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Molecular phylogeny of the genus *Chondrina* (Gastropoda, Panpulmonata, Chondrinidae) in the Iberian Peninsula

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ABSTRACT

Chondrina Reichenbach, 1828 is a highly diverse genus of terrestrial molluscs currently including 44 species with about 28 subspecific taxa. It is distributed through North Africa, central and southern Europe, from Portugal in the West to the Caucasus and Asia Minor in the East. Approximately 70% of the species are endemic to the Iberian Peninsula constituting its main center of speciation with 34 species. This genus includes many micro-endemic taxa, some of them not yet described, confined to limestone habitats (being strictly rock-dwelling species). They are distributed on rocky outcrops up to 2000 m.a.s.l. It is a genus of conical-fusiform snails that differ mainly in shell characters and in the number and position of teeth in their aperture. So far, molecular studies on *Chondrina* have been based exclusively on the mitochondrial Cytochrome Oxidase subunit I region (COI). These studies gave a first view of the phylogeny of the genus but many inner nodes were not statistically supported.

The main objective of the study is to obtain a better understanding of the phylogeny and systematics of the genus *Chondrina* on the Iberian Peninsula, using multilocus molecular analysis. Partial sequences of the COI and 16S rRNA genes, as well as of the nuclear Internal Transcribed Spacer 1 (ITS1-5.8S) and Internal Transcribed Spacer 2 (5.8S-ITS2-28S) were obtained from individuals of all the extant *Chondrina* species known from the Iberian Peninsula. In addition to this, the newly obtained COI sequences were combined with those previously published in the GenBank. Phylogenetic relationships were inferred using maximum likelihood and Bayesian methods. The reconstructed phylogenies showed high values of support for more recent branches and basal nodes. Moreover, molecular species delimitation allowed to better define the studied species and check the presence of new taxa.

1. Introduction

The two main objectives of systematics are delimiting species and reconstructing their phylogenetic relationships (Mayr and Ashlock, 1991; Agapow et al, 2004; Coyne, 1992; Dépraz et al., 2009; Mayden, 1997; Nixon and Wheeler, 1990). These tasks become of mayor relevance when considering that species are the fundamental units in studies focused on biogeography, ecology, evolution and conservation biology (Avise, 2000; Sites and Marshall, 2003; Stanton et al., 2019). However, delimitation of species can be hampered by the presence of cryptic species, phenotypic plasticity, and convergence (Bickford et al., 2007; Losos, 2011; Via et al., 1995).

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Within molluscs, terrestrial gastropods are an interesting group in terms of biodiversity, and are good models to study speciation, historical biogeography, radiation processes and mechanisms generating biodiversity (Chueca et al., 2021; Glaubrecht, 2009; Greve et al., 2010; Neiber et al., 2018). As in other organisms, the classification of molluscs has traditionally been based on morphological characters, with the shell used as the main structure to discriminate taxa at the species level (Ponder and Lindberg, 1997; Welter-Schultes, 2012). Nevertheless, several studies have questioned the usefulness of key shell characteristics to delimitate species and to infer phylogenetic relationships. Indeed, shell variations could be the result of local adaptations to particular environmental conditions as a result of high phenotypic plasticity (Fiorentino et al., 2013; Stankowski and Johnson, 2014; Zając et al., 2020). In addition, homoplasies, are quite frequent in shell form (e.g. flattened versus rounded), composition of apertural barriers (lamellae, plicae and teeth) or presence of periostracal structures, such as hairs or shell microsculpture (Chueca et al., 2018; Motochin et al., 2017; Uit de Weerd and Gittenberger, 2013). Finally, it has been suggested that the number of cryptic species among gastropods may be high which could hide their diversity (Lajus et al., 2015; Matsuda and Gosliner, 2018; Rundell, 2008). Thus, implementing molecular studies can help to delimit species and to further reconstruct their phylogenetic relationships (Elejalde et al., 2008, 2009; Neiber et al., 2017; Neiber and Hausdorf, 2015; Xu and Hausdorf, 2021).

