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Research Article

Phylogeographical patterns of campanula gr. Arvatica, an endemic group of the Cantabrian mountains (NW Iberian peninsula), based on plastid and nuclear DNA polymorphisms

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Endemicity is a key consideration for conservation, and taxa that are endemic to a single country are vaulable conservation targets. In this study, we investigate endemic species of the genus *Campanula* in northern Iberian Peninsula based on sequences of nrDNA (internal transcribed spacer, ITS) and plastid regions (*rbcL, trnL-F* and *trnS-G*) and additionally the secondary structure of ITS2. The main aim of this work was to evaluate the evolutionary history of *Campanula* gr. *arvatica* from the Cantabrian Range (northern Iberian Peninsula) and establish phylogeographical patterns. Our results showed that molecular data from nuclear DNA and plastid regions should be applied for management and conservation strategies to monitor this endemic group from northern Spain. Furthermore, molecular evidence indicates significant differences among the three taxa analysed and shows the importance of the Cantabrian Mountains as a refuge for endemic Iberian flora in which processes of neo-speciation and/or crypto-speciation are under way.

Key words: Campanula, Cantabrian Range, endemism, Iberian Peninsula, molecular systematics, secondary structure

Introduction

The Cantabrian Range is the most south-westerly range with a temperate climate in Europe, extending 700 km between the Pyrenees and the Galician mountains, with a broad diversity of landforms and landscapes. Located in the north of the Iberian Peninsula, between the Atlantic coast and the inner Mediterranean upland of the Duero Basin, the mountains were occupied by ice fields as well as alpine and cirque glaciers during the Pleistocene (Serrano et al., 2013). This refuge of plants located in Cantabrian territories has a high plant diversity and especially endemic plant life (plants exclusively present within the Cantabrian territory, or also present in the Pyrenees and neighbouring mountains). Indeed, around 300 plants from this territory are threatened and/or included in catalogs of current protection, due to their regionality or scarcity (Álvarez García et al., 2007; Fernández Prieto et al., 2014, 2017).

Today a central challenge in conservation is to identify biodiversity rich areas. In this regard, Myers (1988) defined the concept of a 'biodiversity hotspot'. Currently, there are 35 biodiversity hotspots formally defined with new criteria as biogeographic regions with more than 1500 endemic vascular plant species inhabiting less than 30% of their original primary habitat (e.g. Mediterranean Basin; see Myers et al., 2000; Marchese, 2015). However, a successful conservation strategy should not be based merely on the number of taxa present in an ecosystem. On the contrary, there is a need to

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identify conservation priorities at finer scales in order to maximize the effectiveness of conservation investment (Brooks et al., 2006; Marchese, 2015; Schierenbeck, 2017). According to Cañadas et al. (2014), endemic taxa constitute a central group for conservation, since narrowly endemic species are frequently threatened and because endemic-rich areas are also likely to be species-rich. Within this context, the Cantabrian Range is a region that merits attention to carry out studies on the diversity of endemic taxa.

The Campanulaceae Juss. is a nearly cosmopolitan family, chiefly of temperate regions, with a remarkable range of variation in morphology, ecology, pollen and seed morphology. The family includes more than eighty genera divided among five subfamilies, of which subfamily Campanuloideae Burnett, that includes the genus *Campanula* L., is the largest and most widespread (Lammers, 2007; Deyuan et al., 2011). The large clade consisting of *Campanula* and its close relatives contains ca. 600 species (referred to as *Campanula s.l.* in Mansion et al., 2012). The numerous *Campanula* taxa occupy mainly mountainous habitats in temperate and subtropical zones of the Northern Hemisphere, although they extend to east and southern Africa (Lammers, 2007), with their greatest diversity between the Mediterranean and the Caucasus (Deyuan et al., 2011). Many authors have tried to develop a suitable classification of the genus; however, there has been no consensus on interspecific and/or infraspecific ranks, due in part to low taxonomic sampling, geographic biases and a limited number of characters being incorporated into analyses (see Wendling et al., 2011). Recent phylogenetic studies have provided considerable insights, combining classical taxonomic methods with molecular and biogeographical approaches (e.g. Cano-Maqueda & Talavera, 2011; Lakušić et al., 2013), and defining the genus as broadly paraphyletic (Jones et al., 2017). In spite of the number of studies undertaken on inter- and intraspecific diversity in these campanuloids (Losa & Montserrat, 1953; Damboldt, 1966), a solid foundation for their systematics is still lacking.

According to Sáez & Aldasoro (2001), the Iberian flora is represented by twenty-six *Campanula* species, seven of them endemic to the Iberian Peninsula and the Pyrenees; including plants that grow in calcareous rock fissures from the *Campanula arvatica* group (i.e. *C. arvatica* Lag., *C. adsurgens* Leresche & Levier and the recently described *C. mariae-ceballosiae* Fern.Prieto, Arjona, Sanna & Cires). It is important to emphasize this last taxon is not included in the international databases (such as Euro + Med PlantBase, http://www.emplantbase.org; The Plant List, http://www.theplantlist.org/; International Plant Names Index (IPNI), https://www.ipni.org/), likely due to its restricted distribution.

Campanula arvatica is a hairless or sparsely downy tufted perennial with a thick stock; it presents stems that are woody at the base to 20 cm and that have leaf scars and are usually topped by a single flower. The main morphological difference between *Campanula adsurgens* and *C. arvatica* relates to the calyx: the flowers from *C. adsurgens* exhibit a hairy calyx (papillate occasionally), while the calyx of *C. arvatica* is smoothly glabrous (Sáez & Aldasoro, 2001). The new endemic species within the group, *C. mariae-ceballosiae*, is characterized by a corolla tube longer than its teeth, while in *C. adsurgens* & *C. arvatica* it is of equal length or less (Arjona Rodríguez 2012, 2013). *Campanula arvatica* is distributed through the Cantabrian Mountains -from "Puerto de Piedrasluengas" (boundary between Palencia and Cantabria) to "Teverga" (Asturias) and "La Babia" (León)-, *C. adsurgens* can be located from "Peñalba de Santiago" (León) to "Sierra do Caurel" (Lugo), reaching "As Nogais", in the foothills of the "Ancares Lucenses". In the case of *C. mariae-ceballosiae*, its distribution is limited to the east of "Somiedo" (Asturias) (Fig. 1).

The main aim of this work was to evaluate the evolutionary history of endemic species of the genus *Campanula* in northern Iberian Peninsula. In particular, in order to better assess the floristic richness and the conservation priorities of this territory, we study the diversification of the complex *Campanula* gr. *arvatica* using a molecular approach that includes nrDNA ITS and plastid regions (*rbcL*, *trnL-F* and *trnS-G*). Within this framework, we addressed the following goals: (1) perform for the first time a molecular evaluation to test species delimitation and their phylogenetic relationships of these endemic species, and (2) the search for phylogeographical patterns within this endemic group of plants in the Cantabrian Range.

Materials and methods

Plant material and sampling

In the present study, 41 specimens representing the ingroup members of the complex *Campanula arvatica* were sampled (Fig. 1). A total of 164 new molecular sequences were generated. A complete list of specimens sampled, along with their sources, voucher information and corresponding GenBank accession numbers are provided in Table S1 (Supplementary material). Sampling for the *C. arvatica* complex included 17 individuals of *C. adsurgens*, 5 individuals of *C. mariae-ceballosiae* and 18 individuals of *C. arvatica*, broadly covering the geographic distribution and morphological diversity of

