Plastid phylogeography of *Delphinium fissum* subsp. *sordidum* and the series *Fissa* (Ranunculaceae) in the Iberian Peninsula: implications for conservation

Rubén Ramírez-Rodríguez[1*], Juan Francisco Jiménez[2], Francisco Amich[1] & Pedro Sánchez-Gómez[3]

[1] *Departamento de Botánica y Fisiología Vegetal, Universidad de Salamanca, Salamanca, Spain*

[2] *Departamento de Botánica, Universidad de Granada, Granada, Spain*

[3] *Departamento de Biología Vegetal (Botánica), Universidad de Murcia, Murcia, Spain*

*Corresponding Author: Rubén Ramírez-Rodríguez; Departamento de Botánica y Fisiología Vegetal, Universidad de Salamanca, Salamanca, Spain. E-mail: rubenrr@usal.es

Abstract

We study the phylogeography of the series *Fissa* species in the Iberian Peninsula. This group constitutes a complex formed by three Iberian endemic and threatened taxa with restricted distribution ranges and relatively small population sizes. We amplify and sequence two plastid regions, *trn*S-*trn*G and *psb*A-*trn*H from 15 sampling locations (n = 45). A total of 15 haplotypes are detected. The median-joining phylogenetic network shows two main haplotype lineages: one western corresponding to the western taxon, *D. fissum* subsp. *sordidum*, and the other eastern corresponding to eastern taxa *D. bolosii* and *D. mansanetianum*. AMOVA analysis with 94.32% of the total variation and SAMOVA analysis with a Fct value of 0.943 support the presence of these well-defined lineages. We report high values of total gene diversity (h_T = 0.959). Haplotype diversity within populations is low (h_S = 0.311). On the contrary, differentiation among populations (G_{ST} = 0.675) and Fixation Index (F_{ST} = 0.989) are high. These values mean that gene flow among populations is limited. Estimation of divergence times show that the split between *D. fissum* subsp. *sordidum* and the two other taxa (*D. bolosii* and *D. mansanetianum*) took place during the transition between Pliocene and Pleistocene (approximately 2.67 Ma). Negative Tajima's D-values test (Tajima's D = -0.108, P ≥ 0.1) and unimodal mismatch distribution suggest expansion range for *D. fissum* subsp. *sordidum*. We advocate to draw up recovery plans for series *Fissa* species in the Iberian Peninsula, especially for *D. fissum* subsp. *sordidum*.

Keywords: *Delphinium* series *Fissa*; genetic diversity; cpDNA; haplotype network; divergence time estimation; conservation genetics; biodiversity conservation

Introduction

Delphinieae Warming (Ranunculaceae L.) is a tribe mainly of holarctic distribution, ranging from the Mediterranean basin to Korea, Japan, Siberia and North America, with a few perennial species occurring in the tropics: South India (Billore and Singh 1972) and West and East tropical Africa (Milne-Redhead and Turrill 1952; Chartier et al. 2016). This tribe comprises four genera –*Delphinium* L. (included *Consolida* (DC.) S.F. Gray and *Aconitella* Spach), *Aconitum* L., *Staphisagria* J. Hill and *Gymnaconitum* (Stapf) Wei Wang & J.D. Chen− and 650-700 species that correspond to approximately 25% of all Ranunculaceae species (Tamura 1993). Its center of origin is found in South-western China and the Eastern Himalayas, dispersing into the Western Mediterranean basin during the Messinian Salinity Crisis following putative migration patterns pointed out by Bocquet, Wilder, and Kiefer (1978). As a result of such shifts, a few species colonised the Iberian Peninsula, one of the extremes of its distribution range (Blanché 1991).

The series *Fissa* B. Pawl of the genus *Delphinium* is a group with oriental affinities that consists of three endemic and perennial taxa in the Iberian Peninsula. (1) *Delphinium fissum* subsp. *sordidum* (Cuatrec.) Amich, E. Rico & J. Sánchez has the broadest distribution area and is found mainly in the central-western Iberian Peninsula, with a disjunct population in Sierra Mágina (Jaén Province). The subspecies is included in the Red List of Spanish Vascular Flora 2008 (Bañares et al. 2008) under the category EN B2ab(v)c(iv); C2b. At regional level, this subspecies is protected in four Autonomous Communities, with the category “in danger of extinction” in Castile & Leon (BOCYL 2007) and Castile-La Mancha (DOCM 2001), “Special Interest” in Extremadura (DOE 2001) and “Vulnerable” in Andalusia (BOJA 2012). (2) *Delphinium bolosii* C. Blanché & Molero is endemic to Catalonia and is categorized as EN B1ab(iii,iv,v) + 2ab(iii,iv,v); C1 in Bañares et al. (2008) and “in danger of extinction” in the catalogue of threatened flora of Catalonia (DOGC 2008). (3) *Delphinium mansanetianum* Pitarch, Peris & Sanchis has the narrowest distribution area with only one small population in the locality of Mosqueruela (Teruel Province) containing a low number of reproductive individuals. It has not yet been included in any red list (Pitarch García 2002).

The taxonomic key for the identification of this group is based on a combination of morphological and cytogenetic characters (Blanché and Molero 1986; Pitarch García 2002) but this is not sufficient to clearly differentiate the species of the series *Fissa*. Molecular data may be useful to unravel the historical processes and evolutionary relationships between them. Until now, genetic studies about the series *Fissa* species have been limited. The methodology used was based on allozymes (Orellana et al. 2007) or a combination of allozymes and chloroplast DNA (cpDNA) markers (López-Pujol et al. 2014; Bosch et al. 2019). Since then, new scientific data are available. As such, the number of known populations has been increased for *D. bolosii* (Blanché and Bosch 2015) and *D. fissum* subsp. *sordidum* (Ramírez-Rodríguez and Amich 2014; Ramírez-Rodríguez et al. 2016, 2017). A more complete phylogeographic study should provide further insights into the phylogeny of the series *Fissa* species using cpDNA sequencing, a molecular marker characterized by its maternal inheritance, absence of recombination, and high level of genetic diversity (Wheeler et al. 2014).

The main aim of this study was to shed more light on the phylogeography and evolutionary history of the series *Fissa* in the Iberian Peninsula using cpDNA markers. We aimed to: (1) infer the phylogenetic relationships and phylogeographical patterns; (2) provide new data about the unclear taxonomic position of the Iberian System populations; and (3) propose conservation measures and strategies, prioritising in those populations with a unique genetic constitution and/or high genetic diversity, taking into account their current conservation status.

Materials and methods

Sampling and DNA extraction

We sampled 45 individuals from a total of 15 locations (three individuals per location): *Delphinium fissum* subsp. *sordidum* (9), *D. bolosii* (5) and *D. mansanetianum* (1) (Table 1; Figure 1). Wherever possible, transects made in collecting specimens were sufficiently far apart to avoid sampling very closely related individuals. Only 1 or 2 leaves were collected to avoid damaging specimens.

DNA was extracted from silica gel dried leaves using the 2 x cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). Total DNA extracts were quantified using a Nanodrop 2000 spectrophotometer. Two plastid regions, *trn*S-*trn*G and *psb*A-*trn*H, were

amplified and sequenced using the primers *trn*S (GCU)-*trn*G (UCC) (Hamilton 1999), *psb*A3_f (Sang, Crawford, and Stuessy 1997) and *trn*Hf_05 (Tate and Simpson 2003). Polymerase chain reactions were conducted in 50 µl volumes containing approximately 20 ng of genomic DNA, 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 2 units of *Taq* polymerase (Biotools, Madrid, Spain), the buffer provided by the manufacturer, oligonucleotide primers at a final concentration of 0.4 mM and ddH_2O to the final volume. They were performed in an Eppendorf Mastercycler using the following program: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 s, 52 ºC for 30 s, 1 min at 72 ºC, with a single final extension step of 72ºC for 8 min. Amplified products were sent to Macrogen Inc. (Korea) for sequencing. Two ml of the amplification products were visualized on a 1.5% agarose gel, and successful amplifications were cleaned with a GenElute PCR clean-up kit (Sigma-Aldrich). The sequencing was performed with the BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, California) using amplification primers. Finally, amplified products were analysed on an ABI automated sequencer using the Sanger method.

Haplotype network analysis

All sequences were aligned with Clustal-W and then checked manually. GenBank accession numbers are listed in Table S1. The evolutionary relationships between haplotypes and concordance with taxonomic treatment were assessed by constructing a Median Joining network (Bandelt, Forster, and Röhl 1999) using the Pop Art 1.7 software (Leigh and Bryant 2015).

Population genetic analyses

For population genetic analyses, population subdivision was first performed using SAMOVA 1.0 to define groups of locations that are geographically homogeneous and genetically differentiated from each other (Dupanloup, Schneider, and Excoffier 2002). Parameters of within-population diversity (h_S), total gene diversity (h_T), and genetic differentiation (G_{ST}) at species and group levels, as well as those of population subdivision for phylogenetically ordered alleles (N_{ST}), and the fixation index (F_{ST}) were estimated for the whole set of haplotypes and the groups depicted by SAMOVA. These parameters were calculated with PERMUT (Pons and Petit 1996). The mean of the permutated values is used to test if the observed N_{ST} value is larger than G_{ST}. The test is significant when less than 5% of permutated values is less than the observed value of N_{ST}. DnaSP v.5 (Librado and Rozas

2009) estimated the molecular diversity, including the number of segregating sites (S), number of haplotypes (N_H), haplotype diversity (h_D), and nucleotide diversity (Pi). Genetic structure analysis was performed using analysis of molecular variance (AMOVA) to estimate differentiation within and among populations and among the subdivisions which had been detected (Excoffier, Smouse, and Quattro 1992). These analyses were performed using the program ARLEQUIN v.3.5 (Excoffier and Lischer 2009), with significance tests by 10,000 permutations.

