Universitat Autònoma de Barcelona

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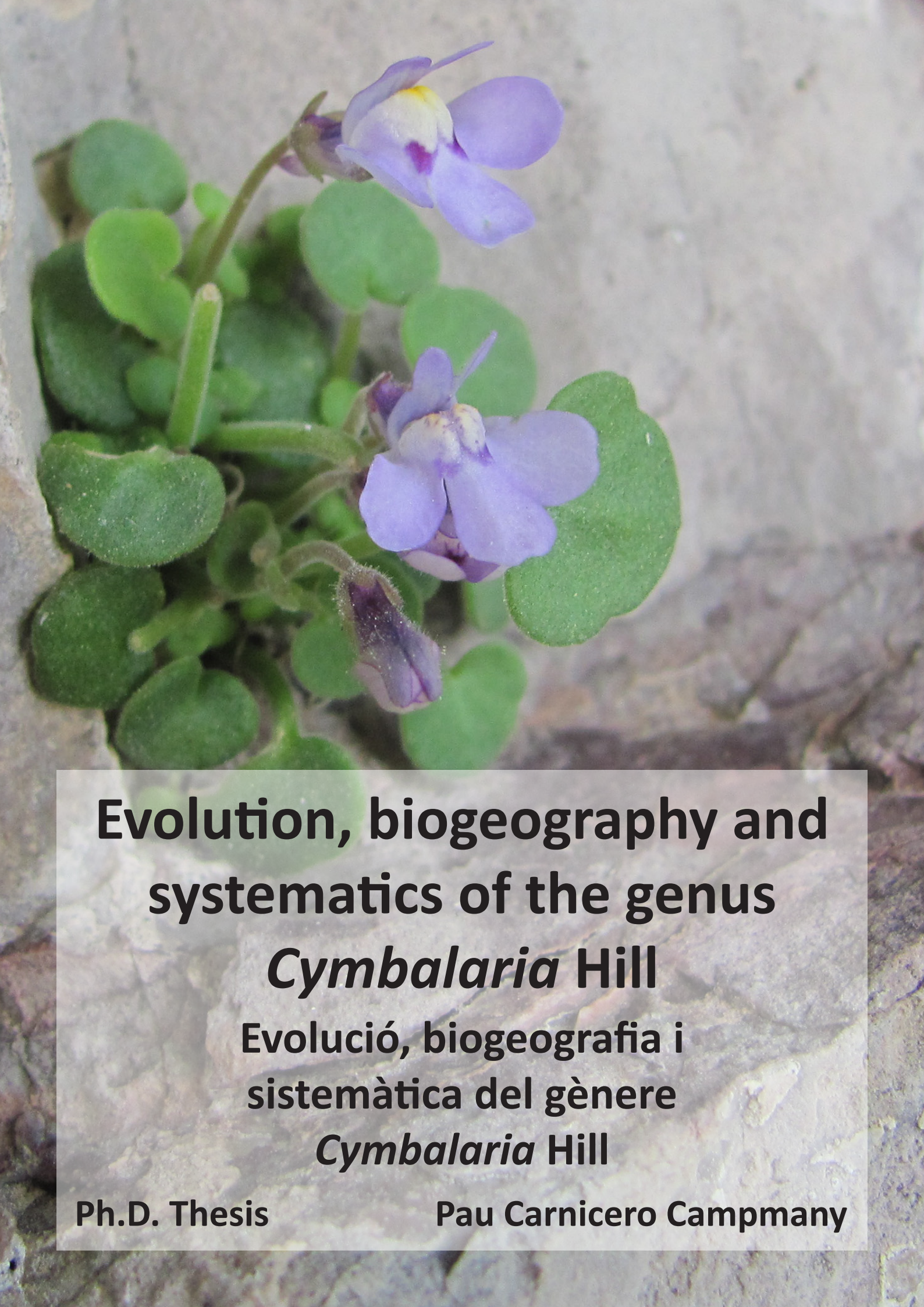
Pau Carnicero Campmany



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Ph.D. Thesis

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Bellaterra, 2017

Programa de doctorat en
Ecologia Terrestre

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Evolution, biogeography and systematics of the genus *Cymbalaria* Hill

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Programa de doctorat en
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"When on board of H. M. S 'Beagle', I was much struck with certain facts in the distribution of inhabitants of South America, and in the geological relations of the present to the past inhabitants of that continent. These facts seemed to me to throw some light on the origin of the species–that mistery of misterys"

C. Darwin, 1859

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Agraïments

Una tesi sol trobar-se envoltada d'una certa aura de transcendència en l'imaginari col·lectiu. Per fer-ne justícia, aquí em trobo, escrivint la part que més persones llegiran en un tren de la línia R3. Abans de res, però, ho dic o rebento: m'ho he passat molt bé fent aquesta tesi.

En aquell penúltim any de carrera, vaig ser víctima de l'entusiasme de la Mercè i el Llorenç per la flora i la distribució dels endemismes. Els vostres estils tan diferents em van inspirar en aquelles memorables jornades de camp en què se'm descobria el tresor que tenia sota els peus. Unes primeres col·laboracions estudiant el gènere *Helichrysum* van relligar la fascinació per la biodiversitat amb una creixent inquietud en l'Evolució. D'aquesta manera vaig conèixer l'Institut Botànic de Barcelona i la Núria, que des de llavors es va atrevir a pensar que alguna cosa se'n podria fer, de mi. La tesi va anar venint com una seqüència natural, i per una sèrie de caramboles les cimbalàries em van, en certa manera, trobar i convertir-se en les protagonistes d'aquesta tesi. Gràcies, directors, per acollir-me, deixar-me provar coses noves i equivocar-me.

Em dec també a tants altres botànics que han anat marcant els meus inicis. Al Ramon Pérez, per dirigir-me a en Llorenç quan et vaig comentar les meves inquietuds. A en Pere Fraga i en Jaume Soler, quin plaer anar al camp amb vosaltres! To all the team from the Evolutionary Systematics Lab in Innsbruck, and specially to Peter, for trusting me and your honesty. The stay in Innsbruck was a milestone in my career. I also thank Pam and Doug Soltis, for being always so positive and open-minded, I learnt a different (and successful!) way of working than we do here. A tot l'equip de la Unitat de Botànica de la UAB i de l'Institut Botànic de Barcelona, entre temps millors i pitjors, m'heu ajudat a anar creixent com a científic.

Com oblidar-me de la més gran, cruel i tendra amiga, la Muntanya. Pels grans paisatges regalats, pels petits racons i aquella brisa fresca, per l'equilibrada dança per la vertical. I per la por a l'abisme que atrau, pel fred extrem dels cims a l'hivern, per l'extenuació on l'aire s'enrareix. Aquesta adrenalina no té preu. Gràcies a tots els que m'hi heu acompanyat en un moment o altre. Llorenç, em vas introduir al món de les crestes i les hores de xerrera alpinística em van descobrir fantàstiques muntanyes i rutes, encara estranyes per mi, mai ho oblidaré. A la Gemma per algunes de les

escalades més fantàstiques de la meva vida. Óscar i Isma, pel gran aprovisionament del vostre camp base. Al Xavi, mestre, referent, sense paraules. A la Núria i el Nico, molt més que companys d'escalada! A la gent de Chile, Tío Lucho, Henry, grans de les alçades. A tots els escaladors calderins amb qui he fet volar coloms, al Joel, Madu, Peies, Piui... i en especial al Martí, per haver anat complint el somni de les hores de pati d'explorar paradisos verticals, sempre fugint endavant. To Peter, I owe you my (limited) ski touring skills, we made such a good team in the mountain! And to Andy and Alex, Giarda, Moritz... just strong people in Innsbruck!

Hi ha tota una corrua de gent fantàstica que no vull oblidar: Uri, Dídac, Cesc, Martí, Peies... Ben tronats! Als companys de militància, la Sandra i l'Anna especialment, quina feinada esteu fent! Als companys de carrera i màster, Marta, Núria, Judit, María... per haver anat compartint penes i alegries (amb la certesa que les alegries han guanyat). A Noe, por los titánicos esfuerzos en provar todas las cervezas artesanas de Gainesville y hacer más llevadera la vida yankee.

No oblido la família, pel mèrit que té recolzar-me sense entendre quin sentit té el què faig! Mama, pel teu recolzament i confiança sincera, no saps quant t'ho agraeixo. Papa, per transmetre'm que sempre es pot anar una mica més enllà. Als avis, exemple admiradíssim de perseverança i il·lusió. Avi, sembla que finalment hi haurà un doctor Carnicero a la família! A la Iaia i la Conxita, per haver-vos enamorat també de les cimbalàries. I des de la sorpresa i la pena de no haver coincidit prou, llegint-te, "Tio" Manel, crec que podríem haver passat hores parlant de les plantes que poblen els volts d'Albinyana tot prenent la fresca al sagrat...

Mar, gràcies per haver-me acompanyat tot aquest temps, per comprendre les meves excentricitats, calmar-me quan m'esverava i escoltar, críticament, els meus projectes i idees. Pels grandíssims moments envoltats de natura i les petites "descobertes", sovint mundanes, que per nosaltres eren com un nou continent. I sobretot, perquè sempre he sentit que creus en mi, i això dóna una embranzida molt bèstia.

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Abstract

Since ancient times the diversity of life forms has fascinated and intrigued humanity. How do species originate? How did they achieve their present geographic distributions? In the last decades, the progress of molecular systematics tools has allowed for successfully answering these questions. In this thesis, we perform a systematic study of the genus *Cymbalaria*, a group of rupicolous plants endemic to the Mediterranean Basin. Mainly, we used phylogenetic and phylogeographic methods and morphological analyses to infer their evolution and biogeographic history, and to propose a new taxonomic classification from the perspective of integrative taxonomy. Our results show that *Cymbalaria* originated ca. 4 million years ago and diversified during and after the establishment of Mediterranean type climate, through allopatric, sympatric and polyploid speciation. At least, two long-distance dispersal events from Corsica-Sardinia to the Balearic Islands occurred, although no apparent adaptations for dispersal exist in this case. Marine barriers successfully interrupted gene flow and allowed allopatric speciation to take place in some cases, while, in others, species successfully maintained gene flow between populations separated by the sea. Pleistocene sea-level fluctuations could have contributed to the present distribution of taxa and triggered speciation. By combining molecular and morphological data, we bring the classification of *Cymbalaria* closer to the integrative taxonomy concept and identify valuable diagnostic morphological characters. Here, we propose a new circumscription of *C. fragilis* to include specimens misidentified as *C. aequitriloba* due to the variability of seed ornamentation. Also, we describe the new paraphyletic subspecies *C. muelleri* subsp. *villosa*, from which *C. muelleri* subsp. *muelleri* originated through anacladogenetic speciation. Thus, we discuss the need for recognizing non-monophyletic taxa, since evolution does not always result in purely dichotomous branching patterns. Finally we suggest a new taxonomic treatment for the eastern Mediterranean species, in which we split *C. microcalyx* into four species, describe the new species *C. spetae* and propose two new combinations.

Resum

Des de temps ancestrals la diversitat d'éssers vius del planeta ha fascinat i intrigat la humanitat. Com s'originen les espècies? Com han assolit la distribució geogràfica actual? En les darreres dècades, el gran avenç de les tècniques de sistemàtica molecular ha permès anar resolent aquests interrogants de manera satisfactòria. En aquesta tesi, desenvolupem un estudi sistemàtic del gènere *Cymbalaria*, un grup de plantes rupícoles endèmiques de la conca Mediterrània. Hem utilitzat principalment tècniques moleculars de filogènia i filogeografia i anàlisis morfològiques per inferir la seva història evolutiva i biogeogràfica, i proposar una nova classificació taxonòmica des de la perspectiva de la taxonomia integrativa. *Cymbalaria* va originar-se al voltant de 4 milions d'anys i va diversificar durant i després de l'establiment del clima mediterrani per mitjà de processos d'especiació al·lopàtrica, simpàtrica i poliploide. Com a mínim, dues dispersions a llarga distància des de Còrsega-Sardenya a les Balears van ocórrer, tot i no observar-se adaptacions específica a la dispersió en aquest cas. Les barreres marines han interromput exitosament el flux genètic en alguns casos, estimulant l'especiació al·lopàtrica, mentre que, en altres, les espècies han aconseguit mantenir el flux genètic entre poblacions separades pel mar. Les fluctuacions del nivell del mar del Pleistocè probablement van contribuir a la distribució actual dels tàxons i van afavorir l'especiació. La combinació de dades moleculars i morfològiques ens han permès apropar la classificació del gènere al concepte de taxonomia integrativa i identificar els caràcters morfològics amb valor diagnòstic. Així doncs, proposem una nova circumscripció per *C. fragilis* per incloure poblacions que, degut a l'ornamentació de les llavors, s'havien identificat com a *C. aequitriloba*. Per altra banda, descrivim la nova subespècie parafilètica *C. muelleri* subsp. *villosa*, de la qual s'originà per especiació anacladogenètica *C. muelleri* subsp. *muelleri*. Així, discutim la importància de reconèixer tàxons no monofilètics, que són el reflex d'una història evolutiva que no sempre produeix relacions purament dicotòmiques entre les espècies. Finalment, proposem un nou tractament taxonòmic per a les espècies de la Mediterrània oriental, en què dividim l'antiga *C. microcalyx* en quatre espècies, descrivim la nova espècie *C. spetae* i proposem dues noves combinacions.

General introduction

Plant systematics

Far are the days when systematics and taxonomy were indistinctly used as synonyms (Stuessy, 2009a). Since C. Darwin and A.R. Wallace's postulates (Darwin & Wallace, 1858; Darwin, 1859) as well as the contribution of many other previous scientists, species became something more than just entities God placed somewhere. Species became something temporary, circumstantial, contrary to immutable, permanent entities. Where do they come from? What is more, what is their ancestor and which are the closest extant species sharing it? Systematists realized they were dealing with a changing discipline, and thus solely classifying the species on the basis of morphology ceased to make sense. Answering the questions on how did they originate and how did they manage to occupy the piece of Earth they do at present became a fundamental part of any systematic study. Taxonomy became just a step in the Systematics approach, the last step.

The study of evolution is crucial for any modern study of systematics, and comprises two faces: the study of the relationships between species (phylogeny), and the processes that originated them (speciation, cf. Stuessy et al., 2014). Advances in molecular techniques represented a great step-forward on studying the evolution, but also pushed part of the scientific community to oversimplification. As a remarkable example, the cladistics school used the molecular techniques to reinforce their principles by advocating for the acceptance of strictly monophyletic clades in taxonomic classification, assuming that all speciation events result in dichotomous branching patterns (Stuessy, 2009b). However, a few well known evolutionary processes result in non-dichotomous branching patterns. Among those, hybridization and polyploidy are processes of main importance in the evolution of plants, and are often linked to each other (Soltis & Soltis, 2009). As a result, they generate incongruence among gene trees, resulting in species relationships that are better explained by networks than by strictly bifurcating phylogenetic trees (Huson & Bryant, 2006). Nowadays, incongruence among gene trees from different sources has been shown to be more the rule than the exception (e.g. Pelser et al., 2010; Hilpold et al., 2014; Sun et al., 2015). Far from considering incongruence a limitation, it has to be considered a source of information to disentangle complex evolutionary processes

(Wendel & Doyle, 1998). In recent years, species tree approaches use DNA from different parts of the genome to build a single phylogenetic tree in the framework of the coalescent theory (Doyle, 1992; Nordborg et al., 2004; Heled & Drummond, 2010). New algorithms are in constant development and improvement to better accommodate for incongruence and higher amounts of data obtained from modern sequencing techniques.

Biogeography is strongly related to the idea of evolution, and the two concepts feed each other. Actually, C. Darwin and A. R. Wallace (specially the latter) are also considered the fathers of modern historical biogeography. The relationship between phylogeny and geography was fundamental for both scientists in developing their postulates: “the more nearly any two forms are related in blood, the nearer they will generally stand to each other in time and space” (Darwin, 1859), and “every species has come into existence coincident both in space and time with a pre-existing closely allied species” (Wallace, 1855). The derived conclusion from this observation was crucial: similar species live close to each other because they descend from the same organism. Biogeographic studies aim to find the origin and causes for the current distribution of organisms, basically by inferring that of their ancestors. This information is essential to understand speciation processes and the role of geographic isolation. When speciation occurs in sympatry, it can be caused or favored by divergent ecologic specialization (Rundle & Nosil, 2005; Schluter, 2009) or by processes such polyploidy, combined or not with hybridization, can result in an immediate interruption of gene flow between the new species and the ancestor/s (Soltis & Soltis, 2009; Wood et al., 2009). The most studied and discussed scenario has been, however, allopatric speciation. Probably, the role of two types of allopatric speciation, vicariance and peripatric speciation, has been the biggest discussion of the last century in biogeography (Wiley, 1988; Queiroz, 2005). The debate became especially harsh for cases in which dispersal barriers were huge: how did organisms without particular adaptations for dispersal reach a given disjunct distribution area? Geology provided the mechanism for the strong vicariance paradigm in biogeography: the continental drift. Its confirmation stimulated the shift from the mostly dispersalist explanation for the distributions of organisms –used in former times by C. Darwin and prevalent idea

until the 1960s– to vicariance. The vicariance school was also fed by the cladistics school, building together a radical thinking that often rejected C. Darwin's ideas, and set dispersalism as an out-of-science discipline (Nelson & Platnick, 1981). However, new pieces of evidence coming from molecular dating strongly advocate against the vicariance paradigm: in most cases, the split of continents is much older than the estimated ages of species (Queiroz, 2005). Thus, we are back to Darwin's message: given enough time, many things that seem unlikely can happen (Darwin, 1859).

Taxonomy

In the modern Systematics concept, taxonomy becomes an integrative discipline that should reflect all the knowledge derived from evolutionary studies as well as any other kind of available data (Stuessy, 2009a; Schlick-Steiner et al., 2010). For us, humans, classification is a need, a basic innate mental activity. We need to store the knowledge in a meaningful way. The first step of any study related to biodiversity, is to identify the components of our study system, and thus the job made by taxonomists becomes crucial. Furthermore, the predictive value of taxonomy is of major interest for applied sciences (Stuessy, 2009a). In front of the unknown, a classification framework based on multiple sources allows for predicting features of an unknown entity based on its similarities with classified knowledge. Predictive value is a powerful consequence of taxonomy with major importance in applied sciences, for example, in the search for new drugs, development of resistance to pests in agriculture or optimization of chemical reactions, among many others.

Relevance of Systematics

Thus, Systematics as a whole has the main objective of understanding the diversity of life, subdivided in three main steps: (1) determine which life forms occur on the planet, (2) understand their origins and (3) organize the knowledge (Stuessy et al., 2014). A profound knowledge of biodiversity has evident direct benefits for society: we depend on a huge proportion on biotic resources from a wide diversity of organisms. Systematics possesses a feature that may shock scientists from other disciplines: often,

a systematic study does not begin with a hypothesis and then seeks for the organism ideally suited to test it. Instead, the organisms to study are often selected first for diverse reasons. For hypothesis testing, model organisms, i.e. well-known organisms that we can easily manipulate in the lab and perform experiments with them (*i.e. Arabidopsis* and *Drosophila*), can be used. However, diversity of life is much bigger; these organisms represent just two final tips of an enormous Tree of Life, and thus can account for a relatively small amount of the processes that originated the whole biodiversity. Instead, the systematic approach has an indisputable good consequence: it results in a higher portion of the diversity of life being studied. Indeed, systematic studies, by exploring an unknown landscape, can result in discoveries "typically of the kind that would not be made otherwise" (Wilson, 1968: 1113). These new discoveries can have a relatively low impact if found in a single organism, but stimulate further research that may end up in the description of processes that represent huge contributions to knowledge on many facets of biology (Greene, 2005). As an example, thermostable polymerases used in PCR procedures, which represented an extreme advance for science, were discovered in a systematic study on bacteria of thermal pools (Saiki et al., 1988).

Systematics tools

Molecular Systematics

Systematics benefits from several different kinds of data, e.g. morphology, anatomy, cytology, molecular biology or ecology, among others. In the last decades, molecular data have played a main role and have provoked a revolution in the field of systematics. This source of information is of main importance in order to explain the evolution, an objective for which morphology as well as other kinds of data have been shown to be limited, mainly due to the high influence of homoplasy. The main advantages of molecular data are (1) the huge amount of available data, only limited by the size of genomes; (2) the ease of interpreting it, since they directly come from genetics and present low ambiguity; (3) their universality; and (4) the higher regularity in their evolution with respect to morphological data (Graur & Li, 1999; Nei & Kumar,

2000). Molecular data have a clear and independent genetic basis, and are thus the best source for testing evolutionary, phylogenetic and biogeographic hypotheses. However, molecular data are also prone to homoplasy, as well as to other artifacts such as concerted evolution or gene duplications, among others. The type of data used must be in accordance with the aims of the study, and combining molecular data with morphology or cytogenetics would be mandatory in some cases. A number of authors have highlighted the importance of a combination of molecular data with morphology and other sources of information for Systematics (e.g. Dayrat, 2005; Will et al., 2005, Stuessy et al., 2014). Here we will shortly describe the main approaches used in our study.

Phylogenetic reconstruction

DNA sequences used for phylogenetic inference in plant systematics are mainly from the chloroplast and the nuclear genome. Although the mitochondrial genome has been widely used in animals, it is not recommended in plant phylogeny due to its high variability in genome structure, size and configuration (Palmer, 1992). Sequences from the nuclear genome are mostly from the nuclear ribosomal DNA (nrDNA). The nrDNA is biparentally inherited and has thousands of copies in most plant genomes, making it easy to amplify and sequence. The concerted evolution phenomenon results in an almost complete homogeneity of all the copies in an individual, and this helps avoiding the effects of events such as gene duplication coupled with lineage sorting and meiotic and sexual recombination to phylogenetic inference (Linder & Rieseberg, 2004). However, the concerted evolution can hide the evidence of reticulate evolution caused by hybridization, by homogenizing the nrDNA towards only one of the parental copies (Wendel et al., 1995), except in cases in which hybrids are recent or of recurrent formation and different parental copies still coexist in the genome (Koch et al., 2003; Kovarik et al., 2005).

Chloroplast DNA (cpDNA) is maternally inherited in most plants (Corriveau & Coleman, 1988). Its genes are essentially single copy and evolve relatively slowly, making them appropriate for deep phylogenetic inference. However, a degree of variation in mutation rates exists and some regions (essentially non-coding regions),

have been successfully used for intraspecific phylogeographic reconstruction (Shaw et al., 2007). Due to the maternal inheritance, cpDNA sequences are useful for reconstructing colonization by seeds (Schaal et al., 1998). An important issue to consider is the possibility of chloroplast transfer after hybridization events (Rieseberg & Soltis, 1991). The combined use of cp and nrDNA regions has been shown to be informative to describe reticulation events (Doyle, 1992).

Among the multiple methods for phylogenetic inference, we chose Maximum Parsimony (MP), an optimality criterion method, and Bayesian Inference (BI), a probabilistic method based on models of evolutionary change. Maximum Parsimony is based in a simple assumption: simpler hypotheses are preferable to more complicated ones. Thus, MP methods operate by selecting trees that minimize the total tree length, the number of nucleotide substitutions required to explain the dataset. The statistical support of branches is generally evaluated with bootstrap pseudoreplicates. Bayesian Inference method needs to assume an explicit model of sequence evolution, which must be previously estimated from the dataset. In the BI process, multiple phylogenetic trees are sampled using a Markov chain Monte Carlo (MCMC) method, and evaluated estimating the probability of the tree given the data and the evolutionary model. A number of trees are kept after the MCMC runs, from where posterior probabilities for each clade in the tree are calculated.

Molecular dating

Dating the divergence of evolutionary lineages is of main importance in systematic studies, since it allows to combine the phylogenetic data with information on the geomorphological and climatic histories, and thus to draw detailed hypotheses on how the evolution of a group took place. The first approach to molecular dating was the assumption of strict clocks, that is, DNA undergoes constant nucleotide substitution rates (Zuckerkanl & Pauling, 1965). However, strict clock hypothesis has been rejected in most cases, since rates have been proved to be different among different DNA regions, through time and even between closely related lineages (Ho & Duchêne, 2014). In the last years, relaxed uncorrelated molecular clocks have gained support as the best approaches for molecular dating (Drummond et al., 2006; Ho & Duchêne,

2014). In relaxed clock models, instead of having a constant nucleotide substitution rate, this is allowed to vary along branches according to a probability distribution. In uncorrelated clocks there is no assumption of correlation between rates in neighboring branches in the phylogeny.

To obtain absolute age estimates, it is crucial to calibrate the clock. Calibration points have a huge influence in dating analyses and can come from (1) geological events, (2) previous independent molecular dating studies (secondary calibration points) and (3) the fossil record. Fossils are considered to be the best source of information for calibration (Forest, 2009). However, a good knowledge on the age of the fossil as well as its phylogenetic position and a complete fossil record are key aspects difficult to fulfill (Forest, 2009; Parham et al., 2012). Moreover, the definition of prior probability distribution for the age of the calibration point is necessary, but often desirable information needed for this purpose is not available and therefore it is uncertain how similar are these distributions to reality (Parham et al., 2012). The importance of using multiple calibration points in relaxed molecular clock dating has been often highlighted for obtaining reliable age estimates (Ho & Phillips, 2009; Sauquet, 2013; Duchêne et al., 2014).

BEAST (Bayesian Evolutionary Analysis by Sampling Trees) is an application that co-estimates phylogeny and divergence times under relaxed-clock models with a Bayesian MCMC method (Drummond et al., 2006). BEAST allows the user to set different clock models, calibration points and speciation models, among other parameters. Moreover, it is possible to calculate the marginal likelihood of each analysis with Stepping Stone and Path Sampling methods, for the purpose of model selection (Baele et al., 2012, 2013). BEAST provides a user-friendly interface, which has probably led to the emergence of hundreds of studies using molecular dating. However, most of these studies lack crucial information or justification on several parameters of the model, or result in huge uncertainty that is even barely mentioned. In recent years, several reviews highlight the importance of a proper selection of model parameters to obtain reliable results (Parham et al., 2012; Sauquet, 2013; Ho & Duchêne, 2014).

Ancestral area estimation

In the last years, biogeographic methods used for historical biogeography inference have switched from event-based parsimony methods to statistical approaches. The most commonly used parsimony method was Dispersal-Vicariance Analysis (DIVA, Ronquist, 1997), which in accordance to the cladistic-vicariance school gave major importance to vicariance processes *versus* dispersal in shaping the biogeographic histories of organisms. Parametric statistical methods are rapidly gaining popularity, and were a major advance in historical biogeography (Ree et al., 2005), since they offer a more flexible general framework for model building, parameter estimation, hypothesis testing and are more flexible to incorporate information of related processes in the biogeographic model (Ronquist & Sanmartín, 2011). Statistical approaches describe biogeographic history in terms of rates of processes and probabilities of events, estimating parameters either by Maximum Likelihood (ML) or BI. The most sounding method in a ML framework is the Dispersal-Extinction-Cladogenesis (DEC) model (Ree & Smith, 2008). Some of the advantages of DEC are the possibility of defining different dispersal probabilities in time and space, and of incorporating dating uncertainty. However, both DEC and DIVA ignore a process considered to be crucial in biogeography: founder event speciation.

BioGeoBEARS is an R package that implements the DEC, DIVA and the Bayesian model BAYAREA in a likelihood framework (Matzke, 2013). Additionally, it implements a version of each model that incorporates a new parameter representing founder-event speciation, a rare dispersal event that "instantaneously" establishes a geographically isolated new lineage (Matzke, 2014). Another advantage of BioGeoBEARS is the common likelihood framework in which the models are implemented that allows statistical model selection procedures to be applied.

Phylogeographic reconstruction

The objective of phylogeography is to explain the spatial distribution of genetic lineages within a species or a group of closely related species (Avise et al., 1987; Avise, 2009). It integrates phylogeny, biogeography and population genetics to find the processes that originated the current genetic structure of populations in the frame of the coalescence theory (Kingman, 1982).

Molecular markers used in a phylogeographic study must present high intraspecific variability and ideally be non-recombining. In plants, some cpDNA regions can meet these requirements, but often show too low variability (Schaal et al., 1998). However, as mentioned before, they are very useful to track colonization by seeds. Amplified fragment length polymorphisms (AFLP) fingerprinting has been widely used in plant phylogeographic studies, since it provides a high amount of data, mostly from the nuclear DNA (Vos et al., 1995). Using short fragments randomly sampled from the whole genome, a fingerprint is generated for each specimen based on the number and length of the fragments obtained. The presence/absence of each fragment is used to compare individuals and infer the relationships between them. One of the disadvantages of this technique is the assumption that fragments of the same size are homologous, since fragments are not sequenced and is therefore not possible to definitely assess homology (Karp et al., 1996). Moreover, AFLPs are dominant markers, since it is only possible to know whether a fragment is present or absent, but it is not possible to distinguish homozygous from heterozygous. However, a number of studies show that phylogenetic signal is present in AFLPs and that they can be used with confidence to infer phylogenies of closely related taxa (e.g. Koopman, 2005; Simmons et al., 2007). The fast development of NGS techniques for phylogeography provides a promising field for overcoming abovementioned disadvantages (McCormack et al., 2013; Cross et al., 2016).

For phylogenetic inference, distance matrices can be constructed from the pattern of presence/absence of fragments in the AFLP output, and afterwards be used to infer phylogenetic trees using a Neighbor-Joining analysis. Phylogenetic networks can also be constructed with the Neighbor-Net method, and can be very informative to represent underlying conflictive or alternative genealogies (Bryant & Moulton, 2004).

Recently, diffusion models have been successfully applied to infer biogeographic histories in the context of phylogeographic studies. Lemey et al. (2010) developed a BI MCMC approach in continuous time and space that coestimates the phylogeny, divergence time and continuous phylogeographic diffusion. cpDNA sequences can be used to produce genealogies with a statistical parsimony method (Templeton et al., 1992).

Morphology

Morphological data have been, and still are most used in plant taxonomy. The current classification system results, basically, from studies based on morphological data, the external form of organisms. Although in the last decades molecular systematics methods have been widely used to revise the classification of a tremendous number of plant groups, morphological characters still have advantages that maintain them as a highly relevant criterion in plant systematics (Stuessy, 2009a). Basically, morphological features can be recorded immediately, and thus allow for a rapid identification of plants in the field, where molecular tools are – still – useless. However, as abovementioned, the combination of the two sources of data, as well others as cytology, anatomy or ecology is highly desirable for the purpose of classification (Schlick-Steiner et al., 2010).

Morphological data can be classified in macromorphological and micromorphological data. Although the former are the most used in keys due to the ease and speed of observation, micromorphological characters have main importance in several groups. The latter have gained importance in the last decades concurrently with the improvement of microscopy techniques, especially scanning electron microscopy (SEM). Among micromorphological characters, those related to seeds and trichomes are worth to mention, due to the high number of recent studies successfully using them as diagnostic characters (e.g. López-Alvarado et al., 2012; Magauer et al., 2014; Vigalondo et al., 2015). Morphological data can be classified too in reproductive and vegetative morphological characters. Reproductive characters belong to highly specialized organs under a high adaptive pressure, and are thus less prone to variation than vegetative characters, that have

varied functions and higher plasticity due to environmental conditions (Stuessy, 2009a).

It is desirable to accompany the description of morphological features with statistical analyses. Multivariate morphometric analyses allow describing the morphological differentiation of organisms in a virtual space and testing the belonging of specimens to predefined classes – taxa – according to several to many morphological characters together (Mc Garigal et al., 2000). When characters are properly defined, this approach allows for rigorously describing the usefulness of traditionally used characters, as well as the discovery of new ones. Principal Components Analysis (PCA) and Canonical Discriminant Analysis (CDA) are amongst the most used methods. PCA is an ordination method that transforms observations into values which will be represented in a spaced defined by new axes that maximizes the variation of the original dataset (Principal Components). Principal Components are ordered so that the first shows the largest possible variance, and each succeeding component in turn has the highest variance possible under the constraint that it is orthogonal to the preceding components. CDA differs mainly from PCA in that it tries to maximize the difference between predefined classes at the same time that it summarizes the variation in new axes (Discriminant Axes). PCA is thus mostly used for exploratory purposes, while CDA allows for specifically testing the fit of original observations to a classification. In recent years, multivariate morphometrics (usually in combination with other data) have been used to clarify the taxonomy of closely related taxa, revise morphologically complex groups, detect hybrids, etc. (e.g. Galbany-Casals et al., 2012; Španiel et al., 2012; Hodálová et al., 2015; Jolles, 2015).

The Mediterranean Basin

The Mediterranean Basin contains *ca.* 25,000 species, of which 63% are endemic (Greuter, 1991), and almost 10% of the world's vascular flora, thus constituting a biodiversity hotspot (Myers et al., 2000). The recent climatic history of the Mediterranean basin is marked by two events thought to have had main importance in shaping the evolution and geographic distribution of plants, namely: the onset of

Mediterranean type climate and the climatic oscillations of the Pleistocene. From a former subtropical climate, during the Miocene gradual cooling and desiccation finally led to the establishment of Mediterranean climate, characterized by a marked seasonality, around 3.2 million years ago (Ma). This event partially coincided with an increase in diversification of several plant lineages, either by favoring speciation by adaptation to the new conditions or by fragmenting formerly wider populations, thus enhancing allopatric speciation events (Fiz-Palacios & Valcárcel, 2013; Thompson, 2005). During the Late Pliocene and the Pleistocene, climatic oscillations associated to glaciations caused remarkable altitudinal and latitudinal shifts in the distribution of plants, as well as extinctions (Comes & Kadereit, 1998; Nieto Feliner, 2014). However, some areas remained more stable during the climatic oscillations, and are thought to have acted as refugia for biodiversity, and therefore to have had a main role as source for recolonization after unsuitable periods (Médail & Diadema, 2009). Sea level oscillations were of main importance for island flora in this period, leading to repeated appearance of land bridges during cold stages between previously isolated areas and to transgressions during interglacials (Vesica et al., 2000). These connections have been often used to explain currently fragmented distribution of several species or groups of related species (Nieto Feliner, 2014).

Cymbalaria Hill (Plantaginaceae)

Cymbalaria Hill is a Mediterranean plant genus that comprises nine species and seven subspecies (Sutton, 1988). Phylogenetic data clearly support it as a member of the tribe Antirrhineae, in the family Plantaginaceae (*sensu* Albach et al., 2005), in a clade composed mostly of New World species (clade *Maurandya*, Ghebrehiwet et al., 2000; Vargas et al., 2004; Guzmán et al., 2015). It is sister to the monotypic Mediterranean genus *Asarina*, from which it was estimated to diverge *ca*. 10 Ma (Vargas et al., 2013)[1]. The distribution pattern of *Cymbalaria* probably reflects a story strongly related to climatically stable areas, as revealed by its presence in several

[1] During the last revision steps of this thesis, the new monotypic genus *Gadoria* Güemes & Mota has been described (Güemes & Mota, 2017). According to a nrDNA phylogenetic analysis, *Gadoria* is the closest extant sister taxon of *Cymbalaria.*

glacial refugia as well as its preference for chasmophytic habitats, known to have often more stable conditions than surrounding areas (Thompson, 2005). The presence of a wide sea barrier between the distribution area of different species or within the distribution of a single species motivated the formulation of vicariant hypotheses linked to old land bridges (Contandriopoulos & Cardona, 1984; Thompson, 2005). However, in the last years the role of sea barriers has been shown to be diverse (Nieto Feliner, 2014), since even species without apparent adaptations for dispersal have managed to cross long distances by long-distance dispersal (LDD, i.e. Guzmán & Vargas, 2009; Piñeiro et al., 2012). The diversity of ploidy levels found in *Cymbalaria* has also motivated biogeographic and phylogenetic hypotheses, based on apparently parallel habitat preferences between pairs of species (Verlaque et al., 1993).

The present taxonomy of the genus is mainly based on morphological traits and ploidy levels, since a detailed molecular study focusing on the genus had not yet been performed. The last complete revision of the genus was conducted by Sutton (1988) in his comprehensive work "A revision of the tribe Antirrhineae". However, it is worth mentioning the important contribution of several authors who performed systematic studies on small groups of species within *Cymbalaria* and often ventured interesting phylogenetic and biogeographic hypotheses beyond taxonomy (Cufodontis, 1936; Greuter & Rechinger, 1967; Contandriopoulos & Cardona, 1984; Speta, 1986; Verlaque et al., 1993; Bigazzi & Raffaelli, 2000). However, taxonomy of *Cymbalaria* seems far from being resolved. Sutton (1988) repeatedly stated the need for profound studies in several cases to discern the most appropriate rank of some taxa, and whether some other entities should or not be recognized. In fact, extant taxonomic studies, checklists and Floras show disagreements in the taxonomic treatments used (e.g. Tutin, 1980; Pignatti, 1982; Güemes, 2009; Dimopoulos et al., 2013), revealing the need of a comprehensive systematic revision of the genus.

As a starting point, we followed Sutton's (1988) taxonomic treatment with few exceptions. On the one hand, *C. pubescens* (C.Presl) Cufod. and *C. fragilis* (J.J.Rodr.) A.Chev. were considered separate species from *C. muralis* G.Gaertn., B.Mey. & Scherb., and *C. aequitriloba* (Viv.) A.Chev., respectively, according to Bigazzi & Raffaelli (2000) and Güemes (2009). Second, we used the name *C. glutinosa* Bigazzi & Raffaelli,

comprising two subspecies, instead of *C. pilosa* auct., non (Jacq.) Grande, which was demonstrated to be an heterotypic synonym of *Sibthorpia europaea* L. (Bigazzi & Raffaelli, 2000). Finally, *Chaenorhinum pluttulum* Rech.f. should not be considered under *Cymbalaria* (*as C. pluttula* (Rech.f.) Speta), since the overall appearance of type material (W!), seed morphology (Sutton, 1988) and preliminary molecular analyses (P. Carnicero et al., unpublished data) showed a strong similarity with iranoturanian *Chaenorhinum* (DC.) Rchb. and the related genera *Holzneria* Speta and *Albraunia* Speta. Accordingly, it has not been not included in this thesis.

Cymbalaria encompasses several features that make it a good candidate for the study of some processes of main importance in plant evolution. On the one hand, its fragmented distribution, the coexistence of species in the same area with different ecological preferences and its diversity of ploidy levels, ranging from diploids to octoploids, suggest the contribution of different speciation types in its evolution. Second, it is distributed mostly in biodiversity hotspots and glacial refugia, areas of main interest in the Mediterranean Basin due to their important role in determining the current biodiversity in the region. The study of particular lineages that occur in these areas is crucial to understand the historic role of biodiversity hotspots and refugia as biodiversity "sources". Third, the paleogeographic and climatic history of the Mediterranean is well known, a crucial requirement for any biogeographical study aiming to combine phylogenetic data with climatic and geographic scenarios to infer evolutionary processes. Fourth, the high number of taxonomic uncertainties advocate for implementing molecular systematics studies combined with detailed studies of morphological features and multivariate statistics analyses to provide new evidence for a renewed taxonomic revision of the genus. Finally, its reduced number of species makes it a relatively simple study system, and its relatively young origin suggest that the evolutionary processes underlying its current diversity may still have a molecular signal.

Structure of the Thesis

This thesis is structured in four chapters formatted as scientific manuscripts. Chapter I is already published in Taxon (Carnicero et al., 2017). Chapter II has been submitted for publication, and chapters III and IV are in different processes of preparation.

In chapter I we aimed to infer the phylogeny of *Cymbalaria* and to describe the speciation processes that originated current species. We used a comprehensive sampling of all taxa accepted in the last taxonomic treatment of the genus (Sutton, 1988), except for *C. pluttula* for the reasons explained before. Two cp- and two nrDNA regions were sequenced for the purpose of molecular analyses. Phylogenetic inference was conducted for the cp- and nrDNA regions separately using MP and BI. We simultaneously inferred divergence times and phylogenetic relationships (BI) for the nrDNA using three calibration points and an uncorrelated lognormal clock. We conducted diversification analyses with the aim of identifying putative changes through time in the diversification rate of *Cymbalaria*. Genome duplication events were estimated using a chromosome evolution model. Historical biogeographic analyses were performed on the dated nrDNA tree and the best model fitting the data was selected with a likelihood test. Combining our results with information on the geomorphological and climatic history of the Mediterranean Basin and on the ecological preferences of species, we discuss the role of different speciation types in the evolution of *Cymbalaria*. Part of these results was presented in a poster in the 4th Meeting of the Spanish Society for Evolutionary Biology the 27th to 29th November, 2013, Barcelona. Chapter I served as a phylogenetic framework for subsequent chapters.

Chapter II is a phylogeographic study of a polyploid lineage identified in Chapter I. It is constituted by four western Mediterranean *Cymbalaria* species and we aimed to infer the number of LDD events between the eastern Balearic Islands and Corsica-Sardinia. Furthermore, we sought the taxonomic identity of some controversial Balearic populations. We used a very complete population sampling to perform

phylogeographic analyses based on AFLP fingerprinting and cpDNA sequences. We conducted multivariate analyses of morphological data (PCA and CDA) to detect morphological groups. Both sources of data (molecular and morphology) were used to evaluate the taxonomic rank and circumscription of the studied taxa. Part of these results was presented in a poster in the Systematics Association Biennial Conference (26^{th} to 28^{th} Agust, 2015, Oxford). They were also presented as a seminar at the Botanical Institute of Barcelona (28^{th} April, 2015) and at the Biosciences Conference celebrated at the Autonomous University of Barcelona (7th June, 2016). The manuscript has been submitted for publication in *Botanical Journal of the Linnean Society*.

In chapter III we aimed to study in detail one of the species studied in chapter II: the Sardinian endemic *Cymbalaria muelleri* (Moris) A.Chev. In the course of our study, we detected two groups of morphologically divergent populations. Using an increased number of populations and a large set of morphological characters, we aimed to test whether the two groups should be considered separate taxa, and to infer their origin. For this purpose, we used AFLP fingerprinting, multivariate analyses on quantitative morphological characters and relative genome size estimation. We used scanning electron microscopic images for detailed examination and description of seed and trichome characters. We finally propose a taxonomic treatment for the two subspecies recognized and an identification key for all Sardinian taxa. The manuscript is prepared for submission in *Plant Systematics and Evolution*.

In chapter IV we focused our attention on eastern Mediterranean taxa. The disagreement between previous taxonomic treatments based on morphology and the uncertainties stated by several authors advocated a revision using molecular data in addition to morphological data. Thus we performed a systematic study of eastern Mediterranean species using cp- and nrDNA sequences, morphological data and relative genome size measures. We specifically aimed to propose a new taxonomic treatment in agreement with new sources of evidence and to ascertain the identity of two populations with exclusive morphological characters that do not match any described taxon. Phylogenetic inference was conducted for the cp- and nrDNA regions separately using MP and BI, and multivariate analyses were

performed on quantitative morphological characters. Testa sculpturing was examined in detail using scanning electron microscopic images. We discuss our results in combination with information on the geomorphological and climatic history of the eastern Mediterranean region to describe the biogeographic history of the studied species. Finally, a new species and a revised taxonomic treatment for the whole group is proposed.

Objectives

- To test the monophyly and phylogenetic position of the genus *Cymbalaria* and to infer the phylogenetic relationships among species using cp- and nrDNA sequences.
- To infer the biogeographic history of the genus by estimating divergence times and ancestral areas in a phylogenetic framework, and discussing the role of climatic and geomorphological events in the diversification and distribution of species.
- To discuss the role of different speciation types in the origin of present diversity in *Cymbalaria* in the light of dating, phylogenetic and biogeographic results.
- To discuss the role of marine barriers and marine transgressions in the current distribution of *Cymbalaria* by studying the frequency of LDD and vicariance events in the context of Mediterranean paleogeographic history. Specifically, we aim to evaluate the validity of previous vicariance hypotheses concerning the distribution of western Mediterranean endemics and the role of previously described land bridges and marine barriers in the Aegean area.
- To study morphological characters and their correspondence with genetic lineages in order to detect diagnostic valuable characters. Specifically, to discuss the high diagnostic value attributed to seed characters.
- To integrate molecular data, morphology and ploidy levels to propose a revised taxonomic treatment, with special focus on poorly studied species and groups with patent incongruence in existing taxonomic treatments.

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Chapter I

Different speciation types meet in a Mediterranean genus: the biogeographic history of *Cymbalaria* (Plantaginaceae)[1]

[1] With Llorenç Sáez Gonyalons, Núria Garcia Jacas and Mercè Galbany Casals. Published in Taxon, 2017, vol. 66

Abstract

Cymbalaria comprises ten species and six subspecies growing in rocky habitats in the Mediterranean Basin. Several features, such as the genus' highly fragmented distribution as well as noticeable ecological differentiation between partially sympatric species and presence of ploidy barriers between species suggest the involvement of different speciation types in its evolution. The aims of this study were to test the monophyly of *Cymbalaria* and to reconstruct infrageneric phylogenetic relationships, to infer the genus' biogeographic history by estimating divergence times and ancestral distribution areas of lineages, and to disentangle the role of different speciation types. To address these issues, we constructed a phylogeny with a complete taxon sampling based on ITS, 3'ETS, *ndh*F and *rpl32-trn*L sequences. We used the nuclear ribosomal DNA data to produce a time-calibrated phylogeny, which served as basis for estimating ploidy level evolution and biogeographic history. *Cymbalaria* was resolved as monophyletic. The genus originated ca. 4 Ma and three lineages segregated rapidly, one comprising solely *C. microcalyx* subsp. *microcalyx* and the other two corresponding to western and central-eastern species, respectively. The main diversification events occurred after the onset of the Mediterranean climate and during Pleistocene climate oscillations. Both founder-event speciation linked to long-distance dispersal events and sympatric speciation were supported by the biogeographic analyses. In addition, at least two polyploid speciation events were inferred. Finally, conflicts between current taxonomy and the phylogeny at the species and subspecies level clearly show the need of more detailed integrative taxonomic studies.

Keywords Ancestral-area estimation, cpDNA, founder-event speciation, long-distance dispersal, molecular dating, nrDNA

Introduction

The Mediterranean Basin contains ca. 25,000 species, of which 63% are endemic (Greuter, 1991), and almost 10% of the world's vascular flora. Three primary types of speciation might have triggered this high diversity (Thompson, 2005). First, allopatric speciation is favoured in fragmented landscapes characterized by temporary events of land connection and isolation both in mainland and between mainland and the numerous islands in the Mediterranean Sea. Allopatric speciation is coupled with the effects of two major climatic events: the establishment of a Mediterranean climate approximately 3.2 million years ago Ma, which marked an increase in the rates of diversification for many plant lineages (Fiz-Palacios & Valcárcel, 2013), and the Pleistocene glaciations, which altered the distributions of species and favoured gene flow among populations in some species, whereas in other cases populations became isolated in climatic refugia (Vargas, 2003; Médail & Diadema, 2009). Second, sympatric ecological speciation has also been documented (Santos-Gally & al., 2011) and is favoured by the great heterogeneity of habitats and altitudinal gradients in relatively small areas. Third, polyploid speciation has been proposed for many Mediterranean plant groups (Thompson, 2005), probably related to the higher success of polyploids in the colonization of new niches (Ramsey, 2011).

Cymbalaria Hill (Plantaginaceae) is a genus of perennial herbs with ten species and six subspecies (Sutton, 1988; Bigazzi & Raffaelli, 2000), distributed throughout the Mediterranean Basin (Fig. 1). *Cymbalaria muralis* G. Gaertn., B. Mey. & Scherb., native to the central Mediterranean Basin, is naturalised almost worldwide in temperate areas (Sutton, 1988) and is therefore the most widespread species. The last complete systematic revision of the genus was carried out by Sutton (1988) who highlighted some taxonomic conflicts, mainly regarding eastern Mediterranean taxa. *Cymbalaria* has been included in molecular studies of tribe Antirrhineae (Ghebrehiwet & al., 2000; Vargas & al., 2004, 2013; Guzmán & al., 2015), but molecular analyses with a comprehensive sampling of the genus have never been performed. All *Cymbalaria* species grow in rocky habitats in a wide range of ecological conditions, from coastal

cliffs to rock crevasses in the subalpine belt. The rocky habitats and most of the areas currently occupied by *Cymbalaria* species are considered to have remained climatically stable during Pleistocene glaciations (Thompson, 2005; Médail & Diadema, 2009), suggesting an important role of climatic refugia in the evolutionary history of *Cymbalaria.* Geographic isolation might have played multiple roles in speciation, since some species are narrow endemics and is therefore likely that geographic isolation has prevented gene flow with other distant populations, while in species with very fragmented, disjunct, but broad distribution areas, geographic distance may have been overcome (Fig. 1). The last pattern could be caused by recent range expansion, extinction in intervening areas or by active gene flow among disjunct populations. Some species occur sympatrically but with well differentiated ecological preferences, likely suggesting the action of sympatric ecological speciation (Fig. 1, Table 1). Ploidy levels vary across species and are often geographically grouped (Fig. 1), ranging from diploids (2*n* = 14) to octoploids (2*n* = 56), supporting an important impact of polyploidy in driving speciation. Diploids mainly occur in the Apennine and Balkan Peninsulas, with one species in the eastern Mediterranean; tetraploids (2*n* = 28) occur in Sicily, the Balkan Peninsula and the eastern Mediterranean basin, and a group of hexa- to octoploids (2*n* = 42, 56) occurs in Corsica, Sardinia and the Balearic Islands. The aforementioned features make *Cymbalaria* an exemplary case for the study of plant

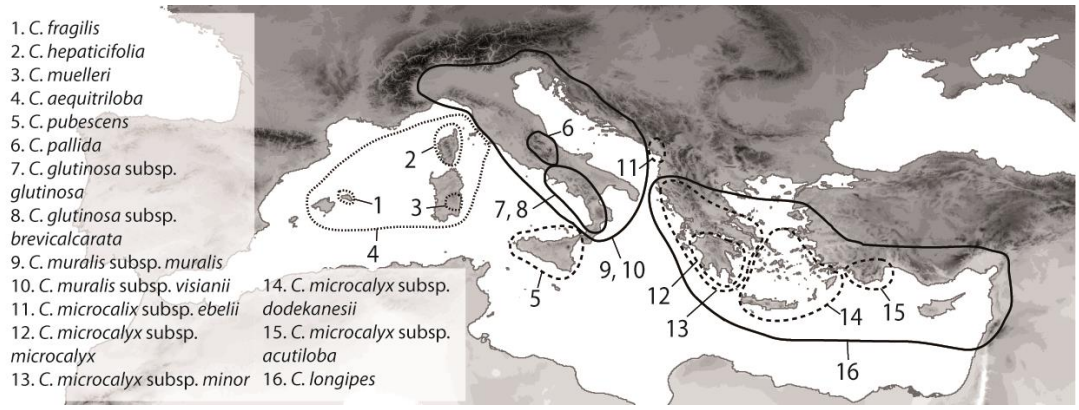


Figure 1. Distribution of *Cymbalaria* taxa, based on Sutton (1988), local Floras, personal field observations and herbarium vouchers. When information on the distribution areas of subspecies was not accurate or overlap was considerable, the general distribution area for the species is shown. For *C. muralis*, which is widely naturalized in temperate regions, the approximate natural distribution is shown. Different line formats indicate ploidy levels: solid line for diploids, dashed line for tetraploids and dotted line for hexa- to octoploids. Ploidy level for *C. microcalyx* subsp. *acutiloba* is not known.