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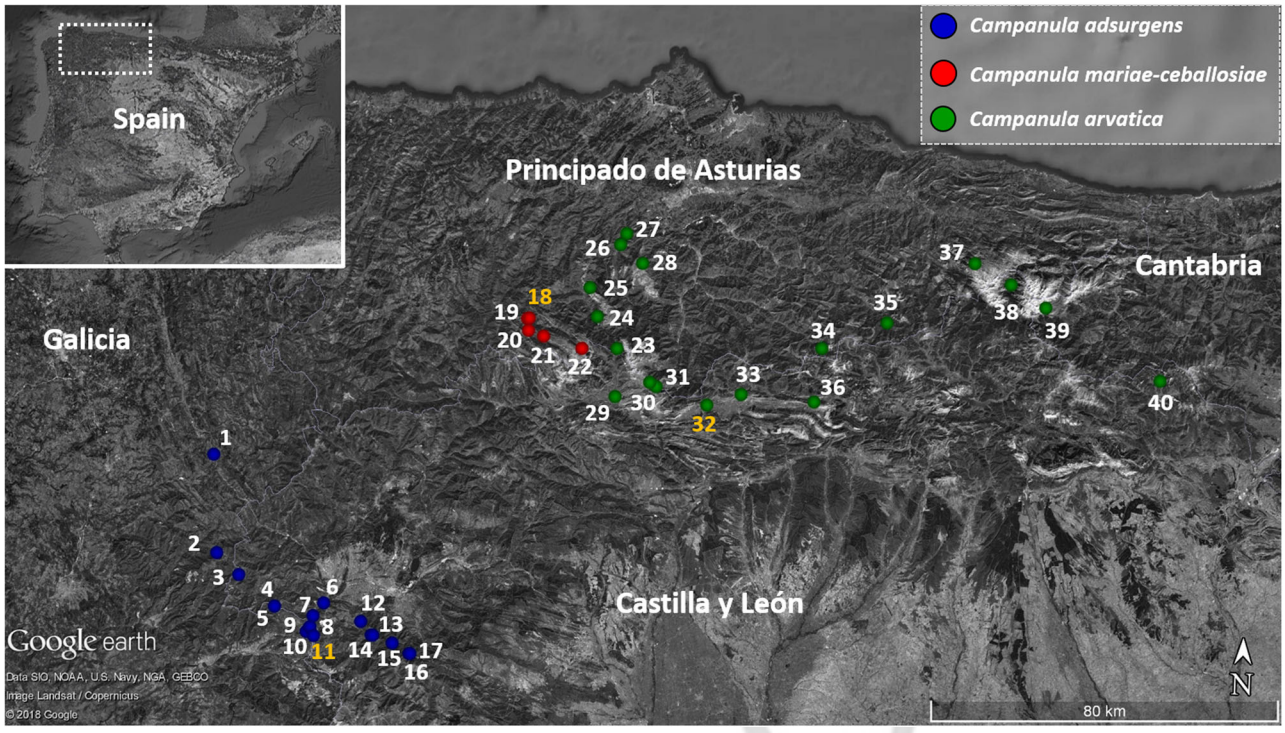

Fig. 1. Distribution of the endemic group *Campanula* gr. *arvatica* in the northwest of the Iberian Peninsula. Orange labels (i.e. 11, 18, 32) denote the classic localities of each species (*C. adsurgens*, *C. mariae-ceballosiae* and *C. arvatica*, respectively).

Table 1. Summary of the nucleotide site variation of the endemic complex *Campanula* gr. *arvatica* (i.e. *C. adsurgens*, *C. arvatica* and *C. mariae-ceballosiae*) from DNA sequences. Results from ITS and plastid regions (*rbcL*, *trnL-F*, *trnS-G*, cpDNA) and combined data (ITS + cpDNA).

	ITS	*rbcL*	*trnL-F*	*trnS-G*	cpDNA	Combined
Length range (bp)	698-701	619	834-836	680-845	2135-2300	2834-2999
Aligned length (bp)	702	619	836	846	2301	3003
Polymorphic sites	50	1	13	169	183	233
Mean G + C content (%)	52.4%	43.1%	34.6%	27.9%	31.7%	46.3%

these three closely related species. In addition, a sample of *Campanula rotundifolia* L. from Cantabria (Spain) was used as an outgroup (see CAM12 referred to Clade 12 in Mansion et al., 2012).

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from dried leaves using the Plant DNeasy Extraction Kit (Qiagen). Published primers that have been successfully employed in other studies of Campanulaceae (e.g. Roquet, 2008; Roquet et al., 2008, García Aloy, 2017), were used for the amplification and sequencing of the ITS (Sun et al., 1994; Fernández Prieto et al., 2013), *rbcL* (McIntosh et al., 1980; Fernández Prieto et al., 2013), *trnL-F* (Taberlet et al., 1991) and *trnS-G* (Hamilton, 1999). For all regions, a standard polymerase chain reaction (PCR) was used for the amplification of double-stranded DNA on a GenAmp PCR System 9700 (Applied Biosystems). The PCR parameters were as follows: 5 min pre-treatment at 94 °C; linked to 40 cycles of 1 min at 94 °C, 1 min at the annealing temperature specific to each primer, 1 min at 72 °C plus a final extension of 10 min at 72 °C. PCR products were run on a 1.5% agarose gel stained with ethidium bromide in order to evaluate the quality and quantity of the amplified templates prior sequencing. PCR products were sequenced at the Macrogen DNA Synthesis and Sequencing Facility (Madrid, Spain). The sequences of all samples were aligned with ClustalW ver. 2.0.10 (Larkin et al., 2007) and the alignment was subsequently corrected manually. The limits of the regions were determined by the positions of flanking primers. IUPAC symbols were used to represent nucleotide ambiguities.

Data analyses

Phylogenetic trees were reconstructed separately for ITS and plastid regions based on maximum-parsimony (MP) and maximum-likelihood (ML) implemented in MEGA 7.0 (Kumar et al., 2016). MP trees were performed using Tree-Bisection-Reconnection (TBR) with the search level setting at 3. ML trees were reconstructed using the nearest neighbor-interchange (NNI) method using all sites and Kimura 2-parameter model (K2P) (Kimura, 1980). Clade supports was calculated based on 1000 bootstrap resamplings (parsimony bootstrap, PB; likelihood bootstrap, LB). A congruence index for testing topological similarity between trees (de Vienne et al., 2007) was calculated to determine whether the two datasets (ITS and cpDNA) differed significantly. To further examine the relationships of chloroplast haplotypes and ITS ribotypes, a median joining network was constructed with Network 5 (Fluxus Technology Ltd.). Polymorphims at variable sites were identified as superimposed nucleotides (additive patterns) by reading the sequence chromatogram in both directions. The analyses were performed assigning a weight 10 for substitutions, 5 for several-nucleotide indels and 1 for differences in number of repeats within microsatellites (e.g. Naciri & Gaudeul, 2007; Cires & Fernández Prieto, 2012). The parameter epsilon was set to its default value (e = 0) in all the analyses. A maximum parsimony (MP) calculation was finally performed to eliminate non-parsimonious links and median vectors (putative haplotypes/ribotypes) from the resulting network, as recommended in the Network's manual.

ITS2 sequences were subjected to secondary structure prediction using tools from the ITS2 database (Koetschan et al., 2012; Ankenbrand et al., 2015). The ITS2 region was identified and delimited based on the Genbank annotation or Hidden Markov Models (HMMs) which was performed through the web server (http://its2.bioapps.biozentrum.uni-wuerzburg.de/). After trimming the 3' and 5' termini of the ribosomal 5.8S and 26S rRNA, the complete ITS2 sequences were aligned with Clustal X (Thompson et al., 1997) and adjusted manually using Geneious v.7.1.3 (www.geneious.com/). ITS2 secondary structures were reconstructed using the 'predict' function of the ITS2 database applying a 75% threshold level for helix transfer. Consensus sequences of *C. adsurgens*, *C. arvatica* and *C. mariae-ceballosiae* were used for the analyses, as well as the type of the genus *Campanula* (i.e. *C. latifolia* L.). In addition, the sequences and structures were also automatically and synchronously aligned with 4SALE 1.7.1 (Seibel et al., 2006, 2008).

Results

The characteristics of nuclear (ITS), plastid regions (*rbcL*, *trnL-F* and *trnS-G*) and combined data are summarized in Table 1. The length of the aligned ITS sequences was 702 bp, with 50 polymorphic sites (7.1%) and a mean GC content of 52.4%. The number of additivities (accessions displaying double peaks of similar height) varied from 1 to 10 (Table S1, Supplementary material). In the case of the combined plastid regions, the length of aligned sequences was 2,363 bp, with a mean GC content of 33.5%, and 486 (20.6%) polymorphic sites within the *Campanula* samples assayed.

Calculation of the congruency index (*Icong*) to assess the existence of topological congruence between nuclear and plastid datasets provides a *P*-value ($P < 0.01$) for the null hypothesis, i.e. two given trees are not more similar than expected by chance ($Icong = 1.2502$). If cpDNA and nrDNA data do indeed reflect different evolutionary histories, their data sets may result in different topologies, and a phylogenetic tree estimated from the simple combined data set would produce an incorrect estimate of the phylogeny or may sometimes represent an oversimplified version of the genetic history. To more accurately reconstruct the phylogeny and evolutionary history of plant groups, the combined analysis of cpDNA and nrDNA data sets should be done with caution, and if incongruence between the data sets exists, it is possible causes should be addressed in detail. Due to the overall phylogenetic incongruence and heterogeneity of the two sequenced datasets, we decided to analyze the data separately but we also provide a combined analysis to get an overview as other previous studies have done within the genus (e.g. Cano-Maqueda et al., 2008).