Population history dynamic analyses

Neutrality tests to estimate Tajima's D (Tajima 1989) and Fu and Li's *D* and *F* statistics (Fu and Li 1993) were conducted using DnaSP v.5 (Rozas et al. 2003) to test for evidence of population expansion or selection in the cpDNA. If these values showed a significantly ($P \leq 0.01$) positive or negative value, we could infer that the populations of that species had experienced a bottleneck or range expansion, respectively. Mismatch distribution analysis was performed using DnaSP v.5 based on pairwise nucleotide differences between any two individuals within a group.

Molecular dating estimation

Previous phylogenetic studies have revealed that *Delphinium* subg. *Delphinastrum* has a close relationship with the genus *Consolida* (Jabbour and Renner 2011; Zhang and Zhang 2012). Based on fossil-calibrated molecular dating in a previous study, two reference dates were used to calibrate our molecular clock: (1) the split between *Consolida* and its *Delphinium* sister clade (*Delphinium* subg. *Delphinastrum*) was dated to 21.7 ± 3 Ma (Jabbour and Renner 2011), and (2) the diversification of five major clades in *Consolida* began at approximately 8.8 (6.2 – 11.8) Ma (Jabbour and Renner 2011). For our dating approach, five species of *Delphinium* subg. *Delphinium*, five species of *Consolida*, two species of the *Delphinium naviculare* group and one species of *Aconitum* were used as outgroups (Jabbour and Renner 2011; Zhang and Zhang 2012) (Table S2).

To obtain an estimate of divergence times among cpDNA haplotypes and the substitution rate for the cpDNA regions (*trn*S–*trn*G and *psb*A-*trn*H), a Bayesian relaxed molecular clock approach was implemented in BEAST version 1.8 (Drummond and Rambaut 2007). BEAST was run under the Yule model. We selected the GTR substitution model using empirical base frequencies. A Markov Chain Monte Carlo (MCMC) approach was conducted

153 with a coalescent-based tree estimation due to the recent temporal relationship of the species.
154 MCMC chains were run for 50,000,000 generations, sampling every 1000 generations. The
155 combined parameters were checked in Tracer version 1.4 (Rambaut and Drummond 2007).

156 **Results**

157 *Geographical distribution and phylogenetic relationships of haplotypes*

158 Alignment of the 45 individuals yielded sequences reaching 983 nucleotides (262
159 nucleotides, *psb*A-*trn*H; 721 nucleotides, *trn*S-G). For both sequences 14 mutations and 7
160 insertions or deletions were detected. A total of 15 different haplotypes were identified from
161 15 analysed populations (Table 1; Figure 1). No haplotypes were shared between the studied
162 taxa. The median-joining network revealed two well-defined haplotype lineages (Figure 2).
163 The western group of haplotypes (8) corresponded to *D. fissum* subsp. *sordidum* and the
164 eastern group of haplotypes (7) corresponded to *D. bolosii* and *D. mansanetianum* (Figures 1
165 and 2). Eight populations had only one haplotype, whereas the remaining seven showed two
166 distinct haplotypes. For *D. fissum* subsp. *sordidum*, the most frequent haplotypes were H4 and
167 H5, the latter occupied a central position and is positioned as internal node in the haplotype
168 network, distributed along the Central System, reaching the northern sub-plateau (Figures 1
169 and 2). In the case of *D. bolosii*, the most frequent haplotype was H14 shared by two
170 populations located in the Iberian System (Figures 1 and 2). Interestingly, the haplotypes H2
171 and H3 were, among the haplotypes detected for *D. fissum* subsp. *sordidum*, the closest ones
172 to *D. bolosii* (Figure 2).

173 *Population genetic analyses*

174 SAMOVA revealed the presence of four groups: (1) VDA, RSV, HER, BEJ, MAS,
175 ADR, MSA; (2) VIL, MAG; (3) COR, TOR1, TOR2; and (4) RUB, ULL, MOS. The
176 differentiation among groups (F_{CT}) was 0.943. Within-population gene diversity (h_S) was
177 0.311, whereas genetic differentiation (G_{ST}) was 0.675, and N_{ST} was significantly higher than
178 G_{ST} (0.948), which indicated a phylogeographic structure of the haplotype distribution in both
179 taxa. F_{ST} was 0.989 and h_T showed a value of 0.959 (Table 1). Haplotype diversity (h_D) was
180 0.921 and nucleotide diversity (Pi) 0.00622 (Table 1). When the two different lineages (*D.*
181 *fissum* subsp. *sordidum* vs *D. bolosii* and *D. mansanetianum*) were considered separately, the
182 number of segregating sites (S) were eight and ten. H_D values were 0.847 and 0.858 and Pi
183 values were 0.002 and 0.004, respectively. AMOVA results showed that 94.32% ($P \leq 0.001$)

of the total variation occurred between these two predefined lineages, while only 4.67% of variation was distributed among populations within the groups.

Population history dynamic analyses

When the whole set of haplotypes was considered, neutrality tests displayed positive non significant values (Fu and Li's D = 1.013, Fu and Li's F = 1.011, Tajima's D = 0.542; $P \geq 0.10$). When both tests were applied to the two lineages separately, only Tajima's D test showed negative values, though non significant, for *D. fissum* subsp. *sordidum* (Tajima's D = - 0.108, $P \geq 0.1$), suggesting population expansion. Mismatch analysis pointed to the distribution of pairwise differences being unimodal only for *D. fissum* subsp. *sordidum* populations, suggesting range expansion (Figure 3). The divergence between *D. fissum* subsp. *sordidum* and the two other species (*D. bolosii* and *D. mansanetianum*) occurred during the late Pliocene and early Pleistocene 2.67 Ma ago (95% HPD: 1.19 - 4.51) (Figure 4). The split of haplotypes for *D. fissum* subsp. *sordidum* (1.61 Ma; 95% HPD: 0.55 – 2.93) and *D. bolosii* together with *Delphinium mansanetianum*) (1.52 Ma; 95% HPD: 0.58 – 2.65) began in the early Pleistocene during the Calabrian stage (1.806 - 0.781 Ma). The diversification events took place mainly in the mid-Pleistocene during the Ionian stage (0.781 - 0.126 Ma) (Figure 4).

Discussion

Geographical distribution and phylogenetic relationships of haplotypes

Phylogenetic analysis showed two main haplotype lineages: one western Iberian corresponding to *D. fissum* subsp. *sordidum*, and the other eastern corresponding to *D. bolosii* and *D. mansanetianum*. Likewise, AMOVA and SAMOVA analyses supported these two well-defined lineages, since almost all the variance (94.32%) occurred between them, with a F_{CT} value of 0.943. In this way, our phylogenetic results are in line with the results provided by other phylogenetic studies (Orellana et al. 2007; López-Pujol et al. 2014; Bosch et al. 2019). Another point worth mentioning is the phylogenetic position of the populations located in the Iberian System, including to *D. mansanetianum*, which are genetically more related to *D. bolosii* than *D. fissum* subsp. *sordidum*. Bosch et al. (2019) pointed that *D. mansanetianum* has genetic affinities with the COR population, reported as *D. fissum* subsp. *sordidum* (Mateo Sanz and Pisco García 1993). In contrast, both populations shared the same haplotype with *D. bolosii* populations and the first genetic barrier detected clearly separated to COR population

from the remaining studied populations of *D. fissum* subsp. *sordidum* (see Bosch et al. 2019). To clarify such inconsistency, new morphological and environmental findings, which sustain our genetic results, will be soon published (Ramírez-Rodríguez et al. unpubl.).

The VIL and MAG populations of *D. fissum* subsp. *sordidum* share the haplotype H2 which is phylogenetically close to *D. bolosii* (Figure 2), being a putatively ancient haplotype which could have been fixed by incomplete lineage sorting when a *Delphinium* ancestor diverged into two well-defined lineages. The present disjunct distribution of *D. fissum* subsp. *sordidum* can be explained by a combination of progressive and long-distance dispersal events from the Central System and Iberian System to Sierra Mágina (see also Bosch et al. 2019). The most widespread and dominant haplotypes were H4 and H5, corresponding to *Delphinium fissum* subsp. *sordidum*. The latter occupied a central phylogenetic position within the haplotype network (Figure 2), in accordance with coalescent theory (Fu and Li 1999). A westward migration of *D. fissum* subsp. *sordidum* along the Central System and northward may have ocurred, supported by a negative Tajima's D-values test (Tajima's D = -0.108, $P \geq 0.1$) and unimodal mismatch distribution (Figure 3). In so doing, it reached the region of the Arribes del Duero, which has unique environmental conditions (mesoclimates) caused partly by its complex orography (Calonge Cano 1990). Isolated, the haplotype H4 was preserved in the MAS population. The VDA, ADR and MSA populations, located in peripheral areas of the species range, show exclusive haplotypes (H1, H6 and H7/H8, respectively) positioned as tip nodes in the haplotype network (Figure 2). This is in accordance with recent colonisation events, as shown in Figure 4.