Table 1. Chromosome number and ecology of the 16 sampled taxa.

Taxon	Chromosome number	Ecology
Cymbalaria aequitriloba (Viv.) A.Chev.	$2n = 56$[1]	Coastal and inland shadowed cliffs, moist rocks on stream banks
C. fragilis (J.J.Rodrig.) A.Chev.	$2n = 56$[2]	Coastal and inland shadowed cliffs
C. glutinosa Bigazzi & Raffaelli subsp. *glutinosa*	$2n = 14$[3]	Coastal and inland shadowed cliffs, walls
subsp. *brevicalcarata* Bigazzi & Raffaelli	$2n = 14$[3]	Coastal and inland shadowed cliffs, walls
C. hepaticifolia Wettst.	$2n = 56$[1]	High-elevation rocks, moist rocks and mountain stream banks
C. longipes (Boiss. & Heldr.) A. Chev.	$2n = 14$[1]	Coastal cliffs, rocks and walls
C. microcalyx (Boiss.) Wettst. subsp. *microcalyx*	$2n = 28$[4]	Inland shadowed cliffs, walls
subsp. *acutiloba* (Boiss. & Heldr.) Greuter	?	Inland shadowed cliffs
subsp. *dodekanesi* Greuter	$2n = 28$[5]	Inland shadowed cliffs
subsp. *ebelii* (Cufod.) Cufod.	$2n = 28$[6]	Inland shadowed cliffs, walls
subsp. *minor* (Cufod.) Greuter	$2n = 28$[1]	Inland shadowed cliffs
C. muelleri (Moris.) A. Chev.	$2n = 42$[7]	Inland overhanging cliffs
C. muralis G. Gaertn., B. Mey. & Scherb. subsp. *muralis*	$2n = 14$[1]	Inland shadowed cliffs, walls
subsp. *visianii* (Ják.) D.A.Webb	$2n = 14$[3]	Inland shadowed cliffs, walls
C. pallida Wettst.	$2n = 14$[1]	High-elevation rocks, mountain stream banks
C. pubescens (J.Presl & C.Presl) Cufod.	$2n = 28$[3]	Inland shadowed cliffs, walls

[1] Sutton (1988) and references therein; [2] Castro & Rosselló (2006); [3] Bigazzi & Raffaelli (2000); [4] Speta (1986); [5] P. Carnicero, unpublished data; [6] Speta (1989); [7] Onnis & Floris (1967).

speciation in the Mediterranean Basin, suggesting that several processes and types of speciation generated its current diversity and distribution.

Multi-locus molecular phylogenies, molecular dating, diversification analyses and ancestral area estimation models can be used to infer the biogeographic history of plants at different taxonomic levels (e.g. Calviño & al., 2016; Cardinal-Mc Teague & al., 2016; Janssens & al., 2016). The well-known geomorphological and climatic history of the Mediterranean Basin, together with the areas' high plant endemism and biodiversity, make it a very suitable and attractive area for reliable reconstructions of the spatio-temporal evolution of plant lineages (e.g., Gaudeul & al., 2016; Hardion &

al., 2016). Here we used plastid and nrDNA sequences to (1) verify the monophyly of *Cymbalaria* and to clarify the phylogenetic relationships among the species, (2) estimate the divergence dates of the lineages and infer the biogeographic history of the genus, and (3) examine the role of the different types of speciation in the evolution of *Cymbalaria.*

Materials and Methods

Plant Material

We sampled 34 individuals of *Cymbalaria*, representing all species and subspecies recognised in the last taxonomic treatments (Sutton, 1988; Bigazzi & Raffaelli, 2000; Appendix 1). Species from 13 additional genera representing the main lineages of the tribe Antirrhineae were also sampled to confirm the placement of *Cymbalaria* within the tribe and to assess its monophyly. *Plantago lanceolata* L. and *Veronica persica* Poir. were used as external outgroups since they have been shown to be closely related to the tribe Antirrhineae (Olmstead & al*.,* 2001).

DNA extraction, amplification and sequencing

To extract the DNA, the CTAB method (Doyle & Doyle, 1987), as modified by Cullings (1992) and Tel-Zur & al. (1999), and the commercial kit NucleoSpin® Plant were used (Macherey-Nagel GmbH & Co., KG, Düren, Germany).

We amplified the ITS region and the conserved 3'ETS region of the nuclear ribosomal DNA (nrDNA) and the *ndhF* region and the *rpl32-trnL*^{UAG} spacer of the plastid DNA (cpDNA). We used the primers ITS1 and ITS4 (Sun & *al.*, 1994) for the ITS region, Ast1 and 18SETS (Markos & Baldwin, 2001) for the 3'ETS region, 3'F (Eldenäs & *al.*, 1999) and +607 (Kim & Jansen, 1995) for the *ndhF* region and rpl32F and trnL^{UAG} (Shaw & *al.*, 2007) for the *rpl32-trnL* spacer. For some specimens, we designed and used specific internal primers for the *ndhF* region: (1) *ndhF* CymbF: 5' TGA ATC GGA CAA TAC CAT GTT ATT 3'; (2) *ndhF* CymbR: 5' ATT CAT ACC AAT TCG TCG AAT CCT 3'; (3) *ndhF* CymbF2: 5' ACG AGT AAT TGA TGG AAT TAC G 3'; and (4) *ndhF* CymbR2: 5' GAG TCT TAT CTG ATG AAT ATC 3'. The profile used for amplification of ITS included 4 min

denaturation at 95°C, followed by 30 cycles of 90 s denaturation at 94°C, 2 min annealing at 55°C and 3 min extension at 72°C, with an additional final step of 15 min at 72°C. The profile used for amplification of the *rpl32-trnL*^{UAG} spacer included 3 min denaturation at 94°C, followed by 30 cycles of 40 s denaturation at 95°C, 2 min annealing at 52°C and 2 min extension at 72°C, with an additional final step of 10 min at 72°C. We followed the PCR profiles described in Galbany-Casals & *al.* (2009) for ETS and Galbany-Casals & *al.* (2012) for *ndhF*. PCR products were purified with Exo-SAP-IT (USB Corp., Cleveland, Ohio, U.S.A.). Direct sequencing was conducted at the DNA Sequencing Core, CGRC/ICBR of the University of Florida, on an ABI 3730xl DNA Analyser (Applied Biosystems) using a Big Dye Terminator v.3.1 kit (Applied Biosystems, Foster City, CA, U.S.A.). See Appendix 1 and electronic supplement Table S1 for information on the vouchers and the sequences.

Phylogenetic analyses

The sequences were examined and aligned by hand using Chromas Lite 2.0 (Technelysium Pty Ltd., Tewantin, Australia) and Mega 6.06 (Tamura & al., 2013). The ambiguous regions of the alignments were manually excluded. Indels were coded as binary characters using the simple indel coding method (Simmons & Ochoterena, 2000) for the cpDNA alignment. The nrDNA alignment provided enough variation and indels were not coded. Plastid and nrDNA regions were analysed separately due to the phylogenetic incongruence found between the two genomes (see Results).

For both cp and nrDNA datasets, Maximum Parsimony (MP) analyses were conducted with PAUP*v.4.0a149 (Swofford, 2002), with 10,000 replicates of heuristic searches with random taxon addition and tree bisection-reconnection (TBR) branch swapping and holding all most parsimonious trees. The indels were coded as missing data, and the uninformative characters were excluded. The bootstrap analyses were performed with 1000 replicates, simple taxon addition and TBR branch swapping. Consistency Index (CI), Retention Index (RI) and Homoplasy Index (HI) were calculated from the consensus tree (Electr. Suppl.: Table S1).

PartitionFinder v.1.1.1 (Lanfear & al., 2012) was used to find the best model of evolution and the best partitioning scheme under the Bayesian information criterion

(BIC; Schwarz, 1978) for the Bayesian Inference (BI) analyses. All loci were defined as unique partitions and the models tested were those implemented in BEAST for nrDNA and MrBayes for cpDNA. A greedy search algorithm was selected for running the analysis for each dataset. The BI analysis of the cpDNA sequences was conducted with MrBayes v.3.2 (Ronquist & al., 2012). For the analysis of the coded indels of the rpl32 the simplest possible model, i.e. the Jukes Cantor model, was used. We generated 10,000 trees running MrBayes for 5,000,000 generations and sampling one of every 500 generations. After ensuring that the Monte Carlo Markov chain (MCMC) reached stationarity, we discarded the first 2500 trees as burn-in.

Divergence time estimation

The dating analysis was performed using the nrDNA sequences because of the low resolution obtained with the cpDNA sequences. The incongruence found between the plastid and nrDNA phylogenies also suggested that a combined analysis was not appropriate, and the lack of multi-individual sampling for some species prevented us from using a species tree approach (Heled & Drummond, 2010). Using a fully resolved multi-locus phylogeny would be desirable (Maddison & Knowles, 2006), but molecular dating based on nrDNA has been successfully used in cases of low levels of polymorphism of cpDNA markers and incongruence between plastid and nrDNA markers (e.g., Gao & al., 2015; Nie & al., 2015; Calleja & al., 2016). After a preliminary analysis using all the specimens sampled (Electr. Suppl.: Fig. S1), we pruned the data set to include only one specimen per taxon to represent the cladogenetic events that resulted in speciation or different genetic lineages. Accordingly, for *C. aequitriloba* (Viv.) A.Chev., we included three individuals representing three genetic lineages (see Results): *C. aequitriloba* 1 represented the Corsican lineage; *C. aequitriloba* 3 the Balearic lineage; and *C. aequitriloba* 5 the Sardinian lineage.

The dating analysis was performed using a relaxed molecular clock as implemented in BEAST v1.8.2 (Drummond & Rambaut, 2007). The importance of using multiple calibration points in relaxed molecular clock dating has been often highlighted as crucial for obtaining reliable age estimates (Ho & Phillips, 2009; Sauquet, 2013; Duchêne & al., 2014). Accordingly, calibration of the tree was conducted based on

three calibration points (CP). CP1: Following the recommendation to use deep calibration points to capture a larger proportion of the overall genetic variation (Duchêne & al., 2014), we defined a secondary calibration point for the root node from a phylogenetic study of the tribe Antirrhineae that used five fossil-based calibration points (Vargas & al., 2013). Accordingly, we defined a normal prior probability distribution with mean 40.1 Ma and a standard deviation of 5 Myr. Since the monophyly of the tribe Antirrhineae is indisputable (Ghebrehiwet & al., 2000; Vargas & al., 2004; Albach & al., 2005), we constrained the tribe as monophyletic. CP2: We used the fossil *Plantaginacearumpollis* (Nagy, 1963) to set an absolute minimum age of divergence between *Plantago* L. and *Veronica* L., following Vargas & al. (2013). It dates to the Sarmatian (Upper Middle-Miocene; 12.8–11.6 Ma, Harzhauser & Piller, 2004) and has been used as a calibration point in previous studies (Thiv & al., 2010; Vargas & al., 2013). We used the upper limit of the stratigraphic interval in which the fossil was found (i.e. 11.6 Ma) as the zero offset of the prior probability distribution following Sauquet (2013). In order to assign the highest point probability for the node age somewhat older than the fossil (Ho & Phillips, 2009), we set a lognormal distribution with mean equal to the fossil age plus 10% (13.2 Ma, Magallón & al., 2015) and a log-standard deviation of 0.59, so that 95% of the probability distribution is younger than a soft maximum bound of 40.1 Ma (age of the root, Ho & Phillips, 2009; Warnock & al., 2011). CP3: We defined a third calibration point in a node close to the origin of *Cymbalaria* in order to obtain better age estimates for the main diversification events in *Cymbalaria* (Linder & al., 2005; Ho & Phillips, 2009; Duchêne & al., 2014). Thus, the divergence time between the clade *Maurandya* [*Epixiphium wislizeni* (A. Gray) Munz, *Maurandya antirrhiniflora* Humb. & Bonpl. and *Lophospermum erubescens* D. Don] and the clade *Asarina procumbens* Mill. – *Cymbalaria* was modelled as a normal distribution with a mean of 20.8 Ma and a standard deviation of 4.4 Myr, as calculated from the posterior distribution of trees from Vargas & al. (2013).

The clock model selection can have a strong impact on the results of a dating analysis, and a rigorous selection of the appropriate model is therefore crucial (Duchêne & al., 2014). Here we tested two types of relaxed uncorrelated models: a lognormal and an exponential clock (Drummond & al., 2006). Strict clocks and

autocorrelated models were initially excluded since relaxed uncorrelated models have been shown to perform well even in datasets simulated under a different model (Ho & al., 2005, Drummond & al., 2006; Brown & Yang, 2011). Additionally, we tested two speciation models: Yule (pure-birth; Yule, 1924) and birth-death process (Gernhard, 2008). For the purpose of model selection, we run preliminary analyses and performed marginal likelihood estimations for each model using two sampling strategies: stepping-stone (Xie & al., 2011) and path sampling (Ogata, 1989; Gelman & Meng, 1998; Lartillot & Philippe, 2006). These have been shown to outperform other marginal likelihood estimators and are implemented in BEAST v1.8.2 (Baele & al., 2012; 2013). We calculated the Bayes factors (BF) from the marginal likelihood estimates (Jeffreys, 1935, 1961) and selected an uncorrelated lognormal model with a birth-death speciation process after comparing the values of 2log(BF) according to Kass & Raftery (1995).

Four MCMCs were run for 20 x 10^6 generations, sampling trees every 1,000 generations. Details of the model are in the BEAST .xml file (Electr. Suppl). An additional run with identical conditions but without sequence data (sample from prior) was performed in order to check the marginal prior distributions of the calibrated nodes (Heled & Drummond, 2012). The marginal and posterior probability distributions for the root and the *Veronica-Plantago* node showed highly coincident distributions. For the third calibration point, distributions were noticeably distinct: the posterior probability distribution shifted towards the present with respect to the marginal probability distribution, although a large overlap between distributions still existed. Accordingly we performed an additional analysis excluding the third calibration point, from which essentially results identical to the previous analysis with three calibration points were obtained (not shown). As this change of a parameter of the model did not alter the result, we assumed that the model is solid and reliable. Moreover, the comparison of the two models using BF (see above) supported the use of the three calibration points, and therefore we preferred to use the three calibration points as recommended in the literature (Linder & al., 2005; Ho & Phillips, 2009; Duchêne & al., 2014). We verified the convergence of runs in Tracer v1.6.0 (Rambaut & al., 2013) by checking that effective sample size values were higher than 200. The trees were

combined with LogCombiner v1.8.2 after discarding the first 25% of the trees as burn-in. We summarized the output in a maximum clade credibility (MCC) tree selecting median ages as node heights with TreeAnnotator v1.8.2.

Diversification analyses

We used 1000 trees randomly sampled from the posterior distribution of trees obtained from the dating analysis and the MCC tree as input files. In order to study the diversification of *Cymbalaria*, we cropped the input trees to contain only the *Cymbalaria* clade, which was monophyletic with high support (see Results). To represent diversification through time, we used the R-package APE 3.3 (Paradis & al., 2004) to construct lineage-through-time (LTT) plots. We used the $dAIC_{RC}$ test (Rabosky, 2006a) as implemented in the R-package LASER (Rabosky, 2006b) to infer whether the diversification rate changed over time. We tested the observed value of $dAIC_{RC}$ against a null distribution of $dAIC_{RC}$ values obtained from 1000 random phylogenetic trees generated under the constant rate pure birth model.

Ploidy data and mapping ploidy change

Chromosome numbers for *Cymbalaria* species were mostly obtained from the literature (Table 1). Although hexa- and octoploid counts have been reported for *C. aequitriloba*, we only considered the octoploid level ($2n$ = 56), since the original reference for the hexaploid counts (Heitz, 1927) does not report any chromosome number for *C. aequitriloba*, and we were unable to trace any other hexaploid count in the literature.

We used chromEvol v2.0 (Glick & Mayrose, 2014) to estimate changes in the ploidy level and their phylogenetic position. We used the MCC tree obtained from the dating analysis as input file after excluding all outgroups. The best chromosome number evolutionary model was selected by obtaining the maximum likelihood scores of ten alternative models, and comparing them using the AIC. The model selected allowed for separate rates of polyploidisation and demi-polyploidisations (multiplication of the chromosome number by a factor of 1.5), as well as separate rates of individual chromosome losses and gains. It was afterwards used to map polyploid events on the tree using both ML and Bayesian approaches, performing 10,000 simulations. We set

the initial parameters as 'gainConstR' 0.5, 'lossConstR' 0.5, 'duplConstR' 0.5 and 'DemiPloidyR' 0.5.

Ancestral-area estimation

We used the MCC tree keeping *Asarina* Mill. as outgroup taxon, since species of the *Maurandya* clade occur only in the New World, and therefore their effect on the ancestral area estimation of *Cymbalaria* would have been negligible. An extra analysis was run with the ingroup only, since the distribution areas of *Asarina* and *Cymbalaria* are completely disjunct, and we expected interferences in the estimates for deep nodes. Although the exclusion of the outgroup can have a negative effect on the estimation of ancestral areas (e.g. Ronquist, 1997), other authors stated that too widespread outgroups may be problematic (Yu & al., 2015), and finally some studies showed little difference between the two approaches (e.g., Xiang & Thomas, 2008; Emata & Hedin, 2016). We considered eight and nine areas, respectively, for the ingroup-only and the outgroup-rooted analyses (Fig. 2, Electr. Suppl.: Fig. S2) based on previously defined biogeographic patterns (Takhtajan, 1986; Rivas-Martínez & al., 2004) and on the endemism and distribution patterns of *Cymbalaria*. These areas are: Balearic Islands (A), Corsica and Sardinia (B), southern Apennine Peninsula (C), Sicily (D), northern Apennine and Balkan Peninsulas (E), southern Balkan Peninsula (F), Aegean Islands (G), Anatolia, Lebanon and Syria shores (H), Eastern Pyrenees and Massif Central (I).

We performed the biogeographic analysis with BioGeoBEARS (Matzke, 2013). This R-package implements six biogeographic models in a common likelihood framework: a likelihood version of Dispersal-Vicariance analysis (DIVALIKE; Ronquist, 1997), LAGRANGE Dispersal and Extinction Cladogensis (DEC) model (Ree & al., 2005; Ree & Smith, 2008), a likelihood version of BayArea (Landis & al., 2013), and an alternative version for each of the models that includes founder-event speciation (+J). BioGeoBEARS has two primary advantages compared with other biogeographical programs: 1) the best model is selected with likelihood ratio tests, and 2) founder-event speciation is included, a process ignored by most other methods.

The maximum number of areas for each node was set to three, which is the

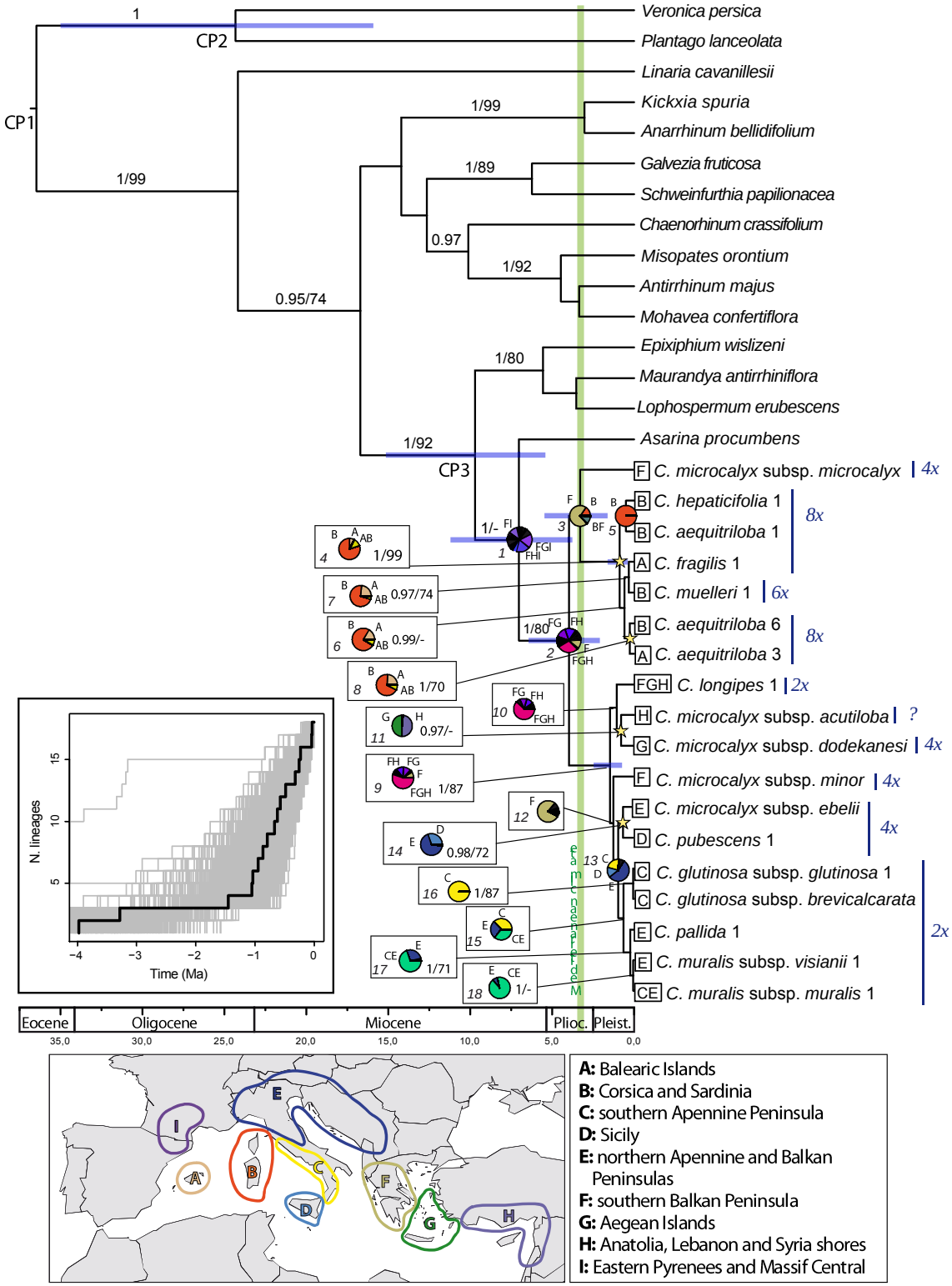
maximum number of areas occupied by extant taxa (Ronquist, 1996; Hilpold & al., 2014). Each terminal node in the tree was coded with the total distribution area of the taxon/lineage, except for *C. muralis* that was only coded for its natural distribution area: the Apennine and northern Balkan Peninsulas (C, E). We defined a dispersal probability matrix to determine the effect of geographic distance on dispersal ability. The rate of dispersal between western (Fig. 2; B, C) and eastern Mediterranean areas (Fig. 2; F, G, H) was set to 0.5 following Hilpold & al. (2014) and was set to 1 for the other cases, to reflect the low probabilities of dispersing from eastern to western Mediterranean areas without an intermediate station in the central Mediterranean areas (Fig. 2; C, D, E). We considered the distribution area of *Asarina* (Fig. 2; I) isolated enough from the rest to set a rate of dispersal of 0.5 between it and all other areas. We ran the six models and after testing them with a likelihood ratio test and the Akaike Information Criterion (AIC), the DEC+J model was selected.

Results

Phylogenetic analyses

The analyses of the nrDNA (Fig. 2, Electr. Suppl.: Table S1, Fig. S1) with MP and BI resulted in congruent phylogenetic tree topologies. *Cymbalaria* was recovered as a monophyletic genus [Fig. 2, Bayesian posterior probability (PP) = 1; bootstrap support (BS) = 80%] sister to *A. procumbens* (PP = 1), and these two genera together were sister (PP = 1; BS = 92%) to the clade *Epixiphium* **–** *Lophospermum* **–** *Maurandya* clade (PP = 1; BS = 80%). Two main lineages were obtained within *Cymbalaria*, composed of the central and eastern Mediterranean species (central-eastern lineage, node 9, PP = 1; BS = 87%) and the western Mediterranean species (western lineage, node 4, PP = 1; BS = 99%), respectively. *Cymbalaria microcalyx* (Boiss.) Wettst. subsp. *microcalyx* was sister to the western lineage without statistical support (PP = 0.68).

The analyses of the cpDNA with MP and BI resulted in a congruent topology (Fig. 3, Electr. Suppl.: Table S1). *Cymbalaria* was monophyletic (PP = 1; BS = 77%) and grouped with *A. procumbens* (PP = 1; BS = 76%) and these two genera with *E. wislizeni* (PP = 1; BS = 95%). The phylogenetic position of *Linaria cavanillesii* Chav. was incongruent with


Veronica persica
Plantago lanceolata
Linaria cavanillesii
Kickxia spuria
Anarrhinum bellidifolium
Galvezia fruticosa
Schweinfurthia papilionacea
Chaenorhinum crassifolium
Misopates orontium
Antirrhinum majus
Mohavea confertiflora
Epixiphium wislizeni
Maurandya antirrhiniflora
Lophospermum erubescens
Asarina procumbens
CP1
CP2
CP3
1
1/99
1/99
1/89
0.97
1/92
0.95/74
1/80
1/92
F C. microcalyx subsp. microcalyx | 4x
B C. hepaticifolia 1
B C. aequitriloba 1
8x
A C. fragilis 1
B C. muelleri 1 | 6x
B C. aequitriloba 6
8x
A C. aequitriloba 3
FGH C. longipes 1 | 2x
H C. microcalyx subsp. acutiloba | ?
G C. microcalyx subsp. dodekanesi | 4x
F C. microcalyx subsp. minor | 4x
E C. microcalyx subsp. ebelii
4x
D C. pubescens 1
C C. glutinosa subsp. glutinosa 1
C C. glutinosa subsp. brevicalcarata
E C. pallida 1
2x
E C. muralis subsp. visianii 1
CE C. muralis subsp. muralis 1
N. lineages
Time (Ma)
Eocene
Oligocene
Miocene
Plioc.
Pleist.
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
A: Balearic Islands
B: Corsica and Sardinia
C: southern Apennine Peninsula
D: Sicily
E: northern Apennine and Balkan Peninsulas
F: southern Balkan Peninsula
G: Aegean Islands
H: Anatolia, Lebanon and Syria shores
I: Eastern Pyrenees and Massif Central

⬅ **Figure 2.** Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA for *Cymbalaria* using BEAST v.1.8.2. Calibration points are indicated and numbered as described in Materials and Methods as CP1, CP2 and CP3. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of the clades that are discussed in the text. Bayesian posterior probabilities ≥ 0.95/bootstrap support values ≥ 70% are indicated. Ploidy levels are indicated to the right of the taxon names. Numbers in italics below nodes indicate the node number. Pie charts at each node show the marginal probabilities of alternative ancestral ranges obtained from the BioGeoBEARS analysis. Letter codes for each area inferred and distribution areas at present are indicated at the nodes and terminals, respectively. Black segments in pie charts represent ancestral ranges with a probability < 10%. Stars show statistically supported clades resulting from polyploid events as inferred in ChromEvol v.2.0. The inset shows a lineage-through-time plot for *Cymbalaria* based on 1000 trees randomly sampled from the posterior distribution of the dating analysis of dataset 3 (see text). The thick line corresponds to the MCC tree.

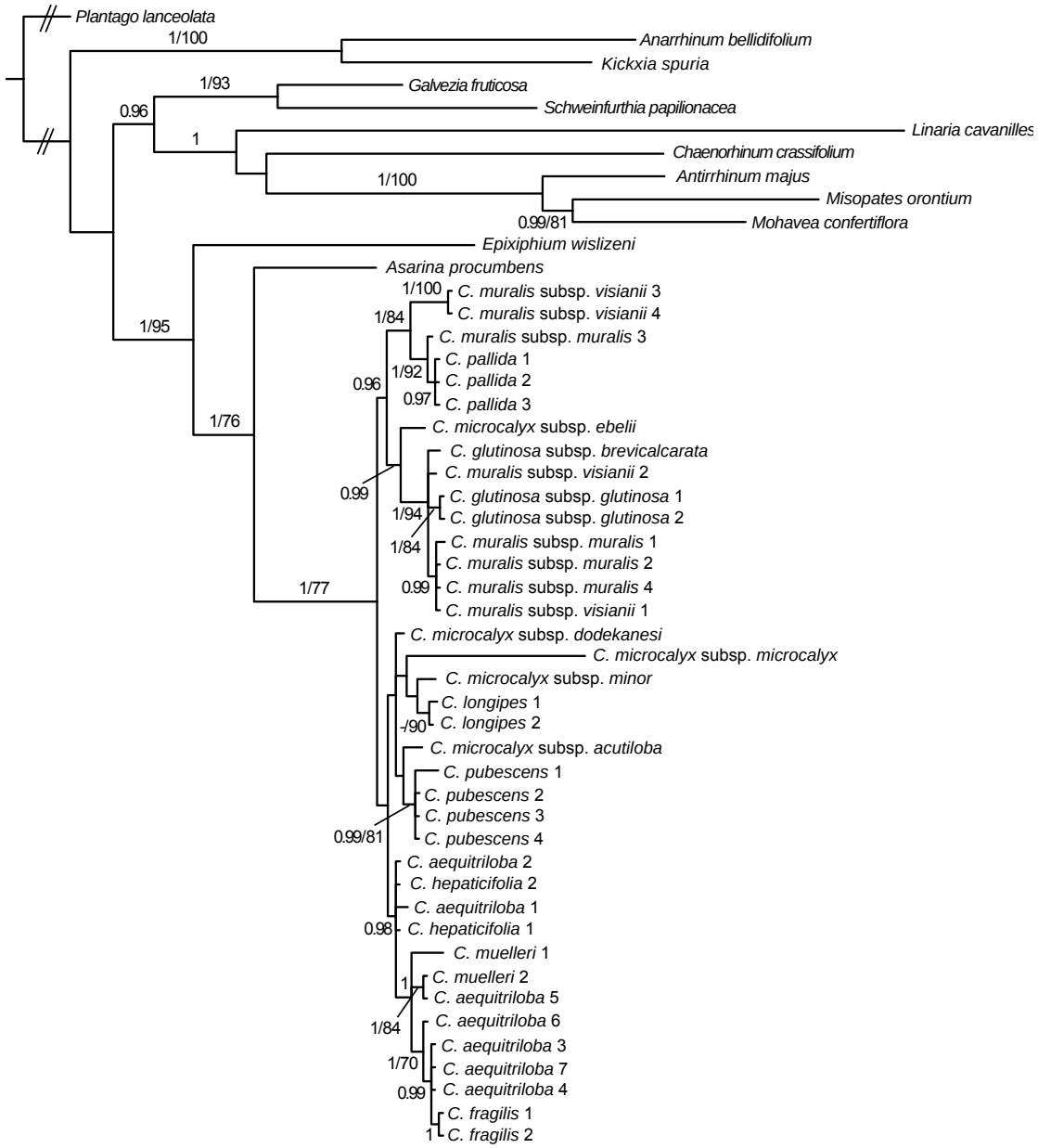


Figure 3. Phylogram from the Bayesian analysis of cpDNA of *Cymbalaria*. Bayesian posterior probabilities ≥ 0.95/bootstrap support values ≥ 70% are indicated. The double slashes at the base of the tree indicate that respective branches have been manually shortened.

the nrDNA analyses, but congruent with previous cpDNA phylogenies (Ghebrehiwet & al., 2000; Vargas & al., 2013). Resolution at the species level was lower compared to the nrDNA analyses and a few incongruences were detected. In the cpDNA analysis *C. microcalyx* subsp. *ebelii* (Cufod.) Cufod. was grouped with *C. glutinosa* Bigazzi & Raffaelli, *C. muralis* and *C. pallida* Wettst., (Fig. 3 PP = 0.96) while in the nrDNA analyses it formed a clade with *C. pubescens* (J.Presl & C.Presl) Cufod (Fig. 2, PP = 0.98; BS = 72%). Slightly incongruent phylogenetic relationships were also obtained in the western lineage. For the taxa with two or more sampled specimens, only *C. glutinosa*, *C. pallida* and *C. pubescens* were monophyletic in both the plastid and nrDNA data sets (Fig. 3, Electr. Suppl.: Fig. S1).

Divergence time estimation and diversification analyses

Cymbalaria diverged from *Asarina* in the upper Miocene (Fig. 2, node 1, 7.01 Ma, 3.77–11.2 Ma 95% HPD). The first diversification within *Cymbalaria* took place 3.97 Ma (node 2, 2.06–6.43 Ma 95% HPD). The first cladogenetic events in the central-eastern and western lineages occurred 1.45 Ma (node 9, 0.73–2.46 Ma 95% HPD) and 0.86 Ma (node 4, 0.37–1.6 Ma 95% HPD), respectively. Although the LTT plot apparently showed an increase of diversification towards the present, the $dAIC_{RC}$ test did not reject a constant rate of diversification (p-value = 0.99).

Mapping ploidy change

According to the model selected, six polyploidisation events were estimated, one of which involved demi-polyploidisation (Electr. Suppl. Fig. S3). However, the lack of statistical support at some deep nodes and lack of knowledge of the reticulate processes affecting the evolution of *Cymbalaria*, strongly suggested to interpret this result with caution. Here we only discuss polyploidisation events coincident with statistically supported nodes.

Ancestral-area estimation

The ingroup-only and outgroup-rooted analyses resulted in almost identical estimations. Here we only comment the results for the statistically supported nodes. However, since several nodes showed low statistical support, area estimation for

surrounding nodes should be interpreted with caution (Nylander & al., 2008). The estimated area with highest probability at each node was the same in both analyses, and only slight differences (≤ 11%) in the probability of each area estimated were detected. Many different areas were recovered with similar probability values for the ancestral area of the MRCA of *Asarina* and *Cymbalaria* (Fig. 2, node 1), but the majority (56%) involved a striking combination of eastern Mediterranean areas with the present distribution area of *Asarina* in the western Mediterranean, a disjunct distribution not observed in any extant taxon. The ancestor of all *Cymbalaria* species was most probably widespread in the eastern Mediterranean area (Fig. 2, node 2, P(FGH) = 28%), although several combinations of narrower eastern areas had also remarkably high probabilities (Fig. 2, node 2, P(FG) = 14%, P(FH) =14%, P(F) = 11%). The MRCA of the west lineage was most probably distributed in Corsica-Sardinia (Fig. 2, node 4, P(B) = 83%), and two dispersal events to the Balearic Islands were inferred at nodes 7 and 8. A combination of the three eastern Mediterranean areas was estimated as the most probable distribution for the ancestor of the centre-east lineage (node 9, P(FGH) = 55%). A dispersal event between the Aegean Islands and Anatolia was inferred for the split between *C. microcalyx* subsp. *acutiloba* (Boiss. & Heldr.) Greuter and *C. microcalyx* subsp. *dodekanesi*, although the direction and origin of the dispersal event was not clearly estimated [node 11, P(H) = 0.49, P(G) = 0.49]. The MRCA of *C. pubescens* and *C. microcalyx* subsp. *ebelii* was probably distributed in the northern Apennine and Balkan Peninsulas (node 14, P(E) = 68%), and subsequent dispersal to Sicily led to the origin of *C. pubescens*. The MRCA of the two *C. glutinosa* subspecies occurred in the southern Apennine Peninsula, the area they currently occupy (node16, P(C) = 100%). The ancestor of *C. muralis* and *C. pallida* was most probably distributed in the Apennine and northern Balkan Peninsulas (node 17, P(CE) = 70%), and subsequent narrow sympatric speciation events gave rise to *C. pallida* and *C. muralis* subsp. *visianii*.

Discussion

The origin of *Cymbalaria* and early diversification

Based on our results, *Cymbalaria* split from *Asarina* in the late Miocene-Pliocene (Fig. 2). This relationship is congruent with the cpDNA analysis (Fig. 3), as well as with previous studies with both plastid and nrDNA (Ghebrehiwet & al., 2000; Vargas & al., 2004, 2013). The east-west disjunct distribution inferred for the ancestor of *Asarina* and *Cymbalaria* is highly questionable. *Asarina* shows features of a relict taxon, i.e. taxonomic isolation (it is a monospecific genus), geographic isolation from its sister taxon (*Cymbalaria*) and low intraspecific morphological variation (Favarger & Contandriopoulos, 1961; Mansion & al., 2008). This may indicate that its closest relatives became extinct and/or that the present distribution is a refugial area derived from range contraction. Therefore, the present distribution of *Asarina* would not be representative enough to describe the ditribution of its ancestor, a problematic situation for biogeographic inference (Lieberman, 2002; Matzke, 2014). The high number of areas estimated with low probability at this node (Fig. 2, node 1) may reflect this uncertainty.

Cymbalaria began to diversify around the establishment of Mediterranean climate, supporting the role of this climatic event as a trigger for diversification of many Mediterranean plant lineages (Fiz-Palacios & Valcárcel, 2013). Eastern Mediterranean areas were estimated as the ancestral distribution of *Cymbalaria*. However, this result could be highly influenced by the low resolution obtained for some deep nodes (Fig. 2, nodes 3, 10 & 12), which resulted in some eastern taxa originating from basal, statistically poorly supported nodes in the phylogeny. Instead, the high number of diploid species found in the Apennine and northern Balkan Peninsulas suggest the central Mediterranean as a plausible area of origin for *Cymbalaria*, since areas with higher ploidy levels have commonly been considered the result of more recent colonization (e.g. Garcia-Jacas & Susanna, 1992). Although there are methods that run biogeographic analyses on multiple trees aiming at accounting for phylogenetic uncertainty (Nylander & al., 2008; Beaulieu & al., 2013), these are not fully implemented in BioGeoBEARS. Moreover, Matzke (2016) pointed to some caveats of

these approaches, mostly concerning the assumptions made about the identity of nodes across phylogenetic trees with different topology. Instead, in the future more effort should be made to obtain fully resolved phylogenetic trees that would provide solid biogeographic estimations.

The low resolution observed at the basal nodes of *Cymbalaria* in the nrDNA tree might reflect rapid diversification periods (Riina & al., 2013; Vitales & al., 2014). However, the $dAIC_{RC}$ test did not reject a constant rate of diversification since the origin of the genus. The pattern of increased diversification towards the present observed in the LTT plot could be explained by the "pull of the present" phenomenon (Nee & al., 1994; Kubo & Iwasa, 1995). A constant extinction rate can result in an excess of recently diverged lineages that could lead to the wrong conclusion of an increase of the diversification rate (Nee, 2001). This phenomenon is also the reason why detecting increases in the diversification rate is more difficult than decreases, and therefore results should be interpreted with caution (Rabosky, 2006a).

The diversification of lineages

The diversification of the two observed lineages occurred after the onset of the Mediterranean climate (Fig. 2). In this particular case, the Mediterranean climate likely favoured isolation of *Cymbalaria* populations in small, relatively humid and/or shadowed areas, favouring allopatric speciation events. This may have been enhanced by the rupestrian habitat occupied by all extant species, which also favours isolation because of the scarcity and discontinuous nature of this type of habitat (Table 1, Thompson, 2005).

The ancestral area of the centre-east lineage was estimated in the eastern Mediterranean, although this can be highly influenced by the weakly-supported grouping of the eastern *C. microcalyx* subsp. *minor* with central species (Fig. 2, node 12). A genetic split between eastern and central species would be expected from phylogeographic studies of plant groups with a similar central-eastern Mediterranean distribution (e.g. *Cerastium dinaricum* Beck & Szyszył.: Kutnjak & al. 2014; *Edraianthus graminifolius* A.DC.: Surina & al., 2014). A high similarity between the northern Balkan Peninsula and the Apennine Peninsula is also suggested by the circumscription of

floristic provinces (Takhtajan, 1986). However, the lack of resolution obtained from our data did not allow for testing this hypothesis.

The central Mediterranean species grouped in three supported clades. The clades *C. glutinosa* (Fig. 2, node 16) and *C. muralis – C. pallida* (Fig. 2, node 17) are diploid taxa of partially sympatric distribution and divergent ecological requirements: *Cymbalaria glutinosa* occurs in warm Mediterranean areas in the southern half of the Apennine Peninsula, whereas *C. pallida* and *C. muralis* occupy northern, wetter and cooler places in the Apennine Peninsula that extend to the northern Balkan Peninsula in the case of *C. muralis* (Pignatti, 1982; Fig. 1, Table 1). In the same line, whereas *C. muralis* occupies humid lowlands, *C. pallida* is endemic to the highest elevations of the Apennine Range (Pignatti, 1982; Fig. 1, Table 1). In both cases, sympatric speciation was inferred in the ancestral area estimation analysis (Fig. 2). The third clade is composed of the tetraploids *C. pubescens* and *C. microcalyx* subsp. *ebelii* (Fig. 2, node 14). Their common ancestor was inferred to have been present in the northern Apennine and Balkan Peninsulas, from where dispersal to Sicily and further isolation led to the origin of *C. pubescens*, a route also proposed for other plant groups (e.g. *Centaurea cineraria* L. group: Hilpold & al., 2011; *Edraianthus graminifolius*: Surina & al., 2014). However, according to the cpDNA phylogeny (Fig. 3), *C. microcalyx* subsp. *ebelii* is closely related to other central Mediterranean taxa, but not to *C. pubescens*.

The subspecies of *Cymbalaria microcalyx*, all endemic to eastern Mediterranean areas, did not form a monophyletic group. The position of *C. microcalyx* subsp. *microcalyx* as weakly supported sister to the west lineage seriously challenges its current taxonomic assigment. Regarding the taxa included in the central-eastern lineage, the only supported monophyletic group was formed by *C. microcalyx* subsp. *dodekanesi* and subsp. *acutiloba* (Fig. 2, node 11). Founder-event speciation was inferred for the split between the two subspecies, although the direction of the dispersal event was not clear. This could be explained by land connections between the Aegean Islands and the mainland during the Pleistocene climatic oscillations, which led to range expansions and subsequent allopatric speciation events when the sea level increased (Polunin, 1980). By contrast, fluctuations in sea level did not have a similar effect on *C. longipes* (Boiss. & Heldr.) A.Chev. This species is widely distributed on

coastal cliffs of the Aegean region with apparent adaptations to marine dispersal (Sutton, 1988), which would lead to continuous gene flow, reducing the effect of marine isolation.

A Corso-Sardinian origin for the west lineage during the Pleistocene was supported (Fig. 2, node 4). Founder-event speciation was reconstructed for *C. fragilis* (J.J.Rodr.) A.Chev. after a long-distance dispersal (LDD) event from Corsica-Sardinia to the eastern Balearic Islands (Fig. 2, node 7). At least one more LDD event was inferred for the range expansion of *C. aequitriloba* to the Balearic Islands (Fig. 2, node 8). These two areas were last connected approximately 20 Ma (Speranza & al., 2002), and therefore, a vicariant alternative to the LDD event (suggested by Verlaque & al., 1993) must be rejected. Long-distance dispersal events were previously invoked to explain the origin of some of the endemic plant species with disjunct Balearic-Corso-Sardinian distribution (e.g. *Thymus herba-barona* Loisel.: Molins & al*.,* 2011). Moreover, Nieto Feliner (2014) reported that LDD events have not been rare in the Mediterranean, even when no particular adaptations for seed dispersal exist. The success in colonization of new areas is more often linked to pre-adaptations of genotypes and availability of suitable habitats than to geographic distance (Alsos & al., 2007). Polyploidy may have been a key trait in the colonization processes because it potentially provided an increased ability to tolerate a wide range of ecological conditions (Ramsey, 2011).

Speciation

Three primary types of speciation likely occurred throughout the evolution of *Cymbalaria*, i.e. allopatric, sympatric and polyploid speciation.

Allopatric speciation is inferred when sister taxa occupy different areas isolated by physical barriers*.* The two main types of allopatric speciation are vicariance and founder-event speciation. In historical biogeography, vicariance has long been recognized as a key process in diversification (Ronquist, 1997), and implies that a widely distributed ancestor gives rise to two or more separate species within its original distribution area when the appearance of a physical barrier promotes their reproductive isolation. However, in *Cymbalaria*, vicariance was not inferred for any statistically supported node. By contrast, founder-event speciation has been a mostly

ignored process in historical biogeographical models but is currently recognized as an essential process in biogeography (Gillespie & al., 2012; Matzke, 2013). It involves rapid divergence of a small, peripheral population of a species originated from a dispersal event (Futuyma, 2005), and is inferred when the area of one of the descendants is not part of the ancestor's distribution area. Indeed, the selection of the DEC+J model indicated that founder-event speciation (parameter J) was important for the model to fit our data. Our results supported founder-event speciation in three cases: the origin of *C. pubescens* (Fig. 2, node 14), the split between *C. microcalyx* subsp. *acutiloba* and *C. microcalyx* subsp. *dodekanesii* (Fig. 2, node 11), and the origin of *C. fragilis* (Fig. 2, node 7). The last case shows the typical structure of a founder-effect speciation event, where the new species (*C. fragilis*) is embedded in a more widely distributed and genetically variable, paraphyletic species (Futuyma, 2005), in this case *C. aequitriloba* (Fig. 2). For *C. fragilis*, LDD was inferred (see subsection: The diversification of lineages), whereas in other cases, the low sea levels during the Pleistocene glaciation periods may have favoured stepping stone dispersal (e.g., Campanulaceae: Cellinese & al., 2009; *Centaurea cineraria* group: Hilpold & al., 2011).

The DEC+J model inferred sympatric speciation in six statistically supported clades (Fig. 2, nodes 4- 6, 16-18). However, geographical and ecological isolation are not mutually exclusive, and their effects are difficult to disentangle (Papadopulos & al., 2014). Most of the inferred cases of sympatric speciation in our results could be interpreted as artefacts of the resolution used when defining the areas. For example, the split between the Corsican lineage (*C. aequitriloba* 1 and *C. hepaticifolia* 1) and the other taxa within the west lineage (Fig. 2, node 3) might have been a case of geographical isolation of this island from Sardinia. Moreover, geographical isolation can also occur at a local scale, particularly for plants that grow in rocky habitats as *Cymbalaria* (Thompson, 2005). However, in groups of taxa where gene flow is possible due to long distance dispersal, the recognition of putative geographic barriers is a difficult task. An additional impediment is that distribution areas can change over time, and currently sympatric species could have originated allopatrically and later expanded their areas to become sympatric. Apart from these limitations, to infer sympatric ecological speciation, it is essential to demonstrate that adaptation to the different

ecological niches exists and that this is the cause of reproductive isolation, and assume that ecological niches have not changed significantly from speciation until the present (Carine & Schaefer, 2009). Local scale environmental data would be required to properly describe ecological niche in the case of *Cymbalaria*, since the habitats where they occur (Table 1) usually have microclimatic conditions very different from the general climatic available data, which makes methods such as species distribution modelling fail (Guisan & Thuiller, 2005; Austin, 2007). In our study group, sympatric ecological speciation could explain the differentiation of *C. muralis* and *C. pallida*, as inferred by the DEC+J model (Fig. 2, node 12). These two species occur in the same region (northern Italy), often within a few hundreds of metres of each other (P. Carnicero & M. Galbany-Casals, personal observations), and occupy different niches (Table 1). However, their distributions at local scale are almost allopatric, given that *C. muralis* mostly occupies the lowlands while *C. pallida* grows at higher elevations. Thus, allopatric speciation cannot be completely ruled out.