Phylogenetic trees of sequences of nuclear (ITS) and plastid regions *(rbcL, trnL-F* and *trnS-G*) built by MP and ML analyses (Fig. 2) placed *Campanula mariae-ceballosiae* as an independent lineage within the complex *Campanula arvatica s.l.* with high bootstrap values. This is one of the most significant result in our work, because it confirms that these three taxa (i.e. *C. adsurgens*, *C. arvatica* and *C. mariae-ceballosiae*) are differentiated at both nuclear and chloroplast level. This is especially important because *C. mariae-ceballosiae* is not recognized as a species in global databases (also, *C. adsurgens* is often traced as a synonym of *C. arvatica*). It should be noted that in the case of ITS analysis, 5 samples from the core area of the *C. adsurgens* distribution (i.e. "C. ads 8-12") constitute an independent clade. Phylogenetic trees of concatenated sequences (ITS + cpDNA) built by MP and ML analyses produced similar results (Fig. S1), distinguishing the three taxa studied on a molecular level. On the other hand, the ribotype/haplotype frequencies are given in Table 1.

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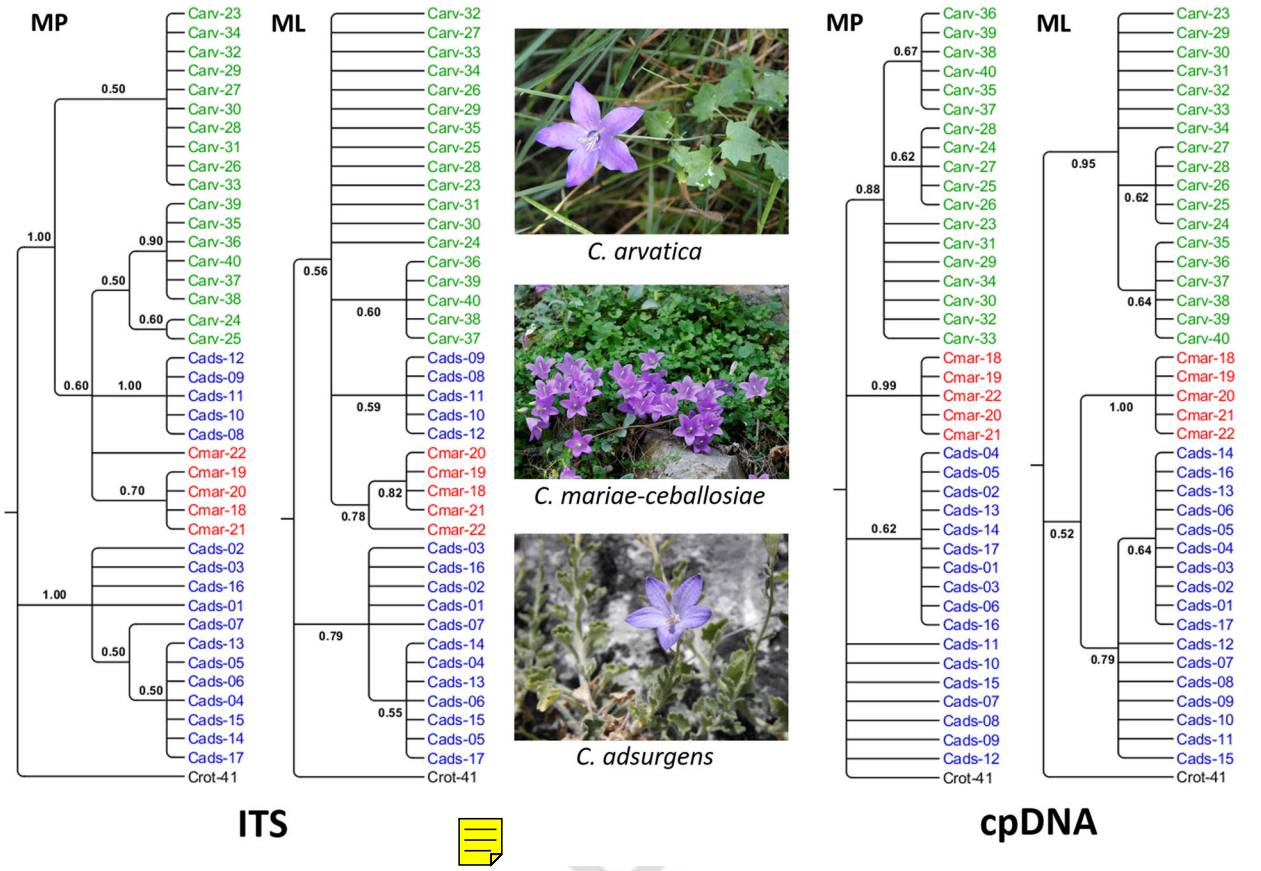

Fig. 2. Maximum parsimony (MP) and maximum-likelihood (ML) analysis of nrDNA (ITS) and plastid DNA (*rbcL*, *trnL-F* and *trnS-G*) regions in *Campanula* gr. *arvatica*. Bootstrap values > 50% are shown above/below the branches. Cads = *C. adsurgens*; Cmar = *C. mariae-ceballosiae*; Carv = *C. arvatica*; Crot = *C. rotundifolia*.

Geographical and network distribution of both datasets are shown in Figs. 3 and 4. Five major groups (A-E) are inferred from the nuclear DNA analysis, including 13 ribotypes, while in the haplotype network three major haplotype groups (formed by two or more haplotypes) are found and related to species assignation. It should be noted that haplotypes from *C. arvartica* are the most diverse and show models of specific geographical distribution.

To identify the effect of the primary sequence divergences, secondary structures of ITS2 were constructed (Fig. 5). All the secondary structures of ITS2 in these species contained a central ring (primary ring) and four similar helices (I, II, III, and IV). The 4SALE alignment resulted in a 51% consensus sequence where the conservation of single base pairs created a robust structure. Single nucleotide polymorphisms (SNPs) occurred in specific helices depending on the species (helices I and II for *C. arvatica*; helix III for *C. mariae-ceballosiae*; helices III and IV for *C. adsurgens*) (Fig. 6). However, ITS2 secondary structures among the different taxa of the complex *Campanula* gr. *arvatica* with respect to the type species (*C. latifolia*) differed significantly in the four helical regions in stem loop number, size, position, and screw angle (Fig. 6).

Discussion

The Iberian flora comprises more than 7000 taxa, approximately 54% of European plant species (Castroviejo, 1986-2018), and the areas with the highest observed species richness (number of species higher than 1600) are located in the Pyrenees and Cantabrian mountains (northern region), and in the Sierra Nevada (southern region). Although richness estimations of the Cantabrian Range showed that regional databases are incomplete (Jiménez-Alfaro, 2009), they permit the estimation of the total floristic richness of the Cantabrian region and 3590 species and subspecies are estimated to occur in this area. According to Aedo et al. (2017), the number of endemic species in the Iberian flora has been estimated at 1258, which is 22.7% of the total native species number. One of the best indicators for

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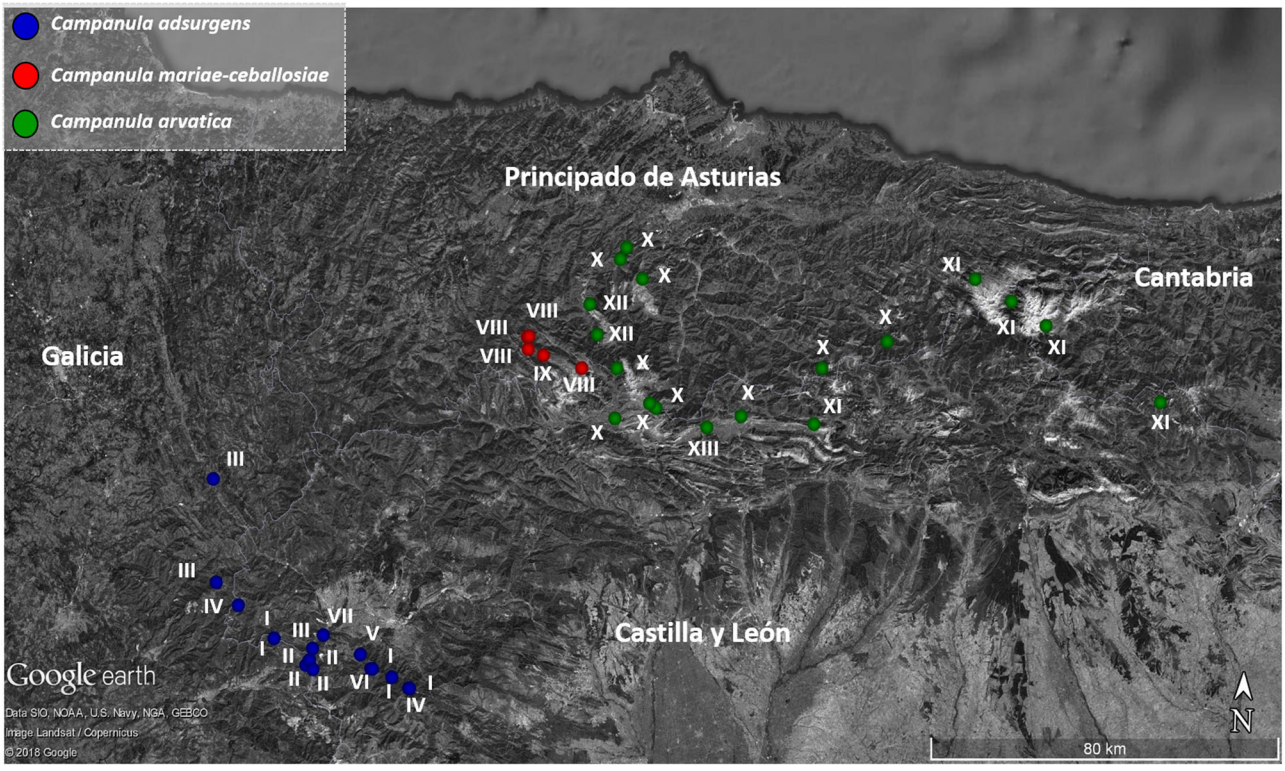