We reported high values of total gene diversity (h_T = 0.959) among 15 populations selected in our study. The haplotype distribution (Figure 1) showed that eight populations (four *Delphinium fissum* subsp. *sordidum* and four *D. bolosii*) contained a single haplotype, indicating a great geographic affinity and marked differentiation through distinct evolutionary processes. Haplotype diversity within populations was low (h_S = 0.311). In contrast, differentiation among populations (G_{ST} = 0.675) and the Fixation Index (F_{ST} = 0.989) were high as welll as N_{ST} (0.948) was higher than G_{ST}. These values indicate that gene flow between populations is limited and the mutation rate is higher than the dispersal rate. In this sense, higher mutation rates are associated to greater diversification rates (in-situ diversification) and low dispersal rates limit the ability to successfully disperse to a suitable habitat when the conditions become unfavourable.

Gene flow among populations depends on pollen and seed dispersal (Petit, Kremer, and Wagner 1993). The species of the series *Fissa* are mainly pollinated by Hymenoptera (specially *Bombus terrestris* and *B. pascuorum*) and Lepidoptera (specially *Macroglossum stellatarum*) (Orellana et al. 2008; Ramírez-Rodríguez and Amich 2017). Maximum foraging distances of *Bombus* spp. range between 550 - 2800 m (Zurbuchen et al. 2010). In contrast, maximum foraging distances of *Macroglossum stellatarum* are up to 32 km (Stockhouse 1973). As a consequence, *M. stellatarum* might contribute significantly to long-distance gene flow, at a nuclear level, among populations (Cánovas et al. 2017).

Seeds of the series *Fissa* species lack adaptive structures for wind dispersal. Seed dispersal occurs by boleochory. Secondary seed dispersal mechanisms may include wind (other than in boleochory) and by animals. Animal trampling or wind force can provoke a catapult effect, throwing the seeds up to 5 metres from the mother plant (Blanché 1991). Long-distance seed dispersal events seem unlikely to occur. However, if they do occur occasionally this may be due to domestic herbivores, wild herbivores or wind (Melendo, pers. com.).

Divergence time estimation

Delphinium fissum or an ancestor of this species might have arrived in the Western Mediterranean from Central Asia during the Messinian salinity crisis (6 - 5.3 Ma), as mentioned by Orellana et al. (2007). It expanded from the Maritime Alps to the Iberian Peninsula, crossing the Pyrenees which was not a strong barrier like the Alps (Hewitt 2000). Range expansions within the Iberian Peninsula may follow the post-Messininan migration patterns proposed by Bocquet, Wilder, and Kiefer (1978). The species might have found suitable conditions in the north-eastern Iberian Peninsula where there was high annual rainfall (~700 - 900 mm) in contrast with the general warm and dry conditions (Fauquette et al. 2006).

The split between *D. fissum* subsp. *sordidum* and the two other species (*D. bolosii* and *Delphinium mansanetianum*) took place during the transition between Pliocene and Pleistocene 2.67 Ma ago (Figure 4), in the late Neogene when climate experienced the onset of a cooling process. As a consequence, the distribution range of *D. fissum* or an ancestor could firstly have been divided. In the course of time, the separated areas could have given rise to two genetically distinct groups by allopatric speciation. The Iberian System could have acted as geographical and ecological barrier delimiting such vicariant areas: one in the

northeastern Iberian Peninsula characterized by having calcareous soils and oceanic temperate and Mediterranean climates, and the other in the central-western characterized by having acidic soils and continental Mediterranean climate. Some individuals managed to cross the Iberian System and spread towards the western region of the Iberian Peninsula. Edaphic adaptation, from calcareous soils to acidic soils, coud be one of the evolutionary drivers in the speciation process. Consequently, the populations of the central-western Iberian Peninsula differentiated to *D. fissum* subsp. *sordidum*. Subsequently, the MAG population may originate by progressive and long-distance dispersal events from Central System and Iberian System to Sierra Mágina (see Bosch et al. 2019).

Implications for conservation

The species of *Delphinium* ser. *Fissa* do not differ substantially in terms of ecology, morphology and cytogenetics. They are especially similar genetically as one can produce hybrids under greenhouse conditions (Bosch 1999). Such characteristics suggest that they have incurred a relatively recent speciation process, without enough time to differentiate (see also Orellana et al. 2007; Bosch et al. 2019). When dealing with this kind of taxa, identification is not as easy as with other taxa where clear differences are evident. This is illustrated by several misidentified cases for series *Fissa* species in the Iberian Peninsula (Blanché 1985; Ascaso and Pedrol 1991; Martín-Blanco and Carrasco 1997) with the subsequent corrections (Carrasco, Martín-Blanco, and Blanché 2003; Simon et al. 1995; Martín-Blanco and Carrasco 2001, respectively). The populations of the Iberian System, including *D. mansanetianum*, are being reviewed and could be new cases of misidentified populations in a near future (Ramírez-Rodríguez et al. unpubl.).

The fixation index (F_{ST}) and the parameter of within-population diversity (h_S) can also indicate the deleterious effects of drift load (Keller and Waller 2002) and inbreeding load (Jaquiéry, Guillaume, and Perrin 2009), respectively. Our results reveal a significant genetic drift and/or inbreeding extinction risk, and therefore, they suggest a need for protection. As such, we recommend the focusing of the conservation efforts on MSA, VDA, ADR, VIL and MAG populations for *D. fissum* subsp. *sordidum*, COR, ULL and RUB populations for *D. bolosii* and MOS for *D. mansanetianum* which display endemic haplotypes, and prioritise those more susceptible to the effects of genetic drift and inbreeding due to fragmentation, isolation and small population sizes (Schemske et al. 1994). If these populations become

extinct, the loss of genetic diversity and evolutionary potential for the series *Fissa* species would be significant.

For *D. fissum* subsp. *sordidum* populations, the MSA population has an important number, at least, of reproductive individuals and its habitat is predominantly constituted with well-conserved *Quercus ilex* forests, which might potentially be affected by fires. However, it represents the only population in Portugal whose conservation status is CR "critically endangered" and no conservation measures have been implemented so far (Ramírez-Rodríguez et al. 2017). Therefore, conservation strategies should be adopted immediately. The VDA population is much larger than ADR but it is subject to a greater number of anthropic threats (Ramírez-Rodríguez and Amich, in press). Even so, monitoring and conservation measures should focus on ADR due to its small population size, the negative impact of wild animals, the limitation of pollinators and low seed production (Ramírez-Rodríguez and Amich 2017). RSV, MAG and VIL populations can be seriously affected by herbivores. MAG has the largest population size, followed by RSV and finally VIL. Large population size can help to maintain population fitness and genetic diversity (Reed 2004). In the first 2 populations, herbivores access is limited by metal fence. In VIL, at present, no conservation measures have been adopted. We recommend immediate implementation of metal fencing to restrict herbivore access and avoid losing this population that is critically threatened (Ramírez-Rodríguez et al. 2016). In the case of the HER population, a high inbreeding rate was reported by Orellana et al. (2007) due to small population size with a very limited number of reproductive individuals. Urgent conservation measures should be taken to avoid its upcoming extinction. Definitely, recovery plan for *D. fissum* subsp. *sordidum* should be drawn up including in situ and ex situ measures not only at the regional level, as in the case of COR population (DOCM 2002), but also at peninsular level.

By comparison, some of the populations of *D. bolosii* (RUB and ULL) have large population sizes with over 1000 individuals (Bosch et al. 1998; Orellana et al. 2007). This is also the case for populations of the Iberian System (COR, TOR1 and TOR2) (Herranz, Ferrandis, and Martínez-Duro 2010; Ramírez-Rodríguez et al. unpubl.). Although the population size was low (only 26 reproductive individuals) in MOS for 2005 (Bosch et al. 2005), this information is actually scarce. We propose to increase the monitoring efforts in this area and adjacent areas in order to discover more populations and obtain more information concerning *D. mansanetianum*. Overall, large population sizes and a dysploid

condition ($2n = 18$) of *D. bolosii* might explain higher values of genetic diversity than *D. fissum* subsp. *sordidum* (Orellana et al. 2007; Bosch et al. 2019). From the data provided in this study, conservation measures and the recovery plan, already reported by Bosch et al. (1998, 2006), should be updated for *D. bolosii*.

Ex situ conservation measures are similar for most plant species. For series *Fissa* species in the Iberian Peninsula it would be advisable to gather seeds in those populations that contain unique haplotypes and/or are present in small population sizes with actual and potential threats, for example MSA, VDA, ADR, VIL, HER and MAG for *D. fissum* subsp. *sordidum*, COR, ULL and RUB for *D. bolosii* and MOS for *D. mansanetianum*. Seeds should be stored in suitable germplasm banks, at the University of Salamanca and the University of Barcelona, in order to preserve their genetic diversity and evolutionary potential.

