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

Running head: Phylogeny and biogeographic history of *Cymbalaria*

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

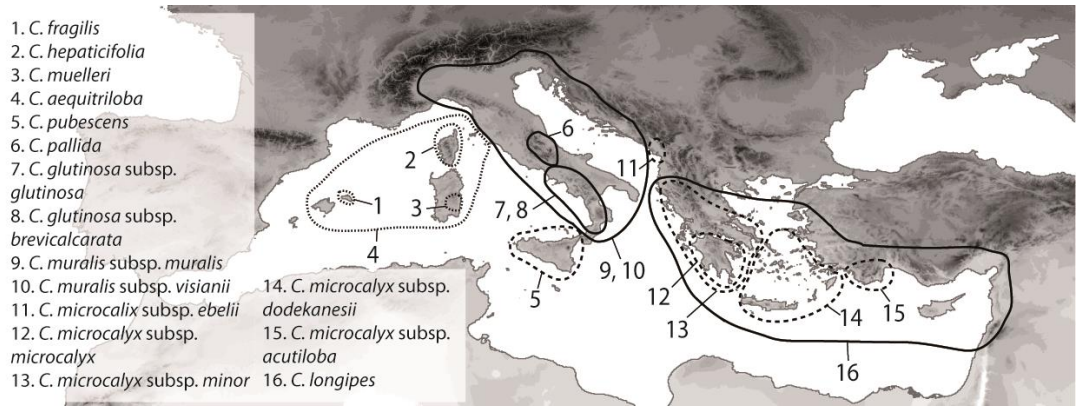


Figure 1. Distribution of *Cymbalaria* taxa, based on Sutton (1988), local Floras, personal field observations and herbarium vouchers. When information on the distribution areas of subspecies was not accurate or overlap was considerable, the general distribution area for the species is shown. For *C. 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).

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 (2*n* = 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