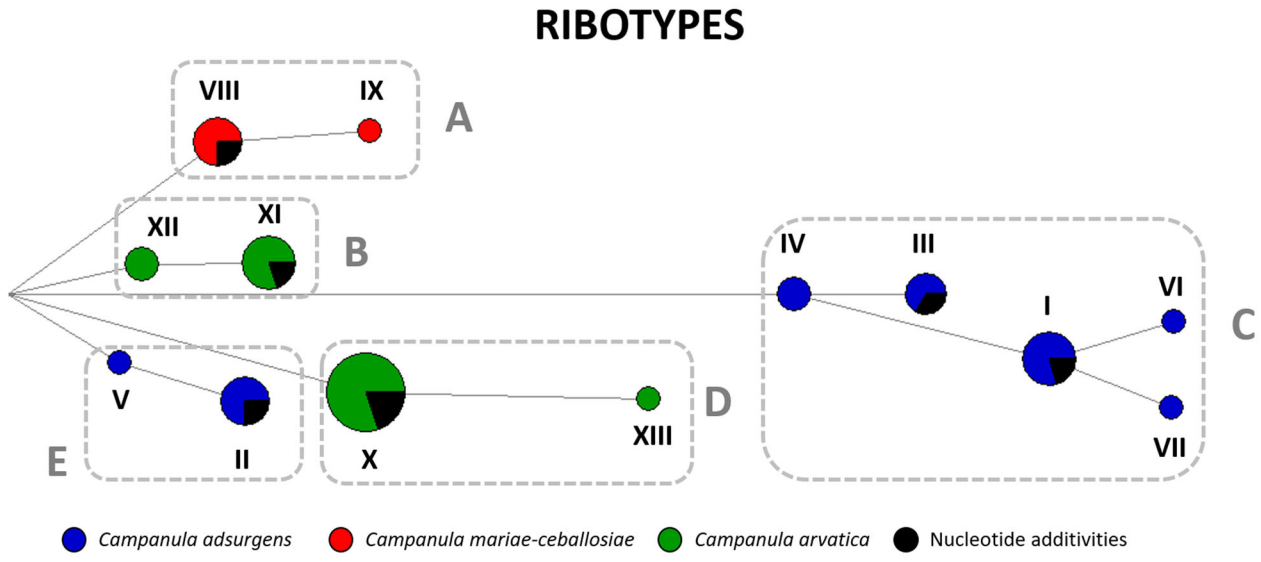

Fig. 3. Geographical distribution and lineage network of 13 ribotypes of *Campanula* gr. *arvatica*. Ribotypes are connected with lines, indicating number of mutations. Colours correspond with the different species analysed and nucleotide additivities (accessions displayed double peaks of similar height). Circles are proportional to the frequency of each ribotype.

determining the distinctiveness of a flora is endemicity. In this aspect, the Iberian Peninsula has endemic and subendemic genera -those who distributions slightly exceeds the Iberian limits (e.g. endemisms: *Gadoria* Güemes & Mota, *Phalacrocarpum* Willk., *Rivasmartinezia* Fern.Prieto & Cires and *Teesdaliopsis* (Willk.) Gand.; for more details see Güemes & Mota, 2017; Nieto Feliner, 1982; Fernández Prieto & Cires, 2014; and www.floraiberica.org, respectively) – (e.g. subendemisms: *Dethawia* Endl., *Petrocoptis* Endl. and *Parapimpinella* Fern.Prieto, Sanna & Arjona).

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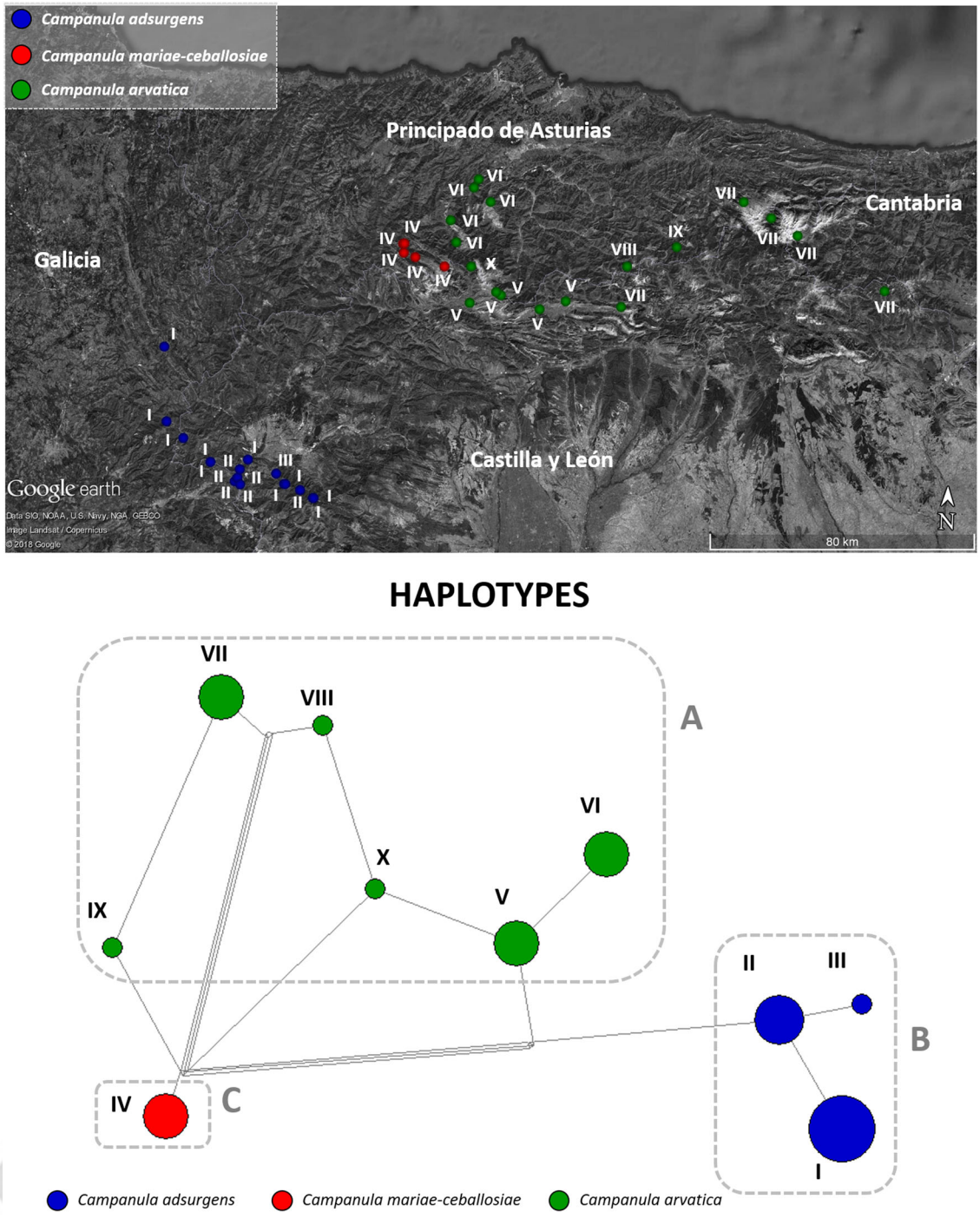

Fig. 4. Geographical distribution and lineage network of 10 haplotypes (plastid data) of *Campanula* gr. *arvatica*. Haplotypes are connected with lines, indicating number of mutations. Colours correspond with the different species analysed. Circles are proportional to the frequency of each haplotype.