Conclusion

Phylogeographic analyses using cpDNA reveal two main haplotype lineages: one western Iberian corresponding to *Delphinium fissum* subsp. *sordidum*, and the other eastern corresponding to *D. bolosii* and *D. mansanetianum*. The phylogenetic position of the populations of the Iberian System, including *D. mansanetianum*, is genetically closely related to *D. bolosii*. Vicariance is assumed to be the main process to account for the present distribution and speciation of species of the series *Fissa* in the Iberian Peninsula whereas the disjunct distribution of *D. fissum* subsp. *sordidum* may be explained by a combination of progressive and long-distance dispersal events from the Central System and Iberian System to Sierra Mágina. Although the split of these well-defined lineages occurred during the late Pliocene and early Pleistocene 2.67 Ma ago, their speciation process was relatively recent. Conservation measures, both in situ and ex situ, are required for series *Fissa* species in the Iberian Peninsula, including such conservation proposals in recovery plans.

Acknowledgments

The authors thank to Maria Bosch for indicating us the accurate geographical coordinates to locate the populations of Ulldemolins and Rubió de Baix (Catalonia province), to António Maria Luis Crespí, João Rocha and Giacomo Colombo for helping us to collect leaf material of the *D. bolosii* populations and to Jesús Riera for collecting material of *Delphinium mansanetianum*. We are also grateful to four anonymous reviewers and the

associate editor, Florian Jabbour, whose relevant suggestions greatly improved the quality of the manuscript.

Notes on contributors

Rubén Ramírez-Rodríguez, PhD Biologist at the University of Salamanca (Spain); sample collection, wrote the manuscript, discussed the results and revised and approved the final draft of the manuscript.

Juan Francisco Jiménez, Professor at the University of Granada (Spain); performed the experiments and analised the data, discussed the results and revised and approved the final draft of the manuscript.

Francisco Amich, Professor at the University of Salamanca (Spain); sample collection, discussed the results and revised and approved the final draft of the manuscript.

Pedro Sánchez-Gómez, Professor at the University of Murcia (Spain): discussed the results and revised and approved the final draft of the manuscript.

References

Ascaso, J., and J. Pedrol. 1991. "De plantis vascularibus praesertim ibericis I [The Iberian Vascular Plants I]." *Fontqueria* 31: 135–140.

Bandelt, H. J., P. Forster, and A. Rhöl. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16(1): 37–48.

Bañares, Á., G. Blanca, J. Güemes, J. C. Moreno, and S. Ortiz, eds. 2008. Lista Roja 2008 de la flora vascular española: 86 [Red List of Spanish Vascular Flora 2008: 86]. Dirección General de Medio Natural y Política Forestal (Ministerio de Medio Ambiente y Medio Rural y Marino). Madrid: SEBICOP.

Billore, K. V., and N. P. Singh. 1972. "On an endemic species of *Delphinium* Linn. from the Western Ghats." *Indian Forester* 98: 407–408.

Blanché, C. 1985. "Una nova localitat de *Delphinium fissum* Waldst. and Kit. subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez. Comentari sobre la seva distribució [A new locality of *Delphinium fissum* Waldst.and Kit. subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez. Remarks about its distribution]." *Collectanea Botanica* 16(1): 230–231.

Blanché, C. 1991. *Revisió biosistemática del gènere Delphinium L. a la Península Ibèrica i a les Illes Balears* [Biosystematic revision of the genus *Delphinium* in the Iberian Peninsula and Balearic Islands]." Institut d'Estudis Catalans, Barcelona.

Blanché, C., and M. Bosch. 2015. "L'esperó de Bolós, retrobat cent anys després [*Delphinium bolossi*, rediscovers one hundred years later]." *Treballs de la SCB* 65: 53.

Blanché, C., and J. Molero. 1986. "*Delphinium* L." In *Flora Iberica* 1, edited by Castroviejo, S., M. Laínz, G. López González, P. Montserrat, F. Muñoz-Garmendia, J. Paiva and L. Villar, 242–251. Real Jardín Botánico, CSIC, Madrid.

Bocquet, G., B. Wilder, and H. Kiefer. 1978. "The messinian model – a new outlook for the floristics and systematics of the Mediterranean area." *Candollea* 38: 269–287.

BOCYL. 2007. "Decreto 63/2007, de 14 de Junio, por el que se crean el Catálogo de Flora Protegida de Castilla y León y la figura de protección denominada Microrreserva de Flora [Decree 63/2007, 14 June, for which they are created the Catalogue of protected Flora in Castile and Leon and the Figure of Protection named Plant Micro-Reserve]." *BOCYL (Boletín Oficial de Castilla y León. España)* 119: 13197–13204.

BOJA. 2012. "Decreto 23/2012, de 14 de Febrero, por el que se regula la conservación y el uso sostenible de la flora y la fauna silvestres y sus hábitats [Decree 23/2012, 14 February, for which it is regulated the conservation and sustainable use of the willd flora and fauna and its habitats]." *BOJA (Boletín Oficial de la Junta de Andalucía España)* 60: 114–163.

Bosch, M. 1999. *Biologia de la reproducció de la tribu Delphinieae a la Mediterrània occidental* [Pollination Biology of the Tribe Delphinieae in Western Mediterranean Area]. Barcelona: Institut d´Estudis Catalans, Arxius de la Secció de Ciències 120.

Bosch, M., S. Herrando-Moraira, A. del Hoyo, J. López-Pujol, S. Massó, J. A. Rosselló, J. Simon, and C. Blanché. 2019. "New conservation viewpoints when plants are viewed at one level higher. Integration of phylogeographic structure, niche modeling and genetic diversity in conservation planning of W Mediterranean larkspurs." *Global Ecology and Conservation* 18: e00580.

Bosch, M., J. Molero, A. Rovira, J. Simon, J. López-Pujol, M. R. Orellana, and C. Blanché. 2006. "Recovery plans for *D. bolosii* and *Thymus loscosii*: results from three-year studies and conclusions." *Bocconea* 19: 89–101.

Bosch, M., M. R. Orellana, R. Pitarch, J. López-Pujol, and C. Blanché. 2005. "Diversidad genética de *Delphinium mansanetianum* [Genetic diversity of *Delphinium mansanetianum*]." Poster presented at the II Conference of Plant Conservation Biology, Gijón, Spain, September 21–23.

Bosch, M., J. Simon, J. Molero, and C. Blanché. 1998. "Reproductive biology, genetic variation and conservation of the rare endemic dysploid *Delphinium bolosii* (Ranunculaceae)." *Biological Conservation* 86: 57–66.

Calonge Cano, C. G. 1990. "La excepcionalidad climática de los Arribes del Duero [The climatic uniqueness of the Arribes del Duero]." *Eria* 21: 45–59.

Cánovas, J. L., J. F. Jiménez, J. F. Mota, and P. Sánchez Gómez. 2017. "Genetic diversity of *Viola cazorlensis* Gand., an endemic species of Mediterranean dolomitic habitats: implications for conservation." *Systematics and Biodiversity* 13(6): 571–580.

Carrasco, M. A., C. J. Martín-Blanco, and C. Blanché. 2003. "*Delphinium emarginatum* subsp. *emarginatum* (Ranunculaceae), nuevo taxón para la Península Ibérica [*Delphinium emarginatum* subsp. *emarginatum* (Ranunculaceae), new taxon for the Iberian Peninsula]." *Anales del Jardín Botánico de Madrid* 60(2): 449–450.

Chartier, M., S. Dressler, J. Schönenberger, A. Rojas Mora, C. Sarthou, W. Wang, and F. Jabbour. 2016. "The evolution of afro-montane *Delphinium* (Ranunculaceae): morphospecies, phylogenetics and biogeography." *Taxon* 65: 1313–1327.

DOCM. 2001. "Decreto 200/2001, de 6 de Noviembre de 2001, por el que se modifica el Catálogo Regional de especies amenazadas de Castilla la Mancha [Decree 200/2001, 6 November of 2001, for which it is modified the regional catalogue of threatened species of Castile-La Mancha]." *DOCM (Documento Oficial de Castilla la Mancha)* 119: 12825–12827.

DOCM. 2002. Decreto 43/2002, de 2 de Abril de 2002, por el que se aprueba el Plan de Recuperación de la especie de flora *Delphinium fissum* subsp. *sordidum*. *DOCM* 47: 6347–6349. DOCM. 2002. "Decreto 43/2002, de 2 de Abril de 2002, por el que se aprueba el Plan de Recuperación de la especie de flora *Delphinium fissum* subsp. *sordidum* [Decree 43/2002, 2 April of 2002, for which it is approved the Recovery Plan for *Delphinium fissum* subsp. *sordidum*]." *DOCM* 47: 6347–6349.

460 DOE. 2001. “Decreto 37/2001, de 6 de Marzo, por el que se regula el Catálogo Regional de
461 Especies Amenazadas de Extremadura [Decree 37/2001, 6 March, for which it is regulated
462 the regional catalogue of threatened species of Extremadura].” *Diario Oficial de*
463 *Extremadura (DOE)* 30: 2349–2364.

464 DOGC. 2008. “Decret 172/2008, de 26 de’agost, de creació del Catàleg de flora amenaçada
465 de Catalunya [Decree 172/2008, 26 August, for which it is created the catalogue of
466 threatened flora of Cataluña].” *DOGC* 5204: 65881–65895.