The family Chondrinidae Steenberg, 1925 includes seven genera (Gittenberger et al., 2016) and together with Truncatellinidae Steenberg, 1925 they constitute the superfamily Chondrinoidea Steenberg, 1925, which is included within Orthurethra along with Azecoidea H. Watson, 1920 and Pupilloidea W. Turton, 1831 (Saadi et al., 2021). *Chondrina* is by far the most diverse genus of this family (Gittenberger, 1973; Kokshoorn and Gittenberger, 2010). It is distributed in North Africa and through Central and southern Europe, from Portugal in the West to the Caucasus and Asia Minor in the East (Gittenberger, 1973). Approximately 70% of the species are endemic to the Iberian Peninsula constituting its main centre of speciation (Bodon et al., 2015; Kokshoorn et al., 2010; Kokshoorn and Gittenberger, 2010). All species of the genus are strictly rock-dwelling, show a strong xerophilous niche conservatism (Kokshoorn et al., 2010), and present a discontinuous distribution pattern, living exclusively in karstic areas (Gittenberger, 1973) up to 2000 m a.s.l. This habitat has promoted speciation in terrestrial molluscs (Schilthuizen et al., 2005). *Chondrina* has been considered to represent an excellent candidate group for evolutionary studies on non-adaptive radiation (Gittenberger, 2004; Kokshoorn, 2008; Solem and van Bruggen, 1984) because speciation events within the genus seem to occur in allopatry and there is speciation in the absence of apparent niche differentiation (Gittenberger, 1991, 2004). As a result of these allopatric speciation processes, this genus includes a high number of micro-endemic taxa, particularly in the Iberian Peninsula. These taxonomic problems also occurred in other land snail genera as *Trochulus* or *Taphrenalla* (Pholyotha et al., 2021; Proćków et al., 2021).

The study of the phylogenetic relationships within the genus *Chondrina* is crucial to provide relevant data for the conservation of its huge biodiversity and to provide insight on the evolutionary complexity of its multiple speciation events. Studies performed by Kokshoorn et al. (2010) gave the first reconstruction of the phylogenetic relationships within *Chondrina* using molecular techniques. Based on these molecular results, Kokshoorn and Gittenberger (2010) updated the classification of the genus, validating most of the morphospecies previously considered by Gittenberger (1973), and assigning full species status to another seven species previously considered (see Table 1) within the synonymy of *Chondrina farinesii*. These authors also described four new species (*Chondrina arigonoides*, *C. ingae, C. marjae* and *C. pseudavenacea*). Moreover, they suggested that another six undescribed putative new species (designated as *Chondrina* spec.1 to *Chondrina* spec. 6), could also be present in the Iberian Peninsula. Therefore, as many as 34 species of *Chondrina*, some of them with several subspecies, have been reported for the Iberian Peninsula (Kokshoorn and Gittenberger, 2010). Although these two works highly contributed to resolve the taxonomy and phylogeny of the genus *Chondrina*, the limited DNA sequence length used did not allow resolving most inner nodes. Moreover, these studies relied solely on a single mitochondrial locus and it has been widely acknowledged that multilocus approaches based on both mitochondrial and nuclear markers is essential for concise reconstruction of the evolutionary processes (Chueca et al., 2018; Rubinoff and Holland, 2005). Therefore, further studies are needed to progress in the knowledge of the systematics of this genus.

Table 1
Chondrina species tested in this study from the Iberian Peninsula. Nomenclature follows the proposal of Kokshoorn and Gittenberger (2010).

C. aguilari Altimira, 1967
C. altimirai Gittenberger, 1973
C. arigonis (Rossmässler, 1859)
C. arigonoides Kokshoorn & E. Gittenberger, 2010
C. ascendens (Westerlund, 1878)
C. avenacea (Bruguière, 1792)
C. bigorriensis (Des Moulins, 1835)
C. calpica (Westerlund, 1872)
C. cantabroccidentalis Somoza-Valdeomillos & Vázquez-Sanz, 2021
C. centralis (Fagot, 1891)
C. cliendentata Gittenberger, 1973
C. dertosensis (Bofill, 1886)
C. farinesii farinesii (Des Moulins, 1835)
C. gasulli Gittenberger, 1973
C. granatensis Alonso, 1974
C. guiraoensis Pilsbry, 1918
C. ingae Kokshoorn & E. Gittenberger, 2010
C. jumillensis (L. Pfeiffer, 1853)
C. kobelti kobelti (Westerlund, 1887)
C. kobelti ordunensis Pilsbry, 1918
C. kobeltoides Gittenberger, 1973
C. lusitanica (Pfeiffer, 1848)
C. maginensis Arrébola & Gómez, 1998
C. marjae Kokshoorn & E. Gittenberger, 2010
C. massotiana massotiana (Bourguignat, 1863)
C. massotiana sexplicata (Bofill, 1886)
C. pseudavenacea Kokshoorn & E. Gittenberger, 2010
C. ripkeni Gittenberger, 1973
C. soleri Altimira, 1960
C. tenuimarginata (Des Moulins, 1835)
C. spec. 1 Kokshoorn & E. Gittenberger, 2010
C. spec. 2 Kokshoorn & E. Gittenberger, 2010
C. spec. 3 Kokshoorn & E. Gittenberger, 2010
C. spec. 4 Kokshoorn & E. Gittenberger, 2010
C. spec. 5 Kokshoorn & E. Gittenberger, 2010
C. spec. 6 Kokshoorn & E. Gittenberger, 2010