In terms of biodiversity in the north of the Iberian Peninsula, one of the most significant contributions of the present study, is the demonstration that within the complex *Campanula arvatica s.l.* there are clearly differentiated groups, supported by robust molecular evidence with pronounced phylogeographical patterns that provide strong support for treatment as different species. Some authors (e.g. Sáez & Aldasoro, 2001)

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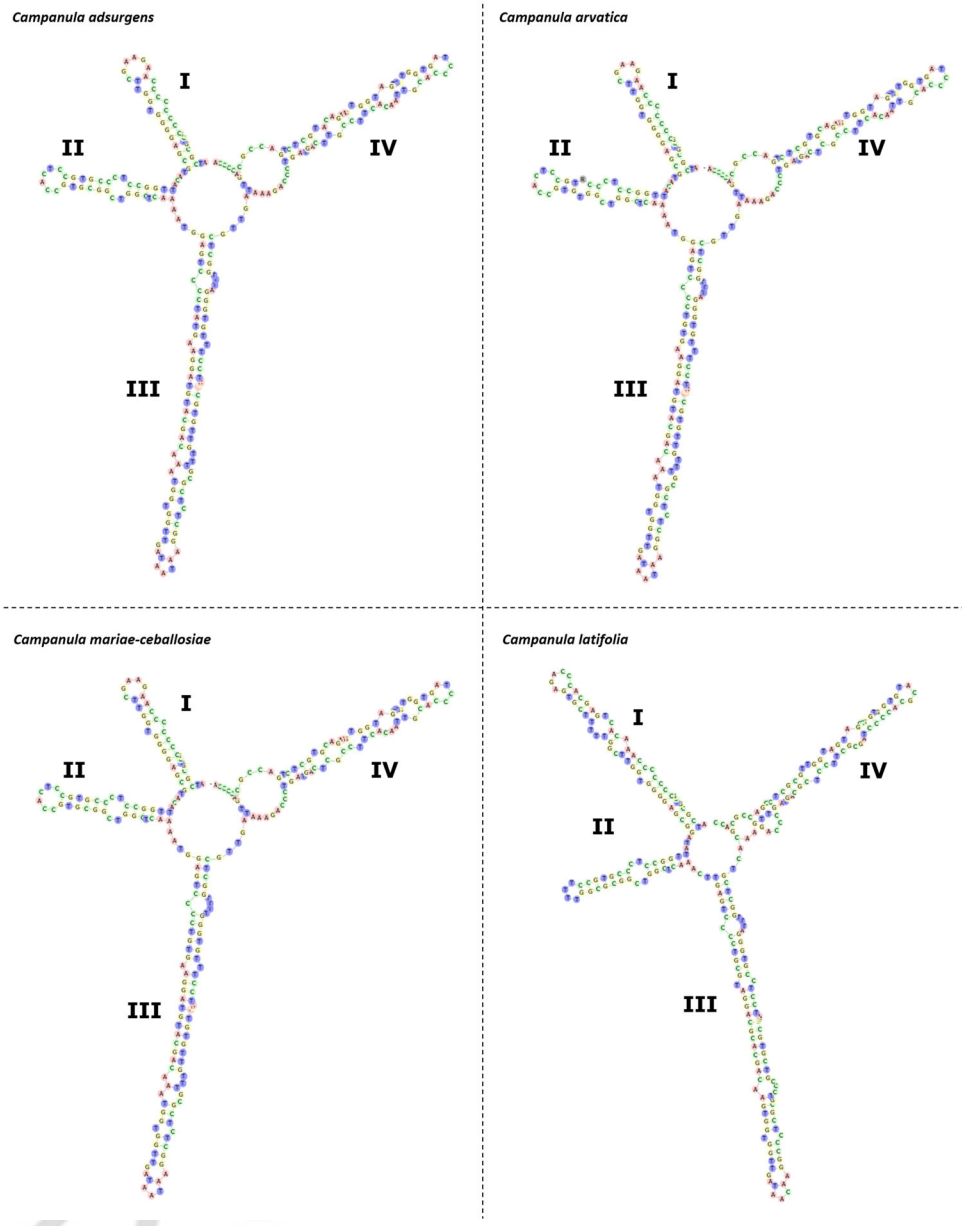

Fig. 5. Secondary structure of different ITS2 types found in *Campanula* gr. *arvatica* compared to the type species (*C. latifolia*).

consider these taxa as separate species: *Campanula arvatica*, distributed in Cantabrian territories such as Palencia, Cantabria and the centre and east of Asturias and León, and *C. adsurgens*, growing in territories of Bierzo (León) and its surroundings (northwest of León and southeast of Galicia (Lugo and Orense)). However, other authors (Damboldt, 1966; Fedorov & Kovanda, 1976; Nieto Feliner, 1985) claim they should be treated as two geographical subspecies: *C. arvatica* subsp. *arvatica* and *C. arvatica* subsp. *adsurgens* (Levier & Leresche) Damboldt. Additionally, Leresche & Levier (1879) described another species from the Picos de Europa within the group: *Campanula acutangula*. Most authors (e.g. Fedorov & Kovanda, 1976; Sáez & Aldasoro, 2001) consider this species as conspecific of *C. arvatica*. However, recent studies (Arjona Rodríguez et al., 2014) have proposed to recognise these populations from the eastern end of the Cantabrian Mountains and the Picos de Europa as *C. arvatica* var. *acutangula* Arjona & Fern.Prieto [incl.: *C. arvatica* fma. *minorifolia* Losa & P.Monts. and fma. *longisepala* Losa & P.Monts.]. From a molecular point of view, the more eastern populations of *C. arvatica* show some exclusive genetic diversity in the var. *acutangula* variety (samples Carv35-40).

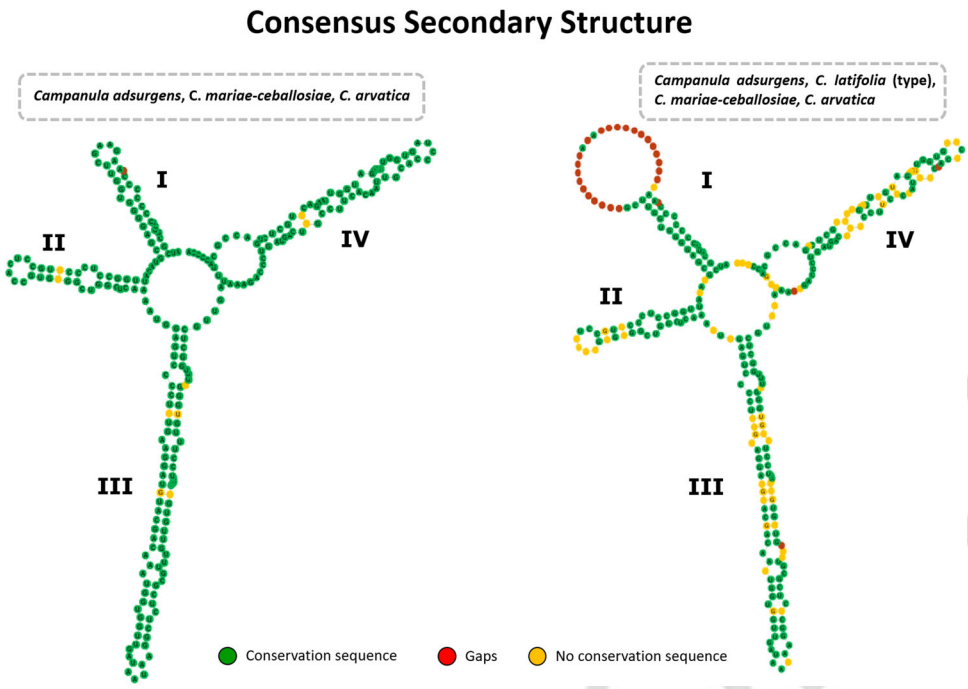

Fig. 6. The predicted ITS2 secondary structure of *Campanula* taxa: consensus secondary structure model of the ITS2 region based on sequences covering the group *Campanula arvatica* (left) and including the comparison with the type species (right). The four helices, each with a stem-loop, are labeled I–IV. Compatible base pairs are colored (green = base pairs conserved, red = insertions/deletions, yellow = base pairs not conserved).

