



Evolutionary origins and species delineation of the two Pyrenean endemics *Campanula jaubertiana* and *C. andorrana* (Campanulaceae): evidence for transverse alpine speciation

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Abstract

Species diversity may be underestimated even in well-explored mountain regions due to the lack of in-depth research in taxonomically intricate groups. Filling such knowledge gap is necessary to optimize conservation management, specially for species occurring in vulnerable ecosystems such as Southern European mountains. *Campanula* sect. *Heterophylla* is a complex group with a high proportion of endemic taxa in European mountain ranges, and whose species delineation is often controversial due to extensive morphological variation. We investigated the phylogenetic relationships and evolutionary entity of its taxa occurring in the Pyrenees, with a special focus on the Pyrenean endemic *C. jaubertiana* (which is sometimes included as a subspecies of or merged with *C. cochleariifolia*); and *C. andorrana*, a taxon allegedly endemic to Andorra (Eastern Pyrenees) and of uncertain taxonomic value. We obtained chloroplast genome and nuclear ribosomal DNA sequences, including several individuals of the three focus taxa, and conducted morphometric analyses. Phylogenetic analyses show that *C. jaubertiana* sensu lato (s. l.; i.e. including *C. andorrana*) constitutes a clearly distinct lineage that is not even closely related to *C. cochleariifolia*; consistent differences in floral morphology were detected between them. Our results support two main evolutionary lineages within *C. jaubertiana* s. l., one corresponding to populations in Central Pyrenees, and another one in Eastern Pyrenees plus Catalan Pre-Pyrenees. Given the degree of genetic and morphological differentiation, we propose the species rank for each lineage (*C. jaubertiana* and *C. andorrana*, respectively) and provide a taxonomic treatment. These two Pyrenean endemics likely diverged through transverse allopatric speciation.

Keywords Allopatric speciation · *Campanula* sect. *Heterophylla* · Integrative taxonomy · Phylogenomics · Pleistocene glacial cycles · Pyrenees

Introduction

On-going biodiversity loss and climate change have surpassed safe limits (Hooper et al. 2012; IPBES 2019). Because these processes continue at an ever-accelerating rate, there is an urgent need to strengthen scientific

knowledge about the origins and maintenance of biodiversity. European mountain floras, especially southern ones, are expected to be particularly affected by climate change (Engler et al. 2011; Steinbauer et al 2018), reducing the area of favourable habitats for alpine species with potential detrimental consequences on mountain ecosystem services

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(Geeta et al. 2014). This is especially worrying for mountain regions such as the Pyrenees, a plant diversity hotspot with 4341 taxa and 5.5% of species endemic to the whole mountain region, whereas this proportion reaches 11% in the alpine vegetation belt (Gómez et al. 2017, 2020). Implementing appropriate conservation actions relies on crucial knowledge about intra- and interspecific biodiversity and the underlying evolutionary mechanisms generating this diversity in alpine ecosystems. Thus, studies contributing to overcome the so-called Linnean shortfall (i.e. the discrepancy between formally described species and the number of species that actually exist; Lomolino 2004) are essential to develop proper conservation strategies given that species are often the operational units of environmental policies (Mace 2004).

Unraveling the evolutionary history of mountain biodiversity is also essential to understand the impact of past and contemporary climatic changes. Pleistocene glacial cycles have generally been considered as a negative factor for biodiversity in mid- and high-latitude regions due to increased extinction rates (Hewitt 2000). On the other hand, such dramatic oscillations may have acted as a “species pump” for mountain lineages through repeated cycles of range expansion and contraction (Muellner-Riehl et al. 2019). A recent synthesis suggested that many endemic alpine lineages may have originated through transverse alpine speciation, i.e. allopatry defined by genetic breaks occurring at right angles (transverse) with the ancestral geographic range oscillations (Wallis et al. 2016); but this speciation model has rarely been tested for alpine plants.

Over the last decade, next generation sequencing (NGS) has revolutionized the field of evolutionary biology by increasing the quantity of data obtained by several orders of magnitude while decreasing its cost (Lemmon and Lemmon 2013). The availability of such huge amounts of data has greatly enhanced phylogenetic resolution at different taxonomic levels. The molecular revolution brought by NGS has been especially significant for previously challenging groups of organisms such as rapidly evolving lineages, which remained unresolved with Sanger sequencing (eg. Herrando-Moraira et al. 2018). However, genetic clusters per se do not inform of species limits given that there is no consensus on the amount of differentiation of that should be used to establish species boundaries (Galtier 2019). The integrative taxonomy approach has emerged in the last decades with the main aim to increase rigour and consensus in species delineation, a crucial issue for biodiversity research (Dayrat 2005; Wiens 2007) and conservation assessment (Stanton et al. 2019). Such approach consists in the combination of multiple sources of evidence: morphological characters, molecular data, ecological niche, reproductive compatibility, geographic distributions, etc. Integrative taxonomy has been successfully applied in taxonomically intricate groups

(eg. Bogdanović et al. 2014; Frajman et al. 2019; Boucher et al. 2021).

The genus *Campanula* L. (Campanulaceae) s. l. comprises ca. 420–600 species (Lammers 2007), most of which inhabit mountainous or steppe-like habitats of the Northern Hemisphere (Fedorov 1957). This genus presents a high degree of endemism, specially in mountain ranges and islands, showing the highest concentration of diversity in two regions: the Mediterranean Basin and the Caucasus (Roquet et al. 2009). The evolutionary and biogeographic history of the main lineages of *Campanula* s. l. have been clarified in the past (Eddie et al. 2003; Roquet et al. 2008; Jones et al. 2017), but our knowledge on the evolutionary history of most infrageneric *Campanula* groups and how their species originated remains highly incomplete. Moreover, infrageneric classification and taxon delimitation in *Campanula* is often difficult and controversial because most morphological characters are evolutionarily labile in this genus (Roquet et al. 2008) and many species show extensive morphological variability. The latter is especially true for *Campanula* sect. *Heterophylla* (Witasek) Tzvelev (commonly known as “harebells”), a taxonomically challenging group with conflicting treatments and whose species delineation is often unclear and controversial.

Campanula sect. *Heterophylla* corresponds to a group of species with dimorphic leaves (sessile linear cauline leaves and petiolate reniform basal ones) and a basal dehiscence of the capsule (De Candolle 1830; Boissier 1875; Fedorov 1957). The majority of taxa belonging to this group are found in mountain areas of the Northern Hemisphere, with a greater concentration in Europe. Some of these species are alpine specialists that are either endemic to a mountain range or widespread, as is the case for the dwarf mountain species *C. cochleariifolia* Lam., which occurs throughout the mountain ranges of the European Alpine System (EAS; Ozenda 1985, 2009). A recent phylogenetic study showed that *Campanula* sect. *Heterophylla* corresponds to a lineage in which a few isophyllous species are also nested (Mansion et al. 2012). Recently, Nicoletti et al. (2014) clarified species boundaries for nine taxa of this group occurring in the southwestern Alps using an integrative taxonomic approach.