The important role of polyploid speciation in the diversification of *Cymbalaria* was previously suggested (Sutton, 1988; Verlaque & al., 1993; Thompson, 2005). Biogeographic analyses do not take polyploid speciation into account; however, we consider that a clade originated from a polyploid speciation event when a genome duplication or demi-duplication predates its origin, as inferred by ChromEvol, as long as the clade has high statistical support. Accordingly, two polyploid speciation events are hypothesized: one for the origin of the west lineage (Fig. 2, node 3) and a second for the origin of the *C. microcalyx* subsp. *ebelii* – *C. pubescens* clade (Fig. 2, node 14). The origin of the polyploids *C. microcalyx* subsp. *microcalyx*, subsp. *dodekanesi* and subsp. *minor* remains uncertain given the low resolution obtained in the phylogenetic analyses and the lack of chromosome number information for subsp. *acutiloba*. The monophyly of the western lineage apparently refutes Verlaque's & al. (1993) hypothesis of independent polyploid origins for *C. hepaticifolia* Wettst. and *C. aequitriloba* from the diploids *C. pallida* and *C. muralis* form the Apennine Peninsula, respectively. However, the existence of two different ploidy levels within this clade (6*x* and 8*x*) may point to less parsimonious hypotheses with at least two independent polyploidisation events. Moreover, a polyploid clade can be the result of interlocus

concerted evolution of the nrDNA, which may hide the genetic information of one of the parental lineages in the case of allopolyploids (Wendel & al., 1995). Although several species may have originated via independent allopolyploid speciation events, concerted evolution could result in homogenization of the nrDNA towards the same parental lineage, and a single common origin would be inferred in the nrDNA phylogenies (Kovarik & al., 2005). Concerted evolution often results in incongruence between plastid and nrDNA phylogenies (Álvarez & Wendel, 2003), as observed in the two polyploid clades. This hypothesis has to be considered especially for the nrDNA clade *C. microcalyx* subsp. *ebelii* **–** *C. pubescens*, given that these two species appear in separate clades in the cpDNA analysis (Fig. 3) and that they occur in separate areas (Fig. 1). However, hybridization resulting in chloroplast capture cannot be ruled out as a possible cause for the incongruence between plastid and nrDNA (Pelser & al., 2010). Indeed, hybridization is often invoked when grouping of specimens from geographically close populations is observed in cpDNA phylogenies (e.g., McKinnon & al., 2004; Lorenz-Lemke & al., 2006). This could be the cause for the grouping of *C. microcalyx* subsp. *ebelii* with the geographically close *C. muralis*, or the grouping of *C. muelleri* and *C. fragilis* with specimens of *C. aequitriloba* occuring in sympatry (Fig. 1). However, differences in ploidy level between the sympatric taxa may hinder hybridization by enhancing reproductive isolation (Husband & Sabara, 2003; Sonnleitner & al., 2013). Additional studies are required to confirm the common origin of polyploids in *Cymbalaria*, to distinguish between auto- and allopolyploidisation events and to identify the parental taxa involved. The support to LDD events found here for the western clade (see subsection: The diversification of lineages) is consistent with the observed pattern of higher probability of LDD events in polyploid groups (Linder & Barker, 2014). This pattern may be associated with the high genetic variability of polyploids but also with their difficulty in succeeding in areas in which the parental species occur (Thompson, 2005; Ramsey, 2011).

Acknowledgements

We thank all herbaria that provided material and colleagues who provided assistance during fieldwork or their own plant material. We also thank M. Fernández-Mazuecos for providing the data on the secondary calibration point for the dating analyses, C. Roquet for assistance in the ancestral-area estimation and P. Schönswetter for valuable comments on the final manuscript. Three reviewers and the editor helped to improve the manuscript considerably. This research was funded in part by the Spanish government (CGL2010-18631/BOS and *Flora iberica* project, CGL2011-28613-C03-01) and the Catalan government (2009-SGR 439 and 2014-SGR 514). Pau Carnicero benefited from the support of a PIF Ph.D. student fellowship from the Universitat Autònoma de Barcelona.

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Appendix 1. Sampled specimens with information on the individual numeric codes used in text and figures, locality, herbarium voucher and accession numbers of the regions analysed. An asterisk (*) indicates sequences newly obtained in this study. A dash (–) indicates sequences that were not obtained in the present study or specimens without individual numeric code.

Taxon, individual number, locality, voucher, GenBank acc. no. ITS, 3'ETS, *ndhF*, *rpl32-trnL*

Cymbalaria Hill: ***C. aequitriloba*** (Viv.) A.Chev., 1, France, Corsica, La Castagniccia, *A. Curcó s.n.* (BCN 86695), KP735225*, KP851084*, KP851014*, KP851100*; ***C. aequitriloba***, 2, France, Corsica, La Castagniccia, *A. Hilpold s.n.* (BOZ 8888), KP735224*, KP851085*, KP851011*, KP851097*; ***C. aequitriloba***, 3, Spain, Balearic Islands, Mallorca, Puig Major, *X. Rotllan s.n.* (no voucher), KP735219*, KP851088*, KP851007*, KP851093*; ***C. aequitriloba***, 4, Spain, Balearic Islands, Mallorca, Formentor, *L. Sáez 7366 & X. Rotllan* (BC 879621), KP735240*, KP851086*, KP851009*, KP851095*; ***C. aequitriloba***, 5, Italy, Sardinia, Nuoro, Badde Salighes, *C. Aedo 9213* (MA 708824), KP735220*, KP851087*, KP851026*, KP851111*; ***C. aequitriloba***, 6, Italy, Sardinia, Cuglieri, Mte. Ferru, *C. Navarro 4683 & al.* (MA 708259), KP735222*, –, KP851006*, KP851092*; ***C. aequitriloba***, 7, Spain, Balearic Islands, Cabrera, *L. Sáez 6196 & L. Guàrdia Valle* (BC 879620), KP735241*, KP851082*, KP851008*, KP851094*; ***C. fragilis*** (J.J.Rodr.) A.Chev., 1, Spain, Balearic Islands, Menorca, Barranc d'Algendar, *P. Carnicero 346 & M. Galbany-Casals* (BC 879636), KP735211*, KP851081*, KP851004*, KP851090*; ***C. fragilis***, 2, Spain, Balearic Islands, Menorca, Barranc d'Algendar, *P. Carnicero 346 & M. Galbany-Casals* (BC 879636), –, –, KP851005*, KP851091*; ***C. glutinosa*** Bigazzi & Raffaelli subsp. ***glutinosa***, 1, Italy, Spigno Saturnia, *P. Carnicero 734 & M. Galbany-Casals* (BC 879627), KP735216*, KP851068*, KP851029*, KP851114*; ***C. glutinosa*** subsp. ***glutinosa***, 2, Italy, Spigno Saturnia, *P. Carnicero 734 & M. Galbany-Casals* (BC 879627), KP735217*, KP851069*, KP851030*, KP851115*; ***C. glutinosa*** subsp. ***brevicalcarata*** Bigazzi & Raffaelli, Italy, Ravello, *P. Carnicero 748 & M. Galbany-Casals* (BC 879626), KP735218*, KP851070*, KP851020*, KP851105*; ***C. hepaticifolia*** Wettst., 1, France, Corsica, Lac du Nino, *A. Hilpold s.n.* (BOZ 8842), KP735223*, KP851079*, KP851022*, KP851107*; ***C. hepaticifolia***, 2, France, Corsica, Castagniccia, *P. Carnicero 444 & M. Galbany-Casals* (BC 879631), KP735215*, KP851078*, KP851013*, KP851099*; ***C. longipes*** (Boiss. & Heldr.) A.Cheval., 1, Greece, Dodecanese Islands, Karpathos, *N. Böhling 8228* (B 10 0138948), KP735232*, KP851064*, KP851038*, KP851123*; ***C. longipes***, 2, Greece, Samos, *E. Gathorne-Hardy 657* (E 629368), –, –, KP851039*, KP851124*; ***C. microcalyx*** (Boiss.) Wettst. subsp. ***microcalyx***, Greece, Peloponnese, Lakonia, *W. Greuter & H. Merxmüller s.n.* (B 10 0460657), KP735238*, KP851063*, KP851041*, –; ***C. microcalyx*** subsp. ***acutiloba*** (Boiss. & Heldr.) Greuter, Turkey, Antalia, Alanya, *P.H. Davis 25847 & O. Polunin* (E 629362), KP735212*, KP851059*, KP851042*, KP851126*; ***C. microcalyx*** subsp. ***dodekanesi*** Greuter, Greece, Rhodes, Archangelos, *P.H. Davis 40310* (E 629364), KP735208*, KP851058*, KP851043*, KP851127*; ***C. microcalyx*** subsp. ***ebelii*** (Cufod.) Cufod., Montenegro, Skadar Lake, *E. Mayer 11192 & M. Mayer* (B 10 0460658), KP735236*, KP851061*, KP851036*, KP851121*; ***C. microcalyx*** subsp. ***minor*** (Cufod.) Greuter, Greece, Kefallinia, Aenos, *J. Damboldt s.n.* (B 10 0460655), KP735237*, KP851060*, KP851037*, KP851122*; ***C. muelleri*** (Moris.) A.Chev., 1, Italy, Sardinia, Seui, Genni d'Acca, *P. Carnicero 406 & M. Galbany-Casals* (BC 879629), KP735210*, KP851080*, KP851012*, KP851098*; ***C. muelleri***, 2, Italy, Sardinia, Ulassai, *P. Carnicero 389 & M. Galbany-Casals* (BC 879630), KP735209*, KP866214*, KP851010*, KP851096*; ***C. muralis*** G.Gaertn., B.Mey. & Scherb. subsp. ***muralis***, 1, Spain, Catalonia, Sant Cugat (naturalized), *P. Carnicero 134* (no voucher), KP735230*, KP851077*, KP851015*, KP851089*; ***C. muralis*** subsp. ***muralis***, 2, Spain, Catalonia, Caldes de Montbui (naturalized), *P. Carnicero 135* (BC 879623), KP735231*, KP851076*, KP851017*, KP851102*; ***C. muralis*** subsp. ***muralis***, 3, Poland, Slask Dolny (naturalized), *Z. Pulawska s.n.* (FI), –, –, KP851018*, KP851103*;

C. muralis subsp. ***muralis***, 4, Italy, Toscana, Albegna, *F. Selvi s.n.* (FI), –, –, KP851019*, KP851104*; ***C. muralis*** subsp. ***visianii*** (Jáv.) D.A.Webb, 1, Italy, Lazio, Palombara, *P. Carnicero 703 & M. Galbany-Casals* (BC 879625), KP735226*, KP851075*, KP851027*, KP851112*; ***C. muralis*** subsp. ***visianii***, 2, Italy, Lazio, Palombara, *P. Carnicero 703 & M. Galbany-Casals* (BC 879625), –, –, KP851028*, KP851113*; ***C. muralis*** subsp. ***visianii***, 3, Italy, Lazio Rocca di Papa, *P. Carnicero 710 & M. Galbany-Casals* (BC 879624), KP735226*, KP851074*, KP851031*, KP851116*; ***C. muralis*** subsp. ***visianii***, 4, Italy, Lazio Rocca di Papa, *P. Carnicero 710 & M. Galbany-Casals* (BC 879624), –, –, KP851032*, KP851117*; ***C. pallida*** Wettst., 1, Italy, Abruzzo, Valle d'Orfenta, *P. Carnicero 780 & M. Galbany-Casals* (BC 879628), KP735234*, KP851072*, KP851033*, KP851118*; ***C. pallida***, 2, Italy, Abruzzo, Valle d'Orfenta, *P. Carnicero 780 & M. Galbany-Casals* (BC 879628), KP735235*, KP851071*, KP851034*, KP851119*; ***C. pallida***, 3, Italy, Abruzzo, l'Aquila, *J. Aldasoro 3276* (MA 698766), KP735233*, KP851073*, KP851035*, KP851120*; ***C. pubescens*** (J.Presl & C.Presl) Cufod., 1, Italy, Sicily, Palermo, La pizzuta, *C. Aedo 5733 & al.* (MA 646152), KP735229*, KP851066*, KP851021*, KP851106*; ***C. pubescens***, 2, Italy, Sicily, Trapani, Erice, *J. Güemes 3085 & al.* (SALA 106642), KP735214*, KP851065*, KP851024*, KP851108*; ***C. pubescens***, 3, Italy, Sicily, Trapani, Mt. Acci, *C. Aedo 5614 & al.* (MA 646631), KP735228*, KP851067*, KP851025*, KP851110*; ***C. pubescens***, 4, Italy, Sicily, Trapani, Mt. Acci, *J. Güemes 3052 & al.* (SALA 106608), KP735213*, –, KP851023*, KP851108*; **Other Antirrhineae:** ***Anarrhinum bellidifolium*** (L.) Willd., Spain, Catalonia, l'Espluga de Francolí, *M. Galbany-Casals 2303* (BC 941028), KP735199*, –, KP851052*, KP851136*; ***Antirrhinum majus*** L., Spain, Catalonia, Alella, *M. Galbany-Casals 2302* (BC 941029), KP735205*, –, KP851048*, KP851132*; ***Asarina procumbens*** Mill., Spain, Catalonia, Montseny massif, *P. Carnicero 253 & L. Sáez* (BC 879635), KP735207*, KP851057*, KP851045*, KP851129*; ***Chaenorhinum crassifolium*** (Cav.) Lange, Spain, Valencian Country, Serra d'Aitana, *P. Carnicero 207 & al.* (BC 879633), KP735203*,–, KP851051*, KP851135*; ***Epixiphium wislizeni*** (A.Gray) Munz, U.S.A., New Mexico, Animas Valley, *G.R. Ballmer s.n.* (RSA 712541), KP735206*, KP851056*, KP851046*, KP851130*; ***Galvezia fruticosa*** J.F.Gmel., Perú, Lima, Yauyos, *M. Weigend 7209 & al.* (B 10 0095831), KP735197*, –, KP851044*, KP851128*; ***Kickxia spuria*** subsp. ***integrifolia*** (Brot.) R.Fern., Spain, Catalonia, Gallecs, *J.M. Blanco s.n.* (BC 939713), KP735200*, –, KP851053*, KP851137*; ***Linaria cavanillesii*** Chav., Spain, Valencian Country, Dènia, *P. Carnicero 197 & al.* (BC 879634), KP735198*, –, KP851050*, KP851134*; ***Lophospermum erubescens***, cult. Botanicher Garten Berlin-Dahlem, *J. Güemes s.n.* (VAL145154); AY731249.1, –, –, –; ***Maurandya antirrhiniflora***, Mexico, Guanajuato, San Miguel de Allende to Dolores km25, *F. Billiet & B. Jadin s.n.* (MA588497), KT187745.1, –, –, –; ***Misopates orontium*** (L.) Rafin., Spain, Valencian Country, Fenestrat, *P. Carnicero 210 & al.* (BC 879632), KP735201*, –, KP851049*, KP851133*; ***Mohavea confertiflora*** A.Heller, U.S.A., California, Colorado desert, *T.R. Stoughton 800* (RSA 778206), KP735202*, –, KP851054*, KP851138*; ***Plantago lanceolata*** L., Spain, Catalonia, Cerdanyola, *P. Carnicero 523* (no voucher), KP735196*, –, KP851055*, KP851139*; ***Schweinfurthia papilionacea*** Boiss., Oman, Nizwa, *A.G. Miller 6657* (E 614757), KP735204*, –, KP851047*, KP851131*; ***Veronica persica*** Poir., Spain, Catalonia, Bellaterra, *P. Carnicero 522* (BC), KX580311*, –, –, –.

Electronic supplement

Table S1. Characteristics of sequences and results of phylogenetic analyses.

Parameter	cpDNA	nrDNA complete dataset[1]	nrDNA "species" data set
Number of sequences	50	45	33
Length of sequences (bp)	2363-2609	531-1044	531-1044
Total number of characters	2715	1071	1071
Maximum Parsimony (MP) informative characters	349	231	227
Number of MP trees	3	1200	28
Number of steps	766	671	667
Consistency Index (CI)	0.59	0.50	0.52
Homoplasy Index (HI)	0.41	0.50	0.48
Retention Index (RI)	0.73	0.73	0.69
Sequence evolution model for Bayesian analyses (BIC criteria)	GTR + G	GTR + I + G	GTR + I + G

[1] Preliminary analysis, shown in Electronic supplement: Fig. S1

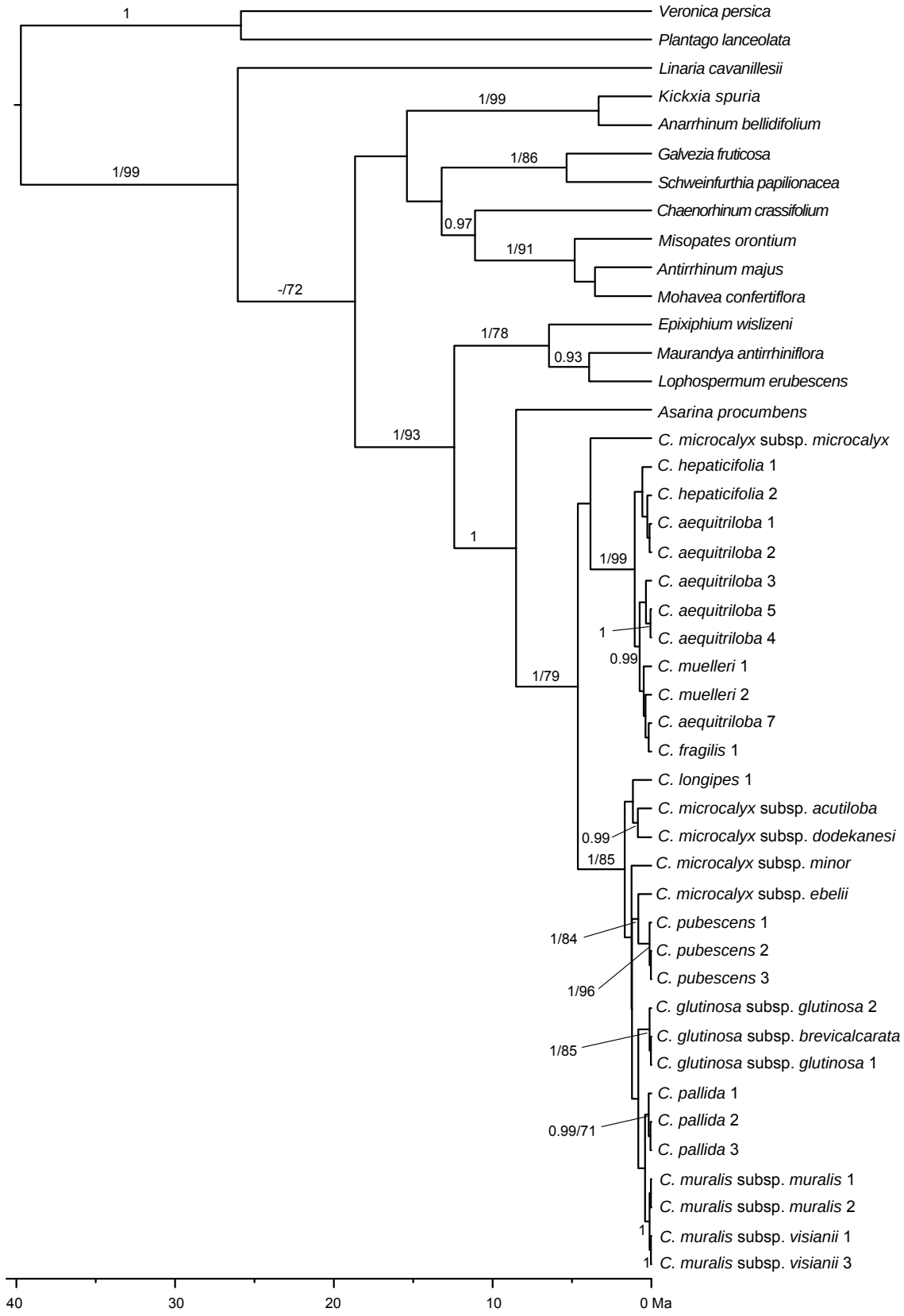

Figure S1. Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA of *Cymbalaria* (complete data set) in BEAST v1.8.2. Bayesian posterior probabilities ≥ 0.95/bootstrap support values ≥ 70% are indicated.

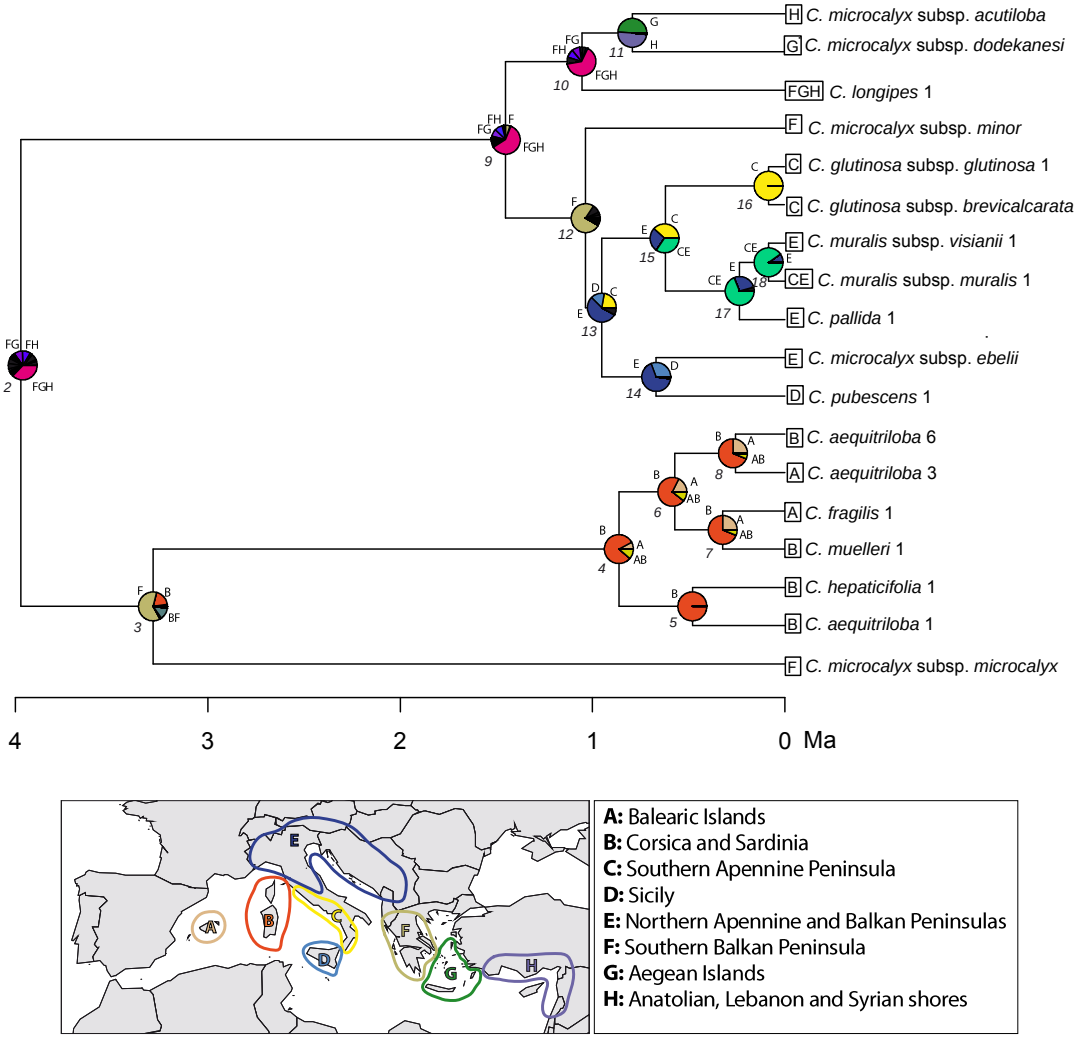

Figure S2. Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA of *Cymbalaria* in BEAST v1.8.2. Pie charts at each node show the marginal probabilities of alternative ancestral ranges obtained from the BioGeoBEARS analysis excluding *Asarina*. Letter codes for each area inferred and distribution areas at present are indicated at the nodes and terminals, respectively. Black segments in pie charts represent ancestral ranges with a probability < 10%. Numbers in italics below nodes indicate the node number.

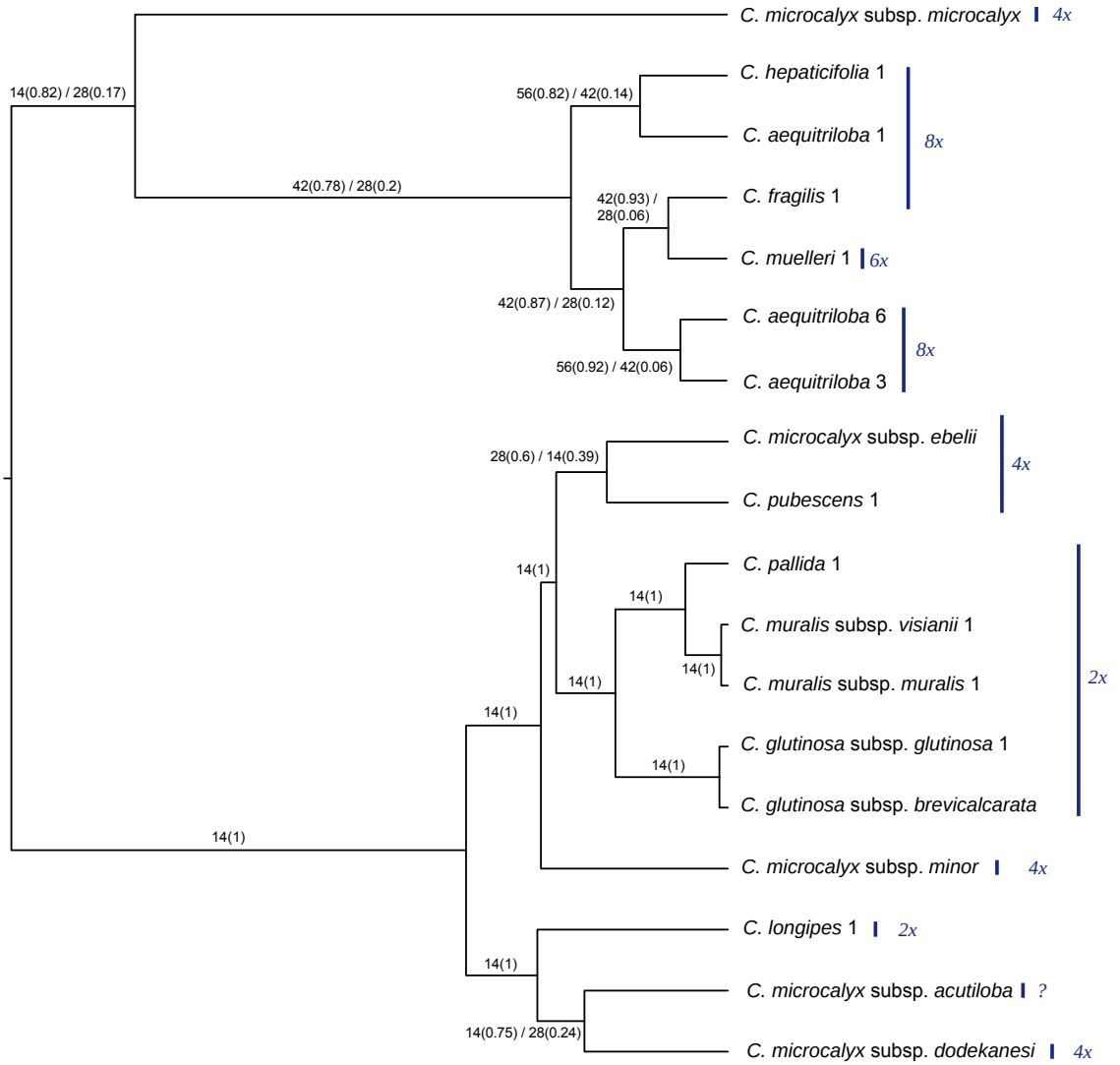

Figure S3. Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA of *Cymbalaria* in BEAST v1.8.2. Chromosome numbers inferred by ChromEvol are shown above branches. Numbers in parenthesis correspond to Bayesian posterior probabilities for each chromosome number. Ploidy levels are indicated to the right of the terminal node names.

Chapter II

Phylogeography of western Mediterranean *Cymbalaria* (Plantaginaceae) reveals two independent long-distance dispersals and entails new taxonomic circumscriptions[1]

[1] With Peter Schönswetter, Pere Fraga, Núria Garcia Jacas, Llorenç Sáez Gonyalons and Mercè Galbany Casals. Submitted to Botanical Journal of the Linnean Society

Abstract

The Balearic Islands, Corsica and Sardinia (BCS) constitute biodiversity hotspots in the western Mediterranean Basin. Oligocene connections and long distance dispersal events have been suggested to cause presence of BCS shared endemic species. One of them is *Cymbalaria aequitriloba*, which, together with three additional species, constitute a polyploid clade endemic to BCS. Combining amplified fragment length polymorphism (AFLP) fingerprinting, plastid DNA sequences and morphometrics, we inferred the phylogeography of the group and evaluated the species' current taxonomic circumscriptions. Based on morphometric and AFLP data we propose a new circumscription for *C. fragilis* to additionally comprise a group of populations with intermediate morphological characters previously included in *C. aequitriloba*. Consequently, we suggest to change the IUCN category of *C. fragilis* from critically endangered (CR) to near threatened (NT). Both morphology and AFLP data support the current taxonomy of the single island endemics *C. hepaticifolia* and *C. muelleri*. The four species had a common origin in Corsica-Sardinia, and two long-distance dispersal events to the Balearic Islands were inferred. Finally, plastid DNA data suggest that interspecific gene flow took place where two species co-occur.

Keywords AFLP, chloroplast DNA, dispersal, island evolution, Mediterranean, morphology

Introduction

Islands in the Mediterranean Basin harbour both high species diversity and endemism. For instance, from the around 5000 islands scattered in the Mediterranean Sea (Vogiatzakis et al., 2008), all the largest ones (i.e. Balearic Islands, Corsica, Crete, Cyprus, Sardinia and Sicily) constitute Mediterranean biodiversity hotspots (Médail & Quézel, 1999). Most of the Mediterranean islands are of continental origin and have been separated from the mainland and from each other through progressive geomorphological processes, often with posterior re-connections due to sea level variation (Thompson, 2005). Such processes are thought to have played an important role in the evolution and diversification of biota (Nieto Feliner, 2014).

The Balearic Islands, Corsica and Sardinia (hereafter termed BCS) originated from progressive splitting and rotation of the Hercynian belt, an Oligocene mountain range linking the Alps with the Iberian Peninsula that started to disintegrate around 30 Million years ago (Ma, Rosenbaum et al., 2002). The split between Corsica-Sardinia and the Balearic Islands occurred around 20 Ma (Speranza et al., 2002). Endemic plant species shared by Corsica, Sardinia and the eastern Balearic Islands (Mallorca and Menorca) have traditionally been considered relict palaeoendemic remnants of the Oligocene connections (Contandriopoulos & Cardona, 1984; Contandriopoulos, 1990; Thompson, 2005). They were formerly named “Tyrrhenian endemisms” (Contandriopoulos & Cardona, 1984), but we prefer to avoid this term given that the Tyrrhenian Sea is situated between Corsica, Sardinia, the Italian Peninsula and Sicily. To our knowledge, an origin consistent with the paleoendemics hypothesis has only been confirmed for *Helicodiceros muscivorus* (L.f.) Engler (Mansion et al., 2008). In most other dated phylogenies of plants showing this distribution pattern (e.g. *Arum pictum* L.f.: Mansion et al., 2008; *Thymus herba-barona* Loisel.: Molins et al., 2011; *Cymbalaria aequitriloba* (Viv.) A.Chev.: Carnicero et al., 2017), or other similar disjunctions in the western Mediterranean Basin (Salvo et al., 2010; Fernández-Mazuecos et al., 2014), palaeoendemism hypotheses had to be rejected (but see Magri et al., 2007). Instead, long-distance dispersal (LDD) was invoked as a possible explanation for the present distribution pattern, since no land connections have been reported between the

Balearic Islands on the one hand and Corsica and Sardinia on the other hand after their split.

Pleistocene climatic oscillations caused massive changes of the sea level that led to repeated appearance of land bridges between some islands during cold stages, and to transgressions reducing the area of islands during interglacials (Vesica et al., 2000; Thompson, 2005). During glaciation periods Corsica-Sardinia and the eastern Balearic Islands (Mallorca, Menorca and Cabrera), respectively, formed single landmasses. The resultant connections have often been used to explain phylogeographic patterns and shared endemisms within each archipelago (e.g. Mansion et al., 2009; Salvi et al., 2010; Mayol et al., 2012).

One of the genera with highest number of endemics in BCS is *Cymbalaria* Hill. (Plantaginaceae), a Mediterranean genus comprising ca. 17 taxa, four of which are endemic to BCS (Sutton, 1988). The BCS endemics form an independent lineage of presumably polyploid origin that diversified during the Pliocene and the Pleistocene, as revealed by a nuclear ribosomal DNA (nrDNA) phylogeny (Carnicero et al., 2017). However, interspecific phylogenetic relationships were poorly resolved, and therefore using a more variable molecular marker such as AFLPs would be desirable. Chromosome counts suggest that the BCS endemics are hexa- to octoploids (2*n* = 42, 56), whereas all other *Cymbalaria* species are diploids or tetraploids (2*n* = 14, 28; Sutton, 1988 and references therein). While *C. aequitriloba* is widely distributed throughout BCS and occurs in all main islands except for the western Balearic Islands Ibiza and Formentera, the other three species are single island endemics according to the current taxonomic treatments (Sutton, 1988; Güemes, 2009). This lineage is therefore an excellent example to understand the mechanisms underlying the endemism patterns in BCS.

The two single island endemics from Corsica and Sardinia have been traditionally considered as clearly distinct species. *Cymbalaria hepaticifolia* Wettst. (2*n* = 56, Sutton, 1988) occurs in the mountains of Corsica, from alpine habitats to fresh and humid forests (Jeanmonod & Gamisans, 2007). *Cymbalaria muelleri* (Moris.) A.Chev., comprising ca. ten known populations, is a hexaploid (2*n* = 42, Onnis & Floris, 1967)

endemic of the mountains of central Sardinia (Arrigoni et al., 1979).

Cymbalaria fragilis (J.J.Rodr.) A.Chev. is a critically endangered (CR) octoploid (2*n* = 56, Castro & Rosselló, 2006) species endemic to Menorca (Bañares et al., 2010). It has been treated either as a subspecies of *C. aequitriloba* (Sutton, 1988; Bolòs et al., 2005) or as a separate species (Güemes, 2009). Furthermore, the circumscription of this taxon is uncertain. According to Güemes (2009), *C. fragilis* is characterized by several distinctive features such as large white flowers, thick leaves and stems, fragility, dense hairiness and finely alveolate to smooth seeds. However, some populations in Menorca and Cabrera exhibit all distinctive features but have alveolate seeds (Güemes, 2009). Seed ornamentation has been regarded as highly relevant in the taxonomy of the tribe Antirrhineae (Elisens & Tomb, 1983; Elisens, 1985; Sutton, 1988; Vigalondo et al., 2015), and was considered sufficient to attribute these populations to *C. aequitriloba* (Güemes, 2009). The latter is the most widespread species among the four BCS endemics, occurring in the eastern Balearic Islands, Corsica, Sardinia and the Tuscan Archipelago. Octoploid counts (2*n* = 56) have been reported from Corsica and the Balearic Islands (Dahlgren et al. 1971, Cardona & Contandriopoulos, 1983; Verlaque et al., 1993); older hexaploid counts (2*n* = 42; Sutton, 1988) were considered erroneous by Verlaque et al. (1993). Indeed, the original reference for the hexaploid counts (Heitz, 1927) does not report any chromosome number for *C. aequitriloba*, and we were unable to trace any other hexaploid count in the literature. *Cymbalaria aequitriloba* exhibits high morphological variability, which is the reason why sparsely hairy forms from Mallorca have sometimes been confused with *C. hepaticifolia* (Pericàs & Roselló, 1983). Some morphological extremes were described as separate taxa, but were later synonymised with *C. aequitriloba* (Sutton, 1988). Sequence data, however, did not support the species' monophyly (Carnicero et al., 2017), and it is thus doubtful whether *C. aequitriloba* is indeed a single taxon. Some authors considered *C. aequitriloba* an example of a palaeoendemism (Contandriopoulos & Cardona, 1984; Contandriopoulos, 1990; Thompson, 2005), although some of its aforementioned features do not fit with the classical definition of the term (Favarger & Contandriopoulos, 1961). Most importantly, its recent origin inferred from a dated phylogeny rejected that hypothesis (Carnicero et al., 2017).

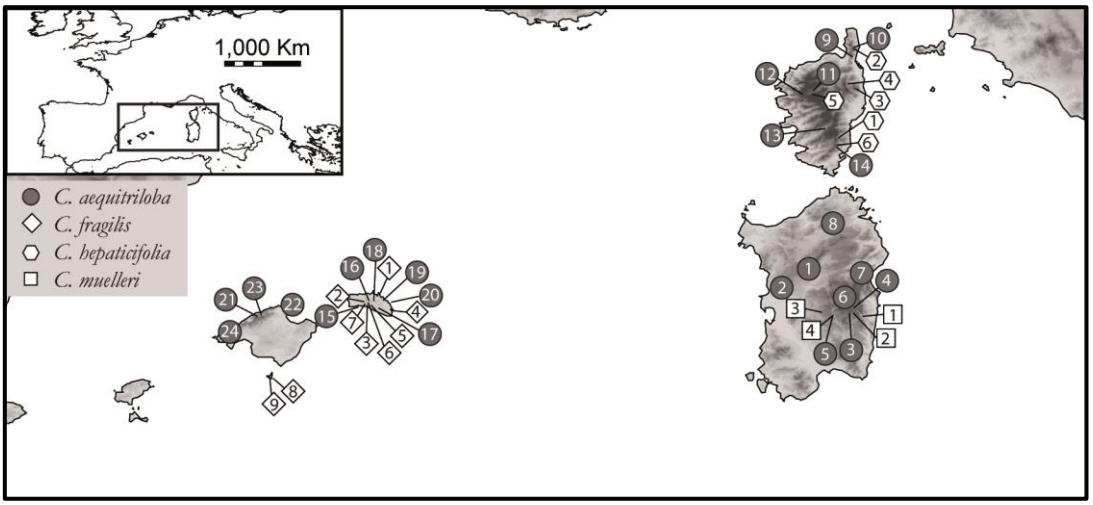

Figure 1. Sampling sites and population numbers. Taxonomy follows the taxonomic concept adopted in the present article, population numbers in the main text are preceded by a letter according to taxonomy (a: *C. aequitriloba*, f: *C. fragilis*, h: *C. hepaticifolia*, m: *C. muelleri*, see Supplementary Table S1).

Here, we focus on the phylogeography and systematics of the BCS endemics of the genus *Cymbalaria*. We use a combination of AFLPs, plastid DNA sequences and morphometrics to tackle the following questions: (1) Are genetic and morphological data congruent and do they support the current taxonomy? (2) Specifically, is *C. aequitriloba* monophyletic based on rapidly homogenizing (Bussel et al., 2005) AFLP data? (3) What is the appropriate taxonomic rank and circumscription of *C. fragilis*? (4) What was the pattern of colonization of the BCS endemics among Sardinia, Corsica and the Balearic Islands?

Materials and Methods

Plant Material and DNA extraction

Leaf material for molecular analyses was collected in the field in 2012 and 2013, dried and stored in silica gel. The sampling localities are shown in Fig. 1 and voucher details are provided in supplementary Table S1. Total genomic DNA was extracted from ca. 10–30 mg silica gel dried leaf material following the CTAB protocol (Doyle & Doyle, 1987) with some modifications (Tel-Zur et al., 1999). When less than 10 mg of dried material was available, no sorbitol washing was applied.

AFLP fingerprinting

AFLP fingerprinting was performed with usually eight to ten individuals per population (supplementary Table S1), using *C. microcalyx* (Boiss.) Wettst. and *C. pubescens* (C.Presl) Cufod.as outgroup taxa. AFLP profiles were generated following established protocols (Vos et al., 1995) with modifications described in Schönswetter et al. (2009) and Rešetnik et al. (2014). Three blanks (DNA replaced by water) were included to test for contamination, and 32 samples (10.5%) were used as replicates between PCR batches to test the reproducibility of AFLP fingerprinting. Based on an initial primer trial the following three selective primer combinations were chosen for selective PCR (fluorescent dye in brackets): *Eco*RI (6-FAM)ACA / *Mse*I-CAT, *Eco*RI (VIC)AGG / *Mse*I-CAC, and *Eco*RI (NED)AAC / *Mse*I-CAG (6-FAM labelled primers: Sigma-Aldrich; NED and VIC labelled primers: Applied Biosystems). Selective PCR products were purified using Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a MultiScreen-HV plate (Millipore, Molsheim, France) in three steps of 200 µl each and packed at 600 g for 1, 1 and 5 min, respectively. Then 0.8 µl of the elution product was mixed with 10 µl formamide (Applied Biosystems) and 0.125 µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130 automated capillary sequencer.

Analyses of AFLP data

Electropherograms were analysed with Peak Scanner version 1.0 (Applied Biosystems) using default peak detection parameters except employing light peak smoothing. The minimum fluorescent threshold was set to 50 relative fluorescence units. Automated binning and scoring of the AFLP fragments were performed using RawGeno 2.0-1 (Arrigo et al., 2009) for R 2.15.0 (R Development Core Team, 2015) with the following settings: scoring range 140–500 bp, minimum intensity 50 relative fluorescence units, minimum bin width 1 bp, and maximum bin width 1.5 bp. Fragments with a reproducibility lower than 80% based on sample-replicate comparisons were eliminated. The error rate (Bonin et al., 2004) was calculated as the ratio of mismatches (scoring 1 versus 0) over phenotypic comparisons in AFLP profiles of replicated individuals. Fragments present/absent in only one individual were excluded.

A Neighbour-Joining (NJ) analysis based on Nei-Li genetic distances (Nei & Li, 1979) was conducted and bootstrapped (2000 pseudo-replicates) with TREECON v.1.3b (van de Peer & De Wachter, 1997). SplitsTree4 12.6 (Huson & Bryant, 2006) was used to produce a Neighbor-Net diagram based on uncorrected P-distances.

A Bayesian phylogeographical diffusion model in continuous space (Lemey et al., 2010) was performed on the AFLP dataset using BEAST v1.8.2 (Drummond et al., 2012) to infer the pattern of colonization among islands. Geographic input data were coordinates of each population (supplementary Table S1). We used a simple substitution model with estimated state frequencies for phylogeny inference and the Bayesian skyline coalescent prior (Drummond et al., 2005) with a piecewise-linear skyline model was set to model population growth. A strict molecular clock was used due to the simple presence-absence structure of AFLP data. The diffusion process was modelled by a lognormal relaxed random walk process, which is an extension of a phylogenetic Brownian motion process that rescales the precision matrix by a branch-specific scalar that is drawn independently from an identical distribution (Nylinder et al., 2014), in our case a lognormal distribution centred on 1. We specified a prior exponential distribution on the standard deviation of the lognormal distribution with a mean of 5. We added random jitter with a window size 1.0 to the tips, as more individuals were sampled from the same location. The analysis of the diffusion inference was run for 200 million generations, logging parameters every 5,000 generations. The performance of the analysis as well as mixing and effective sample size values for all parameters were checked in Tracer 1.6.0 (Rambaut et al., 2013). A maximum clade credibility tree (MCC) was produced and annotated with Tree Annotator (part of the BEAST package) after removing 25% of trees as burnin and visualized with FigTree 1.4.2. The diffused MCC tree with annotated diffusion estimates was visualized in SPREAD v.1.0.6 (Bielejec et al., 2011) and projected together with polygons representing ancestral areas using ArcGIS 10.3.

Analyses of molecular variance (AMOVAs) and genetic diversity were computed with ARLEQUIN 3.5. Groups were defined according to taxa and – for *C. aequitriloba* – to geographic distribution (islands and archipelagos). Average gene diversity over loci (π_n) was obtained for populations and groups after removing populations with fewer than

four sampled individuals. Comparison of gene diversity among groups was performed with Student's t-tests after testing for normality with Shapiro-Wilk normality tests.

A separate dataset for *C. aequitriloba* was prepared and, after independent automated binning and scoring, analysed in the same way. It comprised all populations of the *C. aequitriloba* clade (see Results) plus *C. fragilis* populations f8 and f9 as outgroup, based on the results of the NJ analysis for the entire dataset. In addition, we performed nonhierarchical K-means clustering (Hartigan & Wong, 1979), a model-free clustering approach. We used a script of Arrigo et al. (2010) in R (R Development Core Team, 2015) to identify genetically homogeneous groups in a dataset pruned to *C. aequitriloba*. We performed 100,000 independent runs (i.e., starting from random points) for each assumed value of K (i.e., the number of groups) ranging from 2 to 10.

Amplification and sequencing of plastid DNA markers

We amplified and sequenced the plastid *ndhF* region and the *rpl32-trnL*$^{(UAG)}$ spacer for three individuals per population, with the exceptions of populations a20 and m1 – for which we could only obtain sequences from two individuals –, and the outgroups (*C. muralis* G.Gaertn., B.Mey. & Scherb. and *C. pubescens*) – with a single individual per population. We used the primers 3'F (Eldenäs et al., 1999), +607 (Kim & Jansen, 1995) and the internal specific primers ndhF CymbF and ndhF CymbR (Carnicero et al., 2017) for the *ndhF* region and rpl32F and trnL$^{(UAG)}$ (Shaw et al., 2007) for the *rpl32-trnL* spacer. All reactions were carried out in a MasterCycler Gradient thermocycler (Eppendorf).

For both plastid regions, the reaction mix (total volume 25 µL) contained 9 µL of RedTaq ReadyMix PCR Reaction Mix (Sigma-Aldrich), 1.05 µL bovine serum albumin (BSA; 1 mg mL^{-1}; Promega), 1.05 µL of each primer (10 µM) and 1.5 µL template DNA. We followed the PCR profiles described in Galbany-Casals et al. (2012) for the *ndhF* and Magauer et al. (2014) for the *rpl32-trnL* spacer.

The quality of the PCR products was checked on 1% TBE agarose gels. Subsequently, the amplification products were purified enzymatically using Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas) according to the manufacturer's instructions. Sanger sequencing was conducted by a commercial sequencing facility (Eurofins MWG

Operon, Munich, Germany) using the primers 3'F, +607, rpl32F and trnL$^{(UAG)}$. Sequences were examined and aligned by hand using Chromas Lite 2.0 (Technelysium Pty Ltd, Tewantin, Australia) and Mega 6.06 (Tamura et al., 2013). GenBank accession numbers are given in supplementary Table S1.

Analyses of sequence data

The alignment of the concatenated plastid markers was analysed using statistical parsimony as implemented in TCS 1.21 (Clement et al., 2000) with the connection limit set to 95%. For this analysis, an indel longer than 1 bp was reduced to a single base pair column allowing this structural mutation to be counted as single base pair mutation only. Two overlapping, but clearly non-homologous indels were detected and considered different. Differences on the indel length (1 bp) caused by different lengths of poly-T regions were not considered due to the high likelihood of homoplastic evolution (Aldrich et al., 1988). Specimens with missing data in polymorphic positions were excluded to avoid ambiguous haplotype assignments. After these modifications, gaps were treated as fifth character state in this analysis.

Maximum parsimony (MP) analyses as well as MP bootstrap analyses of both data sets were performed using PAUP 4.0b10 (Swofford, 2002). Indels were coded as binary characters using the simple indel coding method (Simmons & Ochoterena, 2000) using SeqState 1.4.1 (Müller, 2005). The most parsimonious trees were searched heuristically with 1000 replicates of random sequence addition, TBR swapping, and MulTrees on. The swapping was performed on a maximum of 1000 trees (nchuck = 1000). All characters were equally weighted and unordered. The data set was bootstrapped using full heuristics, 1000 replicates, TBR branch swapping, MulTrees option off, and random addition sequence with five replicates. *Cymbalaria pubescens* was used as outgroup based on a previous study (Carnicero et al., 2017).

Bayesian Inference (BI) analysis was performed employing MrBayes v.3.2 (Ronquist et al., 2012) applying the GTR+G substitution model proposed by the Akaike information criterion implemented in JModelTest 0.1.1 (Posada, 2008). Indels were coded as binary characters using the simple indel coding method (Simmons &

Ochoterena, 2000). We generated 10,000 trees running MrBayes for 5,000,000 generations and sampling one of every 500 generations. After ensuring that the Markov chain Monte Carlo (MCMC) reached stationarity, we discarded the first 2500 trees as burn-in.

Morphometric analyses

Twenty-seven characters were scored on the basis of previous studies on the tribe Antirrhineae (Sutton, 1988; Sáez & Crespo, 2005), of which 18 were quantitative and seven were ordinal (supplementary Table S2). Seven corresponded to vegetative features, 11 to floral features, and seven to fruit and seed features. For the vegetative characters, three measurements per specimen were averaged when possible. As it was not possible to score both floral and fruit or seed characters for all populations, we analysed two datasets. Dataset 1 included 11 floral and seven vegetative characters and dataset 2 included seven fruit and seven vegetative characters. Dataset 1 comprised 129 specimens from 31 populations and dataset 2 comprised 85 specimens from 33 populations (supplementary Table S1). All specimens used in these analyses were also included in the molecular analyses, with the exception of *C. muelleri* and some individuals from population a5 of *C. aequitriloba* included in dataset 1, which were added later in order to provide floral measurements. Floral measurements were performed in the field with a caliper. Features of indumentum, calyx, fruits and seeds were examined under a binocular stereoscopic microscope. The other characters were measured on scanned specimens using Image J (Abràmoff et al., 2004). Analyses were conducted using a set of R functions contained in MorphoTools ver. 1.01 (Koutecký, 2015). Pearson and Spearman correlation coefficients were computed to reveal correlation structure among the characters and to ensure that no strong correlations (> |0.95|) were present. After standardization to zero mean and unit variance, principal component analysis (zero-centred PCA based on a covariance matrix) was applied to datasets 1 and 2 to display the overall variation pattern along the first two components. A canonical discriminant analysis (CDA) was performed on dataset 1 to assess the morphological differentiation among the four taxa. Morphological intermediate specimens between *C. fragilis* and *C. aequitriloba* from populations f1, f3, f4, f5, f8 and f9 were included in *C. fragilis* according to the results of the molecular

analyses and the PCA.

Results

AFLPs

In total, 834 AFLP fragments were scored for 304 individuals, of which 779 polymorphic, high-quality, reproducible AFLP-fingerprints were obtained. Fifty-five fragments with singular presences or absences were excluded. The initial error rate (Bonin et al., 2004) before the exclusion of non-reproducible fragments was 3.17%. The final dataset for *C. aequitriloba* comprised 175 individuals and 557 AFLP fragments.