For accurately reconstructing the evolutionary history of plant groups, correct treatment of phylogenetic incongruence is a vital step in the proper analysis of data. The incongruent phylogenetic signal between nuclear and chloroplast markers could be attributed to hybridization events, a common evolutionary force in plants, which can produce disagreement among gene trees based on independent loci (Wendling et al., 2011). With respect to the recently described species, *C. mariae-ceballosiae*, its distribution is limited to the east of Somiedo (Asturias) and inbetween the other two species (i.e. *C. arvatica* and *C. adsurgens*). This taxon was previously identified, as *Campanula arvatica* (Laínz, 1963) or, in other cases, as *C. adsurgens* (Serra & Bueno, 2011). The conflictive placements found for *C. mariae-ceballosiae* in the nuclear and plastid trees (Figs. 2) could indicates a hybrid origin for this member of restricted distribution of the *Campanula* gr. *arvatica* lineage. Therefore, in addition to its intermediate geographical and phylogenetic position, *C. mariae-ceballosiae* also has a morphologically intermediate position which points to its possible hybridogenic origin, a common ev in Campanulacea s reflected in the literature (e.g. Damboldt, 1965; Roquet et al., 2008; Wendling et al. 2011; Röper et al. 2015). Nowadays a central issue in conservation biology is the identification of biodiversity rich areas. In this regard, phylogeographic studies can be very useful in defining evolutionarily significant units for conservation purposes (Moritz, 1994), or even identifying cryptic taxa. The general long-term goal of conservation genetics is to conserve historical genetic variation that may be critical to the long-term evolutionary survival of a species. Therefore, our molecular data from nuclear DNA and plastid regions can be informative for management and should be utilized to maintain the biological diversity of this endemic group from northern Spain.

In recent years, the phylogenetic use of the ITS2 secondary structure has received increasing attention, and many analytical methods and related tools have been proposed (e.g. Feng et al., 2016; Yu et al., 2017; Zhu et al., 2018). Our results confirmed that the ITS2 region can be used as a universal barcode (Han et al., 2013) to distinguish plants from the *C. arvatica* complex. Close inspection of the distribution of mutations in the structure of ITS2 could facilitate the discrimination of true and artefactual polymorphisms. ITS2 structural states contain additional phylogenetic information not found in the primary sequence, so including this information can significantly improve phylogenetic estimates (Keller et al., 2010). For example, ITS2 secondary structure improves discrimination between species when using DNA barcoding (e.g. Zhang et al., 2015). Taking all this information into account, we

recommend including both sequence and secondary structure information together for future studies investigating lower level evolution and infraspecific genetic diversity in plant complexes.

The use of phylogenies in conservation has recently been developed as an effective strategy to maximize diversity in areas of interest (Rolland et al., 2012). According to Faith (1992), phylogenetic diversity is arguably one of the best measures of biodiversity, even better than species richness, and it can be targeted directly in conservation planning (Rodrigues & Gaston, 2002). The evolutionary origins of *Campanula* gr. *arvatica* is an interesting case study to investigate plant evolutionary dynamics in the northwest of the Iberian Peninsula. Although biogeographical and phylogenetic data alone do not provide enough information to fully elucidate the processes driving speciation in the endemic *Campanula* gr. *arvatica*, they allow us to make hypotheses regarding possible drivers of speciation. Considered together, our results make robust inferences about the main mode of speciation in *Campanula adsurgens* and *C. arvatica* as a consequence of allopatric speciation. Our results support the conclusion that geographical separation played a key role in the diversification of these groups. Allopatric speciation does not require divergent selection to act on the ecological characteristics of incipient species, and genetic drift alone can drive speciation (Boucher et al., 2016). A similar scenario has been suggested for many other groups of plants (e.g. Vargas, 2003; Gehrke & Linder, 2011). However, genetic drift need not be the sole driver of speciation and divergent selection on ecological attributes might also be involved in the building of reproductive isolation between incipient species. For example, interactions with pollinators, micro-habitat, or finer characteristics of the substrate used by each species, might have been involved in speciation. Cryptospecies are frequently recognized and described based on DNA sequence data (Bickford et al., 2007), and can also provide valuable information helping to disentangle taxonomically critical groups with potential implications for their conservation (e.g. Molina-Venegas et al., 2017; Roma-Marzio et al., 2017). Genetic distances within traditionally recognized species, often in combination with morphological, geographical and other subtle differences, have revealed cryptic species in most types of organisms and habitats (Bickford et al., 2007), and this seems to be the case for the neoendemic (areas of recent local speciation) *C. mariae-ceballosiae.*

Biodiversity scenarios for the 21st century predict a significant reduction in mountain habitats and the loss of many high mountain plants due to climate change and the impact of human activity. The EU Biodiversity Strategy 2020 aims to halt the loss of biodiversity and ecosystem services in the EU and help stop global biodiversity loss. In this sense, the Cantabrian Range constitutes the principal geomorphological feature of the north of the Iberian Peninsula and presents a series of singularities that make this area an excellent place of refuge for plant biodiversity. For example, the Cantabrian Mountains include areas of high botanical value as a refuge for alpine or Mediterranean species, which represent excellent models to evaluate the effect of climate changes on populations and communities of plants (Barquín Ortiz et al., 2018). There are several causes connected to the presence of high number of endemic species in the Cantabrian Mountains (e.g. *Campanula mariae-ceballosiae*; *Centaurium somedanum* M.Lainz; *Cytisus dieckii* (Lange) Fern.Prieto, Nava, Fern.Casado, M.Herrera, Bueno Sánchez, Sanna & Cires; *Rivasmartinezia vazquezii* Fern.Prieto & Cires; *Saxifraga babiana* T.E.Díaz & Fern.Prieto): (i) the altitudinal range that exceeds 2000 meters, (ii) the annual average rainfall ranging from 1000 to 2000 L/m^2, (iii) the lithology in its western sector corresponds to Palaeozoic siliceous and Precambrian rocks from the former Hercynian range, co-existing with carboniferous calcareous rocks in the central range and pure calcareous rocks scattered in the centre and the eastern part (Picos de Europa), and (iv) this area is the boundary with the Mediterranean region. Given that climatic change has been recognised as the most pervasive threat to biodiversity (Malcolm et al., 2006; Bellard et al., 2014), conservation planners should pay particular attention to preserve areas retaining relatively older/endemic phylogenetic lineages in the Cantabrian Mountains. Therefore, to determine future priorities and strategies for floristic conservation in the north of the Iberian Peninsula it is necessary to incorporate current knowledge about Cantabrian flora. Molecular evidence indicates significant differences among the three taxa analysed and shows the importance of the Cantabrian Mountains as a refuge for endemic Iberian flora in which processes of neo-speciation and/or crypto-speciation are under way.

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No potential conflict of interest was reported by the authors.

Supplemental data

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References

Aedo, C., Buira, A., Medina, L., & Fernández-Albert, M. (2017). The Iberian vascular flora: Richness, endemicity and distribution patterns. In J. Loidi (Ed.), *The vegetation of the Iberian Peninsula. Plant and vegetation* (Vol 12, pp. 101–130). Springer.

Álvarez García, M. A., Farias Arquer, P., Fernánfez Prieto, J. A., & Marquínez García, J. (Eds.) (2007). Prioridades de conservación de la flora cantábrica. *Naturalia Cantabricae*, *3*, 1–101.

Ankenbrand, M. J., Keller, A., Wolf, M., Schultz, J., & Förster, F. (2015). ITS2 database V: Twice as much. *Molecular Biology and Evolution*, *32*(11), 3030–3032. https://doi.org/10.1093/molbev/msv174

Arjona Rodríguez, J. M. (2012). *Filogeografía de dos endemismos cantábricos del género Campanula L. (Campanulaceae Juss.): C. adsurgens Lange ex Boiss. & Levier y C. arvatica Lag. Tesis de Licenciatura*. Facultad de Biología. Universidad de Oviedo.

Arjona Rodríguez, J. M. (2013). *Diversidad, filogeografía y sistemática de Campanula gr. arvatica. Trabajo fin de Master Biotecnología Aplicada a la Conservación y Gestión Sostenible de Recursos Vegetales*. Oviedo.

Arjona Rodríguez, J. M., Sanna, M., Cires Rodríguez, E., & Fernández Prieto, J. A. (2014). Una nueva especie del grupo *Campanula arvatica* Lag. In J. A. Fernández Prieto, V. M. Vázquez, A. Bueno Sánchez, & E. Cires Rodríguez (Eds.), *Notas corológicas, sistemáticas y nomenclaturales para el catálogo de la Flora Vascular del Principado de Asturias. II* (pp. 280–281). Documentos del Jardín Botánico Atlántico 11.