The Pyrenees, like other mountain ranges of the EAS, hold several harebell species, some of which are endemic to this region. The taxonomic rank and relationships of two supposedly Pyrenean endemic harebell taxa is, to date, still unclear: (1) *Campanula jaubertiana* Timb.-Lagr. has been considered as a subspecies (Rivas-Martínez 2002) or as a variety (Bolòs and Vigo 1983, 1996) of *C. cochleariifolia*; and (2) *C. andorrana* Braun-Blanq., a taxon allegedly endemic to Andorra (Eastern Pyrenees), has been considered as a subspecies of *C. jaubertiana* (Losa and Montserrat 1950) or *C. cochleariifolia* (Bolòs and Vigo 1983), or has

been reduced to a synonym of *C. jaubertiana* in modern taxonomic treatments (e.g. Sáez et al. 2001) and in international databases such as Euro+Med-Plantbase (Castroviejo et al. 2010), The Plant List (<http://www.theplantlist.org>), IPNI (2020) or GBIF (<http://gbif.org>). Moreover, it should be noted that the author of *C. andorrana* (Braun-Blanquet 1945) was probably unaware of the publication of the name *C. jaubertiana* (Timbal-Lagrave 1868) given that in his description of *C. andorrana* the author only compared it with the characteristics of *C. cochleariifolia*.

Here, we perform a phylogenetic evaluation and quantitative morphological analyses to delimit species and investigate the evolutionary entity and origins of these taxa, with the main aim to provide an integrative taxonomic treatment. Specifically, we test the following hypotheses: (1) *C. cochleariifolia* and *C. jaubertiana* are clearly distinct species with particular traits; and (2) *C. andorrana* corresponds to a distinct lineage within *C. jaubertiana* s. l. that can be distinguished morphologically. Moreover, we investigate the evolutionary origins of these two Pyrenean endemic taxa by studying their phylogenetic position within the sect. *Heterophylla*. To achieve these goals, we applied an integrative systematic approach by gathering evidence from molecular data, morphological characters, potential ecological differentiation, and geographic distributions. We inferred a robust phylogeny for most of the taxa of Sect. *Heterophylla* occurring in Iberian Peninsula, based on a high number of plastid markers plus nrDNA intergenic regions obtained with low coverage shotgun sequencing. We included several individuals of different populations of *C. andorrana*, *C. cochleariifolia* and *C. jaubertiana*, plus at least one individual from the *locus classicus* for the other taxa of Sect. *Heterophylla*. In addition, we performed a detailed analysis of morphological variation of quantitative and qualitative traits from vegetative and reproductive organs of *C. andorrana*, *C. cochleariifolia* and *C. jaubertiana*. Finally, we provide a well-supported systematic revision of the studied taxa based on the integration of phylogenetic analyses with detailed morphometric data.

Materials and methods

DNA sequencing, phylogenetic inference and molecular dating analyses

We obtained plastome and nuclear DNA sequences for five individuals of *C. andorrana*, three individuals of *C. jaubertiana* sensu stricto (s. str.), and five individuals of *C. cochleariifolia* (see details of each individual locality in Table S1). We also included in the study sequences for one individual of seven species of *Campanula* sect. *Heterophylla* occurring in the Pyrenees and/or Cantabrian Mountains (*Campanula*

adsurgens, *C. arvatika*, *C. cantabrica*, *C. herminii*, *C. hispanica*, *C. precatoria*, *C. scheuchzeri*), *C. rotundifolia* s. str. from Central Europe, and three outgroup species (*C. carpatika*, *C. pyramidalis* and *C. rapunculus*) for tree rooting and fossil calibration.

For each individual, DNA was extracted from silica-dried leaves collected in the field, using a modified protocol of the DNeasy Plant Mini Kit (QIAGEN). Genomic shotgun libraries were prepared and sequenced with the Illumina HiSeq 2000 sequencer (Illumina, USA). The 101-bp paired-end reads obtained for each individual were assembled de novo with The ORGanelle ASSEMBLER (available at <http://metabarcoding.org/org-asm>), setting the read-depth minimum to 5X. The resulting contigs were annotated following the same procedure as in Roquet et al. (2016). In subsequent analyses, we used only genic and intergenic regions that were recovered with the complete sequence within the contigs. The annotated sequences were deposited in GenBank (see accession numbers in Table S2). We extracted the sequences of 48 coding and 7 non-coding plastid regions from annotated contigs using custom Python scripts. We also obtained the internal transcribed spacer (ITS) region of nuclear ribosomal DNA. Each non-coding region was aligned separately with MAFFT (Katoh et al. 2005), and each coding one was aligned separately with MACSE (Ranwez et al. 2011), which explicitly accounts for the underlying codon structure. Resulting alignments were visually checked and quality filtered with Gblocks (Castresana 2000), with codon setup for coding regions and DNA setup for non-coding ones. All plastid regions were concatenated into a single alignment matrix (hereafter “plastid dataset”) with FASconCAT (Kück and Meusemann 2010).

We run Maximum Likelihood (ML) phylogenetic inference analyses with RAxML v. 8.1.3 (Stamatakis 2014); we first analysed separately plastid and nuclear datasets, we checked that resulting topologies were not incongruent, and then we run again the analyses combining these two datasets. Specifically, for each dataset, we first chose the optimal partition scheme for phylogenetic inference with the ‘greedy’ algorithm implemented in PartitionFinder v.1.1.1 (Lanfear et al. 2012). Then, we ran 100 ML independent tree searches with RAxML starting with different trees and applying the GTR-GAMMA model, the rapid hill-climbing algorithm (Stamatakis et al. 2008) and the partition scheme selected with PartitionFinder. Bootstrap searches (BS; 1000 replicates) were executed separately using the standard bootstrap option in RAxML. Bootstrap results were drawn in the best-scoring ML tree (i.e. the one with the highest likelihood) obtained in the previous searches.

We also ran Bayesian inference (BI) analyses with BEAST2 (Bouckaert et al. 2014). Four independent runs were performed for 100 million generations applying a Yule tree prior and lognormal clock; non-coding alignments and

each codon position in coding alignments were modelled with separate averaging-site models as implemented in bModeltest (Bouckaert and Drummond 2017). We used Tracer (Rambaut et al. 2018) to check convergence visually and by checking whether the effective sample size (ESS) was > 100 for all the parameters. We used TreeAnnotator to generate the maximum credibility tree (MCC) with a burn-in of 30%.

The best-scoring ML tree was subsequently time-calibrated both with BEAST2 and with the penalized-likelihood method implemented in treePL v. 1.0 (Smith and O'Meara 2012), with the following two calibrations: a minimum crown age of 16.5 million years ago (mya) to the most recent common ancestor (mrca) of *C. carpatica* and *C. pyramidalis*, based on the fossil seed of *C. palaeopyramidalis* (Łańcucka-Środoniowa 1979); and an upper bound of 56 mya to the root, based on Bell et al. (2010). For treePL, we estimated the optimal smoothing parameter running a cross-validation analysis with values ranging from 0.00001 to 1,000,000, and then set the value that yielded the lowest chi-squared value.

