterranean clade and succulent leaves are present in *Ch. crassifolius* from Malta. A new increase in diversification rates was detected by TreePar at c. 3 Ma (Figure 3), coincident with the establishment of the Mediterranean-type climate around 3.5 Ma [66]. Although the start of diversification within the Mediterranean clade preceded that of the Macaronesian radiation (Figure 3), estimated divergence times for cladogenetic events leading to main subclades or species complexes were surprisingly synchronous. This synchronicity might be explained by the effect of Pleistocene climate oscillations [10], which played an important role in driving plant diversification in the Mediterranean region [67]. A similar pattern of diversification as the one described here, with a slowdown in diversification around 8–7 Ma and a subsequent increase at 3 Ma, has been observed in other Mediterranean plant taxa [68], supporting the hypothesis that Miocene climate changes governed the diversification of these lineages.

Colonization and rapid diversification in the Canary Islands

A single colonization event to the Canary Islands was supported by both the nrDNA and cpDNA trees, in agreement with previous studies based on ITS alone and/or a more restricted sampling [21,22]. Following this initial colonization, *Cheirolophus* seems to have diversified rapidly: with c. 20 species arising in less than 1.8 million years (Figure 3). The high rate of diversification estimated for Macaronesian *Cheirolophus* (0.34–2.84 species Myr^{-1}) is

comparable to those exhibited by other island radiations. For example, Hawaiian *Bidens* (0.3–2.3 species Myr^{-1}) and Macaronesian *Echium* (0.4–1.5 species Myr^{-1}) were considered as the fastest plant radiations on volcanic islands to date [69]. 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 [69-71]. One note of caution, however, must be added here concerning the use of species macroevolutionary models to estimate diversification rates; these models assume complete divergence between taxa [48,50,52,72], whereas in recently diverged groups such as *Cheirolophus* (see also the cases of *Lupinus* in the Andes [71] or *Tetramolopium* in Hawaiian Islands [73,74]) there might not have been enough time for complete sorting of alleles into the diverging lineages. Nevertheless, preliminary AFLP results indicate that all the described Macaronesian species form significantly distinct genotypic clusters (Vitales et al., submitted), thus supporting their taxonomic boundaries.

Which were the mechanisms underlying such rapid diversification? Geographical isolation and allopatric speciation undoubtedly played a significant role. A complex pattern of inter-island colonization events to the east and west was recovered in our phylogeographical analysis, which highlighted Tenerife as the main source area (Figure 5). This agrees with other Canarian studies (e.g., [7,10,60,75]), showing the central island as a major hub for colonization events. Indeed, Tenerife harbours the highest genetic diversity for *Cheirolophus* in the archipelago (Figure 4), a fact observed also in other Canarian genera such as *Bystropogon* [76], *Sideritis* [63] or *Aeonium* [1]. This higher diversity has traditionally been attributed to its ancient and complex palaeogeographic history. Tenerife is composed of three "palaeo-islands", Anaga, Teno, and Roque del Conde, dating back between 4 and 12 Ma [77], which might have acted as a reservoir of relict biodiversity [5]. Most species of *Cheirolophus* in Tenerife are endemic to Teno (*Ch. canariensis*, *Ch. burchardii*) or Anaga (*Ch. anagaensis*, *Ch. tagananensis* (Svent.) Holub, *Ch.* cf. *sp. nova*), although their divergence largely postdates the origin of these ancient massifs. Another explanation is that habitat range fragmentation due to more recent events, such as the collapse of terrains during the last volcanic cycles (1.1–0.17 Ma, [78]) or the climatic fluctuations of the Pleistocene [79], might have contributed to the genetic isolation of populations (Figure 4). Finally, the patterns described here are constrained by the present distribution of the species and it is possible that they were different in the past. Intense volcanic activity and extinction might explain the currently low genetic diversity in older islands like Gran Canaria or the absence of *Cheirolophus* from the eastern islands of Fuerteventura and Lanzarote, which are closer to the mainland and are now too dry for *Cheirolophus* to grow.

Explosive intra-island diversification seems to have also occurred in La Palma, where most species originated after a colonization event from Tenerife less than 0.5 Ma (Figure 5). Limited seed dispersal and the rugged nature of the Canarian landscape have probably promoted rapid allopatric speciation events within the islands. Reduced dispersal potential in island organisms is known to be favoured by selection, as dispersal off the island is likely to result in the loss of the organism and/or propagules in the surrounding ocean [80,81]. Unlike most Centaureinae genera [82], the achenes in *Cheirolophus* can only be dispersed short distances by gravity (i.e. barochory). In addition, the sharp ravines and rocky cliffs which species of *Cheirolophus* inhabit provide deeply fragmented habitats that might have contributed to genetic isolation among populations, and subsequent allopatric speciation.

Nevertheless, several long distance dispersal (LDD) events (e.g. inter-island colonisations) occurred despite the apparently low ability of propagules to be transported. The ease of *Cheirolophus* for LDD is supported not only by the distribution of different species in five of the seven Canary Islands, but also by our haplotype network (Figure 4A) and phylogeographic reconstruction based on the plastid data (Figure 5), showing a double colonization of La Gomera (*Ch. ghomerythus* (Svent.) Holub - *Ch. dariasii* (Svent.) Bramwell and *Ch. sataratensis* (Svent.) Holub) and La Palma (endemic species and *Ch. teydis*). These double colonization events are also suggested by the less informative nrDNA haplotype network (Figure 4B). Given that stochasticity and non-standard transport mechanisms govern LDD in plants, drastic deviations from the usual dispersal distances do sporadically occur [83]. Indeed, evidence of transoceanic LDD has already been found for the Mediterranean *Ch. intybaceus* [84], suggesting that this phenomenon could be recurrent in the genus. Self-compatibility potential in *Cheirolophus* [17] may also have favoured the success of these colonization events, as dispersal of one single seed to a new habitat could establish a sexually reproducing population [85]. Finally, the basic chromosome number in *Cheirolophus* is $x = 15$, implying that the genus is originally polyploid [86,87]. This palaeopolyploidy could result in single fruits carrying higher genetic diversity – due to duplications – than what is expected in a diploid species, thus ameliorating the problem of severe genetic bottlenecks in the founding populations.

Although neutral genetic divergence as a result of restricted gene flow among isolated populations is probably the main force driving evolutionary diversification in Macaronesian *Cheirolophus*, ecological adaptation might be another mechanism responsible for this exceptionally rapid radiation. Even though it is not as spectacular as the

case of the Macaronesian *Argyranthemum* [88] or the Hawaiian silversword alliance [89], some fine examples of intra-island ecological segregation can be found in *Cheirolophus*. For example, species such as *Ch. junonianus* from La Palma and *Ch. falcisectus* Svent. ex Montelongo & Moraleda from Gran Canaria, inhabiting xeric habitats, show clear leaf surface reduction, whereas their sister taxa (i.e. *Ch. arboreus* and *Ch. arbutifolius* (Svent.) G.Kunkel) occupying more humid locations in the same islands, display an arborescent habit and a larger leaf surface. Another example of ecological differentiation could be represented by *Ch. teydis*, the only *Cheirolophus* species inhabiting the subalpine zone (1800–2200 m) and showing morphological adaptations to tougher ecological conditions (i.e. rosette-like disposed leaves with reduced laminas; waxy leaf cover; high number of smaller flowers; annual flowering shoots). Given the short time since the start of the Canarian radiation (Figure 5), we are probably witnessing the initial stages of a process of phenotype-environment driven differentiation, although to demonstrate ecological differentiation and adaptive radiation more stringent tests than simple correlations are needed (see [90]).

Conclusions

In the present study, we sequenced two nrDNA and four cpDNA regions from 57 populations representing the entire specific diversity in *Cheirolophus*. Significant incongruence was found in phylogenetic relationships between nuclear and plastid markers. The origin of *Cheirolophus* diversification was dated in the Mid-Late Miocene, followed by a slowdown in speciation rates at the end of the Miocene (Messinian) and a new increase in the Late Pliocene concurrent with the onset of the Mediterranean climate. Diversification within the Macaronesian clade started in the Early-Mid Pleistocene, with unusually high speciation rates (0.34–2.84 species Myr^{-1}) giving rise to one of the most remarkable examples of explosive plant radiation in oceanic islands so far reported. This exceptionally high diversification rate was probably driven by allopatric speciation (both intra and inter-island diversification), favoured by several intrinsic (e.g. breeding system, polyploid origin, seed dispersal syndrome) and extrinsic factors (e.g. fragmented landscape, isolated habitats, climatic and geological changes) that contributed to the progressive differentiation of populations and resulted in numerous microendemisms. Finally, inter-specific genetic contact via hybridization and chloroplast capture events (see above) and emergent ecological adaptation could be other mechanisms reinforcing the diversification process in Macaronesian *Cheirolophus*.

Data deposition

The data sets supporting the results of this article are available in the TreeBase repository, ID 15742 and http://purl.org/phylo/treebase/phylows/study/TB2:S15742 [91].

Additional files

Additional file 1: Table S1. Origin, collection data and GenBank accession numbers of the studied taxa.

Additional file 2: Table S2. Regions used for DNA sequences, references and polymerase chain reaction (PCR) conditions.

Additional file 3: Table S3. Characteristics of the aligned matrices for the nrDNA and cpDNA regions included in this study The values of the matrices containing all *Cheirolophus* species, only the Canarian *Cheirolophus* species and the *Cheirolophus* species plus the three outgroup species are given.

Additional file 4: Figure S1. Multilocus coalescent analysis of *Cheirolophus* including **(a)** and excluding **(b)** the putative hybrid species *Ch. massonianus*. The analyses are based on the concatenated nrDNA and cpDNA datasets. Branch labels indicate posterior probability values.

Additional file 5: Figure S2. Bayesian ancestral range reconstruction and colonization history of Canarian *Cheirolophus* based on nuclear DNA markers. Numbers above branches are Bayesian posterior probabilities (PP). The colored branch lengths represent the ancestral range with highest marginal probability for each lineage as inferred in BEAST (only branches with PP > 0.5). Node pie charts represent marginal probabilities for alternative ancestral ranges. Colonization routes identified by BSSVS are shown on the map with lines (see text for more details).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS, who has a deep knowledge on the Canarian flora, collected most of the plant material; DV, TG, JP, JV, and IS conceived and designed the experiments; DV performed the experiments; DV and IS analysed the data; DV and IS wrote the paper with relevant contributions of TG, JP and JV. All authors read and approved the final manuscript.

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Processos clau en la diversificació de *Cheirolophus* (*Asteraceae*) en illes oceàniques inferits mitjancant dades d'AFLP

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La radiació del gènere *Cheirolophus* (*Asteraceae*) a la Macaronèsia constitueix un cas espectacular de diversificació ràpida en illes oceàniques. Vint espècies -de les quals nou són incloses en la Llista vermella d'espècies amenaçades de la IUCN- han estat descrites fins el present en els arxipèlags de Canàries i Madeira. Un estudi filogenètic previ revelà que la diversificació de *Cheirolophus* començà fa menys de 2 Ma. Com a resultat d'aquest procés explosiu d'especiació, s'observà una resolució filogenètica limitada, principalment deguda a la baixa variabilitat dels marcadors moleculars usats. En aquest nou estudi, hem emprat marcadors altament polimòrfics AFLP per a i) avaluar els límits específics dels tàxons macaronèsics del gènere, ii) inferir les seves relacions evolutives i iii) investigar els patrons de diversitat genètica en relació amb els processos que potencialment han estat involucrats en la radiació de *Cheirolophus*. Cent setanta-dos individus en representació de totes les espècies macaronèsiques de *Cheirolophus* foren analitzades usant 249 *loci* d'AFLP. Els nostres resultats suggereixen que l'aïllament geogràfic jugà un paper important en aquest procés de radiació. Aquest fou mediat probablement per la combinació d'un flux gènic reduït i una bona habilitat per a fenòmens esporàdics de colonització a llarga distància. A més, també hem trobat traces d'introgressió incipient i d'adaptació ecològica, que podrien haver afavorit aquesta diversificació extraordinària de *Cheirolophus* a la Macaronèsia. Finalment, proposem que les categories d'amenaça actual assignades a les espècies de *Cheirolophus* macaronèsics no reflecteixen la seva rellevància evolutiva, així doncs, le avaluacions futures del seu estat de conservació haurien de tenir en compte els resultats aquí presentats.

Revista: el factor d'impacte d'aquesta revista (corresponent a l'any 2013, segons el JCR de la ISI web of Knowledge) és 3,534, i es troba en la posició 8 de 55 a *Multidisciplinary Sciences* (Q1, primer quartil).

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Key Processes for *Cheirolophus* (Asteraceae) Diversification on Oceanic Islands Inferred from AFLP Data

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Abstract

The radiation of the genus *Cheirolophus* (Asteraceae) in Macaronesia constitutes a spectacular case of rapid diversification on oceanic islands. Twenty species – nine of them included in the IUCN Red List of Threatened Species – have been described to date inhabiting the Madeiran and Canarian archipelagos. A previous phylogenetic study revealed that the diversification of *Cheirolophus* in Macaronesia started less than 2 Ma. As a result of such an explosive speciation process, limited phylogenetic resolution was reported, mainly due to the low variability of the employed molecular markers. In the present study, we used highly polymorphic AFLP markers to i) evaluate species' boundaries, ii) infer their evolutionary relationships and iii) investigate the patterns of genetic diversity in relation to the potential processes likely involved in the radiation of *Cheirolophus*. One hundred and seventy-two individuals representing all Macaronesian *Cheirolophus* species were analysed using 249 AFLP loci. Our results suggest that geographic isolation played an important role in this radiation process. This was likely driven by the combination of poor gene flow capacity and a good ability for sporadic long-distance colonisations. In addition, we also found some traces of introgression and incipient ecological adaptation, which could have further enhanced the extraordinary diversification of *Cheirolophus* in Macaronesia. Last, we hypothesize that current threat categories assigned to Macaronesian *Cheirolophus* species do not reflect their respective evolutionary relevance, so future evaluations of their conservation status should take into account the results presented here.

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Introduction

In the last two decades, the Macaronesian archipelagos (Canary Islands, Cape Verde, Azores, Madeira and Savages) have attracted much interest from researchers studying plant diversification and radiation processes [1]. These volcanic islands provide a wide variety of ecological conditions, geological ages and geographical isolation scales [2–4], which promote the existence of a mosaic of habitats that represent an excellent natural laboratory in which to study selection forces and evolutionary processes. The Macaronesian archipelagos have been recognized as a hotspot of plant diversity [5], and have rapidly become a popular model system for scientists to test many speciation hypotheses, both empirically and theoretically. The wide diversity of habitats found in Macaronesia – spanning from xerophytic coastal cliffs to subalpine belts – has served to demonstrate the role of adaptive radiation in a range of plant groups (e.g. *Argyranthemum* Webb, [6]; *Sonchus* L. alliance, [7]; *Aeonium* Webb & Berthel., [8]; *Echium* L., [9]; *Tolpis* Adans., [10]). Indeed, niche pre-emption through adaptive radiation is the most prevailing hypothesis to explain the high degree of endemism and monophyly within Macaronesian lineages [11,12]. Furthermore, during the geological history of Macaronesian archipelagos, several islands have emerged, disappeared and/or changed their relative geographical position (see [4]), promoting complex isolation-connection and colonisation processes between islands and between islands and the continent. This complexity in volcanic archipelagos has given rise to numerous study cases examining the relative importance of vicariance versus dispersal in shaping insular biotas [13–15]; the role of islands as regions from which taxa might colonise continents and other archipelagos [16,17] and the different stages of colonisation and radiation processes in relation to the ontogeny phases of oceanic islands [18,19].

Occasionally, island radiations occur over a short period of time, resulting in ecologically and morphologically distinct taxa, but leading to poor molecular differentiation [9,20–23]. These

cases, in which explosive species radiation takes place are among the most interesting and least understood evolutionary events, maybe due to the difficulty in carrying out species level analyses [24–26]. Rapid island radiations have been generally associated to some features – such as small population size, release from previous ecological constraints, and adaptation to new niches – recurrently observed in a wide variety of species and island archipelagos [27]. However, since traditional phylogenetic studies usually provide little resolution in delimiting taxonomical boundaries and untangling the relationships among these rapidly evolving species, the precise role of morphological, life history, and physiological traits and their genetic basis in explosive plant radiations remain essentially unsolved [28].

The Macaronesian *Cheirolophus* Cass. (Asteraceae) complex comprises 20 species (out of the 30 constituting the whole genus), with *Cheirolophus massonianus* (Lowe) A.Hansen & Sunding occurring in the Madeiran archipelago, and the remaining species distributed across the western Canary Islands (see Fig. 1). Although they are usually associated with humid basalt cliffs, a few taxa have adapted to inhabit very diverse ecological zones of the archipelago [29,30]. Most of the island *Cheirolophus* are subshrubs, shrubs or even arborescent shrubs, showing a clear increase in woodiness relative to their continental congeners and a general shift towards inflorescences with white to purple flowers arranged in a candelabrum-like pattern. Some species are relatively widespread throughout a single island (e.g. *C. webbianus* (Sch.Bip.) Holub from Tenerife), whereas others are found on two different islands (e.g. *C. teydis* (C.Sm.) G.López from Tenerife and La Palma and *C. massonianus* from Madeira and Porto Santo). However, most species are narrow endemics occurring in a small number of restricted localities with only a few hundred or less individuals. Consequently, nine Macaronesian species have been included in the IUCN Red List of Threatened Species [31] as vulnerable, endangered or critically endangered. In addition, 17 of the species or subspecies endemic to the Canary Islands are included in the 2010 Red List of Spanish Vascular Flora [32]. In those cases where many related taxa are under threat and conservation strategies must be prioritized, taxonomic knowledge becomes of special relevance and efforts should be made to establish accurately the boundaries of the species concept. To achieve this goal, the recent ''unified species concept'' advocates the use of diverse lines of evidence (e.g. monophyly at one or multiple DNA loci, morphological diagnosis, ecological distinctiveness, etc.) so that a higher degree of corroboration in taxonomic delimitation is attained [33]. In recent times, conservation genetics has become an essential approach for evaluating the status and the level of threat at the species and population levels, identifying the genetic basis of processes which may potentially lead into an extinction vortex and informing authorities about management priorities for endangered species and/or populations [34].

Several investigations based on morphological, cytogenetic, isozymes and DNA sequencing data have been previously used to infer evolutionary relationships and taxonomy for the 29 currently recognized species of *Cheirolophus* [35–40]. These studies revealed that the genus arose in the Mediterranean region with subsequent dispersal towards Macaronesia, but they failed to reconstruct the relationships among insular congeners. A recent phylogenetic study featuring relaxed-clock dating analyses and diversification tests (involving sequences of various nuclear and plastid regions and sampling several populations per species, [41]) has evidenced a recent origin of Macaronesian *Cheirolophus* radiation. The age of this group was estimated to be 1.74 Ma (95%HPD 0.82–2.93), implying a diversification rate of 0.34–2.84 species Myr^{-1}. Indeed, this diversification rate is comparable to those exhibited by the 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 documented to date [22]. Bayesian phylogeographic and ancestral range analyses were also applied in the same study, highlighting the major role of allopatric speciation to explain current diversity in Macaronesian *Cheirolophus* [41]. Geographical isolation among populations and long distance dispersal (both intra and inter-island colonisations) were proposed as the main forces driving the rapid diversification of the group, but introgression and emerging ecological adaptation were also suggested as additional factors reinforcing the speciation process. Despite such efforts, the employed nrDNA and cpDNA markers provided very low variability within the Macaronesian species, thus hampering accurate inferences about the role played by those evolutionary mechanisms potentially involved in the radiation of *Cheirolophus*.

Amplified fragment length polymorphism (AFLP) analyses have been found to provide insights into interspecific relationships in diverse animal and plant groups when other methodologies have failed in this attempt (e.g. [42–49]). Indeed, several authors have suggested that this technique can be especially useful in reconstructing phylogenetic relationships among species that have diverged or radiated recently [50–53]. Although some considerations must be taken into account for its use, AFLP are currently widely employed in molecular ecology and evolution research [54–59]. In fact, this DNA fingerprinting approach has proven to be particularly suitable for evaluating the genetic structure of plant species in different oceanic archipelagos, and hence elucidating the potential evolutionary forces – such as gene flow or genetic drift – that influence the distribution of genetic diversity among individuals, populations, and species [60–62].

AFLP fingerprinting – complemented with morphological and other molecular data – was employed here with the main objective of unravelling the explosive radiation that *Cheirolophus* underwent in the Canarian and Madeiran archipelagos. We applied phylogenetic and population genetic approaches to (i) evaluate the taxonomic identity of the Macaronesian species and to (ii) disentangle the evolutionary relationships between them. Additionally, we studied the patterns of genetic diversity within and between populations to (iii) infer the role of the evolutionary processes potentially involved with this explosive radiation (i.e. geographic isolation, ecological adaptation and introgression).

Materials and Methods

Sampling strategy

One hundred and seventy-two individuals from 29 populations of 20 Macaronesian *Cheirolophus* species were sampled, covering the whole taxonomic diversity recognized for the Madeiran and Canarian archipelagos (see Fig. 1 and Table S1 for further geographical details of sampling sites). One to 12 individuals per population were included in the study depending on the material availability and the uneven success of DNA extraction and AFLP procedures. We decided to incorporate those populations containing extremely scarce sampling (one or two individuals) because of their distinctiveness: (1) it was the only material available for the taxon (*C. dariasii* (Svent.) Bramwell; *C. cf. webbianus*); (2) the inclusion of that particular population was essential to understand the distribution of the species (*C. teydis* from La Palma; *C. massonianus* from Porto Santo); or (3) the additional subpopulation completes the sampling of extremely local species (*C. metlesicsii* Parada). Leaf material for DNA extraction was collected from plants in the field, dried in silica-gel

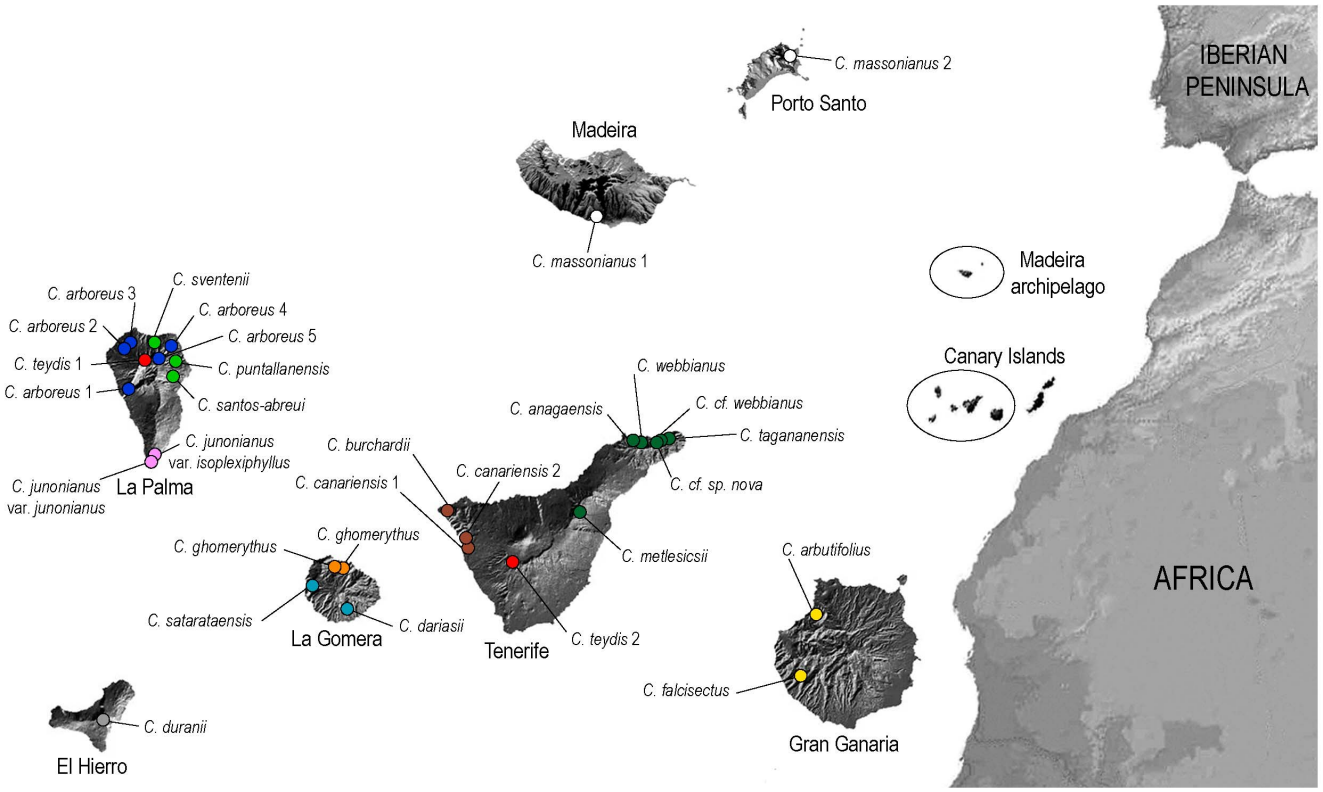

Figure 1. Geographic location of the 29 sampled populations of Macaronesian *Cheirolophus* species. Colour coding circles correspond to genetic structure derived from Bayesian mixture analysis of AFLP markers implemented in BAPS.
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and stored at room temperature. Insular Cabildos of Tenerife, Gran Canaria, La Gomera, La Palma and El Hierro provided all the necessary permits to collect samples from protected natural areas in the respective islands. The Canarian Council of Education, University and Sustainability issued the authorization to collect samples of the protected species listed on Table S1. Samples used of the endangered *C. massonianus* were provided by the Botanical Garden of Madeira.

DNA extraction, AFLP protocol and nrDNA sequencing

Genomic DNA was extracted from fragments of silica-gel dried leaf tissue following the protocol of Doyle and Doyle [63] with slight modifications. DNA samples were cleaned using QIAquick columns (Qiagen, Valencia, CA, USA) and their quality and DNA concentration were determined using NanoDrop ND-1000 spectrophotometry (ThermoScientific, Wilmington, DE, USA). The AFLP technique was carried out following the protocol described in Vos et al. [64] in accordance with the modified AFLP Plant Mapping Protocol (Applied Biosystems Inc. Foster City, CA, USA) using *Eco*RI and *Mse*I with 500 ng of isolated genomic DNA per sample. Eighteen primer pair combinations were tested on six individuals from three different populations to screen those producing the most informative and readable profiles. Three pairs of primers were selected giving the most polymorphic and scorable polymorphic pattern: EcoRI-CTT/MseI-AC; EcoRI-CTC/MseI-AA; and EcoRI-CAG/MseI-AT. The success of each step was tested by running the PCR products on a 1.5% agarose gel. Fragments were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc.) with 10 μL of High Dye (deionized formamide) and 0.2 μL of GeneScan 500 ROX Size Standard per sample. Amplified fragments were genotyped as present/absent using GeneMarker AFLP/Genotyping software (version 1.9; SoftGenetics, LLC., State College, PA, USA).

For an initial scoring, all alleles within a range of 50 to 490 bp were considered. Afterwards, visual correction was carried out to eliminate erroneous peaks (low intensity or no reproducibility). AFLP error rates were calculated following [65]. Twenty-five random samples per primer combination were replicated to ensure reproducibility, repeating all parts of the AFLP protocol (extraction, digestion, pre-selective and selective PCR). All alleles with an error rate >5% were eliminated. In order to test the occurrence of size homoplasy, we also calculated the correlation between AFLP fragment sizes and frequencies using AFLP-SURV 1.0 [66].

We also employed some nuclear ribosomal DNA sequences to examine the potential role of genetic introgression in the radiation of Macaronesian *Cheirolophus*. Only those populations (see Table S2) showing clear evidences of interspecific gene flow according to our AFLP data – together with some of their putative parental species – were studied. Sequencing procedures, GenBank accessions and any other information about the material and methods employed can be found in [41]

AFLP genetic diversity and population differences

The use of AFLP data (dominant markers) for estimating allelic frequencies implies the consideration of an outcrossing mating system and near random mating. This means that those populations would be under Hardy-Weinberg equilibrium [67]. *Cheirolophus* has a predominantly outcrossing mating system and is pollinated by generalist insects, so one expects near random mating in the studied populations.

To estimate genetic diversity in each population showing more than one sampled individual, the following parameters were calculated: a) private alleles; b) rare alleles, present in <10% of the samples; and c) unbiased heterozygosity (Hj), calculated using TFPGA v.1.3 [68]. Further measures of genetic diversity were estimated through: (i) the frequency-down-weighted marker values (DW) index of [69] using AFLPDAT [70]; (ii) the band richness (Br), which is the number of phenotypes expected at each locus, and can be interpreted as an analogue of the allelic richness, ranging from 1 to 2 [71]; and (iii) the percentage of polymorphic loci (PLP) with a significance of 1% (P = 0.99). Br and PLP indices were calculated according to the rarefaction method of Hurlbert [72], and conditioned to the smallest population size (N = 3) allowed by the software AFLPDIV v.1.0 (http://www.pierroton.inra.fr/genetics/labo/Software/Aflpdiv/). As a consequence, Br and PLP were not calculated for populations having less than three individuals. To assess whether genetic diversity indexes (i.e. Hj, DW, Br and PLP) differed among islands, diversity range values were compared in a non-parametric Kruskal-Wallis test using package RCMDR [73] implemented in R software [74].

Pairwise F_{ST} values were estimated for each pair of populations included in this study (Table S1) using AFLP SURV v.1.0 [75]. Significance was evaluated through 10000 permutations.

Phylogenetic analyses

To address questions of species delimitation and evolutionary relationships, AFLP data generated from the three selected primer pairs were combined into one large matrix and analysed together. Phylogenetic trees were reconstructed for this combined dataset using neighbor-joining (NJ) and Bayesian inference methods. NJ trees were built using Nei-Li distances [76] and 1000 replicates with NTSYS PC v.2.1 software [77]. The support for specific nodes for the NJ tree was calculated using bootstrap [78] with 10000 replicates. Bayesian inference was performed in MrBayes v.3.1.2 [79] using a F81-like model for restriction sites [80,81] and running four independent chains each of length 10 million sampling every 100 trees. Convergence was assessed using Tracer v.1.4.1 [82] and a burn-in of 1 million trees was discarded. The remaining trees were used to construct a 50% majority rule consensus tree.

Additionally, non-tree-building approaches have been recommended to avoid conflicting phylogenetic signals in those cases in which the analyses of the data do not exhibit a bifurcating tree-like behaviour [83]. Specifically, network methods have been proposed to resolve the uncertainty of these processes [83,84]. We used the Neighbor-Net method [85] carried out with SplitsTree v.4.10 [83] to construct a distance-based network for the AFLP dataset using the Jaccard coefficient [86], which is restricted to shared band presence rather than shared absence.

Genetic structure: spatial patterns, Bayesian clustering and PCoA

We performed Mantel tests to evaluate the spatial effect in the genetic differences between populations using two similarity matrices. Genetic distance matrices – with and without *C. massonianus* from Madeira – were constructed with F_{ST} values between populations, and geographical matrices were calculated by the spatial distance (X and Y coordinates) between populations using ArcGIS v.9.1 (ESRI, Redlands, CA, USA). Mantel tests were performed with the vegan package [87] implemented in R software [74] using 100000 permutations and considering a *p*-value limit of 0.05. Because most populations were from the Canary Islands and only two of them are from Madeiran archipelago, distant from the remainder (>500 km), the Mantel test analysis was repeated without these populations.