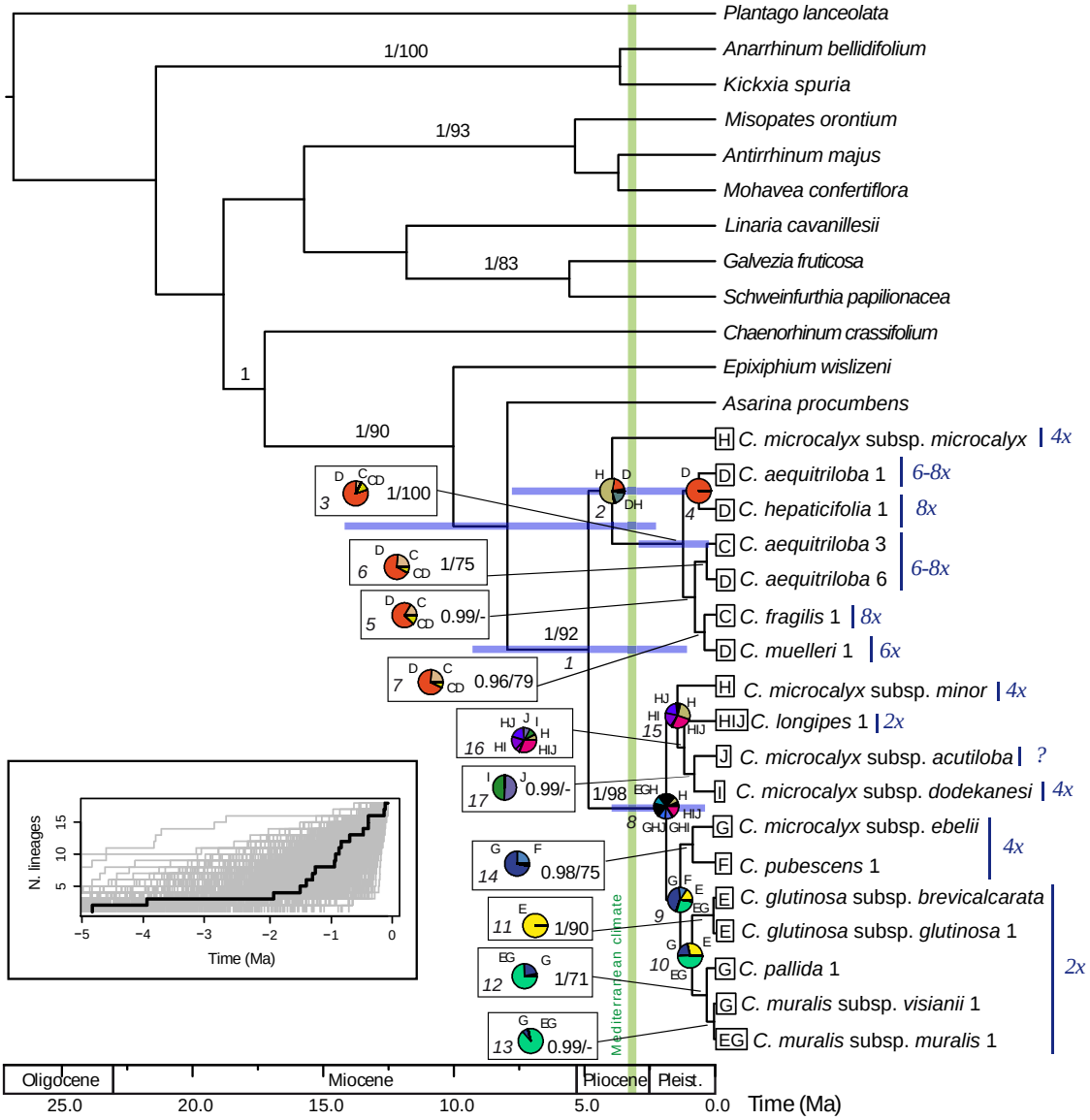
Mediterranean areas without an intermediate station in the central Mediterranean areas (Fig. 2; C, D, E). We considered the distribution area of *Asarina* (Fig. 2; I) isolated enough from the rest to set a rate of dispersal of 0.5 between it and all other areas. We ran the six models and after testing them with a likelihood ratio test and the Akaike Information Criterion (AIC), the DEC+J model was selected.

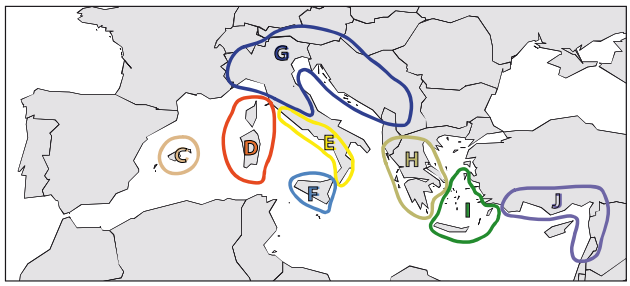
Results

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 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: Balearic Islands
D: Corsica and Sardinia
E: southern Italian Peninsula
F: Sicily
G: northern Italian and Balkan Peninsulas
H: Greek Peninsula
I: Aegean Islands
J: Anatolia, Lebanon and Syria shores

⬅ **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.