Morphometric analyses

Specimens of *C. cochleariifolia* and *C. jaubertiana* s. l. were obtained from four herbaria (BC, BCB, BCN, JACA) and from the author's collections (vouchers deposited in BCB), see Table S3 for a complete list of examined vouchers. In addition, we examined all digitized specimens of these taxa available in the digital platform e-ReColNat (<https://www.recolnat.org>, accessed on June 2019), which gave us access to vouchers from four additional herbaria (LY, MPU, P, TLM), including the type specimens of *C. cochleariifolia*, *C. jaubertiana* and *C. andorrana*. Each specimen of *C. jaubertiana* s. l. was a priori ascribed to one of two groups based on our own observations of field and herbarium specimens and preliminary results of molecular phylogenetic analyses (see next subsection for details): (1) populations from Central Pyrenees and Central Pre-Pyrenees (Aragon region), which would correspond to *C. jaubertiana* s. str.; and (2) populations from Eastern Pyrenees (i.e. those located east of the Noguera Ribagorçana River) and Catalan Pre-Pyrenees, which would correspond to *C. andorrana* (Fig. 1).

We performed morphometric measurements only on well-preserved herbarium specimens to avoid potential distortion of plant features. We selected morphological characters based on taxonomic literature on the studied taxa (Braun-Blanquet 1945; Losa and Montserrat 1950; Timbal-Lagrave 1868) and our own observations. We measured in total 12 vegetative and eleven reproductive quantitative plus two qualitative characters on a total of 142 dry, pressed and well-preserved specimens (Table S3). These specimens were taken from 29 populations of *C. cochleariifolia* (of which 12 corresponded to different locations in the Pyrenees, and 17 to

other European massifs), 8 populations of *C. jaubertiana* s. str., and 7 populations of *C. andorrana*. We consider that this sampling was sufficient to account for morphological variation within each putative taxon. The measure of middle cauline leaves (included in the vegetative quantitative characters dataset) was always scored on the third leaf starting from the upper part of the stem. Measurements of vegetative and reproductive traits were carried out under a Zeiss Stemi DV4 stereo-microscope. The pollen grains were mounted in unstained glycerine jelly after removal from anthers and measurements were made with an OLYMPUS CH2 microscope.

Descriptive statistics (means, standard deviations and ranges) of quantitative characters were computed for all specimens. With the aim to detect morphological differentiation based on such characters, we carried out principal component analyses (PCA) twice: one including all specimens, and another one including only specimens of *C. jaubertiana* s. l. (67 in total). The most discriminant characters resulting from both PCAs were then summarized in the form of box plot graphs. We tested for significant differences between species for these characters with Kruskal–Wallis tests, and post hoc pairwise Mann–Whitney tests with Holm's correction to adjust p values for multiple comparisons. Statistical analyses were performed with the basic packages of R version 3.6.1 (R Core Team 2019), FactoMineR (Lê et al. 2008) to perform the PCA, and factoextra (Kassambara and Mundt 2017) and ggplot2 (Wickham 2016) to plot and save resulting graphs.

Results

Phylogenetic analyses and divergence time estimation

Phylogenetic analyses based on plastome and nuclear sequences yielded congruent results, i.e. supported nodes (BS $> 70\%$; posterior probability, PP > 0.95) reflected the same phylogenetic relationships; we will thus present results obtained with the concatenated dataset only. Bayesian and Maximum Likelihood analyses yielded the same topology. Maximum or high support was recovered for all nodes connecting different taxa (Fig. 2); the only few unsupported nodes correspond to subclades formed by individuals of the same taxon (*C. andorrana*, *C. cochleariifolia*). Concerning phylogenetic relationships, *C. cochleariifolia* is sister to the rest of the ingroup taxa (100% BS; PP = 1.0); the next node (89% BS; PP = 1.0) corresponds to the split of a subclade (100% BS; PP = 1.0) formed by *C. adsurgens*, *C. arvatika* and *C. herminii* from the rest of the taxa; the following node (100% BS; PP = 1.0) corresponds to the split between one subclade formed by the

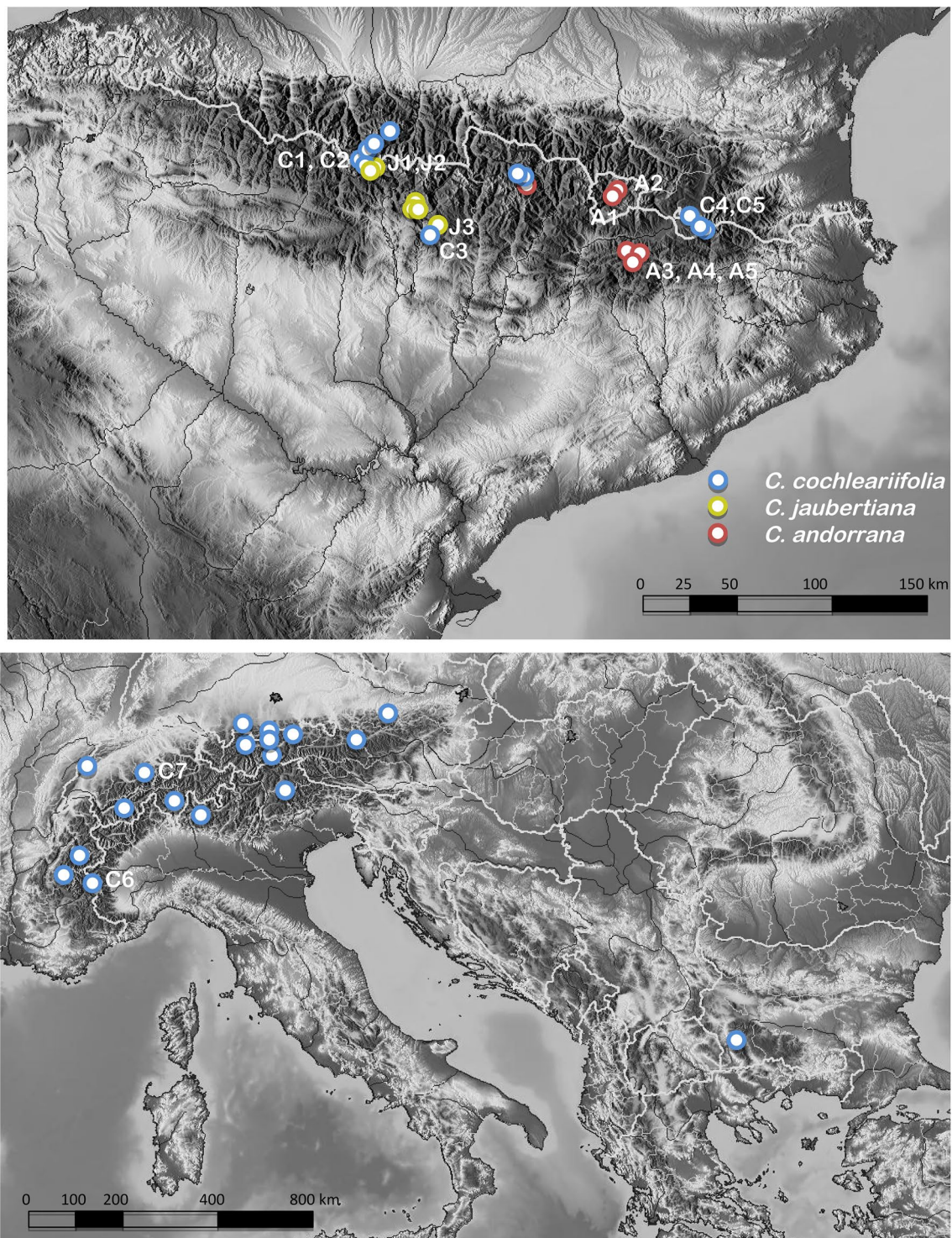


Fig. 1 Geographical distribution of studied specimens of *Campanula andorrana*, *C. cochleariifolia* and *C. jaubertiana*. Alphanumeric codes indicate specimens included in phylogenetic analyses: A, *C. andorrana*; C, *C. cochleariifolia*; J, *C. jaubertiana*

sister taxa *C. jaubertiana* and *C. andorrana*, and another subclade constituted by the sister taxa *C. rotundifolia* and *C. hispanica*, plus *C. scheuchzeri*, *C. precatorea* and *C. cantabrica* (100% BS and PP=1.0 for all nodes connecting

the mentioned taxa). The three taxa for which we included several individuals (*C. andorrana*, *C. cochleariifolia* and *C. jaubertiana*) were recovered each one as monophyletic (100% BS for each taxon lineage). Similar age estimations