Bayesian clustering analysis were carried out using STRUCTURE v.2.3 [88]. We applied the admixture ancestry model and the correlated allele frequencies. Ten independent simulations were run for each possible number of genetic groups (K) from $K = 1$ to 29, using a burn-in period of 10^5 generations and run lengths of 5×10^5. To estimate the number of genetic groups (K) we selected the K value that maximizes the probability of the data L(K). We also considered the criterion proposed by Evanno et al. [89] to estimate the best value of K for our data set, based on the rate of change in the probability between successive K values, ΔK. Bayesian analyses of the genetic structure were also conducted with BAPS (Bayesian Analysis of Population Structure, Spatial Clustering of Groups, [90]), which uses stochastic optimization instead of Markov chain Monte Carlo to find the optimal partition. We performed a mixture analysis of individuals with the geographic origin of the samples used as an informative prior ('spatial clustering of individuals') or without this prior ('clustering of individuals'). BAPS simulations were run with the maximal number of groups (K) set from 1 to 29. Each run was replicated 10 times, and the results were averaged according to the resultant likelihood scores. The output of the mixture analyses were used as input for population admixture analysis [90], with the default settings in order to detect admixture between clusters.

Some authors have warned about a degree of over-splitting in the genetic structure analysed with AFLP markers (e.g. [54]) and have suggested the use of simpler statistical analyses to compare the pattern found by Bayesian methods. Similarities among individuals were also studied via Principal Coordinate Analysis (PCoA, [91]) using the Jaccard distance, in order to detect other possible relations that could not be visualized with assignment methods or phylogenetic analyses. This procedure was carried out with R software [74] using the vegan package [87].

Finally, we conducted analysis of molecular variance (AMOVA; [92]) using ARLEQUIN v.3.5 [93] to estimate genetic differentiation following an alternative and widely used non-Bayesian approach that does not assume Hardy-Weinberg equilibrium or independence of markers. A first AMOVA analysis was implemented without taking regional structure into account. We also carried out four independent AMOVAs grouping the populations of *Cheirolophus* according to: i) their current taxonomic affiliation; ii) their island origin; iii) the most plausible model proposed by STRUCTURE; and, iv) the clustering proposed by BAPS. Pairwise genetic distance between individuals using the square Euclidean distance was used for the AMOVA analyses, considering three levels: within populations, between populations within regions and between regions. Only two levels (within and between populations) were considered when no additional grouping structure was applied.

Morphological revision and analyses

Diagnostic morphological characters classically used in taxonomical treatments of Macaronesian *Cheirolophus* were also analysed in this study. Morphological traits were measured from herbarium vouchers, cultivated individuals and/or gathered from the literature depending on the species. In the present study, morphological data was only considered for a given species when at least three different individuals where available for measurements. We obtained accurate data from 16 species for six morphological variables widely employed to distinguish among Canarian *Cheirolophus* taxa (Table S3) (including leaf length, leaf width, size of capitula, plant size, floral colour and leaf shape). Qualitative traits (i.e. floral colour and leaf shape) were coded

numerically (+1 for whitish and −1 for rose-colored flowers; +1 for entire, −1 for divided and 0 for intermediate/both leaf shapes). All the variables were standardized by subtracting the character mean from each species measure and then dividing by the character standard deviation. The resulting matrix was analysed by Principal Components Analysis (PCA) using RCMDR [73] implemented in R software [74].

In order to correlate genetic AFLP data with morphological traits, we carried out Mantel tests and a generalized analysis of molecular variance (GAMOVA; [94]). Mantel tests were performed with R software [74] and the package vegan [87] computing 100000 permutations and considering a *p*-value limit of 0.05. Complementary to Mantel test, GAMOVA analyses provides a regression-based method of the analysis of molecular variance (AMOVA; [92]) that could be interpreted as a multiple regression. GAMOVA approach is an especially suited tool to identify, associate and evaluate the relationships between the differences in a phenotype or environmental variable of interest between some populations and the genetic variations among the same populations. The significance between genetic differentiation (i.e. F_{ST}) and six morphological traits (see above) was tested by running a regression matrix with a permutation test with 10000 repetitions.

Results

AFLP genotyping and filtering

Initially, 371 alleles were obtained from automatic genotyping. After manual correction, error rates calculation, elimination of small and troublesome alleles and low intensity peaks, a final matrix with 249 (67.1%) alleles were considered for subsequent analyses. The final data set obtained showed an error rate of 3.2%, which is below the maximum error rate percentage accepted for good AFLP reproducibility (5%) [95].

Population genetics: diversity and differentiation

Within-population genetic diversity measures are shown in Table S1. Private fragments were scarce across the studied populations; only three were detected, one each in *C. santos-abreui* A.Santos, *C. sataratаensis* (Svent.) Holub and *C. tagananensis* (Svent.) Holub populations. The frequency of rare fragments was, conversely, much higher (10.6%), but most of these were shared by two or more populations. Hj diversity values were similar in all populations, ranging between Hj = 0.0459 (*C. duranii* (Burchard) Holub), and Hj = 0.1539 (*C. arboreus* (Webb & Berthel.) Holub from Los Tilos) in populations with more than three sampled individuals, with a mean value of Hj = 0.07892. The frequency-down-weighted marker values (DW) index showed overall high values, but considerable differences were observed among populations (ranging from 8.65 in *C. junonianus* (Svent.) Holub var. *isoplexiphyllus* to 19.75 in *C. webbianus*). The percentage of polymorphic loci (PLP %) ranged from 10.1% in *C. duranii* to 77.0% in *C. arboreus* (Los Tilos), and band richness (Br) ranged from 1.08 in *C. junonianus* var. *junonianus* to 1.46 in *C. arboreus* (Los Tilos). The DW was the only diversity index showing significant differences among islands (Kruskal-Wallis chi-squared = 15.02, df = 5, p-value<0.05); La Palma populations showed the lowest DW values. Pair-wise F_{ST} comparisons were significant (Table S4; mean value $F_{ST} \pm SD = 0.259 \pm 0.185$ with all populations, mean value $F_{ST} \pm SD = 0.302 \pm 0.051$ with populations with more than three individuals sampled). The biggest difference was found between *C. puntallanensis* A.Santos and *C. arbutifolius* (Svent.) G.Kunkel ($F_{ST} = 0.577$). The pair-wise comparisons between populations of the same species were much smaller than those between species (e.g. mean $F_{ST} = 0.055$ between *C. ghomerythus* (Svent.) Holub populations).

Phylogenetic analyses of Macaronesian *Cheirolophus* based on AFLP data

Trees constructed with the combined AFLP data using NJ and Bayesian estimation had generally similar topologies. Tree-building analyses of AFLP data resulted in similar fully-resolved assignment of individuals into species classically identified by diagnostic morphological and ecological characters (e.g. Bayesian 50% majority rule tree in Fig. 2; other trees not shown). However, species relationships were poorly resolved and only a few groups were partially identified. All trees included a large, strongly supported clade grouping most species from La Palma plus *C. duranii* from El Hierro (posterior probability, PP = 0.99; Fig. 2). Other supported smaller lineages grouped species from the Taganana peninsula (*C. tagananensis*, *C. anagaensis* A.Santos, *C. cf. webbianus* and *C. cf. sp. nova*; PP = 0.99) or species from the Teno mountains (*C. canariensis*, *C. burchardii* Susanna; PP = 1.00), in both cases from Tenerife. The phylogenetic analyses also confirmed the close relationship between *C. sataratаensis* and *C. dariasii* (PP = 1.00), until recently considered as subspecies of the same species. The two populations of *C. teydis* from Tenerife and La Palma grouped together but were not embedded in a resolved clade. Similarly, *C. arbutifolius* and *C. falcisectus* Svent. ex Montelongo & Moraleda (both from Gran Canaria) appeared closely related (PP = 0.84) but their phylogenetic position in the genus was not fully resolved.

The Neighbor-Net (NN) analysis, although indicating considerable reticulation in the data, resolved most of the species groups according to their current taxonomic boundaries (Fig. 3). In addition, this NJ network approach clustered most of the species in agreement with the Bayesian reconstruction depicted in Fig. 2. The species from La Palma formed a cluster closely related to the species from El Hierro (*C. duranii*). Those inhabiting eastern Tenerife (*C. webbianus*, *C. tagananensis*, *C. anagensis*, *C. metlesicsii* and *C. cf. sp. nova*) and western Tenerife (*C. canariensis* and *C. burchardii*) were also segregated into regional clades already resolved in the Bayesian and NJ phylogenetic analyses. Neighbor-Net analysis also revealed some phylogenetic conflicts not identified in the tree building analyses. For example, the species from El Hierro (*C. duranii*) showed clear reticulation, with an intermediate position between La Palma cluster and a lineage grouping two species from La Gomera (*C. sataratаensis* and *C. dariasii*). Finally, NN analyses suggested a degree of reticulated connection between *C. ghomerythus* (from La Gomera) and *C. massonianus* (from Madeira), but this potential relationship was not supported by any Bayesian approach.

Bayesian clustering and spatial analyses of the genetic structure

Using the matrix of inter-population F_{ST} distances, and the matrix of geographical distances in kilometres, the Mantel test indicated a significant correlation between genetic and geographical distances (r = 0.258; p-value<0.05). Similar results were obtained if the Madeiran populations were excluded from the analysis (Mantel test r = 0.326, p-value<0.05).

The Bayesian analysis of population genetic structure conducted with STRUCTURE found the highest L(K) and ΔK values for $K = 2$. This grouping separated La Palma species (plus *C. duranii* from El Hierro) – cluster A – from the rest of the species from Tenerife, La Gomera, Gran Canaria and Madeira (including the *C. teydis* population from La Palma) – cluster B –, showing high

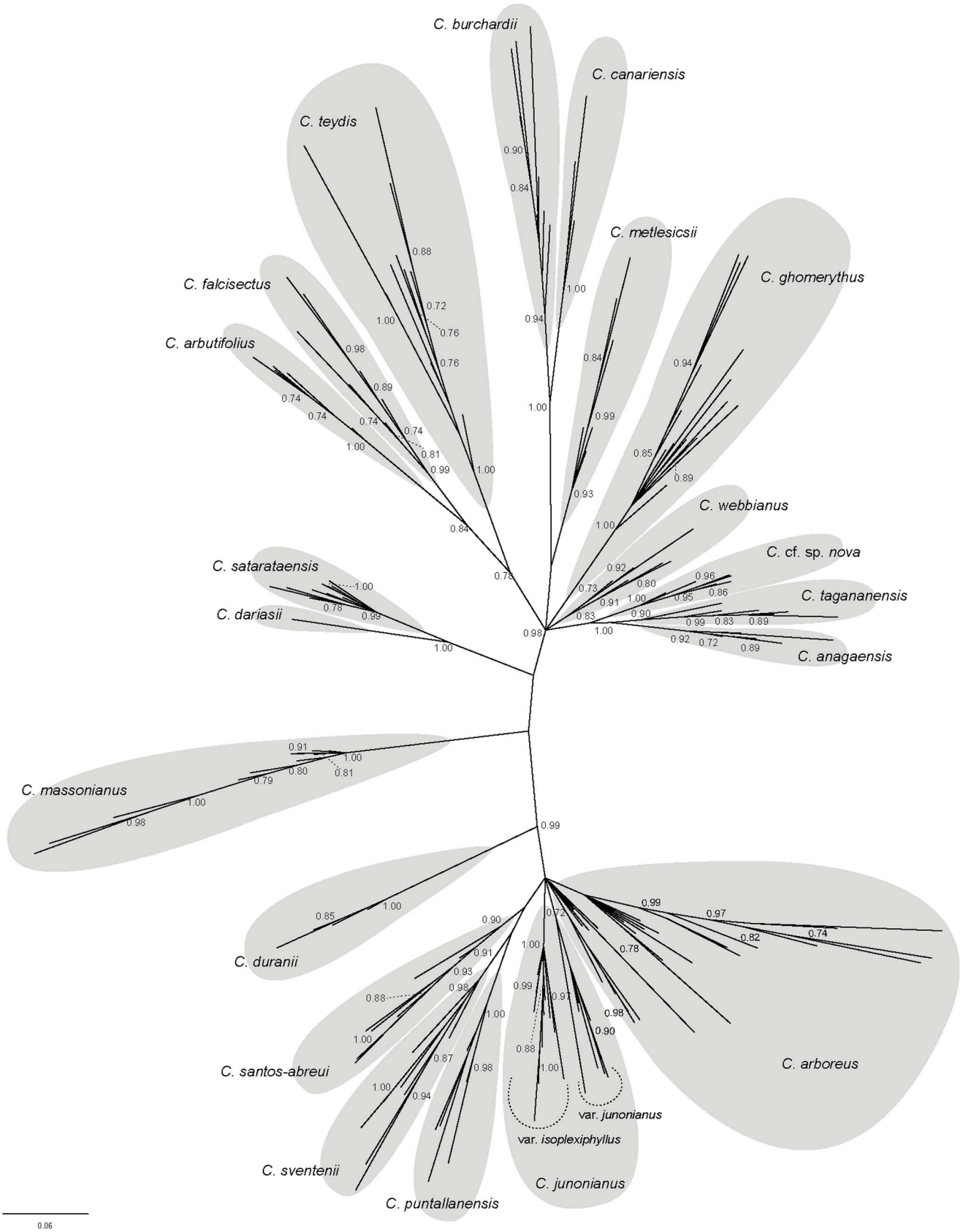

Figure 2. Phylogenetic analysis of AFLP data. Unrooted 50% majority rule tree from Bayesian analysis of the combined AFLP dataset for all Macaronesian species of *Cheirolophus*. Posterior probability values ≥ 70 are shown near each branch.
doi:10.1371/journal.pone.0113207.g002

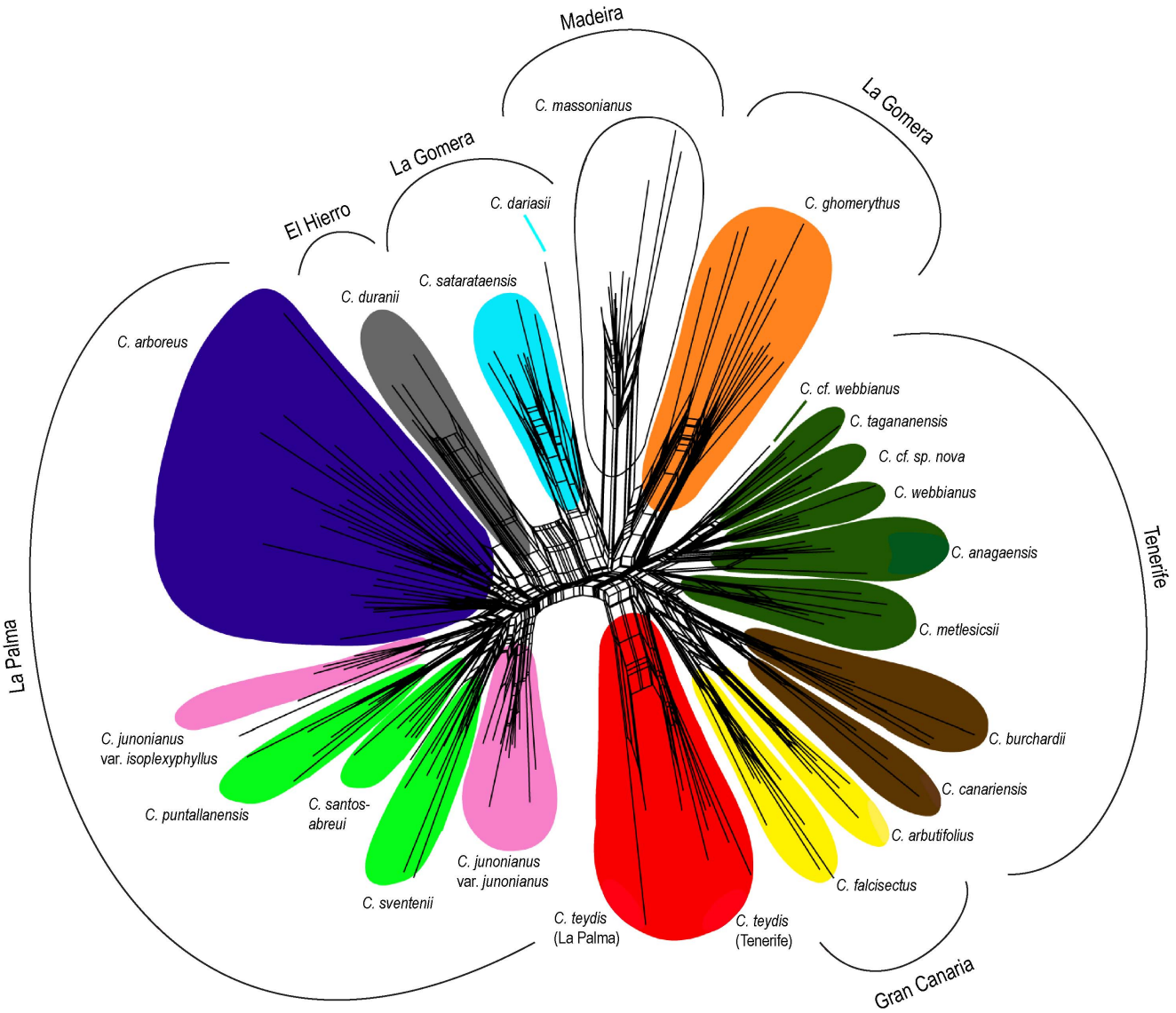

Figure 3. Neighbor-Net of AFLP data obtained from Macaronesian *Cheirolophus*. Colour coding profiles delimitate the different species indicating the genetic clusters assigned by BAPS.
doi:10.1371/journal.pone.0113207.g003

percentages of individual memberships (97% and 98%, respectively) for these predefined groups (Fig. 4). According to the STRUCTURE analyses, the population of *C. teydis* from La Palma, some individuals of *C. arboreus*, *C. duranii* from El Hierro and one specimen of *C. metlesicsii* were the only ones presenting considerable (>10%) levels of admixture among the two genetic groups detected (between 14.9% and 34.3%). BAPS results showed a more fragmented distribution, with $K = 11$ as the most plausible number of clusters ($P = 0.995$; Figs. 1 and 3). The mixture analyses with or without spatially informative priors resulted in congruent assignment of individuals and no individual reassignment between the populations was observed. The genetic structure revealed by BAPS proved to be highly congruent with the phylogenetic analyses carried out (Fig. 3), showing at the same time a clear geographical pattern. Except for the populations of *C. teydis* and *C. massonianus*, the occurrence of genetic clusters determined by BAPS appeared to be limited to single islands and, in most cases, even restricted to particular regions within the islands (Fig. 1). No admixed individuals were detected according to this Bayesian clustering approach.

The results of non-hierarchical AMOVA indicated a highly significant level of genetic structure among populations (56.79%; df = 29; p-value<0.01, Table 1). The hierarchical AMOVA analyses also showed significant genetic differentiation explained at all levels and for all grouping schemes tested (all p-values<0.01, Table 1). The model that explained a larger fraction of variation among groups (38.63%) was the one considering the current taxonomic circumscription ($K = 20$). Using geographical origin (i.e. native island source, $K = 7$) as the grouping criterion revealed slightly lower, but also significant, genetic variance (23.59%) among sites. According to the structure proposed by the Bayesian clustering analyses, genetic differentiation among groups explained 18.73% of variation ($K = 2$; STRUCTURE) and 33.29% of variation ($K = 11$; BAPS).

The PCoA using the first three principal coordinates explained 32.8% of the total variation in the data and confirmed several

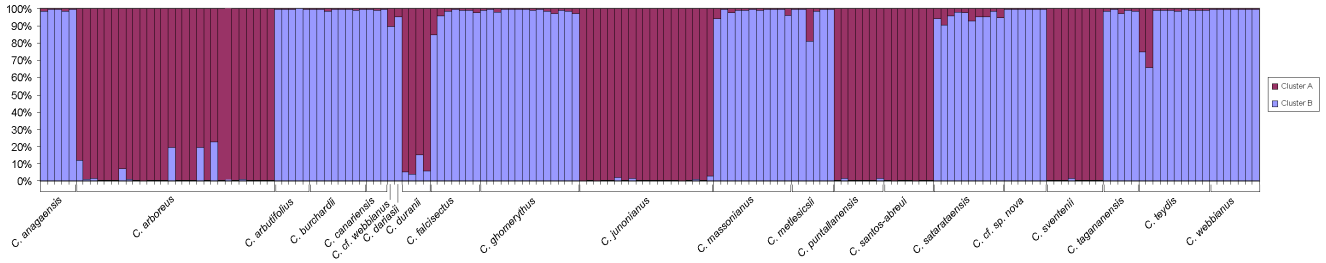

Figure 4. Results of STRUCTURE analyses of the entire AFLP dataset with $K=2$. Bayesian estimation of genetic structure within Macaronesian *Cheirolophus* according to the best model proposed by STRUCTURE ($K=2$).
doi:10.1371/journal.pone.0113207.g004

relationships detected in the phylogenetic and cluster analyses (Fig. S1). The first coordinate (accounting for 18.2% of the total variation) distinguished two main groups of species: the western Canarian taxa occurring on La Palma and El Hierro clearly segregated from another main cluster formed by species inhabiting Tenerife, Gran Canaria, La Gomera and Madeira. The second coordinate (representing 7.9% of variation) segregated *C. ghomerythus* populations in a different cluster, confirming the distinctiveness of this species from the other taxa on La Gomera. The addition of this second axis also separated *C. burchardii*, *C. canariensis* and *C. arbutifolius* accessions in one discrete cluster and *C. massonianus* individuals in another. Finally, plotting a third coordinate (accounting for 6.7% of the total variation) with the first axis, led to the distinction of four discrete clusters of species from: i) Tenerife and Gran Canaria; ii) La Gomera; iii) La Palma and El Hierro; and iv) Madeira.

Morphological analyses and nrDNA sequencing data

The two first principal components represented 60% of the total morphological variation in the data set (Table S3). The first component (PC1) accounted for 31.4% of the total variance, showing the highest coefficients for flower colour and leaf shape traits (Table S3). PC2 represented another 28.4% of the total variance, having the highest coefficients for capitulum and leaf size and plant height traits. No correlation was found between genetic and morphological distances according to Mantel test ($r = 0.00083$, p-value>0.05), nor did any of the GAMOVA analysis show significant results for the morphological variables considered (all p-values>0.05).

Table 1. Analyses of molecular variance (AMOVA) of Macaronesian *Cheirolophus* species based on AFLP markers.

Source of variation	d.f.	Sum of squares	Variance components	Fixation indices	Percentage of variation	P
1. No population structure						
Among populations	28	2758.35	14.84	0.57	56.79	<0.0001
Within populations	143	1614.26	11.29		43.21	<0.0001
2. Species						
Among groups	19	2439.19	10.22	0.39	38.63	<0.0001
Among populations within groups	9	319.16	4.95	0.30	18.70	<0.0001
Within populations	143	1614.26	11.29	0.57	42.68	<0.0001
3. Islands						
Among groups	6	1236.50	6.55	0.24	23.59	<0.0001
Among populations within groups	22	1521.85	9.92	0.47	35.75	<0.0001
Within populations	143	1614.26	11.29	0.59	40.67	<0.0001
4. STRUCTURE						
Among groups	1	557.24	5.38	0.19	18.73	<0.0001
Among population within groups	27	2201.11	12.07	0.52	42.00	<0.0001
Within populations	143	161426	11.29	0.61	39.27	<0.0001
5. BAPS						
Among groups	10	1951.35	9.91	0.33	33.29	<0.0001
Among population within groups	18	807.00	6.58	0.37	24.55	<0.0001
Within populations	143	1614.26	11.29	0.58	42.15	<0.0001

The four possible scenarios considered were: no population structure (1), species delimitation (2), islands groups (3) STRUCTURE clustering (4) and BAPS clustering (5).
doi:10.1371/journal.pone.0113207.t001

The analysis of nrDNA sequences from [41] evidenced a considerable number of heteromorphic sites (double peaks) within potentially introgressed species. Table S2 shows nine positions (four observed in ITS regions and five in ETS region) presenting multiple peaks on the electropherograms in seven Macaronesian species.

Discussion

Species delimitation among Macaronesian *Cheirolophus*

The performance of diverse methods to analyse AFLP data sets has been found to be particularly important in evolutionary studies of plant radiations [45,48,62,96]. Most of the analyses conducted in this study to evaluate the genetic structure of *Cheirolophus* in Macaronesia revealed different but complementary patterns, supporting the complex evolutionary history previously suggested for the genus in the archipelago [41]. Phylogenetic analyses based on AFLP were particularly useful in unravelling taxonomic boundaries among Macaronesian congeners. Both tree-building and network methods provided full support to the current species circumscription mainly based on morphological characteristics. Thus, our results corroborate the distinctiveness of these extraordinarily recent diverged species and support the utility of classical diagnostic characters employed in the taxonomical delimitation of Macaronesian *Cheirolophus*.

Geographic differentiation and long-distance dispersal

AFLP analyses were not able to thoroughly disentangle the phylogeny of Macaronesian *Cheirolophus*, but assignment methods provided further evidence on the evolutionary relationship between different lineages. The model proposed by STRUCTURE ($K = 2$) identified a major geographical division, with the western islands (La Palma and El Hierro) clustering independently from the Central-Eastern ones (La Gomera, Tenerife, Gran Canaria), that grouped together with Madeira (Fig. 4). This genetic barrier has been also found in PCoA analyses (Fig. S1) and is supported by the phylogenetic signal (Figs. 2 and 3). Close evolutionary relationships among species from La Palma and El Hierro have already been reported in some studies concerning diverse animal and plant taxa from Macaronesia [97–99]. However, the majority of phylogeographic studies on Canarian flora and fauna have inferred that endemics from El Hierro appear to be more closely related to species from La Gomera than to those from La Palma [100]. Indeed, plastid DNA phylogeographic analysis has recently revealed a clear evolutionary connexion among El Hierro and La Gomera haplotypes [41], which provides evidence for a series of incongruent patterns among AFLP and cpDNA data (see discussion on introgression section below). A model with more clusters was proposed by BAPS ($K = 11$), suggesting as well significant influence of allopatric speciation in the evolutionary history of Macaronesian *Cheirolophus*. The species from Tenerife are grouped in three clusters corresponding to well differentiated regions of the island. One cluster comprised the taxa inhabiting mainly the eastern part of Tenerife (i.e. *C. webbianus*, *C. tagananensis*, *C. anagaensis*, *C. metlesicsii* and *C. cf. sp. nov.*). Another cluster groups *C. canariensis* and *C. burchardii* from the Teno mountains, at the western end of the island. This eastern/western pattern of geographic distribution in Tenerife has been recovered in other studies of several plant groups (see [101], for a review). In some cases, this segregation has been explained by disjunct evolution of lineages in two palaeo-islands of Tenerife [102,103], currently corresponding to Anaga and Teno massifs at different ends of the island. According to previous phylogeographic and dating analyses [41], this geographic splitting of *Cheirolophus* lineages from eastern and western Tenerife could be related to connection-isolation cycles caused by volcanic activity during the last 2 My [104]. A third cluster included the populations of *C. teydis* from Tenerife (Las Cañadas) and La Palma, all of them living around the subalpine zone (1800–2200 m) on both islands. The representatives from Gran Canaria were placed together by BAPS in a separate cluster. This result may support the recent hypothesis of a single colonisation event for this island and the subsequent diversification process giving rise to *C. arbutifolius* and *C. falcisectus* [41]. Our Neighbor-Net and PCoA analyses pointed to an evolutionary closeness among Gran Canaria and Tenerife lineages, but we were not able to reconstruct their accurate phylogeographic relationship. Nonetheless, in agreement to the BAPS results, the species from La Gomera are grouped in two different clusters showing a clear geographic distribution pattern: one including *C. ghomerythus* from the northern coast of the island and the other one embedding *C. sataratae*nsis and its former subspecies *C. dariasii*, from the south and south-west of La Gomera. Certain evolutionary closeness among the two lineages from La Gomera was detected in the PCoA analyses, but their monophyly could not be confirmed. BAPS method also differentiated four geographically-related clusters of species in La Palma: *C. junonianus* from the south of the island; *C. arboreus* from the north and the west; *C. santos-abreui*, *C. puntallanensis* and *C. sventenii* (A.Santos) G.Kunkel from the north and north-eastern part of La Palma; and *C. teydis* from the summits of this island (and from Las Cañadas in Tenerife). Finally, *C. duranii* from El Hierro and *C. massonianus* from Madeira grouped in two separate clusters, suggesting as well that geographic isolation has been involved in the radiation of the genus.

According to these results, sporadic long-distance dispersal (LDD) events have to be considered to explain the numerous intra and inter-island colonisations resulting in the current distribution of genetic and taxonomic diversity in Macaronesian *Cheirolophus*. Unfortunately, straightforward evidences of LDD are very difficult to obtain [105], and we have not been able to provide direct proofs of these events occurring on our group of study. However, several supporting evidences (discussed below) suggest that Macaronesian *Cheirolophus* might have the potential to undergo successful LDD events. Birds and lizards have been found to be involved in seed long- dispersal of multiple species in the Canary Islands [106,107] and other oceanic archipelagos [108,109]. In the Galapagos and Azores, finches (granivorous birds from family Fringillidae) have been reported as legitimate seed dispersers of dry-fruited plants – including Asteraceae – implicated in LDD events between islands [109–111]. The goldfinch (*Carduelis carduelis* L.) has been observed in the Canary Islands feeding on *Cheirolophus* seeds [112], which suggests that bird-mediated dispersal could also be an important LDD mechanism in Macaronesian species. The role of birds in LDD events in *Cheirolophus* was also pointed by Garnatje et al. [113], who inferred that seabirds could have mediated the re-colonisation event from the Balearic Islands towards the continent in *C. intybaceus* (Lam.) Dostál. Finally, viable seeds from another Centaureinae species have been recovered from lizard guts discarded by predatory birds in the Canarian archipelago [114], supporting this kind of secondary seed dispersal as a likely mechanism involved in the LDD of Macaronesian *Cheirolophus*. All these data suggest that *Cheirolophus* achenes may be able to be transported through long distances but, given the lack of strong direct evidences in our case study and the inherent stochasticity of LDD process [105], speculation about potential role of these or other animals as LDD vectors must be made with extreme caution.

Furthermore, the capacity for successful long distance colonisation in *Cheirolophus* could also be enhanced by certain intrinsic biological features showed by this group of plants. The genus presents a pseudo-self-compatible mating system [115], that may be able to originate a sexually reproducing population from a single propagule, carrying at the same time more genetic variation than a seed from an autogamous population [116]. In summary, the results of our AFLP analysis are consistent with a role of allopatric divergence in the radiation of Macaronesian *Cheirolophus*; a hypothesis apparently supported by additional bibliographic evidences suggesting a certain degree of ability for successful long-distance colonisation events in the genus.

Geographic-genetic correlation and limited gene flow in Macaronesian *Cheirolophus*

Genetic isolation has been proposed as a major factor determining plant speciation on oceanic islands [117]. The significance found in the spatial explicit analysis (Mantel test) suggests that Macaronesian *Cheirolophus* are influenced by geographic-genetic correlation across species. In particular, our results indicate that the more closely evolutionary-related species are as well geographically closer to each other, which is in good agreement with a gene flow scenario dominated by short distance events [118]. Regular seed dispersal in *Cheirolophus* has been reported to be limited to very short distances (see [32] for some species description). This genus shows relatively heavy and pappus-lacking cypselas that are unable to be transported by the wind, falling by gravity beside the mother plant during dissemination [119]. Another important factor enhancing geographic-genetic correlation across species might be topographic isolation [118]. Most *Cheirolophus* taxa inhabit deep ravines, coastal cliffs or steep slopes [30,32], additionally preventing regular seed dispersal through long distances. The large number of Macaronesian *Cheirolophus* species inhabiting a few restricted and isolated locations (e.g. microendemisms such as *C. anagaensis*, *C. burchardii*, *C. dariasii*, *C. falcisectus, C. junonianus*, *C. metlesicsii, C. puntallanensis*, *C. santos-abreui* or *C. tagananensis*) could be related to the additive effect of poorly connected habitats with the limited seed dispersal capacity of *Cheirolophus*.