Thus, the present study aims to advance in the resolution of the taxonomy and phylogeny of the genus *Chondrina* within the Iberian Peninsula, using multilocus molecular analysis including substantially more populations and different methods of species delimitation. With this target, the specific goals of this study were thus to: *i*) elucidate the phylogenetic relationships for the Iberian *Chondrina* species using a multilocus molecular phylogenetic approach, *ii*) investigate the diversity and species limits in this highly diverse land snail genus with conserved morphological characters and *iii*) explore the value of morphological characters for *Chondrina* systematics.

2. Materials and methods

2.1. Taxon sampling

We examined 175 *Chondrina* specimens covering all taxa listed in Table 1, with the exception of *Chondrina* spec. 4 and *C. jumillensis*, and including several samples of presumably new species (specimen data is provided in Supplementary Material S1). Shells and living specimens were collected by hand from rock walls. Whenever possible, they were collected from type localities. Specimens were preserved in 96% ethanol

for DNA isolation and molecular analyses. Adults were classified by shell characters, focusing on the number and position of teeth in the aperture, following the descriptions of previous authors (Alonso Alonso, 1974; Arrébola and Gómez, 1998; Gittenberger, 1973; Raven, 1986; Kokshoorn and Gittenberger, 2010). In some cases, the geographical location of the specimens was also used for species assignment.

2.2. DNA extraction, gene amplification and sequencing

Total genomic DNA was extracted from the whole body using the DNAeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines. During DNA extraction, the aperture of the shell was preserved to allow subsequent morphological identifications.

For the multilocus analyses performed in this study, we selected two mitochondrial gene fragments, the cytochrome *c* oxidase subunit I (COI) and the 16S RNA ribosomal subunit (16S), together with the nuclear rDNA gene cluster divided in two fragments: the 3́ end of the ITS1 region and the 5́ end of 5.8S rRNA gene (ITS1-5.8S) and the 3́ end of the 5.8S rRNA gene, the complete ITS2 region and the 5́ end of the large subunit 28S rRNA gene (5.8S-ITS2-28S). Employed primers are listed in Supplementary Material S2. General PCR conditions used for DNA amplification were as follows: an initial denaturation step at 96 °C for 5 min, 35 cycles of 30 s at 94 °C, 30 s at 55–56 °C (depending on the annealing temperature of the primer pairs, see Supplementary Material S2), and 1 min at 72 °C, and a final extension step at 72 °C for 10 min.

Amplicons were sequenced at Macrogen in The Netherlands and in Spain using an ABI3730XL or ABI3700 sequencer. The resulting forward and reverse sequences were assembled using Geneious 5.1.7 (Kearse et al., 2012). Following automatic assembly, each contig was checked for errors/ambiguities. Double peaks with equally high intensities in the chromatograms were assigned as heterozygous sites. These polymorphic sites were coded as ambiguous nucleotides following IUPAC-IUB code.