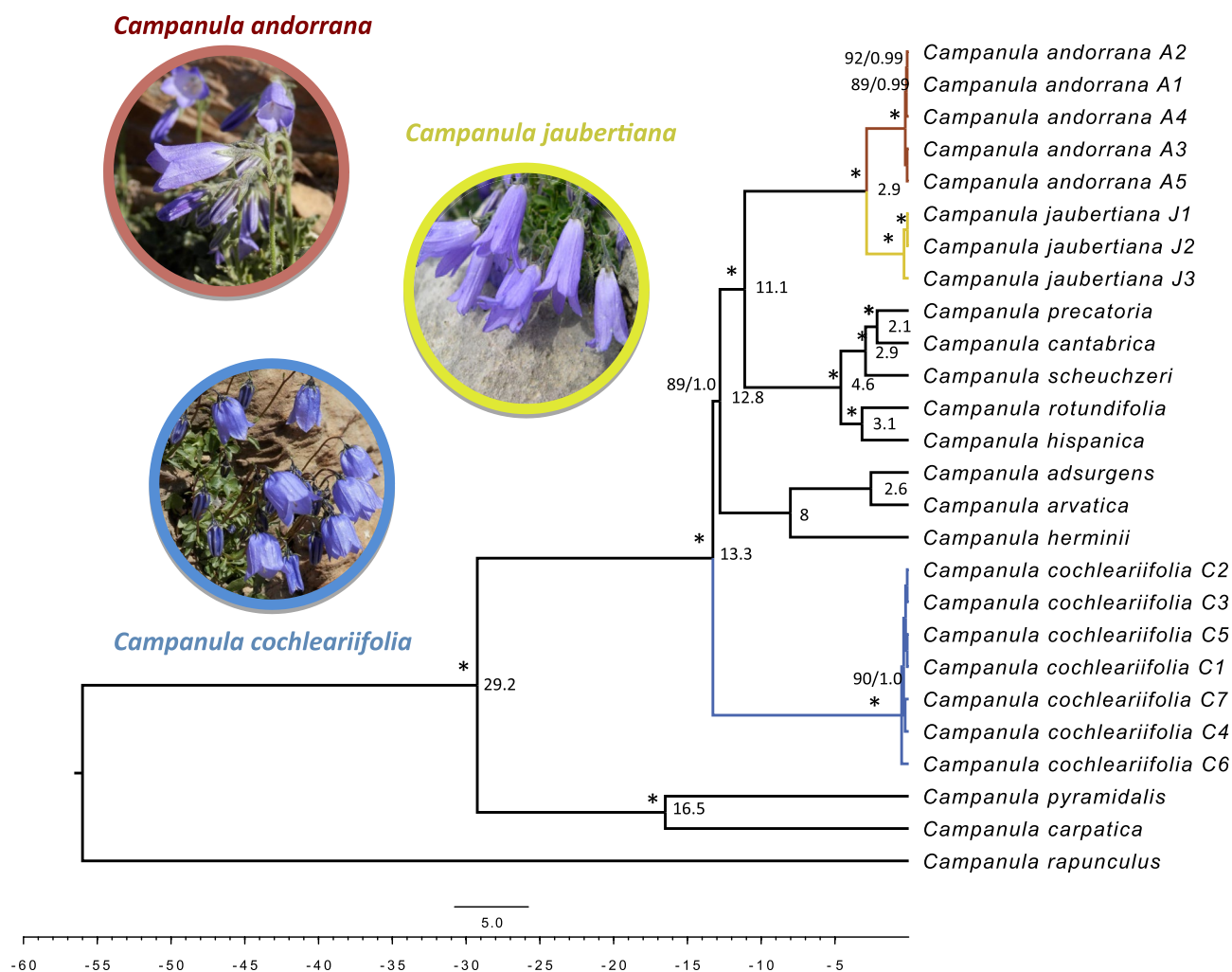


Fig. 2 Time-calibrated phylogenetic tree generated with treePL based on the highest likelihood tree yielded by the maximum likelihood inference analysis conducted with RaxML on the concatenated dataset (plastid plus nrDNA regions). Numbers in black, on the left side of each node, correspond to % of bootstrap support (BS) followed by Bayesian posterior probability (PP). Asterisks indicate nodes that

obtained maximum support in both analyses (100% BS and 1.0 PP). Values are not provided if BS is lower than 70% or PP lower than 0.95. Numbers in grey on the right side indicate estimated age for the node in million years. Individuals of the same species are named as in Fig. 1 and Table S1