Barquín Ortiz, J., Álvarez-Martínez, J. M., Jiménez-Alfaro, B., García, D., García, D., Serrano, E., González-Díez, A., Tejón, S., de Luis Calabuig, E., Taboada, Á., Purroy, F. J., Del Jesus, M., Naves, J., Fernández-Gil, A., Serdio, Á., Lucio, A., Suárez, R., & Araujo, J. (2018). La integración del conocimiento sobre la Cordillera Cantábrica: hacia un observatorio inter-autonómico del cambio global. *Ecosistemas*, *27*(1), 96–104. https://doi.org/10.7818/ECOS.1422

Bellard, C., Leclerc, C., Leroy, B., Bakkenes, M., Veloz, S., Thuiller, W., & Courchamp, F. (2014). Vulnerability of biodiversity hotspots to global change. *Global Ecology and Biogeography*, *23*(12), 1376–1386. https://doi.org/10.1111/geb.12228

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K., & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, *22*(3), 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Boucher, F. C., Zimmermann, N. E., & Conti, E. (2016). Allopatric speciation with little niche divergence is common among alpine Primulaceae. *Journal of Biogeography*, *43*(3), 591–602. https://doi.org/10.1111/jbi.12652

Brooks, T. M., Mittermeier, R. A., da Fonseca, G. A. B., Gerlach, J., Hoffmann, M., Lamoreux, J. F., Mittermeier, C. G., Pilgrim, J. D., & Rodrigues, A. S. L. (2006). Global biodiversity conservation priorities. *Science (New York, N.Y.)*, *313*(5783), 58–61. https://doi.org/10.1126/science.1127609

Cano-Maqueda, J., & Talavera, S. (2011). A taxonomic revision of the *Campanula lusitanica* complex (Campanulaceae) in the Western Mediterranean region. *Anales del Jardín Botánico de Madrid*, *68*(1), 15–47. https://doi.org/10.3989/ajbm.2274

Cano-Maqueda, J., Talavera, S., Arista, M., & Catalán, P. (2008). Speciation and biogeographical history of the *Campanula lusitanica* complex (Campanulaceae) in the Western Mediterranean region. *TAXON*, *57*(4), 1252–1266. https://doi.org/10.1002/tax.574016

Cañadas, E. M., Fenu, G., Peñas, J., Lorite, J., Mattana, E., & Bacchetta, G. (2014). Hotspots within hotspots: Endemic plant richness, environmental drivers, and implications for conservation. *Biological Conservation*, *170*, 282–291. https://doi.org/10.1016/j.biocon.2013.12.007

Castroviejo, S. (coord. gen.). (1986-2018). *Flora iberica Real Jardín Botánico*. CSIC.

Cires, E., & Fernández Prieto, J. A. (2012). The Iberian endemic species *Ranunculus cabrerensis* Rothm.: an intricate history in the *Ranunculus parnassiifolius* L. polyploid complex. *Plant Systematics and Evolution*, *298*(1), 121–138. https://doi.org/10.1007/s00606-011-0529-9

Damboldt, J. (1965). *Campanula tommasiniana* Koch und *C. waldsteiniana* R. et S.: Zur Taxonomie zweier mediterraner Relikstippen. *Österreichische Botanische Zeitschrift*, *112*(3), 392–406. https://doi.org/10.1007/BF01372959

Damboldt, J. (1966). Zur Systematik und Cylologie von *Campanula arvatica* Lagasca. *Berichte Der Deutschen Botanischen Gesellschaft*, *79*, 299–308.

de Vienne, D. M., Giraud, T., & Martin, O. C. (2007). A congruence index for testing topological similarity between trees. *Bioinformatics (Oxford, England)*, *23*(23), 3119–3124. https://doi.org/10.1093/bioinformatics/btm500

Deyuan, H., Lammers, T. G., & Klein, L. L. (2011). Campanulaceae L. *Flora China*, *19*, 505–563.

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological Conservation*, *61*(1), 1–10. https://doi.org/10.1016/0006-3207(92)91201-3

Fedorov, A. A., & Kovanda, M. (1976). *Campanula* L. In G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, & D. A. Webb (Eds.), *Flora*

Europaea., Vol. 4. (pp. 74–93). Cambridge University Press.

Feng, S., Jiang, M., Shi, Y., Jiao, K., Shen, C., Lu, J., Ying, Q., & Wang, H. (2016). Application of the ribosomal DNA ITS region of *Physalis* (Solanaceae): DNA barcoding and phylogenetic study. *Frontiers in Plant Science*, *7*, 1047https://doi.org/10.3389/fpls.2016.01047

Fernández Prieto, J. A., Arjona, J. M., Sanna, M., Pérez, R., & Cires, E. (2013). Phylogeny and systematics of *Micranthes* (Saxifragaceae): an appraisal in European territories. *Journal of Plant Research*, *126*(5), 605–611. https://doi.org/10.1007/s10265-013-0566-2

Fernández Prieto, J. A., & Cires, E. (2014). Phylogenetic placement of *Dethawia*, *Meum*, and *Rivasmartinezia* (Apioideae, Apiaceae): evidence from nuclear and plastid DNA sequences. *Plant Biosystems - an International Journal Dealing with All Aspects of Plant Biology*, *148*(5), 975–987. https://doi.org/10.1080/11263504.2013.819818

Fernández Prieto, J. A., Cires Rodríguez, E., Bueno Sánchez, A., Vázquez, V. M., & Nava Fernández, H. S. (2014). Catálogo de las plantas vasculares del Principado de Asturias. *Documentos del Jardín Botánico Atlántico (Gijón)*, *11*, 7–267.

Fernández Prieto, J. A., Vázquez, V. M., Bueno Sánchez, A., Cires Rodríguez, E., & Nava Fernández, H. S. (Eds.) (2017). Notas corológicas, sistemáticas y nomenclaturales para el catálogo de la Flora Vascular del Principado de. *Asturias. III. Naturalia Cantabricae*, *5*, 1–41.

García Aloy, S. (2017). *Biogeografia i evolució de campanulàcies i geraniàcies en biomes àrids i mediterranis d'Àfrica* [Tesi. Doctorat]. Universitat Autònoma de Barcelona.

Gehrke, B., & Linder, H. P. (2011). Time, space and ecology: why some clades have more species than others. *Journal of Biogeography*, *38*(10), 1948–1962. https://doi.org/10.1111/j.1365-2699.2011.02544.x

Güemes, J., & Mota, J. F. (2017). *Gadoria* (Antirrhineae, Plantaginaceae): A new genus, endemic from Sierra de Gádor, Almería, Spain. *Phytotaxa*, *298*(3), 201–221. https://doi.org/10.11646/phytotaxa.298.3.1

Hamilton, M. (1999). Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology*, *8*(3), 521–523.

Han, J., Zhu, Y., Chen, X., Liao, B., Yao, H., Song, J., Chen, S., & Meng, F. (2013). The short ITS2 sequence serves as an efficient taxonomic sequence tag in comparison with the full-length ITS. *BioMed Res Int*, *2013*, 741476https://doi.org/10.1155/2013/741476

Jiménez-Alfaro, B. (2009). Evaluación del conocimiento florístico de la Cordillera Cantábrica a partir de bases de datos de Biodiversidad. *Pirineos*, *164*(0), 117–133. https://doi.org/10.3989/pirineos.2009.v164.32

Jones, K. E., Korotkova, N., Petersen, J., Henning, T., Borsch, T., & Kilian, N. (2017). Dynamic diversification history with rate upshifts in Holarctic bell-flowers (*Campanula* and allies). *Cladistics*, *33*(6), 637–666. https://doi.org/10.1111/cla.12187

Keller, A., Förster, F., Müller, T., Dandekar, T., Schultz, J., & Wolf, M. (2010). Including RNA secondary structuresimproves accuracy and robustness in reconstruction of phylogenetic trees. *Biology Direct*, *5*, 4. https://doi.org/10.1186/1745-6150-5-4

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution, *16*(2), 111–120. https://doi.org/10.1007/BF01731581

Koetschan, C., Hackl, T., Müller, T., Wolf, M., Förster, F., & Schultz, J. (2012). ITS2 database IV: interactive taxon sampling for internal transcribed spacer 2 based phylogenies. *Molecular Phylogenetics and Evolution*, *63*(3), 585–588. https://doi.org/10.1016/j.ympev.2012.01.026

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Lakušić, D., Liber, Z., Nikolić, T., Surina, B., Kovačić, S., Bogdanović, S., & Stefanović, S. (2013). Molecular phylogeny of the *Campanula pyramidalis* species complex (Campanulaceae) inferred from chloroplast and nuclear non-coding sequences and its taxonomic implications. *Taxon*, *62*(3), 505–524. https://doi.org/10.12705/623.1

Lammers, T. G. (2007). Campanulaceae. In K. Kubitzki (Eds.), *The families and genera of vascular plants: Flowering Plants: Eudicots, Asterales*. Vol. VIII. (pp. 26–59). Springer Verlag.