The NJ analysis revealed that the four taxa analysed constituted a monophyletic group with high bootstrap support (BS 100%, Fig. 2A, supplementary Fig. S1). The main split in the NJ tree separated *C. aequitriloba* and *C. fragilis* from *C. hepaticifolia* and *C. muelleri*. Populations of *C. aequitriloba* formed a clade (BS 62%) excluding all populations with distinctive features of *C. fragilis*, but bearing alveolate seeds (f1, f3, f4, f5, f8 and f9), which instead grouped with *C. fragilis* (BS 100%). One well supported clade each grouped populations of *C. hepaticifolia* (BS 100%) and *C. muelleri* (BS 96%). The NeighborNet (Fig. 2B) showed three clearly distinct groups for *C. fragilis*, *C. hepaticifolia* and *C. muelleri*, coincident with those obtained from the NJ analysis, whereas *C. aequitriloba* populations did not group together. Individuals from the same population – with the exception of populations a14, a23 and h4 – formed monophyletic groups. The *C. fragilis* clade showed a main split between the populations from Menorca (f1 to f7, BS 79%) and those from Cabrera traditionally included in *C. aequitriloba* (f8 and f9, BS 100%).

The results obtained by the Bayesian analyses with BEAST were congruent with the NJ analysis, minor differences concerned nodes with low support (supplementary Fig. S2). The origin of diversification was estimated to be in northern Sardinia (Fig. 3), from where dispersal events to southern Sardinia, Corsica and the Balearic Islands (Menorca) occurred. Later, further dispersals from Sardinia

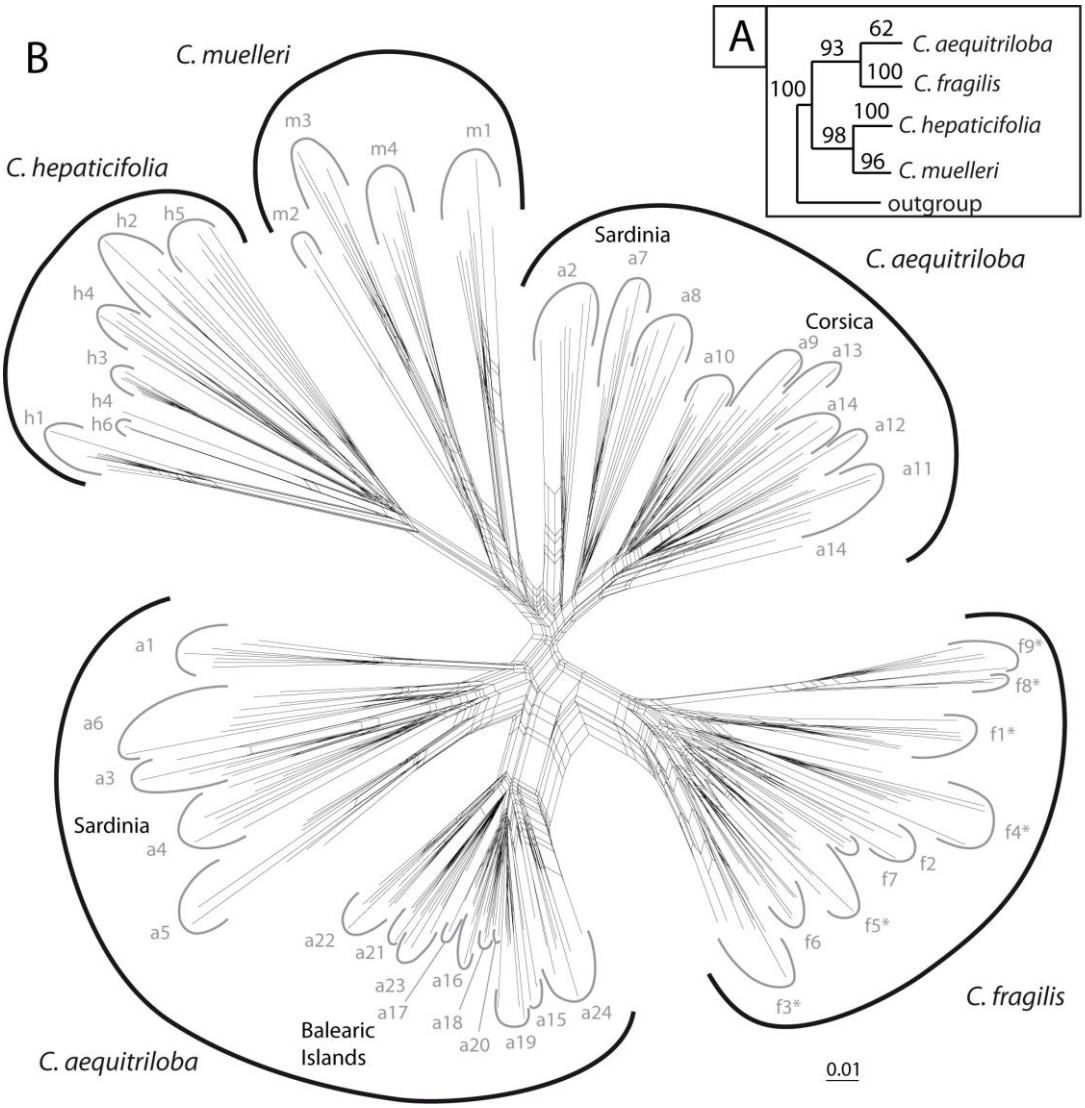

Figure 2. AFLP analyses of *C. aequitriloba*, *C. fragilis*, *C. hepaticifolia* and *C. muelleri*. A, simplified Neighbour-Joining tree. Numbers above branches are bootstrap support values derived from a Neighbour-Joining analysis. B, Neighbor-Net diagram. Asterisks indicate *C. fragilis* populations previously considered *C. aequitriloba*. Populations are numbered as in Fig. 1 and Table S1.

to Corsica and the Balearic Islands (Mallorca) took place.

A hierarchical AMOVA (supplementary Table S3) assigned 28.7% of the entire variation to the among-taxa component. In *C. aequitriloba* the highest variation among populations within an island was found in Sardinia (44.7%), and the lowest in Corsica (27.7%). Genetic diversity expressed as π_n (supplementary Table S4) varied from 0.028 in population a17 of *C. aequitriloba* from Menorca to 0.164 in population h5 of *C. hepaticifolia* from Corsica. No differences were found between taxa. For *C. aequitriloba*, π_n was significantly lower in the Balearic Islands than in Corsica and Sardinia.

In the separate NJ analysis for *C. aequitriloba* (Fig. 4, BS 100%) the populations from the Balearic Islands formed a clade (BS 100%), which contained a subclade comprising populations from Menorca (a15–a20; BS 91%). Populations from Corsica (a9–a14) formed a supported clade (BS 99%) whereas populations from Sardinia were distributed in three main clades (a1, BS 100%; a2, a7 and a8, BS 70%; a3–a6, BS 97%). Nonhierarchical K-means clustering resulted in an optimal separation of the dataset into three groups (Fig. 4) composed by the Sardinian populations a1, a3–a6; all Corsican populations plus the Sardinian populations a2, a7 and a8; and all Balearic populations. At K = 6 populations a1–a8 from Sardinia were split into four clusters, whereas the other two groups included all Corsican and all Balearic populations, respectively (Fig. 4).

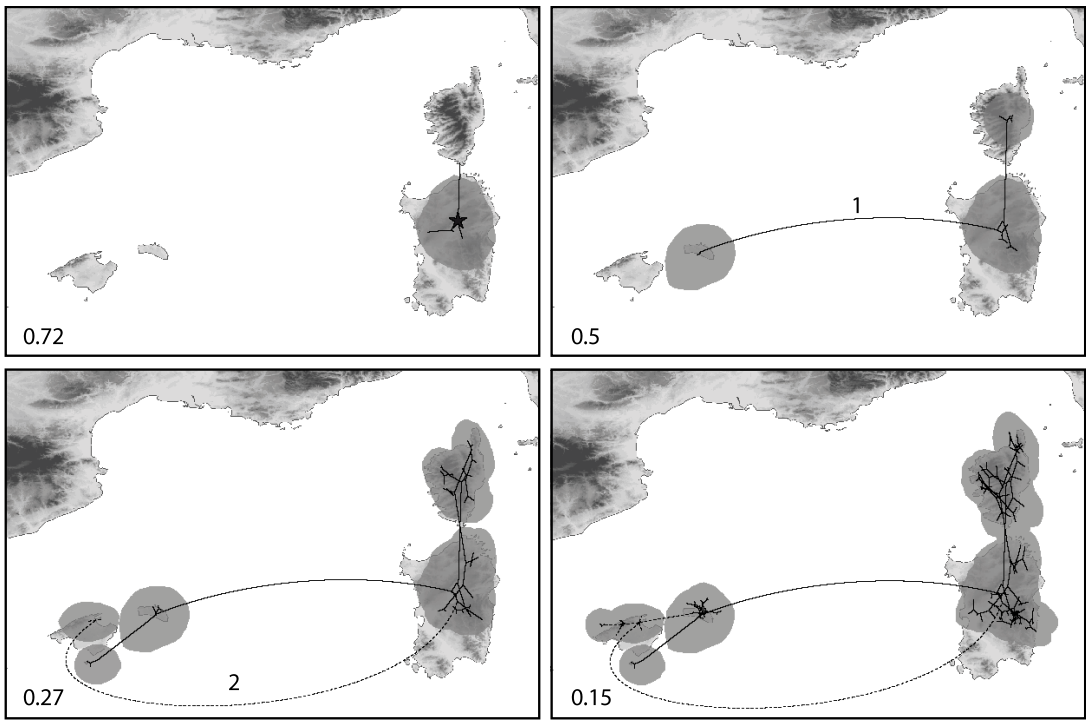

Figure 3. Snapshots of estimated ancestral node areas in the Maximum Clade Credibility tree obtained from the BEAST continuous phylogeographical analysis of AFLP data at different time horizons as visualized using the software SPREAD. The 80% highest posterior density areas for nodes are indicated as grey polygons. The star in the upper left panel indicates the starting point of diversification and the relative time scale of diversification is indicated by numbers (1 = origin, 0 = present). Numbers indicate LDD events to the Balearic Islands leading to (1) the origin of *C. fragilis* and (2) a range expansion of *C. aequitriloba*. Lines indicating the two LDD events were modified to prevent them from crossing.

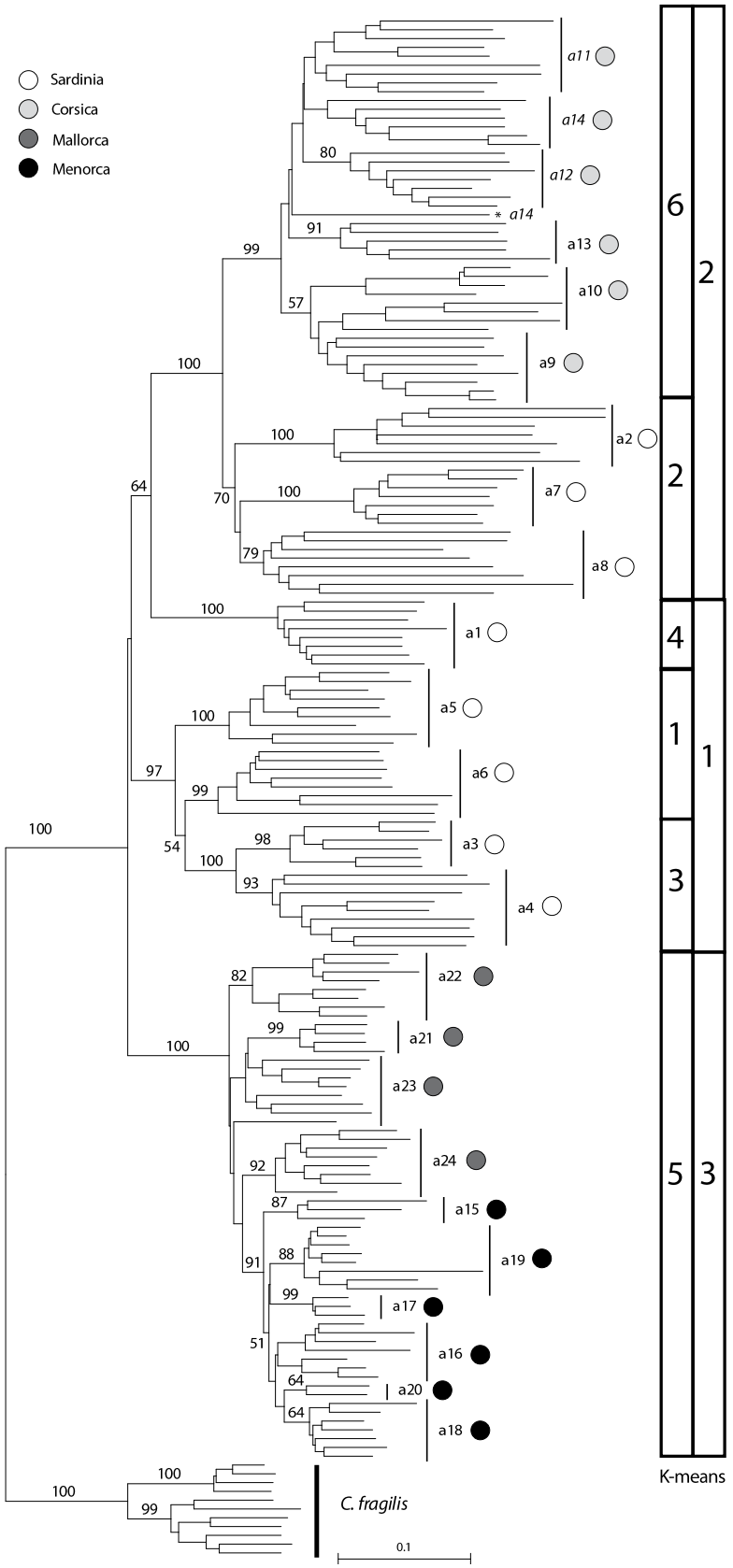

Figure 4. Neighbor-Joining tree based on AFLP data generated for *C. aequitriloba* following the taxonomic concept adopted in the present study. Distributions of clusters identified by nonhierarchical K-means clustering at K = 3 and K = 6 are shown to the right. Circles next to population names indicate the island of origin.

Plastid DNA sequences

The concatenated *ndhF* and *rpl32-trnL*$^{(UAG)}$ sequences consisted of 2776 aligned positions. We found 26 plastid haplotypes that differed by one to 14 substitutions including codified indels (Fig. 5). There was no clear correlation with taxonomy; instead, some geographically close populations of different taxa shared haplotypes. For instance, haplotype 4 was found in almost all populations of *C. aequitriloba* and *C. fragilis* from the Balearic Islands, haplotype 9 was found in two populations of *C. aequitriloba* and one population of *C. hepaticifolia* from Corsica, and haplotype 22 was found in two populations of *C. aequitriloba* and one population of *C. muelleri* from southern Sardinia (Fig. 5). The Balearic Islands clearly showed low haplotype diversity in comparison to Corsica and Sardinia. Relationships inferred by MP and Bayesian analyses were congruent and consequently we only show the topology from the Bayesian analysis (Fig. 5). Two main clades were found in the tree: the first contains the strongly divergent haplotype 26 from Sardinia and a haplotype of *Cymbalaria muralis* (PP 1; BS 100%), and it is sister to all other haplotypes found in BCS (PP 0.89). Phylogenetic relationships among haplotype groups (colour-coded in Fig. 5) were in general statistically weakly supported, whereas relationships among closely related haplotypes reflected the pattern of the parsimony network.

Morphometric analyses

Correlation coefficients did not exceed 0.95 for any pair of characters; therefore, all characters were retained for further analyses (supplementary Table S2).

Dataset 1

In the PCA, the first axis accounted for 34% of the variation and the second axis for 28%. The ordination diagram (Fig. 6A) suggested three clusters, two corresponded to *C. aequitriloba* and *C. hepaticifolia*, respectively, and a third one grouped *C. fragilis* and *C. muelleri*, including the controversial *C. aequitriloba* populations f1, f3, f4, f5, f8 and f9 from Cabrera and Menorca. The characters with most weight in the separation of the three groups were those related with indumentum density. The CDA (Fig. 6B, C) supported morphological differentiation of the four main AFLPs clades. The first two axes (41% and 35% of the explained

variation) supported the three groups observed in the PCA, with some overlap between *C. aequitriloba* and *C. fragilis* plus *C. muelleri*. The third axis (24%) supported the differentiation between *C. fragilis* and *C. muelleri*.

Dataset 2

The first PCA axis accounted for 44% of the variation and the second axis for 14%. The ordination diagram showed a structure similar to that retrieved by the analysis of dataset 1, albeit with stronger overlap of *C. aequitriloba* and the *C. fragilis – C. muelleri* group (supplementary Fig. S3).

Discussion

The combination of molecular and morphological data has been widely used to reevaluate systematics at different taxonomic levels (e.g. Stull et al., 2015; Vigalondo et al., 2015; Carvalho-Sobrinho et al., 2016; Puglisi et al., 2016). Specifically, many studies have used AFLP data and morphometrics to address taxonomic conflicts in groups of closely related plant taxa (e.g., Spaniel et al., 2012, Kuzmanović et al., 2013; Magauer et al., 2014; Ronikier & Zalewska-Gałosz, 2014; Kellner et al., 2014). In the case of *Cymbalaria*, AFLP and morphometric analyses allow for recognising four distinct entities (Fig. 2, supplementary Fig. S1) within a previously identified monophyletic group of *Cymbalaria* species endemic to the Balearic Islands, Corsica and Sardinia (Carnicero et al., 2017). These entities coincide with the current taxonomic circumscription of *C. hepaticifolia* and *C. muelleri*, but require novel circumscriptions of *C. aequitriloba* and *C. fragilis* (Figs 2, 6).

A novel circusmcription of *C. fragilis* and *C. aequitriloba*

Güemes (2009) suggested that *C. fragilis* is restricted to three populations in southern Menorca, and assigned reports from the North and East of the island (populations f1 and f4) as well as those from Cabrera (populations f8 and f9) to *C. aequitriloba*, based on their alveolate seeds. However, these populations exhibit all other distinctive morphological features as well as a late flowering time (Güemes, 2009) characteristic of *C. fragilis* and, interestingly, in population f6 we observed both

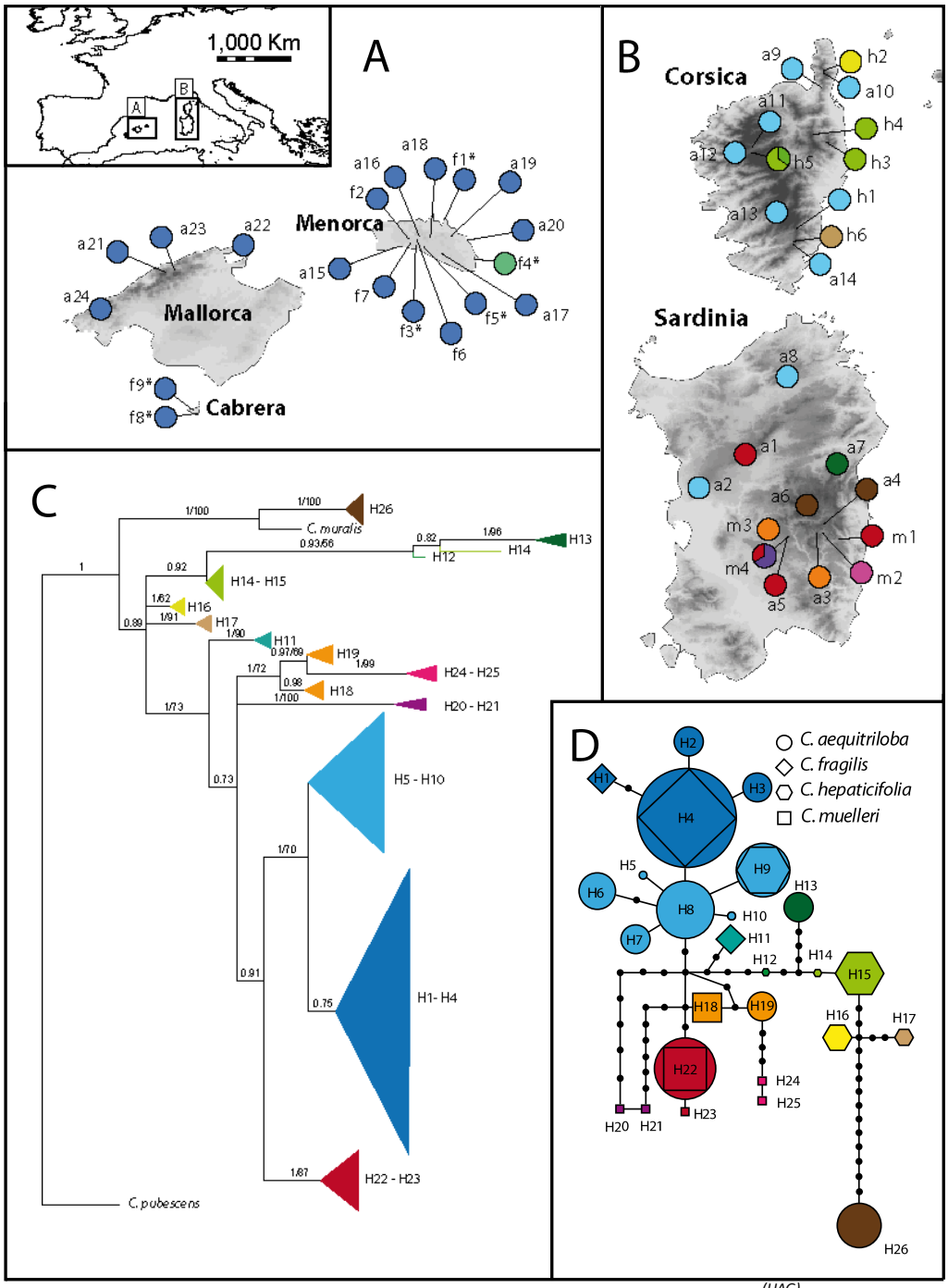

Figure 5. Sampled populations and patterns of plastid DNA (*ndhF* and *rpl32-trnL*$^{(UAG)}$) variation. Colours correspond to groups of closely related haplotypes. Taxonomy follows the taxonomic concept adopted in the present article (a: *C. aequitriloba*, f: *C. fragilis*, h: *C. hepaticifolia*, m: *C. muelleri*). A–B, pie charts indicate the proportions of haplotype groups sampled in each population. Asterisks indicate populations exhibiting mostly distinctive features of *C. fragilis* but previously assigned to *C. aequitriloba* owing to the presence of alveolate seeds. C, Phylogram from the Bayesian Inference analysis. Numbers above branches indicate Bayesian posterior probabilities / Maximum parsimony bootstrap support values. Haplotypes are given for each lineage next to the tips, numbering corresponds to Table S1. D, Statistical parsimony haplotype network. Black dots indicate hypothetical unsampled haplotypes, numbering corresponds to Table S1.

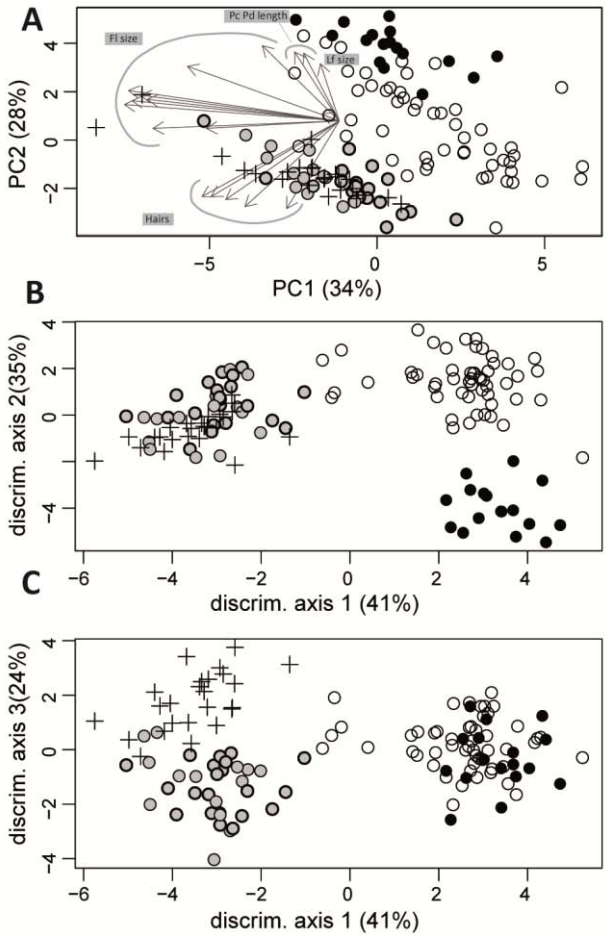

Figure 6. Ordination diagrams of morphometric analyses of 18 floral and vegetative characters obtained for 129 specimens (dataset 1) of *C. aequitriloba* (empty circles), *C. fragilis* (grey circles), *C. hepaticifolia* (black circles) and *C. muelleri* (crosses). Grey circles with thicker outline indicate *C. fragilis* specimens previously considered *C. aequitriloba*. A, Principal component analysis. Scatter plot of principal component scores for the first two components of morphological variation. Grey arrows represent relationships of characters projected in the same ordination space as the samples (Fl: flower, Lf: leaf, Pd: pedicel, Pt: petiole). B–C, Canonical discriminant analysis. Scatter plots of dicriminant scores for the three discriminant axis.

types of seed ornamentation. In the AFLP (Figs 2, 4) as well as in the morphometric analyses (Fig. 6), these populations clustered with *C. fragilis*. Therefore, *C. fragilis* must be redefined to include populations with the before-mentioned distinctive morphological characters from Menorca and Cabrera in spite of their aberrant seeds. Seed ornamentation –in this particular case– must thus be considered a variable character without taxonomic value. The strongly supported monophyly in the AFLP analyses (Fig. 2, supplementary Fig. S1) together with the clear morphological differentiation (Fig. 6) and the overlapping distributions of *C. fragilis* and *C. aequitriloba* in Menorca do not match the definition of subspecies (Van Son, 1955; Hamilton & Reichard, 1992; Stuessy, 2009). In contrast, the available evidence strongly

suggests treating *C. fragilis* as a separate species in accordance with the original description (Rodríguez, 1878), rather than as a subspecies of *C. aequitriloba* (Chevalier, 1936; Sutton, 1988). The proposed change of circumscription brings about a change in the species' threat category according to IUCN (2012) criteria. We sampled all known populations of *C. fragilis* to date, updating the number of populations from three to nine, and therefore suggest that Near Threatened (NT) would be a more adequate category than the formerly applied Critically Endangered (CR; Bañares et al. 2010).

A Corso – Sardinian common ancestor for the BCS endemics

Spatial reconstruction of evolutionary dynamics using relaxed random walks (Fig. 3) suggests a Sardinian origin of the BCS endemic species of *Cymbalaria*. The low haplotype diversity found in the Balearic Islands (Fig. 5) is congruent with a Sardinian origin, but does not allow to reject the alternative hypothesis of a Corsican origin. An east (Corsica-Sardinia) to west (Balearic Islands) pattern of colonization has been also suggested for the BCS endemics *Arum pictum* (Mansion et al., 2008) and *Thymus herba-barona* (Molins et al., 2011).

Cymbalaria hepaticifolia and *C. muelleri* diverged from a common ancestor probably via an allopatric speciation event, as suggested by their current distribution areas (Fig. 1). Our genetic and morphological data confirmed them as two well differentiated species (Figs. 2, 6). However, plastid sequences revealed close relationships among the haplotypes found in the two before-mentioned species and those of sympatric *C. aequitriloba* populations (Fig. 5). Both the strongly reduced haplotype diversity of *C. fragilis* in comparison with Corsican and Sardinian populations of the three other studied species (Fig. 5) and the BEAST continuous phylogeographic analysis (Fig. 3) support the hypothesis that a LDD event to the Balearic Islands led to the origin of *C. fragilis*. Genetic diversity of *C. fragilis* was not significantly lower than that of *C. aequitriloba* (supplementary Table S4), suggesting that the LDD event was ancient enough to allow for a recovery of genetic diversity (Luikart et al., 1998). *Cymbalaria aequitriloba* likely originated in Sardinia (Fig. 3), which is also supported by the highest among-population variation in the AMOVAs (supplementary Table S3), the significantly higher genetic diversity observed in

Sardinian populations as compared to populations from the other islands (supplementary Table S4), the high haplotype diversity (Fig. 5) and the high number of of separable AFLP gene pools (Fig. 4).

Phylogeography of *C. aequitriloba* and *C. fragilis*

Cymbalaria aequitriloba exhibits a higher genetic diversity in Sardinia than in Corsica, as shown by the higher number of plastid haplotypes and AFLP clusters (Figs. 4, 5). This is most probably explained by Pleistocene glaciation periods, which had a stronger effect in Corsica than in Sardinia as illustrated by the large glaciers documented in Corsica during the Last Glacial Maximum (18,000 years ago, Kuhlemann et al., 2005), and could have caused local extinctions (Reille et al., 1997; Salvi et al., 2016). Later range expansion might have occurred from either lowland Corsican or Sardinian refuge areas, via land bridges connecting both islands multiple times during the Pleistocene (Lambeck et al., 2004; Lambeck & Purcell, 2005) or across the narrow Bonifacio strait separating Corsica and Sardinia (Falchi et al., 2009). Populations from northern and western Sardinia showed a high genetic similarity with Corsican populations (Figs 4, 5), supporting contacts between the populations of the two islands as shown for *Cistus creticus* L. (Falchi et al., 2009).

Cymbalaria reached the Balearic Islands via two independent LDD events, as suggested by the BEAST continuous phylogeographic analysis (Fig. 3), the first ultimately leading to the origin of *C. fragilis* and the second causing a range expansion of *C. aequitriloba*. These two events were also supported by the shorter terminal splits depicted in the Neighbor-Net diagram for all Balearic populations indicating strong similarity among individuals (Figs. 2, 4). Significantly lower average genetic diversity of Balearic populations of *C. aequitriloba* as compared to those from Corsica and Sardinia also support the hypothesis of a LDD event (supplementary Table S4). In addition, the strongly reduced haplotype diversity in the Balearic Islands (Fig. 5) may evidence a strong bottleneck resulting from a founder event (e.g., Schönswetter et al., 2008, Burnier et al., 2009). With the exception of haplotype H11, the close phylogenetic relationships among haplotypes from the Balearic Islands (Fig. 5C) suggest a common ancestral haplotype for *C. aequitriloba* and *C. fragilis*. This common ancestor might

have occurred in western and northern Sardinia as shown by the BEAST continuous phylogeographic analysis and the close phylogenetic relationship between H1–H4 from the Balearic Islands and H5–H10 from Corsica and Sardinia (Figs. 3, 5). In the same line, the geographic setting supports a dispersal from western Sardinia, for instance from the surroundings of population a2. It is the closest to the Balearic Islands and thrives on the summit of the Monte Ferru massif, at 1050 m altitude only 12 km from the coast. Alternatively, or in addition, at a later stage hybridization with *C. fragilis* could have led to the sharing of haplotype H4 across species boundaries (Currat et al., 2008).

The history of the Balearic populations of both species was likely influenced by sea level variation during the Pleistocene. Land connections during glaciation periods (Vesica et al., 2000) might have aided *C. aequitriloba* and *C. fragilis* to disperse among the Eastern Balearic Islands until both species achieved their current distributions. However, during early Pleistocene interglacials (1.6–0.7 Ma) the sea rose for up to 100 m above current level, leading to considerable reduction of the surface of Menorca, division of Mallorca into two islands and transgression of most of Cabrera (Gràcia et al., 2001; Mayol et al., 2012). This was likely a major obstacle for the expansion of *C. fragilis* to Cabrera, since only a small area of that island protruded from the sea, suggesting that the colonization most probably occurred after this period. In any case, presence of well-supported clades in Menorca and Cabrera (Fig. 2B, supplementary Fig. S1) supports the current isolation.

In contrast to other members of the tribe Antirrhineae exhibiting winged seeds or adaptation to marine dispersal (e.g. *Linaria* Mill., *Cymbalaria longipes* (Boiss. & Heldr.) A. Cheval.; Sutton, 1988) neither *C. aequitriloba* nor *C. fragilis* show any apparent adaptation for LDD. Moreover, both species are at least partly achorous and actively prevent seed dispersal by positioning mature capsules in rock crevices (Sutton, 1988). However, the importance of non-standard vectors has been recognised (Nathan et al., 2008; Gillespie et al., 2012) and rare stochastic events such as LDDs have been highlighted as important drivers of organisms' histories (Losos & Mahler, 2010; Roquet et al., 2013). In fact, LDD has been invoked in several

cases in spite of the apparent lack of particular adaptations (e.g. Guzmán & Vargas, 2008; Dixon et al., 2009; Escudero et al., 2010; Santos-Gally et al., 2011; Piñeiro et al., 2012). The success in colonization after a LDD is thought to be governed by the availability of adequate niches rather than by dispersal abilities (Nieto Feliner, 2014). In addition, polyploids are better buffered against genetic bottlenecks and subsequent inbreeding resulting from founder effects (Linder & Barker, 2014), a feature that could have helped *C. aequitriloba* and *C. fragilis* to establish in the Balearic Islands.

Finally, our previous results (Carnicero et al., 2017) strongly suggested that *C. aequitriloba* is not a palaeoendemic that originated and expanded its range when Corsica-Sardinia and the Balearic Islands were connected (~20 Ma, Speranza et al., 2002), but rather colonized the Balearic Islands much later, 1–4 Ma. The results presented here support this statement and furthermore show that *C. aequitriloba* does not meet the criteria attributed to relict taxa, i.e., low levels of infraspecific morphological and genetic variation as a result of a long process of adaptation in refugial isolation, and taxonomic isolation due to extinction of close relatives (Favarger & Contandriopoulos, 1961; Mansion et al., 2008). In contrast, *C. aequitriloba* is highly morphologically variable (Fig. 6, Sutton, 1988; Güemes, 2009), genetically equally diverse as its closest relatives (supplementary Table S4) and not taxonomically isolated.

Incongruence between AFLP and sequence data

The monophyly of each of the four species in the AFLP analyses (Fig. 2, supplementary Fig. S1) suggests that reproductive isolation is effective at present. However, interspecific gene flow appears possible due to the close proximity between populations of *C. aequitriloba* and *C. hepaticifolia* in Corsica, and between *C. aequitriloba* and *C. muelleri* in Sardinia (P.C. & M.G., personal field observations). A few incongruities between mostly nuclear-derived AFLPs (Bussell et al., 2005) and maternally inherited plastid DNA sequences (Corriveau & Coleman, 1988), could indicate past reticulation events. Although incomplete lineage sorting (ILS) of ancient polymorphisms cannot be ruled out as cause for these incongruities (Soltis et al., 1998; Degnan et al., 2009; Pelser et al., 2010; Hilpold et al., 2014; Sun et al., 2015), hybridization appears more likely due to the grouping of haplotypes retrieved from

geographically close populations of different taxa (Fig. 4, McKinnon et al., 2004; Lorenz-Lemke et al., 2006). The most obvious case is the occurrence of haplotype 26 otherwise characteristic of the phylogenetically distant (cf. Carnicero et al., 2017) *C. muralis* in populations a4 and a6 of *C. aequitriloba* from Sardinia (Fig. 4). *Cymbalaria muralis*, a diploid species native to the Apennine and northern Balkan Peninsulas, is naturalized almost worldwide in temperate areas (Sutton, 1988). The population of *C. muralis* used here is from the same area in Sardinia where haplotype 26 was found; hybridization resulting in chloroplast capture of a *C. muralis* haplotype is, therefore, likely.

Two statistically supported incongruities were found between the AFLP dataset and the previous phylogeny (Carnicero et al., 2017). The latter retrieved a clade formed by *C. aequitriloba* accessions from Corsica and *C. hepaticifolia*, and inferred a common ancestor for *C. fragilis* and *C. muelleri*; these two clades did not appear in the AFLP analysis (Fig. 2). Although nrDNA and AFLPs have been shown to yield mostly congruent signals (Koopman, 2005), incongruities have also been reported (El-Rabey et al., 2002; Semerikov et al., 2003). Here, we suggest that hybridization followed by concerted evolution of nrDNA towards one parental copy (Álvarez & Wendel, 2003; Soltis et al., 1998) and/or ILS are the most probable causes for the incongruities. Therefore, we consider the aforementioned relationships retrieved from the nrDNA phylogeny to be spurious. In the same line, it has been stated that AFLPs explain better phylogenetic relationships among closely related species, especially when dealing with complex evolutionary processes such as polyploidy and hybridization (Koopman, 2005).

Acknowledgements

We thank all colleagues who assisted during fieldwork or provided plant material. M. Magauer, M. Pacary, D. Pirkebner and R. Vilatersana provided valuable help with lab work. We also thank B. Frajman, M. Magauer and I. Rešetnik for assistance in the BEAST continuous phylogeography and K-means clustering analyses. J. M. Blanco-

Moreno contributed to the morphometric analyses with valuable comments. This research was funded in part by the Spanish government (CGL2010-18631/BOS and the *Flora iberica* project, CGL2011-28613-C03-01) as well as the Catalan government (2014-SGR 514). Pau Carnicero benefited from the support of a PIF PhD student fellowship from the Universitat Autònoma de Barcelona and an Erasmus+ traineeship mobility fellowship, which supported a research stay at the Institute of Botany of the University of Innsbruck.

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Table S1. List of sampled populations with information on the population codes used in the text and figures, locality, herbarium voucher, number of individuals included in each analysis and accession numbers. Superscripts next to the haplotype code indicate the number of individuals owing the corresponding haplotype.

species	population code	voucher	locality	Sampled specimens				Accession numbers	
				AFLPs	Haplotypes	Morphology dataset 1	Morphology dataset 2	*rpl32-trnL*	*ndhF*
Cymbalaria aequitriloba	a1	*P. Carnicero 380 & M. Galbany Casals* (BC 5777)	IT, Sardinia, Badde Salighes, Ortachis	8	H22[3]	7	1		
Cymbalaria aequitriloba	a2	*P. Carnicero 385 & M. Galbany-Casals* (BC 955774)	IT, Sardinia, Badde Urbara, Mt Pertusu	7	H8[3]	5	1		
Cymbalaria aequitriloba	a3	*P. Carnicero 396 & M. Galbany-Casals* (BC 955775)	IT, Sardinia, Seui, Funtana dorada	6	H19[3]	0	1		
Cymbalaria aequitriloba	a4	*P. Carnicero 412 & M. Galbany-Casals* (BC 955776)	IT, Sardinia, Seui, Perda Liana	9	H26[3]	1	3		
Cymbalaria aequitriloba	a5	*P. Carnicero 415 & M. Galbany-Casals* (BC 955781)	IT, Sardinia, Gadoni, Foresta comunale	9	H22[3]	0	4		
	a5	*P. Carnicero 1232 & M. Unzeta* (BC 955767)				2	0		
Cymbalaria aequitriloba	a6	*P. Carnicero 421 & M. Galbany-Casals* (BC 955768)	IT, Sardinia, Bruncu Spina, ski resort	8	H26[2]	4	1		
Cymbalaria aequitriloba	a7	*P. Carnicero 424 & M. Galbany-Casals* (BC 955768)	IT, Sardinia, Oliena, Sorgenti del Gologone	7	H13[3]	1	3		
Cymbalaria aequitriloba	a8	*P. Carnicero 425 & M. Galbany-Casals* (BC 955779)	IT, Sardinia, Témpio Pausania, Mt Limbara	8	H5[1], H8[2]	2	3		
Cymbalaria aequitriloba	a9	*P. Carnicero 430 & M. Galbany-Casals* (BC 955780)	FR, Corsica, Bastia, summit of Pigno	8	H6[1], H8[2]	7	5		

Cymbalaria aequitriloba	a10	*P. Carnicero 432 & M. Galbany-Casals* (BC 955778)	FR, Corsica, Sisco, W of Mte Corvu	8	H7[3]	0	1		
Cymbalaria aequitriloba	a11	*P. Carnicero 446 & M. Galbany-Casals* (BC 955773)	FR, Corsica, 3km above Albertacce, left edge of Golo	8	H8[2], H10[1]	1	1		
Cymbalaria aequitriloba	a12	*P. Carnicero 448 & M. Galbany-Casals* (BC 955772)	FR, Corsica, Paisolu Aitone, Foret Demaniale d'Aitone	7	H9[3]	2	1		
Cymbalaria aequitriloba	a13	*P. Carnicero 451 & M. Galbany-Casals* (BC 955771)	FR, Corsica, from Zicavu to Guitera les Bains	5	H9[2]	0	1		
Cymbalaria aequitriloba	a14	*P. Carnicero 460 & M. Galbany-Casals* (BC 955770)	FR, Corsica, Bocca Illarata	8	H6[3]	0	0		
Cymbalaria aequitriloba	a15	*P. Carnicero 544 et al.* (BC 955766)	SP, Balearic Islands, Menorca, Ciutadella, Cala en Turqueta	3	H4[3]	0	0		
Cymbalaria aequitriloba	a16	*P. Carnicero 562 et al.* (BC 955762)	SP, Balearic Islands, Menorca, Ferreries, Ermita	7	H4[3]	1	0		
Cymbalaria aequitriloba	a17	*P. Carnicero 563 et al.* (BC 955761)	SP, Balearic Islands, Menorca, Es Mercadal, Llinàritx Nou	3	H4[3]	0	0		
Cymbalaria aequitriloba	a18	*P. Carnicero 571 et al.* (BC 955764)	SP, Balearic Islands, Menorca, Es Mercadal, Toro	7	H2[3]	1	1		
Cymbalaria aequitriloba	a19	*P. Carnicero 574 et al.* (BC 955763)	SP, Balearic Islands, Menorca, Binifabini	8	H4[2]	0	0		

Cymbalaria aequitriloba	a20	*P. Carnicero 579 et al.* (BC 955765)	SP, Balearic Islands, Menorca, Es Grau	2	H4[2]	1	0	
Cymbalaria aequitriloba	a21	*L. Sáez 7365* (BC 955759)	SP, Balearic Islands, Mallorca, Sóller, Gorg Blau	4	H4[3]	3	2	
Cymbalaria aequitriloba	a22	*L. Sáez 7366 & X. Rotllan* (BC 879621)	SP, Balearic Islands, Mallorca, Formentor	8	H4[3]	0	4	KP851095 KP851009
Cymbalaria aequitriloba	a23	*L. Sáez 7368* (BC 955758)	SP, Balearic Islands, Mallorca, Sóller, Puig Major	8	H3[3]	5	4	
Cymbalaria aequitriloba	a24	*L. Sáez 7385* (BC 955760)	SP, Balearic Islands, Mallorca, Calvià, Puig de Galatzó, vessant NE	8	H4[3]	10	0	
Cymbalaria fragilis	f1	*P. Carnicero 353 & M. Galbany-Casals* (BC 955790)	SP, Balearic Islands, Menorca, Fornells, Mola de Fornells	10	H4[3]	5	0	
Cymbalaria fragilis	f2	*P. Carnicero 800 & P. Fraga* (BC 955791)	SP, Balearic Islands, Menorca, Ferreries, Barranc d Algendar, Pas d en Revull	6	H4[3]	4	2	
Cymbalaria fragilis	f3	*P. Carnicero 805 & P. Fraga* (BC 955792)	SP, Balearic Islands, Menorca, Es Migjorn, Barranc Albranca	10	H4[3]	5	0	
Cymbalaria fragilis	f4	*P. Carnicero 810 & P. Fraga* (BC 955793)	SP, Balearic Islands, Menorca, Maó, Mola de Maó	10	H11[3]	5	4	

Cymbalaria fragilis	f5	*P. Carnicero 814 & P. Fraga* (BC 955794)	SP, Balearic Islands, Menorca, Barranc de Binigaus, St. Agustí Vell	10	H4[3]	5	5		
Cymbalaria fragilis	f6	*P. Carnicero 816 & P. Fraga* (BC 955795)	SP, Balearic Islands, Menorca, Barranc de Trebalúger, Son Marcer de Baix	10	H4[3]	5	4		
Cymbalaria fragilis	f7	*P. Carnicero 818 & P. Fraga* (BC 955796)	SP, Balearic Islands, Menorca, between Binimassó and Algendaret, Canal d'en Curt	3	H1[3]	4	3		
Cymbalaria fragilis	f8	*L. Sáez 7329* (BC 955797)	SP, Balearic Islands, Cabrera, Imperialet	4	H4[3]	0	1		
Cymbalaria fragilis	f9	*L. Sáez 7377* (BC 879620)	SP, Balearic Islands, Cabrera, Es Llenegall	7	H4[3]	2	4		
Cymbalaria hepaticifolia	h1	*P. Carnicero 427 & M. Galbany-Casals* (BC 955753)	FR, Corsica, NE of Col de Bavella	8	H9[3]	0	0		
Cymbalaria hepaticifolia	h2	*P. Carnicero 433 & M. Galbany-Casals* (BC 955757)	FR, Corsica, Sisco, Mt Corvu	8	H16[3]	4	3		
Cymbalaria hepaticifolia	h3	*P. Carnicero 441 & M. Galbany-Casals* (BC 955754)	FR, Corsica, Castagniccia, Felce	8	H15[2]	0	1		
Cymbalaria hepaticifolia	h4	*P. Carnicero 444 & M. Galbany-Casals* (BC 879631)	FR, Corsica, Castagniccia, road from Croce to Porta	7	H15[3]	6	3	KP851099	KP851013

Cymbalaria hepaticifolia	h5	*P. Carnicero 447 & M. Galbany-Casals* (BC 955755)	FR, Corsica, Albertacce, Foresta de Valdo niello, close to sorgenti di mezzanule	8	H12[4], H14[1], H15[1]	7	5		
Cymbalaria hepaticifolia	h6	*P. Carnicero 456 & M. Galbany-Casals* (BC 955756)	FR, Corsica, N of Bocca d'Illarata	2	H17[2]	0	1		
Cymbalaria muelleri	m1	*P. Carnicero 389 & M. Galbany-Casals* (BC 879630)	IT, Sardinia, Ulassai, Bruncu Matzeu	8	H22[1]	0	3	KP851096	KP851010
	m1	*P. Carnicero 1263 & M. Unzeta* (BC 955784)				6	0		
Cymbalaria muelleri	m2	*P. Carnicero 406 & M. Galbany-Casals* (BC 879629)	IT, Sardinia, Seui, Foresta de Montarbu, SE cliffs of Genni d'Acca	2	H24[1], H25[1]	0	0	KP851098	KP851012
	m2	*P. Carnicero 1251 & M. Unzeta* (BC 955783)				6	0		
Cymbalaria muelleri	m3	*P. Carnicero 414 & M. Galbany-Casals* (BC 955485)	IT, Sardinia, Laconi, Corona Sa Guardia, S'aza du Ziu Chiccu	8	H18[3]	0	2		
	m3	*P. Carnicero 1223 & M. Unzeta* (BC 955789)				6	0		
Cymbalaria muelleri	m4	*P. Carnicero 416 & M. Galbany-Casals* (BC 955786)	IT, Sardinia, Gadoni, Foresta comunale	8	H20[1], H21[1], H23[1]	0	1		
	m4	*P. Carnicero 1231 & M. Unzeta* (BC 955788)				6	0		

Outgroups									
Cymbalaria microcalyx		*P. Carnicero 1074* (BC 955798)	GR, Peloponnese, Lakonia, road from Trypi to Kalamata	1					
Cymbalaria muralis		*P. Carnicero 420 & M. Galbany-Casals* (BC 955752)	IT, Sardinia, Aritzo						
Cymbalaria pubescens		*M. Galbany-Casals 2311 & N. Garcia-Jacas* (BC 955799)	IT, Sicily, from Portella della Páglia to La Pizzuta	2					

Table S2. Characters studied in morphometric analyses.

Character	Code	Type	dataset
Upper lip width (mm)	UlpW	Quantitative	1
Upper lip length (mm)	ULpL	Quantitative	1
Upper lip sinus (mm)	UlpS	Quantitative	1
Lower lip width (mm)	LLW	Quantitative	1
Lower lip length (mm)	LLpL	Quantitative	1
Corolla length (mm)	CrL	Quantitative	1
Corolla tube width (mm)	CrTW	Quantitative	1
Spur length (mm)	SpL	Quantitative	1
Spur width (mm)	SpW	Quantitative	1
Indumentum of capsule	ICp	Ordinal (0 = glabrous; 1 = subglabrous, sparsely distributed hairs; 2 = non-overlapping uniformly distributed hairs; 3 = overlapping hairs)	2
Indumentum of stem	ISt		1, 2
Indumentum of adaxial leaf surface	ILfA		1, 2
Indumentum of abaxial leaf surface	ILfR		1, 2
Indumentum of calyx	ICx		1, 2
Indumentum of upper part of pedicel	Ipd		1, 2
Number of leaf lobes	NLb	Quantitative	1, 2
Maximum leaf width (mm)	MLfW	Quantitative	1, 2
Pedicel length at anthesis (mm)	PdLFl	Quantitative	1
Pedicel length, fruiting (mm)	PdLFr	Quantitative	2
Petiole length of bracts at anthesis (mm)	PcLFl	Quantitative	1
Petiole length of bracts, fruiting (mm)	PcLFr	Quantitative	2
Calyx length (mm)	CxL	Quantitative	2
Capsule length (mm)	CpL	Quantitative	2
Seed surface	SdS	Ordinal (1 = smooth; 2 = alveolate; 3 = cristate-alveolate; 4 = cristate; 5= tuberculate)	2
Seed length (mm)	SdL	Quantitative	2

Table S3. Hierarchical AMOVAs. BI: Balearic Islands, Co: Corsica, Sa: Sardinia.

	Groups	Among groups		Among populations		Within populations	
		d.f.	% variation	d.f.	% variation	d.f.	% variation
Western Mediterranean *Cymbalaria*	4 (species)	3	28.77	39	36.47	257	34.76
C. aequitriloba	2 (BI, Co-Sa)	1	24.71	21	33.55	133	41.74
	3 (BI, Co, Sa)	2	28.08	20	27.88	133	44.05
C. aequitriloba (BI)	-	-	-	9	35.53	48	64.47
C. aequitriloba (Co)	-	-	-	5	27.65	38	72.35
C. aequitriloba (Sa)	-	-	-	7	44.66	54	55.34

Table S4. Average gene diversity (π_n) and Student's t-tests for comparisons of gene diversity among groups. BI: Balearic Islands, Co: Corsica, Sa: Sardinia.