467 Doyle, J. J., and J. L. Doyle. 1987. “A rapid DNA isolation procedure from small quantities of
468 fresh leaf tissues.” *Phytochemical Bulletin* 19: 11–15.

469 Drummond, A. J., and A. Rambaut. 2007. “BEAST: Bayesian evolutionary analysis by
470 sampling trees.” *BMC Evolutionary Biology* 7: 214.

471 Dupanloup, I., S. Schneider, and L. Excoffier. 2002. “A simulated annealing approach to
472 define the genetic structure of populations.” *Molecular Ecology* 11: 2571–2581.

473 Excoffier, L., and H. Lischer. 2009. “Arlequin Ver. 3.5: An integrated software package for
474 population genetics data analysis.” Available at: http://cmpg.Unibe.Ch/software/arlequin3.

475 Excoffier, L., P. Smouse, and J. Quattro. 1992. “Analysis of molecular variance inferred for
476 metric distances among DNA haplotypes: application to human mitochondrial DNA
477 restriction data.” *Genetics* 131: 479–491.

478 Fauquette, S., J. P. Suc, A. Bertini, S. M. Popescu, S. Warny, N. B. Taoufiq, M. J. Perez
479 Villa, H. Chikhi, N. Feddi, D. Subally, G. Clauzon, and J. Ferrier. 2006. “How much did
480 climate force the Messinian salinity crisis? Quantified climatic conditions from pollen
481 records in the Mediterranean region.” *Palaeogeography, Palaeoclimatology,*
482 *Palaeoecology* 238: 281–301.

483 Fu, Y. X., and W. H. Li. 1993. “Statistical tests of neutrality of mutations.” *Genetics* 133:
484 693–709.

485 Fu, Y. X. and W. H. Li. 1999. “Coalescing into the 21st century: an overview and prospectus
486 coalescent theory.” *Theoretical and Populational Biology* 58: 1–10.

487 Hamilton, M. B. 1999. “Four primers pairs for the amplification of chloroplast intergenic
488 regions with intraspecific variation.” *Molecular Ecology* 8: 521–523.

Herranz, J. M., P. Ferrandis, and E. Martínez-Duro. 2010. "Seed germination ecology of the threatened endemic Iberian *Delphinium fissum* subsp. *sordidum* (Ranunculaceae)." *Plant Ecology* 211: 89–106.

Hewitt, G. M. 2000. "The genetic legacy of the Quaternary ice ages." *Nature* 405: 907–913.

Jabbour, F., and S. S. Renner. 2011. "*Consolida* and *Aconitella* are an annual clade of *Delphinium* (Ranunculaceae) that diversified in the Mediterranean basin and the Irano-Turanian region." *Taxon* 60: 1029–1040.

Jaquiéry, J., F. Guillaume, and N. Perrin. 2009. "Predicting the deleterious effects of mutation load in fragmented populations." *Conservation Biology* 23: 207–218.

Keller, L. F., and D. M. Waller. 2002. "Inbreeding effects in wild populations." *Trends in Ecology & Evolution* 17: 230–241.

Leigh, J. W., and D. Bryant. 2015. PopART: full-feature software for haplotype network construction. *Methods in Ecology and Evolution* 6(9): 1110–1116.

Librado, P., and J. Rozas. 2009. "DnaSp v5: a software for comprehensive analysis of DNA polymorphism data." *Bioinformatics* 25(11): 1451–1452.

López-Pujol, J., M. Bosch, S. Massó, J. Molero, A. M. Rovira, and C. Blanché. 2014. *Estudi preliminary de variabilitat isoenzimàtica de Delphinium bolosii al Parc Natural de Sant Llorenç del Munt i l'Obac* [Preliminary study about the isoenzimatic variability of *D. bolosii* at the Sant Llorenç del Munt i l' Obac Natural Park]. Barcelona: Diputació de Barcelona.

Martín-Blanco, C. J., and M. A. Carrasco. 1997. "Fragmenta Chorologica Occidentalia, 5846-5893." *Anales del jardín Botánico de Madrid* 55(1): 146-149.

Martín-Blanco, C. J., and M. A. Carrasco. 2001. "*Delphinium fissum* subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez (Ranunculaceae), taxón a excluir de la flora de Ciudad Real [*Delphinium fissum* subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez (Ranunculaceae), taxon to exclude of the Flora of Ciudad Real]." *Anales del Jardín Botánico de Madrid* 58(2): 355.

Mateo Sanz, G., and J. M. Pisco García. 1993. "*Delphinium fissum* subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez en el Sistema Ibérico [*Delphinium fissum* subsp. *sordidum* (Cuatrec.) Amich, Rico and Sánchez in the Iberian System]." *Acta Botanica Malacitana* 18: 286–287.

520 Milne-Redhead, E., and W. B. Turrill. 1952. "*Delphinium*." In *Flora of tropical East Africa:*
521 *Ranunculaceae* edited by Turrill, W.B., and E. Milne-Redhead Pp. 19–22 in: Turrill,
522 W.B. & E. Milne-Redhead, 19–22. London: The Crown Agents for the Colonies.

523 Orellana, M. R., J. López-Pujol, C. Blanché, and M. Bosch. 2007. "Genetic diversity in the
524 endangered dysploid larkspur *Delphinium bolosii* and its close diploid relatives in the
525 series *Fissa* of the Western Mediterranean area." *Biological Journal of the Linnean*
526 *Society* 92: 773–784.

527 Orellana, M. R., A. Rovira, C. Blanché, and M. Bosch. 2008. "Effects of local abundance on
528 pollination and reproduction in the narrow endemic endangered species *Delphinium*
529 *bolosii* (Ranunculaceae)." *Orsis* 23: 27–46.

530 Petit, R. J., A. Kremer, and D. B. Wagner. 1993. "Finite island model for organelle and
531 nuclear genes in plants." *Heredity* 71: 630–640.

532 Pitarch García, R. 2002. *Estudio de la flora y vegetación de las sierras orientales del Sistema*
533 *Ibérico: La Palomita, Las Dehesas, El Rayo y Mayabona (Teruel)* [Study about the flora
534 and vegetation of the Eastern Sierras of the Iberian System]. Serie Investigación n. º 38.
535 Consejo de Protección de la Naturaleza de Aragón, Zaragoza.

536 Pons, O., and R. J. Petit. 1996. "Measuring and testing genetic differentiation with ordered
537 versus unordered alleles." *Genetics* 144: 1237–1245.

538 Rambaut, A., and A. J. Drummond. 2007. "TRACER V1.4." Available at:
539 http://beast.bio.ed.ac.uk/Tracer.

540 Ramírez-Rodríguez, R., and F. Amich. 2014. "Notes on rare and threatened flora in western
541 central Iberia". *Lazaroa* 35: 221–226.

542 Ramírez-Rodríguez, R., and F. Amich. 2017. "Effects of local abundance on pollination and
543 reproduction in *Delphinium fissum* subsp. *sordidum* (Ranunculaceae)." *Botany Letters*
544 164: 371–383.

545 Ramírez Rodríguez, R., F. Amich, S. Fuentes Antón, and D. Rodríguez de la Cruz. 2016.
546 "Una nueva localidad del endemismo Ibérico amenazado *Delphinium fissum* subsp.
547 *sordidum* (Ranunculaceae) [A new locality of the threatened endemic Iberian *Delphinium*
548 *fissum* subsp. *sordidum*]." *Acta Botanica Malacitana* 41: 265–267.

549 Ramírez-Rodríguez, R., L. Medina, M. Menezes de Sequeira, C. Aguiar, and F. Amich. 2017.
550 "*Delphinium fissum* subsp. *sordidum* (Ranunculaceae) in Portugal: distribution and
551 conservation status." *Anales del Jardín Botánico de Madrid* 74(1): e056.

552 Reed, D. 2004. "Relationship between population size and fitness." *Conservation biology* 19:
553 563–569.

554 Rozas, J., J. C. Sánchez-Del Barrio, X. Messeguer, and R. Rozas. 2003. "DnaSP, DNA
555 polymorphism analyses by coalescent and other methods." *Bioinformatics* 19: 2496–2497.

556 Sang, T., D. Crawford, and T. Stuessy. 1997. "Chloroplast DNA phylogeny, reticulate
557 evolution, and biogeography of *Paeonia* (Paeoniaceae)." *American Journal of Botany*
558 84(8): 1120.

559 Schemske, D. W., B. C. Husband, M. H. Ruckelshaus, C. Goodwillie, I. M. Parker, and J. G.
560 Bishop. 1994. "Evaluating approaches to the conservation of rare and endangered plants."
561 *Ecology* 75: 584–606.

562 Simon, J., M. Bosch, C. Blanché, and J. Molero. 1995. "*Delphinium*." *Flora Mediterranea* 5:
563 323–331.

564 Stockhouse, R. E. 1973. "Biosystematic studies of Oenothera L. subgenus Pachylophus."
565 Ph.D. diss., Fort Collins, Colorado, USA: Colorado State University.

566 Tajima, F. 1989. "Statistical method for testing the neutral mutation hypothesis by DNA
567 polymorphism." *Genetics* 123: 585–595.