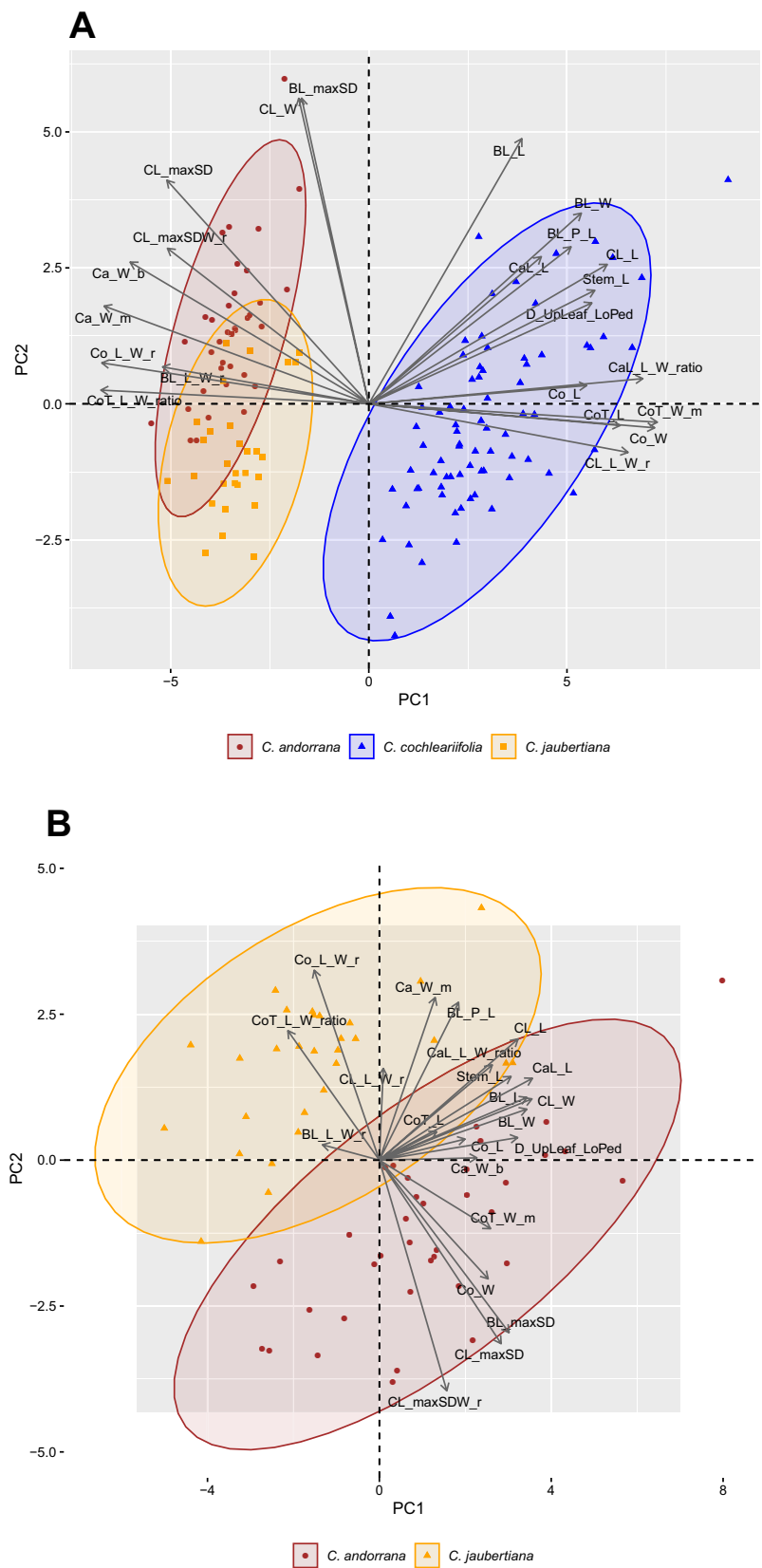
were obtained with the programs treePL and BEAST for all nodes. Branch lengths of the time-calibrated tree (Fig. 2) show that *C. cochleariifolia* split from the rest of the ingroup taxa c. 13.3 mya according to treePL (12.8 mya according to BEAST; 9.9–16 mya 95% confidence interval, hereafter C. I.), whereas the lineages of *C. jaubertiana* and *C. andorrana* split c. 2.9 mya (3.1 mya according to BEAST; 1.4–5.3 mya 95% C.I.).

Morphometric analyses

The PCA including all measured specimens suggested two clusters: one corresponding to specimens of *C. cochleariifolia*, and another one to individuals of *C. jaubertiana* s. l., with a clear discontinuity between them (Fig. 3A). The first

two axes of the PCA represented 67% of the overall morphometric variability (54.3% corresponded to the first axis). The five variables that contributed the most to the first axis were all reproductive traits, by order of contribution: corolla tube width at middle length, maximum width of corolla tube, calyx lobe length-to-width ratio, corolla tube length-to-width (at middle length) ratio, corolla tube length-to-width ratio (Fig. S1). Box plot graphs (Fig. 4) also show that these variables are useful to discriminate *C. cochleariifolia* from *C. andorrana* and *C. jaubertiana*. Concerning the qualitative characters examined, we detected that all specimens of *C. cochleariifolia* presented glabrous calyx lobes and a glabrous ovary, whereas they are hairy in *C. jaubertiana* s. l. Morphological characters that are useful to distinguish these taxa are provided in Table 1.

Fig. 3 **A** Principal component analysis (PCA) biplot for specimens of *C. cochleariifolia*, *C. jaubertiana* and *C. andorrana*. **B** PPCA biplot including only specimens of *C. jaubertiana* and *C. andorrana*. Abbreviations of variables: BL_L, basal leaf length; BL_L_W_r, basal leaf length-to-width ratio; BL_maxSD, maximum sinus depth of basal leaf lobes; BL_P_L, length of the petiole in basal leaf; BL_W, basal leaf width; CaL_L, calyx lobe length; CaL_L_W_ratio, calyx lobe length-to-width ratio; Ca_W_b, calyx lobe width at the base; Ca_W_m, calyx lobe width at middle length; CL_L, cauline leaf length; CL_L_W_ratio, cauline leaf length-to-width ratio; CL_maxSD, maximum sinus depth of cauline leaf lobes; CL_maxSDW_r, maximum sinus depth-to-width ratio of cauline leaf lobes; CL_W, cauline leaf width; Co_L, corolla length; Co_L_W_r, corolla length-to-width ratio; Co_W, corolla width; CoT_L, corolla tube length; CoT_W_m, corolla tube width at middle length; CoT_L_W_r, corolla tube length-to-width ratio; D_UpLeaf_LoPed, distance between the upper leaf to the lowest pedicel; Stem_L, length of the stem (flower excluded)



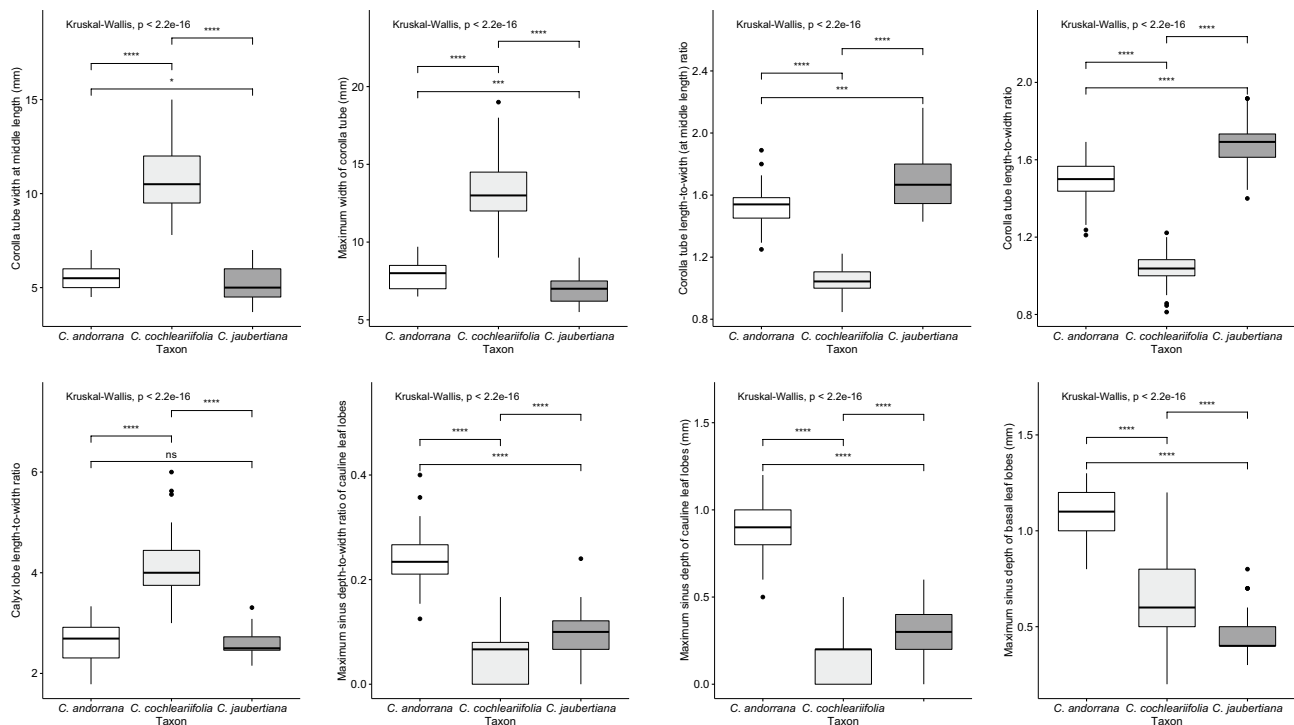


Fig. 4 Box plots for the most discriminant morphological characters between *C. andorrana*, *C. cochlearifolia*, and *C. jaubertiana*. The p-value obtained with Kruskal–Wallis test is indicated at the top of