Our analysis across the whole distribution range of Macaronesian *Cheirolophus* revealed overall low levels of genetic diversity. This result is in accordance with the general expectation that endemic species, and particularly island endemics [120], exhibit lower levels of genetic diversity than widespread species [121,122]. Generally, microendemics occupying restricted and isolated populations (e.g. *C. anagaensis*, *C. duranii*, *C. junonianus*, *C. massonianus*, *C. cf. sp. nova*, or *C. tagananensis*) show lower genetic diversity levels than species with numerous, widely distributed populations (e.g. *C. arboreus*, *C. ghomerythus*, *C. sventenii*, or *C. teydis*). However, this expected pattern is contradicted by certain populations of widely distributed species (e.g. *C. arbutifolius*, *C. sataratae nsis*) also showing lower genetic diversity values. Considering the limited number of sampled populations per species and the low number of individuals available, these indices could be underestimating genetic diversity, particularly in these widespread species. We found only three populations showing private fragments, probably due to the low sampling size within some populations and the strict choice of polymorphic bands.

Some of the genetic diversity indices studied in the Macaronesian *Cheirolophus* can also be compared with the values reported by Garnatje et al. [113] in the Mediterranean complex of *C. intybaceus*, which includes some taxa distributed in the eastern Iberian Peninsula and the Balearic Islands. Diversification in the *C. intybaceus* complex presumably started in the same period as the Macaronesian radiation [41], but in the Mediterranean group it resulted in only four doubtful species. Heterozygosity levels detected in populations of Macaronesian species are significantly lower than in Mediterranean populations of the *C. intybaceus* complex. Lower genetic variation has been associated with species showing limited geographical distribution, smaller populations and exhibiting little gene flow [123–125]. Our results could be reflecting that Macaronesian *Cheirolophus* – distributed in small populations across islands with steeply dissected topography – have been comparatively more isolated than Mediterranean ones, thus contributing to their progressive genetic divergence. As it has been proposed for other plant groups by Ellis et al. [118] and Knope et al. [22], the combination of certain ability for sporadic long-distance colonisation – see the section above – and poor gene flow capacity – due to both intrinsic and geographic characteristics – could have played an important role enhancing the explosive diversification of *Cheirolophus* in Macaronesia.

Ecological adaptation

Geographic isolation may have been important for enhancing diversification but ecological adaptation is as well a common mechanism contributing – either in allopatry [126] or in sympatry [127] – to island plant speciation. According to Whittaker and Fernández-Palacios [1], the diversification of *Cheirolophus* in the Macaronesian archipelagos can be considered an example of non-adaptive radiation occurred on oceanic islands. Indeed, most species of this group exploit very similar niches in different islands, showing minor morphological differences. However, Macaronesian *Cheirolophus* present as well a few cases of taxa that have apparently adapted their morphology to the significantly diverse ecological conditions found on these archipelagos. *Cheirolophus teydis* is the only species of the genus that occupies the subalpine habitat, showing morphological adaptations to tougher ecological conditions (i.e. rosette-like disposed leaves with reduced laminas; waxy leaf cover; high number of smaller flowers; annual flowering shoots). The species from Gran Canaria – *C. arbutifolius* and *C. falcisectus* – are the result of a diversification process originated after a single colonisation of the island (see above). These species have diverged into different niches allopatrically within the same island – *C. falcisectus* inhabits more xeric habitats and shows clear leaf reduction while *C. arbutifolius* occupies more humid locations and present an arborescent habit and a larger leaf surface – thus suggesting an additional example of ecological differentiation. A similar case of ecological adaptation can be found in La Palma, where *C. junonianus* – from the south of the island and inhabiting significantly more arid localities than the rest of species from the northern part of this island – shows parallel morphological adaptation in size and leaf shape to drought conditions. Equivalent eco-morphological responses have already been reported in other Macaronesian plant taxa that have undergone an adaptive radiation process (e.g. *Argyranthemum* [2]). Interestingly, our morphology-genetics correlation analyses indicate that morphological similarity across the different Macaronesian *Cheirolophus* species is not affected by genetic similarity. Certainly, phylogenetic and clustering analyses are not congruent with a relationship between the main lineages and ecology: some species occupying different niches share the same or close genetic groups/lineages (e.g. *C. falcisectus* and *C. arbutifolius*; or *C. junonianus* and *C. arboreus*), whilst species showing similar habitats and morphological features frequently belong to very different genetic clusters/lineages (e.g. *C. duranii* and *C. tagananesis*; or *C. ghomerythus* and *C. burchardii*). These patterns suggest that the few cases of ecological adaptation (cited above) do not seem to be the result of a

single eco-morphologic shift occurring at initial stages of the Macaronesian *Cheirolophus* diversification, but they may correspond to more recent, multiple and independent phenotype-environment differentiation processes.

Clearly, there are not enough data here to discard a vital role of selection in the radiation of *Cheirolophus*. We only measured a few morphological traits usually employed to delimitate taxonomically the Macaronesian species (Table S3), but other potentially important morphological and physiological features could have been missed. In addition, there is no accurate evaluation of the niches occupied by the different species, so there could also be fine-scale ecological variables differentiating habitats formerly considered as equivalent. Therefore, more precise inferences about the relative importance of ecological adaptation in this radiation process would require additional intraspecific sampling, supplementary morphological, physiological and ecological measurements as well as more appropriate tests (see [126,128,129]). Further studies applying these methodologies will improve our understanding of the role played by selection versus neutral differentiation in islands diversifications.

Evidence of interspecific gene flow

Introgression is another mechanism formerly proposed to have played a role in the evolutionary history of Macaronesian *Cheirolophus* [41]. For instance, the species from Madeira, *C. massonianus*, was proposed to have come into contact with a continental congener, resulting in a chloroplast capture event. The genetic imprint of this hybridization event was not detected in the nrDNA regions sequenced in that former study, grouping *C. massonianus* within the Canarian clade and suggesting that introgression – via plastid transfer (see [130]) – did not affect the nuclear genome. From our AFLP data, pair-wise F_{ST} comparisons between Madeiran and Canarian populations showed similar genetic differentiation values to comparisons within Canarian populations (Table S4), apparently supporting the overall closeness among nuclear genomes of species from both archipelagos. Unfortunately, we did not include in this AFLP study any continental species putatively involved in *C. massonianus* hybridization, so our analyses do not allow assessment of whether introgression was limited to the chloroplast genome or also affected the nuclear genome.

In contrast, traces of genetic admixture were detected from AFLP data by STRUCTURE analysis in a population of *C. teydis* from La Palma (Fig. 4). Our results suggest that *C. teydis* originated in Tenerife and colonised subsequently La Palma, where genetic contact with other species from this island may occur. Plastid DNA analyses [41] support this hypothesis, pointing to a process of plastid capture to explain haplotypic differentiation among *C. teydis* populations from Tenerife and La Palma. In the same way, few *C. arboreus* individuals from populations in North-West La Palma show some genetic introgression among both genetic clusters defined by STRUCTURE. It has been reported that some specimens of *C. arboreus* from this part of the island exhibit morphological traits significantly distinct from the type [32]. Indeed, one of these *C. arboreus* populations from NW La Palma (Bco. Briestas) shows a different cpDNA haplotype from the rest of populations [41]. Another potential case of introgression could be affecting the species from El Hierro, *C. duranii*. This species is grouped together with the rest of taxa from La Palma according to our phylogenetic, PCoA and clustering analyses (Figs. 2, 4 and S1). In contrast, plastid DNA phylogenetic analysis [41], revealed a clear evolutionary closeness among *C. duranii* and the species from La Gomera. The Neighbor-Net analysis of our AFLP data mainly supports the evolutionary relationship among *C. duranii* from El Hierro and the rest of species from La Palma, but it also shows a faint reticulation signal between *C. duranii* and *C. sataraetaensis* from La Gomera (Fig. 3). In this case, certain genetic admixture – albeit very weak – can also be perceived from the STRUCTURE analysis, suggesting as well that this species from El Hierro may present genetic traces from both La Palma and La Gomera *Cheirolophus* species. Finally, the occurrence of introgression events during the radiation process could be also supported by the polymorphic sites observed in nrDNA sequences of the species here mentioned (see Table S2).

These results suggest that introgression might have played certain role in the evolutionary history of *Cheirolophus* in Macaronesia. However, similar patterns can be generated by ancestral polymorphisms and incomplete lineage sorting [131]. The STRUCTURE analysis does provide indication of admixed genotypes in some species, but at $K = 2$ the parent species for introgression are impossible to determine, being perhaps a vestige from some ancestral polymorphisms. According to the phylogeographic analysis performed by Vitales et al. [41], La Palma and El Hierro were colonised by *Cheirolophus* from Tenerife Island, so the genetic cluster A – mainly found in La Palma and El Hierro – proposed by STRUCTURE should be derived from the putative older cluster B, predominant in Tenerife, La Gomera and Gran Canaria (see Fig. 4). The admixture signal observed in *C. duranii* or in NW *C. arboreus* populations fits well with this alternative scenario considering certain retention of ancestral polymorphisms. In contrast, the pattern observed in *C. teydis* from La Palma – showing considerable admixture signal from the derived cluster B – seems more difficult to explain by the only action of ancient polymorphism retention. Heteromorphic positions found in nrDNA are consistent with some genetic reticulation but they can also be attributed to retention of polymorphisms and incomplete concerted evolution [132], especially considering the rapidity of this radiation process. In summary, there are some evidence for genetic introgression during the diversification of Macaronesian *Cheirolophus*, but in most cases there are alternative possible explanations (e.g. ancestral polymorphisms), thus limiting our conclusions about the relative importance of reticulation in the evolutionary history of the group.

Conservation recommendations

The genus *Cheirolophus* is illustrating one of the largest plant radiations in the Canary Islands [133]. Having nine of the 20 extant insular species included in the IUCN Red List [31], *Cheirolophus* shows the highest proportion of endangered taxa for any species-rich lineage in this archipelago. Thus, attending to the major conservational interest of the group, we consider capital to analyse the results of this study from a conservation point of view. Even though Macaronesian *Cheirolophus* are the result of an exceptionally recent diversification, phylogenetic analyses based on AFLP confirmed the evolutionary identity of currently described endemics. Generally, the genetic diversity indexes calculated for the different populations and species of the group were found to be relatively heterogeneous, reflecting the complexity of this radiation process (see above). However, the frequency-down-weighted fragment values per population (DW) provided interesting and useful information about conservation biology of the numerous endangered representatives of the genus. This rarity index (DW), has been employed to assess the genetic distinctiveness of populations and species [134], and can also be used as an indicator of uniqueness and evolutionary relevance for conservation. Previous works suggest that species with high levels of unique genetic information are more likely to be threatened (e.g. [135,136]). However, according to our results, current threat

categories assigned to Macaronesian *Cheirolophus* species do not reflect their uneven evolutionary differentiation. *Cheirolophus* taxa considered in higher extinction risk categories (CR and EN) in the Spanish Red List of Endangered Flora [32] and IUCN Red List of Threatened Species [31] have relatively low DW values (e.g. *C. arboreus*, *C. duranii*, *C. metlesicsii*, *C. santos-abreui*). In contrast, those species showing higher DW values - therefore considered more genetically distinct – are assigned to lower extinction risk categories (VU) or even considered unthreatened (e.g. *C. canariensis*, *C. sataratae nsis*, *C. webbianus*). As resources for conservation are limited, their optimal allocation is essential [137]. Prioritization may be especially important when dealing with rich groups of closely related endemics inhabiting biodiversity hotspots [138–140], as is the case of Macaronesian *Cheirolophus*. We suggest that future evaluations of the endangered status of Macaronesian *Cheirolophus* should take into account the evolutionary distinctiveness results presented here. Predictably, the prioritization in resources allocation among *Cheirolophus* species would change if their genetic differentiation level is considered during conservation assessment process.

Supporting Information

Figure S1 Principal coordinates (PCoA) plot of AFLP data for the Macaronesian *Cheirolophus* populations included in this study. Different symbols correspond to different populations as shown in the legend in the right side. Some populations groups that are well-differentiated and/or mentioned in the text are circled and named.
(TIF)

Table S1 Sampling information and genetic diversity indexes assessed. Taxa, locality, code, number of sampled individuals (N), and genetic diversity indexes assessed by AFLP in 29 populations of *Cheirolophus* from Macaronesia. Genetic indices: number of private fragments (f_u); heterozygosity (Hj); percentage of polymorphic loci for a standardised sample size of three (*PLP* 1%); band richness for a standardised sample size of three (*Br*); and frequency-down-weighted marker values index (DW).
(DOC)

Table S2 Polymorphic positions in nrDNA sequencing of some Canarian *Cheirolophus* taxa. Positions in ITS and ETS sequences of some Canarian *Cheirolophus* specimens where more than one base is represented in a single amplification product, seen as subequal multiple peaks on the electropherograms. Data of sequences from Vitales et al. (2014).
(DOC)

Table S3 Data from diagnostic morphological characters of Canarian *Cheirolophus* species. Character loadings in first two principal components for the analysis of *Cheirolophus* morphological data (high loadings are highlighted in boldface type).
(DOC)

Table S4 Pair-wise F_{ST} distances based on AFLP data calculated among the Macaronesian *Cheirolophus* populations included in this study.
(XLS)

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Author Contributions

Conceived and designed the experiments: TG JV JP DV. Performed the experiments: RSC MFF DV. Analyzed the data: AG-F OH DV. Contributed reagents/materials/analysis tools: AS-G MFF RSC OH. Wrote the paper: AG-F TG JV JP DV. Reviewed and improved the manuscript: AS-G OH MFF RSC.

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- **Botanical Journal of the Linnean Society (en revisió)**

Genètica de la conservació de l'endemisme Ibèric rar *Cheirolophus uliginosus* (*Asteraceae*)

Daniel Vitales, Alfredo García-Fernández, Teresa Garnatje, Joan Vallès, Robyn S. Cowan, Michael F. Fay i Jaume Pellicer

Cheirolophus uliginosus és una espècie rara, endèmica del sud-oest de la península Ibèrica i enumerat com a tàxon característic dels bruguerars atlàntics temperats, un hàbitat prioritari per a la conservació en la Unió Europea. L'estat de conservació d'aquesta espècie en la major part de la seva area de distribució és poc conegut. No obstant, recentment la desaparició de poblacions i la reducció del nombre d'individus en algunes d'elles ha estat reconegut. En aquest context, analitzàrem l'efecte de la grandària de les poblacions en la diversitat genètica, tot revelant que l'erosió genètica i la depressió per autogàmia podrien tenir un impacte significatiu en les poblacions de mida reduïda. Seguidament, estudiàrem els patrons d'estructura genètica i variabilitat específica, observant un nivell de diversitat genètica intrapoblacional sorprenentment baix, combinat amb una alta diferenciació genètica entre poblacions. Finalment, les anàlisis de l'estructura genètica suggereixen una història filogeogràfica llarga i complexa de *Ch. uliginosus* a la regió, d'acord amb l'estatus de relicte climàtic que ha estat proposat per aquesta espècie.

Revista: el factor d'impacte d'aquesta revista (corresponent a l'any 2013, segons el JCR de la ISI web of Knowledge) és 2,669, i es troba en la posició 46 de 199 a *Plant Sciences* (Q1, primer quartil).

Conservation genetics of the rare Iberian endemic *Cheirolophus uliginosus* (Asteraceae)

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Running title: Conservation genetics of *Cheirolophus uliginosus*

Abstract

Cheirolophus uliginosus is a rare species, endemic to the south-western Iberian Peninsula and listed as a characteristic taxon from the temperate Atlantic wet heaths, a priority habitat for conservation by the European Union. The conservation status of this species in most of its distribution area is poorly known. However, in recent times the disappearance of populations and a reduction in the number of individuals on some of them has been noticed. In this context, we analysed the effects of population size on genetic diversity, revealing that genetic erosion and inbreeding depression could be having a significant impact on smaller populations. Subsequently, we studied the patterns of genetic structure and variability at the species level, and observed a strikingly low within-population diversity and high among-population genetic differentiation. Finally, the genetic structure analyses suggest a long and complex phylogeographic history of *Ch. uliginosus* in the region, in agreement with the climate relict status proposed for this species.

Keywords: AFLP-cpDNA--endangered species-genetic diversity-genetic structure-Habitats Directive-phylogeography-population genetics-rare species

Introduction

Wet heathlands are extraordinarily valuable habitats because of the biodiversity they harbor and the important ecological functions and services that they provide (Webb, 1998; ALFA, 2004; Muñoz *et al*., 2012). Like many other wetland habitats, these are also very sensitive ecosystems to natural and anthropogenic disturbances (Cristofoli, Monty & Mahy, 2010; LePage, 2011). For those reasons, temperate Atlantic wet heaths with *Erica ciliaris* L. and *Erica tetralix* L. are ranked as priority habitats for conservation by the European Union (Habitats Directive 92/43/EEC, code 4020). These humid heathlands typically occur along the Atlantic shores of Spain, Portugal, France and some localities in south-west England (MacSharry, 2009). In Portugal and south-western Spain they represent a naturally fragmented distribution (Ojeda, 2009), occupying a few scattered and rare areas where the specific environmental conditions required for this ecosystem take place at such lower latitudes. From the point of view of biodiversity value, these southern wet heaths are particularly interesting, showing comparatively higher species richness and a larger amount of narrow endemics than in the northern representatives (Andres & Ojeda, 2002). However, given the high dependence on water availability as well as their occurrence in the Mediterranean climate, the Iberian heathlands are recognised as especially vulnerable to global change (Schröter *et al*., 2005). For this reason, studying the diversity, phylogeographical patterns and the conservation biology of the endemic flora that constitutes these threatened habitats can help understanding their importance and vulnerability.

Cheirolophus uliginosus (Brot.) Dostál (Asteraceae) is listed as a characteristic species of temperate Atlantic wet heaths according to the Interpretation Manual of European Union Habitats–EUR28 (2013). However, the geographic distribution of *Ch. uliginosus* is limited to the south-western and western Iberian Peninsula (Ruiz de Clavijo & Devesa, 2013), so that this species could be considered as a distinctive component of the Mediterranean-climate wet heathland flora. This Iberian endemic plant is a hemicryptophyte –the only herbaceous member of the genus– showing vegetative reproduction through rhizomes and an allogamous breeding system (Bañares *et al*., 2010). Flowers are hermaphroditic, arranged in capitula and most likely pollinated by

generalist hymenoptera. Seeds have a deciduous pappus and they are gravity-dispersed. The natural rarity of the habitat where *Ch. uliginosus* occurs has probably prevented this species from becoming as widespread as other more generalist relatives (e.g. *Ch. sempervirens* Pomel) (Susanna, 1993). In addition, these ecosystems are under increasing anthropogenic pressures, such as forest planting, grazing, urbanization, drying out, pollution, mismanagement of water levels and competition with ruderal vegetation (ALFA, 2004; Ojeda, 2009). Consequently, numerous populations of *Ch. uliginosus* cited during the early and middle 20th century and compiled by Rivas-Martínez *et al*. (1980) or Susanna (1993), have not been confirmed during recent field surveys or have undergone a significant reduction in the number of individuals and/or extinction. Furthermore, the reduced size of some of the remaining populations and their fragmentation might have triggered pernicious effects for the species, such as inbreeding or genetic drift (Frankham, Ballou & Briscoe, 2002). This species is not included in the current Spanish legislation of endangered species (Catálogo Español de Especies Amenazadas, Real Decreto 139/2011), but it is classified in the Spanish red book of threatened vascular flora (Bañares *et al*., 2010) as CR ("Critically Endangered") according to IUCN criteria. In Portugal, where most of the populations of *Ch. uliginosus* occur, its conservation status is poorly known since no red list of threatened vascular flora exists for this country. Nevertheless, according to the Sociedade Portuguesa de Botânica, it is recognised as a rare species (Porto *et al*., 2010).

The genus *Cheirolophus* Cass. has been comprehensively studied from an evolutionary point of view (Garnatje *et al.*, 1998, 2012; Garnatje, Garcia & Canela, 2007; Susanna, Garnatje & Garcia-Jacas, 1999; Vitales *et al.*, 2014a). In a recent phylogenetic reconstruction (Vitales *et al*., 2014a), *Ch. uliginosus* is partially resolved as an early-diverged lineage of the genus, suggesting a climate relict status for this species in the Atlantic coast of the Iberian Peninsula. Additional molecular cytogenetic studies (Garnatje *et al.,* 2012) support the significant evolutionary distinctiveness of this species and hence highlighting its important conservation value (Cadotte & Davies, 2010). At the same time, in a previous study focusing on the reproductive features of this species (Vitales *et al*., 2013), a preliminary genetic survey evidenced certain inter-

population variability in some plastid DNA regions suggesting that plastid markers could be also a helpful complement to understand the phylogeographic history of this rare species. DNA fingerprinting methods –e.g. amplified fragment length polymorphism (Vos *et al*., 1995; AFLP)– have also proven helpful in studying the phylogeography and population genetics in different *Cheirolophus* species (Garnatje *et al*., 2013; Vitales *et al*., 2014b).

In the present work we use both plastid DNA sequences and AFLP markers to study *Ch. uliginosus* from a conservation genetics perspective and to gain an understanding of the phylogeographic history of this endemic species to the southern heathlands of the Iberian Peninsula. Specifically, we aim to: i) examine the genetic diversity at the population and species levels, checking whether small populations are being affected by a particular loss of genetic diversity; ii) discuss the patterns of genetic structure among populations in relation to the early and ancient evolutionary history of *Ch. uliginosus* in the context of the habitat where it occurs; and iii) infer conservation management strategies for this species according to the results obtained.

Materials and methods

Sampling strategy

Cheirolophus uliginosus was sampled from 17 populations located in the south-western Iberian Peninsula. These were all the populations in the area we were able to find consulting the herbarium records and experts in the local flora (E. Sánchez-Gullón, V. Girón, J. Paiva & M. Porto, pers. comm.). Details of locations and number of sampled individuals of each population are listed in Table 1 and Fig. 1. To avoid sampling clones, we only collected plants distanced by five or more metres. Rhizomes of *Ch. uliginosus* are small and vegetative reproduction is expected to occur just a few centimetres away from the mother plant. In the case of some small populations, the reduced number of individuals limited the distance among sampled plants as well as the optimal sampling size. Leaf tissue was immediately dried in silica gel and stored at room temperature until DNA extraction.

The number of individuals in each population was visually counted (Table 1). However, as this species can reproduce vegetatively, the accurate determination of population size at the genet level (i.e. group of genetically identical individuals) may be difficult (e.g. Luijten *et al*., 1996). Indeed, during fieldwork, it was difficult to distinguish between individuals of *Ch. uliginosus* occurring in dense clusters of rosettes. In addition, in several populations, poor accessibility and/or leafy vegetation did not allow visual contact with all individuals so the approximate size was estimated according to apparent density and extension of populations.

DNA isolation, AFLP fingerprinting and DNA sequencing

Total genomic DNA was extracted from silica-gel-dried leaf tissue following the protocol of Doyle & Doyle (1987) with slight modifications. DNA samples were cleaned using QIAquick columns (Qiagen, Valencia, CA, USA) and their quality and DNA concentration was determined using NanoDrop ND-1000 spectrophotometry (ThermoScientific, Wilmington, DE, USA).

The AFLP technique was carried out following the protocol described in Vos *et al*. (1995) in accordance with the modified AFLP® Plant Mapping Protocol of PE Applied Biosystems Inc. using EcoRI and MseI with 500 ng of isolated genomic DNA per sample. After a preliminary trial involving 12 selective primers, three primer pairs were finally chosen: EcoRI-AC/MseI-CTT; EcoRI-AG/MseI-CTC; and EcoRI-AT/MseI-CAG. The success of each step was tested by running the PCR products on a 1.5% agarose gel. Fragments were run on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with 10 µL High Dye (deionized formamide) and 0.2 µL GeneScanTM 500 ROXTM Size Standard per sample. Amplified fragments were scored using GeneMarker®AFLP/Genotyping software (version 1.9; SoftGenetics, LLC., State College, PA, USA). AFLP error rates were calculated following Bonin *et al*. (2004). Twenty random samples per primer combination were replicated to ensure reproducibility, repeating all parts of the AFLP protocol. All alleles with an error rate >5% were eliminated, following the recommendations for high quality AFLP development (Crawford, Koscinski & Keyghobadi, 2012). In addition, those individuals that did not

produce scorable patterns for all three primer combinations were excluded. Finally, 122 out of 175 attempted individuals (70 %) produced scorable and reproducible patterns for all three primer combinations and were consequently analysed.

For DNA sequencing, we conducted a previous screening test involving nuclear (ITS and ETS) and plastid (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*) markers that were sequenced for a few individuals of different and distanced populations. Of the six regions tested, we selected the ones providing the highest levels of polymorphism: *rpl32-trnL* and *trnS-trnC*. Both regions were amplified and sequenced following protocols in Vitales *et al.* (2013), except for some accessions that were obtained from GenBank. The number of individuals finally analysed per population ranges between four and 13 due to the plant material availability and PCR success (Table 1). Nucleotide sequences were edited using Chromas LITE v2.01 (Technelysium Pty, Tewantin, Australia) and subsequently aligned manually with BioEdit v.7.0.5.3 (Hall, 1999).

Data analysis

The use of AFLP data (dominant markers) for estimating allelic frequencies implies the consideration of an outcrossing mating system and near random mating. This means that those populations would be under Hardy-Weinberg equilibrium (Lynch & Clarke, 1994). *Cheirolophus* has a predominantly outcrossing mating system and is pollinated by generalist insects, so one expects near-random mating in the studied populations. Although the sampling strategy was designed to avoid the collection of clone individuals, this issue was investigated using the function Clones in the AFLPdat software (Ehrich, 2006). A histogram of the number of pair-wise differences among individuals within populations was constructed to explore the occurrence of several plants belonging to the same clonal lineage. In this case, the distribution of pairwise differences within populations is expected to be bimodal. While the main peak represents the pair-wise differences among genotypes, the second peak at low values may represent the differences among plants belonging to a single clonal lineage (Ehrich *et al.,* 2008). The number of genotypes per population and the Nei's gene

diversity among genotypes (excluding the putative clones) was also measured using the same software.

To estimate genetic diversity in each population, the following parameters were calculated: a) private alleles (*Npriv*); b) rare alleles (where present in < 10% of the samples); and c) Nei's unbiased heterozygosity within populations (*Hj*) and average gene diversity within populations (*Hw*) calculated using TFPGA v. 1.3 (Miller, 1997). A rarefacted measure of Nei's unbiased heterozygosity was also estimated, randomly resampling the populations to *N* = 4 and recalculating the index [*Hj* (4)] with the same software. Further measures of genetic diversity were estimated through: (i) the band richness (*Br*), which is the number of phenotypes expected at each locus, and can be interpreted as an analogue of the allelic richness, ranging from 1 to 2 (Coart *et al*., 2005); and (ii) the percentage of polymorphic loci (*PLP*) with a significance of 1% (P = 0.99). *Br* and *PLP* indices were calculated according to the rarefaction method of Hurlbert (Petit & Mousadik, 1998), and conditioned to the smallest population size (*N* = 4) with the software AFLPDIV v. 1.0. The frequency-down-weighted marker values (*DW*) index of Schönswetter & Tribsch (2005) was calculated as ratio of means, making the measure less sensitive to large differences in sample size between localities, using AFLPDAT (Ehrich, 2006). Linear regression analyses were performed to study the effect of population size on genetic variation. Population size was log-transformed in all analyses. Total gene diversity in the species (*Ht*) and the unbiased derived estimate ϑ_I (analogue of Wright's F_{ST} coefficient) were calculated using Hickory (Holsinger, Lewis & Dey, 2002). Pairwise F_{ST} values were estimated for each pair of populations studied with AFLP SURV 1.0 (Weir & Cockerham, 1984). Significance was evaluated through 10000 permutations. In addition, estimates of inbreeding coefficient were calculated with the software I4A (Chybicki *et al.,* 2011). The measures were obtained after 60,000 steps, after 10,000 burnin steps, as recommended by the author. Because the method requires initial guesses on the priors, analyses were conducted starting from three initial sets of parameters [α=β= (0.1,1,5)] to avoid a dependence of final results on these guesses.

Population genetic structure revealed by AFLP was investigated using phylogenetic and clustering analysis. We used the Neighbor-Net method (Bryant & Moulton, 2004)

carried out with SplitsTree v.4.10 (Huson & Bryant, 2006) to construct a distance-based network using the Jaccard coefficient (Jaccard, 1901), which is restricted to shared band presence rather than shared absence. Bayesian clustering analyses were carried out using STRUCTURE 2.3 (Hubisz *et al*., 2009). We considered the admixture ancestry model and the correlated allele frequencies. Ten independent simulations were run for each possible number of genetic groups (from K = 1 to 17), using a burn-in period of 10^5 generations and run lengths of 5×10^5. To estimate the number of genetic groups (K) we selected the K value that maximizes the probability of the data $L(K)$. We also considered the criterion proposed by Evanno, Regnaut & Goudet (2005) to estimate the best value of K for our data set, based on the rate of change in the probability between successive K values, ΔK. Bayesian analyses of the genetic structure were also conducted with BAPS (Bayesian Analysis of Population Structure; Corander & Marttinen, 2006), which uses stochastic optimization instead of Markov chain Monte Carlo to find optimal partition. We performed a mixture analysis of individuals with the geographic origin of the samples used as an informative prior (spatial clustering of individuals) or without this prior (clustering of individuals). BAPS simulations were run with the maximal number of groups (K) set at the number of sampled populations in each species. Each run was replicated 10 times, and the results were averaged according to the resultant likelihood scores. The output of the mixture analyses were used as input for population admixture analysis (Corander & Marttinen, 2006), with the default settings in order to detect admixture between clusters. Finally, we conducted AMOVA analyses by using ARLEQUIN 3.5 (Excoffier, Laval & Schneider, 2005) to estimate genetic differentiation following an alternative and widely used non-Bayesian approach that does not assume Hardy-Weinberg equilibrium or independence of markers. Three independent AMOVA analyses were carried out i) without taking regional structure into account (i.e. among and within population variance only), ii) considering the clusters proposed by STRUCTURE and iii) considering the clusters proposed by BAPS.

To further characterize the spatial genetic distribution of *Ch. uliginosus*, we performed Mantel tests and a spatial autocorrelation test to evaluate the existence of isolation by distance patterns. In order to execute these analyses, genetic distance matrices were

constructed with F_{ST} values between populations, and geographical matrices were calculated by the spatial distance (X and Y coordinates) between populations using ArcGIS 9.1 (ESRI, Redlands, CA, USA). Mantel tests were performed on ARLEQUIN 3.5 with 100000 permutations and considering a *p*-value limit of 0.05. The additional spatial genetic structure calculation was estimated with SPAGeDi software (Hardy & Vekemans, 2002) considering the kinship multilocus coefficient (F_{ij}) with dominant markers. Inbreeding coefficient (F_{IS}) was set according to the estimate obtained with the software I4A (other values were also used in a preliminary stage, but results were similar), and 20000 permutations were run for the test.

Plastid DNA haplotypes of *Ch. uliginosus* were determined using the number and position of nucleotide substitutions and indels from the aligned sequences. A statistical parsimony haplotype network was also constructed using TCS v1.21 (Clement, Posada & Crandall, 2000). For this latter analysis, insertions/deletions longer than one base pair were re-coded as single base pair mutations, and sequence gaps were treated as a fifth character state. Total haplotype diversity (*Hd*), average haplotype diversity within populations (*Hs*) of Nei (1973) and genetic differentiation among populations (G_{ST}; Nei, 1973) were calculated for the combined matrix of plastid sequences using DNASP v. 5 (Librado & Rozas, 2009).

Results

AFLP profile, genetic diversity, structure and isolation by distance.