2.3. Phylogenetic analyses

We analysed two different datasets for phylogenetic reconstruction. On the one hand, in order to get strong support in phylogenetic inference, we analysed the concatenated matrix of the new sequences obtained in this work for COI, 16S rRNA, ITS1-5.8S and 5.8S-ITS2-28S loci (hereafter multilocus dataset). This dataset consisted of 169 *Chondrina* specimens and three species were used as outgroups (GenBank accession numbers are in Supplementary Material S1): *Abida secale* (Draparnaud, 1801), *Rupestrella rhodia* (J. R. Roth, 1839) and *Rupestrella dupotetii* (Terver, 1839). On the other hand, we analysed a dataset combining the new COI sequences generated in this study with the COI sequences published by Kokshoorn et al. (2010) for the Iberian *Chondrina* specimens (hereafter COI dataset), taking into account the *corrigendum* of Somoza-Valdeolmillos et al. (2019). These analyses allowed us to compare the results of that publication with our COI dataset information. The COI dataset included sequences of 234 *Chondrina* individuals with ten outgroup samples belonging to 5 species (Supplementary Material S1): *Rupestrella dupotetii*, *Rupestrella occulta* (Rossmässler, 1839), *Pyramidula pusilla* (Vallot, 1801), *Abida secale secale*, *Abida secale ateni* Gittenberger, 1973 and *Abida bigerrensis* (Moquin-Tandon, 1856).

Sequences were aligned with MAFFT 7.313 online version (Katoh et al., 2017) using the L-INS-I strategy for the COI gene fragments and the Q-INS-i algorithm for the 16S rRNA, ITS1-5.8S and 5.8S-ITS2-28S loci. For each codon position in COI, substitution saturation was assessed following the entropy-based information method (Xia et al., 2003) as implemented in DAMBE v.6.1.19 (Xia, 2013). Gene partition schemes for the two datasets were obtained with Partition Finder V1.1.1 (Lanfear et al., 2012). The best evolutionary model for each gene partition was estimated with jModelTest 3.7 (Darriba et al., 2012) according to the Bayesian Information Criterion (BIC) employing the CIPRES Science Gateway (Miller et al., 2010). Molecular characters statistics including parsimony informative sites and base frequency were calculated with MEGA 7 (Kumar et al., 2016) for each gene partition.

Phylogenetic analyses on the different datasets were conducted using both Bayesian Inference (BI) and Maximum Likelihood (ML) methods and applying the partition schemes obtained with Partition Finder (see Supplementary Material S3). Bayesian search of tree space was performed with MrBayes 3.2.2 (Ronquist et al., 2012) at CIPRES Science Gateway cluster specifying for each partition the best evolutionary model obtained with jModelTest. MrBayes was programmed to run for 95 million generations in two parallel runs, sampling every 1,000 generations with the first 25% of trees being discarded as burn-in. Convergence between runs was assessed by comparing the traces using Tracer v1.7.1 (Rambaut et al., 2018). Maximum Likelihood analysis, was conducted partitioned (see Supplementary Material S3) with RAxML 8.2.10 (Stamatakis, 2014) at CIPRES Science Gateway, under the GTRGAMMA model, with 1,000 non-parametric bootstrap replicates to assess node support. For the different topologies obtained, we interpreted Posterior Probability (PP) values from the BI analysis above 0.95 as significant statistical support and values above 70% from bootstrapping procedures (BS) as meaningful support.

2.4. Species delimitation analyses

Species boundaries were explored using different approaches employing three species delimitation methods: i) the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012a), ii) the STACEY package v.1.2.5 (Jones, 2017) for BEAST2 v.2.5 (Bouckaert et al., 2014), and iii) BPP v.3.4 program (Yang, 2015; Yang and Flouri, 2018).

Because the ABGD method was specifically designed for single locus data sets (specifically for the COI gene, see Puillandre et al., 2012b), we applied it only to the COI sequence data from the multilocus dataset. This method is based on the assumption that within the distribution of pairwise differences between sequences, a gap could be observed or not between intraspecific and interspecific diversity which can be used as a threshold for delimiting species under the premise that individuals within species are more similar than between species (Roy et al., 2014). The data matrix used for the ABGD analysis consisted of 162 COI sequences. The sequences were uploaded at https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html and the method was run under the default settings (Pmin = 0.001, Pmax = 0.1, Steps = 10, Nb = 20) and a relative gap width of 0.4. Despite it was not the best fitting evolutionary model for our dataset (Supplementary Material S3), the analysis was carried out under Kimura (K80) model as the best approach (TS/TV = 2.0, calculated with MEGA 7), because ABGD only implements Jukes-Cantor (JC69) and Kimura (K80) models for distance calculation.