each plot. Significance levels obtained with post hoc pairwise Mann–Whitney test applying Holm’s correction are indicated as follows: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns, $p \geq 0.05$

Table 1 Morphological characters that enable to distinguish *Campanula andorrana*, *C. cochlearifolia* and *C. jaubertiana*.

Character	<i>C. andorrana</i>	<i>C. jaubertiana</i>	<i>C. cochlearifolia</i>
Margin of basal leaves	Undulate to crispate	Flat to slightly undulate	Flat to slightly undulate
Base of basal leaves	Usually truncate	Cuneate to cordate	Atenuate to cordate
Middle cauline leaves shape	Oblanceolate to broadly elliptical	Lanceolate to narrowly elliptical	Linear to narrowly lanceolate, rarely elliptical
Margin of cauline leaves	incise-dentate	shallowly incise-dentate; teeth up to 0.5(0.6) mm long	entire to remotely crenate; teeth up to 0.2 mm long
Teeth length of cauline leaves (mm long)	0.5–1.2	up to 0.5(0.6)	up to 0.2
Calyx and ovary	Usually densely hairy	Hairy	Glabrous
Ratio length/width of calyx lobes	1.7–3.3	2.1–3.3	3–6
Corolla	Narrowly campanulate or cylindrical campanulate to campanulate	Narrowly campanulate to campanulate	Broadly campanulate
Corolla hairs at base (mm long)	0.1–0.5	0.1–0.3	Absent

The second PCA, based only on specimens of *C. jaubertiana* s. l., showed again two clusters: one corresponding to individuals of *C. jaubertiana* s. str., and another one corresponding to specimens of *C. andorrana* (Fig. 3B). The first two axes explained 45.6% of the variability (the first axis alone represented 28.3%). The most useful variables for discriminating *C. andorrana* and *C. jaubertiana* were, by order

of contribution: the ratio between maximum teeth length and maximum sinus width of cauline leaf lobes, maximum teeth length of cauline leaf lobes, and maximum teeth length of basal leaf lobes, as also reflected by the box plots (Fig. 4, Fig. S2; Table 1).

Kruskal–Wallis tests indicated that there were significant differences between species for all the discriminant traits

identified with the two PCAs ($p < 0.0001$), and pairwise Mann–Whitney tests showed significant differences in all comparisons except for the trait «Calyx lobe length-to-width ratio» when comparing *C. andorrana* vs. *C. jaubertiana* (significance levels provided in Fig. 4).

Discussion

The Pyrenees host a remarkable concentration of plant biodiversity and endemism due to a number of intertwined factors driving strong habitat heterogeneity and species turnover over sometimes very small spatial scales. These factors are: strong abiotic gradients associated with ample altitudinal ranges (> 3000 m), climatic influences (oceanic, continental, sub-Mediterranean), and tremendous bedrock variability (granite, limestone, sandstone, schist, conglomerate, volcanic rocks). However, such noteworthy biodiversity may still be underestimated because of taxonomically intricate groups and potential cryptic species that remain to be investigated thoroughly (Bickford et al. 2007; Wiens 2007). The failure to detect this cryptic, so-far unnoticed, species diversity may even be exacerbated by the fact that such hidden diversity may result from recent speciation events. Our study shows that the combination of different sources of evidence enables to unravel the evolutionary entity of taxa belonging to controversial taxonomic groups (Dayrat 2005). As we discuss below in detail, our results support our two initial hypotheses: (1) *C. cochleariifolia* and *C. jaubertiana* s. l. belong to distinct lineages that are not closely related, and they can be differentiated morphologically by several reproductive traits; and (2) *C. jaubertiana* s. l. is constituted by two clearly defined evolutionary lineages (which diverged c. 2.9 mya) that can be distinguished morphologically mostly by vegetative traits (Figs. 5 and 6), and which are distributed in separated geographic areas of the Pyrenees.

Our phylogenetic analysis provides compelling evidence that the widespread *C. cochleariifolia* and the Pyrenean endemic *C. jaubertiana* are not closely related: the sister lineage of *C. jaubertiana* s. l. is a clade constituted by narrow mountain endemics (the Pyrenean *C. precatorea* and the Cantabrian *C. cantabrica*) and widespread species (*C. hispanica*, *C. rotundifolia* s. str., *C. scheuchzeri*). At the morphological level, previous authors suggested that *C. jaubertiana* (Losa and Montserrat 1950) or *C. andorrana* (Braun-Blanquet 1945) could be distinguished from *C. cochleariifolia* by the following characters: plant's hairiness, shape and disposition of cauline leaves, corolla shape, and shape of the calyx lobes; but they did not provide quantitative evidence of whether these traits are readily discriminant. Our results show that *C. cochleariifolia* and *C. jaubertiana* s. l. (as circumscribed by Sáez and Aldasoro 2001) can be differentiated by at least two qualitative and one quantitative

traits (hairiness of calyx lobes and ovary, corolla shape), as we propose in the taxonomic treatment provided in Appendix S1.

Concerning *C. jaubertiana* s. l., the highly congruent cpDNA and nrDNA data clearly show that populations from the Eastern Pyrenees and Catalan Pre-Pyrenees are genetically divergent from populations in Central Pyrenees. According to our time-calibrated tree, the time of their divergence (2.9 mya according to treePL; 3.1 mya according to BEAST; 1.4–5.3 mya 95% CI) is of similar age than other species divergences of well-established species of the Sect. *Heterophylla* such as *C. adsurgens* and *C. arvatica*, which split 2.6 mya (treePL) or 2.4 mya (BEAST; 1–4.5 mya 95% C.I.); and such as *C. precatorea* and *C. cantabrica*, which dates back to 2.1 mya (treePL) or 2.4 mya (BEAST; 1.5–4.2 mya 95% CI). Furthermore, our morphological quantitative analysis brings evidence that the two lineages of *C. jaubertiana* s. l. can be distinguished by the degree of leaf denticulation (Fig. 5). This diagnostic character was suggested among others by Losa and Montserrat (1950), who proposed that *C. andorrana* should be considered as a subspecies of *C. jaubertiana*, but our study provides unique evidence on the discriminant power of this trait. Thus, given the evolutionary differentiation of the two lineages of *C. jaubertiana* s. l. together with their consistent and divergent leaf characters, we propose in the taxonomic treatment here provided (Appendix S1) that they should be treated as two separate species: *C. andorrana* (which corresponds to the populations in Eastern Pyrenees and Catalan Pre-Pyrenees) and *C. jaubertiana* (Central Pyrenees). Regarding the geographic distribution of *C. andorrana* (Fig. 1), it should be noted that our extensive study of herbarium specimens and field work has revealed that it is larger than what was described in previous taxonomic studies, which considered it as endemic to Andorra (Braun-Blanquet 1945; Losa and Montserrat 1950).