Initially, 183 alleles were obtained from automatic genotyping of *Ch. uliginosus* AFLP profiles. After manual correction, error rates calculation, elimination of small and troublesome alleles and low intensity peaks, a final matrix with 157 (85.8%) alleles was considered for subsequent analyses. The final data set showed an error rate of 2.3%. The distribution of the number of pairwise differences among plants within populations did not present a bimodal shape (Fig. 2), but certain increase in the frequency of individuals differing by zero alleles could be observed. Therefore, to explore the potential effect of clonality in our sampling, individuals showing the same

genotype were tentatively considered as possible ramets belonging to the same clonal lineage. Fifteen putative clones were detected in populations DO1 (3), AG1 (6), AL1 (1), AL3 (4) and AA1 (1). Nei's gene diversity among genotypes (excluding the potential clones) ranged among *D* = 0.0170 for population AG1 to *D* = 0.0960 for population BM4 (Table 1), averaging 0.0561 ± 0.0251.

Additional population genetic diversity measures are shown in Table1. Average gene diversity per population (*Hw*) was 0.0572 ± 0.0279. Nei's unbiased heterozigosiy ranged (*Hj*) ranged from 0.0112 (AG1) to 0.1073 (BM4), whereas the rarified measure [*Hj*(4)] varied among 0.0109 (AG1) and 0.0928 (BM4). The percentage of polymorphic loci [*PLP*(4)] ranged from 22.9 (BM4) to 2.5 (AG1) among populations, and band richness [*Br*(4)] varied between 1.178 (BM4) and 1.017 (AG1). The frequency-down-weighted marker values (*DW*) demonstrated large variation among populations, ranging between 332.8 (AL3) and 141.9 (DO5). Private fragments were scarce across the studied populations; only four populations exhibited one or two of them (Table 1). Ten rare alleles were detected: five of them being exclusive of one population [i.e. AL2 (1); SP1 (2); AL3 (1); AG2 (1)], four of them shared by a couple of populations [i.e. AL2 and AL3 (3); DO3 and SP1 (1)] and another one shared by several northern populations (i.e. AA1, BM2, BM3, BM4 and BV1). We detected significant positive relationships between most of the genetic diversity indexes and the size of populations in *Ch. uliginosus* (*Hj*, $R^2 = 0.498$, $p < 0.05$; *Hj*(4), $R^2 = 0.3097$, $p < 0.05$; *PLP*(4), $R^2 = 0.481$, $p < 0.05$; *Br*(4), $R^2 = 0.357$, $p < 0.05$; *D*, $R^2 = 0.3113$, $p < 0.05$). Conversely, there was not a significant relationship between the rarity index *DW* and the population size in this species ($R^2 = -0.0463$; $p > 0.05$).

Total genetic diversity at the species level (*Ht*) was 0.208 and the average population differentiation due to genetic structure (Wright's F_{ST}) was 0.582 (SD = 0.022). All three inbreeding analyses executed with different alpha and beta prior distributions converged to almost the same posterior distributions of the inbreeding coefficient (mean value = 0.0169; Table S1). Also the likelihood behaviour across different priors (e.g. similar average and standard error values of Log L) proved that the model was stable. Results of AMOVA analyses are depicted in Table 2. Genetic variation between the populations contributed at least 42.08% to overall genetic diversity in *Ch.*

uliginosus. Using the matrix of inter-population F_{ST} distances, and the matrix of geographical distances in kilometres, the Mantel test indicated a significant correlation between genetic and geographical distances of the different populations (r = 0.308, $p < 0.05$). Significant effects of isolation by distance in the kinship coefficient among the studied populations were also found with SPAGeDi (Fig. S1), especially at distance intervals up to 150 km, where populations were more similar than expected by random.

The Bayesian analysis of population genetic structure conducted with STRUCTURE for *Ch. uliginosus* dataset found the highest $L(K)$ and ΔK values for $K = 2$. This grouping separated Doñana populations (Andalusia) from the rest of the populations located in Portugal, showing high percentages of individual memberships for these predefined groups (Fig. 3B). Alternatively, BAPS results supported a more fragmented distribution, with $K = 4$ as the most plausible number of clusters (P = 0.97; Fig. 3A). This clustering analysis segregated: Doñana area (DO1-5) populations (Cluster I); south-western seaside populations (Algarve, AG1-2; Alentejo Litoral, AL1-2; and Setubal Península, SP1; Cluster II); population AL3 from inland Alentejo Litoral (Cluster III); and northern populations (Alto Alentejo, AA1; Baixo Mondengo, BM1-4 and Baixo Vouga, BV1; Cluster IV). The mixture analyses with or without spatially informative priors resulted in congruent assignment of individuals, and no individual reassignments between the populations were observed. The hierarchical AMOVA analyses showed a significant differentiation among groups defined by both clustering approaches (Table 2), but the grouping proposed by BAPS explained better (24.86%) than STRUCTURE (21.96%) the overall genetic diversity found in the species. Although the phylogenetic network constructed with SplitsTree (Fig. 3A) resulted in a poorly resolved genetic structure of *Ch. uliginosus* populations, the four clusters proposed by BAPS could be identified.

The two plastid markers were successfully sequenced for 139 samples from 17 different populations resulting in an alignment of 1783 bp. We detected 16 polymorphic sites –including a 22 bp indel– representing ten different haplotypes (Fig. 1 and Table 1). All populations contained one single haplotype ($Hs = 0$), with the exception of the two large populations that held two (BM4, $Hs = 0.282$; and SP1, $Hs = 0.536$). Haplotypes C, D, F, H and J were each one restricted (i.e. private haplotypes) to

a single population (Table 1; Fig. 1). Phylogenetic relationship between haplotypes inferred by the parsimony network is shown in Fig. 1. Different haplotypes of *Ch. uliginosus* distinguished from adjacent haplotypes by one, two or three evolutionary events (substitutions or indels) and extinct or unsampled haplotypes were represented as black dots in the parsimony network. Total haplotype diversity resulted to be considerably high (*Hd* = 0.759) and plastid DNA among-population differentiation was large (G_{ST} = 0.903).

Discussion

Genetic diversity patterns within Ch. uliginosus *populations*

Declining gene diversity is a typical pattern found in rare plant species generally showing small population sizes (see Frankham *et al*., 2002 for a review). According to our AFLP results, average within-population gene diversity in *Ch. uliginosus* (*Hw* = 0.0572) is extremely low; notably below the values reported for endemic plant species using dominant markers [mean *Hw* = 0.20 in (Nybom, 2004)]. We have also found that *Ch. uliginosus* exhibits a significantly lower within-population diversity than its widespread Mediterranean congener *Ch. intybaceus* (Lam.) Dostál (*Hw* = 0.134; Garnatje *et al*., 2013). Gene diversity has been reported to be lower in data sets containing clones relative to clone-corrected data (Ellstrand & Roose, 1987; McLellan *et al.,* 1997). However, we did not found significant differences between the overall gene diversity within populations (*Hw* = 0.0572 ± 0.0279) and the gene diversity among genotypes (*D* = 0.0561 ± 0.0251), so an underestimation of genetic diversity due to sampling of clonal individuals can be rejected. We tried to avoid collecting the same genetic individual twice and indeed putative clonal individuals were only identified in small sized populations where the distance among sampled plants was limited. Therefore, our study also indicates that single genets of *Ch. uliginosus* are not able to grow >5 m in diameter, as supposed when we chose the sampling design.

The genetic diversity of *Ch. uliginosus* has resulted to be significantly lower in smaller populations than in larger ones. All the studied genetic diversity indexes –including

rarefacted and clone corrected ones– show a significant positive relationship with population size. In contrast, we have not found an association among the genetic rarity index *DW* and the size of populations, reinforcing the hypothesis that genetic diversity indexes are better indicators of contemporary demographic processes than rarity ones, which may perform better explaining phylogeographic patterns (Comps et al., 2001; Widmer & Lexer, 2001; Paun *et al.*, 2008). Plastid DNA diversity recovered in our study is not large enough to construct statistically supported inferences at the intrapopulation level. Nevertheless, we observe that the only populations showing haplotypic diversity are the two large-sized ones (BM4, *Hs* = 0.282; and SP1, *Hs* = 0.536), therefore suggesting an effect of population size also in the plastid genetic diversity of *Ch. uliginosus*. The relationship between genetic variation and population size has already been reported in numerous studies involving different plant species and employing diverse molecular markers (Ellstrand & Elam, 1993; Frankham *et al*., 2002; Jadwiszczak *et al*., 2012; Ilves *et al*., 2013). Bottlenecks, genetic drift and inbreeding are usually the main causes proposed to explain the reduction of genetic diversity levels (Soulé, 1986). In this way, vegetative reproduction reported in the species may have acted as an enhancer of genetic drift by further reducing the effective size of local populations (Chung & Kang, 1996; Jones & Gliddon, 1999).

In some cases the genetic erosion can also lead to a reduction in plant performance in small populations (Reed & Frankham, 2003). Certainly, lower plant performance in small *Ch. uliginosus* populations has already been documented in previous research (Vitales *et al*., 2013). This former study showed that seed germination rate was significantly reduced in small populations, whereas medium and large populations did not show any noticeable germination constraint. Correlation between small population size and reduction in reproductive fitness traits –such as germination capacity– has been reported in several studies (see Reed, 2005 for a review) as a consequence of inbreeding depression. In the case of *Ch. uliginosus*, factors related to demographic stochasticity (such as pollen limitation) and habitat deterioration (see the Conservation remarks section) could also be detrimental, leading to reduced plant performance in small populations (Vergeer *et al*., 2003). However, given the significantly lower genetic diversity found in the smaller populations of this species, inbreeding depression

appears to be the most likely explanation for the fitness reduction reported by Vitales *et al*. (2013).

Among-population genetic differentiation in Ch. uliginosus

Heterozygosity at the whole species level (*Ht* = 0.208) in *Ch. uliginosus* does not seem to be as impoverished as it is at the population level, especially when compared with total heterozygosity in the common *Ch. intybaceus* (*Ht* = 0.211; Garnatje *et al*., 2013). These results are in agreement with AMOVA analysis, suggesting that much of the genetic diversity in *Ch. uliginosus* is distributed among the different populations. Certainly, this species shows high levels of genetic differentiation among populations (Wright's F_{ST} = 0.582), far greater than the values found by Nybom (2004) for endemic species analysed using dominant markers (mean Wright's F_{ST} = 0.26). These patterns of genetic diversity showed by AFLP data totally agree with those found in plastid DNA markers. Our survey based on two plastid DNA spacers (*rpl*32-*trn*L and *trn*S-*trn*C) revealed ten different haplotypes, similar to the values found in other more widespread Mediterranean species (e.g. Quintela-Sabarís *et al*., 2011; Mráz *et al*., 2012). Consequently, *Ch. uliginosus* shows as well considerable plastid diversity at the species level (*Hd* = 0.759). In contrast, all except two populations had a single fixed haplotype, which implies overall low within-population diversity and high genetic differentiation among populations (G_{ST} = 0.903). Thus, both AFLP and plastid DNA data suggest that *Ch. uliginosus* shows notably low intra-population heterozygosity but substantial inter-population diversity, being a large proportion of the genetic variance found in the species attributed to differences between populations.

A high degree of among-population genetic differentiation is –like the declining within-population diversity– a characteristic pattern observed in rare and endangered plants (see Hamrick & Godt, 1996; Gitzendanner & Soltis, 2000; or Cole, 2003 for a review). Small population size, restricted distribution range, geographical isolation, reproduction features and limited seed dispersal have been reported as common factors contributing to the low genetic diversity and high population genetic differentiation in several species (e.g. Mousadik & Petit, 1996; Gong *et al.,* 2010;

Lauterbach, Ristow & Gemeinholzer, 2011; Kolb & Durka, 2013). In the case of *Ch. uliginosus*, several of these elements may be shaping the genetic variability of this species. First, as it has been stated above, we must consider the reduced number of individuals found in most of the sampled populations. In addition, according to the historical citations of *Ch. uliginosus* collated by Susanna (1993), the distribution of this species appears to be originally partitioned as a consequence of the naturally fragmented habitat it occupies. Finally, the significant correlation found among genetic and geographical distances (Mantel test) certainly suggests that *Ch. uliginosus* is influenced by limited gene flow. Pollen exchange between populations of *Ch. uliginosus* separated by more than 10 km is not likely since generalist pollinators cannot travel such long distances (Kwak, Velterop & van Andel, 1998; Pasquet *et al*., 2008). The dispersal ability of seeds in this species is also restricted by lack of morphological adaptation for wind or animal transport, their dispersal occurring simply by gravity (Bañares *et al*., 2010). Thus, regular gene flow among the majority of the populations –in most cases separated by more than 10 km attending to our field survey– must be limited. In summary, several factors such as small population sizes, natural habitat fragmentation, intrinsic low gene flow abilities and/or vegetative reproduction possibly contribute to the strikingly high genetic differentiation observed in this species.

Genetic structure and phylogeography of Ch. uliginosus

In this study, four groups of populations are proposed according to the genetic clustering inferred by BAPS (Fig. 3A), and no genetic admixture has been observed among individuals belonging to different groups. A similar geographic distribution of genetic diversity, identifying the same four clusters of populations, is also recovered by Neighbor-Net analysis (Fig 3A). AMOVA analysis implemented taking into account the genetic structure found by BAPS supports as well a significant and notable differentiation among those groups (Table 2). Some of these clusters (e.g. clusters II and IV) contain populations distanced by > 100 km, far beyond what regular gene flow in *Ch. uliginosus* is able to connect. Spatial autocorrelation test also indicate that

populations separated by distances up to 150 km are more similar to each other than expected by random sampling (Fig. S1). These results suggest that other factors apart from current gene flow alone must be shaping the genetic structure observed in this species. Therefore, the observed subdivision of genetic diversity may be better explained by a pre-existing genetic structure related to the ancient phylogeographic history of this species.

The distribution of haplotypes among the population clusters supports the hypothesis that those genetic clusters may have a long evolutionary history. For instance, different and unconnected haplotypes can be found within northern (BAPS Cluster IV) or south-western (Cluster II) populations, indicating that both groups retain a large amount of the genetic diversity found in *Ch. uliginosus*. This pattern also suggests that both clusters of populations possess a long evolutionary history, sufficient to generate and maintain the broad variability observed in the relatively slowly evolving plastid DNA (Lynch, Koskella & Schaack, 2006). The case of Doñana populations (DO1-5, Cluster I) and AL3 (Cluster III) seems to be different. Populations within both clusters share the same haplotype A (Fig. 1), suggesting that any of the two genetic groups could have be originated from a recent long-distance-dispersal or a contemporary fragmentation event. However, DO populations not only form a genetic group according to BAPS (Cluster I) but they also constitute the only separate cluster defined by STRUCTURE (Fig. 3B) and a clearly separate group according to the Neighbor-Net analysis (Fig. 3A). These results point to a relatively ancient isolation of Andalusian populations. Meanwhile, population AL3 possesses the largest value for the frequency-down-weighted marker (*DW*) in relation to the other sampled *Ch. uliginosus* populations (Table 1). This genetic rarity index is expected to be high in long-term isolated populations where rare markers should accumulate due to mutations (Schönswetter & Tribsch, 2005; Lihová, Kudoh & Marhold, 2010), thus suggesting that AL3 population could also be well the result of a long evolutionary history. Therefore, attending to our data, the main genetic and geographic subdivision of *Ch. uliginosus* does not seem to be the result of recent fragmentation, extinction or colonisation events, but it could be the result of an ancient and independent evolutionary history in different isolated groups of populations.

According to recent phylogenetic and molecular cytogenetic studies of the genus (Garnatje *et al.,* 2012; Vitales *et al.,* 2014a; Vitales *et al.,* in prep.), *Ch. uliginosus* has been proposed to be an early diverging species within *Cheirolophus*. These authors hypothesised that this species may possess a long in-situ evolutionary history in the humid Atlantic coast of the Iberian Peninsula, where the lineage probably arrived seeking refuge from the progressive aridification of the Mediterranean climate during the Plio-Pleistocene (Thompson, 2005). Subsequently, *Ch. uliginosus* must have survived to the climatic oscillations associated to Pleistocene glaciations that profoundly affected to the European and the Mediterranean flora (Weiss & Ferrand, 2007). The long-term ability of species to persist along repeated episodes of climate oscillation has been associated to both intrinsic (e.g. asexual propagation) and extrinsic (e.g. particularly stable ecological habitats) features (Hampe & Jump, 2011). In this way, the geographic distribution of the four genetic clusters of *Ch. uliginosus* populations proposed by AFLP analyses virtually overlaps with different putative glacial refugia previously identified by Médail & Diadema (2009) in the region (i.e. Beira Litoral, Extremadura, Algarve and Cadiz regions). The narrow ecological niche of this species might concur with particularly stable areas from a climatic point of view, possibly contributing to the survival of this climate relict species in separated groups with long and independent evolutionary histories. The particular phylogeographic pattern showed by *Ch. uliginosus* might be the result of this ancient segregation in different groups of populations.

Conservation remarks

Our results demonstrate the need for accurate censuses of *Ch. uliginosus* populations, particularly in Portugal, where no previous data about the conservation status of the species exist. In relation to the conservation of the smallest populations of this species, showing particularly low values of genetic diversity, additional measures could be adopted to avoid the effects of possible inbreeding depression and the resultant risk of extinction. The conservation of populations DO1, AG1, AA1 and AL1, showing very small population sizes (< 50 individuals) and the lowest values of genetic diversity

should be a priority. First, seed collection –conducted according with the genetic structure detected in our results– and storage in germplasm banks will ensure the maintenance of the genetic diversity even if they eventually disappear. Moreover, specific demographic monitoring on these extremely small populations should be carried out to evaluate the risk derived from inbreeding depression on their long-term survival. If conservation managers decide to reinforce these impoverished populations with individuals from other localities to increase their heterozygosity levels and overcome the effects of inbreeding depression, our results may serve to choose the best candidate populations to transfer some individuals based on their genetic closeness.

From the point of view of the protection of the species as a whole, considering that much of the genetic variability showed by this species is distributed among populations, we believe that conservation measures must focus on the preservation of the maximum number of different populations. At least all the plastid DNA haplotypes and the genetic clusters inferred by AFLP data should be well represented when defining conservation strategies for *Ch. uliginosus*. Due to the valuable ecosystem where this species typically occurs, most of the populations are included in natural protected areas (see Table S2). However, some of these habitats are still affected by threats derived from the human activity occurring inside or next to the protected areas [e.g. agriculture and overexploitation of water resources in Sudoeste Alentejano e Costa Vicentina and Doñana natural parks (Bañares *et al.,* 2010; LPN, 2011)]. In addition, other populations are located in areas without any legal protection so their conservation cannot be currently guaranteed in the long term. In these latter cases, the creation of botanical reserves –small protected areas for wild plants already working on Portugal (Laguna, 2001)– may be appropriate for the conservation of these currently unprotected populations of *Ch. uliginosus*. Finally, attending to the suggested refugial role of these southern heathlands in the conservation of the genetic diversity of this species –together with the occurrence of other evolutionary and floristic interesting taxa usually sharing the same habitat of *Ch. uliginosus* (e.g. *Euphorbia uliginosa* Welw. ex Boiss.; *Genista ancistrocarpa* Spach)–, we agree with Ojeda (2009) about the particular ecologic and biogeographic value of temperate Atlantic wet

heaths from the Iberian Peninsula, thus deserving further protection and supplementary studies.

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Figures

Figure 1. (A). Geographical distribution of cpDNA haplotypes and studied populations of *Ch. uliginosus*. The population codes correspond to those in Table 1, and the pie charts represent the percentage of each haplotype in each population. (B) Statistical parsimony network showing relationships of the ten plastid haplotypes. Each line between haplotypes indicates a mutational step, and black dots represent extinct or unsampled haplotypes

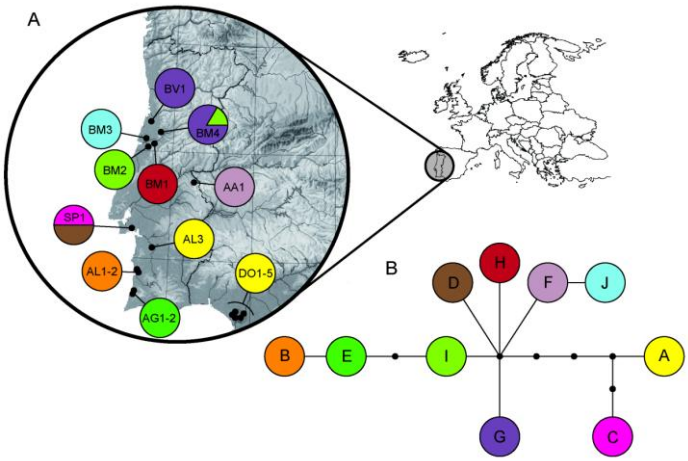

Figure 2. Distribution of the number of pairwise differences among *Cheirolophus uliginosus* plants within populations.

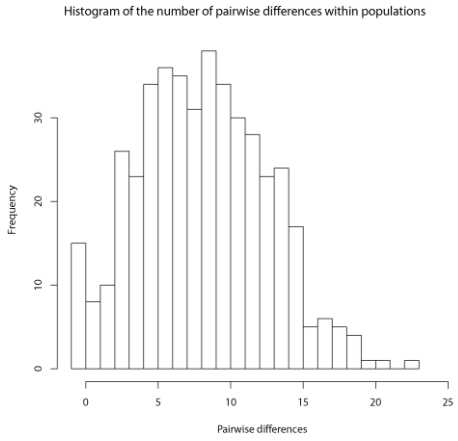

Figure 3. A. Neighbor-Net of AFLP data obtained from the 17 sampled populations of *Ch. uliginosus*. Colour coding profiles delimitate the different clusters assigned by BAPS. B. Bayesian estimation of genetic structure within *Cheirolophus uliginosus* inferred from AFLP according to the best model proposed by STRUCTURE (*K* = 2).

Table1. Sampling information and genetic diversity indexes assessed. Population code, locality (see Table S2 for details), estimated population size, number of analysed individuals with AFLP and cpDNA [*N* (AFLP) and *N* (cpDNA)], number of genotypes [*N* (geno)] and genetic diversity indexes assessed in 17 populations of *Cheirolophus uliginosus*. Genetic indexes: gene diversity among genotypes (*D*); *heterozygosity (Hj); heterozigosity rarefacted to four individuals [Hj(4)]; frequency-down-weighted marker values index (DW); band richness for a standardised sample size of four [Br(4)]; percentage of* polymorphic loci for a standardised sample size of four [*PLP*(4)]; number of private alleles (*Npriv*); haplotypes found and average haplotype diversity within populations (*Hs*).

Population code	**Locality**	**Estimated Size**	***N* (AFLP)**	***N* (geno)**	***D***	***Hj***	***Hj* (4)**	***Br* (4)**	***PLP* (4)**	***DW* (means)**	***Npriv***	***N* (cpDNA)**	**Haplotypes**	***Hs***
DO1	Doñana: Acebrón	10	5	3	0.0212	0.0131	0.0144	1.029	0.032	150.900	0	5	A	0
DO2	Doñana: Palacio Acebrón trail	100	8	8	0.0569	0.0675	0.0372	1.106	0.159	150.874	0	9	A	0
DO3	Doñana: Estero Domingo Rubio	300	9	9	0.0248	0.0326	0.02	1.046	0.07	155.464	0	10	A	0
DO4	Doñana: Estero Las Madres	50	5	5	0.0611	0.0573	0.0526	1.115	0.134	143.347	0	4	A	0
DO5	Doñana: Forestal house	200	9	9	0.0573	0.0776	0.043	1.108	0.185	141.884	0	7	A	0
AG1	Algarve, Faro, Ocedeixe south	30	6	3	0.0170	0.0112	0.0109	1.017	0.025	162.010	0	4	E	0
AG2	Algarve, Faro, Ocedeixe north	200	9	9	0.0485	0.0515	0.0373	1.088	0.121	230.385	1	11	E	0
AL1	Alentejo Litoral: Almograve creek	25	5	4	0.0403	0.0308	0.033	1.065	0.07	158.174	0	8	B	0
AL2	Alentejo Litoral: Almograve, beach dunes	75	11	11	0.0651	0.0764	0.0547	1.118	0.185	255.836	1	8	B	0
AL3	Alentejo Litoral, Alcácer do Sal, Arez	50	10	7	0.0522	0.0503	0.0433	1.093	0.115	332.794	1	10	A	0
SP1	Setubal Peninsula, Calhariz	400	10	10	0.0722	0.0833	0.0771	1.131	0.178	297.374	2	8	C/D	0.536
AA1	Alto Alentejo, Reguengo	4	4	3	0.0255	0.0187	0.0187	1.038	0.038	150.513	0	4	F	0
BM1	Baixo Mondego, Paul Madriz	300	4	4	0.0934	0.0802	0.0802	1.172	0.172	151.489	0	8	H	0
BM2	Baixo Mondego, Figueiró do Campo	100	4	4	0.0828	0.0644	0.0644	1.146	0.146	153.529	0	10	J	0
BM3	Baixo Mondego, Mata da Foja	40	5	5	0.0879	0.0855	0.0825	1.159	0.178	146.970	0	7	I	0
BM4	Baixo Mondego, Valdoeiro	1000	6	6	0.0960	0.1073	0.0928	1.178	0.229	147.001	0	13	G/H	0.282
BV1	Baixo Vouga: Fermentelos	100	12	12	0.0523	0.0651	0.0294	1.096	0.166	151.140	0	13	G	0

Table 2. Analyses of molecular variance (AMOVA) of *Cheirolophus uliginosus* based on AFLP markers. Three independent AMOVA analyses were carried out: 1) without taking regional structure into account (i.e. among and within population variance only), 2) considering the clusters proposed by STRUCTURE and 3) considering the clusters proposed by BAPS.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Fixation indices	Percentage of variation	P
1. No population structure						
Among populations	16	422.69	3.11	0.42	42.08	<0.001
Within populations	105	449.56	4.28		57.92	<0.001
2. STRUCTURE clustering						
Among groups	1	116.05	1.85	0.22	21.96	<0.001
Among populations within groups	15	306.63	2.29	0.35	27.17	<0.001
Within populations	105	449.56	4.28	0.49	50.87	<0.001
3. BAPS clustering						
Among groups	3	223.82	1.95	0.25	24.86	<0.001
Among populations within groups	13	198.86	1.62	0.27	20.65	<0.001
Within populations	105	449.56	4.28	0.45	54.49	<0.001

- **Journal of Systematics and Evolution (en revisió)**

Evidències filogeogràfiques en l'espècie de terres baixes *Cheirolophus sempervirens* en el sud-oest de la península Ibèrica

Daniel Vitales, Alfredo García-Fernández, Teresa Garnatje, Jaume Pellicer i Joan Vallès

El sud-oest de la península Ibèrica és una regió biogeogràfica important, que mostra alts nivells de biodiversitat i allotja diversos suposats refugis glacials per a la flora europea. Aquí, estudiem la diversitat i l'estructura genètica de la planta termòfila mediterrània *Cheirolophus sempervirens* al llarg del seu rang de distribució en el SO d'Ibèria, com una eina per a donar a conèixer alguns dels patrons biogeogràfics que han contribuït a la configuració d'aquest punt calent de refugis. Es va observar una diversitat genètica nul·la en el cribratge de seqüenciació de DNAcp. No obstant això, les dades d'AFLP revelaren alts nivells de diferenciació genètica entre poblacions, correlacionada amb la seva ubicació geogràfica. Els nostres resultats suggereixen una llarga persistència d'espècies en refugis ibèrics en el sud durant els períodes glacials i efectes fundadors posteriors cap al nord a causa de la colonització en èpoques més càlides (és a dir, el patró "riquesa al sud, puresa al nord"). A més, les nostres anàlisis filogeogràfiques indiquen la presència de dos llinatges genètics separats dins de *Ch. sempervirens*, recolzant doncs la hipòtesi de múltiples refugis menors en el SO d'Ibèria, d'acord amb el model de "refugis dins de refugis".

Revista: el factor d'impacte d'aquesta revista (corresponent a l'any 2013, segons el JCR de la ISI web of Knowledge) és 1,648, i es troba en la posició 81 de 196 a *Plant Sciences* (Q2, segon quartil).

Phylogeographic insights of the lowland species *Cheirolophus sempervirens* in the southwestern Iberian Peninsula

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Running title: Phylogeography of *Ch. sempervirens* in Iberia

Abstract The southwestern Iberian Peninsula is an important biogeographic region, showing high biodiversity levels and hosting several putative glacial refugia for European flora. Here, we study the genetic diversity and structure of the Mediterranean, thermophilous plant *Cheirolophus sempervirens* across its whole distribution range in SW Iberia as a tool to disentangle some of the general biogeographic patterns shaping this southern refugia hotspot. Null genetic diversity was observed in the cpDNA sequencing screening. Nonetheless, AFLP data revealed high levels of among-population genetic differentiation correlated to their geographic location. Our results suggest longer species persistence in southern Iberian refugia during glacial periods and subsequent founder effects northwards due to colonizations in warmer stages (i.e. the "southern richness to northern purity" pattern). Additionally, our phylogeographic analyses indicate the presence of two separate genetic lineages within *Ch. sempervirens,* supporting the hypothesis of multiple minor refugia for SW Iberia in agreement with the refugia within refugia model.

Keywords AFLP, genetic diversity, genetic structure, glacial refugia, Mediterranean, Pleistocene climatic oscillations, southern Europe

Introduction

The Iberian Peninsula is a widely recognized hotspot of biodiversity (Médail & Quezél, 1999), and represents a key area from a biogeographic point of view (Nieto Feliner, 2014). In this way, the scientific community has largely demonstrated the significant role played by this region as a major refugium for flora and fauna during Pleistocene glaciations (Taberlet et al., 1998; Hewitt, 2011). Until recently, it was widely agreed that the entire Iberia acted -similarly to other Mediterranean peninsulas such as the Italian or the Balkan- as a single refugium for biodiversity during the coldest periods of climatic oscillations (e.g. Hewitt, 2001). However, in recent times, the idea of multiple refugia occurring within the Iberian Peninsula (i.e. the refugia within refugia model) has been largely supported by several works reviewing phylogeographic patterns of different plant and animal taxa (e.g. Olalde et al., 2002; Gómez & Lunt, 2007; Médail & Diadema, 2009). Indeed, even considering multiple independent refugia would not adequately reflect the complexity of the biogeographic processes that occurred in Southern European peninsulas during Pleistocene climatic oscillations (Nieto Feliner, 2011), and the relationship among minor refugia nested within the so-called larger refugia (e.g. big peninsulas) still remains under discussion (Hewitt, 2001).

Recent studies dealing with the evolutionary history of different plant species occurring along regional refugia in the Balkan (e.g. Surina et al., 2011; Grdiša et al., 2014) or the Italian (e.g. Španiel et al., 2011; Hardion et al., 2014) peninsulas have deeply contributed to better understanding the biogeographic patterns that influenced these refugia hotspots during Pleistocene glaciations. Sanz et al. (2014) also highlighted the role of these peninsulas (Iberian, Italian and Balkan) as glacial refugia for alpine floras. Focusing on the Iberian Peninsula, similar phylogeographic studies have focused on the patterns and processes affecting endemic and subendemic taxa from refugia-rich areas such as the Pyrenees (e.g. Segarra-Moragues et al., 2007), the Central Range (e.g. García-Fernández et al., 2013) or the Iberian eastern coast (e.g Garnatje et al., 2013). According to recent studies (Gómez & Lunt, 2007; Médail & Diadema, 2009), the southwestern half of the Iberian Peninsula (hereafter SW Iberia) also hosts multiple regional Pleistocene refugia (see Fig. 1). In addition, many endemic species (García-Barros et al., 2002) –as well as numerous subendemic plants whose only Eurasian populations occur on this particular area (Rodriguez-Sanchez et al., 2008)– inhabit

along the various minor refugia distributed along the SW coast of Spain and Portugal. However, very scarce phylogeographic attention has been paid on this region to date, so the effects of climatic oscillations on the flora from SW Iberia are still poorly understood.