Both STACEY and BPP are multispecies coalescent species delimitation methods that accommodate lineage sorting due to ancestral polymorphism to estimate the probability of different delimitation hypotheses in a Bayesian approach. We used the multilocus sequence data including COI, 16S rRNA and ITS2 sequences for these methods. For ease of analyses, we avoided using the ITS1 data because of the low number of obtained sequences and the 5.8S and 28S data because of their low variability. Similarly, due to computation limitations and the differences in the composition of each of the clades, these analyses were conducted partitioning the data matrix into the main clades (C1–C5). Thus, the analyses were performed independently for each of the clades except for C1, which was not tested because it includes a single species, *C. maginensis*, with a great genetic divergence with respect to the other groups of the genus.

For the STACEY analysis, it is not mandatory to assign individuals to species. However, we provided some information to the method by grouping some individuals by population or by their close relationship according to their geographical distribution and the phylogenetic tree (Supplementary Material S4). A single tree and a single relaxed log-normal molecular clock were specified for mitochondrial genes. For the nuclear marker, individual strict clock and tree were defined. The *collapseweight* (ω) values specified for each clade are shown in Table 2.

Table 2
Values for the C2, C3, C4 and C5 clades of ω and *collapseheights* for STACEY and θ and τ for BPP.

Parameter	Clade			
	C2	C3	C4	C5
		Stacey		
collapseweight (ω)	0.84	0.63	0.5	0.8
collapseheights	0.09	0.03	0.09	0.09
		BPP		
θ mean	0.2	0.025	0.03	0.04
θ ~ IG(α, β)	(3, 0.4)	(3, 0.05)	(3, 0.06)	(3, 0.08)
τ mean	0.002	0.0175	0.0075	0.0075
τ ~ IG(α, β)	(3, 0.004)	(3, 0.035)	(3, 0.015)	(3, 0.015)

Finally, a Yule speciation tree prior and a *collapseheight* of 0.0001 were set and two independent runs of 50,000,000 generations were carried out using BEAST through the CIPRES Science Gateway (Miller et al., 2010) with 10% burn-in for the C2 and C4 clades and 16% for the C3 and C5 clades. To assess the statistical support of species delimitation, the posterior tree distribution was analysed using SpeciesDelimitationAnalyser v.1.8.0 (Jones et al., 2015), setting different *collapseheights* for each clade (Table 2).

BPP, on the other hand, requires *a priori* assigning samples to candidate species and, therefore, we used the information obtained from ABGD to define the species hypothesis for each of the main clades specified above. The specimen composition of the putative species tested with BPP are shown in Supplementary Material S5. The prior distributions of the ancestral population size (θ) and root age (τ_0) can affect models' posterior probabilities. To assess if the prior means of these parameters were reasonable for the data, we checked the posterior distribution created for the parameters under the coalescent model when the species phylogeny was fixed (A00 analysis). The final inverse gamma priors and their means for each of the main clades are summarised in Table 2. Since the datasets often contain more information about species delimitation than about their relationships (Yang, 2015), we carried out the BPP analysis A11 which do not require a guide species tree. Each species delimitation model was assigned equal prior probability, cleandata was set to 0, all finetune settings to 0.01 and Γ to 3. For each of the rjMCMC algorithms (0 and 1) implemented in the program, running the rjMCMC analyses for 300,000 generations (sampling interval of three) with a burn-in period of 30,000, produced consistent results across separate analyses initiated with different starting seeds.

3. Results

3.1. Dataset characteristics

The information of both datasets concerning alignment length, parsimony informative sites and average base frequencies for each marker are listed in Supplementary Material S3. The multilocus dataset included 169 new *Chondrina* individuals and the COI dataset incorporated 175 sequences in addition to the 68 sequences of Iberian *Chondrina* species from GenBank published by Kokshoorn et al. (2010). The best evolutionary models are also shown in Supplementary Material S3. All new sequences were deposited in GenBank and their accession numbers are provided in Supplementary Material S1.

No stop codons were detected in the COI sequences. Neither overall COI nor any codon position in COI showed signs of saturation, indicated by an Iss (index of substitution saturation based on 32 OTUs with 1,000 replicates) significantly lower than the Iss.c (critical substitution saturation index).