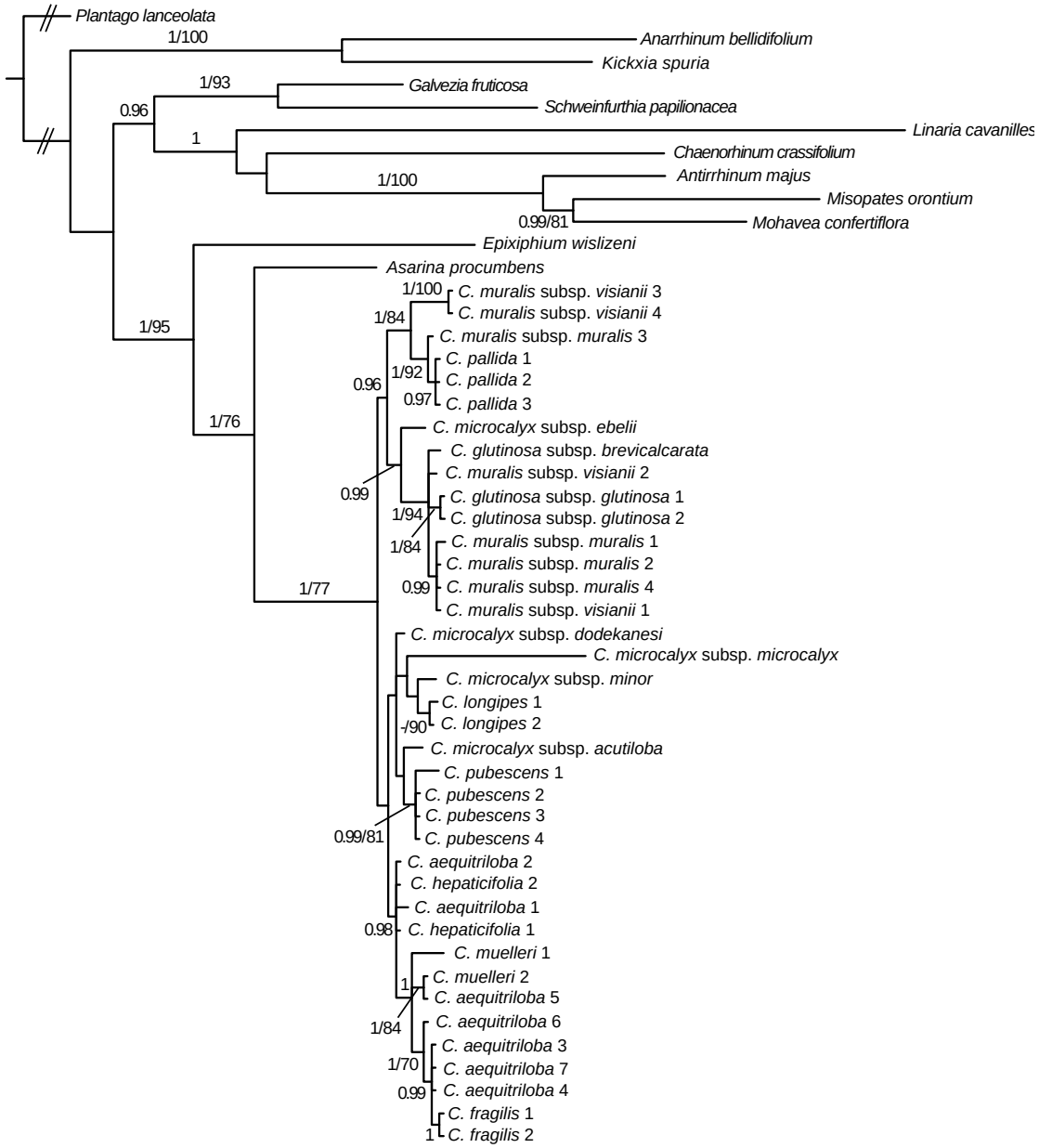


Figure 3. Phylogram from the Bayesian analysis of cpDNA of *Cymbalaria*. Bayesian posterior probabilities ≥ 0.95/bootstrap support values ≥ 70% are indicated. The double slashes at the base of the tree indicate that respective branches have been manually shortened.

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 (node 16, 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*** (Ják.) 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.