Taxon (group of populations)	Average gene diversity (π_n)	t-test		
		vs.	T	p-value
C. aequitriloba	0.090			
C. aequitriloba (BI)	0.067	*C. aequitriloba* (Sa)	-5.536	0.000 *
		C. aequitriloba (Co)	-3.528	0.005 *
C. aequitriloba (Co)	0.101	*C. aequitriloba* (Sa)	-0.566	0.58
C. aequitriloba (Sa)	0.107			
C. fragilis	0.079	*C. aequitriloba*	1.439	0.17
		C. aequitriloba (BI)	-1.532	0.15
C. hepaticifolia	0.104	*C. aequitriloba*	-0.540	0.62
	0.104	*C. aequitriloba* (Co)	-0.119	0.91
C. muelleri	0.092	*C. aequitriloba*	-0.244	0.82
		C. aequitriloba (Sa)	1.6	0.19

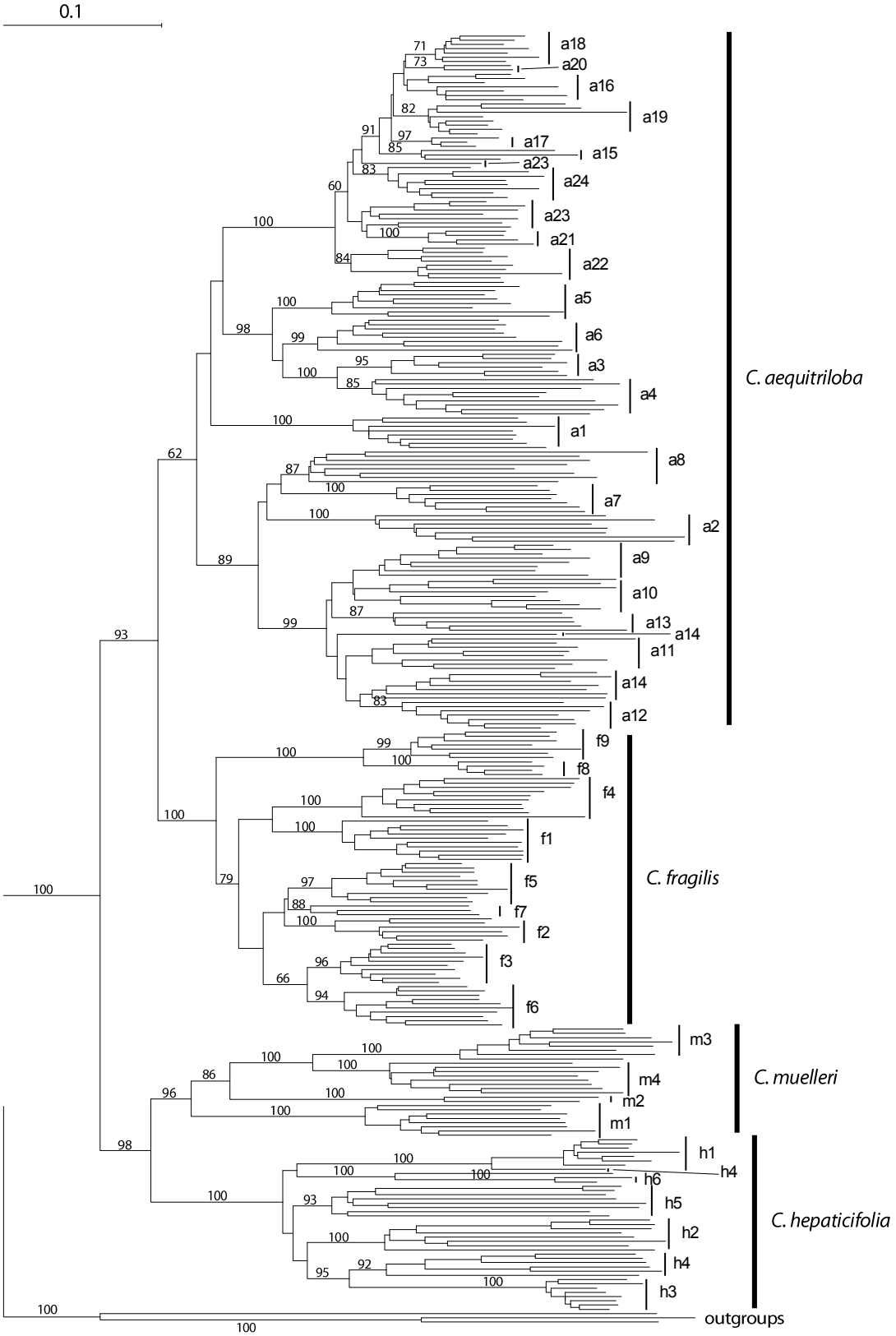

Figure S1. Phylogenetic tree resulting from Neighbour-Joining analysis of AFLP data for *C. aequitriloba*, *C. fragilis*, *C. hepaticifolia* and *C. muelleri*. Populations are numbered as in Fig. 1 and Table S1. Numbers above branches are bootstrap support values ≥ 60%.

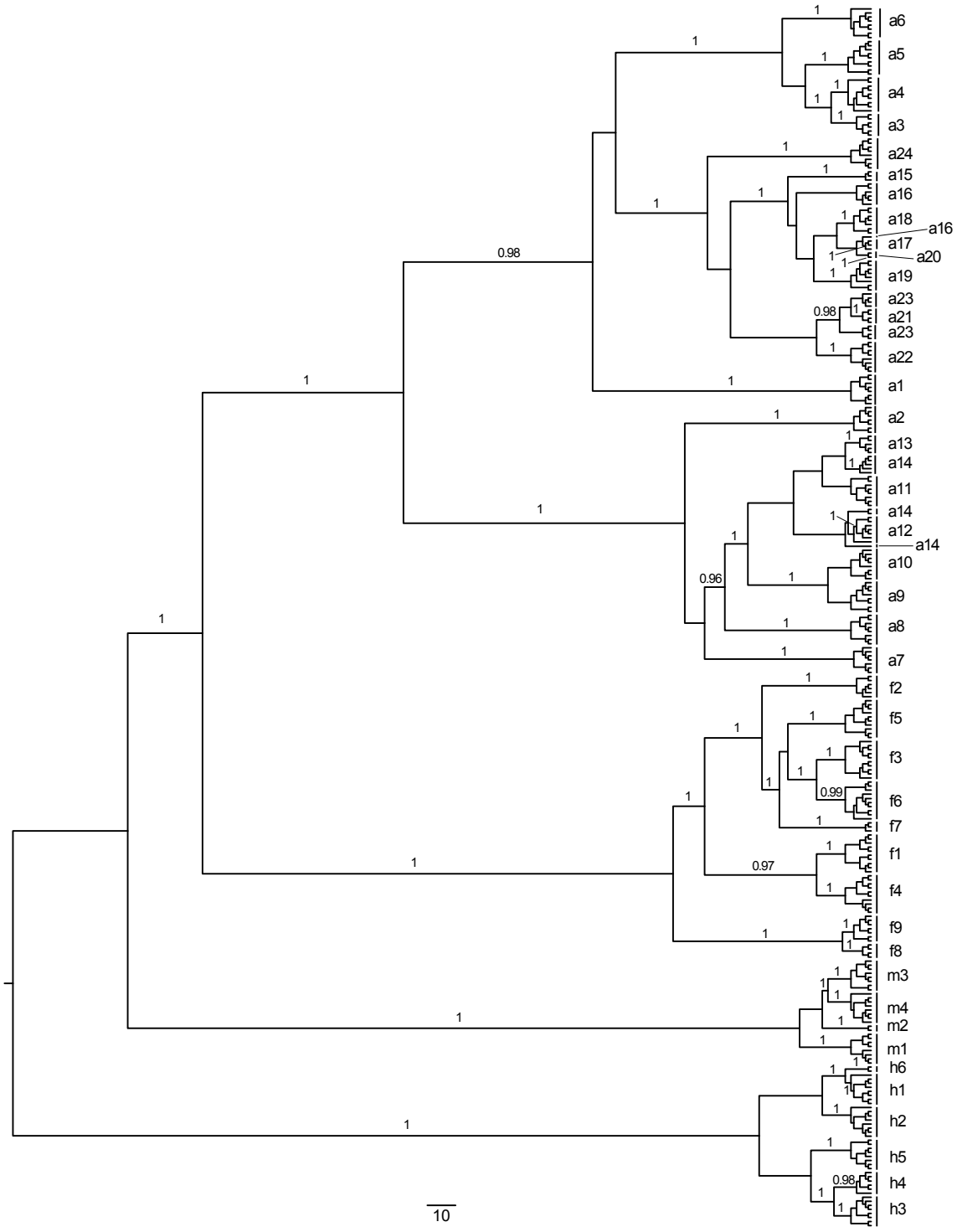

Figure S2. Maximum clade credibility (MCC) tree resulting from the Bayesian phylogeographical diffusion model in continuous space performed on the AFLP dataset using BEAST v1.8.2. Populations are numbered as in Fig. 1 and Table S1. Bayesian posterior probabilities ≥ 0.95 are indicated above branches.

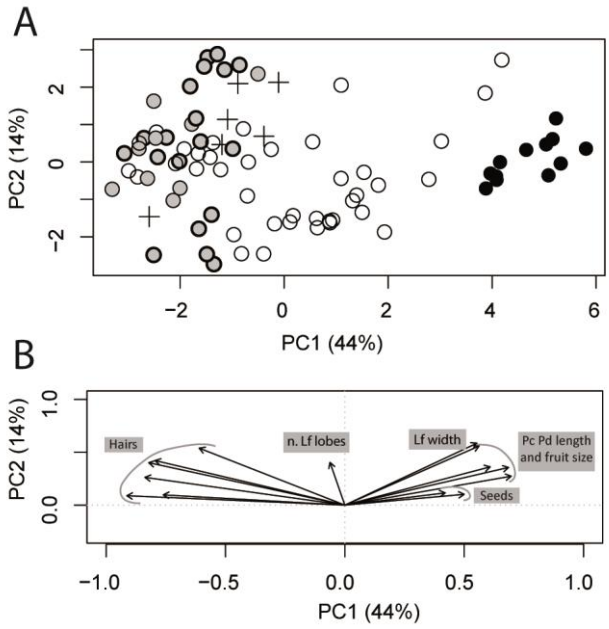

Figure S3. Ordination diagrams of principal component analysis of 14 floral and vegetative morphometric characters obtained for 85 specimens (dataset 2) of *C. aequitriloba* (empty circles), *C. fragilis* (grey circles), *C. hepaticifolia* (black circles) and *C. muelleri* (crosses). Grey circles with thicker outline indicate *C. fragilis* specimens previously considered *C. aequitriloba*. A, Scatter plot of principal component scores for the first two components of morphological variation. B, Relationships of characters projected in the same ordination space as the samples (Lf, leaf; Pd, pedicel; Pt, petiole).

Chapter III

Cymbalaria muelleri subsp. *villosa* subsp. nov., a new morphologically and genetically divergent Sardinian endemic[1]

[1] With Peter Schönswetter, Núria Garcia Jacas, Llorenç Sáez Gonyalons and Mercè Galbany Casals. Prepared for submission to *Plant Systematics and Evolution*

Abstract

Cymbalaria muelleri (Plantaginaceae) is a chasmophytic species endemic to Sardinia, a major island in the Mediterranean Basin. Its distribution range is divided into two well delimited geographic groups. Here, we used a combination of morphology, molecular data (amplified fragment length polymorphisms fingerprinting) and relative genome size estimation to ascertain whether the two geographic groups should be considered two separate taxa. Morphological analyses support the differentiation of the two population groups, as they revealed strong differences in indumentum and seed coat sculpturing. Therefore, the eastern populations exhibiting distinctive long hairs and cristate-alveolate seeds are formally described as *C. muelleri* subsp. *villosa*. The new taxon is shown to be paraphyletic according to the molecular analyses, and has given rise to a monophyletic group composed by all western populations (*C. muelleri* subsp. *muelleri*). Thus, an anacladogenetic (sub)speciation process is proposed for the origin of *C. muelleri* subsp. *muelleri*. Alongside morphology and genetics, slightly divergent habitat preferences and the disjunct distribution of the two subspecies advocate the recognition of two taxa. Genome size data obtained for the two subspecies are consistent with the previously established hexaploidy of *C. muelleri*.

Keywords AFLP, anacladogenetic speciation, *Cymbalaria muelleri* subsp. *villosa*, endemism, Sardinia

Introduction

The Mediterranean Basin is one of the 25 biodiversity hotspots (Myers et al. 2000). It harbors ca. 25,000 species, around 10% of the world's vascular flora, of which 63% are endemic (Greuter 1991). Mediterranean islands importantly contribute to the area's high diversity and endemism. Indeed, the largest islands Corsica, Crete, Cyprus, Sicily and Sardinia constitute biodiversity hotspots (Médail and Quézel 1997) as they provided climatically stable areas where species and genetic diversity were preserved during glaciation periods (Médail and Diadema 2009). These features, together with an often well-known history of land connections between islands or with the continent due to tectonic movements and climatic oscillations (Thompson 2005) render these islands an important study area for phylogeographic studies in the Mediterranean Basin (Nieto Feliner 2014).

Sardinia, situated in the western Mediterranean Basin, is the second-largest Mediterranean island. It originated from the splitting and rotation of the Hercynian belt during the Oligocene (~30 Ma, Rosenbaum et al. 2002). Its isolation, abrupt relief and geological diversity have triggered a high endemism rate, especially in the mountain areas (Médail and Quézel 1997). Of the 2,408 taxa occurring in Sardinia, 168 are endemic to the island (Bacchetta et al. 2012a); this number rises to ca. 350 if endemics shared between Sardinia on the one hand and Corsica, the Balearic Islands and/or the Tuscan Archipelago on the other hand are considered (Bacchetta et al. 2005). In particular, chasmophytes contribute to the high endemism rate due to the antiquity and high abundance of limestone cliffs (Bacchetta et al. 2007). Chasmophytes represent 135 taxa, of which 41 are endemic to Sardinia and 27 to Sardinia and surrounding areas (Bacchetta et al. 2007).

Cymbalaria muelleri (Moris) A. Chev. (Plantaginaceae) is a chasmophytic species endemic to Sardinia. It comprises twelve known populations distributed across a limited mountainous area spanning ca. 40 × 20 km south of the Gennargentu Massif (Arrigoni et al. 1979). In spite of its limited distribution and the presence of apparent anthropic threats due to grazing and occurrence in climbing areas (P.C. and M.G. field observations), it is not

considered endangered (Bacchetta et al. 2012; Camarda et al. 2015). From an evolutionary point of view, *C. muelleri* is part of a polyploid lineage composed by three more species endemic to Corsica, Sardinia, the eastern Balearic Islands and the Tuscan Archipelago (Carnicero et al. 2017). According to a recent phylogeny mainly based on amplified fragment length polymorphism (AFLP) fingerprinting (Carnicero et al. in prep., Chapter II), *C. muelleri* forms a monophyletic lineage, which is sister to the Corsican endemic *C. hepaticifolia* Wettst. The morphological variability of *C. muelleri* is remarkably high and geographically structured. Our field observations and herbarium studies revealed that plants from the type locality (Laconi, Corona Sa Guardia, S'aza du Ziu Chiccu) and other western populations show a dense indumentum composed of short patent hairs, whereas eastern populations exhibit a less dense indumentum of long hairs. This divergence

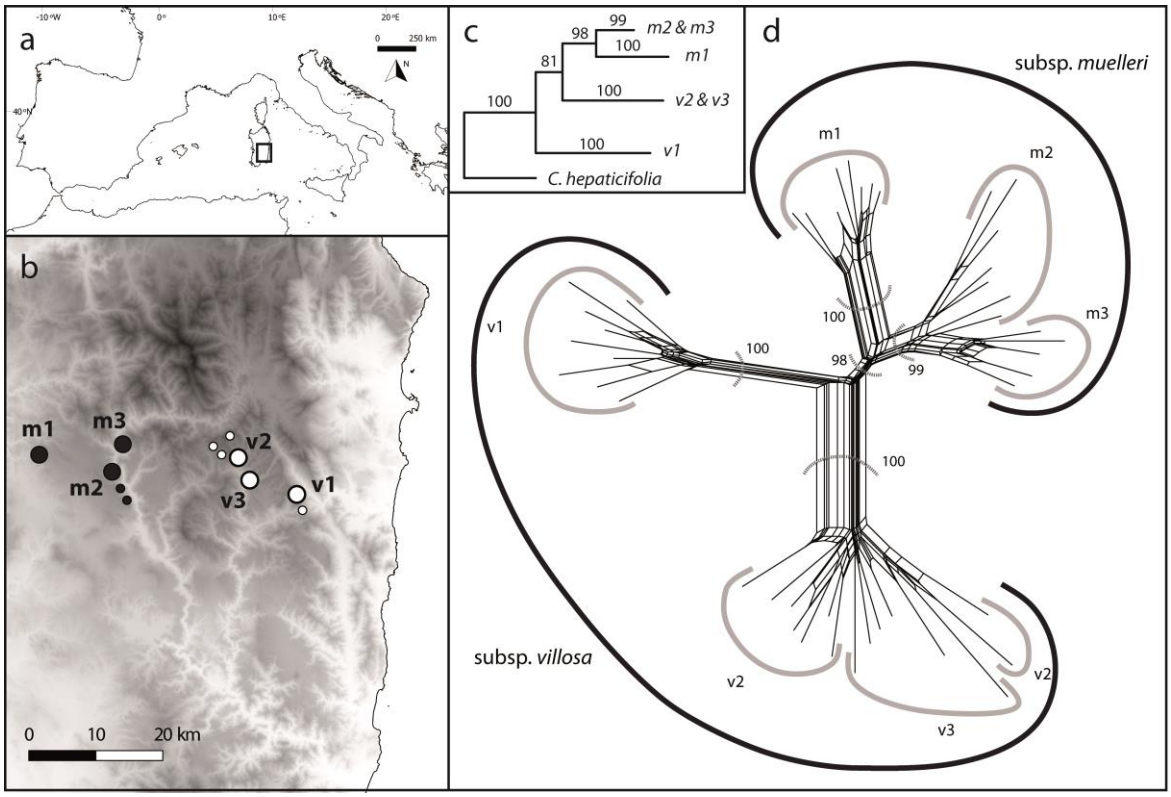

Figure 1. a, b Distribution map of *Cymbalaria muelleri*. Big circles represent sampled populations with population codes next to them as in Supplementary Table S1. Small circles show non-sampled population based on additional herbarium specimens examined. Forms filled in black correspond to *C. muelleri* subsp. *muelleri* and symbols filled in white correspond to *C. muelleri* subsp. *villosa*. **c, d** AFLP analyses. **c** simplified Neighbour-Joining tree. Numbers above branches are bootstrap support values derived from a Neighbour-Joining analysis. **d** Neighbour-Net diagram. Numbers above branches are bootstrap support values derived from a Neighbour-Joining analysis.

coincides with a clear gap in the species' distribution. Arrigoni et al. (1979) reported the presence of two types of indumentum, but did not provide any detail regarding the variability of the character on a populational or geographic scale. In addition, we observed a certain habitat differentiation, as western populations exclusively occur in vertical to overhanging limestone cliffs, whereas eastern ones can occupy less steep cliffs or big shadowed rocks.

As our previous study focusing on western Mediterranean *Cymbalaria* species included only a few individuals of *C. muelleri* (Carnicero et al. in prep., Chapter II), we here use a combination of AFLPs, flow cytometry and morphology to test if *C. muelleri* should be divided into two taxa. As we detected congruent morphological and genetic divergence, we split the species into two subspecies and provide a taxonomic treatment for them, as well as an identification key to all the Sardinian *Cymbalaria* species.

Material and methods

Plant material

Leaf material for molecular analyses was collected in the field in 2012 and 2015, dried and stored in silica gel. We sampled six *C. muelleri* populations, including three populations from the eastern and three from the western partial distribution areas. After checking the species' total known distribution and the morphological affinity of voucher specimens from non-sampled populations (see Taxonomic treatment, additional specimens studied), we are convinced that our sampling is representative of the overall morphological variation. Generally, non-sampled populations occurred less than 5 km from sampled populations in the same mountain range. The sampling localities as well as non-sampled populations are shown in Fig. 1 and voucher details are provided in Supplementary Table S1 and in the Taxonomic treatment.

Morphological analyses

Fifteen quantitative characters (Table 1) were scored on the basis of previous studies

on the tribe Antirrhineae (Sutton 1988; Sáez and Crespo 2005) and the variability we observed within *C. muelleri*. Six corresponded to vegetative characters and nine to floral characters. For the vegetative characters, three measurements per specimen were averaged when possible. Fruit and seed characters were excluded from the morphometric analyses due to a high amount of missing data. The dataset comprised 35 specimens from six populations. For populations m1, m2, v1 and v2, individuals included in molecular and morphometric analyses were sampled in different years in order to provide floral measurements. Floral measurements were performed in the field with a caliper. We used microscopic images taken with an Olympus UC 30 wide zoom camera on an Olympus SZX9 binocular stereoscopic microscope at 50–200× magnification to determine the density and length of trichomes. The remaining characters were measured on scanned specimens using Image J (Abràmoff et al. 2004). Analyses were conducted using a set of R functions contained in MorphoTools ver. 1.01 (Koutecký 2015). Pearson and Spearman correlation coefficients were computed to reveal correlation structure among the characters and to ensure that no strong correlations (> |0.95|) were present. After standardization to zero mean and unit variance, principal component analysis (zero-centred PCA based on a covariance matrix) was applied to display the overall variation pattern along the first two components. A canonical discriminant analysis (CDA) was performed to assess the morphological differentiation between the two groups of populations m1–m3 and v1–v3.

After checking the uniformity of testa sculpturing and trichomes length and type under a Zeiss Stemi DV4 binocular stereoscopic microscope within each of the two groups of populations, scanning electron microscopic (SEM) images were taken with the aim to better describe and illustrate the observed differences in these characters. We analysed mature seeds and trichomes from the stem of a specimen of populations m1 (type locality of *C. muelleri*) and v1. Material was glued on SEM aluminum stubs and sputter-coated with 40–50 nm gold. Samples were later examined with an Evo MA 10 Scanning Electron Microscope (Zeiss) at 15–25 kV. Terminology of seed shape and testa sculpturing follows Sutton (1988).

Table 1. Characters studied in morphometric analyses.

Character	Code	Type
Upper lip width (mm)	UlpW	Floral
Upper lip length (mm)	ULpL	
Upper lip sinus (mm)	UlpS	
Lower lip width (mm)	LLW	
Lower lip length (mm)	LLpL	
Corolla length (mm)	CrL	
Corolla tube width (mm)	CrTW	
Spur length (mm)	SpL	
Spur width (mm)	SpW	
Trichome density of upper leaf surface (n/mm^2)	TDLfA	Vegetative
Length of stem trichomes (μm)	TLSt	
Number of leaf lobes	NLb	
Maximum leaf width (mm)	MLfW	
Pedicel length in flowers (mm)	PdLFl	
Peciole length (bracteal leaf in flowers, mm)	PcLFl	

Molecular analyses

Total genomic DNA was extracted from ca. 10–30 mg silica gel dried leaf material following the CTAB protocol (Doyle and Doyle 1987) with some modifications (Tel-Zur et al. 1999). AFLP fingerprinting was performed for seven to ten individuals per population of *C. muelleri* (five for the outgroup *C. hepaticifolia*; Supplementary Table S1). AFLP profiles were generated following established protocols (Vos et al. 1995) with modifications described in Schönswetter et al. (2009) and Rešetnik et al. (2014). One blank (DNA replaced by water) was included to test for contamination, and six random samples were used as replicates between PCR batches to test the reproducibility of AFLP fingerprinting. Based on an initial primer trial and a previous study (Carnicero et al. in prep., Chapter II) the following three selective primer combinations were chosen for selective PCR (fluorescent dye in brackets): *Eco*RI (6-FAM)ACA / *Mse*I-CAT, *Eco*RI (VIC)AGG / *Mse*I-CTG, and *Eco*RI (NED)AAC / *Mse*I-CAG (6-FAM labelled primers: Sigma-Aldrich; NED and VIC labelled primers: Applied Biosystems). Selective PCR products were purified using Sephadex G-50 Fine (GE Healthcare Bio-Sciences, Uppsala, Sweden) applied to a

MultiScreen-HV plate (Millipore, Molsheim, France) in three steps of 200 µl each and packed at 600 g for 1, 1 and 5 min, respectively. Then 0.8 µl of the elution product was mixed with 10 µl formamide (Applied Biosystems) and 0.125 µl GeneScan 500 ROX (Applied Biosystems) and run on an ABI 3130 automated capillary sequencer.

Electropherograms were analysed with Peak Scanner version 1.0 (Applied Biosystems) using default peak detection parameters except employing light peak smoothing. The minimum fluorescent threshold was set to 50 relative fluorescence units. Automated binning and scoring of the AFLP fragments were performed using RawGeno 2.0-1 (Arrigo et al. 2009) for R 2.15.0 (R Development Core Team, 2015) with the following settings: scoring range 140–490 bp, minimum intensity 50 relative fluorescence units, minimum bin width 1 bp, and maximum bin width 1.5 bp. Fragments with a reproducibility lower than 80% based on sample-replicate comparisons were eliminated. The error rate (Bonin et al. 2004) was calculated as the ratio of mismatches (scoring 1 versus 0) over phenotypic comparisons in AFLP profiles of replicated individuals. Fragments present/absent in only one individual were excluded.

A Neighbour-Joining (NJ) analysis based on Nei-Li genetic distances (Nei and Li 1979) was conducted and bootstrapped (2000 pseudo-replicates) with TREECON v.1.3b (van de Peer and De Wachter 1997). *Cymbalaria hepaticifolia* was used as outgroup based on Carnicero et al. (in prep., Chapter II). SplitsTree4 12.6 (Huson and Bryant 2006) was used to produce a Neighbor-Net diagram based on uncorrected P-distances after excluding the outgroup.

Flow cytometry

For relative DNA content (RGS) measurements, fresh leaf material from germinated seeds from populations m1, v1 and v2 was used. As a reference, diploid *C. muralis* G. Gaertn., B. Mey. & Scherb., growing in the Botanical Garden of the University of Innsbruck, was measured. Flow cytometry (FCM) of 4',6-diamidino-2-phenylindole (DAPI; final concentration 0.036 M) stained nuclei was used to estimate RGS and to assess DNA ploidy levels of fresh *C. muelleri* samples (Suda and Trávníček 2006). The standard used to

determine DNA amounts was *Solanum pseudocapsicum* (2C= 2.58 pg; Temsch et al. 2010). Fresh leaf tissue was chopped with leaf material of the reference and processed as described in Suda et al. (2007). The relative fluorescence intensity of 3,000 particles was recorded using a Partec CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Partec FloMax software was used to evaluate the histograms, which were manually gated. RGS was calculated as ratio between the relative fluorescence of sample and standard. The reliability of the measurements was assessed by calculating coefficients of variation (CV) for the G1 peaks of both the analysed sample and the reference standard. Analyses yielding a CV threshold of > 5 % were discarded and the samples measured again. Ploidy levels were estimated by comparing RGS of samples with that of diploid *C. muralis* (2*n* = 14; Sutton 1988).

Results

Morphometric analyses

Correlation coefficients did not exceed 0.95 for any pair of characters; therefore, all characters were retained for further analyses (Table 1). In the PCA, the first axis accounted for 43% of the variation and the second axis for 15%. The ordination diagram (Fig. 2a) showed no overlap between two main groups of populations: v1–v3 and m1–m3. The characters with most weight in the differentiation of the two groups were trichome density of upper leaf surface and length of stem trichomes (Fig. 2a) as plants from populations m1–m3 showed shorter and more densely distributed trichomes (Fig. 3a, b) than those from populations v1–v3 (Fig. 3f, g). These two characters showed no overlap between populations v1–v3 and m1–m3 (Fig. 2c), and are therefore excellent diagnostic characters. Other characters related to flower size had an important weight in explaining the entire morphological variability but contributed little to differentiation (Fig. 2a). The CDA also showed strong morphological differentiation between the same two groups of populations v1–v3 and m1–m3 (Fig. 2b).

Qualitative inspection of testa sculpturing under the binocular stereoscopic microscope

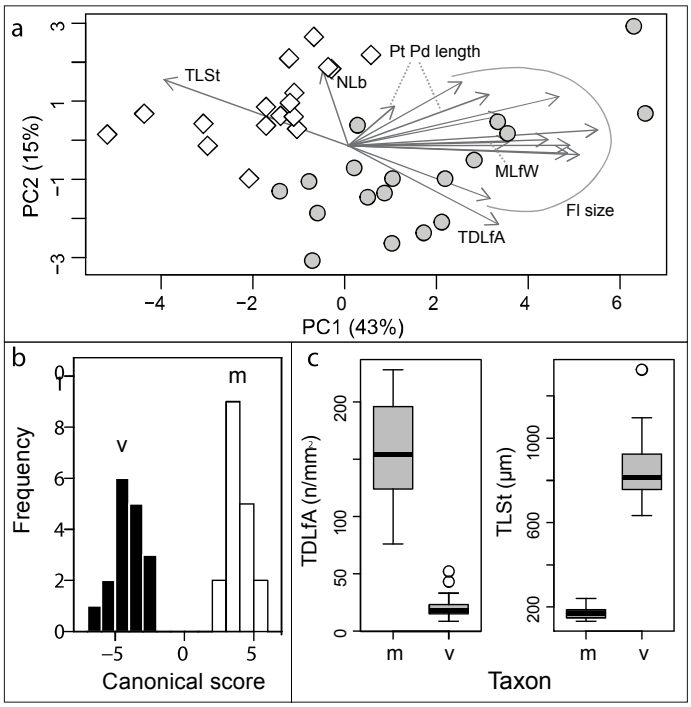

Figure 2. Morphometric analyses of fifteen floral and vegetative characters obtained for 35 specimens of *C. muelleri* subsp. *muelleri* (m) and *C. muelleri* subsp. *villosa* (v). **a** Principal component analysis. Grey circles represent *C. muelleri* subsp. *muelleri* and white diamonds *C. muelleri* subsp. *villosa*. Grey arrows represent relationships of characters projected in the same ordination space as the samples (Fl: flower, MLfW: maximum leaf width, NLb: number of leaf lobes, Pd: pedicel, Pt: petiole, TDLfA: trichome density of upper leaf surface, TLSt: Length of stem trichomes). **b** Canonical discriminant analysis. **c** Boxplots showing median, upper and lower quartiles, minimum and maximum values (excluding outliers) and outliers for the two characters showing the greatest differences between the two subspecies *C. muelleri* subsp. *muelleri* (m) and *C. muelleri* subsp. *villosa* (v). TDLfA: trichome densitiy of upper leaf surface, TLSt: length of stem trichomes.

revealed a strong differentiation between populations v1–v3 and m1–m3. In populations m1–m3 seeds were covered by big hemispherical tubercles (Fig. 3c) whereas populations v1–v3 showed some tubercles mainly on the dorsal side, and the ventral side was shallowly cristate-alveolate (Fig. 3h, i). At higher magnification, SEM images showed testa cells of both populations with thickened periclinal walls and a papilla in the middle of the anticlinal wall (Fig. 3d, e, j). The papilla was more prominent in population m1 (Fig. 3d, e) than in population v1 (Fig. 3j).

AFLPs

In total, 407 AFLP fragments were scored for 49 individuals, of which 365 polymorphic, high-quality, reproducible AFLP-fingerprints were obtained. Thirty-eight fragments with singular presences or absences were excluded. The initial error rate (Bonin et al. 2004)

before the exclusion of non-reproducible fragments was 2.94%.

The NJ analysis (Fig. 1c) revealed that populations m1–m3 constituted a monophyletic group with high bootstrap support (BS 99%). It was sister (BS 81%) to a clade comprising populations v2 and v3 (BS 100%). The consecutive sister was population v1, rendering populations v1–v3 paraphyletic. Individuals from the same population grouped together for populations m1 (BS 89%), m3 (BS 100%) and v1 (BS 100%), while other populations were not monophyletic. The Neighbor-Net diagram showed an almost identical pattern, one clade for the eastern populations (m1–m3) and two clades for the western populations (v1–v3, Fig. 1d).

Flow cytometry

Mean RGS for populations m1–m3 and v1–v3 was 1.16 (1.15–1.17), and 1.10 (1.09–1.11), respectively. Both geographic groups were estimated to be hexaploid as populations m1–m3 and v1–v3 exhibited 5.87 and 5.56 times the RGS of diploid *C. muralis*, respectively. These values are lower than expected if genome size increased in direct proportion with polyploidy, which can be explained by genome downsizing, an often reported phenomenon in non-recently formed polyploids (Leitch and Bennet 2004; Frajman et al. 2015; Lazarević et al. 2015). Hexaploidy corresponds to the reported chromosome number of *C. muelleri* (*2n* = 42, Onnis and Floris, 1967). The RGS values translate into a genome size of 2.99 pg and 2.84 pg for western and eastern populations, respectively. These values fit well to the previously reported *C*-value of *C. muralis* (1C = 0.49 pg, Siljak-Yakovlev et al., 2010; Benneth and Leitch, 2012).

Discussion

A novel taxon endemic to eastern central Sardinia

Eastern and western populations of *C. muelleri* (Fig. 1b), which are separated by a gap of ca. 15 km (Arrigoni et al. 1979), are morphologically distinct: they differ in the length of stem trichomes, trichome density of the upper surface of mid stem leaves (Figs. 2c, 4) and

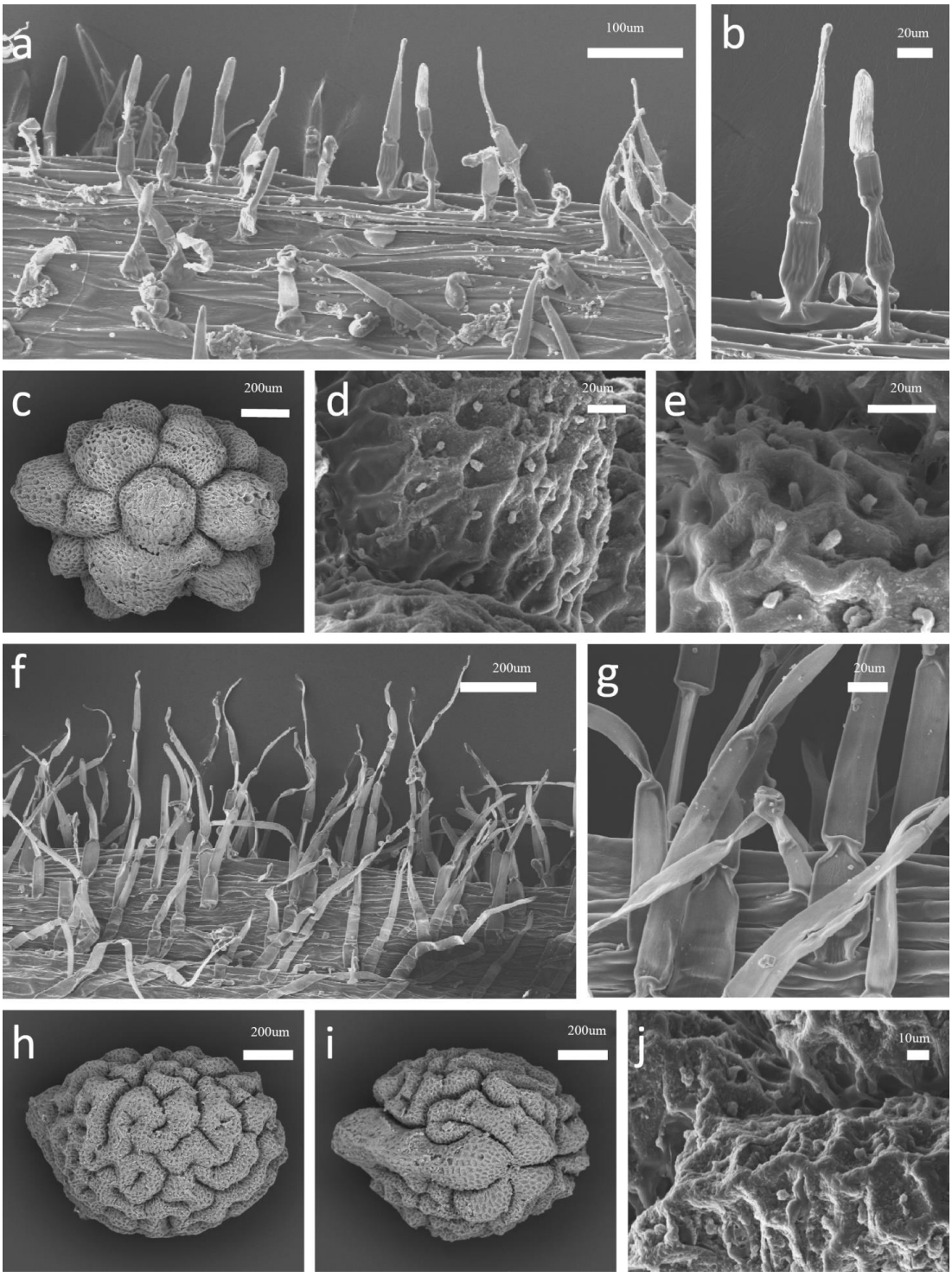

Figure 3. Scanning electron micrographs of trichomes and seeds of *Cymbalaria muelleri*. Scale bars are indicated in each image. **a**–**e** *Cymbalaria muelleri* subsp. *muelleri*, Italy, Sardinia, Laconi, Corona Sa Guardia, S'Aza du Ziu Chiccu, 680 m, 09/05/2015, *P. Carnicero 1223 & M. Unzeta* (BC 955789). **f–j** *Cymbalaria muelleri* subsp. *villosa*, Italy, Sardinia, Ulassai, Bruncu Matzeu, 770 m, 11/05/2015, *P. Carnicero 1263 & M. Unzeta* (BC 955784). **a, f** Stem trichomes, general view, note the different scale. **b, g** Detail of stem trichomes. **c, h** Seeds, ventral side. **i** Seed, dorsal (hilum) side. **d, e, j** Detail of testa cells with a papilla in the anticlinal wall.

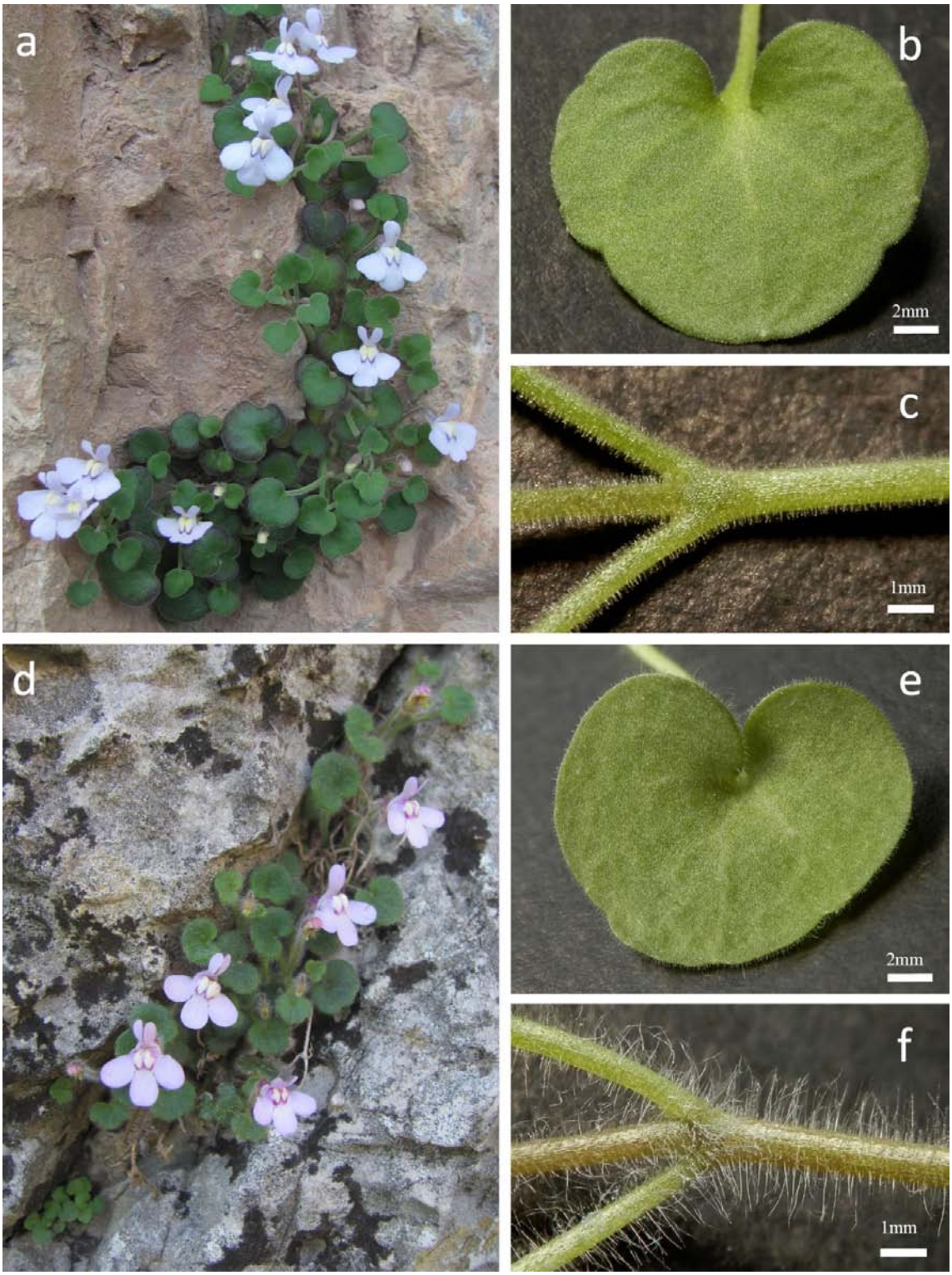

Figure 4. Overall appearance of *Cymbalaria muelleri* subspecies. **a**–**c** *Cymbalaria muelleri* subsp. *muelleri*. **d–f** *Cymbalaria muelleri* subsp *villosa*. **a, d** specimens in their natural habitat. **b, e** upper surface of a mid stem leaf. **c, f** trichomes on stem, note the difference in trichome length.

testa sculpturing (Fig. 3c, h, i). The eastern populations exhibit a less dense, villous indumentum composed of long trichomes (Figs. 3f, 4d–f), and have shallowly cristate-alveolate seeds with few, or lacking, big hemispherical tubercles (Fig. 3h, i). The western populations exhibit a puberulent indumentum composed of short densely distributed trichomes (Figs. 3a, 4a–c) and seeds completely covered by big hemispherical tubercles (Fig. 3c). Examination of herbarium specimens from six non-sampled populations (see Taxonomic treatment, Additional specimens examined) revealed that the diagnostic characters are constant within each geographic group. Below, the eastern populations are described as *C. muelleri* subsp. *villosa*, given that the type specimen of *C. muelleri* is from one of the western populations. As only characters corresponding to *C. muelleri* subsp. *muelleri* are mentioned in identification keys (Tutin 1980; Pignatti 1982; Sutton 1988), it was impossible to correctly identify specimens of *C. muelleri* subsp. *villosa*. Thus, we propose a new identification key for Sardinian *Cymbalaria* species in order to account for the entire intraspecific variation and to include the new subspecies.

Molecular analyses also support the recognition of two subspecies in *C. muelleri*. *Cymbalaria muelleri* subsp. *muelleri* is monophyletic (BS 98%) and nested in *C. muelleri* subsp. *villosa*, which is consequently resolved as paraphyletic (Fig. 1c, d). This pattern clearly supports an anacladogenetic (sub)speciation process (Stuessy et al. 1990), where subsp. *muelleri* evolved from the ancestral, non-extinct subsp. *villosa* by budding (Mayr and Bock 2002). Although monophyly of taxa is desirable from a cladistic perspective, important evolutionary processes such as hybridization, anagenetic and anacladogenetic speciation almost necessarily result in non-dichotomous branching patterns (Hörandl 2006). Moreover, paraphyletic groups often constitute natural transition states in the origin of a holophyletic taxon (*C. muelleri* subsp. *muelleri*) from its ancestor (*C. muelleri* subsp. *villosa*; Hörandl and Stuessy 2010). Thus, the recognition of paraphyletic taxa can reflect better the evolutionary processes, as shown at various taxonomic levels (e.g. *Ranunculus alpestris* L. subsp. *alpestris*: Paun et al. 2008; *Pozoa coriacea* Lag.: López et al. 2012; *Saxifraga oppositifolia* L.: Winkler et al. 2013; *Helichrysum* Mill.: Galbany-Casals et al. 2014).

Altogether, the strong morphological differentiation, supported by molecular divergence, and an allopatric distribution (Stuessy et al. 2014) strongly support to segregate the eastern populations of *C. muelleri* as a new taxon, which is formally described below. We choose the rank of a subspecies, because the level of morphological and molecular differentiation between the two taxa is not enough to deserve the species rank, as used in *Cymbalaria*. Moreover, the integrity of *C. muelleri* as a species is supported by several morphological characters such as thick, fleshy leaves and a relatively robust habit, as compared to the sympatric and morphologically similar *C. aequitriloba* and other related species that are also part of the polyploid western clade (Carnicero et al. in prep., Chapter II). Moreover, its strongly supported monophyly based on AFLP data (Carnicero et al. in prep., Chapter II) and its hexaploidy support the species' integrity and differentiation from its sister taxon, the octoploid *C. hepaticifolia* (Sutton 1988).

Conservation

Adding previous reports (Arrigoni et al. 1979, see Taxonomic treatment, Additional specimens examined) to our sampling allowed us to preliminarily evaluate the two subspecies' state of conservation. We exclude a recent report of *C. muelleri* from Monte Perda 'e Liana (Loi et al. 2004), which was considered the northernmost locality of this taxon. These authors did not report *C. aequitriloba* from this locality, although the only specimens we found growing in that mountain were demonstrated to belong to *C. aequitriloba* in a previous molecular study (Carnicero et al. in prep., Chapter II). Therefore, we consider the report of *C. muelleri* to be erroneous. We used GeoCAT (Bachman et al. 2011) to calculate the extent of occurrence (EOO) and area of occupancy (AOO) on a 2 × 2 km grid for both subspecies of *C. muelleri*. The results indicate that *C. muelleri* subsp. *muelleri* should be considered vulnerable (VU D2) and *C. muelleri* subsp. *villosa* near threatened (NT) according to IUCN (2012) criteria. However, as we detected direct anthropogenic threats (climbing routes, grazing) and counted a low number of individuals in some populations (P.C. and M.G., personal field observations), a higher extinction risk probably applies. A rigorous conservation study is needed to definitely assess the conservation status of the two subspecies and to adopt appropriate measures to protect

these narrow endemics.

Our study clearly shows the necessity of conducting critical surveys and systematic studies also in areas with a long history of botanical exploration, such as Sardinia. Specifically, habitats with difficult accessibility in areas with high diversity, such as glacial refugia and biodiversity hotspots, may still harbor new taxa that remain undiscovered, or are considered part of another entity due to the lack of a critical study at populational level (e.g. *Aquilegia cremnophylla* Bacch., Brullo, Congiu, Fenu, J. Garrido & Mattana: Bacchetta et al. 2012b; *Androsace komovensis* Schönswetter & Schneew.: Schönswetter and Schneeweiss 2009). Mountainous areas in the Mediterranean Basin, and specifically chasmophytic habitats, usually meet these requirements, and therefore we here stress the need for active research in these areas. Moreover, these still unknown taxa may be important from a conservation point of view, since their rarity is likely the reason why they remain undiscovered.

Taxonomic treatment

Cymbalaria muelleri (Moris) A. Chev. in Bull. Soc. Bot. France 83: 645. 1937. ≡ *Linaria muelleri* Moris, Diagn. Stirp. Sard. Nov.: 1-2. 1857. —HOLOTYPE: '*Linaria mulleri* Nob., affinis *Linaria pallida* Tenor! Guss.! Chavann.! At seminibus […] charact differt / a Mullero' (TO* [web!]). Isotypes: '*Linaria aequitriloba* Spr., Ad rupes prope Laconi, Unio itineraria, Jul 1827 / Müller' (B, K [web!] and W!).

≡ *Linaria pilosa* var. *pauciloba* Benth. in DC., Prodr. 10: 267. 1846. ≡ *Linaria pilosa* subsp. *muelleri* (Moris) Nyman, Consp. 3: 543. 1881. ≡ *Linaria aequitriloba* subsp. *muelleri* (Moris) Nyman, Consp. Suppl. 2(2): 235. 1890.

* Sutton (1988) already indicated the specimens kept in B and W as type material, but he was unaware of the existence of the holotype.