3.2. Phylogenetic inference

The phylogenetic reconstructions obtained from the analyses of both datasets, multilocus and COI, are shown in Figs. 1, 2, 3 and 4. For the multilocus dataset containing all genes (Figs. 1 and 2, hereafter multilocus tree), the topologies obtained through ML and BI phylogenetic analyses were identical and differed only in support values, such that the topology of the phylogeny shown is based only on BI. The topology of the tree obtained from the COI dataset (Figs. 3 and 4, hereafter COI tree) is also based on BI since the results of both ML and BI were congruent and differ only in weakly supported relationships. A tree relaying on the mitochondrial information (COI + 16S rRNA) and another one using nuclear loci (ITS1-5.8S + 5.8S-ITS2-28S) of the multilocus dataset have been included as Supplementary Material S6 and S7, respectively. Below we use the obtained multilocus tree to describe the phylogenetic relationships within the genus, with comments about the COI tree, when necessary.

The *Chondrina* genus was recovered as monophyletic (PP = 1.00; BS = 89%) and the results yielded five well supported main phylogroups, named clades C1–C5 (Figs. 1–4). The geographical distribution of the samples included in the multilocus tree are represented in Fig. 5 (Clades C1, C2 and C4) and Fig. 6 (Clades C3 and C5)..

The fully supported clade C1 (PP = 1.00; BS = 100%) included only *C. maginensis* specimens and was recovered as the sister group of the remaining species (PP = 1.00; BS = 64%). The sequences published by Kokshoorn et al. (2010) for *C. maginensis* joined within this clade in the COI tree.

Clade C2 joined with strong support (PP = 1.00; BS = 92%) several species distributed along the northeastern quarter of the Iberian Peninsula, from the East Cantabrian Mountains in the West, to Alicante province in the South (Fig. 3). It contained five subclades (subclades C2a–e). The most divergent group (subclade C2a) joined with strong support (PP = 1.00; BS = 99%) four specimens of the morphotype *C. farinesii* collected from the Central Iberian System (Soria, Zaragoza and Guadalajara provinces), here referred to as *Chondrina* spec. A. Subclade C2b (PP = 0.97; BS = 57%) grouped two specimens of *C. farinesii* morphotype from the Northeast Iberian Peninsula. Subclade C2c (PP = 0.98; BS = 67%) joined other specimens of *C. farinesii* morphotype from the Northeast Iberian Peninsula together with the topotypes collected in La Preste (eastern Pyrenees, France). Consequently, subclade C2c corresponded to *C. farinesii s.str.* Subclade C2d joined with full support (PP = 1.00; BS = 100%) a few populations living in Castellón and Cuenca provinces, including specimens collected from Los Cloticos, Bejís (Castellón). We have provisionally called these populations *Chondrina* spec. 5, the name proposed by Kokshoorn and Gittenberger (2010) for the specimens collected from Los Cloticos (Castellón) which were also placed within this subclade in the COI tree (Fig. 2). Subclade C2d was recovered with full support as the sister group of the bigger subclade C2e. *Chondrina ascendens* was recovered as paraphyletic within subclade C2e. *Chondrina massotiana massotiana* and *C. m. sexplicata* were also paraphyletic and intermingled with *Chondrina* spec. 6 *sensu* Kokshoorn and Gittenberger (2010). On the contrary, a clade joining *C. arigonis*, *C. dertosensis* and *Chondrina* spec. 1 sensu Kokshoorn and Gittenberger (2010) within subclade C2e was fully supported. Some specimens of the *C. farinesii* morphotype were also included within the C2e subclade.

Clade C3 was strongly supported (PP = 1.00; BS = 94%) and recovered as the sister group of clade C2 (PP = 1.00; BS = 81%). The C3 phylogroup was divided into two supported sister groups, C3a (PP = 1.00; BS = 100%) and C3b (PP = 1.00; BS = 97%). Subclade C3a joined the species *C. aguilari*, *C. soleri* and *Chondrina* spec. 2 *sensu* Kokshoorn and Gittenberger (2010), all of them strongly supported and with the latter two taxa recovered as sister groups (PP = 1.00; BS = 90%). These species are restricted to Catalonia and adjoining parts of Aragón (Northeast Iberian Peninsula). The COI tree also grouped the sequences published by Kokshoorn et al. (2010) for *C. aguilari* and *Chondrina* spec. 2 within this group. *Chondrina soleri* was not included in the molecular study of Kokshoorn et al. (2010). Subclade C3b was subdivided into three main lineages. Subclade C3bi was supported only by BI analysis (PP = 0.98; BS = 43%) and it joined *C. granatensis*, *C. guiraoensis*,