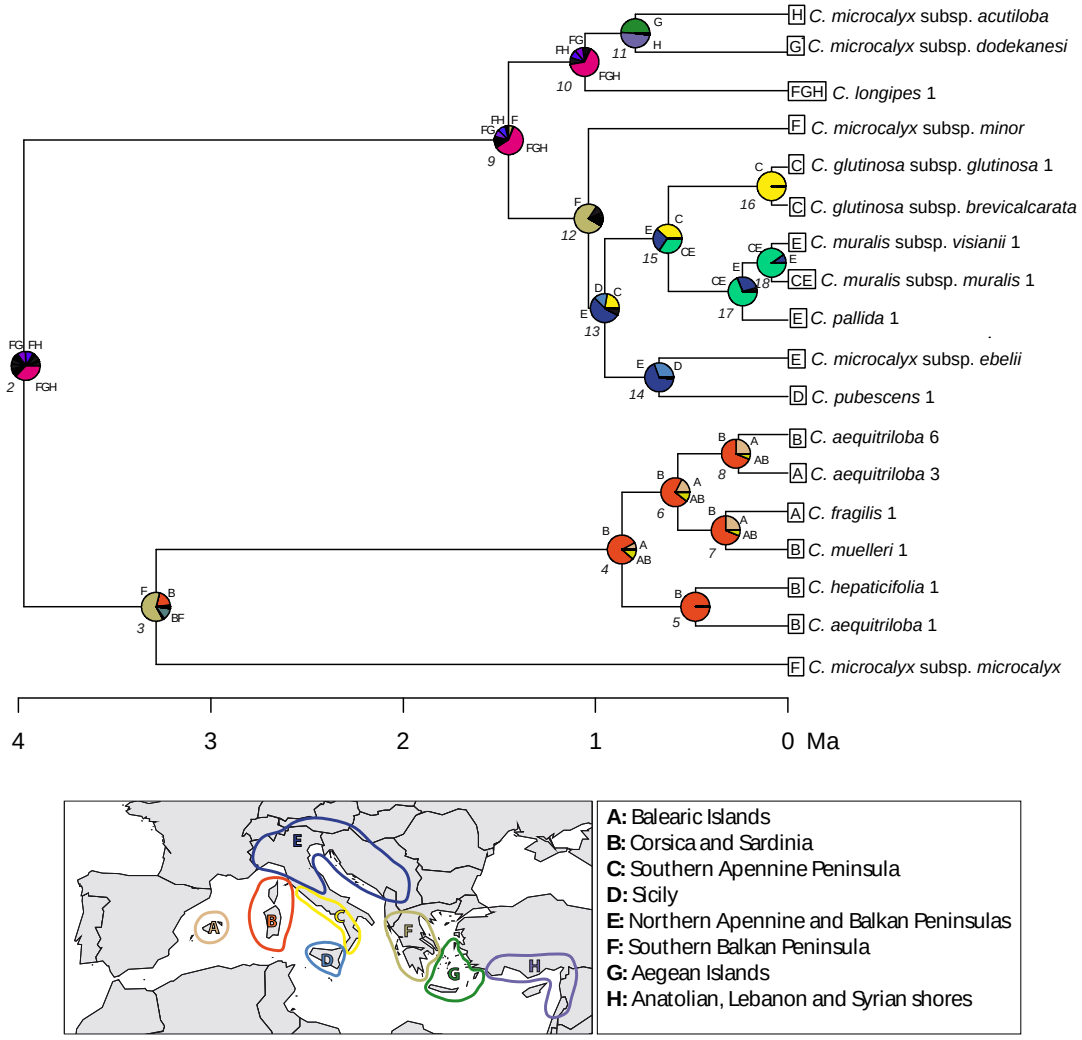

Figure S2. Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA of *Cymbalaria* in BEAST v1.8.2. Pie charts at each node show the marginal probabilities of alternative ancestral ranges obtained from the BioGeoBEARS analysis excluding *Asarina*. Letter codes for 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.