Given that the ranges of *C. andorrana* and *C. jaubertiana* s. str. do not overlap and that their habitat is similar, it is highly likely that they diverged by allopatric speciation, an important driver of alpine plant diversification (Kadereit et al. 2004; Boucher et al. 2016). It also provides striking evidence in favour of the model of transverse speciation across the Pyrenees (Wallis et al. 2016) driven by the onset of Pleistocene glaciation, both in terms of spatial arrangement and timing of speciation (around 2–3 mya). To our knowledge, our study is the first one to provide evidence of allopatric speciation between Central and Eastern Pyrenees originating two endemic alpine plants (*C. andorrana* and *C. jaubertiana*). A recent review suggested that in situ diversification in the Alps has been very rare (c. 1.2% of the native and c. 9% of the endemic flora of the Alps, Kadereit 2017) as a result of Quaternary climatic oscillations; a similar pattern might be found in other mountain regions of the EAS

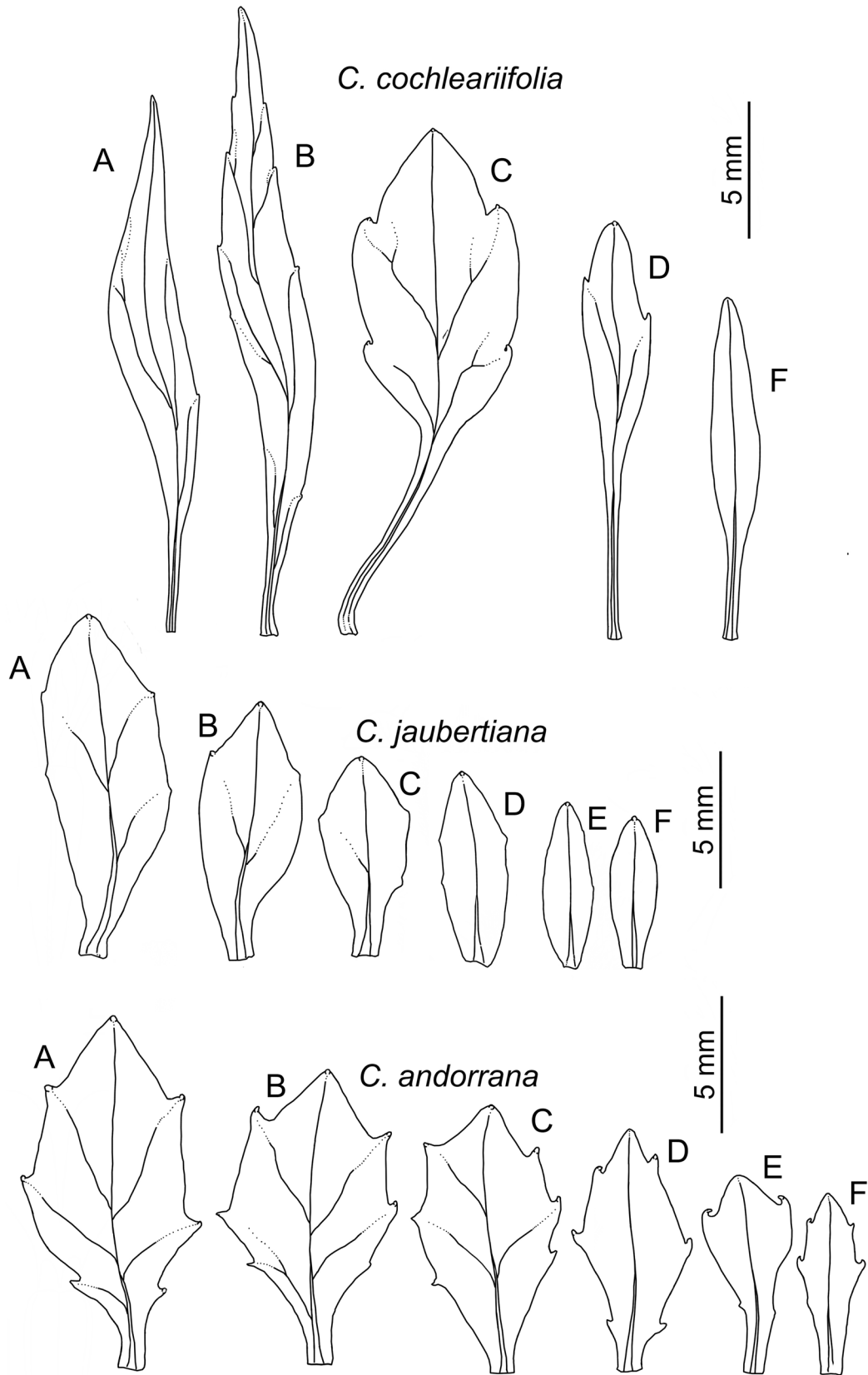
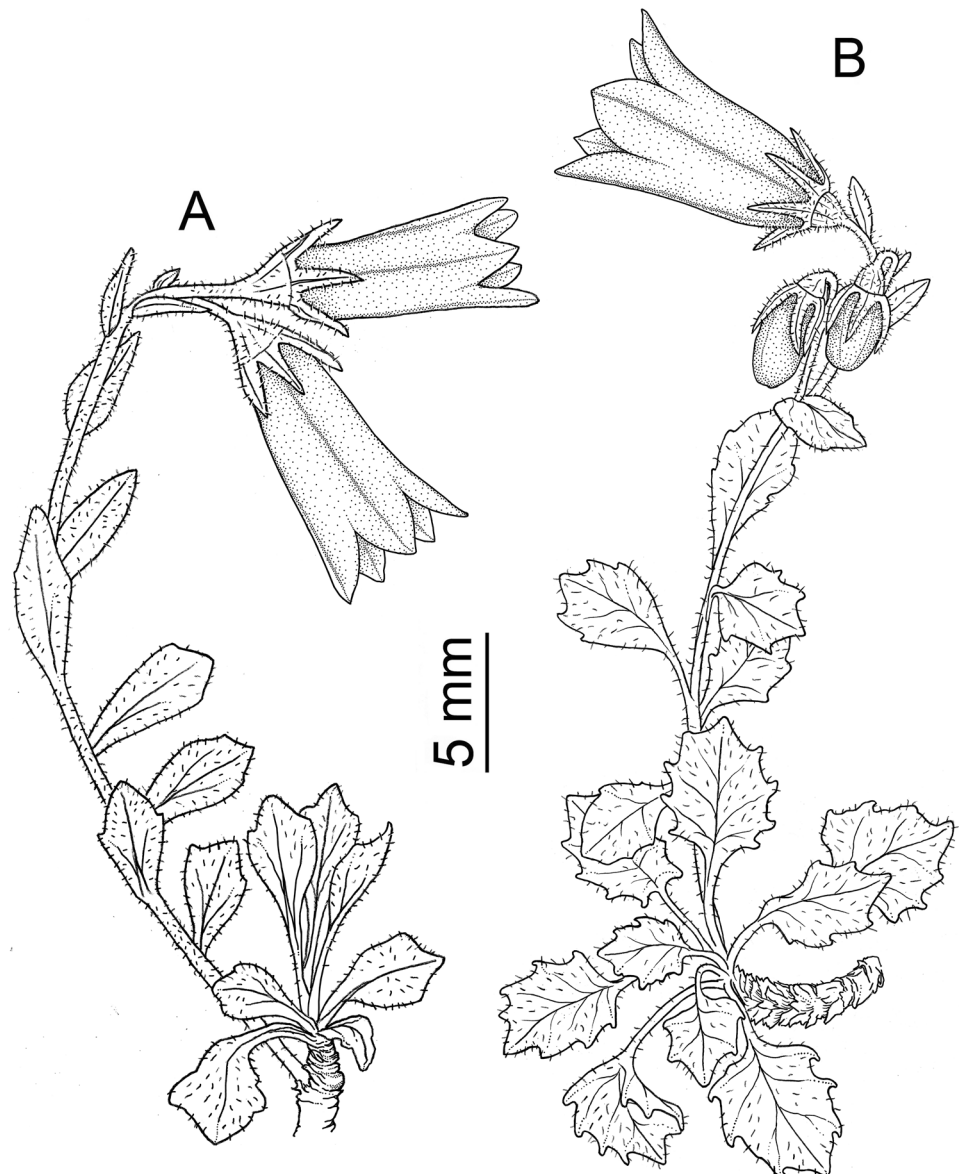