The effects of Quaternary climatic oscillations in the flora of southern European refugia have been proposed to be less drastic and more complex than in northern regions, where extinction was the dominant process (Birks & Willis, 2008; Nieto Feliner, 2011). In this regard, thermophilous plants are considered to be particularly interesting for the study of the climatically-buffered Mediterranean refugia, since they are likely to be more severely affected by glaciations than cold-adapted taxa (Hewitt, 2000; Stewart et al., 2010). Recent biogeographical works investigating the complex effects of climatic oscillations on various lowland southern refugia successfully employed thermophilous plants [e.g. *Arundo plinii* Turra (Hardion et al., 2014); *Cheirolophus intybaceus* (Lam.) Dostál (Garnatje et al., 2013); *Tanacetum cinerariifolium* Sch.Bip. (Grdiša et al., 2014)] as models of study. Thermophilous species from lowland or coastal habitats are likely to respond to climatic oscillations with geographical (mostly latitudinal) range shifts – contributing to the so-called "southern richness to northern purity" (hereafter SR-NP; Hewitt, 2000) genetic diversity pattern. In contrast, cold-resistant or alpine taxa are believed to respond with elevation range shifts limited by their island-like environment, resulting on genetic structure distributed in different isolated refugia with equivalent diversity levels (Surina et al., 2011). Consequently, lowland and coastal flora from Mediterranean regions such as SW Iberia should fit better to the SR-NP genetic pattern rather than to the "equivalent isolated refugia" pattern. However, the response to this phylogeographical and microevolutionary processes in southern European species yet remains poorly studied (Hewitt, 2011).

Cheirolophus sempervirens (L.) Pomel (Asteraceae) is a species particularly well suited for testing biogeographical hypotheses related to Pleistocene glacial refugia from SW Iberia. This is a thermophylous shrub with a narrow cold tolerance, inhabiting humid valleys and low mountain stage with clear maritime influence (Susanna, 1991). The species is a subendemism occurring exclusively in the SW Iberian Peninsula, excepting for a few isolated populations cited from the northern mountains of Algeria. Interestingly, the distribution of *Ch. sempervirens* in the Iberian Peninsula shows a

linear distribution, following moist and warm locations close to the coast from Malaga to Coimbra (see Fig. 1). This distribution range crosses over five putative refugia identified by Médail & Diadema (2009) and extends along 500 km of latitudinal range. Additional isolated locations have been cited in more inland regions -i.e. Salamanca- or in northern latitudes -i.e. Galicia- of the Iberian Peninsula, but these citations are thought to be either misidentifications (Susanna, 1991) or have not been found in recent field surveys (D. Vitales, pers. obs.). Similarly to the rest of the members of the genus, *Ch. sempervirens* has an outcrossing pollination and produces seeds that disperse by gravity very close to the mother plant (Ruiz de Clavijo & Devesa, 2013). The evolutionary history of *Cheirolophus* has been recently studied (Vitales et al., 2014b) and -as reported for other Mediterranean taxa (e.g. Migliore et al., 2012; Besnard et al., 2013; Fiz-Palacios & Valcárcel, 2013)- the time-calibrated phylogeny describes the first divergence of the main Mediterranean lineages of the genus close to the onset of the Mediterranean climate (c. 3.1 Ma, late Pliocene) and their diversification during the Quaternary (around 1.3 Ma). Therefore, we may hypothesize that the genetic diversity showed by *Ch. sempervirens* should have been shaped by Pleistocene glaciations, the most important climatic changes affecting the Mediterranean region during this period of earth history (Thompson, 2005).

In this context, the main goal of our study was to analyze the phylogeography of *Ch. sempervirens* across its whole distribution range in SW Iberia as a complement to disentangle some general biogeographic patterns affecting this southern refugia hotspot. Specifically, we proposed to i) test whether *Ch. sempervirens* shows signs of the SR-NP genetic diversity pattern reported, based on other plant taxa, in other southern European refugia and ii) investigate whether the phylogeographical structure of this thermophylous species fits the 'refugia within refugia' model proposed for the Iberian Peninsula.

Materials and methods

Sampling strategy

Cheirolophus sempervirens was sampled from 10 populations located on southwestern Iberian Peninsula, covering a most of the distribution range of the species. The number of analysed individuals per population ranged between 5 and 10 depending on

population size and uneven success of laboratory procedures. Details of locations and number of sampled individuals of each population were listed in Table 1 and Fig. 1. Leaf material was immediately dried in silica gel and stored at room temperature (20 - 25ºC) until DNA extraction.

DNA isolation, AFLP fingerprinting and DNA sequencing

Total genomic DNA was extracted from fragments of silica-gel dried leaf tissue following the protocol of Doyle and Doyle (1987) with slight modifications. DNA samples were cleaned using QIAquick columns (Qiagen, Valencia, CA, USA) and their quality and DNA concentration was determined using NanoDrop ND-1000 spectrophotometry (ThermoScientific, Wilmington, DE, USA).

The AFLP technique was carried out following the protocol described in Vos et al. (1995) with some modifications (see Vitales et al., 2014a). After a primer trial involving 12 selective primers, three primer pairs were finally chosen: EcoRI-AC/MseI-CTT; EcoRI-AG/MseI-CTC; and EcoRI-AT/MseI-CAG. The success of each step was tested by running the PCR products on a 1.5% agarose gel. Fragments were run on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with 10 µL High Dye (deionized formamide) and 0.2 µL GeneScanTM 500 ROXTM Size Standard per sample. Amplified fragments were genotyped using GeneMarker®AFLP/Genotyping software (version 1.9; SoftGenetics, LLC., State College, PA, USA). AFLP error rates were calculated following Bonin et al. (2004). Twenty random samples per primer combination were replicated to ensure reproducibility, repeating all parts of the AFLP protocol. All alleles with an error rate >5% were eliminated. In addition, those individuals that did not produce scorable patterns for all three primer combinations were also excluded. Out of the 93 attempted individuals, 85 (91 %) were retained in further data analysis.

We also conducted a screening test for DNA sequencing involving four highly variable chloroplast markers (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*), which were sequenced for a few individuals of different populations. All these regions were newly amplified and sequenced for nine individuals from Andalucia, Algarve, Centro and Coimbra regions following protocols in Vitales et al. (2013).

Data analysis

Based on AFLP data, the unbiased heterozygosity within populations (*Hj*), the average

gene diversity within populations (*Hw*) and total gene diversity in the species (*Ht*) were calculated using the software TFPGA v. 1.3 (Miller, 1997). We also conducted measures of genetic rarity: the number of private alleles in each population; and the frequency-down-weighted marker values (*DW*) index of Schönswetter & Tribsch (2005), calculated as ratio of means -which makes the measure less sensitive to differences in sample size between localities- using AFLPDAT (Ehrich, 2006). R software (R Development Core Team, 2012) was used to perform Spearman rank correlation analyses between the genetic diversity (*Hj*) of populations and their latitude, as well as between the *DW* index of populations and their latitude. Pairwise F_{ST} values were estimated for each pair of populations studied with AFLP SURV 1.0 (Weir & Cockerham, 1984). Significance was evaluated through 10000 permutations. Finally, we conducted AMOVA analyses by using ARLEQUIN 3.5 (Excoffier et al., 2005) to estimate genetic differentiation attributable to population subdivision. To further characterize the spatial genetic distribution in this species, we performed Mantel tests based on genetic distance matrices constructed with F_{ST} values between populations and on geographical matrices calculated by the euclidean distance (X and Y coordinates) between populations using ArcGIS 9.1 (ESRI, Redlands, CA, USA). Mantel tests were performed on ARLEQUIN 3.5 with 100000 permutations and considering a p-value limit of 0.05.

Population genetic structure revealed by AFLP was investigated using phylogenetic, clustering and multivariate analysis. We used the Neighbor-Net method (Bryant & Moulton, 2004) carried out with SplitsTree v.4.10 (Huson & Bryant, 2006) to construct a distance-based network using the Jaccard coefficient (Jaccard, 1901), which is restricted to shared band presence rather than shared absence. A Neighbor-Joining (NJ) analysis of the same matrix, with 1000 bootstrap replicates, was also performed using SplitsTree 4.10. Bayesian clustering analyses were carried out using STRUCTURE 2.3 (Hubisz et al., 2009). We considered the admixture ancestry model and the correlated allele frequencies. Ten independent simulations were run for each possible number of genetic groups (*K*) (from *K* = 1 to 10), using a burn-in period of 10^5 generations and run lengths of $5x10^5$. STRUCTURE HARVESTER (Earl & vonHoldt, 2011) was employed to estimate the number of genetic groups (*K*): we selected the *K* value that maximizes the probability of the data *L(K)* and we also considered the criterion

proposed by (Evanno et al., 2005) based on the rate of change in the probability between successive *K* values, *ΔK*. Similarities among individuals were also studied via Principal Coordinate Analysis (PCoA, Gower (1966)) using the Jaccard distance, in order to detect other possible relations that could not be visualized with assignment methods or phylogenetic analyses. This procedure was carried out with R software (R Development Core Team, 2012) using the vegan package (Oksanen et al., 2008).

Results

Initially, 221 alleles were obtained from automatic genotyping of *Ch. sempervirens* AFLP profiles. After manual correction, error rates calculation, elimination of small and troublesome alleles and low intensity peaks, a final matrix with 195 (88.2%) alleles was considered for subsequent analyses. The final data sets showed an error rate of 2.6%, which is below the maximum error rate percentage accepted for good AFLP reproducibility (5%) (Pompanon et al., 2005). None of the chloroplastid regions analysed in the screening test yielded any variability among or within *Ch. sempervirens* populations.

Population genetic diversity measures are shown in Table1. Average gene diversity per population (*Hw*) was 0.0455 ± 0.0245. Private alleles were irregularly distributed across the studied populations: AND population showed five private fragments; ALG, ODE and MIL presented two; SET and COI showed one; and no private alleles were found in the rest of populations. The frequency-down-weighted marker values (*DW*) also demonstrated considerable variation among populations, ranging between 567.466 (AND) and 164.459 (NAZ). Total genetic diversity for AFLP markers resulted to be *Ht* = 0.1602, and the AMOVA analysis revealed that most of the variability in *Ch. sempervirens* was attributable to differences among populations (73.62%, P < 0.001; Table 2). Using the matrix of inter-population F_{ST} distances, and the matrix of geographical distances (in kilometres), the Mantel test indicated a significant correlation between genetic and geographical distances (r = 0.516, $p < 0.05$). Spearman rank correlation analyses revealed that within-population genetic diversity (*Hj*) in *Ch. sempervirens* was not associated to the latitude of populations ($p > 0.05$; Fig. 2A). In contrast, the same test indicated a significant negative correlation between the *DW* index and the latitudinal distribution of this species (r = -0.903, P < 0.05; Fig. 2B).

According to the Bayesian analysis of population genetic structure conducted with STRUCTURE, the populations of *Ch. sempervirens* showed the highest *L(K)* and *ΔK* values for *K* = 2 and *K* = 5 (see Fig. S1). For *K* = 2, populations from south-western Portugal (Algarve and Baixo Alentejo) clustered separately from the rest of populations, whereas the one from Setubal Peninsula (SET) showed a considerable level of admixture among the two genetic groups (Fig. 3A). For *K* = 5, populations north of the river Tagus remained together while most of the others -excepting both populations located in Baixo Alentejo (ODE and MIL), which remained linked- constituted independent clusters (Fig. 3B). The Neighbor-Net (NN) and the Neighbor-Joining (NJ) analyses showed considerably resolved phylogenetic reconstructions (NN, Fig. 4; NJ, Fig S2), highly congruent with the genetic structure revealed by STRUCTURE.

The PCoA using the first two principal coordinates explained 52.2% of the total variation in the data and confirmed several relationships detected in the phylogenetic and cluster analyses (Fig. S3). The first coordinate (accounting for 38.6% of the total variation) distinguished two main groups of species: the south-western populations occurring on Algarve and Baixo Alentejo clearly segregated from the rest of populations, being SET from Setubal Peninsula intermediate between the two groups. The second coordinate (representing 13.6% of variation) mainly segregated the population from Andalucia (AND) in a different cluster. The five genetic groups proposed by STRUCTURE could also be recognized in this PCoA analysis (Fig. S3).

Discussion

In contrast to the null genetic diversity observed in the cpDNA sequencing survey, AFLP analyses provided considerable information on the magnitude and pattern of genetic variation existing in ten populations covering the whole distribution range of *Ch. sempervirens* in SW Iberian Peninsula. This result reinforces the idea that AFLP is a particularly suitable tool to perform phylogeographic analyses when other molecular markers (such as cpDNA sequencing) provide insufficient information (Després et al., 2003; Meudt et al., 2007). Overall, the data revealed high levels of genetic differentiation among populations and low genetic diversity within populations. This pattern indicates a strong genetic structure among *Ch. sempervirens* populations, which seems to be correlated to their geographic distance according to the Mantel

test.

Southern richness versus northern purity in southwestern Iberian Peninsula

The results of our study on *Ch. sempervirens* support the hypothesis of longer species persistence in southern Iberian refugia and founder effects in northward colonizations (i.e. the SR-NP pattern). A significant association between the genetic rarity index *DW* and the latitude of populations was clearly observed in our analyses (Fig. 2B). Similarly, private alleles were much more frequent in the five southernmost populations (12 private alleles in total) than in the five northern populations (only one private allele). The abundance of rare and private alleles has been proposed as a characteristic signal of populations with a long *in situ* history, most probably going back to the last glaciation (Schönswetter & Tribsch, 2005; Ehrich et al., 2008). Indeed, genetic rarity measures such as the *DW* or the private alleles have been successfully employed to infer the glacial refugia patterns in numerous plants from different parts of the globe [e.g Mráz et al. (2007) in the Alps; Pérez-Collazos et al. (2009) in the Iberian Peninsula; Trometsberger et al. (2009) in South America; Li et al. (2011) in Asia]. Conversely, intra-population heterozygosity did not show any correlation with the latitude of populations in *Ch. sempervirens* (Fig. 2A). Genetic rarity has been seen as a better indicator of historical processes rather than patterns of genetic diversity, which mirror contemporary processes such as connectivity of populations and population sizes (Comps et al., 2001; Widmer & Lexer, 2001; Paun et al., 2008). In this way, differences in heterozygosity between *Ch. sempervirens* populations could have been recently blurred due to the differences in regional abundance and population size between southern and northern localities along the distribution range (D. Vitales, pers. obs.).

A similar outcome suggesting the southern survival of the species during the Pleistocene glaciations and the more recent formation of the northern populations was also supported by the genetic structure of *Ch. sempervirens*. The five genetic clusters proposed by STRUCTURE (Fig. 3B) and recovered as well in the PCoA (Fig. S3) and the networking (Fig. 4) analyses showed a marked SR-NP distribution pattern. Specifically, the southernmost populations constituted four of these genetic groups (almost one group per population), whereas the five northern populations were grouped in one single genetic cluster. Populations located in refugial regions are expected to present greater genetic structuring than those located in recolonized areas (Hampe & Petit,

2005; but see also de Lafontaine et al. (2013) for a contrasting pattern). Indeed, the occurrence of larger number of genetic lineages in areas rich in glacial refugia has been reported in former studies analyzing the phylogeography of different plant species (e.g. Schönswetter et al., 2003; Picó et al., 2008). In sumamry, both within-population indexes (i.e. genetic rarity) and genetic structure inferences suggest that *Ch. sempervirens* from SW Iberia responded to Pleistocene climatic oscillations with latitudinal range shifts, somewhat mirroring the performance of lowland flora from other southern European refugia.

Refugia within refugia in the southwestern Iberian Peninsula

As reported for several organisms from worlwide distributed refugia hotspots (see Weiss & Ferrand, 2007; Shafer et al., 2010; Qiu et al., 2011 for some reviews) and particularly from Southern European refugia (Gómez & Lunt, 2007), the current genetic structure of *Ch. sempervirens* suggest the survival of the species in various glacial refugia across SW Iberia (i.e. the refugia within refugia model). This multiple refugia pattern is clearly depicted by one of the best clustering model (K = 2) proposed by STRUCTURE (Fig. 3A) and supported as well by the other methodological approaches employed to study the phylogeography of *Ch. sempervirens*. At K = 2, the populations from Algarve and Baixo Alentejo clustered in a differentiated group (lineage 1), a genetic segregation further supported with the high bootstrap (97%) assigned by the NJ analysis to the branch segregating those three southern Portuguese populations (Fig. 4). This phylogeographic split -together with the high genetic rarity levels showed by these populations- indicate that SW Iberian corner may be acting as an isolated glacial refugium for *Ch. sempervirens*, a pattern already reported in other Iberian endemic plants such as *Senecio gallicus* Chaix (Comes & Abbott, 1998). The complementary genetic cluster at K = 2 groups the five northernmost populations with the southernmost one from Malaga (Andalucia), constituting an additional genetic lineage within this species (lineage 2). In this case, according to the higher genetic rarity values showed by Andalucian population (Table 1), the refugial area of this second plylogeographic lineage should be found in this southern region of Spain. Therefore, our data suggest that at least two independent refugia -probably located in southern Portugal and in southern Spain- may have played a primary role in the evolutionary history of *Ch. sempervirens* during Pleistocene climatic oscillations.

Notwithstanding, several authors have recently warned that the model of multiple independent refugia might be an oversimplification (Nieto Feliner, 2011; de Lafontaine et al., 2013), postulating that recurrent isolation and admixture processes would have shaped a more complex phylogeographic scenario in Pleistocene refugia hotspots. According as well to the *K* = 2 model of STRUCTURE, the population from Setubal Peninsula (SET), located halfway from Algarve-Baixo Alentejo localities (lineage 1) and from the rest of northern populations (lineage 2), showed significant admixture between both genetic lineages. Likewise, the NN and the PCoA results also indicated that SET population shows an intermediate position between both main genetic clusters. Thus, this result suggest that Tagus river may act in *Ch. sempervirens* both as a soft barrier and as a secondary contact zone of lineages diversified in different phases of climatic oscillation cycles. Indeed, river basins have been proposed as genetic barriers in other plants affected by Pleistocene glaciations in southern European refugia (e.g. Picó et al., 2008; Grdiša et al., 2014). In our work, the limited sampling has shed light into the general phylogeographic patterns showed by this lowland plant from SW Iberia, but prevent us to infer more precise details about its evolutionary history. Therefore, caution must be taken when hypothesizing on the mode and location of particular phylogeographic events that affected this species during Pleistocene glaciations. Further studies in the southwestern Iberian refugia will be necessary to test whether the phylogeographic history of *Ch. sempervirens* is idiosyncratic or represent more general patterns.

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Figures

Fig. 1. Map of geographic distribution and sampled populations of *Cheirolophus sempervirens* in the Iberian Peninsula. Dashed line indicates distribution range of *Ch. sempervirens*. Red-colored areas correspond to putative glacial refugia identified by Médail & Diadema (2009) in this region.

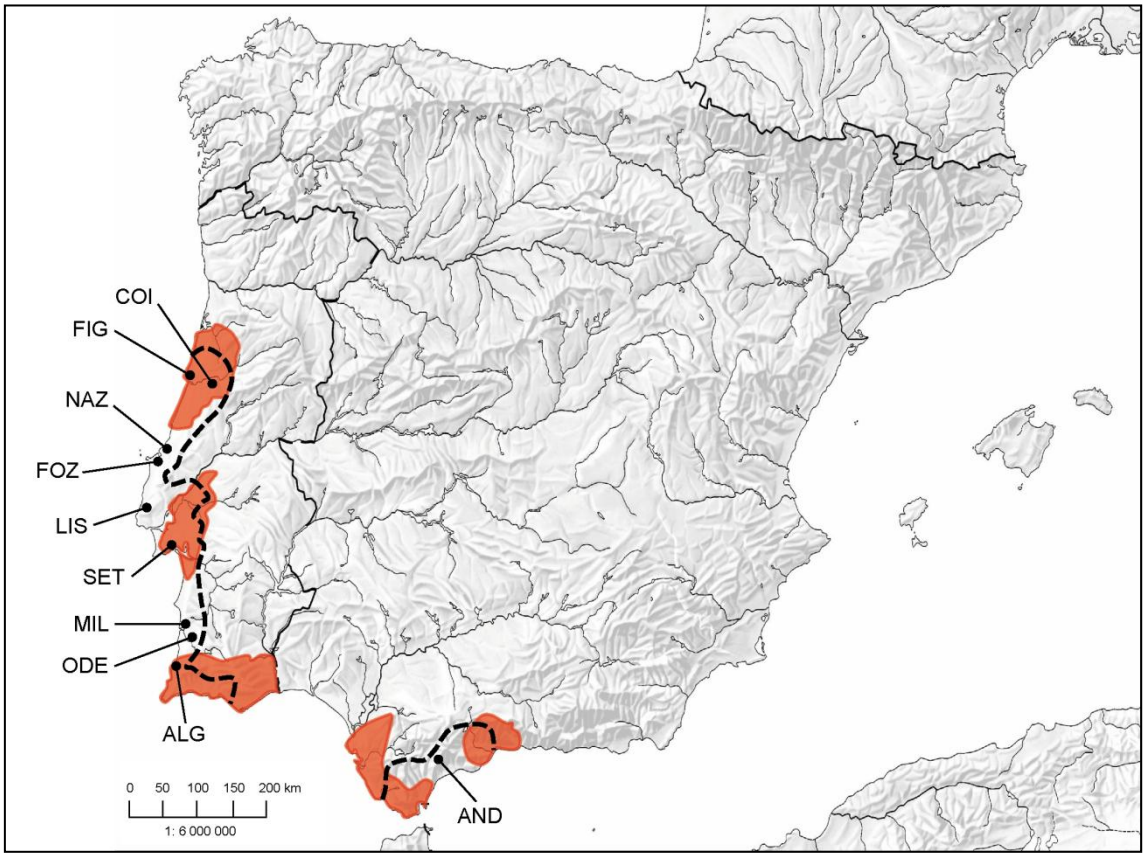


Fig. 2. A The genetic diversity (*Hj*) and **B** the genetic rarity (*DW*) of each sampled population of *Cheirolophus sempervirens* along their latitudinal distribution in the Iberian Peninsula, calculated using amplified fragment length polymorphism (AFLP).

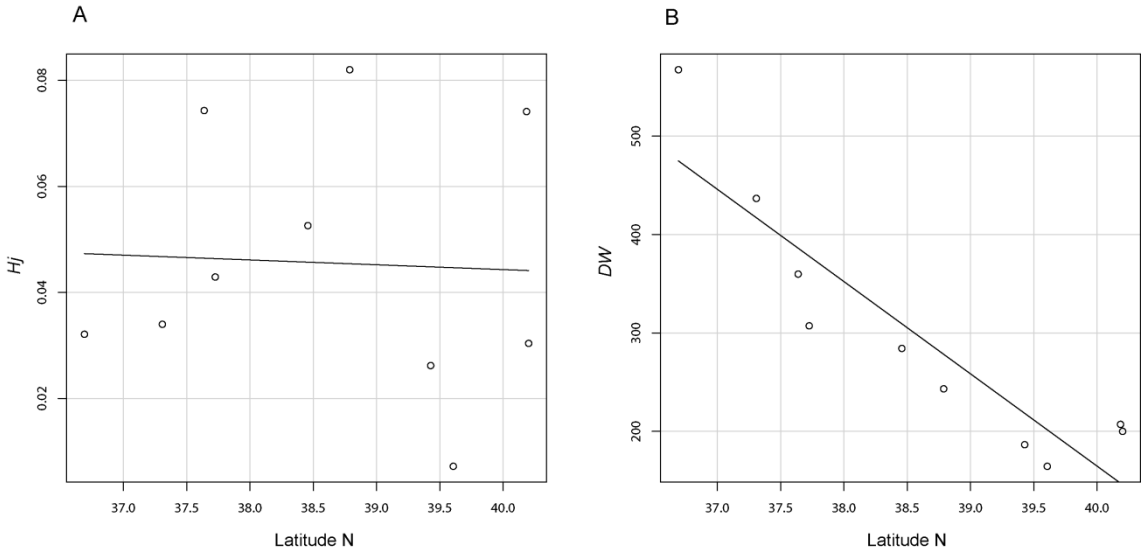


Fig. 3. Bayesian estimation of genetic structure within *Cheirolophus sempervirens* inferred with STRUCTURE from AFLP data, according to the best models [**(A)** *K* = 2 and **(B)** *K* = 5] proposed by STRUCTURE HARVESTER.

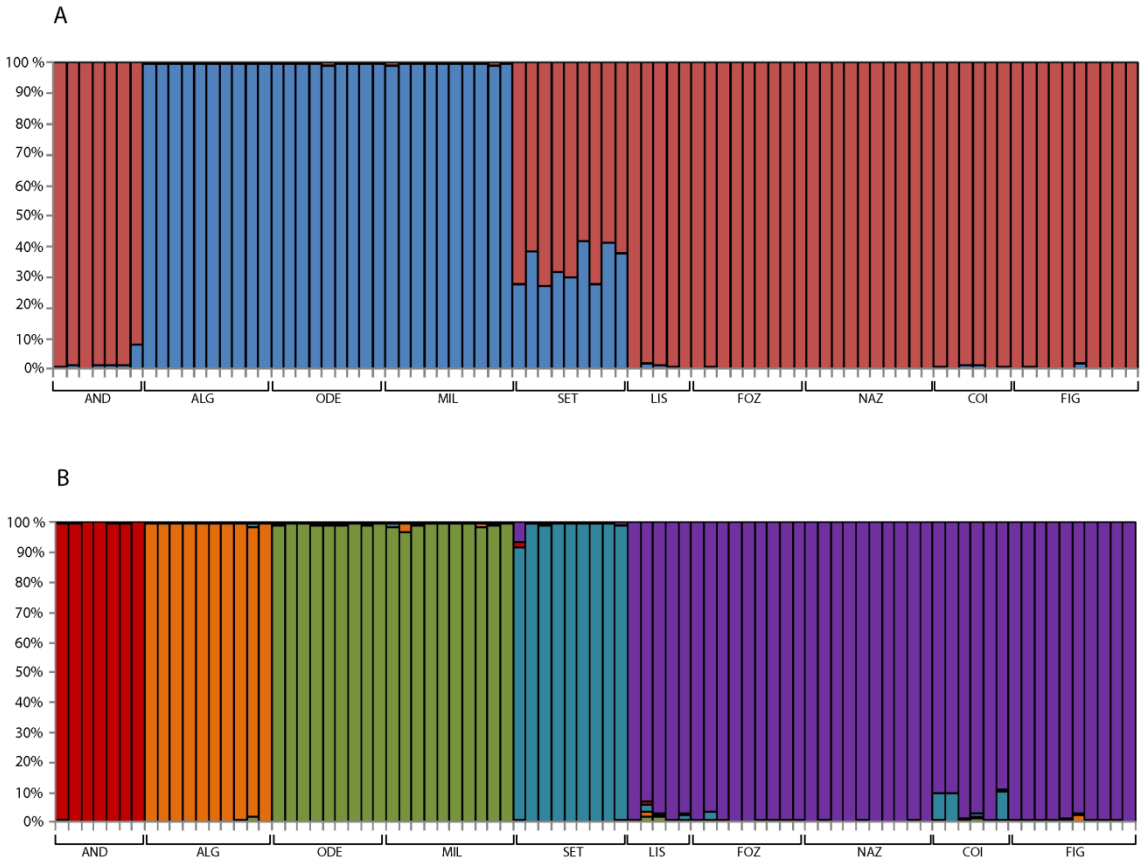
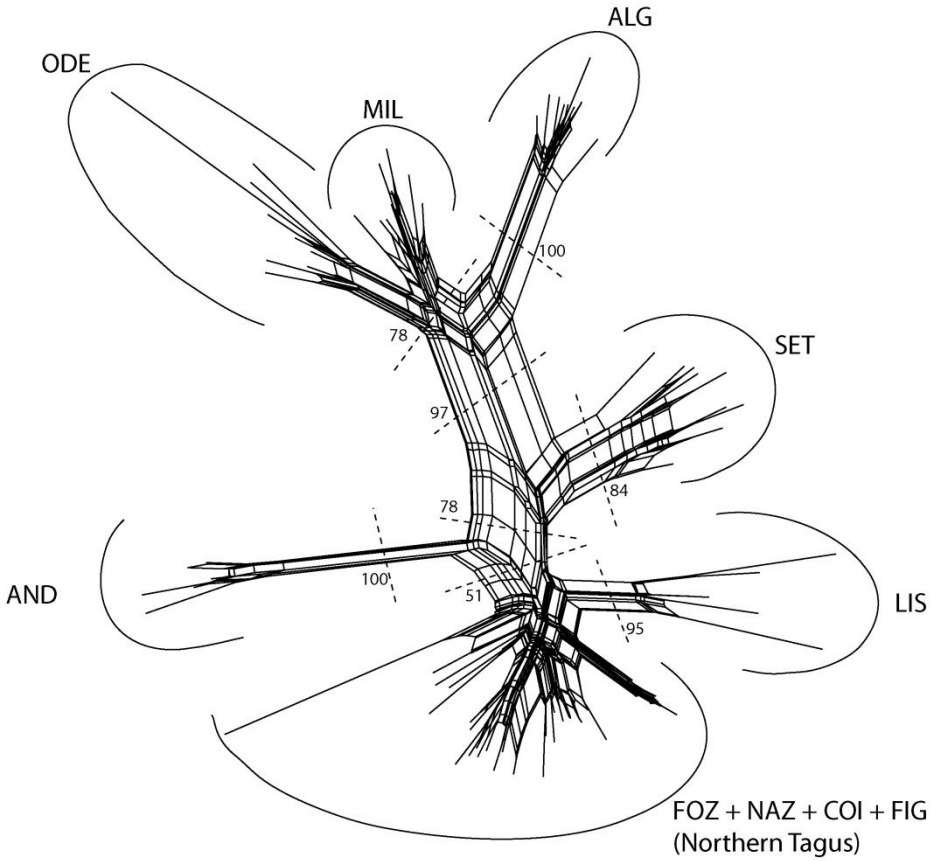


Fig. 4. Neighbor-Net based on Jaccard distance obtained from 85 individuals of ten sampled populations of *Cheirolophus sempervirens*. Bootstrap values above 50% derived from a Neighbor-Joining analysis are given for the main branches.



Tables

Table 1 Sampling information and genetic diversity indexes of *Cheirolophus sempervirens* from the Iberian Peninsula**.** Code, locality, geographical coordinates, number of sampled individuals (*N*), and genetic diversity indexes assessed by AFLP in 10 populations of *Cheirolophus sempervirens* from the Iberian Peninsula. Genetic indices: heterozygosity (*Hj*); frequency-down-weighted marker values index (*DW*); and private alleles.

Code	Location	Latitude N	Longitude W	*N*	*Hj*	*DW*	Private alleles
AND	Andalusia: Málaga, Ronda	36.692	5.266	7	0.0321	567.466	5
ALG	Algarve: Aljezur	37.308	8.802	10	0.0340	436.645	2
ODE	Alentejo Litoral: Odemira	37.638	8.620	9	0.0743	359.830	2
MIL	Alentejo Litoral: Milfontes	37.726	8.769	10	0.0429	307.222	2
SET	Centro: Setubal, Sesimbra	38.458	9.113	9	0.0526	284.285	1
LIS	Centro: Lisboa, Sintra	38.788	9.403	5	0.0820	243.147	0
FOZ	Leiria: Foz do Arelho	39.428	9.187	9	0.0262	186.496	0
NAZ	Leiria: Nazaré	39.607	9.079	10	0.0072	164.459	0
COI	Baixo Mondego: Coimbra	40.185	8.436	6	0.0741	207.150	1
FIG	Baixo Mondego: Figueira da Foz	40.202	8.899	10	0.0304	199.991	0

Table 2 Analyses of molecular variance (AMOVA) of *Cheirolophus sempervirens* populations based on AFLP markers.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	P
Among populations	9	805.76	10.15	73.62	<0.001
Within populations	75	272.80	3.64	26.38	<0.001
Total	84	1078.56	13.79		

Supplementary information

Fig. S1. Plots with the estimates of the number of *K* groups based on A the ΔK statistic of Evanno et al. (2005) and B the mean likelihood *Ln(K)* calculated with STRUCTURE HARVESTER.