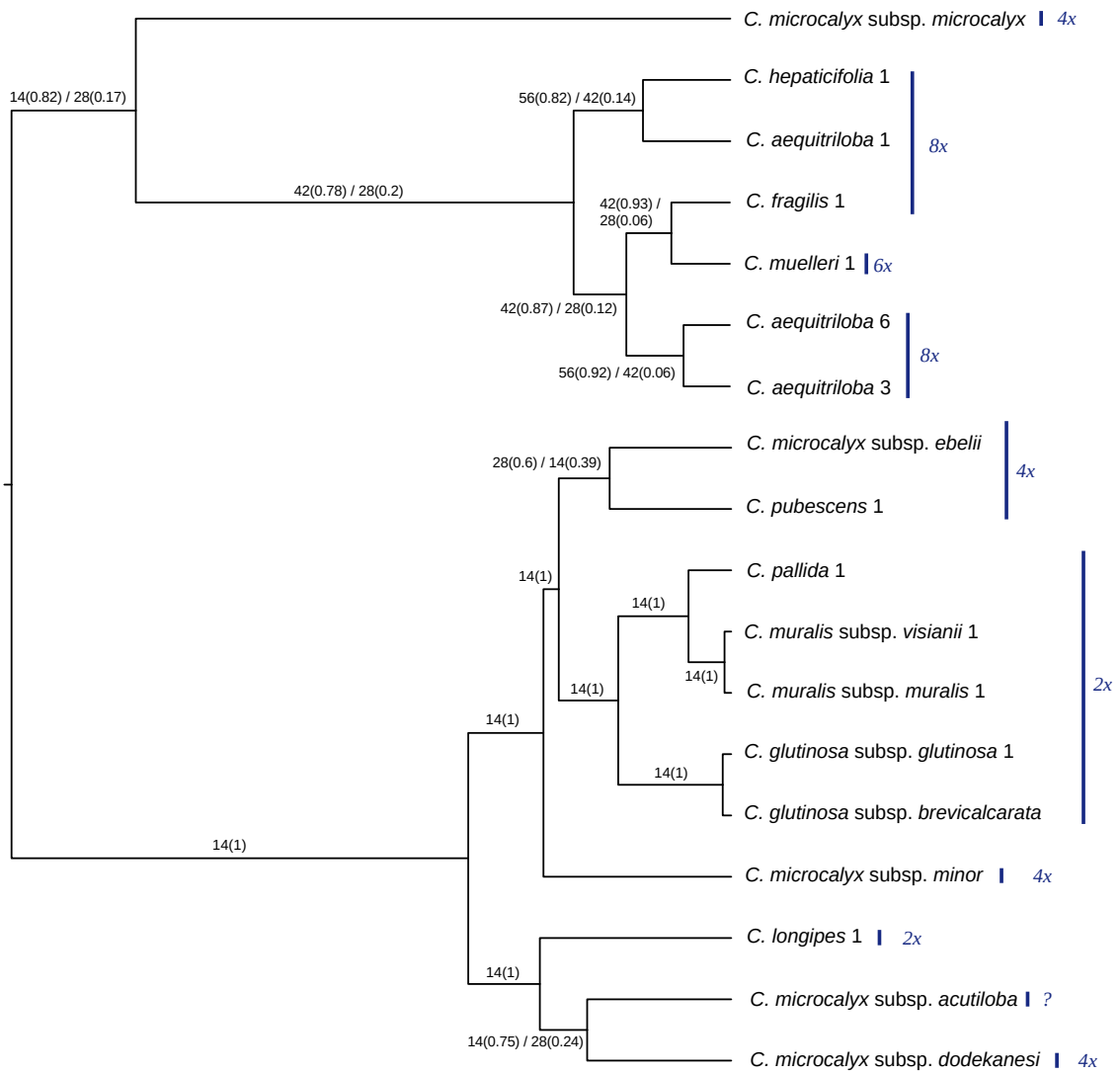

Figure S3. Maximum clade credibility (MCC) tree produced with a relaxed molecular clock analysis of nrDNA of *Cymbalaria* in BEAST v1.8.2. Chromosome numbers inferred by ChromEvol are shown above branches. Numbers in parenthesis correspond to Bayesian posterior probabilities for each chromosome number.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