Laínz, M. (1963). Aportaciones al conocimiento de la flora cántabro-astur, VII. *Boletín del Instituto de Estudios Asturianos*. *Suplemento de Ciencias*, *7*, 35–81.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics (Oxford, England))*, *23*(21), 2947–2948. https://doi.org/10.1093/bioinformatics/btm404

Leresche, L., & Levier, E. (1879). Decas plantarum novarum in Hispania collectarum. *The Journal of Botany*, *17*, 196–201.

Losa, M. T., & Montserrat, P. (1953). Nueva aportación al estudio de la flora de los montes cántabro-leones. *Anales del Instituto Botánico A. J. Cavanilles*, *11*, 385–462.

Malcolm, J. R., Liu, C., Neilson, R. P., Hansen, L., & Hannah, L. (2006). Global warming and extinctions of endemic species from biodiversity hotspots. *Conservation Biology*, *20*(2), 538–548. https://doi.org/10.1111/j.1523-1739.2006.00364.x

Mansion, G., Parolly, G., Crowl, A. A., Mavrodiev, E., Cellinese, N., Oganesian, M., Fraunhofer, K., Kamari, G., Phitos, D., Haberle, R., Akaydin, G., Ikinci, N., Raus, T., & Borsch, T. (2012). How to handle speciose clades? Mass taxon-sampling as a strategy towards illuminating the natural history of *Campanula* (Campanuloideae). *Plos One*, *7*(11), e50076https://doi.org/10.1371/journal.pone.0050076

Marchese, C. (2015). Biodiversity hotspots: A shortcut for a more complicated concept. *Global Ecology and Conservation*, *3*, 297–309. https://doi.org/10.1016/j.gecco.2014.12.008

McIntosh, L., Poulsen, C., & Bogorad, L. (1980). Chloroplast gene sequence for the large subunit of ribulose bisphosphate carboxylase of maize. *Nature*, *288*(5791), 556–560. https://doi.org/10.1038/288556a0

Molina-Venegas, R., Aparicio, A., Lavergne, S., & Arroyo, J. (2017). Climatic and topographical correlates of plant palaeo- and neoendemism in a Mediterranean biodiversity hotspot. *Annals of Botany*, *119*(2), 229–238. https://doi.org/10.1093/aob/mcw093

Moritz, C. (1994). Defining 'Evolutionarily Significant Units' for conservation. *Trends in Ecology & Evolution*, *9*(10), 373–375. https://doi.org/10.1016/0169-5347(94)90057-4

Myers, N. (1988). Threatened biotas: "hot spots" in tropical forests. *The Environmentalist*, *8*(3), 187–208. https://doi.org/10.1007/BF02240252

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A., & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature*, *403*(6772), 853–858. https://doi.org/10.1038/35002501

Naciri, Y., & Gaudeul, M. (2007). Phylogeography of the endangered *Eryngium alpinum* L. (Apiaceae) in the European Alps. *Molecular Ecology*, *16*(13), 2721–2733. https://doi.org/10.1111/j.1365-294X.2007.03269.x

Gutiérrez Larena, B., Fuertes Aguilar, J., & Nieto Feliner, G. (2004). El género *Phalacrocarpum* Willk. (Compositae). *Anales del Jardín Botánico de Madrid*, *61*(1), 53–60. https://doi.org/10.3989/ajbm.2004.v61.i1.64

Nieto Feliner, G. (1985). Estudio crítico de la flora orófila del suroeste de León: Montes Aquilianos, Sierra del Teleno y Sierra de la Cabrera. *Ruizia*, *2*, 1–240.

Rodrigues, A., & Gaston, K. J. (2002). Maximising phylogenetic diversity in the selection of networks of conservation areas. *Biological Conservation*, *105*(1), 103–111. https://doi.org/10.1016/S0006-3207(01)00208-7

Rolland, J., Cadotte, M. W., Davies, J., Devictor, V., Lavergne, S., Mouquet, N., Pavoine, S., Rodrigues, A., Thuiller, W., Turcati, L., Winter, M., Zupan, L., Jabot, F., & Morlon, H. (2012). Using phylogenies in conservation: New perspectives. *Biology Letters*, *8*(5), 692–694. https://doi.org/10.1098/rsbl.2011.1024

Roma-Marzio, F., Najar, B., Alessandri, J., Pistelli, L., & Peruzzi, L. (2017). Taxonomy of prickly juniper (*Juniperus oxycedrus* group): A phytochemical-morphometric combined approach at the contact zone of two cryptospecies. *Phytochemistry*, *141*, 48–60. https://doi.org/10.1016/j.phytochem.2017.05.008

Röper, A. C., Lütken, H., Christensen, B., Boutilier, K., Petersen, K. K., & Müller, R. (2015). Production of interspecific *Campanula* hybrids by ovule culture: exploring the effect of ovule isolation time. *Euphytica*, *203*(3), 64[illegible]7. https://doi.org/10.1007/s10681-014-1296-9

Roquet, C. (2008). *Evolució, sistemàtica i biogeografia de Campanula L. i relacions filogenètiques amb gèneres afins de Campanulàcies* [Tesi. Doctorat]. Universitat Autònoma de Barcelona.

Roquet, C., Sáez, L., Aldasoro, J. J., Susanna, A., [illegible]cón, M. L., & Garcia-Jacas, N. (2008). Natural del[illegible]ion, molecular phylogeny and floral evolution in Campanula. *Systematic Botany*, *33*(1), 203–217. https://doi.org/10.1600/036364408783[illegible]65

Sáez, L., Aldasoro, J. J. (2001). *Campanula* L. In J. Paiva, F. Sales, I. C. Hedge, C. Aedo, J. J. Aldasoro, … M. Velayos (Eds.), *Flora Ibérica*. (Vol. *XIV*, pp. 105–136). CSIC.

Schierenbeck, K. A. (2017). Population-level genetic variation and climate change in a biodiversity hotspot. *Annals of Botany*, *119*(2), 215–228. https://doi.org/10.1093/aob/mcw214

Serrano, E., González Trueba, J. J., Pellitero, R., González García, M., & Gómez Lende, M. (2013). Quaternary glacial evolution in the Central Cantabrian Mountains (Northern Spain). *Geomorphology*, *196*, 65–82. https://doi.org/10.1016/j.geomorph.2012.05.001

Seibel, P. N., Müller, T., Dandekar, T., Schultz, J., & Wolf, M. (2006). 4SALE - A tool for synchronous RNA sequence and secondary structure alignment and editing. *BioMed Central Bioinformatics*, *7*, 498.

Seibel, P. N., Müller, T., Dandekar, T., & Wolf, M. (2008). Synchronous visual analysis and editing of RNA sequence and secondary structure alignments using 4SALE. *BMC Research Notes*, *1*, 91. https://doi[illegible]10.1186/1756-0500-1-91

Serra, L., & Bueno, A. (201[illegible] *Campanula adsurgens*, novedad para Asturias. *Flora Montibérica*, *47*, 27–28.

Sun, Y., Skinner, D. Z., Liang, G. H., & Hulbert, S. H. (1994). Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. *TAG. Theoretical and Applied Genetics. Theoretische und angewandte Genetik*, *89*(1), 26–32. https://doi.org/10.1007/BF00226978

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, *17*(5), 1105–1109. https://doi.org/10.1007/BF00037152

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, *25*(24), 4876–4882. https://doi.org/10.1093/nar/25.24.48[illegible]

Vargas, P. (2003). Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe. *TAXON*, *52*(3), 463–476. https://doi.org/10.2307/3647383

Wendling, B. M., Galbreath, K. E., & DeChaine, E. G. (2011). Resolving the evolutionary history of *Campanula* (Campanulaceae) in Western North America. *Plos One*, *6*(9), e23559. https://doi.org/10.1371/journal.pone.0023559

Yu, N., Wei, Y., Zhang, X., Zhu, N., Wang, Y., Zhu, Y., Zhang, H., Li, F., Yang, L., Sun, J., & Sun, A. (2017). Barcode ITS2: A useful tool for identifying *Trachelospermum jasminoides* and a good monitor for medicine market. *Scientific Reports*, *7*(1), 5037. https://doi.org/10.1038/s41598-017-04674-w

Zhang, W., Yuan, Y., Yang, S., Huang, J., & Huang, L. (2015). ITS2 secondary structure improves discrimination between medicinal "Mu Tong" species when using DNA Barcoding. *PLoS One*, *10*(7), e0131185. https://doi.org/10.1371/journal.pone.0131185

Zhu, S., Li, Q., Chen, S., Wang, Y., Zhou, L., Zeng, C., & Dong, J. (2018). Phylogenetic analysis of *Uncaria* species based on internal transcribed spacer (ITS) region and ITS2 secondary structure. *Pharmaceutical Biology*, *56*(1), 548–558. https://doi.org/10.1080/13880209.2018.1499780

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