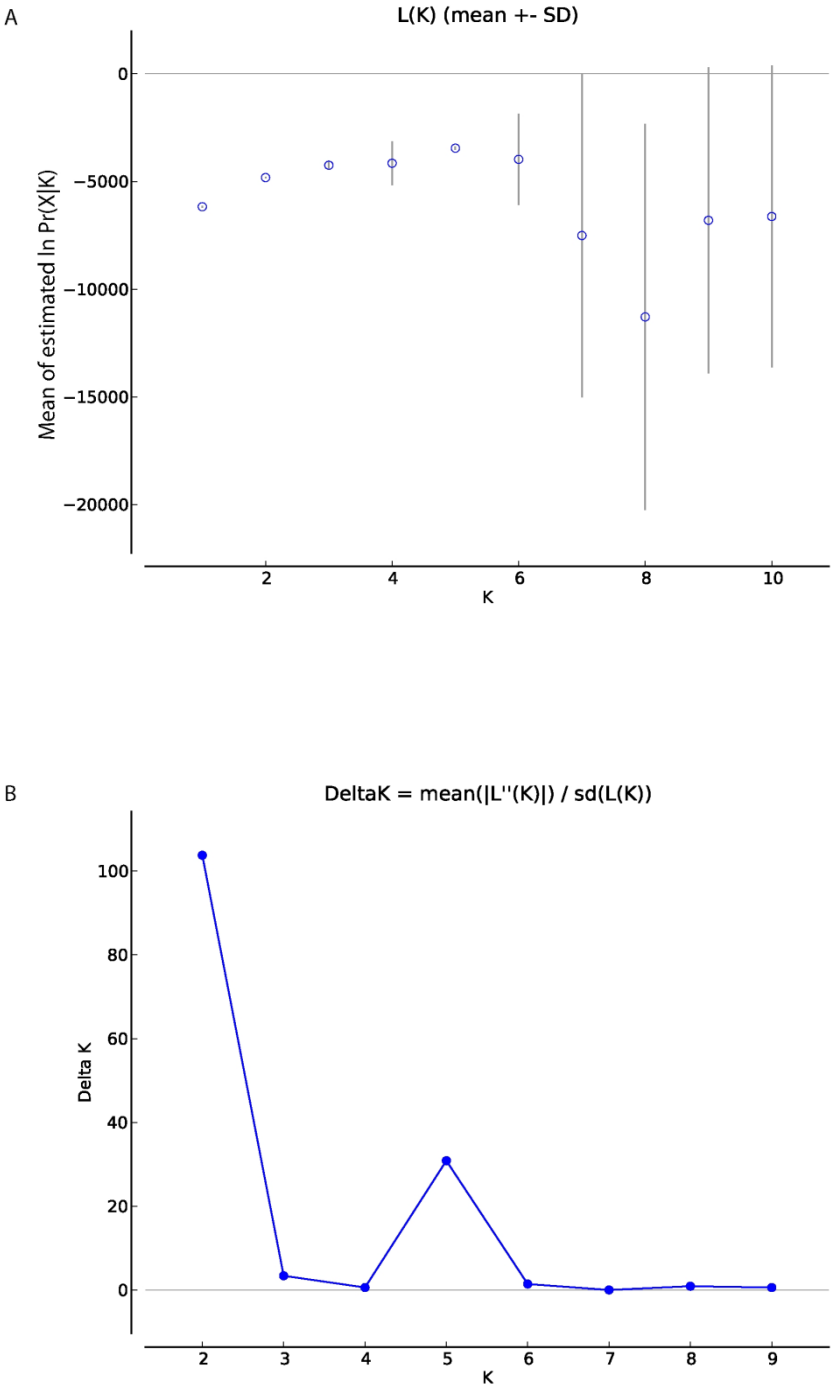


Fig. S2. Neighbor-Joining tree of 85 *Cheirolophus sempervirens* individuals from the 10 sampled populations. Bootstrap values greater than 50% that were obtained after 1000 permutations are indicated on the branches.

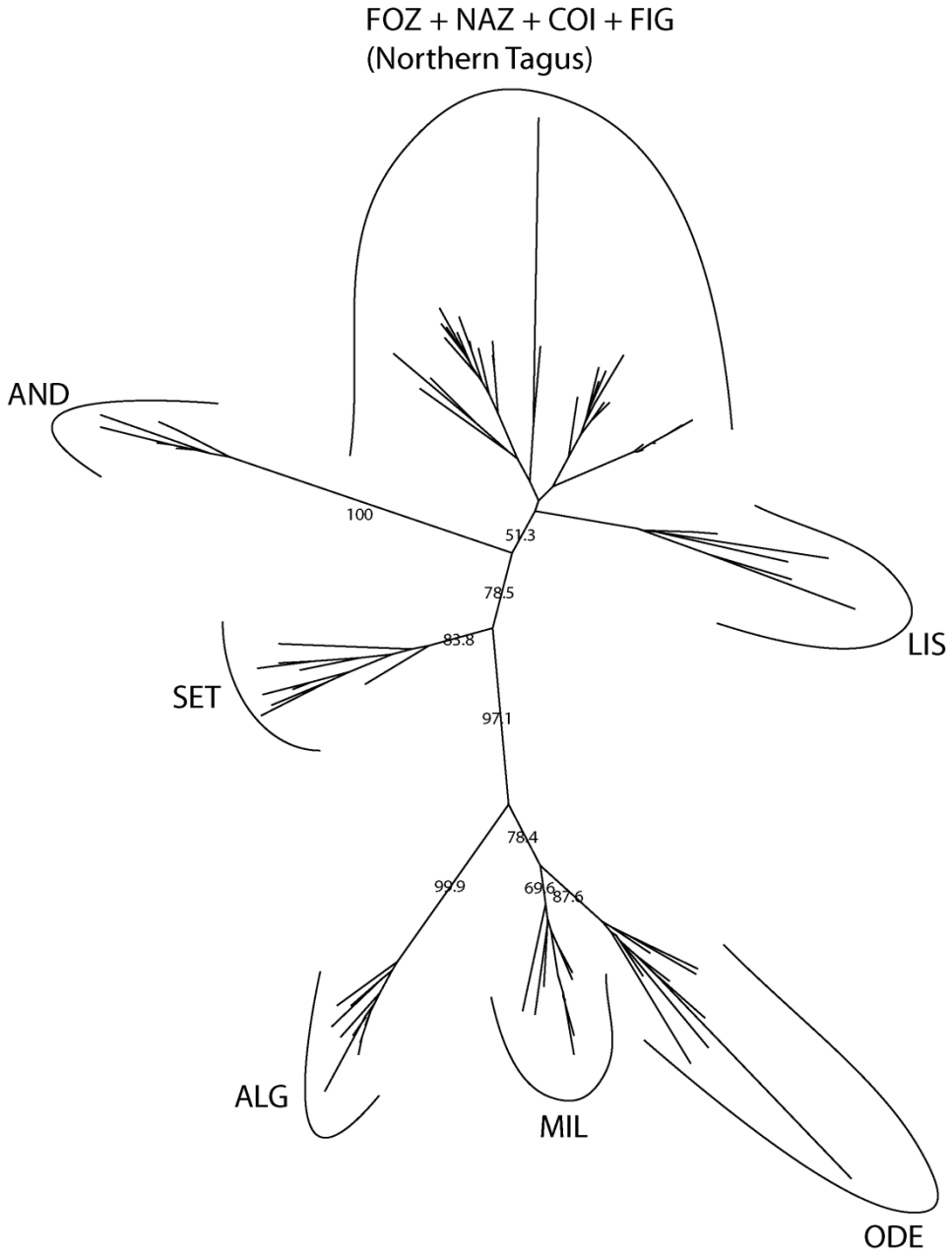
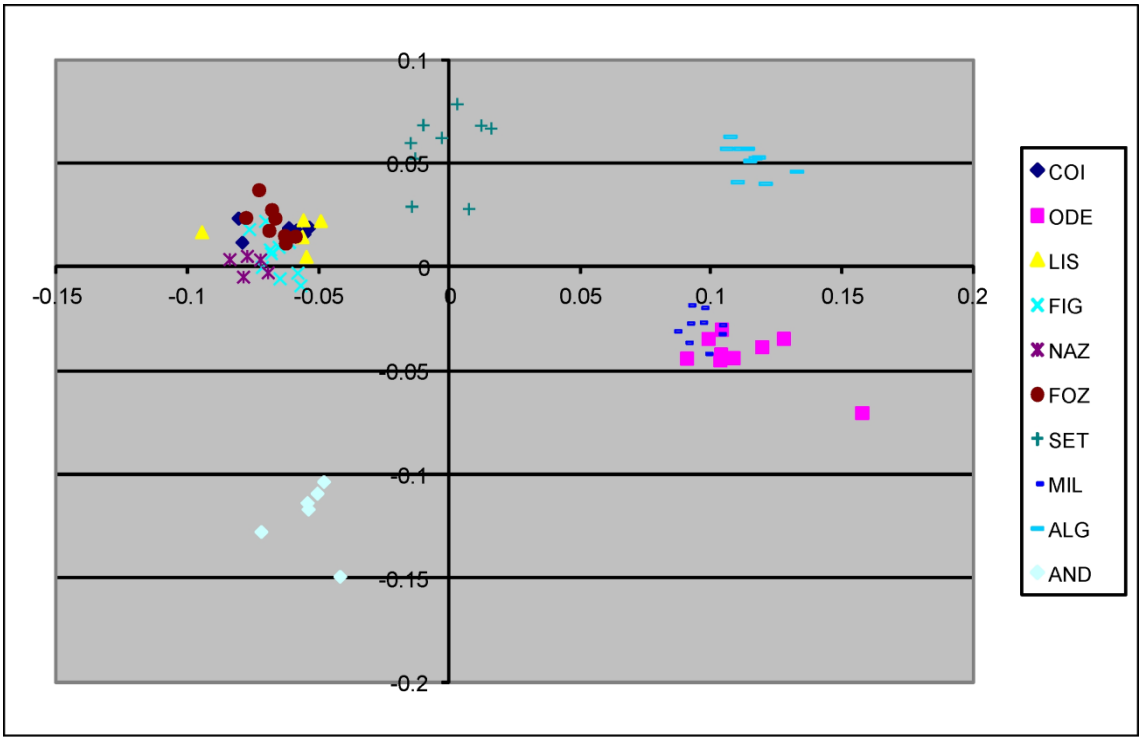


Fig. S3. Principal coordinates (PCoA) plot of AFLP data for the *Cheirolophus sempervirens* populations included in this study. Different symbols correspond to different populations as shown in the legend in the right side.



- **AoB PLANTS (en preparació)**

Evidència genòmica en la radiació insular oceànica: reforç de trets preexistents en *Cheirolophus* (*Asteraceae*, *Centaureinae*)

Daniel Vitales, Oriane Hidalgo, Joan Vallès, Teresa Garnatje, Sonja Siljak-Yakovlev i Jaume Pellicer

El gènere *Cheirolophus* constitueix un dels casos més sorprenents de radiació a la Macaronèsia, on es va diversificar en un llinatge d'aproximadament 20 espècies endèmiques, a un ritme d'entre els més ràpids observats en illes oceàniques. Estudis previs sobre la mida del genoma i altres aspectes citogenètics revelaren valors C significativament menors en les espècies macaronèsiques respecte a les continentals, i un nombre excepcionalment alt de *loci* de DNAr 35S tant en representants insulars com continentals. En aquest treball hem construït un perfil citogènetic del gènere, que inclou nous nombres cromosòmics i mapatge físic de *loci* de DNAr 35S, i hem analitzat l'evolució de la mida del genoma en un context filogenètic. Els nostres resultats revelaren que l'evolució de *Cheirolophus* ha estat governada per una reducció gradual de la mida del genoma, que començà ja en estadis primerencs de la seva història evolutiva, tot i que tingué lloc de forma més accentuada durant la colonització macaronèsica. S'ha evidenciat també un contrast en el nombre de *loci* de DNAr 35S entre l'espècie que divergí primer, *Ch. crassifolius*, que presenta sols quatre *loci*, i la resta d'espècies que presenten un increment remarcable, de set a 10 *loci*, i les macaronèsiques amb almenys vuit *loci*. Aquests resultats evidencien un elevat dinamisme genòmic en *Cheirolophus*, fins i tot abans de la seva radiació a la Macaronèsia, que de fet podria haver jugat un paper clau com a motor d'aquest procés.

Revista: el factor d'impacte de la revista (corresponent a l'any 2013, segons el JCR de la ISI web of Knowledge) a la que tenim previst enviar aquest treball és 1,743, i es troba en la posició 72 de 199 a *Plant Sciences* (Q2, segon quartil).

Genome insights into oceanic island radiation: enhancement of pre-existing traits in *Cheirolophus* (Asteraceae, Centaureinae)

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Abstract. The genus *Cheirolophus* constitutes one of the most striking cases of radiation in Macaronesia, where it diversified into a lineage of ca. 20 endemic species, at a rate amongst the fastest for oceanic islands. Previous genome size and cytogenetic surveys revealed significantly lower C-values in Macaronesian species than in their continental counterparts, and an exceptional high number of terminal 35S rDNA loci in both continental and island species. In this study we have built a more comprehensive cytogenetic profile for the genus, including new chromosome number and physical mapping of 35S rDNA loci, and modelled the evolution of genome size within a phylogenetic context. Our results revealed that *Cheirolophus* evolution has been governed by gradual genome downsizing, which started early in its evolutionary history, albeit accentuated during the Macaronesian colonisation. A contrasting pattern of 35S rDNA loci number is also evidenced with the earliest diverged species, *Ch. crassifolius*, bearing just four loci while the remaining species display a remarkable increase with seven to 10 loci, and the Macaronesian ones with at least eight loci. These results highlight a high genomic dynamism in *Cheirolophus*, even before its radiation across Macaronesia, which could have been indeed playing a key role as the engines promoting such process.

Keywords: *C-value*, *chromosome number*, *constitutive heterochromatin*, *genome size*, *oceanic island radiation*, *rDNA loci*, *speciation*

Introduction

The genus *Cheirolophus* Cass. (Asteraceae, Centaureinae) stands out for constituting a remarkable case of plant radiation in Macaronesian archipelagos. As a result of just one single colonisation event, most likely from the Iberian Peninsula 1.7 Mya, the diversification of the genus in the archipelagos has reached to the fastest rates amongst documented oceanic island radiations, leading to ca. 20 endemic species (Vitales *et al.*, 2014a). In contrast to this oceanic explosive diversity, only nine species are known from the continent and continental islands (Balearic and Maltese Islands) along the Western Mediterranean region (see Appendix 1 for the list of *Cheirolophus* species).

Cheirolophus are typically shrubby perennial plants (except the hemicryptophyte species *Ch. uliginosus*), and show a tendency towards increased height and woodiness in Macaronesia. Certainly, enhanced arborescence and inflorescences disposed in a candelabrum-like structure evolved in the archipelagos probably as the result of secondary environmental adaptations. Shrubs -and particularly arborescent shrubs- are indeed rare in Centaureinae, where they constitute secondary-evolved habit otherwise scattered in the phylogeny of the subtribe (e.g. *Centaurodendron* Johow, *Centaurothamnus* Wagenitz & Dittrich, *Ochrocephala* Dittrich and *Centaurea ptosimopappa* Hayek) (Hidalgo *et al.*, 2006). In this sense, *Cheirolophus* represents an exception within the subtribe for combining, in Macaronesia, treelet habit and high species diversity. Whether the pre-existing woody nature of *Cheirolophus* has promoted its radiation subsequent to the colonisation of Macaronesia yet remains to be demonstrated, however, it should be noted that aside this increase in size and woodiness, *Cheirolophus* radiation has been characterised by moderate morphological divergence (Susanna *et al.*, 1999). This pattern is consistent with the current theory suggesting that geographic isolation and long distance dispersal were likely the main forces driving diversification in the genus, while ecological adaptation, usually related to adaptive radiation with high levels of morphological divergence, played a secondary role in the process (Vitales *et al.*, 2014b).

Early studies considered *Cheirolophus* as part of the Tertiary pre-glacial circum-

Mediterranean stock that originated the Canarian flora (Bramwell, 1976). However, this assumption was argued by isozyme analysis that provided convincing evidence suggesting a younger age for *Cheirolophus* species (this study did not include *Ch. crassifolius*, Garnatje *et al.*, 1998). In fact, the most recent phylogenetic data (Vitales *et al.*, 2014a) accommodated both assumptions by establishing a pre-glaciation origin for *Ch. crassifolius* (ca. 10.4 Mya), while the westward expansion towards the Mediterranean basin (3.08 Mya) and subsequent Macaronesian diversification (1.7 Mya) are contemporaneous to the glaciation period. In turn, the enigmatic *Ch. uliginosus*, appears isolated in an unresolved trichotomy along with the Macaronesian and Mediterannean clades. The identity of the species -even the lineage- responsible for the colonisation of Macaronesia remains unclear, however, although with weak signal, phylogenetic approaches suggest that this process likely took place from Iberia rather than Africa (Vitales *et al.*, 2014b).

Whether a given trait has promoted, accompanied or is a consequence of the radiation, or even is unrelated or impede the process, has to be evaluated in tight connection with the history of lineage diversification. This effort has largely been concentrated on the processes underpinning changes in morphology, which can be dramatically apparent in radiating lineages, such as the evolution of woodiness (e.g. *Aeonium* Webb & Berthel., *Echium* L., *Sonchus* L.; Mort *et al.*, 2002; García-Maroto *et al.*, 2009; Kim, 2012). However, other aspects of plant speciation like the genomic ones are receiving increased attention over recent years, and this is contributing to significantly achieve valuable insights into our understanding of plant radiation phenomena. There is little available on the evolution of karyotype traits during oceanic plant radiations, and evidence suggest that while this process might not be associated with major chromosomal rearrangements (Stuessy and Crawford, 1998), rDNA loci might escape this apparent stasis and vary among related congeners (e.g. *Pachycladon* Hook.f., Mandakova *et al.*, 2010). Indeed, a previous study by Garnatje *et al.* (2012) in *Cheirolophus* revealed that while chromosome number and the number of 5S rDNA loci remained constant across the genus, the number of 35S loci was unusually high and variable. Regarding genome size (GS) evolution, species involved in island colonisations have been found to have smaller genomes than their continental

counterparts in various plant groups, including those involved into a radiation (e.g. *Cheirolophus*, *Schiedea* Cham. & Schltdl.; Garnatje *et al.*, 2007; Kapralov *et al.*, 2009). However, it is still unclear whether this trend is due to the selection of colonisers with smaller genomes or alternatively, that genome skimming is produced by the colonisation process itself (Kapralov and Filatov, 2011). Current evidence suggests that in the course of radiation, colonisation could be followed by genomic expansion as e.g. in *Schiedea* (Kapralov *et al.*, 2009), where species from younger islands present larger genomes than species from older ones, a trend that could be the result of an activation of transposable elements in reduced populations after colonisation events.

Here we served of the most recent phylogeny of the genus (Vitales *et al.*, 2014a), together with an extended comprehensive survey of nuclear DNA contents and physical mapping of 35S rDNA loci distribution including key species, such as the early diverged *Ch. crassifolius*, to analyse the evolution genomic traits in the course of oceanic radiations.

Materials and methods

Materials

Table 1 shows the provenance of *Cheirolophus* populations sampled and 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 with the exception of *Ch. crassifolius*, which cultivated plants were provided by the Orto Botanico dell'Universita di Catania (Palermo, Sicily). Note that in Appendix 1, previous published results concerning karyological, cytogenetic and GS data available for *Cheirolophus* and used in subsequent analyses have been collated.

Chromosome counts and protoplast preparation

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 during 24 h, transferred to ethanol 70 % and stored at -4 °C. For chromosome counts, fixed root tips were hydrolysed in 1 M hydrochloric 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% aceto-orcein for microscope observations. Protoplasts were prepared with the air-drying technique of Geber and 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% cellulose Onozuka R10, 1% pectolyase Y23 and 4% hemicellulase) 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.

Chromomycin A3 banding and fluorescent *in situ* hybridisation (FISH)

We followed the protocols of Schweizer (1976) and Cerbah *et al.* (1995) for CMA banding (i.e. GC-rich DNA), and Heslop-Harrison *et al.* (1991) and Cerbah *et al.* (1998) for FISH experiments, with the modifications described in Garnatje *et al.* (2004). The probe used for 35S rDNA detection was a 0.8 kb TaqI fragment of 18S gene of *Triticum aestivum* L. (Richard *et al.*, 1995) isolated from pTa71 and cloned in pUC18; this probe was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) through polymerase chain reaction.

DNA content assessment

Nuclear DNA contents were estimated by propidium iodide (PI) flow cytometry using the internal standard *Petunia hybrida* Vilm. 'PxPc6' (2C = 2.85 pg; Marie and Brown, 1993). Seeds of the standards were provided by the Institut des Sciences du Végétal, Gif-sur-Yvette (France). Leaf tissue together with the standard were chopped in 600 µl of LB01 isolation buffer (Doležel *et al.*, 1989) with a razor blade and supplemented with 100 µg/ml of ribonuclease A (RNase A, Boehringer). Samples were filtered through a 30 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 extracted per individual. Further technical details on the procedure can be found in (Garnatje *et al.*, 2007). Measurements were carried out at the Centres Científics i Tecnològics of the Universitat de Barcelona.

Reconstruction of ancestral genome size values

From the Bayesian inference conducted in Vitales *et al.* (2014a), a sample of 1000 post-burnt trees was 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 fitted model for analysis of continuous characters (i.e. random walk vs. directional) was selected by running BayesFactor test using the logarithm of the harmonic mean estimated from five independent runs under 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 = δ, κ and λ. Parameter values were inspected with Tracer v.1.5 (Rambaut and Drummond, 2009) to ensure they had reached a stationary dynamic. The random walk model was supported in most of the runs, and the posterior distribution of the scaling parameters generated was used as a model-setting for the second phase of the analysis in which GS of specific nodes was estimated using the addMRCA (most recent common ancestor) command. Ancestral GS were also reconstructed with maximum parsimony (MP) for continuous traits in Mesquite v.2.73 software (Maddison and Maddison, 2007).

Results

Chromosome counts

Somatic chromosome counts made by Feulgen squash technique in various species revealed a stable chromosome number of $2n = 32$, and are illustrated in Figure 1. Further chromosome numbers were also obtained using protoplast preparations from this and a previous study (Figure 2; Garnatje *et al.*, 2012; results summarised in Appendix 1).

Chromomycin A3 banding and fluorescent *in situ* hybridisation (FISH)

Results of GC-rich heterochromatin fluorochrome banding and physical mapping of

35S rDNA loci (10 populations of nine species analysed) are presented in Figure 2A–T. Schematic illustrations of loci number are depicted along branches of the phylogram in Figure 3 for clearer interpretation, including previous records of Garnatje *et al.* (2012). Briefly, the number of rDNA loci ranges between four in the early branched *Ch. crassifolius* to 10, found in both continental (i.e. *Ch. intybaceus*) and in several Macaronesian endemics (e.g. *Ch. massonianus*, *Ch. puntallanensis* and *Ch.* cf. *webbianus*).

DNA content assessments

New nuclear DNA contents are provided for 11 species (Table 2), with values showing relatively moderate diversity among congeners [1.35-fold, including those from Garnatje *et al.* (2007)]. *Cheirolophus duranii* is the species with the smallest GS, with a 2C = 1.33 pg. At the upper end, the species *Ch. crassifolius* represents the largest estimate, with a 2C = 1.80 pg.

Reconstruction of ancestral genome size values

Reconstruction of ancestral 2C-values is indicated in Table 3, and illustrated on the phylogeny (Figure 3), which evidences a trend towards genome downsizing during the diversification of the genus, somewhat more noticeable at early stages of the Macaronesin colonisation (Figure 3).

Discussion

2*n* = 32 is more frequent than previously thought and likely the ancestral chromosome number for *Cheirolophus*, with further independent transitions to 2*n* = 30

Chromosome counts are currently available for 22 out of 28 *Cheirolophus* species, showing the presence of two chromosome numbers, 2*n* = 30 and 32, with some few mentions to B chromosomes for both (Appendix 1; Watanabe, 2002; 2004). Although very unusual, there are also reports of 2*n* = 45 in *Ch. tagananensis* and 2*n* = 24 in *Ch. uliginosus*, which represent quite divergent numbers and hence might be doubtful since they coexist with counts of 2*n* = 30 in both species (Watanabe, 2002; 2004).

While 2*n* = 30 is predominant in published records, our results suggest that 2*n* = 32 could be more widely represented across the main lineages of *Cheirolophus* than previously thought (Appendix 1). *Cheirolophus* chromosomes are very small (mean size 2-4 μm) and not easily observable, so it could be hypothesised that such additional chromosome pair counted in 2*n* = 32 reports is indeed a product of a technical artefact as the result of a chromosome fragmentation (due to centromeric fragility) during squashing. This issue has been already evidenced in other groups of basal Centaureinae, such as *Oligochaeta* (DC.) K.Koch and *Rhaponticum* Vaill. (Hidalgo *et al.*, 2007). Notwithstanding, chromosome morphology was perfectly observable in CMA and FISH preparations of *Ch. crassifolius* demonstrating the existence of 2*n* = 32, with clearly visible centromeres in all chromosomes, thus discarding potential fragmentation and the presence of B chromosomes (Figure 2). Conventional squash technique was also applied and confirmed this number (Figure 1), although previous reports using this technique were of 2*n* = 30 (Watanabe, 2002; 2004).

According to our results, and based on the extant diversity known for the genus, the presence of 32 chromosomes in *Ch. crassifolius* (the earliest-diverged species) indicates that this number is the likely ancestral state. Altogether, these new results combined with Vitales *et al.* (2014a) phylogenetic hypothesis suggested that derived 2*n* = 30 chromosome numbers could have arisen independently several times during the evolutionary history of the genus, which is consistent with the overall trend found within the Centaureinae. Indeed, since 2*n* = 32 is the highest and hypothesised ancestral chromosome number for the Centaureineae, aside of polyploidy, recurrent episodes of chromosomal losses have been therefore predominant in the subtribe, with the lowest number (2*n* = 14) found in *Centaurea* (Garcia-Jacas *et al.*, 1996). Whether chromosome number reduction in *Cheirolophus* occurred through chromosomal fusions, losses, or both mechanisms is difficult to address given the very small size of the chromosomes and the resulting difficulty to observe the karyotype in details. However, in at least the case of *Ch. webbianus*, CMA and FISH preparations depicted a much longer chromosome pair bearing a rDNA loci close to the centromere (Garnatje *et al.*, 2012), suggesting that the shift to 2*n* = 30 was likely the result of a Robertonian translocation.

Swarm of 35S rDNA loci: before Macaronesian radiation but after the branching of *Ch. crassifolius*

Our results confirmed the exceptional high number of 35S rDNA loci previously evidenced for *Cheirolophus* (Garnatje *et al.*, 2012). This swarm of 35S happened to be shared by both Macaronesian and Mediterranean lineages, which is also confirmed by the present study through an extended sampling. However, while previous findings allowed determining that unusually high number of 35S preceded the radiation to Macaronesia, they were unable to provide further insights concerning where and when in *Cheirolophus* evolution this feature appeared. By complementing available FISH data with that of *Ch. crassifolius*, we revealed that the earliest-diverged species within the genus is bearing just four 35S rDNA loci, a number similar to those found in other basal Centaureinae lineages, such as the *Rhaponticum* group (Hidalgo *et al.*, 2008). This result indicates that the ancestral condition for *Cheirolophus* was probably a moderate number of 35S rDNA loci inherited from its basal Centaureinae-like ancestor, and that the huge increase of 35S loci number happened later in the diversification of the genus.

As mentioned above, the species from both the Macaronesian and Mediterranean lineages presented higher number of 35S rDNA loci, from 8 to 10, which evidenced a punctuated species-specific pattern in contrast with the gradual GS decrease found in the genus. Novel rDNA units can be produced either by locus duplication or by the amplification of orphaned rDNA loci previously generated by transposon-mediated insertions (Matyasek *et al.*, 2012). Our results revealed a constant terminal position of *Cheirolophus* 35S rDNA loci, which suggests that locus duplication could be the more likely mechanism producing such rDNA proliferation within the genus. While this study highlights that bursting of 35S rDNA units is tightly linked with the diversification of the genus, it is still unclear whether it happened once in the common ancestor of the Macaronesian and Mediterannean lineages, or independently in each of the crown clades. Only a qualitative characterisation of the rDNA repeats would provide strong evidence supporting either of the alternatives. Likewise, yet remains to be clarified if the increment of rDNA loci number is just a consequence of a genome reshuffling occurring during radiation or if indeed played an

active role in the radiation process providing increased fitness, which would help explaining this surprising 35S rDNA loci proliferation in an overall context of GS decrease. To date, there are little data available to explain the putative advantages of harbouring numerous rDNA loci, however, the evidence suggests that the rate of genome rDNA homogenisation slows down when increasing the number of rDNA loci, hence indicating a possible a relation between loci number and rDNA sequence diversity (Matyasek *et al.*, 2012).

The analysis of the number of rDNA loci also proved useful in providing systematic insights in some species, such as e.g. *Ch. uliginosus*, a species whose evolutionary position in previous phylogenetic reconstructions (Vitales *et al.*, 2014a) had not been determined with certainty. This taxon had been positioned either at the basis of the genus -together with *Ch. crassifolius*- according to the cpDNA data or in an unresolved polytomy at the split of Macaronesian and Mediterranean clades according to the ITS-ETS tree. FISH analyses revealed that *Ch. uliginosus* presents six (Garnatje *et al.*, 2012) or seven 35S rDNA loci (this study), a higher number than *Ch. crassifolius* but lower than any other species within the Macaronesian or the Mediterranean clades. Similarly, the genome size estimations reported for *Ch. uliginosus* are somewhat intermediate between the values showed by the basal *Ch. crassifolius* and those found in the rest of the species of the genus (see below). These results suggest that the divergence of *Ch. uliginosus* ocurred after the split of *Ch. crassifolius* but at an earlier stage than the separation of the Macaronesian and the Mediterranean lineages. Therefore, *Ch. uliginosus* could have experienced a long *in situ* differentiation throughout its evolutionary history, contributing probably to the diversity in loci number that we found in this study as well as the strong and complex genetic structure reported in recent phylogeographic work of this species (Vitales *et al*., submitted).

A remarkable genome downsizing during the diversification of *Cheirolophus*

A previous study of nuclear DNA content evolution in the genus *Cheirolophus* evidenced the existence of significantly smaller GS in Macaronesian species (Garnatje *et al.*, 2007). The reconstruction of ancestral GS values onto a more comprehensive phylogeny supported the hypothesis of a gradual downsizing (Figure 3, Table 3), which already started early in the evolutionary history of the genus. According to this

inference, the most recent common ancestor of the genus was reconstructed to have a 2C = 1.65-1.59 pg (Figure 3, node 1), and the MRCA of the Macaronesian lineage with just a 2C = 1.41-1.39 (Figure 3, node 3). The C-values estimated in extant representatives established *Ch. crassifolius* (2C = 1.80 pg) as the largest record for the genus, followed by *Ch. uliginosus* (2C = 1.62 pg) and the mean values for Mediterranean (2C = 1.55 pg) and Macaronesian clades (2C = 1.40 pg).