Fig. 5 Middle cauline leaves of *Campanula cohleariifolia*, *C. jaubertiana* and *C. andorrana*. Provenience of the specimens: *C. cohleariifolia*, **A, D** (Estanyeres, BCN 59,189), **B–D** (Fontalba, BCB, L. Sáez, Herb. Pers.); *C. jaubertiana*, **A, D** (Bielsa, BCN 104,944); **B, C** (Bielsa, BCN 104,945), **E, F** (Turbó, BCN 104,945); *C. andorrana*, **A** (Cassamanya, BCN 106,126), **B** (Torrent Gran BCN 25,617), **C** (Tancalaporta, BCN 25,618), **D** (Encantat, BCN 104,947), **E** Serrat de la Muga BCN 104,946), **F**: Pedraforca (BCB, L. Sáez, Herb. Pers.). Illustrations done by L. Sáez

such as the Pyrenees. However, this type of events may still be underestimated due to cryptic diversity in alpine environments: for instance, three novel strictly alpine species of *Androsace* L. were recently described within the Alps (Boucher et al. 2021). In addition, our data suggest that allopatric speciation in the same period also likely originated two sister species endemic of the Cantabrian Range,

Fig. 6 **A** *Campanula jaubertiana* from Huesca province, Pineta (BCN 57,221); **B** *C. andorrana* from Barcelona province, Pedraforca (BCB, L. Sáez, Herb. Pers.). Illustrations done by L. Sáez



C. arvatica and *C. adsurgens* (which share the same type of habitat—cracks and ledges in calcareous rocks from 500 to 2000 m a.s.l., and their geographic ranges do not overlap); and indeed, a very recent study corroborates this latter point and suggests in addition that a third Cantabrian endemic taxon recently described (*Campanula mariaceballii* Fern. Prieto & Arjona) also deserves the species rank (Fernández Prieto et al. 2020).

Perspectives

Integrative approaches have successfully been applied in our study as well as in previous studies to unravel evolutionary entities of intricate plant groups with cryptic or pseudo-cryptic species (i.e. species for which morphological

differentiation has been overlooked, Knowlton 1993), such as in the genus *Androsace* (Boucher et al. 2021), *Euphorbia* L. (Frajman et al. 2019) and *Linaria* Mill. (Vigalondo et al. 2015). However, the list of taxonomic groups that need further scrutiny to unveil hidden diversity is still long. Overcoming the Linnean shortfall is specially urgent for plant alpine lineages given that ongoing climate change is rapidly reducing their habitat (Engler et al. 2011). To our knowledge, at least the following vascular plant genera comprise several complex species groups distributed in the European Alpine System that deserve further research to clarify their taxonomic diversity: *Festuca* L., *Linaria*, *Pedicularis* L., *Pinquicula* L., *Saxifraga* L., and *Soldanella* L. Integrative taxonomic studies focused on these taxa should be performed to: (1) improve biodiversity assessment of European mountain floras, (2) properly assess their conservation status, and (3) ultimately implement appropriate conservation strategies.

Our study allowed accurate identification of species boundaries in the studied taxa of sect. *Heterophylla*, a crucial requirement for biodiversity conservation since the species is the most often unit for legal protection statuses. During our study, we identified conservation issues that we detail below, along with aspects that need further field research to implement appropriate conservation actions. First of all, three populations of the narrow endemic *C. andorrana* (including the *locus classicus*) are located in highly frequented areas by hikers and climbers (Pedraforca, Pic de Casamanya, Encantat Gran). Given that the popularity of outdoor activities such as rock climbing has hugely increased in the recent years (deCastro-Arrazola 2021) and that this activity can alter cliff plant communities (Lorite et al. 2017), it would be highly recommended to study the potential impact of human disturbance in these populations and propose management guidelines accordingly. Second, *C. andorrana* should be added in the Red List of Catalonia (Sáez et al. 2010). Finally, biodiversity action plans should be elaborated for *C. andorrana* and *C. jaubertiana*, and in the context of such plans, it would be highly recommended to study the population genetic structure and diversity of both species to enlighten whether and where ex-situ conservation and population reinforcement actions may be needed.

Conclusions

Our study shows that species diversity in supposedly well-known regions may be underestimated due to the lack of integrative studies focused on taxonomically intricate groups such as *Campanula* Sect. *Heterophylla*. Here, we demonstrate that *C. cochleariifolia* and *C. jaubertiana* are two distinct species (not even sister ones); and that within *C. jaubertiana* s. l. there are two clearly differentiated lineages

supported by robust phylogenetic evidence and morphological differentiation, which altogether provide strong support for treatment as two different species: *C. jaubertiana* and *C. andorrana*. These two Pyrenean endemics likely originated through transverse allopatric speciation driven by the onset of Pleistocene glaciations. A taxonomic treatment for the studied taxa is provided in Appendix S1. Our study also highlights the importance of Pyrenees as a hotspot of alpine endemic plants in Europe, and the need of further research in intricate taxonomic groups to improve mountain biodiversity assessment and propose adequate conservation actions.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00035-021-00257-8>.

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Author contribution C.R. and L.S. designed the research; L.S., C.R., J.S., S.L., C.P. and B.K. conducted field work; M.B. and A.A. performed laboratory analyses; F.D., E.C. and J.S. performed sequence data curation; C.R. and L.S. conducted the research and analyzed the data; C.R., L.S. and S.L. wrote the article with contributions of all co-authors.

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Data availability The molecular data generated for this study will be available in Genbank upon acceptance. Other datasets and code generated during the current study are available from the corresponding author on demand.

Declarations

Conflict of interest The authors declare no competing interests.

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