568 Tamura, M. 1993. "Ranunculaceae." In *The Families and Genera of Vascular Plants II*,
569 edited by Kubitzki, K., J. G. Rohwer, and V. Bittrich, 563–583. Springer, Berlín.

570 Tate, J. A., and B. B. Simpson. 2003. "Paraphyly of *Tarasa* (Malvaceae) and diverse origins
571 of the polyploid species." *Systematic Botany* 28: 723–737.

572 Wheeler, G. L., H. E. Dorman, A. Buchanan, L. Challagundla, and L. E. Wallace. 2014. A
573 review of the prevalence, utility, and caveats of using chloroplast simple sequence repeats
574 for studies of plant biology. *Applications in Plant Sciences* 2: 1400059. doi:
575 10.3732/apps.1400059.

576 Zhang, H. X., and M. L. Zhang. 2012. "Genetic structure of the *Delphinium naviculare*
577 species group tracks Pleistocene climatic oscillations in the Tianshan Mountains, arid
578 Central Asia." *Palaeogeography, Palaeoclimatology, Palaeoecology* 353–355: 93–103.

579 Zurbuchen, A., L. Landert, J. Klaiber, A. Müller, S. Hein, and S. Dorn. 2010. “Maximum
580 foraging ranges in solitary bees: only few individuals have the capability to cover long
581 foraging distances.” *Biological Conservation* 143: 669–676.

Table 1. Population names, code of population (COD), coordinates (UTM 10x10) (coor.), elevation (m.a.s.l.) (Elev.), haplotype diversity (h_D), within-population diversity (h_S), total gene diversity (h_T), nucleotide diversity (Pi), Fixation Index (F_{ST}), genetic differentiation between populations (G_{ST} and N_{ST}) for the studied populations of the series *Fissa* species: *D. fissum* subsp. *sordidum* (Dfs), *D. bolosii* (Db) and *D. mansanetiaum* (Dm).

	Population	**COD**	**Coor.**	**Elev.**	**Haplotype**	**h_D**	**h_S**	**h_T**	**Pi**	**F_{ST}**	**G_{ST}**	**N_{ST}**
Dfs	Teso de San Cristóbal, Villarino de los Aires, Salamanca	VDA	29TQF17	660	H1	0			0			
Dfs	Sierra de Mágina, Jaén	MAG	30SVG57	1707	H2, H3	0,6667			0,00068			
Dfs	Sierra de San Vicente, El Real de San Vicente, Toledo	RSV	30TUK54	1130	H4	0			0			
Dfs	Tranco del Diablo, Béjar, Salamanca	BEJ	30TTK67	810	H4, H5	0,6667			0,00068			
Dfs	Hervás, Cáceres	HER	30TTK55	1003	H4, H5	0,6667			0,00068			
Dfs	Los Ceños, Aldeadávila de la Ribera, Salamanca	ADR	29TQF06	480	H6	0			0			
Dfs	Minas de Santo Adrião, Caçarelhos, Vimioso, Bragança, Portugal	MSA	29TQG10	590	H7, H8	0,6667			0,00068			
Dfs	Cascada del Pinero, Masueco de la Ribera, Salamanca	MAS	29TQF06	480	H4, H5	0,6667			0,00068			
Dfs	Puerto de Villatoro, Villatoro, Ávila	VIL	30TUK18	1400	H2	0			0			
Population of Dfs						0,847	0,37	0,898	0,00213		0,588	0,806
Db	Ulldemolins, Priorat, Tarragona	ULL	31TCF27	630	H11	0			0			
Db	Rubió de Baix, La Noguera, Lérida	RUB	31TCG34	290	H12, H13	0,6667			0,00068			
Db	Barranco de la Hoz, Corduente, Guadalajara	COR	30TWL81	1050	H10	0			0			
Db	Río Gallo, Dehesa Boyal, Tordellego, Guadalajara (1)	TOR1	30TXL11	1235	H14	0			0			
Db	Río Gallo, Dehesa Boyal, Tordellego, Guadalajara (2)	TOR2	30TXL11	1246	H14, H15	0,6667			0,00068			
Dm	Masía Matorrilo, Mosqueruela, Teruel	MOS	30TYK17	1640	H16	0			0			
Populations of Db						0,858	0,222	0,956	0,00432		0,767	0,953
TOTAL						0,921	0,311	0,959	0,00622	0,989	0,675	0,948

Eliminado: 9

Table S2. Sequences of the *trn*S-*trn*G plastid region were downloaded from the GenBank database, for those species which were used to calibrate the molecular clock.

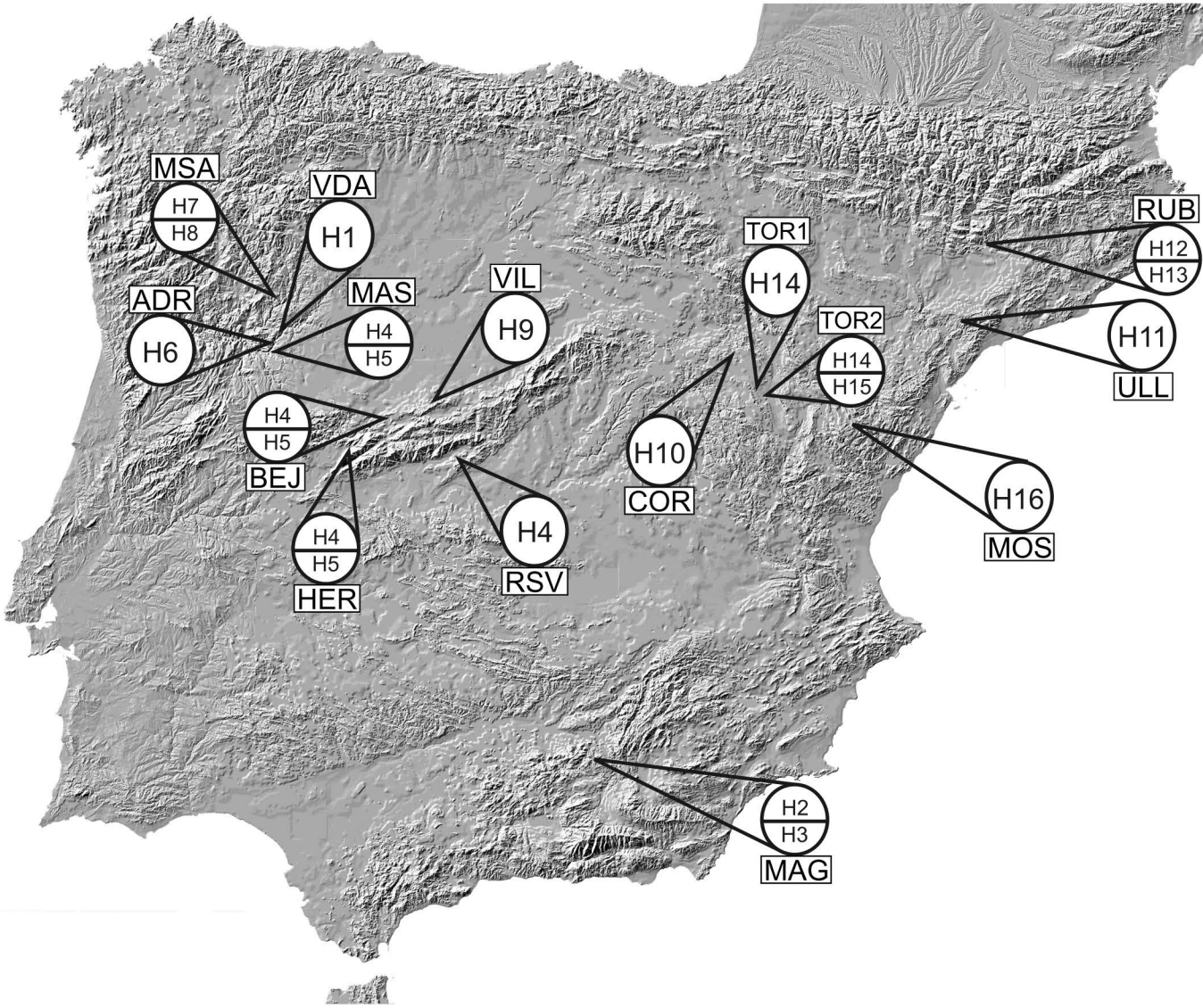
Species	***trn*S-*trn*G genbank accession**
Aconitum gymnandrum	JF331856
Aconitella aconiti	JF331810
Consolida flava	JF331825
Consolida mauritanica	JF331833
Consolida regalis	JF331842
Consolida orientalis	JF331836
Delphinium elatum-1	JX026689
Delphinium elatum-2	JX026690
Delphinium favargeri	JF331864
Delphinium halteratum	JF331866
Delphinium macropetalum	JF331868
Delphinium naviculare-1	JX026683
Delphinium naviculare-2	JX026685
Delphinium naviculare-3	JX026686
Delphinium naviculare-4	JX026687
Delphinium naviculare-5	JX026688
Delphinium tiansthanicum-1	JX026691
Delphinium tiansthanicum-2	JX026692