Some of the most frequently invoked phenomena producing small deletions, and hence having a role in genome downsizing, are unequal homologous and illegitimate recombination (see Leitch and Leitch, 2013; for a review in the field), which could in turn affect rDNA loci distribution in the chromosomes. In *Cheirolophus*, however, it is noteworthy that apparently any of the 35S loci position at chromosome terminal region is affected despite the above reported GS decrease. By contrast, a completely different pattern has been evidenced in *Oligochaeta* (another basal Centaureinae), where a huge GS loss was associated with the random physical scattering of 35S loci from terminal to intercalary positions, as the result of deep chromosome restructuring (Hidalgo *et al.*, 2008). In the case of *Cheirolophus*, the consistent position of 35S rDNA loci suggests that the GS decrease most likely happened through mechanisms that would affect the chromosome structure to a lesser extent.

In addition to that, within-clade GS values were found relatively stable (Figure 4), suggesting limited or counterbalancing DNA gains and losses during the radiation process, in contrast to the genome upsizing pattern reported in *Schiedea* (Kapralov *et al.*, 2009). *Cheirolophus* radiation, estimated to have started ca. 1.7 Mya (Vitales *et al.*, 2014a), largely postdates the formation of most Canary Islands, dating from 15 Mya for Gran Canaria to 1.1 Mya for El Hierro (Fernández-Palacios *et al.*, 2011). While it is well established that this radiation was originated by a single colonisation event from the continent, it is unclear whether it occurred quicly after *Cheirolophus* first reached Macaronesia. Alternatively, it is possible that there was a large time lapse between the first colonisation event and the subsequent radiation, during which former island to island colonisations and extinctions could have happened. Such extinction scenario,

supported by molecular data (Vitales *et al.*, 2014a), should be taken into account to explain the remarkable genome downsizing observed in extant Macaronesian species.

Conclusions

The present study has contributed to better understand the evolution of cytogenetic traits in the genus *Cheirolophus* from a phylogenetic perspective. It is now confirmed that this contrasting trend, which consists in an overall GS reduction and unusually high number of 35S rDNA loci is key in the evolution of the genus. Indeed, the Macaronesian radiation has been characterised by an enhancement of these pre-existing traits, a dynamic that seemed 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 and Filatov, 2011), yet remains to be fully demonstrated as to whether rDNA loci enhancement has been relevant for the colonisation and subsequent explosive radiation of *Cheirolophus* in the Canary Islands and Madeira archipelago.

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Catalonia) and the co-fund of Marie Curie Actions (European Union 7th R&D Framework Programme).

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Figures.

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

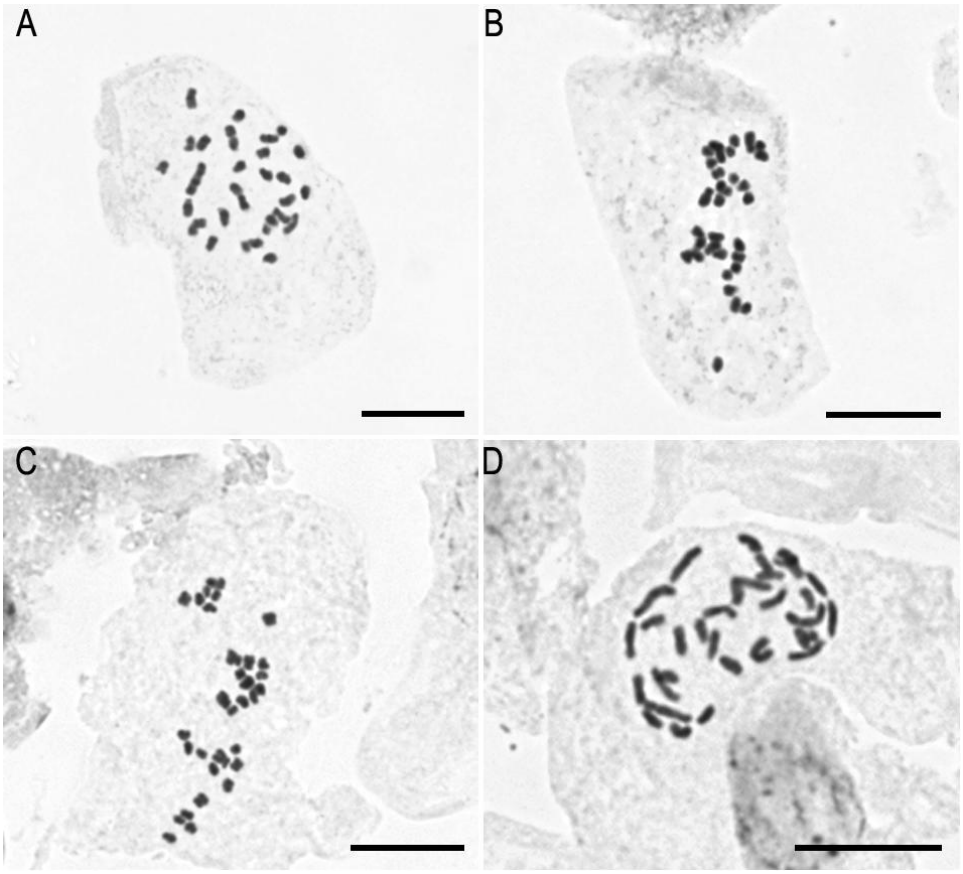

Figure 2. Somatic metaphases of *Cheirolophus* species. A–J: Chromomycin A_3 banding. K–T: Fluorescent in situ hybridisation of 35S rDNA loci (marked in red). (Number of 35S loci). (A,K) *Ch. arbutifolius* (10), (B,L) *Ch. crassifolius* (4), (C,M) *Ch. duranii* (9), (D,N) *Ch. intybaceus* (10), (E,O) *Ch. puntallanensis* (10), (F,P) *Ch. santos-abreui* (9), (G,Q) *Ch. sempervirens* [1] (8), (H,R) *Ch. sempervirens* [2] (8), (I,S) *Ch. uliginosus* (10), (J,T) *Ch.* cf. *webbianus* (7). Scale bar = 10 µm.

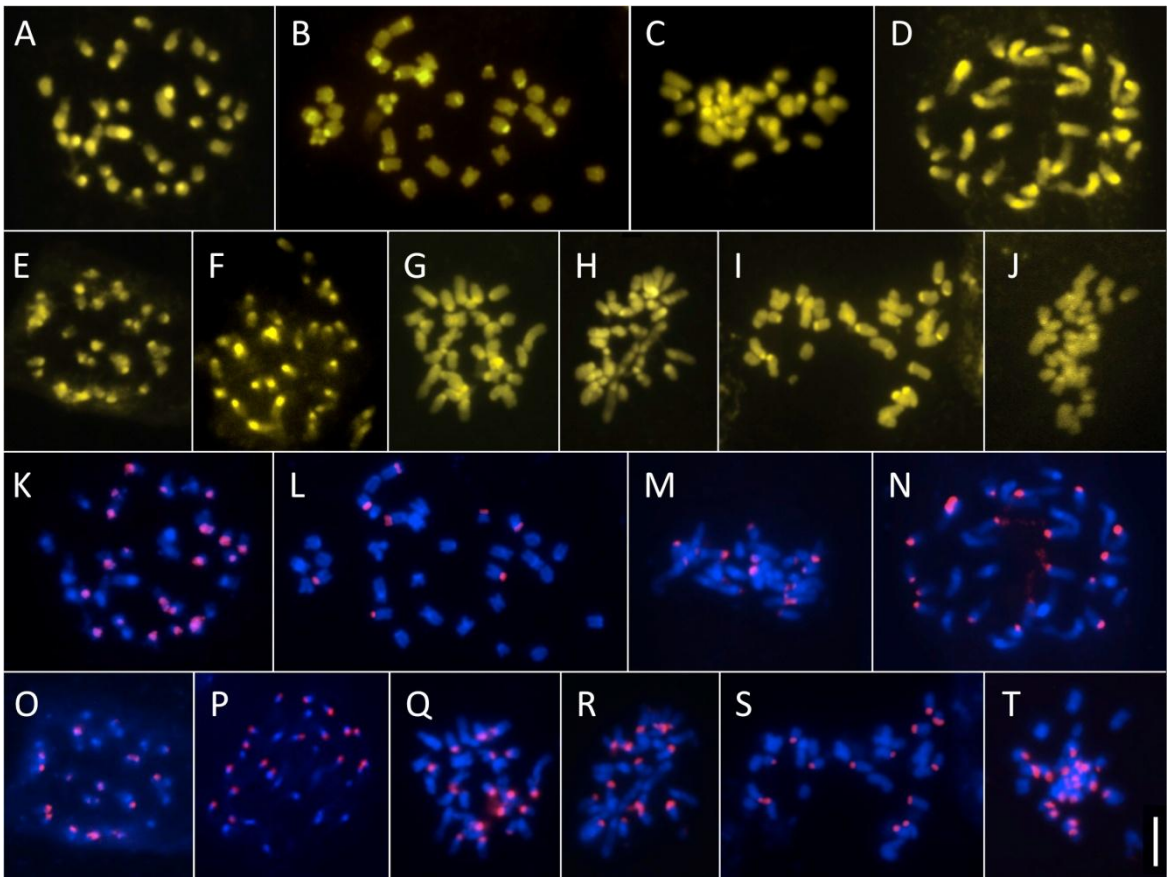

Figure 3. Ancestral genome size (2C) reconstruction in *Cheirolophus*. The most recent common ancestors (MRCAs) of selected nodes are indicated in Table 3. Note that the number of 35S rDNA loci obtained here and GS data have been combined with the results from Garnatje *et al.* (2007, 2012) and are depicted in the tree.

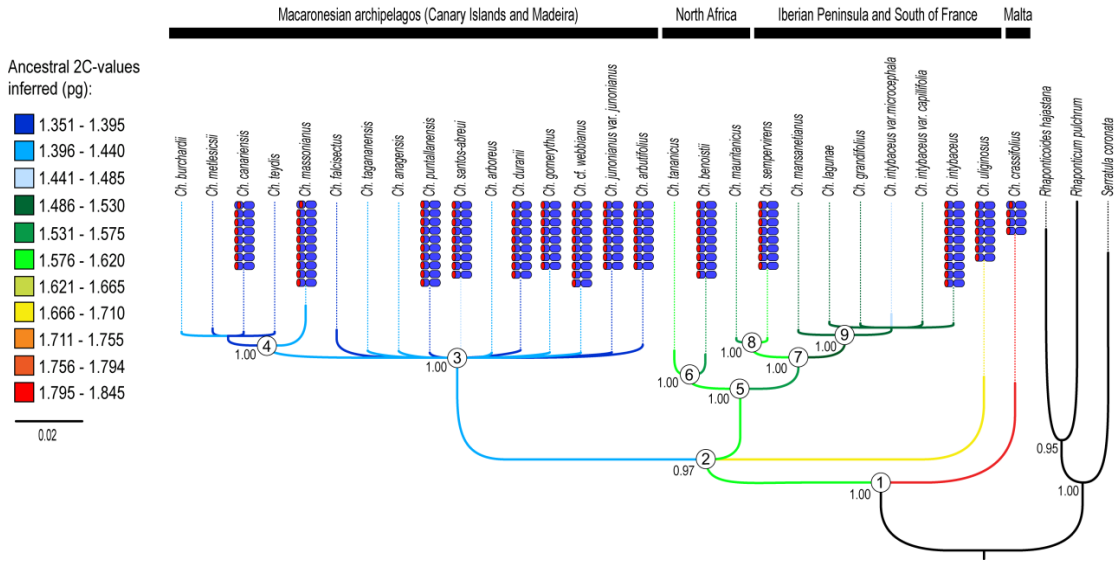

Figure 4. Box-plot of the distribution of genome size values grouped by phylogenetic clades.

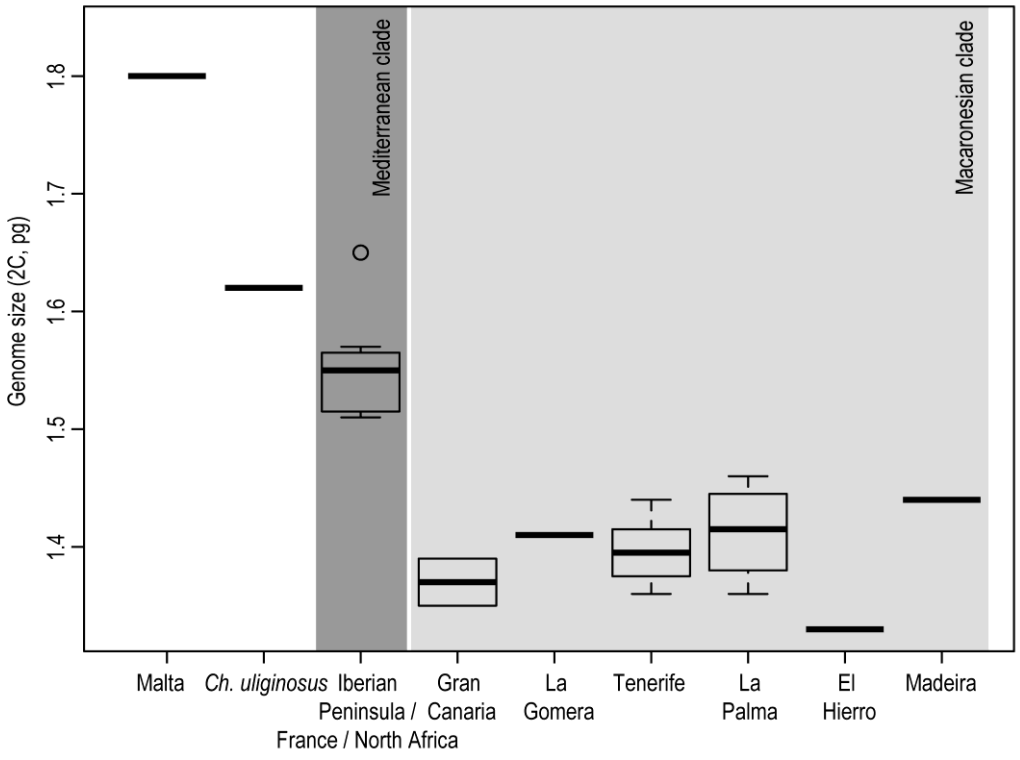

Table 1. Taxa and collection data of the studied *Cheirolopus* populations.

Species	**Locality**
Ch. anagaensis	Spain, Canary Islands: Tenerife, Anaga, Roque de los Pinos, Santos-Guerra 14.V.08 (ORT)
Ch. arbutifolius	Spain, Canary Islands: Gran Canaria, Agaete, Santos-Guerra 17.II.09 (ORT)
Ch. canariensis	Spain, Canary Islands: Tenerife, Los Carrizales, Santos-Guerra 17.VIII.08 (ORT)
Ch. crassifolius	Italy, Sicily: Palermo, Orto Botanico dell'Università di Catania (cultivated from Malta), Vitales (BC)
Ch. duranii	Spain, Canary Islands: El Hierro, Temijiraque, Barranco Balcón, Santos-Guerra 23.VII.09 (ORT)
Ch. intybaceus	Spain: Pedralba, Garnatje & Pellicer 01.XI.2006 (BC)
Ch. junonianus var. *junonianus*	Spain, Canary Islands: La Palma, Fuencaliente, Teneguía, Santos-Guerra 26.VI.09 (ORT)
Ch. puntallanensis	Spain, Canary Islands: La Palma, Puntallana, Barranco Nogales, Santos-Guerra 17.II.08 (ORT)
Ch. santos-abreui	Spain, Canary Islands: La Palma, Barranco Madera, Santos-Guerra 15.II.08 (ORT)
Ch. sempervirens	[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 & Vitales (BC)
Ch. taganananensis	Spain, Canary Islands: Tenerife, Taganana, Roque de las Ánimas, Santos-Guerra 07.IX.09 (ORT)
Ch. uliginosus	Portugal, Beira Litoral: Pateira de Fermentelos, Vitales 13, Pellicer and Garnatje (BC).
Ch. webbianus	Spain: Tenerife, Anaga, Chinamada, Santos-Guerra 14.V.08 (ORT)
Ch. cf. *webbianus*	Spain: Canary Islands, Tenerife: Taganana, at the base of Roque de las Ánimas, Garnatje 3 and Luque (BC)

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

Species	**Distribution**	**2C (SD) [pg]**	**1C*x*[1] [pg]**	**2C [Mbp][2]**
Ch. anagaensis	Canary Islands	1.42 (0.03)	0.71	1389
Ch. canariensis	Canary Islands	1.36 (0.01)	0.68	1330
Ch. crassifolius	Maltese Islands	1.80 (0.04)	0.90	1760
Ch. duranii	Canary Islands	1.33 (0.05)	0.67	1301
Ch. junonianus var. *junonianus*	Canary Islands	1.48 (0.05)	0.74	1447
Ch. puntallanensis	Canary Islands	1.36 (0.02)	0.68	1330
Ch. santos-abreui	Canary Islands	1.46 (0.05)	0.73	1428
Ch. sempervirens [2]	Iberian Penisula	1.53 (0.04)	0.77	1496
Ch. taganananensis	Canary Islands	1.41 (0.01)	0.71	1379
Ch. uliginosus	Iberian Penisula	1.55 (0.04)	0.78	1516
Ch. webbianus	Canary Islands	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 *et al.,* 2003).

Table 3. Ancestral genome size (2C, in pg) inferences for the MRCAs of selected nodes inferred under parsimony and Bayesian (MCMC) approaches (node numbers are depicted in Figure 3).

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)

Supplementary information.

Appendix 1. List of *Cheirolophus* species, with indication of their distribution, and when available their chromosome number, genome size and number of AT-rich heterochromatin band and rDNA sites. Distribution code: 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 subspecies of *Ch. junonianus* are likely to be considered as constituting two different species (Vitales *et al.*, 2014a, b). [2]This population has been first attributed to *Ch. tagananensis* (Susanna *et al.*, 1999; Garnatje *et al.*, 2007), but morphological and genetic divergence indicate that it may constitute a separate taxon (D. Vitales, unpublished data). Grey-highlighted chromosome numbers are thoses new or reassessed in this study. In addition to the data provided by the present study we gathered chromsome count and genome size data from the Index to Chromosome numbers in Asteraceae (Watanabe, 2002, 2004) and GSAD (Garcia *et al.*, 2014) databases, respectively.

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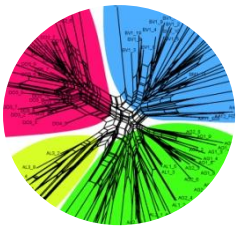
SÍNTESI I DISCUSSIÓ DELS RESULTATS



SÍNTESI I DISCUSSIÓ DELS RESULTATS OBTINGUTS

En aquest apartat es presenta un resum de la discussió dels resultats més rellevants obtinguts en el marc d'aquesta tesi, tots ells recollits en els articles disponibles en l'apartat del compendi de publicacions.

1. Filogènia molecular i reconstrucció filogeogràfica del gènere *Cheirolophus*

La reconstrucció filogenètica del gènere *Cheirolophus* obtinguda durant aquesta tesi doctoral ha aportat novetats rellevants respecte als anteriors treballs filogenètics existents (Susanna et al., 1999; Garnatje et al., 2007). Les noves anàlisis es basen en la seqüenciació de dues regions del DNA nuclear (ITS i ETS) i quatre del DNA cloroplàstic (*rpl32-trnL, rpoB-trnD, rps16-trnK i trnS-trnC*) seleccionades després d'un cribratge de 14 regions, que han servit per a analitzar 32 tàxons (és a dir, tota la diversitat específica i pràcticament tota la infraespecífica del gènere). Els resultats han mostrat una incongruència significativa entre els dos conjunts de marcadors (nuclears i cloroplàstics), tot i que no dins de cadascun d'ells. Aquest tipus de senyal incongruent pot ser degut a diversos fenòmens, entre ells l'ILS (*incomplete lineage sorting*), la duplicació o la pèrdua de gens, la captura cloroplàstica, la poliploïdia i la hibridació (Wendel & Doyle, 1998). En el present estudi, sembla que diversos d'aquests processos podrien explicar els diferents punts d'incongruència detectats.

Dintre del clade mediterrani s'observen diferents posicions incongruents entre la reconstrucció basada en marcadors nuclears -que és coherent amb la delimitació taxonòmica de les espècies- i la filogènia basada en marcadors cloroplàstics -que presenta algunes poblacions de la mateixa espècie segregades en branques diferents. Aquests casos es deurien més aviat al fet que les regions del DNA cloroplàstic presenten molta menys variabilitat que les nuclears, i per tant, aquests marcadors d'orgànuls no haurien pogut rastrejar prou acuradament els esdeveniments d'especiació. Això podria constituir una explicació plausible tant de l'artefacte de les posicions d'algunes espècies del clade mediterrani, com de la manca de resolució en

els clades basals de la filogènia basada en les regions cloroplàstiques. Un cas diferent és el de *Ch. massonianus* de Madeira, que presenta una posició filogenètica absolutament discordant entre la reconstrucció basada en DNA nuclear -dintre del clade macaronèsic- i la inferida a partir del DNA cloroplàstic -a la base de la filogènia, juntament amb *Ch. crassifolius* i *Ch. uliginosus*. Aquí, la hibridació, que implica una captura cloroplàstica és la hipòtesi més plausible, suportada per la posició allunyada d'aquest tàxon a les dues anàlisis independents i per l'increment del suport en els clades que contenen els possibles progenitors quan s'elimina l'espècie que considerem híbrida en l'anàlisi de coalescència amb els dos tipus de marcadors combinats. La quantitat de DNA de *Ch. massonianus*, a mig camí entre els valors 2C de les espècies continentals i la major part de les insulars (Garnatje et al., 2007), constituiria una altra prova d'aquesta possible hibridació. Finalment, com es veurà més endavant a l'apartat de radiació insular, episodis recurrents d'hibridació també podrien explicar la polifília d'algunes espècies canàries en l'arbre obtingut a partir de l'anàlisi del DNA cloroplàstic.

Malgrat aquestes incongruències, ambdós tipus de marcadors s'han revelat d'utilitat en la reconstrucció filogenètica del gènere quan s'han analitzat de manera independent (vegeu el treball 3 del compendi de publicacions). Així doncs, com ja s'havia proposat en altres estudis (p. e. Comes & Abbott, 2001), mentre el DNA nuclear ha aportat resolució a la vertebració dels grans clades i ha introduït una dimensió temporal a la història evolutiva del gènere, el cloroplàstic ha proporcionat més informació sobre les relacions filogeogràfiques en grups d'espècies estretament relacionades. Tots dos conjunts de dades, nuclear i cloroplàstic, donen suport a la monofília del gènere i també a l'existència d'un clade insular ben diferenciat de la resta, que inclou l'espècie endèmica de Madeira, *Ch. massonianus*, però només quan es tracta de DNA nuclear. Aquesta mateixa reconstrucció nuclear mostra un clade mediterrani ben definit i que reflecteix afinitats morfològiques i geogràfiques entre les espècies, fet que no observem en la reconstrucció cloroplàstica. *Cheirolophus crassifolius*, l'espècie endèmica de les illes de Malta i Gozo, apareix en les dues anàlisis esmentades com un llinatge que va divergir de manera primerenca, en posició germana a la resta dels representants del gènere. Pel que fa a *Ch. uliginosus*, l'únic hemicriptòfit del gènere, també es situa en posicions força basals -tot i que no del tot

resoltes- a les dues reconstruccions filogenètiques basades en DNA nuclear i en DNA cloroplàstic.

Com ja s'ha comentat anteriorment, l'anàlisi de les regions nuclears ha resultat particularment útil en l'estudi de les primeres etapes de la història evolutiva de *Cheirolophus*, així com per a establir un marc temporal per a la seva filogènia. L'origen de la diversificació del gènere s'ha datat a mitjan miocè, quan la regió mediterrània encara gaudia d'un clima de característiques tropicals, molt més humit que l'actual (Thompson, 2005). Segons la reconstrucció de la història biogeogràfica de la tribu *Cardueae* de Barres et al. (2013), l'origen de la subtribu *Centaureinae* es troba a l'oest d'Àsia, des d'on els diferents llinatges basals -com ara *Cheirolophus*- s'haurien dispersat durant el miocè cap a la regió mediterrània. Aquesta història concorda amb els nostres resultats, que mostren l'espècie més oriental del gènere com a germana de tota la resta de tàxons del grup, la qual cosa suggereix una colonització de la regió en direcció est-oest. La progressiva aridificació que va començar afectant la zona oriental de la conca mediterrània durant aquella època (Fortelius et al., 2006) podria haver empès *Cheirolophus* cap a l'oest de la regió, on actualment trobem la major part de les espècies del gènere. L'aparició de nous llinatges dintre de *Cheirolophus* hauria continuat fins a finals del miocè, quan les nostres dades indiquen una aturada en sec de la taxa d'especiació que es perllonga durant aproximadament 5 milions d'anys (Ma). El procés de diversificació no s'hauria recuperat fins fa uns 3 Ma, coincidint amb l'establiment del clima mediterrani a la regió. Aquest patró de cladogènesi no hauria succeït únicament a *Cheirolophus*, sinó que s'ha observat darrerament en molts altres grups de plantes de distribució mediterrània [vegeu-ne una revisió a Fiz-Palacios & Valcárcel (2013)]. Segons els mateixos autors, els efectes climàtics de la crisi salina del messinià haurien estat responsables de l'extinció de molts llinatges de plantes mediterrànies, deixant una empremta característica -com la que presenta *Cheirolophus*- a la filogènia dels grups que van aconseguir sobreviure.

Pel que fa la radiació del gènere a la Macaronèsia, la nostra filogènia calibrada temporalment indica que la diversificació en aquests arxipèlags oceànics hauria començat fa aproximadament 1,7 Ma. Això suposa que les aproximadament 20 espècies de *Cheirolophus* reconegudes actualment haurien aparegut a un ritme

trepidant, al voltant de 0,34-2,84 espècies per Ma, sols comparable a nivell insular amb altres grups com *Bidens* o *Echium*, els processos de radiació dels quals han estat considerats fins al moment present com els més ràpids coneguts en plantes en illes (Knope et al., 2012). En aquest sentit, les regions cloroplàstiques s'han mostrat particularment resolutives a l'hora d'estudiar el procés explosiu de diversificació de *Cheirolophus* a la Macaronèsia. Les nostres anàlisis filogeogràfiques indiquen que l'illa de Tenerife sembla haver estat el lloc més probable d'origen de la diversificació, amb un rol pivotant en les successives colonitzacions cap a l'est (des de Tenerife cap a Gran Canària) i cap a l'oest (des de Tenerife cap La Gomera i la Palma, per un costat i cap a La Gomera i El Hierro, per l'altre). Durant aquests esdeveniments, les anàlisis suggereixen que La Gomera podria haver estat colonitzada dues vegades. Aquests resultats concorden amb altres estudis filogeogràfics a les illes Canàries (p. e. Allan et al., 2004; Goodson et al., 2006; Guzmán & Vargas, 2010), que proposen que aquesta illa central podria servir com a node per a la colonització de tot l'arxipèlag. De fet, Tenerife conté la major part de la diversitat genètica del gènere *Cheirolophus* a les illes Canàries, un patró que també s'ha trobat a altres gèneres com *Bystropogon* (Trusty et al., 2005), *Sideritis* (Barber et al., 2007) o *Aeonium* (Jorgensen & Olesen, 2001). Aquesta major diversitat genètica i taxonòmica de Tenerife s'ha explicat per la particular i complexa història geològica d'aquesta illa (Carracedo et al., 2007), que hauria contribuït a processos de fragmentació successius i seguits de reconnexió entre els hàbitats, i hauria propiciat la diferenciació al·lopàtrica de les poblacions.

Subseqüentment, a les illes més grans -especialment a Tenerife i a La Palma- haurien tingut lloc diversos processos de diversificació intrainsular, probablement impulsats per fenòmens ja comentats, com l'aïllament genètic, però també potser deguts a processos com l'adaptació ecològica incipient i la hibridació. Aquest és un model de radiació molt similar al trobat en altres grups de plantes i animals que s'han diversificat -en major o menor grau- a les illes Canàries [vegeu Sanmartín et al. (2008) per a una revisió]. Per tant, els nostres resultats semblen indicar que el patró de colonització, dispersió i diferenciació de *Cheirolophus* no explica per si sol la rapidíssima radiació del grup a la Macaronèsia; altres factors intrínsecs i extrínsecs deuen haver contribuït en aquest espectacular procés.

2. Implicacions evolutives de la grandària del genoma i del DNA ribosòmic en la diversificació del gènere *Cheirolophus*

La història evolutiva de *Cheirolophus* també ha estat estudiada en aquesta tesi des dels punts de vista cariològic i citogenètic. L'anàlisi de la grandària del genoma i de l'organització del DNA ribosòmic (DNAr) ha demostrat la seva utilitat filogenètica en nombroses ocasions (vegeu p. e. Stace, 2000; Leitch et al., 2005; Stuessy, 2009; Garcia et al., 2010 i referències contingudes en aquests treballs), i com veurem més endavant, també ens ha ajudat a inferir la posició filogenètica d'algunes espècies del nostre grup d'estudi. Tanmateix, l'objectiu principal dels experiments de cariologia i citogenètica portats a terme en aquest gènere ha estat investigar si existeixen factors genòmics intrínsecs que podrien haver fet alguns tàxons més susceptibles que d'altres a iniciar un procés de radiació.

Cheirolophus és l'únic gènere de les *Centaureinae* que ha radiat a les illes Canàries i, com la majoria de gèneres filogenèticament propers (*Callicephalus*, *Myopordon*, *Oligochaeta*, *Rhaponticum* i *Centaurea*, vegeu Hidalgo et al., 2006, 2008; Dydak et al., 2009 per a més detalls), presenta una organització del DNAr 35S i 5S en *loci* separats físicament. En canvi, així com la majoria de gèneres relacionats presenta un nombre relativament baix de *loci* de DNAr 35S (Hidalgo et al., 2008), els resultats de la hibridació *in situ* fluorescent (FISH) en *Cheirolophus* han revelat que tant els representants insulars com els continentals presenten un nombre sorprenentment alt de bandes de cromomicina (CMA+) coincidents amb *loci* 35S majoritàriament en posició terminal. En aquest sentit, s'observa una certa tendència a l'increment del nombre de *loci* 35S en les espècies macaronèsiques i, per tant, aquests resultats ens podrien dur a pensar que aquest nombre inusual de marcatges podria haver tingut lloc durant el procés de radiació. No obstant, l'elevat nombre de marcatges comparables també observats en espècies continentals com *Ch. benoistii* o *Ch. intybaceus* ens han fet descartar aquesta hipòtesi, de manera que si existeix alguna relació entre el nombre de marcatges 35S i la radiació, només pot ser que es tracti d'una acció facilitadora del DNAr, és a dir, que hi pugui haver un elevat nombre de còpies diferents

per *locus* i que aquesta diversitat en el DNAr ajudi a promoure la radiació insular (vegeu el treball 1 del compendi de publicacions).