Description: Perennial herb, puberulent to villous in all vegetative organs, indumentum composed of eglandular trichomes 130–1100 µm long and short glandular trichomes up to 0.5 mm long. *Stems* trailing, procumbent or decumbent, up to 20 cm long, puberulent to

villous. *Leaves* 6–25.3 mm in diameter, opposite to alternate, petiolate, thick and fleshy, reniform to orbicular, entire to five-lobed, puberulent to villous on upper and lower surfaces. *Flowers* zygomorphic, pedicellate, solitary in leaf axils; *pedicels* 8–33(47) mm long in flower, 15–52 mm long in fruit, puberulent to villous, with decreasing density of eglandular trichomes towards the apex and high density of short glandular hairs in the upper part. *Calyx* lobes subequal, 2.0–3.3 mm long in flower, 2.3–4.2 mm long in fruit, lanceolate, puberulent to villous. *Corolla* (7.2)9.1–14.2 mm long from the palate to the tip of the spur, pale violet, violet or pink, palate yellow and occasionally with purple veins; tube 1.7–2.7 mm wide; upper lip (4.0)5.5–8.4(9.5) × 2.7–7.7 mm with purple veins, sinus 1.4–5.2 mm long; lower lip 7.4–16.6(17.7) mm wide, sinus 2.1–5.7 mm long; spur 1.8–5.2 × 0.7–1.8 mm. *Capsule* 2.5–5.0 mm long, spherical, glabrous to densely glandular-pubescent, glandular trichomes up to 0.5 mm long, loculi equal, each loculus dehiscing by irregular valves. *Seeds* 1.0–1.3 mm long, ovoid, black; surface either shallowly cristate to alveolate with low rounded ridges, in this case occasionally with large hemispherical tubercles on the dorsal (hilum) side, or with large hemispherical tubercles on the entire surface; testa cells polygonal with anastomosed anticlinal walls, periclinal walls bearing a median papilla.

Cymbalaria muelleri (Moris) A. Cheval. **subsp. *muelleri***

Description: Stems puberulent, covered by eglandular trichomes 130–250 µm long. *Leaves* with 50–230 eglandular trichomes per mm^2 in their upper surface. *Seeds* surface entirely covered by large hemispherical tubercles.

Chromosome number: 2*n* = 42 (Onnis and Floris, 1967)

Habitat and altitudinal range: vertical to overhanging north facing limestone cliffs, between 600 and 800 m a.s.l.

Distribution area: central Sardinia; Oristano, Nuoro and Cagliari provinces; south of Gennargentu massif, western "Tacchi" area. The area of occupancy is ca. 16 km^2 and the extent of occurrence is ca. 25 km^2.

Additional specimens examined: Italy, Sardinia. Pareti rocciose sotto Regione Coringiu

Irau (Villanovatulo), 15 Giugnio 1963, *P.V. Arrigoni* (FI 050334); Guturru Forreddu (Villanovatulo), 21 May 1964, *P.V. Arrigoni* (FI 050335); S'aza du Ziu Chiccu (parets N NW de la Corona Sa Guardia) sobre Laconi, sota la torre de vigilància forestal, 690 m, sobreploms i fissuretes, abundant, 14 Jul 2012, *P. Carnicero 414 & M. Galbany* (BC 955485); a poca alçada a les grans parets de la Foresta comunale de Gadoni, 690 m, parts pelades lleument extraplomades de la paret, 14 Jul 2012, *P. Carnicero 416 & M. Galbany* (BC 955786); Laconi, Corona Sa Guardia, S'Aza du Ziu Chiccu, 680 m, 09 May 2015, *P. Carnicero 1223 & M. Unzeta* (BC 955789); Gadoni, Foresta Latinazzu, 690 m, 09 May 2015, *P. Carnicero 1231 & M. Unzeta* (BC 955788); Gadoni, Taccu di Tornulu, 750, parets verticals i sobreplomades calcàries exp. N, 10 May 2015, *P. Carnicero 1246 & M. Unzeta* (BC 955787).

Conservation status: "Vulnerable" (VU D2).

Cymbalaria muelleri (Moris) A. Chev. **subsp. *villosa*** Carnicero, **subsp. nov.** — HOLOTYPE Italy, Sardinia, Ulassai, Bruncu Matzeu, 770 m, 11 May 2015, *P. Carnicero 1263 & M. Unzeta* (BC 955784, Fig. 5); isotypes: TO, W.

Etymology: This subspecies is named in reference to its most apparent diagnostic character, the villous indumentum present in stems and leaves.

Description: Stems pubescent to villous, covered by eglandular trichomes (530)630–1100 µm long. Upper surface of mid stem *leaves* with 11–33 eglandular trichomes per mm^2. *Seeds* surface shallowly cristate to alveolate with low rounded ridges, occasionally with large hemispherical tubercles on the dorsal (hilum) side surface.

Diagnosis: The new subspecies differs from subsp. *muelleri* in its less dense indumentum and longer trichomes in leaves and stems. The testa is partly covered by big hemispherical tubercles on the dorsal side (rarely absent) and shallowly cristate-alveolate elsewhere.

Chromosome number: it is likely $2n = 42$, as hexaploidy was inferred from RGS.

Habitat and altitudinal range: cliffs and big rocks, mostly shadowed faces, between 700 and 1000 m a.s.l.

Figure 5. Holotype of *C. muelleri* subsp. *villosa*. Italy, Sardinia, Ulassai, Bruncu Matzeu, 770 m, 11/05/2015, *P. Carnicero 1263 & M. Unzeta* (BC 955784)

Distribution area: central-eastern Sardinia; Ogliastra Province; south of Gennargentu massif, eastern "Tacchi" area. The area of occupancy is ca. 24 km^2 and the extent of occurrence is ca. 43 km^2.

Additional specimens examined: Italy, Sardinia. Margini rocciosi di Sud-Ouest della Foresta Demaniale di Monte Arbu (Seui), 23 May 1964, *P.V. Arrigoni* (FI 050338); FF. DD. [Foresta Demaniale] di Montarbu (Seui), Rocce sotto Serra Middai, 23 May 1964, *P.V. Arrigoni* (FI 050337); Rocce calcaree sopra Porcu e Ludu (Jerzu), 15 Jul 1964, *P.V. Arrigoni* (FI 050336); Seui, Foresta di Montarbu: rupi calcaree dei Tonneri, all'altezza di Bruncu Arrascialé. Esp. NE. m 1000 ca., 27 Jun 1972, *P.V. Arrigoni & E. Nardi* (FI 050339, FI 050340); v. N Bruncu Matzeu, Ulassai, 770 m, bretxa molt ombrejada i parets verticals de la base. 12 Jul 2012, *P. Carnicero 389 and M. Galbany-Casals* (BC 879630); Seui, Genni d'Acca, 13 Jul 2012, *P. Carnicero 406 & M. Galbany-Casals* (BC 879629); Seui, Foresta di Montarbu, Genni d'Aca, 900 m, 10 May 2015, *P. Carnicero 1251 & M. Unzeta* (BC 955783); Ussassai, Nuraghe Urseri, 870 m, 10 May 2015, *P. Carnicero 1257 & M. Unzeta* (BC 955782).

Conservation status: "Near Threatened" (NT).

Identification key to Sardinian *Cymbalaria* taxa

1a. Leaves and stems glabrous or only with occasional hairs on young organs, most leaves 5–9 lobed ..... ***C. muralis*** subsp. ***muralis***

1b. Leaves and stems hairy, most leaves 1–3 lobed..... 2

2a. Leaves thin, not fleshy. Seeds 0.8–1.2 mm long..... ***C. aequitriloba***

2b. Leaves thick and fleshy. Seeds 1.1–1.3 mm long ..... ***C. muelleri***

- Stems and leaves puberulent, eglandular trichomes 130–250 µm long, shorter than half the diameter of the stem. Seeds tuberculate ..... subsp. ***muelleri***

- Stems and leaves pubescent to villous, eglandular trichomes (530)630–1100 µm long, longer than the diameter of the stem. Seeds shallowly cristate-alveolate, often with

some hemispherical tubercles on the dorsal side ..... subsp. ***villosa***

Acknowledgements

M. Gassner, M. Magauer, M. Pacary and D. Pirkebner provided valuable assistance in lab work. This research was funded in part by the Spanish government (CGL2010-18631/BOS and the *Flora iberica* project, CGL2011-28613-C03-01) as well as by the Catalan government (2014-SGR 514). We thank P.V. Arrigoni, I. Camarda and E. Farris for their help in organizing collecting trips, and M. Unzeta for her valuable assistance during field work. We also thank J.M. Blanco-Moreno for the valuable comments on multivariate statistics. We are grateful to the herbaria that provided material for examination (FI, TO, W). Pau Carnicero benefited from the support of a PIF Ph.D. student fellowship from the Universitat Autònoma de Barcelona and an Erasmus+ traineeship mobility fellowship, which partly supported a research stay at the Institute of Botany of the University of Innsbruck. We thank P. Daniel Schlorhaufer from the Botanical Garden of the University of Innsbruck for successfully cultivating accessions of *Cymbalaria*.

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Table S1. List of sampled populations with information on the population codes used in the text and figures, herbarium voucher, locality and number of individuals included in each analysis.

Taxon	Population code	Voucher	Locality	Coordinates		Investigated individuals		
				Longitude	Latitude	AFLPs	Morphology	RGS
Cymbalaria muelleri subsp. *muelleri*	m1	*P. Carnicero 414 & M. Galbany-Casals* (BC 955485)	IT, Sardinia, Laconi, Corona Sa Guardia, S'aza du Ziu Chiccu	9.05	39.87	8		2
	m1	*P. Carnicero 1223 & M. Unzeta* (BC 955789)					6	
Cymbalaria muelleri subsp. *muelleri*	m2	*P. Carnicero 416 & M. Galbany-Casals* (BC 955786)	IT, Sardinia, Gadoni, Foresta di Latinazzu	9.18	39.84	8		
	m2	*P. Carnicero 1231 & M. Unzeta* (BC 955788)					6	
Cymbalaria muelleri subsp. *muelleri*	m3	*P. Carnicero 1246 & M. Unzeta* (BC 955787)	IT, Sardinia, Gadoni, Taccu di Tornulu	9.2	39.88	10	6	
Cymbalaria muelleri subsp. *villosa*	v1	*P. Carnicero 389 & M. Galbany-Casals* (BC 879630)	IT, Sardinia, Ulassai, Bruncu Matzeu	9.5	39.81	8		1
	v1	*P. Carnicero 1263 & M. Unzeta* (BC 955784)					6	
Cymbalaria muelleri subsp. *villosa*	v2	*P. Carnicero 406 & M. Galbany-Casals* (BC 879629)	IT, Sardinia, Seui, Foresta de Montarbu, SE cliffs of Genni d'Acca	9.4	39.86	8		3
	v2	*P. Carnicero 1251 & M. Unzeta* (BC 955783)					6	
Cymbalaria muelleri subsp. *villosa*	v3	*P. Carnicero 1257 & M. Unzeta* (BC 955782)	IT, Sardinia, Ussassai, Nuraghe Urseri	9.42	38.83	7	5	
Cymbalaria hepaticifolia	h1	*P. Carnicero 447& M. Galbany-Casals* (BC 955755)	FR, Corsica, Albertacce, Forêt de Valdu Niellu	8,93	42,29	5		

Chapter IV

Disentangling a knot, the reorganization of eastern Mediterranean *Cymbalaria* (Plantaginaceae)[1]

[1] With Llorenç Sáez Gonyalons, Núria Garcia Jacas, Theophanis Constantinidis and Mercè Galbany Casals. In prep.

Abstract

The eastern Mediterranean houses a remarkably high plant diversity. Historical connections between currently isolated areas across the Aegean region have been invoked to explain current distribution patterns of species. According to most recent treatments, two *Cymbalaria* species occur in this area: *Cymbalaria microcalyx* and *C. longipes*. The former comprises several intraspecific taxa, treated at different ranks by different authors, evidencing the need of a taxonomic revision. Additionally, some populations of *C. microcalyx* show exclusive morphological characters that do not match any described taxon. Some taxa are distributed across currently isolated areas in the Aegean, constituting thus good candidates for testing the role of paleogeographic changes in the region in the origin and current distribution of species. In our study we aim to resolve the systematics of eastern Mediterranean *Cymbalaria* and to propose a classification congruent with molecular, morphological and ploidy level data. We performed phylogenetic analyses using ITS, 3'ETS, *ndhF* and *rpl32-trnL* sequences. We estimated the ploidy level of some species performing genome size measures. Molecular data combined with morphology support the division of traditionally delimited *C. microcalyx* in four different species, three of them with two subspecies each. Unidentifiable specimens constituted a well-defined phylogenetic and morphological entity, and are described here as a new species: *Cymbalaria spetae*. *Cymbalaria longipes* is polyphyletic, but it is allegedly characterized by being glabrous and diploid, unlike other eastern species that are hairy and tetraploid. In *C. microcalyx* and *C. minor*, that have a wide distribution in currently isolated areas, west to east colonization of the Aegean region is inferred, probably facilitated by Pleistocene land bridges but also dispersing across marine barriers.

Keywords Aegean region, cpDNA, dispersal, marine barriers, morphology, nrDNA

Introduction

The Mediterranean Basin constitutes a biodiversity hotspot (Myers et al., 2000; Thompson, 2005). Specifically, the eastern Mediterranean houses two major centres of biodiversity: the Balkans-Aegean area with 6500–7000 species, of which ~1500 are endemic, and the Mediterranean part of the Anatolia Peninsula, with ca. 5000 species and a 30% of endemism (Thompson, 2005). This is in part attributable to moderate consequences of Pleistocene glaciation periods in this area (Fady-Welterlen, 2005; Wu et al., 2007), and the complexity of its geography, with several mountain ranges, cliffs and islands (Polunin, 1980; Thompson, 2005). Further causes of the high diversity and endemism of the eastern Mediterranean Region are the entrance of Irano-Turanian floristic elements to this area (Zohary, 1973; Manafzadeh et al., 2014), as well as the fact that several Mediterranean lineages (e.g. *Campanula* L.: Roquet et al., 2009, *Centaurea* L. subgenus *Centaurea*: Hilpold et al., 2014; *Aegilops geniculata* Roth.: Arrigo et al., 2010; *Haplophylum* A. Juss.: Manafzadeh et al., 2014) have their origin of diversification here. Finally, a higher degree of single island endemism compared to western Mediterranean also contributes to the high diversity and singularity of eastern Mediterranean (Thompson, 2005).

The Mediterranean parts of the Balkans-Aegean area and the Anatolian Peninsula can be divided in three major fitogeographic units (Rechinger, 1950; Takhtajan, 1986): the northwestern part of the Balkan Peninsula, the southern part of the Balkan Peninsula (or western Aegean region, comprising most Aegean islands), and the eastern Aegean islands-Anatolia. Two main biogeographic barriers limited the plant species exchange among these three regions. From the West to the East, a first major biogeographic barrier divides the northwestern part from the southern part of the Balkan Peninsula. In fact, classic biogeographic provinces (Takhtajan, 1986) as well as modern systematic studies (Bardy et al., 2010; Hilpold et al., 2014) show closer affinities of the northwestern Balkan peninsula with the Apennine Peninsula than with the southern Balkan Peninsula. The main causes for this barrier are: 1) the severe central European climate of the continental part of the Balkans, that restrict the Mediterranean climate area to a series of isolated patches in the coast, and 2) a stronger influence of glaciations that isolated northern from southern Balkan

Peninsula, as shown by the traces of glaciers at relatively low altitudes in the north of what is actually Albania (Polunin, 1980). Further east, a second major biogeographic break is the sea barrier between the western Aegean region and eastern Aegean region-Anatolia (Rechinger, 1943, 1950; Strid, 1996). In former times, these two regions were connected by land via three ancient ranges: the Bosphorus Strait in the North; a central range that included the Cyclades islands; and a southern range that included the southern Aegean Islands Crete, Karpathos and Rhodes. Around ten million years ago (Ma), the southern range collapsed, originating the island of Crete and the Karpathos archipelago, while Rhodes was still connected to Anatolia (Polunin, 1980). In the early Pliocene (ca. 5 Ma) sea expanding northwards broke the connection via the central range, isolating the Cyclades from the eastern Aegean islands-Anatolia (Strid & Tan, 1997; Chatzimanolis et al., 2003; Thompson, 2005). The imaginary line east of Karpathos and the Cyclades has been named Rechinger's line (Strid, 1996) and is considered the phytogeographical borderline between Europe and Asia (Strid & Tan, 1997). That sea barrier persisted even in Pleistocene glaciations, when sea levels were 150–200 m lower than nowadays, thus connecting most of the Cyclades to each other and the eastern Aegean Islands to Anatolia (Van Andel & Shackleton, 1982).

Cymbalaria Hill (Plantaginaceae) is a Mediterranean genus with at least two well delimited genetic lineages in the eastern Mediterranean region (Carnicero et al., 2017). Taking Sutton (1988) as a reference, two species occur in this area: the glabrous *C. longipes* (Boiss. & Heldr.) A.Chev. and the hairy *C. microcalyx* (Boiss.) Wettst. *Cymbalaria longipes* occurs in most coastal areas from the Ionian Islands in the west up to Lebanon in the east (Fig. 1). There is little concern on its taxonomic status due to some distinctive features: 1) its characteristic habitat in cliffs and pebbles close to the sea; 2) it is glabrous in all vegetative organs; 3) it produces big seeds that conform a concrescent mass in an indehiscent capsule; and 4) its ploidy level (2*n* =14, diploid, Sutton, 1988).

On the other hand, *C. microcalyx* is an aggregate of taxa with as many taxonomic combinations as the number of systematic studies that have dealt with them (e.g. Cufodontis, 1936; Greuter, 1979; Speta, 1986). *Cymbalaria microcalyx* inhabits cliffs from the sea level up to ca. 2000 m (Maroulis & Georgiadis, 2005), from the Balkans to

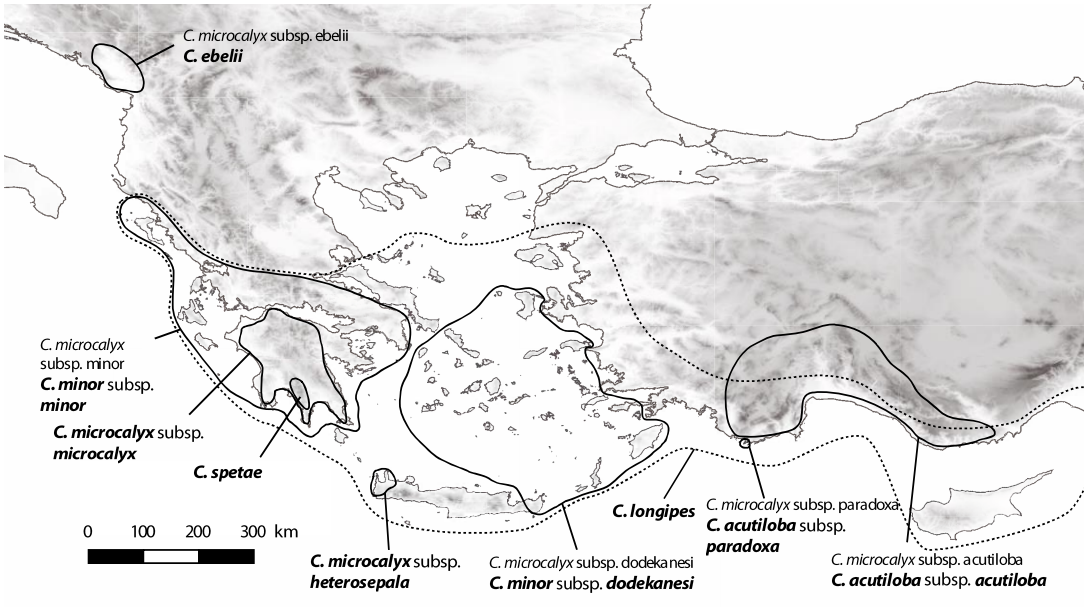

Figure 1. Distribution of eastern Mediterranean *Cymbalaria* taxa, based on Sutton (1988), local Floras, personal field observations and herbarium vouchers. Previous taxonomic treatment and final taxonomic treatment (in bold) are given. Only one name in bold is shown when they coincide.

western Anatolia. Sutton (1988) recognized five subspecies (Fig. 1), but also mentioned other entities not studied in depth but that, in his opinion, could deserve taxonomic recognition. The westernmost subspecies of *C. microcalyx* is *C. microcalyx* subsp. *ebelii* (Cufod.) Cufod., a narrow endemic that has distinctive seeds with very low ridges. *Cymbalaria microcalyx* subsp. *microcalyx* refers to a group of populations from the Peloponnese with allegedly unequal-sized seeds, all with prominent wing-like ridges. *Cymbalaria microcalyx* subsp. *minor* Cufod. is a second taxon from the Peloponnese and immediate islands, characterized by small, equal-sized, alveolate seeds without wing-like expansions. However, descriptions of this taxon are somewhat confusing, since the size of seeds reported by Sutton (1988) does not coincide with the original description of this taxon (Cufodontis, 1936). Moreover, Speta (1986) mentioned two apparently very different forms of *C. microcalyx* subsp. *minor*, differing notably in the size of their seeds, but he did not propose a formal taxonomic recognition for them. A third taxon, *C. microcalyx* subsp. *dodekanesi* Greuter, is distributed throughout numerous Aegean Islands: the Cyclades, eastern Aegean Islands, eastern Crete, Karpathos and Rhodes. Its seeds are similar to those of *C. microcalyx* subsp. *minor*, from which it can be distinguished by the lanate indumentum of the capsule. *Cymbalaria microcalyx* subsp. *acutiloba* (Boiss. & Heldr.) Greuter occurs in the mountains of southwestern Anatolia and bears unequal-sized seeds with prominent

wing-like ridges (Speta, 1986; Sutton, 1988). However, Speta (1986) referred to two different forms in the protologue, probably because he included *C. microcalyx* subsp. *dodekanesi* from Rhodes in his concept of *C. acutiloba* (Boiss. & Heldr.) Speta. Finally, *C. microcalyx* subsp. *paradoxa* Greuter, from Kastellorizo island, and *C. microcalyx* subsp. *heterosepala* (Cufod.) Speta, from western Crete, are two narrow endemic taxa that Sutton (1988) did not study in depth, but which were included as accepted taxa in a recent checklist of the Flora of Greece (Dimopoulos et al., 2013), as well as *C. microcalyx* subsp. *alba* (Voliotis) Kit Tan. The latter is a Peloponnese endemic that allegedly differs from *C. microcalyx* subsp. *microcalyx* by its white flowers (Voliotis, 1990). A recent phylogenetic study showed that *C. microcalyx* is not monophyletic (Carnicero et al., 2017). Indeed, subspecies of *C. microcalyx* were found in at least three distinct lineages. Regarding the ploidy levels, available data suggest that *C. microcalyx* subsp. *microcalyx*, *C. microcalyx* subsp. *ebelii*, *C. microcalyx* subsp. *dodekanesii*, and *C. microcalyx* subsp. *minor* are tetraploids (Speta, 1986, 1989; Sutton, 1988; P. Carnicero, unpubl. data), whereas for C. *microcalyx* subsp. *acutiloba*, *C. microcalyx* subsp. *alba, C. microcalyx* subsp. *heterosepala* and *C. microcalyx* subsp. *paradoxa* there is no data available.

In the course of our studies on the genus *Cymbalaria*, two specimens kept in the herbaria SALA and MA and identified as *C. microcalyx* caught our attention. These specimens were collected from the same locality in the Taygetos mounts (Peloponnese) and showed extremely different characters from any taxon described in the region, i.e. small cristate-alveolate seeds, big flowers with a short and wide spur, and a higher number of leaf lobes. In later field prospection, we found a second population showing the same characters in the Taygetos mounts (Peloponnese).

The high amount of infraspecific taxa described within eastern Mediterranean *Cymbalaria* species and the incongruence in taxonomic treatments between authors suggest that a comprehensive study combining molecular systematics tools, morphology and ploidy data would help to clarify the taxonomy of the group. From a modern systematics perspective, taxonomy feeds from molecular data as well other kinds of data to produce useful classifications that reflect the evolutionary history of lineages (Stuessy, 2009). Moreover, the high diversity of eastern Mediterranean

Cymbalaria, combined with extremely different species distributions areas, and the coexistence of at least two ploidy levels, suggest that its study can be a relevant contribution to the knowledge on plant evolution in the Mediterranean and the role of biogeographic barriers.

Using a combination of plastid (cpDNA) and nuclear ribosomal (nrDNA) sequences, morphological data and genome size, we aim to clarify the systematics of eastern Mediterranean *Cymbalaria* species, by identifying genetic lineages, inferring their phylogenetic relationships and finding morphologically homogeneous groups. Specifically, we aim to test whether the unclassifiable populations from the Taygetos mounts belong to a new taxon and its phylogenetic affinities. Finally, we aim to propose a revised taxonomic treatment for the eastern Mediterranean *Cymbalaria* species congruent with all the available data.

Material and Methods

Plant material

We sampled 65 individuals of *Cymbalaria* comprising at least one specimen of all described species, with the aim of building a complete phylogenetic backbone to infer the phylogenetic position of the eastern Mediterranean taxa. Eastern Mediterranean taxa were more intensively sampled with the aim of studying their phylogenetic relationships and to capture as much geographic genetic variation as possible. In total, 65 specimens were used in molecular analyses, of which 28 were sequenced for the present study; seven in genome size measurements and 62 in morphological studies. The sampling localities, voucher details, as well as specimens used in each analysis, are provided in supplementary material, Table S1. *Asarina procumbens* Mill. was sampled and used as outgroup, based on previous studies (Ghebrehiwet et al., 2000; Vargas et al., 2004; Guzmán et al., 2015; Carnicero et al., 2017).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from ca. 10–30 mg silica gel dried leaf material or herbarium vouchers following the CTAB protocol (Doyle & Doyle, 1987) with some modifications (Tel-Zur et al., 1999). When less than 10 mg of dried material was

available, no sorbitol washing was applied.

We amplified the ITS region and the conserved 3'ETS region of the nuclear ribosomal DNA (nrDNA) and the *ndhF* region and the *rpl32-trnL*^{UAG} spacer of the plastid DNA (cpDNA). We used the primers ITS1 and ITS4 (Sun et al., 1994) for the ITS region, the primers Ast1 and 18SETS (Markos & Baldwin, 2001) for the 3'ETS region, the primers 3'F (Eldenäs et al., 1999) and +607 (Kim & Jansen, 1995) and the internal primers ndhF CymbF and ndhF CymbR (Carnicero et al., 2017) for the *ndhF* region, and finally the primers rpl32F and trnL^{UAG} (Shaw et al., 2007) for the *rpl32-trnL* spacer. The profile used for amplification of ITS included 4 min denaturation at 95°C, followed by 30 cycles of 90 s denaturation at 94°C, 2 min annealing at 55°C and 3 min extension at 72°C, with an additional final step of 15 min at 72°C. The profile used for amplification of the *rpl32-trnL*^{UAG} spacer included 3 min denaturation at 94°C, followed by 30 cycles of 40 s denaturation at 95°C, 2 min annealing at 52°C and 2 min extension at 72°C, with an additional final step of 10 min at 72°C. We followed the PCR profiles described in Galbany-Casals et al. (2009) for ETS and Galbany-Casals et al. (2012) for *ndhF*. Direct sequencing was conducted by the Macrogen Sequencing Service (Seoul, South Korea). See supplementarymaterial, Table S1 for information on the vouchers and the sequences.

Phylogenetic analyses

The sequences were first examined and edited using Mega 6.06 (Tamura et al., 2013) and Finch TV 1.4 (Geospiza). Sequences were initially aligned using Clustal X 2.0.9 (Larkin et al., 2007) and adjusted manually in Mega 6.06. Ambiguous regions in cpDNA alignment were detected and excluded using Gblocks v.0.91 (Castresana, 2000; Talavera & Castresana, 2007) with relaxed conditions in order to preserve as much information as possible: “Minimum Number Of Sequences For A Conserved Position” and “Minimum Number Of Sequences For A Flank Position” were half the number of sequences, “Minimum Number Of Contiguous Nonconserved Positions” was 5, “Maximum Number Of Contiguous Nonconserved Positions” was 10, “Minimum Length Of A Block” was 5, and “Allowed Gap Positions” was “With Half”. We kept the entire nrDNA matrix since we detected no ambiguously aligned regions after a visual inspection. Indels were coded as binary characters using the simple indel coding

method (Simmons & Ochoterena, 2000). Plastid and nrDNA regions were analysed separately due to the phylogenetic incongruence found between the two genomes (see Results).

Maximum Parsimony (MP) analyses were conducted with PAUP*v.4.0a149 (Swofford, 2002) for both cp and nrDNA datasets. We performed 10,000 replicates of heuristic searches with random taxon addition and tree bisection-reconnection (TBR) branch swapping and holding all most parsimonious trees, uninformative characters were excluded. The bootstrap analyses were performed with 1000 replicates, TBR branch swapping and random taxon addition with 10 replicates. Consistency Index (CI), Retention Index (RI) and Homoplasy Index (HI) were calculated from the consensus tree (supplementary material, Table S2).

PartitionFinder (Lanfear et al., 2012) was used to find the best model of evolution and the best partitioning scheme under the Bayesian information criterion (BIC; Schwarz, 1978) for the Bayesian Inference (BI) analyses. The models tested were those implemented in MrBayes. A greedy search algorithm was selected for running the analysis for each dataset. Unique partitions were found for the cp and nrDNA respectively, and therefore no partitions were defined in further analyses. The BI analysis was conducted with MrBayes v.3.2 (Ronquist et al., 2012). Coded indels were defined as separate partitions and analysed with the simplest possible model, i.e. the Jukes Cantor model. We generated 10,000 trees running MrBayes for 5,000,000 generations and sampling one of every 500 generations. After ensuring that the Monte Carlo Markov chain (MCMC) reached stationarity, we discarded the first 2500 trees as burn-in.

Morphological analyses

Twelve characters were scored on the basis of previous studies on *Cymbalaria* and the tribe Antirrhineae (Sutton, 1988; Sáez & Crespo, 2005; Vigalondo et al., 2015; Carnicero et al., in prep., Chapter II) and our observed variability within studied taxa (Table 1). Six corresponded to vegetative characters and six to reproductive characters. Two extra characters were calculated as ratios of different corolla size characters, in order to summarize information on the shape of the corolla rather than just absolute measures. For the vegetative characters, three measurements per specimen were

Table 1. Morphometric characters studied.

Character	Type
Corolla length (mm)	Quantitative
Corolla tube width (mm)	Quantitative
Spur length (mm)	Quantitative
Spur width (mm)	Quantitative
Ratio spur length (mm) / corolla width (mm)	Quantitative (ratio)
Ratio spur length (mm) / spur width (mm)	Quantitative (ratio)
Calyx length (mm)	Quantitative
Indumentum of stem	Semiquantitative (0 = glabrous; 1 = subglabrous, sparsely distributed hairs; 2 = non-overlapping uniformly distributed hairs; 3 = overlapping hairs; 4 = lanate)
Indumentum of adaxial leaf surface	
Indumentum of abaxial leaf surface	
Indumentum of calyx	
Indumentum of upper part of pedicel	
Number of leaf lobes	Quantitative
Maximum leaf width (mm)	Quantitative
Number of seeds / capsule*	Quantitative
Heterocarpy*	Qualitative
Concrescence of seeds *	Qualitative
Testa sculpturing*	Qualitative
Seed length (mm)*	Quantitative

*Characters excluded from the analyses due to the high amount of missing data.

averaged when possible. Six semiquantitative indumentum characters were examined under a binocular stereoscopic microscope. The remaining characters were measured on scanned specimens using Image J (Abràmoff et al., 2004). Unfortunately, *C. microcalyx* subsp. *heterosepala* was not included in the analyses because flowers were not available in the specimens examined. *Cymbalaria ebelii* was excluded from quantitative analyses due to its clear differentiation using seed characters (Speta, 1986, 1989), its phylogenetic position (Carnicero et al., 2017, and results of the present paper), and previous studies pointing to its independence from *C. microcalyx* (Cufodontis, 1936, 1947; Sutton, 1988). The final dataset comprised 62 specimens from 37 populations. Sixty nine additional herbarium specimens were also examined for the purpose of the taxonomic treatment (see Appendix: additional specimens examined).

Quantitative analyses were conducted using a set of R functions contained in MorphoTools ver. 1.01 (Koutecký, 2015). Pearson and Spearman correlation coefficients

were computed to reveal correlation structure among the characters and to ensure that no strong correlations (> |0.9|) were present. After standardization to zero mean and unit variance, principal component analysis (zero-centred PCA based on a covariance matrix) was applied to display the overall variation pattern along the first two components. A canonical discriminant analysis (CDA) was performed to assess the morphological differentiation between the taxa which we propose to accept as a combination morphological (PCA) and phylogenetic results (see taxonomic treatment). We performed CDA at two different taxonomic levels: at the species level (5 groups) and at the subspecies level (7 groups).

Several seed characters were also studied under a binocular stereoscopic microscope and scored (Table 1), but they were excluded from the morphometric analysis due to the high amount of missing data. Specifically we measured seeds length, in addition to establishing other characters: the number of seeds per capsule, testa sculpturing, heterocarpy (i.e. remarkable differences in seed size within a single capsule) and whether or not the seeds converged in a compact concrescent mass. Scanning electron microscopic (SEM) images were taken with the aim to better describe and illustrate the observed differences in testa sculpturing of seeds. We analysed mature seeds of a subset of five specimens, with special focus on taxa lacking seed micrographs in previous studies (i.e. *Cymbalaria acutiloba* subsp. *paradoxa*, *C. microcalyx* subsp. *heterosepala* and the putative new taxon from the Taygetos Mounts). Material was glued on SEM aluminum stubs and sputter-coated with 40–50 nm gold. Samples were later examined with an Evo MA 10 Scanning Electron Microscope (Zeiss) at 15–25 kV. Terminology of seed shape and testa sculpturing follows Sutton (1988).

Flow cytometry

Relative DNA content (RGS) measurements were performed with the aim of inferring the ploidy level of taxa without published chromosome counts. However, due to the lack of fresh mature seeds able to germinate and the low quality of measurements performed on dry leaf material, only four taxa were analysed. Fresh leaf material from germinated seeds from *C. microcalyx* subsp. *paradoxa* and the putative new taxon from the Taygetos Mounts was used (supplementary Table S1). Additionally, *C. microcalyx* subsp. *microcalyx* and *C. microcalyx* subsp *dodekanesi* were also

measured in order to confirm previously documented ploidy levels (Sutton, 1988; P. Carnicero unpubl. data). The diploid *C. muralis* G. Gaertn., B. Mey. & Scherb. from the Botanical Garden of the University of Innsbruck, was also measured as a reference. Flow cytometry (FCM) of 4',6-diamidino-2-phenylindole (DAPI; final concentration b 0.036 M) stained nuclei was used to estimate RGS and to assess DNA ploidy levels (Suda & Trávníček, 2006). The standard used to determine DNA amounts was *Solanum pseudocapsicum* L. (2C = 2.58 pg; Temsch & Greilhuber, 2010). Fresh leaf tissue was chopped with leaf material of the reference and processed as described in Suda et al. (2007). The relative fluorescence intensity of 3,000 particles was recorded using a Partec CyFlow Space flow cytometer (Partec GmbH, Münster, Germany). Partec FloMax software was used to evaluate the histograms, which were manually gated. RGS was calculated as ratio between the relative fluorescence of sample and standard. The reliability of the measurements was assessed by calculating coefficients of variation (CV) for the G1 peaks of both the analysed sample and the reference standard. Analyses yielding a CV threshold of > 5 % were discarded and the samples measured again. Ploidy levels were estimated by comparing RGS of samples with that of diploid *C. muralis* (2*n* = 14; Sutton, 1988).

Results

Phylogenetic analyses

The analyses of the nrDNA with MP and BI resulted in congruent phylogenetic tree topologies. However, the results from the cpDNA presented several incongruities with the nrDNA analysis (Figs. 2, 3).

The nrDNA analyses (Fig. 2) retrieved three main lineages. The eastern *Cymbalaria* taxa appeared in two of these three lineages: one was composed only by eastern Mediterranean species (eastern lineage, clade MS, BS = 100%, PP = 1) and a second one was composed of central and eastern Mediterranean species (central-eastern lineage, BS = 100%, PP = 1). The eastern lineage contained three main clades. One was composed by all specimens from the Taygetos mounts (as *C. spetae*; BS = 100%, PP = 1);

a second clade was composed by all specimens of *C. microcalyx* subsp. *heterosepala* and one specimen of *C. microcalyx* subsp. *microcalyx* (BS = 71%, PP = 0.99); and the third clade was composed by the rest of specimens of *C. microcalyx* subsp. *microcalyx* (BS = 74%, PP = 1). Relationships between these three mains clades in the eastern lineage received low statistical support. Within the central-eastern lineage, *C. microcalyx* subsp. *ebelii* formed a clade (BS = 97%, PP = 1) sister to *C. pubescens* (C. Presl) Cufod., a species endemic to Sicily, with moderate statistical support (BS = 60%, PP = 0.91), and the remaining eastern species constituted an independent monophyletic group (clade ALM, PP = 0.98). Within clade ALM, *C. microcalyx* subsp. *minor* constituted a monophyletic group (BS = 53%, PP = 0.98) sister to a moderate statistically supported clade containing *C. microcalyx* subsp*. dodekanesi*, *C. microcalyx* subsp. *acutiloba*, *C. longipes* and *C. microcalyx* subsp. *paradoxa* (PP = 0.90). *Cymbalaria microcalyx* subsp*. dodekanesi* formed two supported clades respectively constituted by the specimens from Karpathos (specimens 5 and 6, BS = 100%, PP = 1) and by the specimens from Rhodes (specimens 1–4, BS = 100%, PP = 1). Finally, a clade with low statistical support grouped all specimens of *C. longipes* with the specimens of *C. microcalyx* subsp. *acutiloba* and *C. microcalyx* subsp *paradoxa* (clade AL). Clade AL was constituted by two statistically supported subclades congruent with geography: 1) *C. longipes* 3 and *C. microcalyx* subsp. *acutiloba*, both from Anatolia (PP = 1), and 2) *C. longipes* 4–9 and *C. microcalyx* subsp. *paradoxa*, all from eastern Aegean Islands (BS = 74%, PP = 1). *Cymbalaria longipes* 1, from Karpathos, was sister with moderate statistical support to the second subclade (PP = 0.92).

In the cpDNA analyses, *Cymbalaria* was divided in two clades (Fig. 3). One clade grouped all specimens from the Taygetos mounts (as *C. spetae*; BS = 96%, PP = 1) with some of the central Mediterranean species (BS = 79%, PP = 1) and the other main clade grouped together all remaining *Cymbalaria* specimens (PP = 0.95). Statistical supports for the basal branches in the second clade were in general low, and therefore phylogenetic relationships between taxa were poorly inferred. Within the eastern taxa, only *C. microcalyx* subsp. *minor* and *C. microcalyx* subsp. *paradoxa* (three specimens from a single population) were monophyletic (BS = 77%, PP = 0.96 and PP = 0.98, respectively). Most eastern taxa were split in two or more clades instead. The most

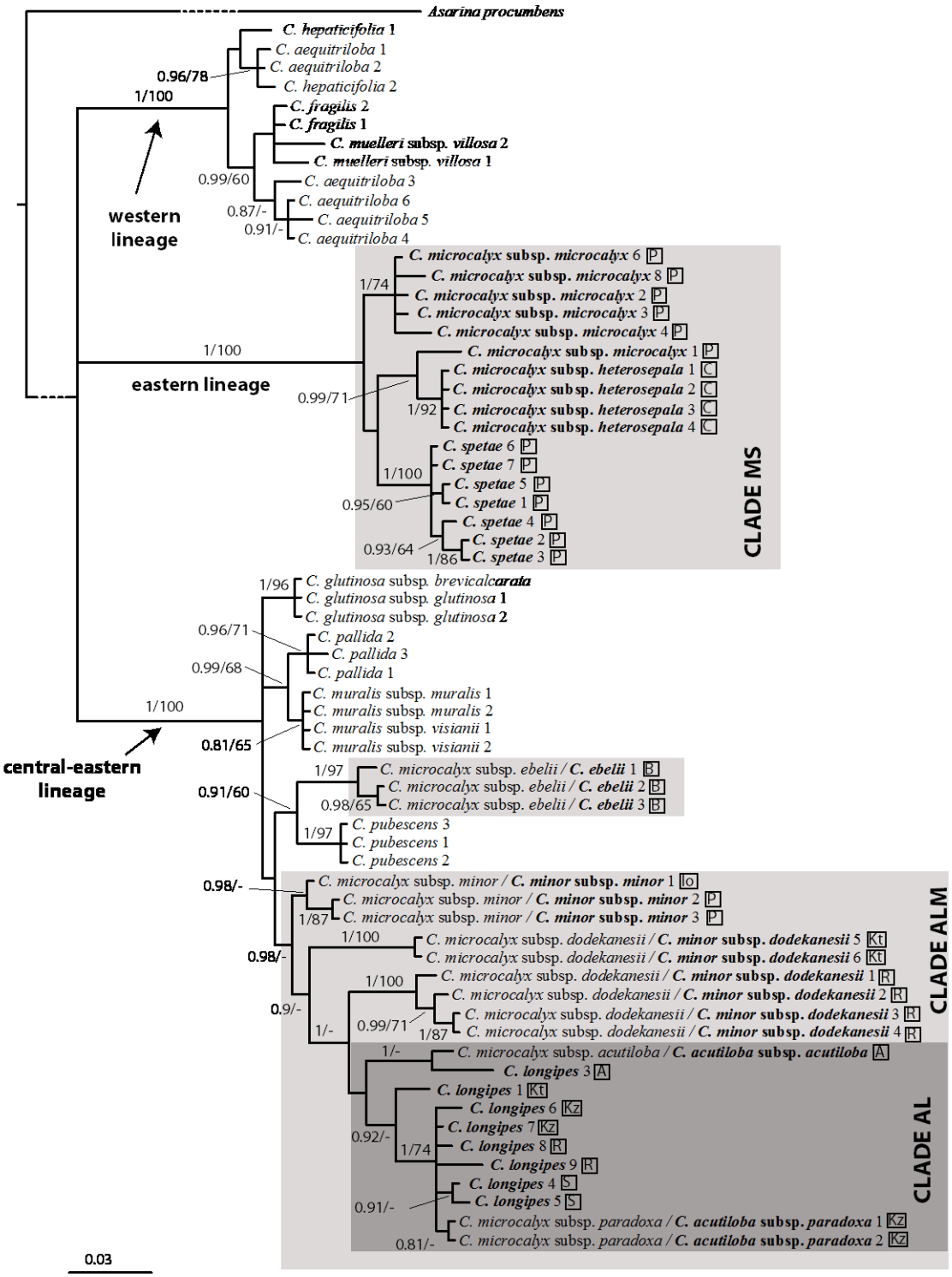

Figure 2. Phylogram from the Bayesian analysis of nrDNA of *Cymbalaria*. Dotted lines indicate branches that have been manually shortened. Bayesian posterior probabilities ≥ 0.80/bootstrap support values ≥ 60% are indicated. Previous taxonomic treatment and final taxonomic treatment (in bold) are given. Only one name in bold is shown when they coincide. Grey boxes indicate clades composed only by eastern Mediterranean species. Clade MS: clade *C. microcalyx–C. spetae*. Clade ALM: clade *C. acutiloba–C. longipes–C. minor.* Clade AL: clade *C. acutiloba–C. longipes*. Letters next to the species names indicate the geographic origin of eastern Mediterranean specimens (A: Anatolia, B: Balkans, C: Crete, Kt: Karpathos, Kz: Kastelorizo, P: Peloponnese, R: Rhodes, S: Samos).

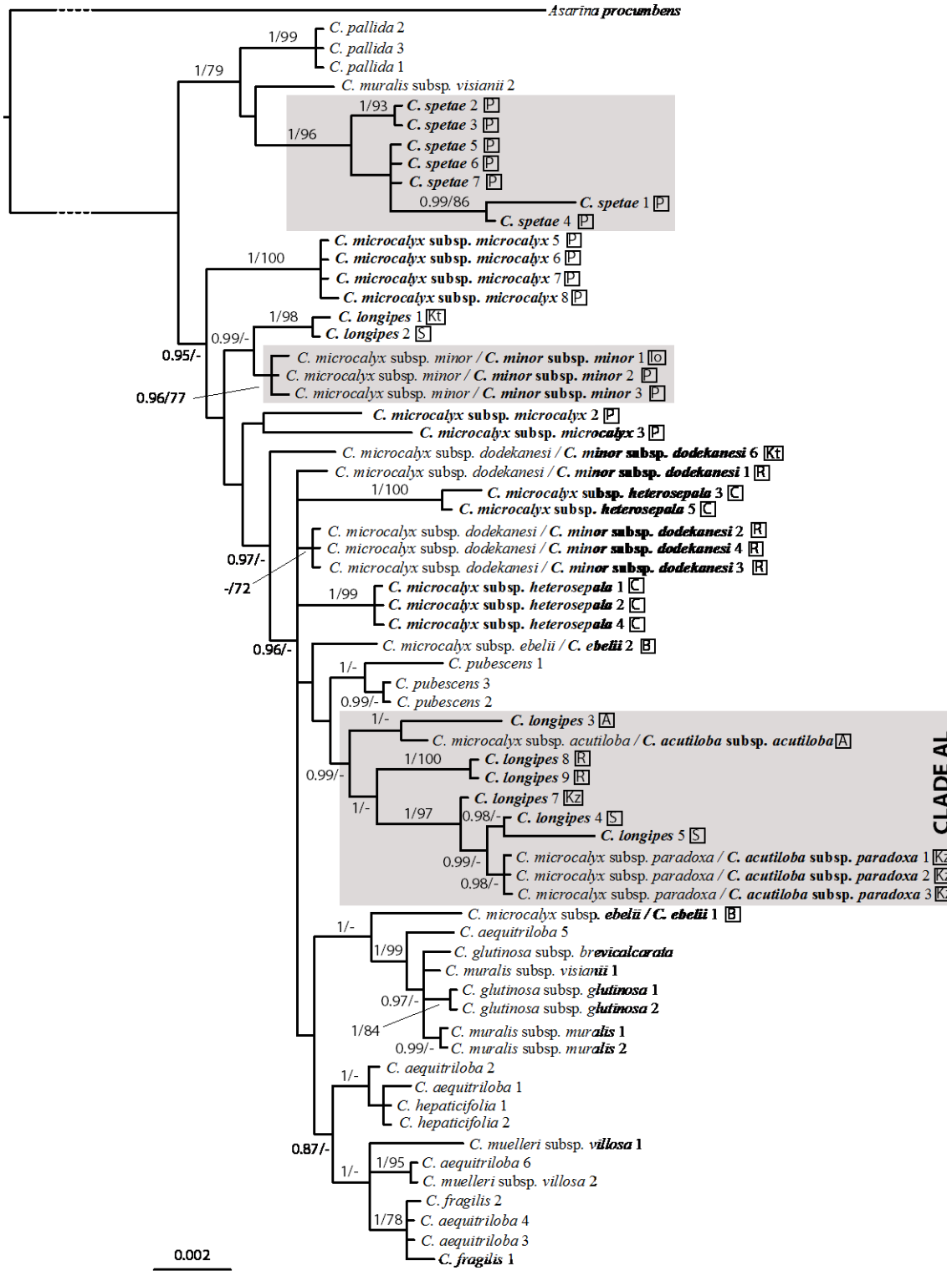

Figure 3. Phylogram from the Bayesian analysis of nrDNA of *Cymbalaria*. Bayesian posterior probabilities ≥ 0.80/bootstrap support values ≥ 60% are indicated. Previous taxonomic treatment and final taxonomic treatment (in bold) are given. Only one name in bold is shown when they coincide. Grey boxes indicate main congruent clades with the nrDNA. Clade AL: clade *C. acutiloba–C. longipes*. Letters next to the species names indicate the geographic origin of eastern Mediterranean specimens (A: Anatolia, B: Balkans, C: Crete, Io: Ionian Islands; Kt: Karpathos, Kz: Kastelorizo, P: Peloponnese, R: Rhodes, S: Samos).

remarkable relationships coincident with the nrDNA analysis were: 1) the respectively monophyletic *C. microcalyx* subsp. *minor* and *C. spetae*; 2) clade AL, that included the same members with the exception of *C. longipes* 1, that formed a clade with *C. longipes* 2 instead (BS = 98%, PP = 1), and this clade was sister to *C. microcalyx* subsp. *minor* (PP = 0.99); and 3) *Cymbalaria microcalyx* subsp. *ebelii* did not group with eastern Mediterranean taxa.

Morphological analyses

Correlation coefficients did not exceed 0.95 for any pair of characters; therefore, all characters were retained for further analyses (Table 1). In the PCA, the first axis accounted for 50% of the variation and the second axis for 17%. The ordination diagram (Fig. 4A) showed a strong separation of specimens from the Taygetos mounts (as *C. spetae*) from remaining eastern species. Its clear independence was shown in boxplots for selected characters (Calyx length and the ratio spur length / corolla length, Fig. 4B). *Cymbalaria longipes* also constituted a well delimited group. *Cymbalaria microcalyx* specimens were in a cloud with less clear separation among infraspecific entities. However, three groups with low overlap could be observed: 1) *Cymbalaria microcalyx* subsp. *acutiloba*-*C. microcalyx* subsp. *paradoxa*; 2) *Cymbalaria microcalyx* subsp. *minor*-*C. microcalyx* subsp. *dodekanesi*; and 3) *Cymbalaria microcalyx* subsp. *microcalyx* (including subsp. *alba*).

The examination of seeds under the binocular stereoscopic microscope, SEMimages (Fig. 5) and previous studies (Speta, 1986, 1989; Sutton, 1988) supported the groups visualized in the PCA. Details on seed morphology are summarized in Table 2.

According to phylogenetic results, the PCA analysis of quantitative characters, traits of seed morphology and results of flow cytometry, a taxonomic classification was proposed and used to define groups in CDA. The first three axes in the CDA at the species level accounted for 83% of the total variation (Fig. 6A). Here, *C. spetae*, *C. longipes* and *C. microcalyx* (see taxonomic treatment) were strongly supported. Although showing less compact clouds, *C. acutiloba* (Boiss. & Heldr.) Speta and *C. minor* (Cufod.) Speta also formed non-overlapping groups. At the subspecies level, the first three axes accounted for 69% of the total variation (Fig. 6B). Amongst the taxa traditionally comprised within *C. microcalyx*, the newly proposed *C. acutiloba* subsp.