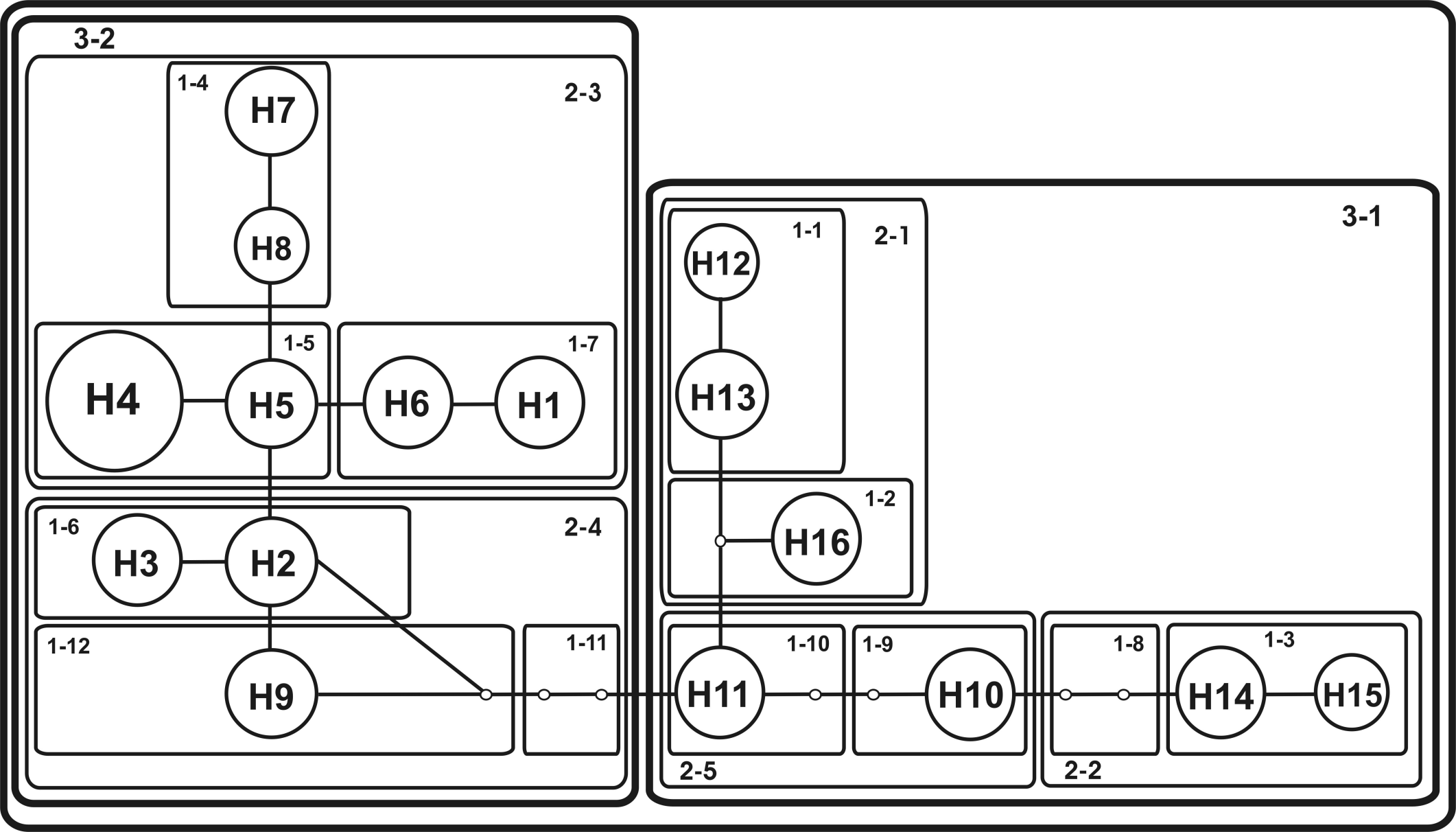
Figure 1. Locations and haplotype distribution from 15 studied populations of *Delphinium* series *Fissa* in the Iberian Peninsula. Population numbers and haplotypes correspond to those shown in Table 1.

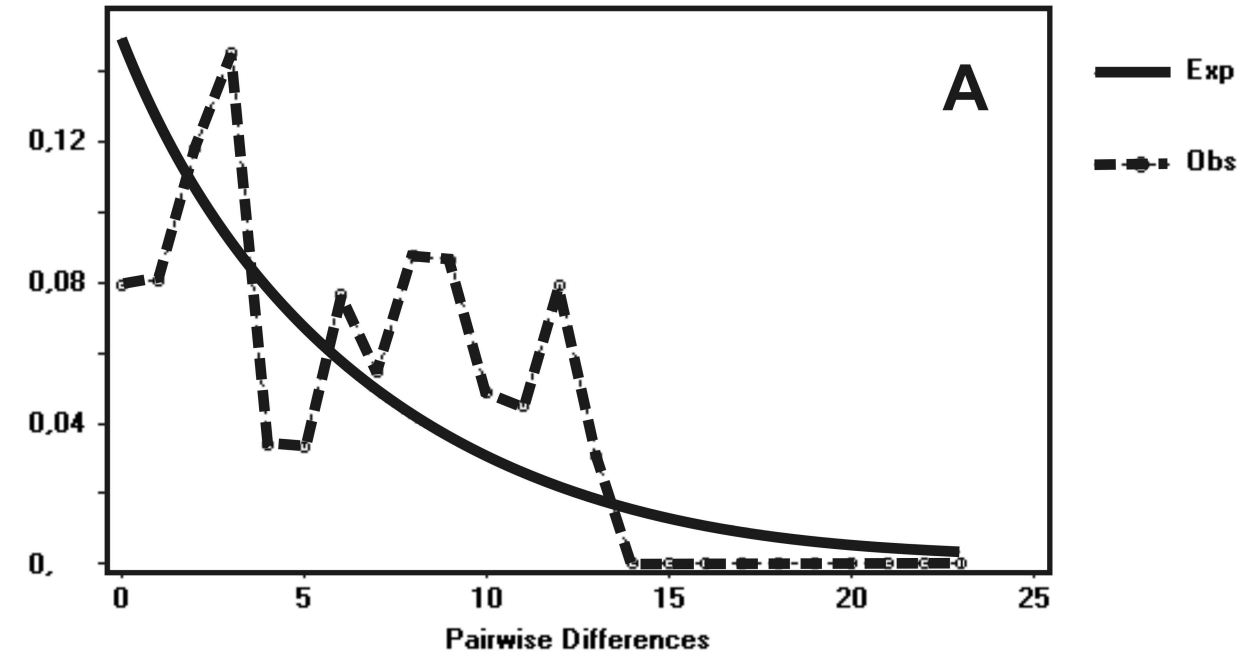
Figure 2. Median-joining network analysis, based on two cpDNA regions (trnS-trnG and psab-trnH), of 15 haplotypes identified for the series *Fissa* species in the Iberian Peninsula.

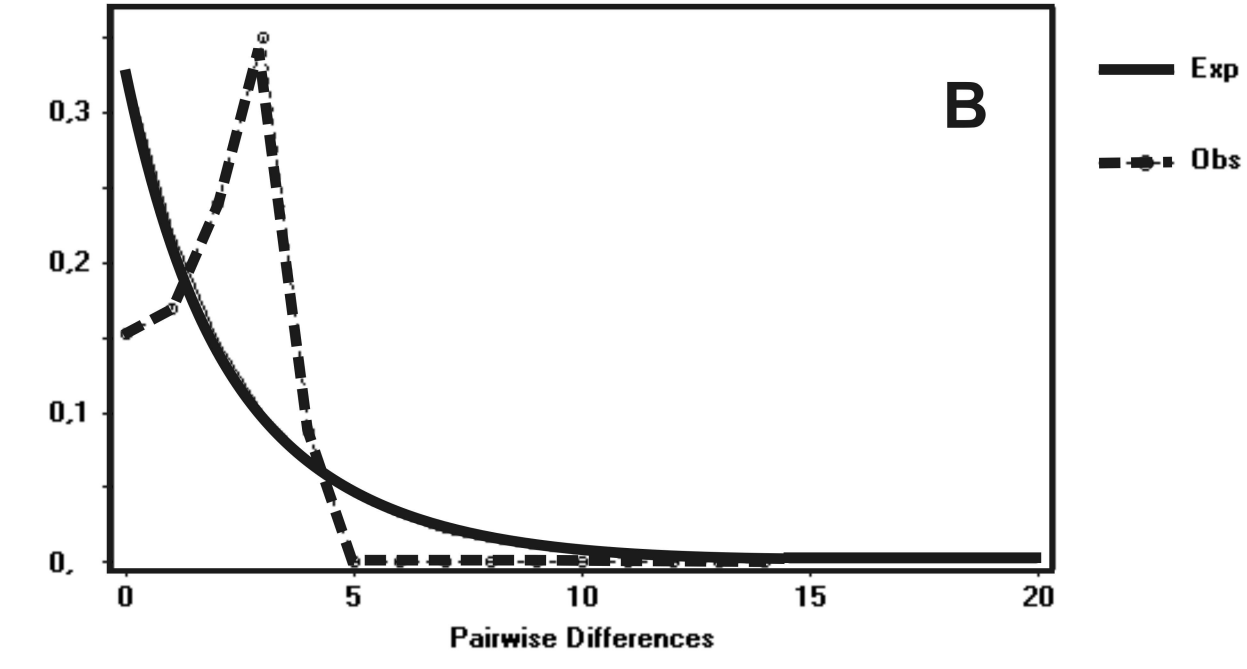
Figure 3. Mismnatch distribution analysis of *Delphinium* series *Fissa* for (A) all studied populations, (B) *D. fissum* subsp. *sordidum*, and (C) *D. bolosii*.