Posteriorment, gràcies a un altre treball que ha incorporat més dades d'hibridació *in situ* fluorescent, s'ha pogut demostrar que aquest augment espectacular del nombre de *loci* 35S hauria tingut lloc abans de la radiació macaronèsica, començant just després de la divergència de *Ch. crassifolius* (vegeu el treball 7 del compendi de publicacions). En aquest estudi s'ha pogut determinar, respecte a l'altre treball previ, que *Ch. crassifolius* sols presenta quatre *loci* 35S, un nombre similar a l'observat en altres llinatges de *Centaureinae* basals, com en el cas del gènere *Rhaponticum* (Hidalgo et al., 2008; Hidalgo *et al.,* en preparació). Aquest resultat indicaria, per una banda, que el caràcter ancestral de *Cheirolphus* era un nombre moderat de *loci* 35S de DNAr, heretat probablement dels ancestres de les *Centaureinae* basals i, per altra, que l'increment en el nombre de 35S va tenir lloc durant la diversificació del gènere a la Mediterrània occidental. Les dades citogenètiques que hem generat també han servit per a aclarir la posició de *Ch. uliginosus* dins la filogènia del gènere. El nombre de marcatges 35S en aquest hemicriptòfit és més gran que a *Ch. crassifolius* però clarament inferior al que presenten els altres membres dels clades mediterrani i macaronèsic. Igualment, la mida del genoma a *Ch. uliginosus* presenta valors intermedis entre els de l'espècie basal *Ch. crassifolius* i els de la resta de tàxons del gènere. Aquest patró sembla indicar que la divergència d'aquesta espècie hauria tingut lloc després de la separació de *Ch. crassifolius*, però abans de la diferenciació dels llinatges mediterranis i macaronèsics. Així, la variabilitat en el nombre de marcatges 35S que s'ha trobat analitzant individus de diferents poblacions de *Ch. uliginosus* podria explicar-se pel seu caràcter relicte i per una possiblement llarga història evolutiva *in situ* d'aquesta espècie.

Tot i que el nombre de cromosomes roman estable dintre del gènere, les espècies macaronèsiques i la resta -mediterrànies- presenten entre vuit i 10 *loci* de DNAr 35S (en alguns casos més del doble que en *Ch. crassifolius*); aquest patró contrasta amb la disminució de la quantitat de DNA nuclear observada durant l'evolució del gènere. Es poden generar nous *loci* de DNAr per duplicació o per l'amplificació de *loci* existents mitjançant la inserció de transposons (Matyášek et al., 2012), però el fet que la majoria

de marcatges es trobin en una posició terminal suggereix que la duplicació podria ser el mecanisme més freqüent responsable de la proliferació de DNAr dins del gènere. En qualsevol cas, caldrà dur a terme més estudis per tal d'aclarir si l'increment del nombre de *loci* de DNAr és només conseqüència d'una reorganització del genoma ocorreguda durant la diversificació de *Cheirolophus* o si va jugar un paper actiu en el procés de radiació, la qual cosa ajudaria a explicar aquesta sorprenent proliferació de *loci* 35S en un context general de disminució de la quantitat de DNA. Fins ara, hi ha poques dades disponibles per a explicar els avantatges de presentar nombrosos *loci* de DNAr, però l'evidència suggereix una relació entre el nombre de *loci* i la diversitat de còpies de DNAr (Matyášek et al., 2012), reduint per tant la taxa d'homogeneïtzació del DNAr en el genoma.

Com ja s'ha comentat en paràgrafs anteriors, d'acord amb els resultats obtinguts per altres autors (Mandáková et al., 2010), la radiació de *Cheirolophus* no tingué lloc associada a canvis en el nombre cromosòmic ni en el nivell de ploïdia. Tot i així, els models de distribució del DNAr i el decrement de la quantitat de DNA nuclear si que han posat en evidència un cert grau de dinamisme genòmic en les espècies macaronèsiques del grup. En tot cas, a nivell genèric, i en tota l'àrea de distribució, el fet que tant 2*n* = 30 com 2*n* = 32 hagin estat reportats en diverses espècies, ha produït certa discrepància a l'hora de determinar quin és el nombre de cromosomes putatiu en *Cheirolophus.* Els nostres resultats indiquen que el nombre cromosòmic 2*n* = 32 és més freqüent del que s'havia proposat en reports anteriors, que donaven 2*n* = 30 com a nombre majoritari (Watanabe, 2002, 2004), i de fet, podria ser el nombre cromosòmic ancestral del gènere. Malgrat que el nombre 2*n* = 32 de vegades ha estat atribuït a problemes tècnics -de preparació i/o de resolució- atesa la mida petita (de 2 a 4 µm) dels cromosomes i la seva fragilitat centromèrica, també observada en *Oligochaeta* i *Rhaponticum* (Hidalgo et al., 2007), la morfologia cromosòmica és perfectament observable en les preparacions de CMA i FISH, que demostren l'existència de 2*n* = 32 a *Ch. crassifolius* (l'espècie que va divergir primer), malgrat que 2*n* = 30 havia estat prèviament reportat per a aquest tàxon (Watanabe, 2002, 2004). Aquesta troballa confirma que 2*n* = 32 constitueix el nombre cromosòmic ancestral i que el nombre 2*n*

= 30 hauria aparegut diverses vegades de manera independent durant la història evolutiva del gènere.

Un estudi anterior sobre la grandària del genoma dins del gènere *Cheirolophus* va evidenciar l'existència de quantitats de DNA nuclear significativament menors en les espècies de la Macaronèsia (Garnatje et al., 2007) respecte de les continentals. La reconstrucció dels valors C ancestrals duta a terme mitjançant diferents mètodes estadístics i usant una filogènia representativa, donen suport a la hipòtesi d'una reducció gradual que ja va començar a principis de la història evolutiva del gènere. Alguns dels mecanismes evolutius coneguts que més freqüentment s'han determinat com a inductors de petites delecions, i per tant tenen un paper en la reducció de la mida del genoma, són la recombinació homòloga desigual i la recombinació il·legítima (vegeu Leitch & Leitch, 2013, per a una revisió), que al seu torn podrien afectar també la distribució dels *loci* de DNAr als cromosomes. A *Oligochaeta divaricata* K.Koch (un altra espècie inclosa en les *Centaureinae* basals i pròxima al gènere *Cheirolophus*) una gran pèrdua de quantitat de DNA es va associar amb la dispersió aleatòria de *loci* 35S de posicions terminals a posicions intercalars, com a resultat d'un procés de reestructuració cromosòmica profunda (Hidalgo et al., 2008). A *Cheirolophus*, però, cal destacar que les posicions terminals del 35S no semblen veure's afectades per la reducció de la mida del genoma.

3. Genètica de poblacions en els *Cheirolophus* endèmics dels arxipèlags macaronèsics

El clade macaronèsic de *Cheirolophus* ha merescut un estudi especial amb l'objectiu d'investigar la radiació d'aquest grup que, amb unes 20 espècies aparegudes en menys de dos milions d'anys, constitueix un dels casos més espectaculars de diversificació en illes oceàniques. Com ja s'ha comentat al primer apartat de la síntesi i discussió dels resultats, la resolució obtinguda a la filogènia del gènere va ser força limitada en aquest complex insular, precisament per la rapidesa del procés radiatiu i la poca variabilitat dels marcadors moleculars utilitzats. Per aquest motiu es va decidir

dur a terme un treball de genètica poblacional amb marcadors hipervariables (AFLPs, vegeu el treball 4 del compendi de publicacions), que han demostrat àmpliament la seva utilitat filogenètica en grups de diversificació recent (p. e. Richardson et al., 2003; Meudt & Clarke, 2007; Joyce et al., 2011) i quan altres tècniques moleculars han fracassat (p. e. Després et al., 2003; Guo et al., 2005).

En primer lloc, aquesta aproximació poblacional va servir per a estudiar la delimitació taxonòmica de les espècies macaronèsiques de *Cheirolophus*, una qüestió que ja havia estat objecte de debat en els primers treballs del gènere (Garnatje, 1995). Les anàlisis filogenètiques de les dades d'AFLPs donen suport a l'actual circumscripció taxonòmica de les seves espècies, basada fonamentalment en caràcters morfològics. Per tant, els nostres resultats corroboren la diferenciació d'aquestes espècies d'aparició recent i confirmen la utilitat dels caràcters diagnòstics clàssics emprats per a la delimitació taxonòmica dels *Cheirolophus* macaronèsics.

Tot i que no es van poder reconstruir en detall les relacions filogenètiques entre totes les espècies macaronèsiques, les anàlisis dutes a terme amb aquests marcadors ajuden a entendre de manera més consolidada la història evolutiva dels diferents llinatges insulars. L'estructura genètica de les poblacions mostra l'existència d'una segregació geogràfica entre les illes de l'oest (La Palma i El Hierro) i les del centre-est (La Gomera, Tenerife i Gran Canària), que s'agrupen amb Madeira. Les anàlisis que tenen en compte més *clusters*, posen de manifest la importància de l'especiació al·lopàtrica en els *Cheirolophus* macaronèsics: la major part de les poblacions i les espècies s'agrupen geogràficament, ja sigui entre illes com també dintre de les illes. És interessant tornar a remarcar l'estructura de grups que apareix a l'illa de Tenerife, que ja havia estat reportada en altres grups de plantes anteriorment (Trusty et al., 2005), que s'explicaria per la disjunció dels llinatges corresponents a cadascuna de les dues paleoilles que actualment representen els massissos d'Anaga (complex de *Ch. webbianus*) i de Teno (*Ch. burchardii* i *Ch. canariensis*), situats en el NE i NO de l'illa, respectivament (Ancochea et al., 1999).

Tot i el limitat mostreig a nivell poblacional, aquest estudi també ha posat de manifest el pobre flux gènic existent entre les poblacions macaronèsiques del gènere *Cheirolophus*. El fort senyal d'aïllament per distància, la baixa heterozigosi de les

poblacions i els valors alts en els índexs de fragmentació gènica indiquen un intercanvi genètic limitat entre les poblacions. Aquests resultats concorden amb la baixa capacitat de dispersió de les llavors, l'aïllament geogràfic de les poblacions i la seva mida reduïda, contribuint possiblement a un procés progressiu de diferenciació per deriva genètica. Tanmateix, el patró filogeogràfic observat en aquest grup -que inclou nombrosos esdeveniments de colonització i recolonització interinsulars i intrainsulars- suggereix que els *Cheirolophus* macaronèsics presentarien una bona capacitat per a la dispersió a llarga distància de forma esporàdica (vegeu Crawford et al., 2009 per a algunes hipòtesis al respecte). En aquest sentit, autors com Ellis et al. (2006) o Knope et al. (2012) han proposat que aquesta combinació de baixa capacitat de flux gènic amb una certa predisposició per a la dispersió ocasional a llarga distància podria jugar un paper clau en els processos de radiació de certs grups de plantes.

Com ja s'ha comentat a la introducció, els *Cheirolophus* macaronèsics han estat proposats com a exemple de radiació no adaptativa en illes (Whittaker & Fernández-Palacios, 2007). Respecte a això, les nostres anàlisis de correlació entre la similitud genètica i la morfològica indiquen que no hi ha una associació clara entre els llinatges genètics i les característiques ecomorfològiques considerades. Òbviament, les dades i les anàlisis utilitzades en aquest estudi són preliminars i insuficients per a descartar un rol essencial de la selecció adaptativa en la radiació d'aquest grup. Tot i així, aquests resultats semblen indicar que l'adaptació ecològica no hauria dirigit els estadis inicials de la diversificació dels *Cheirolophus* macaronèsics. Els exemples d'adaptacions a condicions ecològiques concretes que presenten algunes espècies d'aquest grup (p. e. *Ch. junonianus* del sud de l'illa de La Palma o *Ch. teydis* de l'estatge subalpí de Tenerife i La Palma) correspondrien més aviat a processos relativament recents i independents de diferenciació ecomorfològica.

Un altre dels mecanismes que pot haver jugat un paper clau en la història evolutiva dels *Cheirolophus* macaronèsics és la introgressió (Vitales et al., 2014). A banda de *Ch. massonianus* de Madeira, que, com ja s'ha comentat, podria haver experimentat un procés de captura cloroplàstica amb algun tàxon continental, les nostres anàlisis també suggereixen altres esdeveniments d'introgressió gènica a les espècies de les illes Canàries. Concretament, s'han trobat evidències de barreja

genètica a *Ch. teydis* i *Ch. arboreus* de La Palma, o a *Ch. duranii* d'El Hierro. En alguns casos, aquestes empremtes genètiques semblen suportades per dades morfològiques i posicions heteromòrfiques trobades al DNAr nuclear d'aquestes espècies. No obstant, alguns d'aquests senyals també es podrien explicar per la retenció de polimorfismes ancestrals, fenòmens de separació incompleta de llinatges (*incomplete lineage sorting*, ILS), especialment si tenim en compte la rapidesa amb la qual ha tingut lloc el procés de radiació. Tot plegat ens obliga a ser cauts a l'hora de determinar la importància de la introgressió gènica en la radiació de *Cheirolophus* a la Macaronèsia.

4. Patrons filogeogràfics en tàxons de la península Ibèrica: *Ch. sempervirens* i *Ch. uliginosus*

Cheirolophus sempervirens és una espècie ideal per a comprovar certes hipòtesis filogeogràfiques relacionades amb la resposta que la flora termòfila de les penínsules del sud d'Europa va experimentar durant les glaciacions del plistocè. La seqüenciació d'algunes regions del DNA cloroplàstic per a diversos individus de diferents poblacions d'aquesta espècie no ha mostrat gens de variabilitat genètica. En canvi, les anàlisis basades en AFLPs han mostrat alts nivells de diferenciació genètica entre poblacions i baixos dins de les poblacions, la qual cosa indica una forta estructura genètica a *Ch. sempervirens*, que sembla ben correlacionada amb la distància geogràfica segons el test de Mantel (vegeu el treball 6 del compendi de publicacions per a més detalls). S'ha trobat una forta correlació negativa entre la latitud de les poblacions estudiades i el seu nivell de raresa genètica, un índex que s'associa amb l'antiguitat de les poblacions (Schönswetter & Tribsch, 2005; Ehrich et al., 2008). Aquests resultats suggereixen una llarga persistència de l'espècie en els refugis glacials del sud de la península Ibèrica i el posterior efecte fundador de les colonitzacions cap al nord. Aquest patró filogeogràfic correspon al model anomenat de riquesa al sud i puresa al nord (*southern richness - northern purity*, SR-NP), que ha estat proposat per tal d'explicar la història biogeogràfica d'altres plantes de terra baixa refugiades durant les glaciacions del plistocè al sud d'Europa (Hewitt, 2001). De fet, un patró similar ja s'havia trobat al complex d'espècies de *Ch. intybaceus* de l'est de la península Ibèrica (Garnatje et al.,

2013), i això suggeria que aquest podria ser un patró filogeogràfic habitual tant dels *Cheirolophus* ibèrics com d'altres plantes termòfiles de la península Ibèrica. Les nostres anàlisis també apunten que la meitat meridional de l'àrea de distribució de *Ch. sempervirens* acumularia múltiples llinatges genètics, mentre que només un agruparia totes les poblacions del nord. Igualment, els resultats obtinguts indiquen que aquesta espècie podria presentar diverses zones de refugi a la part sud de la seva àrea de distribució, suggerint un patró designat com refugis dins de refugis (Gómez & Lunt, 2007) per al sud-oest de la península Ibèrica.

Cheirolophus uliginosus és una altra de les espècies continentals del gènere, que presenta una àrea de distribució restringida al sud-oest de la península Ibèrica, força coincident amb la de *Ch. sempervirens.* Com també s'ha trobat en aquesta darrera espècie, la diversitat genètica intrapoblacional obtinguda per a *Ch. uliginosus* mitjançant els estudis d'AFLPs és molt baixa, mentre que la variabilitat genètica es troba majoritàriament distribuïda entre les poblacions (vegeu treball 5 del compendi de publicacions per a més detalls). Aquests resultats concorden amb els 10 haplotips obtinguts de forma paral·lela en l'anàlisi de dues regions cloroplàstiques*, rpl32-trnL i trnS-trnC,* que han mostrat una important diversitat genètica a nivell específic, però una heterozigosi baixíssima dintre de les poblacions. També de manera similar al que es va trobar a *Ch. sempervirens*, l'estructura genètica de *Ch. uliginosus* basada en les dades d'AFLPs suggereix que la meitat sud de l'àrea de distribució d'aquesta espècie presenta múltiples llinatges genètics, mentre que només un agrupa totes les poblacions del nord. Tot i així, l'estructura filogeogràfica a *Ch. uliginosus* no és tan clara com la que es va inferir per a *Ch. sempervirens*. Per exemple, els índexs de diversitat i raresa gèniques de les poblacions d'aquest hemicriptòfit no presenten un patró nord-sud significatiu. En el mateix sentit, l'estructura genètica dels haplotips cloroplàstics també és força complexa a *Ch. uliginosus,* on són repartits entre les diferents poblacions sense una clara pauta geogràfica. Les dades filogenètiques i citogenètiques indiquen que aquesta és una espècie relicta dintre del gènere i que podria presentar una història evolutiva llarga i complexa a la seva area de distribució. A més, com es discuteix a l'apartat següent, la diversitat genètica d'algunes poblacions de *Ch. uliginosus* es podria haver vist afectada per esdeveniments recents de fragmentació i

pèrdua d'individus, relacionats amb l'estat de conservació d'aquesta espècie. Tots aquests factors -entre d'altres- podrien contribuir al patró filogeogràfic relativament complicat que presenta aquest tàxon en comparació amb el que s'ha trobat a *Ch. sempervirens*.

5. Biologia de la conservació de les espècies amenaçades de *Cheirolophus*

La desaparició d'alguns hàbitats, especialment de zones humides, durant les últimes dècades ha captat la nostra atenció pel que fa a les possibles conseqüències sobre la supervivència de *Ch. uliginosus*. Com ja hem vist a l'apartat anterior, aquesta és una espècie endèmica del sud-oest de la península Ibèrica, que creix a bruguerars humits, un hàbitat prioritari pel que fa a la conservació per part de la Unió Europea. Concretament, la desaparició de poblacions i el descens del nombre d'individus per població que s'ha reportat en els darrers temps (Susanna, 1993; Bañares et al., 2010) han fet que ens interessem per la conservació d'aquesta espècie des del punt de vista de la genètica de poblacions.

Els resultats de les nostres anàlisis basades en AFLPs indiquen que les poblacions més petites de *Ch. uliginosus* presenten valors significativament més baixos de diversitat genètica que les poblacions amb un nombre més gran d'individus (vegeu treball 5 del compendi de publicacions per a més detalls). En el cas dels marcadors cloroplàstics analitzats, la diversitat intrapoblacional detectada és massa petita per a poder inferir conclusions sòlides en aquest nivell, però les dades obtingudes mostren igualment certa associació entre la variabilitat haplotípica i la mida de les poblacions. Aquesta relació també ha estat trobada en altres espècies per diferents autors (p.e. Soulé, 1986; Ellstrand & Elam, 1993; Frankham et al., 2002; Jadwiszczak et al., 2012; Ilves et al., 2013), que han proposat la deriva genètica, els colls d'ampolla i l'endogàmia com a algunes de les principals causes que explicarien la reducció de la diversitat genètica. Paral·lelament, a *Ch. uliginosus* es va observar que la taxa de germinació es redueix significativament en les poblacions de mida petita, mentre que aquesta baixada no té lloc a la resta de poblacions amb una mida mitjana i/o

relativament gran (vegeu treball 2 del compendi de publicacions per a més detalls). Tot i que el deteriorament de l'hàbitat podria contribuir a reduir l'eficiència de les plantes en poblacions petites (Vergeer et al., 2003), atesa la baixa diversitat trobada en les poblacions de mida petita, la depressió per endogàmia és l'explicació més plausible per a la reducció de l'eficiència reproductiva (Vitales et al., 2013).

El nostre estudi assenyala que la major part de la variabilitat genètica de *Ch. uliginosus* es troba repartida entre les diferents poblacions, més que no pas dintre de les poblacions. Així doncs les estratègies de conservació haurien de dirigir-se cap a la protecció del major nombre possible de poblacions diferents. Les principals amenaces per a la conservació de *Ch. uliginosus* estan relacionades amb l'eliminació i/o la degradació de l'hàbitat que ocupa (Susanna, 1991; Bañares et al., 2010), i per tant, les accions principals de conservació s'haurien de centrar en la protecció de les zones atlàntiques humides del sud-oest de la península Ibèrica. Mentre que algunes de les poblacions de l'espècie ja es troben en àrees protegides i haurien de tenir garantida la seva conservació a llarg termini, d'altres es troben localitzades en àrees sense cap mesura de protecció. Finalment, en relació a la conservació les poblacions més petites de *Ch. uliginosus,* que presenten valors particularment baixos de diversitat genètica intrapoblacional, però contenen una part molt important de la variabilitat genètica total de l'espècie, s'haurien d'adoptar mesures addicionals per a evitar els possibles efectes de la depressió per endogàmia i el subseqüent risc d'extinció.

Pel que fa als *Cheirolophus* macaronèsics, la conservació de les nombroses espècies amenaçades d'aquest grup insular no ha estat objecte de cap estudi en particular. No obstant això, les dades de genètica poblacional generades per a estudiar el procés de radiació a les illes oceàniques ens han permès extreure algunes conclusions interessants sobre la biologia de la conservació d'aquestes espècies. D'una banda, les anàlisis filogenètiques basades en AFLPs ens han servit per a confirmar la delimitació taxonòmica d'aquests microendemismes (vegeu treball 4 del compendi de publicacions per a més detalls). D'altra banda, les mesures de diversitat genètica obtingudes han pogut ser utilitzades com a estimadors aproximats de la rellevància evolutiva dels diferents tàxons (Redding & Mooers, 2006). Els nostres resultats suggereixen que les espècies de *Cheirolophus* macaronèsics amb uns valors més alts

dels índexs de raresa gènica -considerades per tant com a evolutivament més diferenciades- es troben assignades a categories d'amenaça de menor risc que aquelles que presenten valors baixos de raresa. El nombre de poblacions analitzades en aquest estudi és massa limitat per a emetre conclusions particulars sobre la biologia de la conservació d'aquestes espècies. Tanmateix, sembla clar que la prioritat de conservació dintre d'aquest grup podria ser diferent -i, en definitiva, més clarament atribuïble- si durant el procés d'avaluació del grau d'amenaça s'hi incorporés aquesta informació evolutiva.

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CONCLUSIONS

CONCLUSIONS (ENGLISH)

1. Molecular phylogeny and phylogeographic patterns in the genus *Cheirolophus*

- The use of nuclear and chloroplast DNA sequences has proven useful to disentangle the evolutionary relationships in *Cheirolophus*, although with limitations. While nuclear DNA provides an overall resolution at the backbone of the phylogeny, the plastid markers used have contributed to better understand the interspecific relationships in Macaronesia.
- Conflicting signals between nuclear and chloroplast markers may be attributed to hybridization events in some cases (e.g. phylogenetic position of *Ch. massonianus*) and to low variability of the markers in other cases (e.g. within the Mediterranean clade).
- With *Ch. crassifolius*, endemic to Malta and Gozo, as sister species to the extant diversity of the genus, climate-driven diversification likely explains the early evolutionary history of the genus in the Mediterranean basin.
- The phylogeographic reconstruction suggests that Macaronesian diversification started around 1.74 Ma, followed by an explosive event of radiation during the late Pleistocene and particularly at the Holocene (0.5 Ma).

2. Genome size and ribosomal DNA evolution

- The high incidence of 2*n* = 32 as the main chromosome number is confirmed as well as its ancestral status, since it is reported here in the early diverged *Ch. crassifolius*. Besides, 2*n* = 30 has arisen in several episodes during the evolution of the genus, probably due to mechanisms of chromosomal fusion.
- Just after the diversification of the genus, the number of 35S ribosomal DNA loci has undergone a significant increase (from 4 to 7-10). However, since this has happened in both insular and continental taxa, we cannot confirm a direct cause-effect link between such genomic reorganization and the successful radiation into Macaronesia.

- The surprisingly high number of 35S rDNA loci contrasts with the gradual genome downsizing observed during the evolution of the genus, especially in the course of Macaronesian colonization. This trend, however, agrees with current views suggesting a potential limiting effect of genome size in insular biotas.
- As found in other plant groups, a high number of loci might underlie higher diversity of ribosomal DNA copies, hence we hypothesize that this (among other factors such as genome downsizing) could have had a role in the adaptability of species, and hence in the colonization success.

3. Phylogeography and population genetics in the Macaronesian *Cheirolophus*

- According to our reconstruction, Tenerife has played an important biogeographic role in the Macaronesian radiation, with dispersals from this island both eastwards (towards Gran Canaria) and westwards (towards La Gomera and la Palma, and La Gomera and El Hierro).
- Tenerife island has revealed a complex genetic structure, which can be attributed to the existence of disjunct lineages between the former paleo-islands, nowadays part of the Anaga and Teno peninsulas in the North, and the subsequent connections and isolation events due to volcanic activity in the last 2 Ma.
- The use of AFLP markers in Macaronesian taxa suggests that the combination of poor gene flow capacity and certain ability for sporadic long-distance colonisation could have played an important role enhancing the explosive diversification of *Cheirolophus* in Macaronesia.
- As for the phylogenetic reconstruction, population genetic analysis has revealed traces of introgression and incipient ecological adaptation, which could have also enhanced the extraordinary diversification of the genus in the archipelagos.

4. Phylogeographic patterns in the Iberian peninsula

- *Cheirolophus sempervirens* probably survived in southern Iberian refugia during glacial periods and subsequently recolonized northwards in warmer stages (i.e. the "southern richness to northern purity" pattern).
- The existence of two separate old genetic lineages within *Ch. sempervirens* supports the hypothesis of multiple minor refugia for SW Iberia (i.e. the "refugia within refugia" model).
- The genetic structure of *Ch. uliginosus* is similar to the one presented by *Ch. sempervirens*, but in the first case a more complex phylogeographic history is inferred.
- Contemporary demographic events related to conservation issues and a long *in situ* evolutionary history might have contributed to the complex phylogeographic pattern showed by *Ch. uliginosus.*

5. Insights and recommendations for the conservation of endangered *Cheirolophus*

- Very low levels of intrapopulation genetic diversity are observed in *Ch. uliginosus*, with higher incidence in the smallest populations. This is probably the result of bottlenecks, genetic drift and high levels of endogamy.
- Lower plant performance (in terms of seed germination) has been significantly detected in small populations of *Ch. uliginosus*, being inbreeding depression the most likely explanation for the reduction in the reproductive fitness.
- It is hypothesized that current threat categories assigned to Macaronesian *Cheirolophus* do not reflect their respective evolutionary relevance, so further conservation strategies and category revaluations should be implemented.

CONCLUSIONS (CATALÀ)

1. Filogènia molecular i patrons filogeogràfics en el gènere *Cheirolophus*

- L'ús de seqüències d'ADN nuclear i cloroplàstic ha demostrat ser útil per a aclarir les relacions evolutives en *Cheirolophus*, encara que amb limitacions. Mentre que el DNA nuclear proporciona una resolució general a la columna vertebral de la filogènia, els marcadors cloroplàstics utilitzats han contribuït a fer comprendre millor les relacions interespecífiques a la Macaronèsia.
- Els senyals contradictoris entre els marcadors nuclears i cloroplàstics es poden atribuir a esdeveniments d'hibridació en alguns casos (per exemple, la posició filogenètica de *Ch. massonianus*) i a la baixa variabilitat dels marcadors en altres casos (per exemple, en el clade mediterrani).
- Amb *Ch. crassifolius*, endèmica de Malta i Gozo, com a espècie germana de la diversitat existent del gènere, la diversificació regulada per factors climàtics probablement explica la història evolutiva inicial del gènere a la conca mediterrània.
- La reconstrucció filogenètica suggereix que la diversificació macaronèsica va començar al voltant de fa 1,74 Ma i va ser seguida per un esdeveniment explosiu de radiació durant el plistocè tardà i en particular a l'holocè (0,5 Ma).

2. Evolució de la grandària del genoma i del DNA ribosòmic

- Es confirma l'alta incidència de 2*n* = 32, com a nombre cromosòmic principal, així com el seu estatus ancestral, ja que es reporta aquí en *Ch. crassifolius*, tàxon que va divergir inicialment. A més, 2*n* = 30 ha sorgit en diversos episodis durant l'evolució del gènere, probablement a causa de mecanismes de fusió cromosòmica.
- Just després de la diversificació del gènere, el nombre de *loci* del DNA ribosòmic 35S va experimentar un augment significatiu (de 4 a 7-10). No obstant això, com que aquest fet ha passat tant en tàxons insulars com en continentals, no podem

confirmar una relació directa de causa-efecte entre aquesta reorganització genòmica i la radiació amb èxit a la Macaronèsia.

- El nombre sorprenentment alt de *loci* 35S del DNAr contrasta amb la reducció gradual de la mida del genoma observada durant l'evolució del gènere, especialment en el curs de la colonització macaronèsica. Aquesta tendència, però, està d'acord amb punts de vista actuals que suggereixen un efecte limitant potencial de la mida del genoma en biotes insulars.
- Com s'ha trobat en altres grups de plantes, un gran nombre de *loci* podria ser a la base d'una més alta diversitat de còpies de DNA ribosòmic, per la qual cosa plantegem la hipòtesi que aquest fet (entre d'altres factors, com ara la reducció del genoma) podria haver tingut un paper en la capacitat d'adaptació de les espècies, i per tant en l'èxit de la colonització.

3. Filogeografia i genètica de poblacions en els *Cheirolophus* macaronèsics

- D'acord amb la nostra reconstrucció, Tenerife ha jugat un paper biogeogràfic important en la radiació macaronèsica, amb dispersions d'aquesta illa, tant cap a l'est (cap a Gran Canària) com cap a l'oest (cap a La Gomera i La Palma, i La Gomera i El Hierro).
- L'illa de Tenerife ha revelat una estructura genètica complexa, que pot atribuir-se a l'existència de llinatges disjunts entre les antigues paleoilles, avui dia part de les penínsules d'Anaga i Teno al nord, i les posteriors connexions i els posteriors esdeveniments d'aïllament a causa de l'activitat volcànica en els darrers 2 Ma.
- L'ús de marcadors AFLP en tàxons macaronèsics suggereix que la combinació de la poca capacitat de flux de gens i una certa aptitud per a la colonització esporàdica a llarga distància podrien haver jugat un paper important en l'augment de la diversificació explosiva de *Cheirolophus* a la Macaronèsia.
- Quant a la reconstrucció filogenètica, l'anàlisi de genètica de poblacions ha revelat rastres d'introgressió i adaptació ecològica incipient, la qual cosa podria també haver millorat l'extraordinària diversificació del gènere en els arxipèlags.

4. Patrons filogeogràfics a la península Ibèrica

- *Cheirolophus sempervirens* va sobreviure probablement en refugis del sud ibèric durant els períodes glacials i posteriorment va recolonitzar-ne la zona nord en fases més càlides (és a dir, va seguir el patró de "riquesa al sud, puresa al nord").
- L'existència de dos llinatges genètics antics separats dins de *Ch. sempervirens* dóna suport a la hipòtesi de múltiples refugis menors per al sud-oest ibèric (és a dir, el model de "refugis dins dels refugis").
- L'estructura genètica de *Ch. uliginosus* és similar a la que presenta *Ch. sempervirens*, però en el primer cas s'infereix una història filogeogràfica més complexa.
- Els esdeveniments demogràfics contemporanis relacionats amb aspectes de conservació i una llarga història evolutiva *in situ* podrien haver contribuït al complex patró filogeogràfic mostrat per *Ch. uliginosus*.

5. Perspectives i recomanacions per a la conservació d'espècies amenaçades de *Cheirolophus*

- Els nivells de diversitat genètica intrapoblacional que s'observen en *Ch. uliginosus* són molt baixos, amb major incidència d'aquest fet en les poblacions més petites. Aquest és probablement el resultat de colls d'ampolla, deriva genètica i alts nivells d'endogàmia.
- El nivell més baix d'èxit (en termes de germinació de llavors) s'ha detectat de manera significativa en poblacions de *Ch. uliginosus* petites, i la depressió per consanguinitat és l'explicació més probable per a la reducció de l'eficiència reproductiva.
- Es planteja la hipòtesi que les categories actuals d'amenaça assignades als *Cheirolophus* de la Macaronèsia no reflecteixen la seva respectiva rellevància evolutiva, de manera que convindria implementar noves estratègies de conservació i fer una reavaluació de les categories.