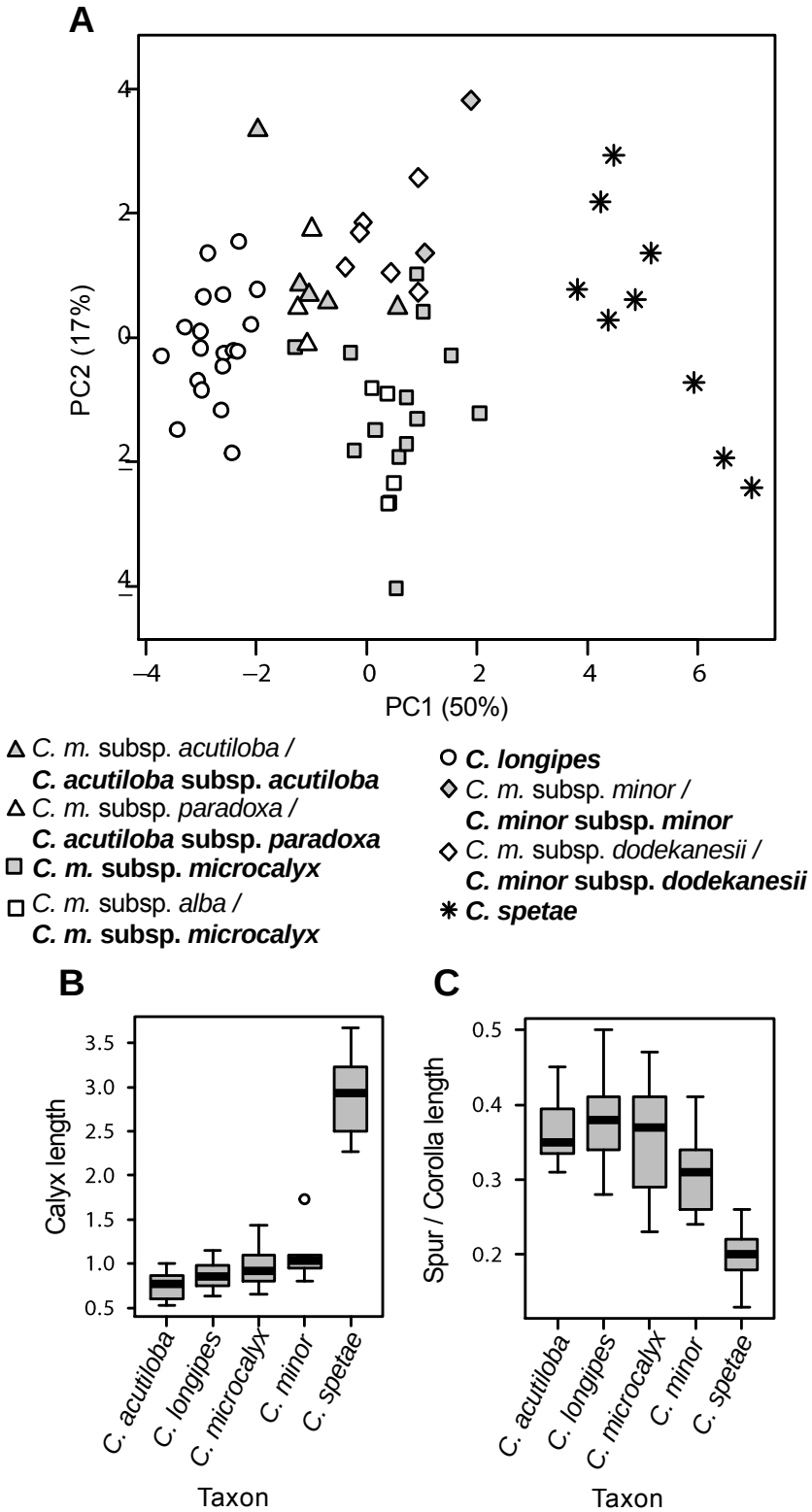

Figure 4. A: Principal component analysis of twelve floral and vegetative characters obtained for 62 specimens. Previous taxonomic treatment and final taxonomic treatment (in bold) are given. Only one name in bold is shown when they coincide. m.: *microcalyx*. B, C: Boxplots showing median, upper and lower quartiles, minimum and maximum values (excluding outliers) and outliers for the two characters showing the greatest differences between *Cymbalaria spetae* and other eastern Mediterranean taxa according to the taxonomic treatment adopted in this study.

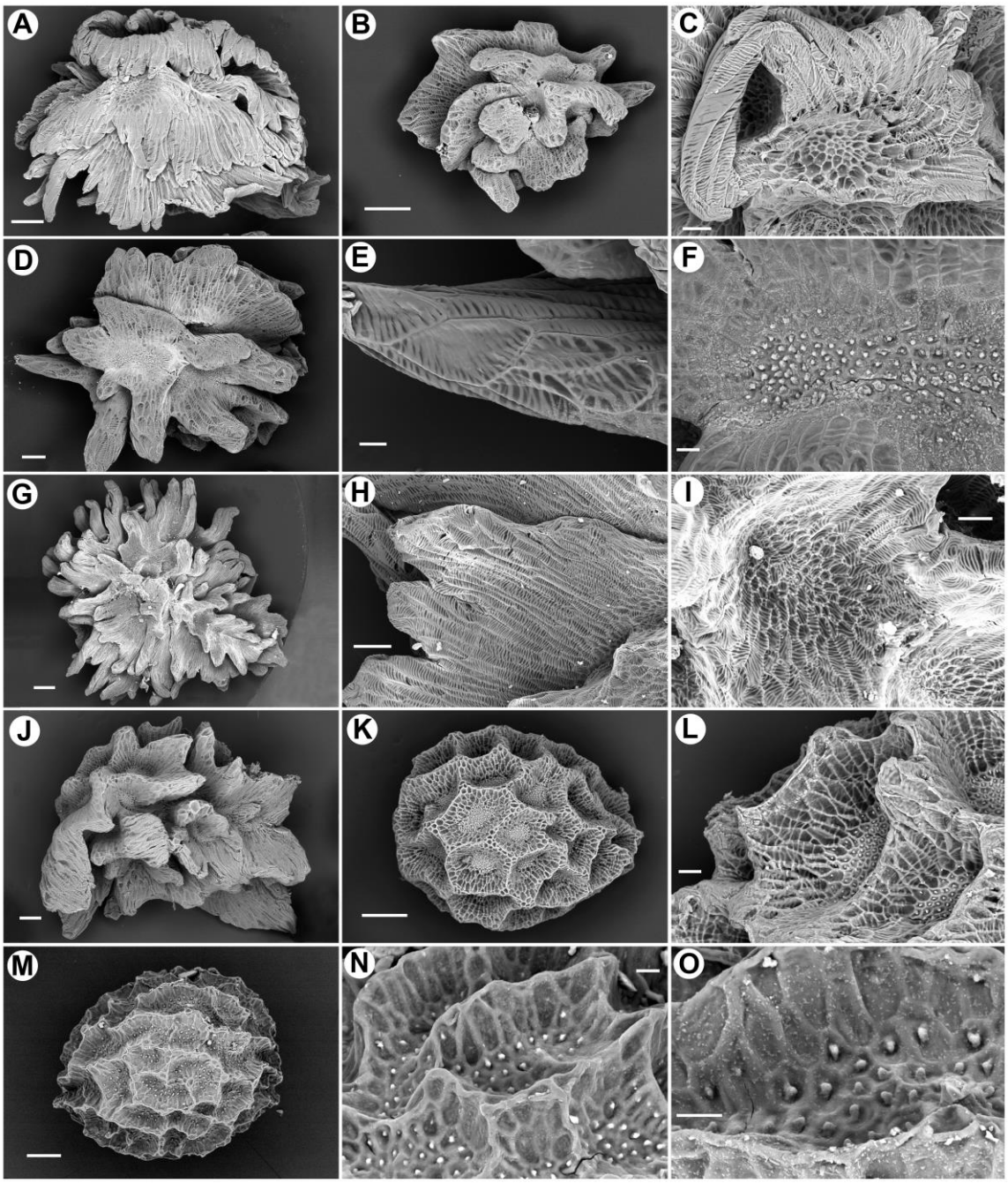

Figure 5. Scanning electron micrographs of seeds of eastern Mediterranean *Cymbalaria* taxa. A-C: ***C. microcalyx* subsp. *heterosepala*,** Greece: Crete, Falasarna, cape above ruins, cracks in limestone rocks, cliffs above the sea, 50 m, *P. Carnicero 1022* (BC); D-F: ***C. microcalyx* subsp. *microcalyx*,** Greece: Peloponnese, Lakonia, Mystras, gorge, trail to Taygeti, Cracks in limestone cliffs facing N and shady blocks, 400-430 m, *P. Carnicero 1063* (BC); G-I: *C. microcalyx* subsp. *paradoxa* / ***C. acutiloba* subsp. *paradoxa*,** Greece: Kastelorizo, above Megisti, stears climbing the cliff, Cracks in limestone rocks facing N, 100-130 m, *P. Carnicero 1039* (BC); J-L: *C. microcalyx* subsp. *dodekanesi* / ***C. minor* subsp. *dodekanesi*,** Greece: Rhodes, Arcangelos, Mount Profitis Ilias S of vilage, following the road towards the hermitage, cracks in limestone cliff facing NW, 430 m, *P. Carnicero 1062* (BC); M-O: ***C. spetae*** Greece: Peloponnese, Lakonia, Trypi, road to Kalamata, tunnel excavated in the rock, cracks in shady limestone rocks, 780 m, *P. Carnicero 1074* (BC 955798). Scale bars: A, B: 400 µm; C, I, M: 100 µm; D, H, J, K: 200 µm; E, F: 40 µm; G: 600 µm; L: 50 µm; M, N: 20 µm.

Table 2. Seed characters and ploidy level of studied species. Taxon names follow the final taxonomic treatment adopted here.

Taxon	Chromo-some number	N. seeds / capsule	Seed length range (smallest and biggest seed observed, mm)	Seeds equal in size / disposition within the capsule	Concres-cence of seeds	Testa
C. acutiloba subsp. *acutiloba*	unknown	few[1]	2–3[1]	unequal / irregular	unknown	irregularly cristate, ridges wing-like, with numerous triangular tips[2]
C. acutiloba subsp. *paradoxa*	$2n = 28$[3]	4-6	3.8–4.6	unequal / irregular	yes	irregularly cristate, ridges wing-like, deeply divided in digitate expansions
C. ebelii	$2n = 28$[4]	2–10[4]	1.5–2.5	equal	no	cristate, ridges low and scarce, not wing-like
C. longipes	$2n = 14$[2]	2–6[2]	3.5–5.6	unequal / irregular	yes	irregularly cristate, ridges wing-like, deeply divided in digitate expansions
C. microcalyx subsp. *microcalyx*	$2n = 28$[1,3]	4–10	1.3–4	unequal / irregular	no	irregularly cristate, ridges wing-like
C. microcalyx subsp. *heterosepala*	*unknown*	4–6	2.2–4.8	unequal / irregular	yes	irregularly cristate, ridges wing-like, strongly compressed, only shallowly divided in rounded segments
C. minor subsp. *minor*	$2n = 28$[2]	12	0.8–1.3	equal	no	alveolate, ridges low, not wing-like
C. minor subsp. *dodekanesi*	$2n = 28$[3,5]	8–14	0.9–3.3	unequal / decrea-sing towards the apex	no	alveolate, ridges high, decreasing towards the apex, not wing-like
C. spetae	$2n = 28$[3]	17–24	0.6–0.8	equal	no	shallowly cristate-alveolate, ridges low, not wing-like

[1] Data partially or totally obtained from Sutton (1988)
[2] Data partially obtained from Greuter & Rechinger (1967)
[3] Data inferred from flow citometry measures
[4] Data obtained from Speta (1989)
[5] P. Carnicero, unpublished chromosome count

paradoxa was the most clearly differentiated, while other taxa never formed absolutely well delimited groups.

Flow cytometry

Mean RGS for *C. microcalyx* subsp. *dodekanesi* and *C. microcalyx* subsp. *microcalyx* were respectively 0.63 (0.62–0.64) and 0.60. Both exhibited, respectively, 3.15 and 2.99 times the RGS of the diploid *C. muralis*, although chormosome counts confirmed them as tetraploids (Speta, 1986; Carnicero, unpubl. data). *Cymbalaria spetae* and *C. microcalyx* subsp. *paradoxa* showed a mean RGS of 0.61 and 0.7 (0.70–0.71), respectively, 3.1 and 3.5 times the RGS of the diploid *C. muralis*. They are therefore estimated to be tetraploids too. These values are lower than expected if genome size increased in direct proportion with polyploidy, which can be explained by genome downsizing, an often reported phenomenon in non-recently formed polyploids (Leitch and Bennett, 2004; Frajman et al., 2015; Lazarević et al., 2015).

Discussion

Our data support the split of a variable and widely distributed *C. microcalyx*, as recognized in most recent revisions (Sutton, 1988; Dimopoulos et al., 2013), into four species with allegedly distinctive seed characters: *Cymbalaria acutiloba*, *C. ebelii*, *C. microcalyx* and *C. minor* (see taxonomic treatment). This treatment was already adopted by Speta (1986), although he admitted that these species were hardly identifiable without mature seeds. They all share several distinctive vegetative and floral characters: they are hairy throughout including the capsule, they have 3–5 lobed leaves and a short calyx. This was probably the reason why Cufodontis (1936) and Sutton (1988) considered them to constitute a single species. Here we describe the new species *C. spetae* from the Taygetos mounts in the Peloponnese, with several distinctive morphological characters and a well-defined genetic identity. Finally, we propose to keep *C. longipes* as a single widespread species, although strongly related to *C. acutiloba* as supported by molecular data.

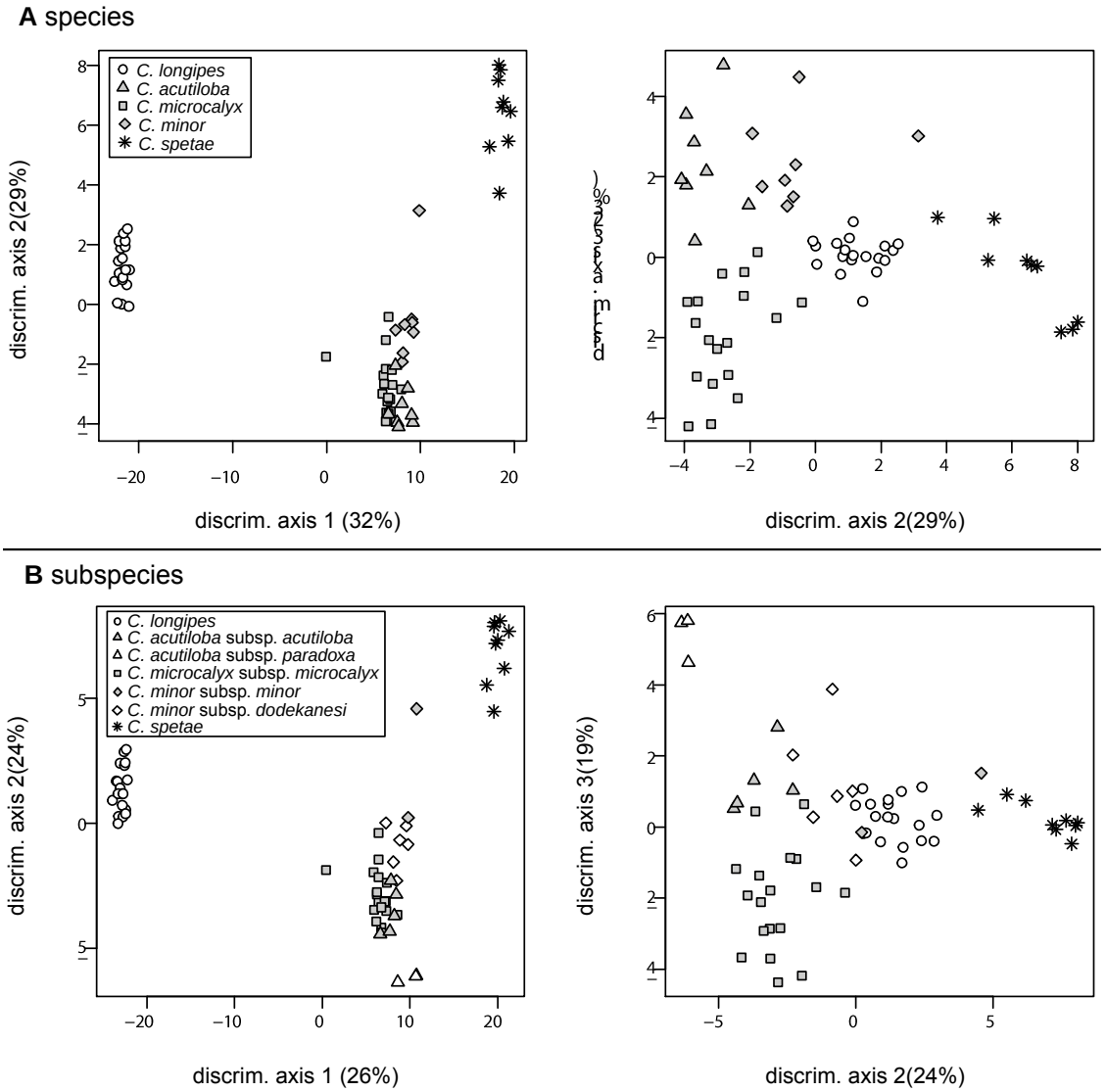

Figure 6. Canonical discriminant analyses. Taxa are identified according to the final taxonomic treatment adopted in this study. A: Analysis at the species level. B: Analysis at the subspecies level.

Our treatment reflects better the high morphologic and phylogenetic diversity found in the region than previous treatments (Figs 2, 3, 4, 6). The Peloponnese is revealed as a diversity centre for *Cymbalaria*, with four species corresponding to two early divergent genetic lineages within the genus. Moreover, considering taxonomy as a last step in the systematic approach, we tried to make a treatment that reflect the evolutionary history of lineages (Stuessy et al., 2014). However, it is also indispensable to find diagnostic characters that allow the identification of the species. In the tribe Antirrhineae, as well as other plant groups, seeds morphology have been traditionally considered of great taxonomic value (e.g. Elisens, 1985; Vigalondo et al., 2015). In eastern *Cymbalaria*, several authors highlighted the crucial importance of seeds as a

diagnostic character (Greuter & Rechinger, 1967; Speta, 1986, 1989; Sutton, 1988). However it has also been demonstrated that it has no diagnostic value for the western Mediterranean species *C. fragilis* J.J.Rodr. (A.Chev.), which has two types of testa sculpturing (Carnicero et al., in prep., Chapter II). Instead, in eastern Mediterranean, we demonstrate that seed morphology, as well as other identified diagnostic characters, is congruent with the genetic lineages found and have therefore high taxonomic value.

***Cymbalaria ebelii*: a species related to central Mediterranean taxa**

Cymbalaria ebelii did not group with any eastern Mediterranean species in the molecular analyses (Figs. 2, 3). Instead, it grouped with moderate statistical support with *C. pubescens* (BS = 60%, PP = 0.91), a tetraploid from Sicily, in the nrDNA analysis. In the cpDNA analysis, the phylogenetic position of *C. ebelii* 2 was not statistically supported, while *C. ebelii* 1 grouped with high statistical support (PP = 1) with central Mediterranean species. The presence of *C. aequitriloba* (Viv.) A.Chev. in this clade is probably due to introgression with *C. muralis*, as shown by Carnicero et al. (in prep., Chapter II). According to phytogeographic provinces, the area occupied by *C. ebelii* has closer floristic affinities with the Apennine Peninsula and the northern Balkan coast than with the southern Balkan Peninsula, Aegean area and Anatolia Peninsula (Takhtajan, 1986). This disjunction could be caused by a climatic barrier, as evidenced by a stronger effect of Pleistocene glaciations and currently more humid and cold climate in the border between the two areas (Polunin, 1980). Our molecular results agree with other phylogenetic and phylogeographic studies in the area showing a strong biogeographic split between the northern and southern Balkans (Bardy et al., 2010; Surina et al., 2011; Hilpold et al., 2014) and with the hypothesis of polyploid speciation for the origin of *C. ebelii* and *C. pubescens* from a central Mediterranean ancestor (Carnicero et al., 2017).

Morphologically, *C. ebelii* has unique oblong seeds with few low longitudinal ridges (Speta, 1986), as we have observed in the type material. Moreover, Speta (1989) also reported distinctive capsule traits: the walls of the capsule are folded so that there is little space left for the few seeds contained, and it opens along undulated longitudinal ridges. Thus, both molecular and morphological data support *C. ebelii* as a separate

species. Sutton (1988) also pointed to the possibility of recognizing *C. ebelii* at species level but he could not observe mature capsules and provisionally kept it at the subspecies level under *C. microcalyx*.

Cymbalaria spetae: a new phylogenetically and morphologically well delimited species

All *C. spetae* specimens sampled constituted a monophyletic group in molecular analyses (Figs. 2, 3), thus strongly supporting its genetic uniqueness. Morphologically, it showed several divergent characters with respect to all other eastern species, namely a higher number of leaf lobes, a longer calyx, a proportionally shorter spur, a high number of seeds per capsule and much smaller seeds (Figs. 5, 7, Table 2). In fact, the most similar species to *C. spetae* is *C. glutinosa* Bigazzi & Raffaelli, from the southern Apennine Peninsula, from which it can be clearly distinguished by having a longer calyx (*C. spetae*: 2.3–3.7 mm *vs. C. glutinosa ca*. 2 mm, Bigazzi & Raffaelli, 2000) and often a bigger corolla (*C. spetae*: 9–14.2 mm *vs. C. glutinosa* 6–12(13) mm, Bigazzi & Raffaelli, 2000). Moreover, these two species show always distant phylogenetic positions and have different ploidy levels: *C. spetae* is presumably a tetraploid as inferred from RGS estimation, while *C. glutinosa* is a diploid (Bigazzi & Raffaelli, 2000).

At present, *C. spetae* is only known from two populations in the Taygetos mounts in the Peloponnese. Although it has clearly different characters, the specimen *C. spetae* 1 had been identified in herbaria as *C. microcalyx*, probably because it is the only hairy eastern Mediterranean *Cymbalaria* included in present Floras (e.g. Webb, 1972; Strid & Tan, 1997; Dimopoulos et al., 2013). Therefore, it is plausible that other populations of *C. spetae* might have been misidentified and, consequently, the distribution area of the species may be bigger.

Cymbalaria microcalyx and *C. minor* have parallel mainland-island subspecies

The recognition of *C. microcalyx* and *C. minor* as separate species is supported by the nrDNA gene tree (Fig. 2). Morphologically, the most divergent characters are related to seeds. While *C. microcalyx* has 4–10 big seeds per capsule, and the testa has wing-like ridges, *C. minor* has 8–14 smaller seeds per capsule, with the testa more or less deeply alveolate, but never forming wing-like expansions (Table 2, Fig. 5).

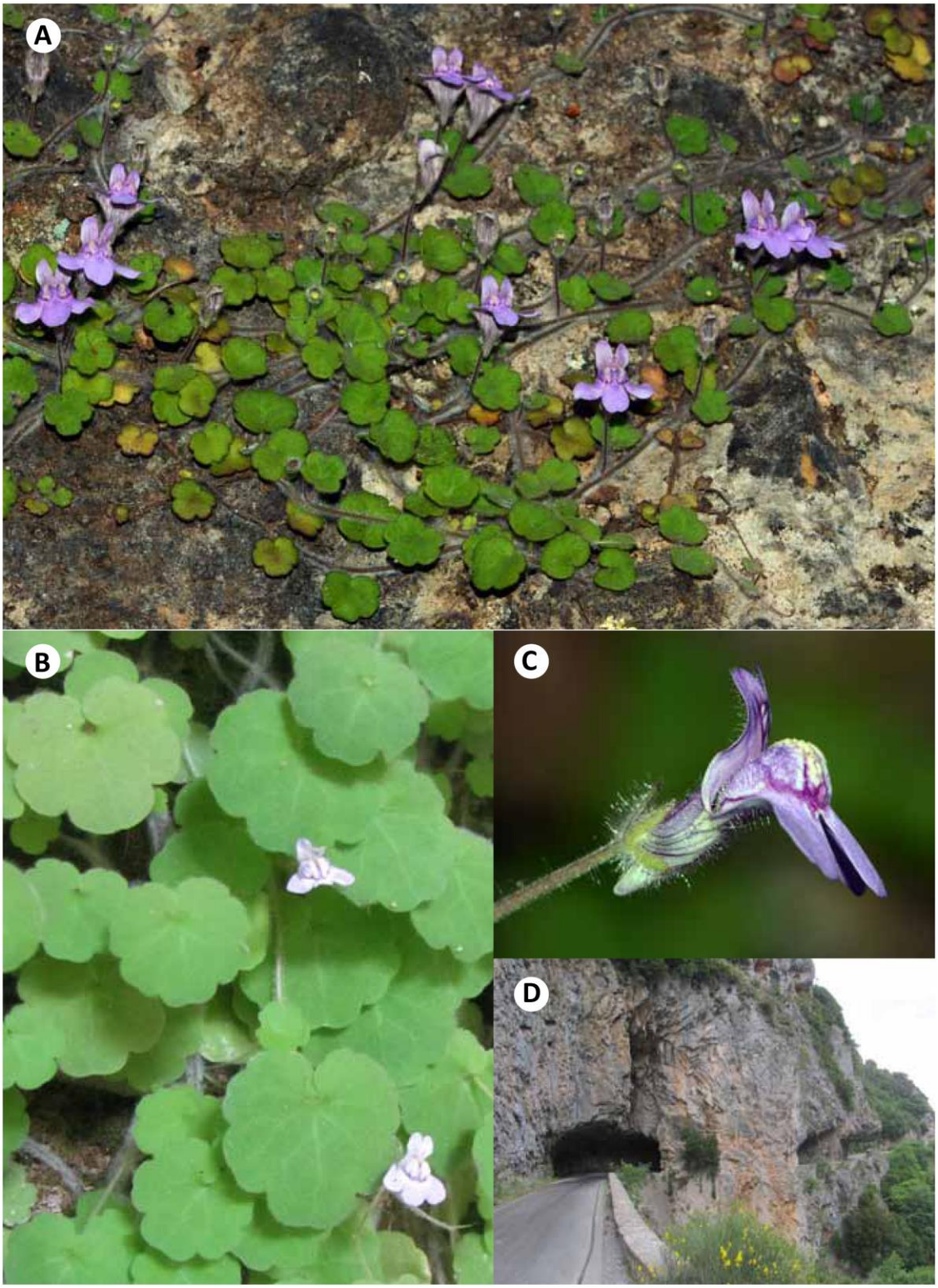

Figure 7. *Cymbalaria spetae* in its natural habitat (A–C) and type locality (D).

Available data support the split of *C. microcalyx* in two allopatric subspecies: subsp. *microcalyx*, from the Peloponnese and subsp. *heterosepala*, from western Crete. These are distinguishable mainly by seed characters. *Cymbalaria microcalyx* subsp. *heterosepala* has all seeds concrescent in a compact mass, similar to the seeds disposition of *C. longipes* and *C. paradoxa*, while subsp. *microcalyx* has the seeds free, individually separated (Fig. 5). Subsp. *heterosepala* was originally described as different from *microcalyx* based on the different length observed among lobes of the same calyx (Rechinger, 1943). However, we observed that this character is not even constant in all flowers of the holotype. Moreover, Greuter & Rechinger (1967) reported that some individuals from Peloponnese populations can also have unequal calyx lobes. The nrDNA tree (Fig. 2) showed a close relationship between the two subspecies, congruent with the paleogeographic history of the region. The marine distance between the Peloponnese and Crete was considerably reduced in two periods: during sea transgressions in the Messinian Salinity Crisis (5.96–5.33 Ma., Meijer & Krijgsman, 2005) and during the phases of low sea level in Pleistocene Glaciation periods (Van Andel & Shackleton, 1982), thus facilitating dispersal between the two regions. Previous land connections existed (10 Ma, Polunin, 1980), but are irrelevant to eastern *Cymbalaria* because they originated much later, in the Pliocene-Pleistocene (Carnicero et al., 2017). The paraphyly of subsp. *microcalyx* (Fig. 2) suggests the Peloponnese as the ancestral area for the species followed by further dispersal to Crete. Interestingly, two independent clades were found in the cpDNA tree for *C. microcalyx* subsp. *heterosepala*, although a single small population was sampled. This could indicate a higher haplotype diversity than expected in the case of a single dispersal event (Schönswetter et al., 2008; Burnier et al., 2009), which points to persistence of gene flow between the continent and Crete until land masses became geographically distant and subsp. *heterosepala* differentiated. The fact that subsp. *heterosepala* is monophyletic in the nrDNA tree (Fig. 2) supports the idea that gene flow is not occurring at present. The fragmented distribution of other species in the Peloponnese, Crete and southwestern Anatolia (along the southern Aegean ancient mountain range) also support the idea of effective gene flow across sea barriers (e.g. *Ranunculus brevifolius* Ten., *Atraphaxis billardieri* Jaub. & Spach, *Potentilla speciosa* Willd., Strid & Tan, 1997). Few molecular systematic studies have been performed in the region, but a

similar case has been revealed in *Campanula* subgenus *Roucela* (Crowl et al., 2015). Their analyses support vicariance for the origin of *C. creutzburgii* Greuter (endemic to Crete) and *C. drabifolia* Sm. (endemic to continental Greece and the Cyclades), driven by the breakup of the southern Aegean range (ca. 10 Ma) which isolated Crete from the Peloponnese (Polunin, 1980).

The nrDNA tree (Fig. 2), morphometric analyses (Figs. 4, 6) and further coincidences in seed characters (Table 2) showed a close relationship between former *C. microcalyx* subsp. *minor* and *C. microcalyx* subsp. *dodekanesi*. Thus we combine both under *C. minor*. Both subspecies can be distinguished by characters of the seeds and capsule (Sutton, 1988; Table 2): *Cymbalaria minor* subsp. *dodekanesi* have seeds of different size in each loculus and a lanate capsule, while in *C. minor* subsp. *minor* capsules are pubescent and all seeds are approximately of the same size, similar in size and shape to the smallest seeds of subsp. *dodekanesi*. *Cymbalaria minor* subsp. *minor* is found in the Ionian Islands and continental Greece whereas subsp. *dodekanesi* grows in most Aegean Islands (Fig. 1). Land bridges connected the Aegean Islands (the Cyclades) with the continent during Pleistocene glaciation periods (Van Andel & Shackleton, 1982), thus facilitating dispersal and further differentiation of the two subspecies when sea level rose again. The nrDNA phylogenetic relationships between the two subspecies (Fig. 2) suggest a continental origin followed by dispersal to islands, probably through the Cyclades. The low sea level during Pleistocene glatiations also resulted in reduced distances between the Cyclades and surrounding areas such the Eastern Aegean Islands, Crete and Karpathos, where *C. minor* subsp. *dodekanesi* is also found at present. A strong connection between the Cyclades, Crete and Karpathos has been previously suggested from the high number of endemics shared (e.g. *Nigella doerfleri* Vierh., *Onosma graeca* Boiss., Rechinger, 1950; Strid & Tan, 1997; Comes et al., 2008). More surprising is the presence of *C. minor* subsp. *dodekanesi* in eastern Aegean Islands, that are considered to be quite isolated from the western Aegean region by the Rechinger line (Rechinger, 1950; Strid, 1996). Specifically, the nrDNA tree (Fig. 2) suggests a former colonization of Crete and Karpathos from the Cyclades, followed by dispersal across the Rechinger line to Rhodes and the remaining eastern Aegean Islands. However, this hypothesis and its details would need to be confirmed with a

phylogeographic study with a comprehensive sampling of *C. minor* subsp *dodekanesi* populations. Although this phytogeographic break has been partly confirmed with molecular data for some groups (Bittkau & Comes, 2005; Crowl et al., 2015), Rechinger (1950) reported some taxa that occur at both sides of the barrier (e.g. *Campanula delicatula* Boiss., *Helichrysum orientale* Gaertn.).

The cases of *C. microcalyx* and *C. minor* show parallel subspeciation events between continental areas and islands and highlight the important role of eustatic changes in the Pleistocene in the Mediterranean Basin and specifically in the Aegean Area. However, the hypothesis of longer dispersal events should not be automatically discarded in any case, since long-distance dispersal in plants has received increasing evidence in recent years (e.g. Nathan, 2006; Guzmán & Vargas, 2009; Santos-Gally et al., 2011). Moreover, polyploids seem to be specially well suited for success after long-distance dispersal (Linder & Barker, 2014; Ogutcen & Vamosi, 2016).

The clade AL: *Cymbalaria acutiloba* and *C. longipes*

The nrDNA supported clade AL, embedded in *C. minor*, thus suggesting an anacladogenetic origin for the taxa contained in this clade (Stuessy et al., 1990; Mayr & Bock, 2002). This could partly support the hypothesis of Greuter & Rechinger (1967) considering *C. longipes* a "young" taxon of the *C. microcalyx* aggregate, and thus its combination as a subspecies of a broad concept of *C. microcalyx* (Greuter, 1979). However, ploidy data seriously challenges this statement: *Cymbalaria minor* and *C. acutiloba* subsp. *paradoxa* are tetraploids while *C. longipes* is diploid, which would imply a ploidy level reduction. Although aneuploid chromosome number reduction is not rare (Lysak et al., 2007; Soltis et al., 2009; Fawcett & Peer, 2010), we could not find any case of whole ploidy level reduction in plants (but see Spangler et al., 1999). Moreover, the sampling in this clade is incomplete: *Cymbalaria longipes* has been only sampled from the eastern Aegean region, including Rhodes, Karpathos and southwestern Anatolia, but its distribution expands west till the Peloponnese and east as far as the coast of Lebanon (Sutton, 1988). Additional genetic variation might arise if a wider sampling was included. However, the morphological uniformity of the taxon – the only glabrous taxon in the eastern Mediterranean region– as observed from the herbarium material studied (see appendix: additional specimens examined) suggests

that the species level is appropriate. It is remarkable that it has maintained a low morphological variation across several sea barriers that enhanced speciation in other eastern species. Cufodontis (1947) probably provided the clue to this issue: the convergence of seeds in a compact mass in an indehiscent capsule may be helpful for marine dispersal. Taking into account the habitat of *C. longipes*, cliffs and pebbles close to the sea, it is a plausible option.

In the case of *C. acutiloba*, although not monophyletic, the close phylogenetic relationship of *C. acutiloba* subsp. *actutiloba* and *C. acutiloba* subsp. *paradoxa*, as well as morphology, support the recognition of a single species with two subspecies. *Cymbalaria acutiloba* subsp. *paradoxa* is a tetraploid endemic to Kastellorizo, an island close to the Anatolian coast with strong phytogeographic affinities with the Anatolian element (Constantinidis, 2013). It is hairy throughout, unlike *C. longipes*, and the capsules with mature seeds are very similar to those of *C. longipes*. For the Anatolian *C. acutiloba* subsp. *acutiloba* we could not examine any complete capsule and information in the bibliography is scarce. Later, Speta (1986) described two different types of seed for the species, but he included populations from Rhodes as *C. acutiloba*, from where only *C. minor* subsp. *dodekanesii* has been reported (Sutton, 1988; Dimopoulos et al., 2013). Moreover, its ploidy level is unknown. Thus, a phylogeographic study including a wide sampling with morphological analyses and ploidy level estimation would be desirable to clarify the taxonomic identity and phylogenetic relationships among taxa included in clade AL. In fact, often nrDNA data are not sufficient to resolve phylogenetic relationships among closely related species, and geography has often a strong weight in final results (e.g. Hilpold et al., 2014; Herrando-Moraira et al., in prep.). In the case of *Cymbalaria*, AFLP data used in a phylogeographic study successfully resolved phylogenetic relationships among western taxa (Carnicero et al., in prep., Chapter II), which had not been inferred in detail with nrDNA data (Carnicero et al., 2017).

Taxonomic treatment

Cymbalaria ebelii (Cufod.) Speta in Phyton (Horn) 26(1): 50 (1986) ≡ *Linaria microcalyx* subsp. *ebelii* Cufod. in Arch. Bot. (Forlì) 12 (3-4): 237 (1936) ≡ *C. microcalyx*

subsp. *ebelii* (Cufod.) Cufod. in Bot. Not. 2: 151 (1947) – *Ind. loc*.: Jugoslavia. Dalmatia merid. Alte Feldmeuren in Sutomore bei Spizza, Kalk, 60 m., 15.4.1911, fl. (Latzel, -S-) [78, L. pil.]. Montenegro merid. Boljevici (=Bogliovich) pr. Virpazar, fl. (comm. 1841 Clementi, -GG-) – Insula Wranina im Skutarisee, Anf. 6.41, fl. fr. (Ebel, -VM-) [33, 52, L. Cymb.] – Seljani am sw.-Ende d. Skuteri-Sees, alte Mauern, 21.6.95, fl. fr (Reiser, -S-) [78, L. pil.] – (cfr: 63) – Lectotype (designated by Sutton, 1988, Appendix 9.3: 109): Montenegro, Insel Wranina, June 1841, *Ebel s. n.* (W!).

Cymbalaria minor (Cufod.) Speta in Phyton (Horn) 26(1): 51 (1986) subsp. ***minor*** ≡ *Linaria microcalyx* var. *minor* Maire & Petitmengin ex Cufod. in Arch. Bot. (Forlì) 12(3-4): 235 (1936) ≡ *C. microcalyx* subsp. *minor* (Cufod.) Greuter in Boissiera 13: 107 (1967) – *Ind. loc*.: Graecia: Ins. Levkas, Ins. Cephalonia, Acarnania, Achaia, Argolis. – Lectotype (designated by Strid & Tan, 1992: 328): Leucade, roches calcaires maritimes et fente des murailles d'une tour au cap i souana, 14 July 1906, *Maire & Petitmengin 311* (WU-Hal; isolectotype B [web!]).

Cymbalaria minor (Cufod.) Speta subsp. ***dodekanesi*** (Greuter) Carnicero, L. Sáez, N. Garcia & Galbany, **comb. nov.** ≡ *C. microcalyx* subsp. *dodekanesi* Greuter in Boissiera 13: 108 (1967) ≡ *C. acutiloba* subsp. *dodekanesi* (Greuter) Speta in Phyton (Horn) 26(1): 51 (1986) – *Ind. loc*.: Kárpathos, mons Aj. Ilías prope Ólimbos – Holotype: Karpathos, Olympos. Mte. Hagios Ilias, 19 May 1886, *Major 140* (G [web!]).

=*Linaria toplouensis* Coustrier & Gand. in Gand., Fl. Cret.: 85 (1916) – *Ind. loc*.: Sitia: in cavis[?] rupium ad coenobium Toplou – Type material (Sutton, 1988, Appendix 9.3: 107): Crete: Sitia, Toplou, 25 April 1914, *Gandoger 549* (iso? K).

Cymbalaria longipes (Boiss. & Heldr.) A. Chev. in Bull. Soc. Bot. France 83: 641 (1937) ≡ *Linaria longipes* Boiss. & Heldr. in Boiss., Diagn. pl. Or. Nov. ser. 1(12): 40 (1853) ≡ *L. cymbalaria* subsp. *longipes* (Boiss. & Heldr.) Hayek in Feddes Repert. (Beih.) 30(2/2): 143 (1929) ≡ *C. microcalyx* subsp. *longipes* (Boiss. & Heldr.) Greuter in Willdenowia 8(3): 580 (1979) – *Ind. loc*.: ad. muros et rupes circà *Adalia* Pamphyliae (Heldr.). – Type: ad muros et rupes circa Adalia [Antalya] Pamphyliae, 12 March 1845, *Heldreich 474* (holotype BM 000997823 [web!]; isotypes CGE, E [web!], FI!, G [web!], W!).

= *Linaria cymbalaria* var. *crassifolia* D'Urv. in Mém. Soc. Linn- Paris 1: 330 (1822) ≡ *Cymbalaria crassifolia* (D'Urv.) Grande in Bull. Ort. Bot. Napoli 4: 170 (1914) – *Ind. loc*.: In rupibus maritimis Astypalaeae, ad muros insulae Leri – Type material: unknown.

= *Linaria microcalyx* var. *glabrescens* Maire in Bull. Soc. Bot. France 68: 74 (1921) – *Ind. loc*.: Dans les galets de marbre de la plage du port Tristomon (Treboukhi) au Sud de l'île de Skyros – Type material: unknown, probably at MPU**.**

=*Linaria cymbalaria* var. *sieberi* Chav., Monogr. Antirrh.: 99 (1833) – *Ind. loc*.: [Crete] Not stated – Type material: unknown.

Cymbalaria acutiloba (Boiss. & Heldr.) Speta in Phyton (Horn) 26(1): 50 (1986) subsp. ***acutiloba*** ≡ *Linaria microcalyx* var. *acutiloba* Boiss & Heldr. in Boiss., Diagn. Pl. Or. Nov. ser. 1 2(12): 40 (1853) ≡ *C. microcalyx* subsp. *acutiloba* (Boiss. & Heldr.) Greuter in Boissiera 13:108 (1967) – *Ind. loc*.: ad rupes promontorii Alaya Pamphyliae – Type: ad rupes promontorii Alaya [Alanya], 18 April 1845, *Heldreich 565* (holotype G-BOIS; isotypes BM [web!], CGE, FI!, G [web!], K [web!], W!).

Cymbalaria acutiloba (Boiss. & Heldr.) Speta subsp. ***paradoxa*** (Greuter) Carnicero, L. Sáez, N. Garcia & Galbany, **comb. nov.** ≡ *C. microcalyx* subsp. *paradoxa* Greuter in Willdenowia 8: 580 (1979) – *Ind. loc*.: K [Kastellorizo]; local in shady cliff crevices.– Holotype: Dhodhekanisos; Kastellorhizo island group, Mejisti, S Harbous, 80–120 m, 15 April 1974, *Greuter 11859* (W).

Cymbalaria microcalyx (Boiss.) Wettst. in Engler & Prantl, Natürl. Pflanzenfam. ed. 1 4(3b): 58 (1891) subsp. ***microcalyx*** ≡ *Linaria microcalyx* Boiss., Diagn. Pl. Or. Nov. ser. 1 1(4): 72 (1844) – *Ind. loc*.: in fissuris rupium in faucibus Taygeti propè Mistra – Type: in fissuris rupium in faucibus Taygeti prope Mistra, April 1842, *Boissier s. n.* (holotype G-BOIS; isotypes B, FI!, K [web!], W!).

= *Antirrhinum pilosum* Bory & Chaub. in Bory, Expéd. Sci. Morée 3: 175 (1832), *nom. illeg., non* Jacq. – *Ind. loc*.: Sur les murs des conduits du molin de Gortys et sur les murs du château de Mistra; le mont Dia, à Naxie, où il nous a paru assez rare – Type material: unknown.

= *Linaria microcalyx* var. *orphanidiana* Cufod. in Arch. Bot. (Forlì) 12 (3-4): 235

(1936) ≡ *Cymbalaria microcalyx* var. *orphanidiana* (Cufod.) Cufod. in Bot. Not. (2): 151 (1947) – *Ind. loc*.: Graecia: Laconia (Acarnania?) – **Lectotype (designated here):** in locis umbrosis: montis Malevo Laconiae prope Platanos (rarissima), alt 3500', 22 April – 04 May 1857, *T. G. Orphanides 712* (W!; isolectotypes B, F, FI!, FR, GE, K[web!], W!).

= *Cymbalaria microcalyx* var. *alba* Voliotis in Ann. Mus. Goulandris 8: 224 (1990) ≡ *C. microcalyx* subsp. *alba* (Voliotis) Kit Tan, Endemic Pl. Greece, Peloponnese: 328 (2001) – *Ind. loc*.: Mystras-Ruinenstadt, 6 km von der Stadt Sparti am Ostabhang des Taygetos–Gebirges; auf Kalkfelsunterlage. – Holotype: Nomos Lakonias, Eparchia Lakedemonos, Mystras–Ruinenstadt, 6 km von der Stadt Sparti am Ostabhang des Taygetos-Gebirges; auf Kalkfelsunterlage, 8 May 1983, *Voliotis 2428* (ACA).

Cymbalaria microcalyx (Boiss.) Wettst. subsp. ***heterosepala*** (Cufod.) Speta in Phyton (Horn) 26(1): 50 (1986) ≡ *Linaria microsepala* var. *heterosepala* Cufod. in Rech. fil., Denkschr. Akad. Wiss. Math. -Nat. Kl. (Wien) 105(1): 110 (1943) ≡ *C. microcalyx* var. *heterosepala* (Cufod.) Cufod. in Bot. Not. 2: 151 (1947) – *Ind. loc*.: Kissamos: Insel Grabusa Agria, Kalkfelsritzen. – Holotype: Creta: Distr. [?] Kissamos, in fissuris rupium calc. insulae Grabusa Agria, Kalkfelsritzen, 20 April 1942, *Rechinger 12095* (W!).

Cymbalaria spetae Carnicero, L. Sáez, N. Garcia & Galbany, **sp. nov.** – Holotype (Fig. 8): Greece: Peloponnese, Lakonia, Trypi, road to Kalamata, tunnel excavated in the rock, cracks in shady limestone rocks, 780 m, 08 June 2014, *P. Carnicero 1074* (BC 955798, isotype W).

Etymology: dedicated to Franz Speta, in recognition of his outstanding work on the taxonomy of *Cymbalaria*.

Description: Perennial herb, villous to lanate in all vegetative organs, indumentum composed of eglandular trichomes and glandular trichomes. *Stems* trailing, procumbent or decumbent, up to 100 cm long, villous to lanate. *Leaves* 13.4–42 mm in diameter, opposite to alternate, petiolate, reniform to orbicular, (5)7–11 lobes, villous on both surfaces. *Flowers* zygomorphic, pedicellate, solitary in leaf axils; *pedicels* 15–29(37) mm long in flower, 25–37 mm long in fruit, puberulent to villous, with decreasing density of eglandular trichomes towards the apex and high density of short glandular hairs in the upper part. *Calyx* lobes subequal, 2.3–3.7 mm long in flower, 3.3–

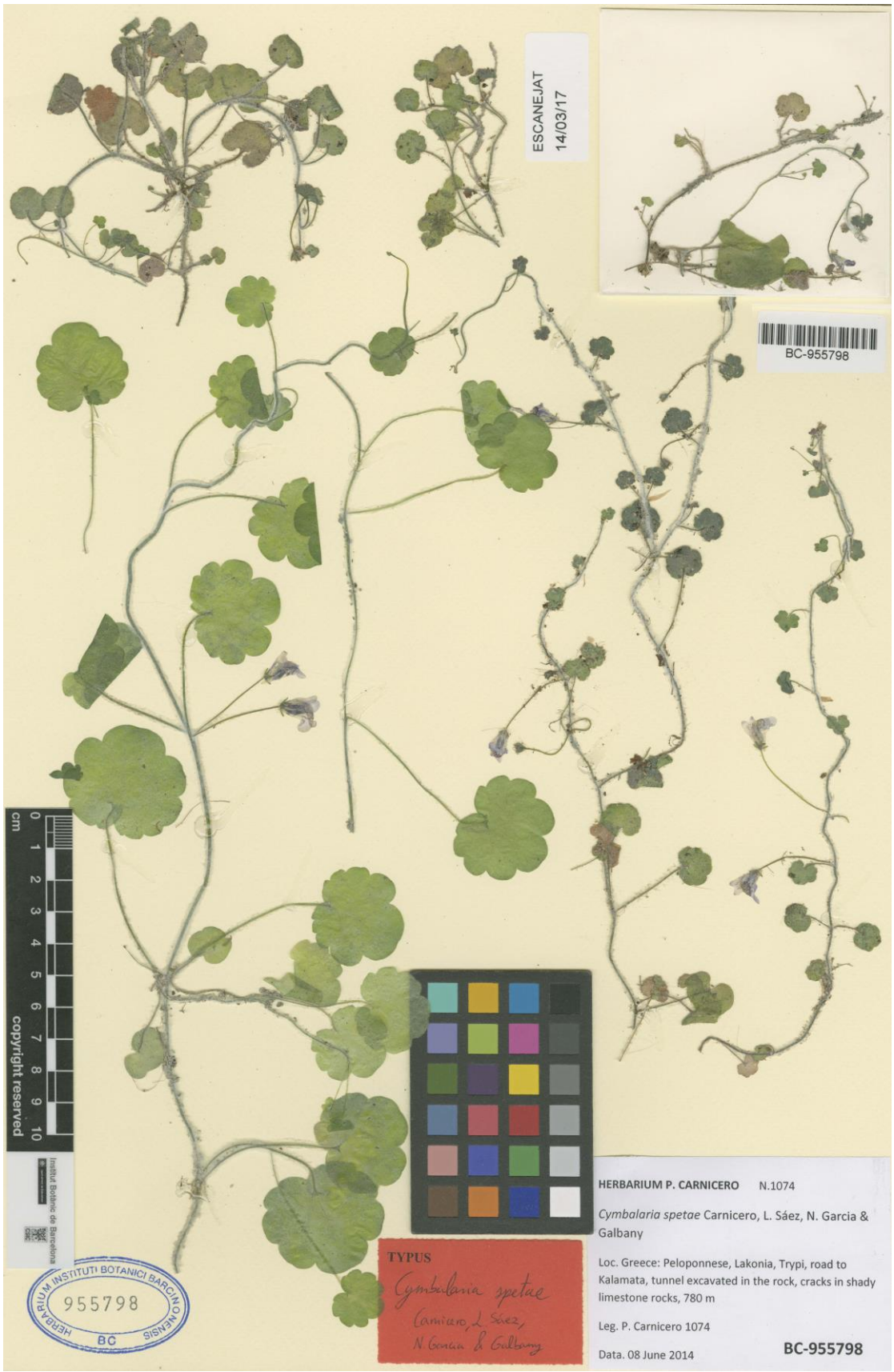

Figure 8. Holotype of *C. spetae*. Greece: Peloponnese, Lakonia, Trypi, road to Kalamata, tunnel excavated in the rock, cracks in shady limestone rocks, 780 m, 08 June 2014, *P. Carnicero 1074* (BC 955798).