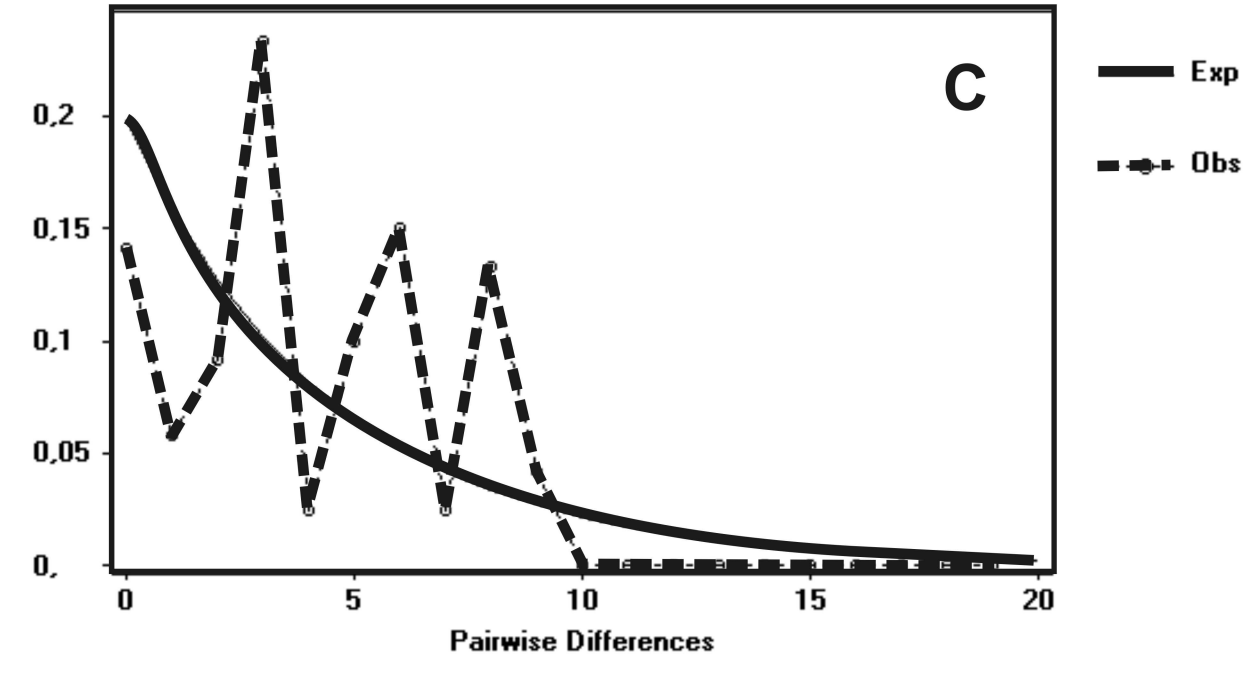
Figure 4. Divergence time estimated, based on two cpDNA regions, trnS–trnG and psab-trnH, using the Bayesian relaxed clock methodology with node calibration in BEAST version 1.8.

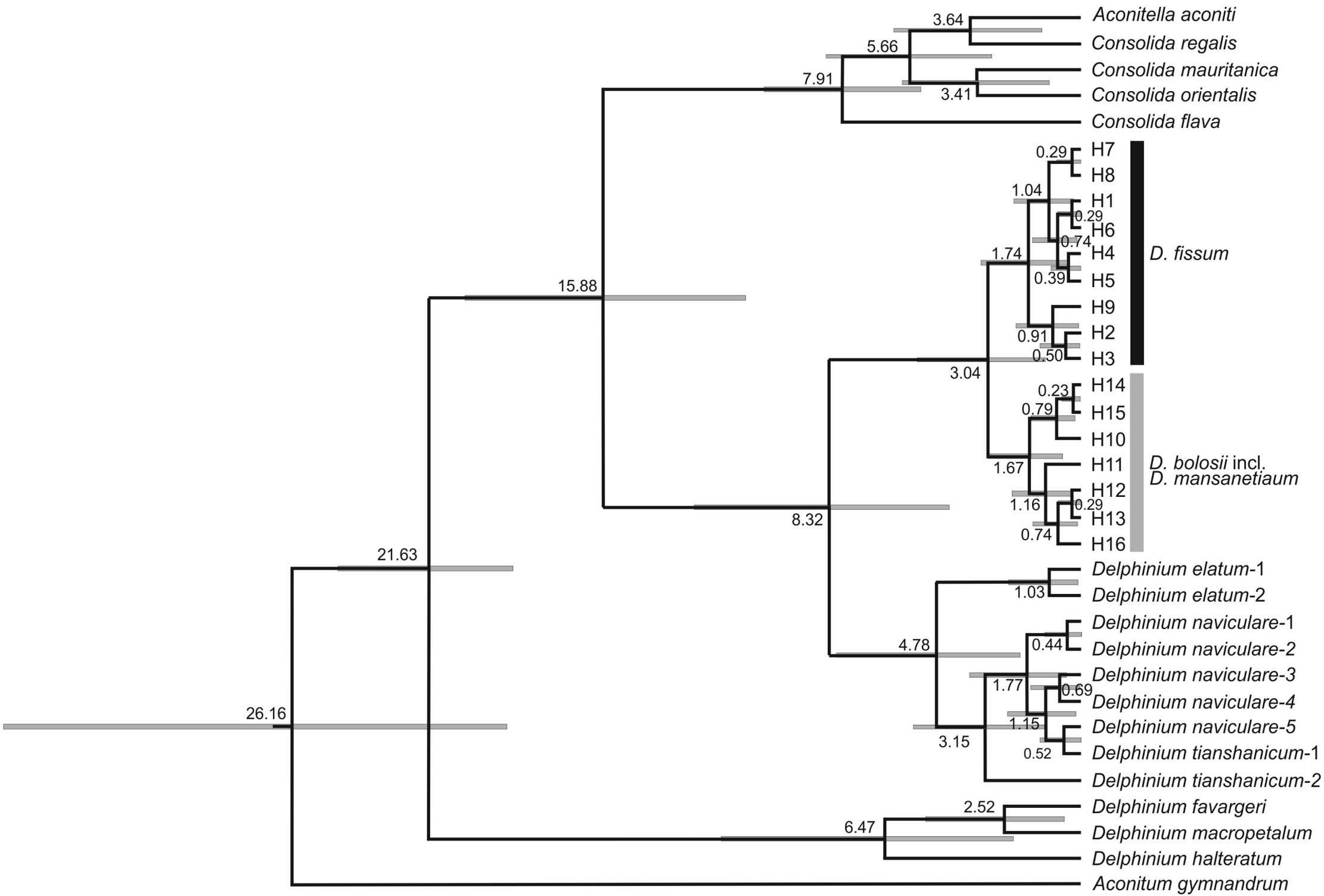

MSA
H7
H8
VDA
H1
ADR
H6
MAS
H4
H5
VIL
H9
H4
H5
BEJ
H4
H5
HER
H4
RSV
H10
COR
TOR1
H14
TOR2
H14
H15
RUB
H12
H13
H11
ULL
H16
MOS
H2
H3
MAG


3-2
1-4
H7
2-3
H8
1-5
1-7
H4
H5
H6
H1
1-6
H3
H2
2-4
1-12
1-11
H9
3-1
1-1
2-1
H12
H13
1-2
H16
1-10
1-9
1-8
1-3
H11
H10
H14
H15
2-5
2-2


A
Exp
Obs
0,12
0,08
0,04
0,
0
5
10
15
20
25
Pairwise Differences


B
Exp
Obs
0,3
0,2
0,1
0,
0
5
10
15
20
Pairwise Differences


C
Exp
Obs
0,2
0,15
0,1
0,05
0,
0
5
10
15
20
Pairwise Differences


Aconitella aconiti
Consolida regalis
Consolida mauritanica
Consolida orientalis
Consolida flava
H7
H8
H1
H6
H4
H5
H9
H2
H3
D. fissum
H14
H15
H10
H11
H12
H13
H16
D. bolosii incl.
D. mansanetiaum
Delphinium elatum-1
Delphinium elatum-2
Delphinium naviculare-1
Delphinium naviculare-2
Delphinium naviculare-3
Delphinium naviculare-4
Delphinium naviculare-5
Delphinium tianshanicum-1
Delphinium tianshanicum-2
Delphinium favargeri
Delphinium macropetalum
Delphinium halteratum
Aconitum gymnandrum
3.64
5.66
7.91
3.41
0.29
1.04
0.29
0.74
1.74
0.39
0.91
0.50
15.88
3.04
0.23
0.79
1.67
1.16
0.29
0.74
8.32
21.63
1.03
4.78
0.44
1.77
0.69
1.15
0.52
3.15
26.16
2.52
6.47