3.9 mm long in fruit, lanceolate, villous. *Corolla* 9–14.2 mm long from the palate to the tip of the spur, pink to violet, palate yellow to pale violet and occasionally with purple veins; tube 2.5–4.9 mm wide; upper lip 6.2–9.6 × 5–6.8 mm with purple veins, sinus 1.7–3.1 mm long; lower lip 9.4–12.2(16.8) mm wide, sinus 3–4.7 mm long; spur 1.3–3.2 × 0.9–2.2 mm. *Capsule* 2.2–3.0 × 2.5–3.3 mm, spherical, glandular-pubescent, glandular trichomes up to 0.5 mm long, loculi equal, each loculus dehiscing by irregular valves. *Seeds* 17–25 per capsule, 0.6–0.8 mm long, equal-sized, spherical to ovoid, not constituting a concrescent mass, black; surface shallowly cristate-alveolate; testa cells polygonal with anastomosed anticlinal walls, periclinal walls of cells of the alveolus bearing a median papilla.

Diagnosis: The new species differs from other eastern Mediterranean species (*C. acutiloba*, *C. ebelii*, *C. longipes*, *C. microcalyx* and *C. minor*) in its longer calyx lobes (2.3–3.7 mm long in flower, 3.3–3.9 mm long in fruit), smaller and more numerous seeds per capsule (0.6–0.8 mm long, 17–25 seeds/capsule) and a proportionally shorter and wider spur. It differs from its most similar species *C. glutinosa* in having a longer calyx (*C. spetae*: 2.3–3.7 mm *vs. C. glutinosa ca.* 2 mm, Bigazzi & Raffaelli, 2000: 200) and an generalle bigger corolla (*C. spetae*: 9–14.2 mm long *vs. C. glutinosa*: 6–12(13) mm long, Bigazzi & Raffaelli, 2000: 200).

Chromosome number: it is likely 2*n* = 28, as tetraploidy was inferred from RGS.

Habitat and altitudinal range: Rock crevices of shady limestone cliffs, 200–800 m.

Distribution area: Local endemic to the Taygetos Mounts in the Peloponnese (only two populations known).

Identification key to eastern Mediterranean *Cymbalaria* taxa

1a. Leaves and stems glabrous or only with occasional hairs on young organs 2

2a. Capsule dehiscent. More than 10 seeds per capsule. Seeds 0.9–1.3 mm long, free from each other .....***C. muralis*** subsp. ***muralis***

2b. Capsule indehiscent. 2–6 seeds per capsule. Seeds 3.5–5.6 mm long, forming a concrescent mass in each loculus .....***C. longipes***

1b. Leaves and stems hairy 3

3a. Calyx lobes 2.3–3.7 mm long in flower. 17–25 seeds per capsule. Seeds 0.6–0.8 mm long..... ***C. spetae***

3b. Calyx lobes 0.5–2 mm long in flower. 2–14 seeds per capsule. Seeds 0.8–5.6 mm long.....4

4a. Capsule dehiscing along undulated longitudinal ridges. Seeds cristate with low longitudinal ridges .....***C. ebelii***

4b. Capsule dehiscing differently. Seeds alveolate or cristate with wing-like ridges.....5

5a. 8–14 seeds per capsule. Seeds equal-sized or regularly decreasing in size towards the apex of the capsule, 0.8–3.3 mm long. Seeds alveolate, sometimes the basal seed with more or less pronounced ridges.....***C. minor***

5b. 4–10 seeds per capsule. Seeds of different size, 1.3–4.8 mm long. Seeds irregularly cristate with wing-like ridges. 6

6a. Seed ridges deeply divided in digitate expansions with rounded apex or with numerous triangular tips.....***C. acutiloba***

6b. Seed ridges shallowly divided, segments drawn into a rounded apex .....***C. microcalyx***

Identification key to *C. minor* subspecies

- Seeds alveolate with regularly low ridges. Seeds more or less equal-sized, each seed 0.8–1.3 mm long..... ***C. minor* subsp*. minor***

- Seeds alveolate, the basal seed with pronouced ridges, ridges descreasing towards the apex of the capsule. Size of seeds decreasing towards the apex of the capsule, each seed 0.9–3.3 mm long ..... ***C. minor* subsp*. dodekanesi***

Identification key to *C. acutiloba* subspecies

- Seed expansions drawn out into numerous triangular tips.....

.....***C. acutiloba* subsp*. acutiloba***

- Seed expansions with a rounded apex .....***C. acutiloba* subsp*. paradoxa***

Identification key to *C. microcalyx* subspecies

- Seeds clearly separated from each other, never constituting a concrescent mass.....

.....***C. microcalyx* subsp*. microcalyx***

- At least some seeds hard to separate from each other, constituting a concrescent mass .....***C. microcalyx* subsp*. heterosepala***

Acknowledgements

This research was funded in part by the Spanish government (CGL2010-18631/BOS and the *Flora iberica* project, CGL2011-28613-C03-01) as well as by the Catalan government (2014-SGR 514). We are grateful to the herbaria that provided material or kindly sent scanned images for examination (B, BM, E, G, K, FI, W) and specially to the team of BC. We are also grateful to R. Vilatersana for collecting material from the Aegean Islands. Pau Carnicero benefited from the support of a PIF Ph.D. student fellowship from the Universitat Autònoma de Barcelona. We thank M. Gassner from the Institute of Botany of Innsbruck for performing relative genome size measures. We thank the gardeners team from the Botanical Garden of Barcelona for successfully cultivating accessions of *Cymbalaria*.

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Supplementary Material

Appendix: Specimens examined

Specimens used in morphometric analyses

Cymbalaria acutiloba (Boiss. & Heldr.) Speta ***subsp. acutiloba***, **Turkey;** Ad muros et rupes Alaya [?], April 1845, *Heldreich* (K000806697); ad rupes umbrosis promontorii Alaya Ciliciae, April 1845, *Heldreich* (BM000997821); ad rupes promont. Alaya, April 1845, *Heldreich* (BM000997822); Prov. Mugla, dist. Fethiye: Kalkan, 30, Shady limestone rocks. Perennial, 30 March 1956, *Davis & O. Polulin* (E629366); Prov. Antalya. Alanya promentory, 100 m, Shady limestone rocks. Perennial. Fls. Lavender., 15 April 1956, *Davis 25847 & O. Polunin* (E00629362); ***Cymbalaria longipes*** (Boiss. & Heldr.) A.Chev., **Greece;** Dodecanese, Tilos, among shingle at edge of shore at Livadia, 25 April 1962, *Gathorne-Hardy 125* (E629369); Ep. Sitia: b. der Hafenbucht am Kap Sidero, 10-20 m, Plattenkalk. Felsiger N-Hang, in Menge in schattigen Ritzen, kaum mehr blühend; Fr. in Ritzen hineinwachsend, schwer zu kriegen, May 1962, *Greuter* (B10 0138948); Samos, Shingle just E. of Tigani, 20 April 1964, *Gathorne-Hardy* (E629254); Samos. Shingle just E. of Tigani, 20 April 1964, *Gathorne-Hardy 657* (E629368); Kykladen: Amorgos: Prof. Ilias, NW-Seite, ca. 500-600 m, Poterium-, Euphorbia- Kugelpolster Marmor, 19 May 1988, *W. Burri & F. Krendl* (W2008-0009149); **Turkey;** ad mures et rupes Adalia / ad rupes circa Altaliam Pamphyliae, March 1845, *Heldreich 474* (E00629371); Ad muros et rupes Antalia [?], March 1845, *Heldreich* (W); ad muros et rupes Adalia[?], March 1845, *Heldreich* (BM000997824); Ad muros et rupes Adalia, April 1845, *Heldreich* (E346170); Prov Antalya. Antalya. 5 m. Limestone rocks, 5 April 1956, *Davis 25693 & O. Polunin* (E629370); ***Cymbalaria microcalyx*** (Boiss.) Wettst. subsp. ***microcalyx***, **Greece;** Arcadiae m. Olenos, 5000-6000', ad rupes umbrosas in veg. alpina, 20–24 July 1848, *Heldreich* (B10 0460657); ***Cymbalaria minor*** (Cufod.) Speta subsp. ***minor***, **Greece**; Epirus: In faucibus calc. prope Emin Agha, ca. 35 km a Joannina meridiem versus, 600 m, 12 May 1961, *K.H. Rechinger*, (B10 0460656); Griechenland: Ion. Insel Kefallinia, Aenos, Kalkfelsen beim Megas Soros, 1600 m, 1969, *LJ. Damboldt* (B10 0460655); ***Cymbalaria minor*** (Cufod.) Speta subsp. ***dodekanesii*** (Greuter in Greuter & Rech. fil.) Carnicero, **Greece;** Karpatos. Mte. Kalolimni, 24 May 1883, *W. Barbey 491* (E629283); Amorgos: Leugadha [?], shady rocks, 10 April 1940, *Davis 1385K* (E629290); Khalki, N. facing smallcliffs on top of hill about 2 km. N.E. of town, 03 May 1964, *Gathorne Hardy 748* (E629363); ***Cymbalaria spetae*** Carnicero, L. Sáez, N. Garcia & Galbany, **Greece;** Lakonia, Taigetos mts., pr. Arghia Marina, 37º05'08''N 22º18'59''E, 790 m, roquedo, June–July 2007, *C. Aedo 14341 et al.* (MA760738).

Additional specimens examined

Cymbalaria acutiloba (Boiss. & Heldr.) Speta ***subsp. acutiloba***, **Turkey;** ad rupes Promont. Alaya, April 1845, *Heldreich* (FI-Webb136502); Ad rupes promont. Alaya, April 1845, *Heldreich* (E346184); Ad rupes prom. Alaya, April1845, *Heldreich* (BM000997820); Ad rupes promont. Alaya, April 1845, *Heldreich* (G00356709); Cap Alaya, Cilician occidental/ ad rupes promont. Alaya, April 1845, *Heldreich 565* (W); Karpatos. Mte. Kalolimni, 24 May 1883, *W. Barbey 491* (E629283); Amorgos: Leukadha, shady rocks, 10 April 1940, *Davis 1385K* (E629290); Prov. Antalya. Alanya promentory, 100 m Shady limestone rocks. Perennial. Fls. lavender, 100, Shady limestone rocks. Perennial. Fls. lavender, 11 April 1956, *Davis & O. Polulin* (E629362); Karpathos: cliffs at Mesochorio, 06 May 1963, *Gathorne-Hardy 442* (E629284); Khalki, N. facing smallcliffs on top of hill about 2 km. N.E. of town, 03 May 1964, *Gathorne Hardy 748* (E629363); 13km W Antalya, C3 Antalia, Fels in *Pinus brutia* wald, 820, 27 June 1965, *F. Sorger* (W1991-06258); Türkei, C4 Jael, 15 km NW Siliske, Gölesu [?] Uber bereirh [?] felsiger Hang, 80 m, 10 April 1985, *F. Sorger 85-48-30* (W1991-0008064); ***Cymbalaria ebelii*** (Cufod.) Speta, **Montenegro;** Insel Wranina, Montenegro, Anfangs June 1841 *Ebel* (W); Jugoslavia: Montenegro (Crna Gora): Skadarsko jezero: ad muros vetustos pag. Vranjina (Loc. Class.!)- 10 m, 12 May

1984, *E. 11192 & M. Mayer* (B10 0460658); ***Cymbalaria longipes*** (Boiss. & Heldr.) A.Chev., **Cyprus;** ad littora Ins Cyprus, Billard, *Jacquin Fil* (FI-Webb136268); **Greece;** Ad muros et rupes Atalia, March 1845, *Heldreich* (FI); In fiss. rup. ins Salamis, Graecia, March 1876, *Pichler* (FI); Halki ins., April 1886, *F. Major* (W1991-0006256); Argolis: inter lapides trachyticos ad litora maris prope Vromolimni paeninsulae Methanaeae, cespites inextricabiles efformans., 11 May 1887, *Heldreich* (E629255); Argolis: inter lapides trachyticos ad litora maris prope Vromolimni paeninsulae Methanaeae, cespites inextricabiles efformans., 11 May 1887, *Heldreich 964* (K000806706); Argolis: inter lapides trachyticos ad litora maris prope Vromolimni paeninsulae Methanaeae, cespites inextricabiles efformans., 11 May 1887, *Heldreich 964* (K000806707); Chio, Rhodes, *Aucher-Eloy 1905* (FI-Webb136483); Isola di Rodi, roccie marittima della spiaggia, 16 May 1912, *Vaccari*, (FI); Isola di Rodi, Cattavia, 26 April 1922, *N. Mazzocchi-Alemanni* (FI); Dodecanese, Parmos, beach in Karo Merika bay on the side of island across narrow neck from Scala, in shingle close to sea; forming tangles nets, 05/04 May 1962, *E. Gathorne-Hardy* (W1991-0006257); Tokmakia Isles (N.E. of Lesvos): Apronisos, ion pebble beach, 07 May 1978, *J.R. Edmondson & M.A.S. McClintock* (E629371); Karpathos-Gr., Nomos Dodekanisou, Eparchia Karpathou Saria, Palatia, 5, trockene Kalkstein-FluBgerölle, 08 May 1998, *N. Böhling* (E629368); **Turkey;** ad muros et rupes pr Adalia Pamphyliae, March 1845, *Heldreich* (BM000997823); Ad muros et rupes Adalia, March 1845, *Heldreich*, (G00356711); Ad muros et rupes Adalia, March 1845, *Heldreich* (G00356712); Ad muros et rupes Adalia, March 1845, *Heldreich* (G00356713); Ad muros et rupes Antalia, March 1845, *Heldreich* (K000979892); C3 Antalya, Konya Alti. W Antalia, Felsswand/Stwand [?], 23 May 1963, *F. Sorger 63-26-18* (W1991-0006256); C1 Mugla, NE Knidas, 0-1 m, Stwand [?], 17 April 1984, *F. Sorger 84-5-1* (W1991-0006257); ***Cymbalaria microcalyx*** (Boiss.) Wettst. subsp. ***microcalyx***, Greece; Taygeti ad rupes in reg. infer., April 1842, *Boissier* (FI-Webb136500); Taygeti ad rupes in reg. infer., April 1842, *Boissier* (W1889-0287745); Taygeti ad rupes in reg. Inter., April 1848, *E. Boissier* (K000806698); M. Olenos Arcadiae ad rupes umbrosas, alt 6000', Jul 1848, *Heldreich* (K000806699); in locis umbrosis montis Maleva Laconiae prope Platanos (rarissima!), 3500', 22 April - 4 May 1857, *T. G. Orphanides* (FI-Webb136501); In Locis umbrosis: montis Malevo Laconiae prope Platanos (rarissima), alt 3500', 22 April - 4 May 1857, *E. Boissier* (K000874007); in locis umbrosis, montis Malevo Laconicae prope Platanos (rarissima) Fl. Apr. Majo. alt 3500', 22 April - 4 May 1857, *T. G. Orphanides 712* (W); Felsen au SW Burg o Mistra, 2 April 1891, *Boissier* (W1998-0003784); Peloponnesus: Laconia: Penins. Malea In promont. Supra Neapolis, substr. Calc., in fissuris rupium, 08 July 1958, *K.H. Rechinger 20045* (W1981-0010318); LACONIA: Mistras prope sparti, substr. Calc., 400-500 m, in fissuris rupium umbros., 9-10 May 1964, *K.H. Rechinger 24741* (W1981-0010590); Peloponnisos: Lakonia: ca. 2km S Mistras: Flaumeichenwald 600 m, 2 April 1972, *P. Krendl* (W2003-0011868); Peloponnes, Lakonien, Ep. Epidhavros Limirias: Hügel Alona südlich Elliniko, bei der Kapelle Ajios Sotirios. Nordexponierte Kalkfelswände; 650-700 m, 10 April 1979, *W. Greuter 17056 & H. Merxmüller* (B10 0460657); Peloponnes, Lakonien, Ep. Epidhavros Limirias: Hügel Alona südlich Elliniko, bei der Kapelle Ajios Sotirios, 650-700 m, Nordexponierte Kalkfelswände, 10 April 1979, *W. Greuter & H. Merxmüller* (FI); Peloponnes: Lakonien: Mistras, von der Burg durch die Schlucht zum Ort Mistras, ca. 400-600 m; Schluchtwald, Platanen, Felsen, Kalk, 22 April 1986, *W. Burri & F. Krendl* (W2008-0008683); ***Cymbalaria microcalyx*** (Boiss.) Wettst. subsp. ***heterosepala*** (Cufod.) Speta, **Greece**, **Crete**; Creta: Distr. Kissamos, in fissuris rupium calc. insular Grabusa Agria, 20 April 1942, *K.H. Rechinger 12095* (W0013280).***Cymbalaria minor*** (Cufod.) Speta subsp. ***minor***, **Greece;** Limestone cliffs above lake Pheneus (Arcadia), 6 May 1883, *Lacaita 7725* (BM); Grevès maritimes a Lefkas (Leucade), Summer 1906, *R. Maire & M. Petitmengin 311* (B10 0673677, ISOTYPUS); Flora Graeca, Levkas, Frini, Strand, 22 April 1929, *Th. Just* (W1930-0012462); Peloponnesus: Arcadia, in saxosis calcareis 6km a Vitina septentrionem versus, circa 800 m, 14 June 1958, *K.H. Rechinger 20441* (W1981-0010317); Graecia (Epirus): In faucibus calc.

Prope Emia Agha, ca. 35 km a Joannina meridiem versus, ca. 600 m, 12 May 1961, *K.H. Rechinger 23293* B10 0460656; Griechenland: Ion. Insel Kefallinia, Aenos, Kalkfelsen beim Megas Soros, ca. 1600 m, 1969*, J. Damboldt* (B10 0460655); ***Cymbalaria minor*** (Cufod.) Speta subsp. ***dodekanesi*** (Greuter in Greuter & Rech. fil.) Carnicero, **Greece;** Insula Rhodos (Rodi): In fissuris rupium calc. Montis Hag. Elias prope Archangelos, ca. 400 m, 25 June 1935, *K.H. & F. Rechinger 8405* (W1936-0010263); Karpathos: cliffs at Mesochorio, 06 May 1963, *Gathorne-Hardy 442* (E629284); EPIROS: Inter Paramythia et Glyky. in fissuris rupium calc., 28 May 1964, *K.H. Rechinger 25644* (W1994-0005692); EPIRUS: In fissuris rupium calc. supra Paramythia, 28 May 1964, *K.H. Rechinger 25635* (W1994-0005691); Olimbos, 1km östl. des Ortes, Felsbänder und steinige Hänge am NrdfuB des Profitis Ilias, Kalkgestein, 200 m, 19 April 1982, *Raus & Pleger* (B10 0560659); Kykladen: Amorgos: Prof. Ilias, Nw-Seite, ca.500-600 m, Poterium-, Euphorbia-Kugelpolster, Marmor, 19 May 1988, *W. Burri & F. Krendl* (W2000-0007949); Kykladen: Amorgos: Prof. Ilias, Nw-Seite, ca.500-600 m, Poterium-, Euphorbia-Kugelpolster, Marmor, 19 May 1988, *W. Burri & F. Krendl* (W2008-0009154); Dodekanes: Karpathos: zwischen Volada und Lastos, ca. 600-800 m, Felsen, Blockhalden, Kalk, 7 May 1990, *W. Burri & F. Krendl* (W2000-0007944); Dodekanes: Karpathos: zwischen Volada und Lastos, ca. 600-800 m, Felsen, Blockhalden, Kalk, 7May 1990, *W. Burri & F. Krendl* (W2008-0009151); Dodekanes: Karpathos: N-Seite des Lastos, Niederungen N Lastos, ca.600-700 m, Kulturen, Mauern, Wegränder, Flysch, Kalk, 21 May 1990, *W. Burri & F. Krendl* (W2000-0007943); Dodekanes: Karpathos: N-Seite des Lastos, Niederungen N Lastos, ca. 600-700 m, Kulturen, Mauern, Wegränder, Flysch Kalk, 21 May 1990, *W. Burri & F. Krendl* (W2008-0009150);

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Taxon	Specimen code usedin trees	Type of analysis and number of studied specimens	Locality	Voucher	Accession number			
					ITS	3'ETS	*ndhF*	*rpl32-trnL*
***Cymbalaria* Hill**								
Eastern Mediterranean *Cymbalaria*								
C. acutiloba (Boiss. & Heldr.) Greuter subsp. *acutiloba*			Turkey, Antalya, Alanya	*P. H. Davis 25847 & O. Polunin* (E629362)	KP735212	KP851059	KP851042	KP851126
subsp. *paradoxa* (Greuter) Carnicero, L. Sáez, N. Garcia & Galbany	1, 2	FC^5, M^3	Greece, Kastelorhizo, Megisti	*P. Carnicero 1039* (BC)				
C. ebelii (Cufod.) Cufod.	1		Montenegro, Skadar Lake	*E. Mayer 11192 & M. Mayer* (B10 0460658)	KP735236	KP851061	KP851036	KP851121
	2,3		Crna Gora, Skadarsko jezero, Virpazar	*P. Janaćković s. n.* (BC)				
C. longipes (Boiss. & Heldr.) A.Cheval.	1	M^1	Greece, Karpathos	*N. Böhling 8228* (B10 0138948)	KP735232	KP851064	KP851038	KP851123
	2	M^1	Greece, Samos	*E. Gathorne-Hardy 657* (E629368)	-	-	KP851039	KP851124
	3	M^1	Turkey, Antalia	*S.G. Senol 41796* (BC)				
	4, 5		Greece, Samos, along coast Mykali, among peebles	*Th. Constantinidis 13485 et al.* (ATHU)				

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Taxon	Specimen code usedin trees	Type of analysis and number of studied specimens	Locality	Voucher	Accession number			
					ITS	3'ETS	*ndhF*	*rpl32-trnL*
C. longipes (Boiss. & Heldr.) A.Cheval.	6, 7	M^3	Greece, Kastelorhizo, Megisti	*P. Carnicero 1057* (BC)				
	8, 9	M^1	Greece, Rhodes, Lindos, Cape Soumani	*Del Rey & Vilatersana 2538* (BC)				
C. microcalyx (Boiss.) Wettst. subsp. *microcalyx*	1	M^1	Greece, Peloponnese, Lakonia	*W. Greuter & H. Merxmüller s. n.* (B 10 0460657)	KP735238	KP851063	KP851041	-
	2	M^5	Greece, Peloponnese, Platanos	*Th. Constantinidis 13435 & E. Kalpoutzakis* (ATHU)				
	3, 4	M^2	Greece, Peloponnese, Arkadia, between Agios Panteleimon and Kastanitsa	*Th. Constantinidis 13447 & E. Kalpoutzakis* (ATHU)				
	5, 6	FC^3, M^5	Greece, Peloponnese, Lakonia, Mystras	*P. Carnicero 1063* (BC)				
[subsp. *alba* (Voliotis) Kit Tan]	7, 8	M^5	Greece, Peloponnese, Lakonia, Mystras	*P. Carnicero 1069* (BC)				
subsp. *heterosepala* (Cufod.) Speta	01-may		Greece, Crete, Falasarna	*P. Carnicero 1022* (BC)				

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Taxon	Specimen code usedin trees	Type of analysis and number of studied specimens	Locality	Voucher	Accession number			
					ITS	3'ETS	*ndhF*	*rpl32-trnL*
C. minor (Cufod.) Speta subsp. *minor*	1	M[1]	Greece, Kefallinia, Aenos	*J. Damboldt s. n.* (B10 0460655)	KP735237	KP851060	KP851037	KP851122
	2, 3		Greece, Peloponnese, Achaia, Tsapournia	*P. Carnicero 1080* (BC)				
subsp. *dodekanesi* (Greuter) Carnicero, L. Sáez, N. Garcia & Galbany	1		Greece, Rhodes, Archangelos	*P. H. Davis 40310* (E629364)	KP735208	KP851058	KP851043	KP851127
	2	FC[6]	Greece, Rhodos, between Kolympia i Arcangelos, pr. Tsampika monastery	*P. Carnicero 1061* (BC)				
	3, 4		Greece, Rhodos, Mount Profitis Ilias	*P. Carnicero 1062* (BC)				
	5, 6	M[2]	Karpathos, Cap Castello	*Del Rey & Vilatersana 2531* (BC)				
C. spetae Carnicero, L. Sáez, N. Garcia & Galbany	1	M[1]	Greeece, Peloponnese, Lakonia, Taygetos	*C. Aedo 14341 et al.* (SALA140867)	KP735239	KP851062	KP851040	KP851125
	2, 3	M[3]	Greece, Peloponnese, Kambos	*Th. Constantinidis 13405 & E. Kalpoutzakis* (ATHU)				
	04-7	FC[2], M[5]	Greece, Peloponnese, Lakonia, Trypi	*P. Carnicero 1074* (BC955798)				

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					ITS	3'ETS	*ndhF*	*rpl32-trnL*
Other *Cymbalaria*								
C. aequitriloba (Viv.) A. Chev.	1		France, Corsica, La Castagniccia	*A. Curcó* (BCN86695)	KP735225	KP851084	KP851014	KP851100
	2		France, Corsica, La Castagniccia	*A. Hilpold s. n.* (BOZ8888)	KP735224	KP851085	KP851011	KP851097
	3		Spain, Balearic Islands, Majorca, Puig Major	*X. Rotllan* (no voucher)	KP735219	KP851088	KP851007	KP851093
	4		Spain, Balearic Islands, Majorca, Formentor	*L. Sáez 7366 & X. Rotllan* (BC879621)	KP735240	KP851086	KP851009	KP851095
	5		Italy, Sardinia, Fonni, Gennargentu	*S. Castroviejo 17018 et al.* (SALA142359)	KP735221	KP851083	KP851016	KP851101
	6		Italy, Sardinia, Nuoro, Badde Salighes	*C. Aedo 9213* (MA708824)	KP735220	KP851087	KP851026	KP851111
	7		Italy, Sardinia, Cuglieri, Mte. Ferru	*C. Navarro 4683 et al.* (MA708259)	-	-	KP851006	KP851092
C. fragilis (J. J. Rodr.) A. Chev.	1		Spain, Balearic Islands, Minorca, Barranc d'Algendar	*P. Carnicero 346 & M. Galbany-Casals* (BC879636)	KP735211	KP851081	KP851004, KP851005	KP851090, KP851091
	2		Spain, Balearic Islands, Cabrera	*L. Sáez 6196 & L. Guàrdia Valle* (BC879620)	KP735241	KP851082	KP851008	KP851094

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					ITS	3'ETS	*ndhF*	*rpl32-trnL*
C. glutinosa Bigazzi & Raffaelli subsp. *glutinosa*	1, 2		Italy, Spigno Saturnia	*P. Carnicero 734 & M. Galbany-Casals* (BC879627)	KP735216, KP735217	KP851068, KP851069	KP851029, KP851030	KP851114, KP851115
subsp. *brevicalcarata* Bigazzi & Raffaelli			Italy, Ravello	*P. Carnicero 748 & M. Galbany-Casals* (BC879626)	KP735218	KP851070	KP851020	KP851105
C. hepaticifolia Wettst.	1		France, Corsica, Lac du Nino	*A. Hilpold s. n.* (BOZ8842)	KP735223	KP851079	KP851022	KP851107
	2		France, Corsica, Castagniccia	*P. Carnicero 444 & M. Galbany-Casals* (BC879631)	KP735215	KP851078	KP851013	KP851099
C. muelleri (Moris.) A. Chev.	1		Italy, Sardinia, Seui, Genni d'Acca	*P. Carnicero 406 & M. Galbany-Casals* (BC879629)	KP735210	KP851080	KP851012	KP851098
	2		Italy, Sardinia, Ulassai	*P. Carnicero 389 & M. Galbany-Casals* (BC879630)	KP735209	KP866214	KP851010	KP851096
C. muralis G. Gaertn., B. Mey. & Scherb. subsp. *muralis*	1		Spain, Catalonia, Sant Cugat (naturalized)	*P. Carnicero 134* (no voucher)	KP735230	KP851077	KP851015	KP851089
	2		Spain, Catalonia, Caldes de Montbui (naturalized)	*P. Carnicero 135* (BC879623)	KP735231	KP851076	KP851017	KP851102

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					ITS	3'ETS	*ndhF*	*rpl32-trnL*
C. muralis G. Gaertn., B. Mey. & Scherb. subsp. *muralis*	3		Poland, Slask Dolny (naturalized)	*Z. Pulawska s. n.* (FI)	-	-	KP851018	KP851103
	4		Italy, Toscana, Albegna	*F. Selvi s. n.* (FI)	-	-	KP851019	KP851104
subsp. *visianii* (Jáv.) D. A. Webb	1		Italy, Lazio, Palombara	*P. Carnicero 703 & M. Galbany-Casals* (BC879625)	KP735226	KP851075	KP851027, KP851028	KP851112; KP851113
	2		Italy, Lazio Rocca di Papa	*P. Carnicero 710 & M. Galbany-Casals* (BC879624)	KP735226	KP851074	KP851031, KP851032	KP851116, KP851117
C. pallida Wettst.	1		Italy, Abruzzo, l'Aquila	*J. Aldasoro 3276* (MA698766)	KP735233	KP851073	KP851035	KP851120
	2, 3		Italy, Abruzzo, Valle d'Orfenta	*P. Carnicero 780 & M. Galbany-Casals* (BC879628)	KP735234; KP735235	KP851072, KP851071	KP851033, KP851034	KP851118, KP851119
C. pubescens (C. Presl) Cufod.	1		Italy, Sicily, Palermo, La pizzuta	*C. Aedo 5733 et al.* (MA646152)	KP735229	KP851066	KP851021	KP851106
	2		Italy, Sicily, Trapani, Erice	*J. Güemes 3085 et al.* (SALA106642)	KP735214	KP851065	KP851024	KP851108
	3		Italy, Sicily, Trapani, Mt. Acci	*C. Aedo 5614 et al.* (MA646631)	KP735228	KP851067	KP851025	KP851110

Table S1 List of specimens used in molecular analyses with information on the specimen codes used in the text and figures, locality, herbarium voucher and accession numbers. The number of specimens used in morphological (M) and genome size (FC) analyses from each locality are indicated with a superscript.

Taxon	Specimen code usedin trees	Type of analysis and number of studied specimens	Locality	Voucher	Accession number			
					ITS	3'ETS	*ndhF*	*rpl32-trnL*
Other Antirrhineae								
Asarina procumbens Mill.			Spain, Catalonia, Montseny massif	*P. Carnicero 253 & L. Sáez* (BC879635)	KP735207	KP851057	KP851045	KP851129

Table S2 Characteristics of sequences and results of phylogenetic analyses

Parameter	**cpDNA**	**nrDNA**
Number of sequences	66	66
Length of sequences (bp)	1967-2731	452-1040
Total number of characters	2731	1048
Number of indels coded	25	16
Maximum Parsimony (MP) informative characters	123	161
Number of MP trees	96195	8631
Number of steps	206	271
Consistency Index (CI)	0.62	0.68
Homoplasy Index (HI)	0.38	0.33
Retention Index (RI)	0.85	0.95
Sequence evolution model for Bayesian analyses (BIC criteria)	GTR + G	SYM + G

General discussion

Our studies have shown various processes that originated the current taxa of *Cymbalaria* Hill and their distributions. Our results allowed us to make some taxonomic contributions that approximate the classification of *Cymbalaria* to the concept of integrative taxonomy (Dayrat, 2005; Will et al., 2005). *Cymbalaria* originated recently, approximately with the onset of the Mediterranean climate suggesting that *Cymbalaria* is well adapted to Mediterranean climatic conditions. In contrast with its sister genus *Asarina* Mill. and *Gadoria* Güemes & Mota, with a single relict species with a restricted distribution area in the western Mediterranean area, *Cymbalaria* diversified and conquered a wide area in the Mediterranean Basin.

Biogeography

The fragmented distribution of *Cymbalaria* taxa allowed us to study the impact of marine barriers in their current distribution. Most Mediterranean islands are of continental origin, and land connections between them, and between islands and continental areas during Pleistocene eustatic changes are well known (Thompson, 2005). However, the Mediterranean has ancient deep basins too that kept some areas permanently isolated since their separation. This results in the presence of two main models: species or groups of species occurring in areas that were connected or very close to each other during low sea level periods, and others that occur in areas where eustatic changes did not significantly reduce the marine distance between land patches.

The western Mediterranean species group (*C. aequitriloba* (Viv.) A.Chev., *C. fragilis* (J.J.Rodr.) A.Chev., *C. hepaticifolia* Wettst. and *C. muelleri* (Moris) A.Chev.) originated *ca.* 15 million years later that the last connection between the two main areas where they occur, the Balearic Islands and Corsica-Sardinia (BCS, Chapter I). At least two long distance dispersal events (LDD) should be invoked to explain 1) the origin of the eastern Balearic Islands endemic *C. fragilis* and 2) the distribution area of *C. aequitriloba* in both archipelagos (Chapter II). We found reduced gene diversity in the Balearic Islands, thus supporting the bottleneck expected after LDD. Our study strengthens the hypothesis of LDD as an important process in explaining species

distributions (de Queiroz, 2005; Nathan, 2006; Roquet et al., 2013). Moreover, LDD is not restricted to organisms specially adapted to travel long distances, but there are several examples in which no apparent adaptations exist (e.g. Escudero et al., 2010; Santos-Gally et al., 2011; Piñeiro et al., 2012), as seem to be the case in most *Cymbalaria* species. The change from a vicariance based paradigm to present situation is not new, however, and recent documented examples of LDD events confirm C. Darwin, A. R. Wallace and other former biogeographers' hypothesis, skeptical with the increasing prevalence of vicariance in that moment (Darwin, 1859; Wallace, 1880).

Despite the LDD events inferred, the marine distance separating Corsica and Sardinia from the Balearic Islands is revealed as an effective barrier to gene flow since it allowed *C. fragilis* to become a separate taxon (Chapter II). Whether it is effective for *C. aequitriloba* remains to be seen, since in this case we are in front of a relatively recent colonization that would putatively end up in the differentiation of Balearic populations in the future. The monophyly of the Balearic populations of *C. aequitriloba* in the AFLPs analyses and their low genetic diversity support the effectiveness of the barrier (Chapter II). In contrast, Rechinger's line, described as a major phytogeographic barrier in the eastern Aegean area (Strid & Tan, 1997), seems to have had little effectiveness in preventing gene flow in *C. minor* (Cufod.) Speta subsp. *dodekanesi* (Greuter) Carnicero, L.Sáez, N.Garcia & Galbany and *C. longipes* (Boiss. & Heldr.) A.Chev. (Chapter IV). The two species occur at both sides of the barrier, which exists since *ca.* 5 Ma., earlier than the origin of both taxa. Although *C. minor* subsp. *dodekanesi* shows no apparent adaptations for dispersal, Cufodontis (1947) described that the indehiscent capsules of *C. longipes* were probably adapted to marine dispersal. An enhanced hability for marine dispersal could be the reason for the wide, disjunct distribution of *C. longipes* in the coasts of the eastern Mediterranean region, from the southern Balkans to Lebanon.

However, we show how reduced distance or land bridges between islands have presumably facilitated the migration of species in several cases. These connections could explain the occurrence of *C. aequitriloba* and *C. fragilis* in the eastern Balearic Islands, which were a single island during Glaciation periods, but separated from western Balearic Islands, where these species are not present. Similarly, land bridges

could have facilitated plant migration between Corsica and Sardinia, and thus allowed relatively high genetic diversity values in both islands (Chapter II). Reduced distances, added to a climatic barrier that limited the floristic exchange between northern and southern Balkan peninsula (Polunin, 1980), are also invoked to explain the closer phylogenetic affinity of *C. ebelii* (Cufod.) Speta to plants from the Apennine Peninsula rather than to the eastern Mediterranean species. Finally, sea transgressions in the Pleistocene seem to have played an important role in the distribution of Aegean taxa, especially for the colonization of Crete from the Peloponnese by *C. microcalyx* (Boiss.) Wettst. and for the colonization of Aegean Islands through the Cyclades by *C. minor* (Chapter IV). However, as above mentioned, since some taxa in the eastern and western Mediterranean managed to disperse across marine barriers, it remains thus uncertain whether marine transgressions have been or not essential for the current distribution of species.

These results support the coexistence of vicariance and dispersal hypothesis. The abovementioned land bridges could have allowed ancestors with widespread distributions that originated their descendants after the appearance of a marine barrier. Although vicariance was not specifically inferred for any supported clade (Chapter I) it is congruent with further data obtained for 1) the origin of *C. hepaticifolia* and *C. muelleri* respectively from Corsica and Sardinia (Chapter II) and 2) the split between the two subspecies of *C. minor* in the eastern Mediterranean (Chapter IV). Therefore, it should not be interpreted that vicariance hypothesis is dead, as we demonstrate that when species coexist in time with new land connections, these usually facilitate their dispersal. However, our results reinforce the need for testing these hypotheses in a temporal frame (Nathan, 2006; Salvo et al., 2010), since increasing evidence shows that the lack of land connections does not exclude the possibility of plant dispersal (Alsos et al., 2007; Ogutcen & Vamosi, 2016).

Speciation

Allopatric speciation occurs at very different scales in *Cymbalaria*. The origin of *C. fragilis* after founder-event speciation is well explained by the big distance from its sister species at the moment of speciation (Chapter II). However, for the closely related *C. muelleri*, no apparent reproductive barrier and just *ca.* 15 km distance between the two distribution areas allowed for the origin of two subspecies (Chapter III). The latter is a clear case of anacladogenetic speciation, thus resulting in one paraphyletic subspecies (Stuessy et al., 1990; Mayr & Bock, 2002). In fact, paths taken by evolution often result in non-dichotomous patterns (Hörandl, 2006), and if the classification process should reflect the evolution, monophyly cannot be a *sine qua non* condition.

Sympatric speciation, although inferred by biogeographic analyses, needs a critical discussion since the geographical scale used in each study can have great influence in its inference (Schliewen et al., 2006; Papadopulos et al., 2014). In the previous example, the origin of the two subspecies of *C. muelleri* could have easily been identified as sympatric speciation, but on the field one can observe that the distribution of the two taxa never overlaps (Chapter III). In *Cymbalaria*, as well as in most chamophytes, populations can easily be very isolated from each other due to the discontinuity of their habitat (Thompson, 2005). Thus, additional data and a profound knowledge on the species biology are needed to certainly attribute sympatric speciation to an event. Biogeographic analyses pointed to sympatric ecological speciation for *C. muralis* and *C. pallida* Wettst., and their divergent ecological specialization supports it, but fine scale allopatric speciation could not be ruled out (Chapter I).

Finally, the role of polyploid speciation, which has been shown to play a major role in Angiosperm evolution (Soltis et al., 2009; Wood et al., 2009; Fawcett & Peer, 2010), seems to have been crucial in the evolution of *Cymbalaria*. We detected at least two genome duplications preceding a clade, i.e. the clade composed by the western species, and the clade composed by the two central Mediterranean polyploids *C. ebelii* and *C. pubescens* (J. Presl & C. Presl) Cufod (Chapter I). In both cases, polyploids occur far from the distribution area of their sister diploids, thus supporting the idea of

enhanced success of polyploids in colonization of new areas (Linder & Barker, 2014). A third clade of putative polyploid origin is constituted by the tetraploids *C. microcalyx* and the new species *C. spetae* Carnicero, L.Sáez, N.Garcia & Galbany, but the origin of the remaining tetraploids from the eastern Mediterranean remains unclear (Chapter IV). It remains unknown too whether the polyploids are allo- or autopolyploids, which would need further studies. Current evidence found in the western Mediterranean suggests that hybridization –and thus allopolyploidy– is possible. In two *C. aequitriloba* populations, we detected a strongly divergent haplotype from remaining western species, but closely related to *C. muralis* haplotypes form a close area (Chapter II). Moreover, we also observed morphologically intermediate specimens between *C. muralis* and *C. pallida* in the field that deserve further studies. Thus, hybridization should also be considered as a force originating new taxa, combined or not with genome duplication (e.g. Soltis & Soltis, 2009; Pavarese et al., 2013; Meeus et al., 2015).

Taxonomy

We have taken taxonomy as an integrative discipline, the last step of a systematic study, and pretend that the proposed taxonomic treatment reflects the evolution of the genus (Stuessy et al., 2014). Thus, we always tried to assign the species level to groups of populations with a common origin, the same ploidy level and that are morphologically diagnosable. When morphological differences between two putative taxa are more subtle, they are closely related in the phylogeny and their geographic distributions do not overlap, we proposed the subspecies level (Stuessy, 2009). As stated in previous section, we did not consider monophyly a requisite for recognizing a taxon since some evolutionary processes can result in non-monophyletic entities (Hörandl & Stuessy, 2010). In fact, we described the new subspecies *C. muelleri* subsp. *villosa* Carnicero (Chapter III) and recognized *C. minor* and *C. longipes* (Chapter IV), the three of them paraphyletic taxa according to our data. After all, the classification process has a necessarily subjective component, since we are trying to apply discrete units to the dynamic phenomenon of evolution, and current subspecies could be

Darwin's "incipient species", as well as just forms that will disappear in the future (Darwin, 1859).

Although always subject to a certain degree of subjectivity, it is important to keep congruent species and subspecies delimitation criteria within a taxonomic group. Until present, however, the most followed taxonomic treatment for the genus (Sutton, 1988) presented a rather analytic criterion for western and central Mediterranean taxa, while the eastern Mediterranean *C. microcalyx* was presented as a morphologically variable taxon with a wide distribution area, in which many subspecies were recognized. In contrast, our molecular and morphological analyses supported to split of *C. microcalyx* in four species. Our results mostly coincide with the treatment proposed by Speta (1986, 1989). Sutton (1988) probably adopted a less analytic treatment due to a lack of in-depth study, as he highlighted the need for further studies in several occasions for some taxa considered under *C. microcalyx*. For western and central Mediterranean species, Sutton's (1988) treatment has been more often followed, although we choose to recognize *C. fragilis* as a separate species from *C. aequitriloba*, due to clear morphological and phylogenetic differentiation (Chapter II).

Molecular tools in combination with morphology allowed us to determine diagnostic reliable characters. Our study on seeds resulted in contradictory results and prevented us from trying to find unique valid characters for the whole genus. On the one hand, seed ornamentation characters correspond to genetic lineages in eastern taxa and in the subspecies of *C. muelleri* (Chapters III, IV). On the other hand, we have shown how these characters are variable within *C. fragilis* and, instead, characters traditionally considered of low taxonomic value, such flower size and color, and leaf thickness, could successfully be used to identify the species (Chapter II).

The combination of molecular and morphological tools allowed us to describe a new subspecies under *C. muelleri* and a new species from the eastern Mediterranean region: *C. spetae* (Chapters III, IV). In both cases, the detailed study of herbarium specimens and field observations inspired the hypotheses that were further tested, the appropriate process following the integrative taxonomy concept (Schlick-Steiner et al., 2010). That reinforces the importance of accumulating a wide knowledge of the species included in a systematic study.

Concluding remarks

Cymbalaria encompasses several features that made it an attractive candidate for systematic studies. Our studies suggested a remarkable diversity of processes and responses to paleogeographic events in shaping its evolutionary history. *Cymbalaria* contributes to refute previously well-established paradigms, such as the main role of vicariance in biogeography and the strong diagnostic power attributed to seed ornamentation in the Antirrhineae. However, our results in any case suggest these rules are never working: they do work for some *Cymbalaria* species and for several other published studies (e.g. vicariance: Mansion et al., 2008; Salvo et al., 2010; Crowl et al., 2015; seeds as diagnostic character: Elisens, 1985; Vigalondo et al., 2015). Instead, our studies in *Cymbalaria* prevent us from assuming hypotheses that fit well with current distribution of species without specifically testing them. This remarks the importance of using all available tools to study every life form on Earth, keeping in mind that systematic studies can result in discoveries "typically of the kind that would not be made otherwise" (Wilson, 1968: 1113).

Our studies meant a significant increase in the knowledge of the genus *Cymbalaria*, and a modest contribution to plant systematics and evolution in the Mediterranean region. However, they also revealed some unknowns that should be tackled in the future. Among those, *Cymbalaria* provides an interesting but complex model for studying recent polyploid speciation. Furthermore, systematic studies should still be undertaken to provide satisfactory taxonomic classification for the whole genus. Special attention should be payed to central Mediterranean specie, where some uncertainties arose in the course of our field work; and on the eastern Mediterranean, where a study with comprehensive population sampling would be helpful to confirm our phylogeographic hypotheses and the taxonomic treatment adopted.

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Conclusions

Conclusions

- *Cymbalaria* Hill is monophyletic as confirmed with cp- and nrDNA sequences.
- *Cymbalaria* originated *ca.* 4 Ma and rapidly diverged in three lineages according to nrDNA phylogeny, supporting the role of Mediterranean climate establishment as a trigger for plant diversification.
- The three main lineages found in *Cymbalaria* were: 1) the western lineage, constituted by hexa- to octoploids from the western Mediterranean; 2) the central – eastern lineage, constituted by all central Mediterranean species (all diploids) and part of the eastern taxa (diploids and tetraploids); and 3) the eastern lineage, constituted by two tetraploid species from the eastern Mediterranean.
- Biogeographic analyses estimated the eastern Mediterranean as the area of origin of *Cymbalaria*, although ploidy data suggest the central Mediterranean as a more plausible area.
- Allopatric, sympatric and polyploid speciation types are suggested for the genus *Cymbalaria*:
    - Allopatric, founder-event speciation was inferred for the origin of *C. acutiloba* (Boiss. & Heldr.) Speta, *C. fragilis* (J.J.Rodr.) A.Chev., and *C. pubescens* (J. Presl & C. Presl) Cufod. Vicariance speciation was not inferred by our analyses, but is congruent with available data for the origin of *C. hepaticifolia* Wettst. and *C. muelleri* (Moris) A.Chev., and the split of *C. minor* (Cufod.) Speta in two subspecies.
    - Sympatric speciation was inferred for six cases, although most of them could also be caused by allopatric speciation at a local scale. Sympatric ecological speciation could have caused the differentiation of *C. muralis* G. Gaertn., B. Mey. & Scherb. and *C. pallida* Wettst.
    - Polyploid speciation had a main role in the diversification of *Cymbalaria* originating at least three clades: the western lineage, the clade *C.*

pubescens – *C. ebelii* (Cufod.) Speta and the eastern lineage. In the first two cases, polyploidy was linked to colonization of new areas.

- The western lineage had a single common origin in Corsica – Sardinia and colonized the eastern Balearic Islands later via at least two LDD events: 1) a first event originating *C. fragilis* and 2) a second one resulting in the expansion of the distribution of *C. aequitriloba* (Viv.) A.Chev. to the west.
- All western Mediterranean species were resolved as monophyletic according with AFLP data. *Cymbalaria aequitriloba* is the sister species of *C. fragilis* and *C. hepaticifolia* is sister of *C. muelleri*.
- *Cymbalaria aequitriloba* originated in Sardinia and dispersed later to Corsica and the Balearic Islands.
- Emerged land bridges among eastern Balearic Islands during Pleistocene glaciations probably facilitated dispersal and explain the present distribution of *C. aequitriloba* in Mallorca and Menorca and *C. fragilis* in Cabrera and Menorca.
- In the light of molecular and morphological data, *C. fragilis* and *C. aequitriloba* are re-circumscribed:
    - Testa sculpturing is variable within *C. fragilis* and caused the erroneous identifications. Alternative vegetative (leaf and stems thickness and hairiness) and floral (corolla size and color) diagnostic characters are proposed.
    - *C. fragilis* should be considered at the rank of species and also include specimens with shallowly alveolate seeds, previously identified as *C. aequitriloba*.
    - The threat category of *C. fragilis* should change from critically endangered (CR) to near threatened (NT) as a consequence of the new circumscription.
- Incongruence between AFLP and sequence data support hybridization events where species occurs geographically close to each other.

- The Sardinian endemic *C. muelleri* is split in two subspecies congruent with molecular (AFLPs), morphological and geographical data: *Cymbalaria muelleri* subsp. *muelleri* and *C. muelleri* subsp. *villosa* Carnicero.
- *Cymbalaria muelleri* subsp. *muelleri* originated via anacladogenetic (sub)speciation, thus making subsp. *villosa* paraphyletic.
- Testa sculpturing and trichome length and density were the most valuable diagnostic characters for differentiation of *C. muelleri* subspecies.
- The threat category of *C. muelleri* subsp. *muelleri* is vulnerable (VU) and *C. muelleri* subsp. *villosa* is near threatened (NT).
- The eastern Mediterranean species *C. microcalyx* (Boiss.) Wettst.should be divided in four species according to morphology and molecular data: *Cymbalaria acutiloba*, *C. ebelii*, *C. microcalyx* and *C. minor*.
- Seed characters have high diagnostic value for identifying eastern Mediterranean taxa.
- The new species *C. spetae* Carnicero, L.Sáez, N.Garcia & Galbany from the Peloponnese is described based on divergent mophological characters and cp- and nrDNA genealogies. Diagnostic characters are the calyx length, and the size of corolla and seeds.
- Pleistocene marine transgressions favored the colonization of islands from the west to the east by *C. microcalyx* and *C. minor*. Subsequent regressions caused the differentiation of a continental and an islander subspecies in both cases. On the basis of molecular, morphological and geographic data, *C. microcalyx* subsp. *dodekanesi* Greuter is combined as a subspecies of *C. minor*.
- *Cymbalaria longipes* (Boiss. & Heldr.) A.Chev.and *C. acutiloba* constituted a clade of anacladogenetic origin, although ploidy level data contradict the molecular hypothesis.
- According to molecular, morphological and geographical data, *C. microcalyx* subsp. *paradoxa* Greuter is combined as a subspecies of *C. acutiloba.*

- Dispersal across the Rechinger line, a major phytogeographic barrier in the eastern Mediterranean, is inferred for two taxa, *C. longipes* and *C. minor* subsp. *dodekanesi* (Greuter) Carnicero, L.Sáez, N.Garcia & Galbany.