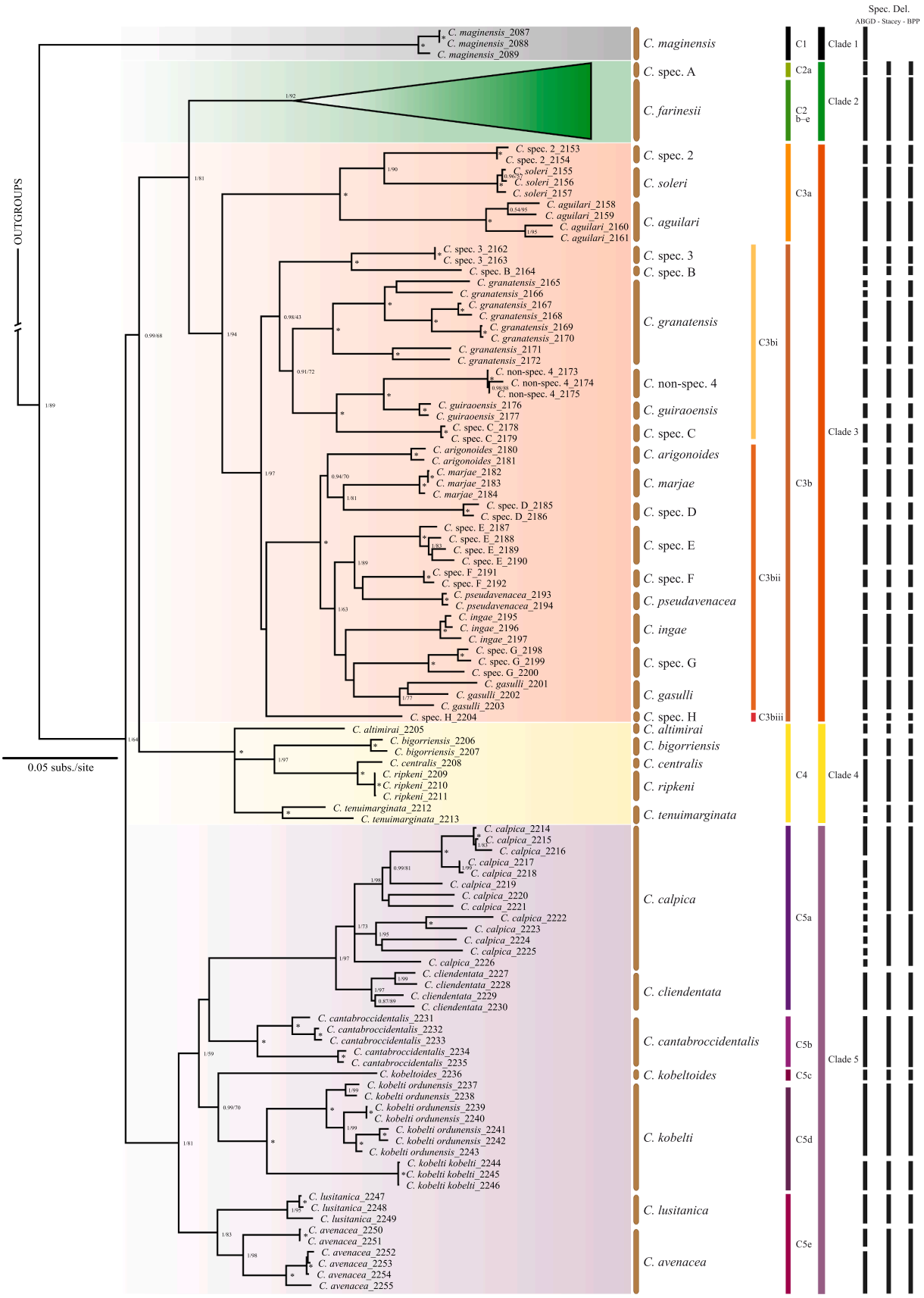

Fig. 1. Phylogenetic reconstruction obtained from the multilocus dataset by Bayesian inference where clade C2 is collapsed. Numbers on the nodes correspond to BI posterior probabilities and ML bootstrap values, respectively. Fully supported nodes are marked with an asterisk. The tree is coloured to distinguish the five main phylogroups. Brown bars correspond to specimens' assignation to nominal species and new putative species according to morphology. Coloured bars represent the main clades and their subclades. Black bars on the right summarize species delimitation results.

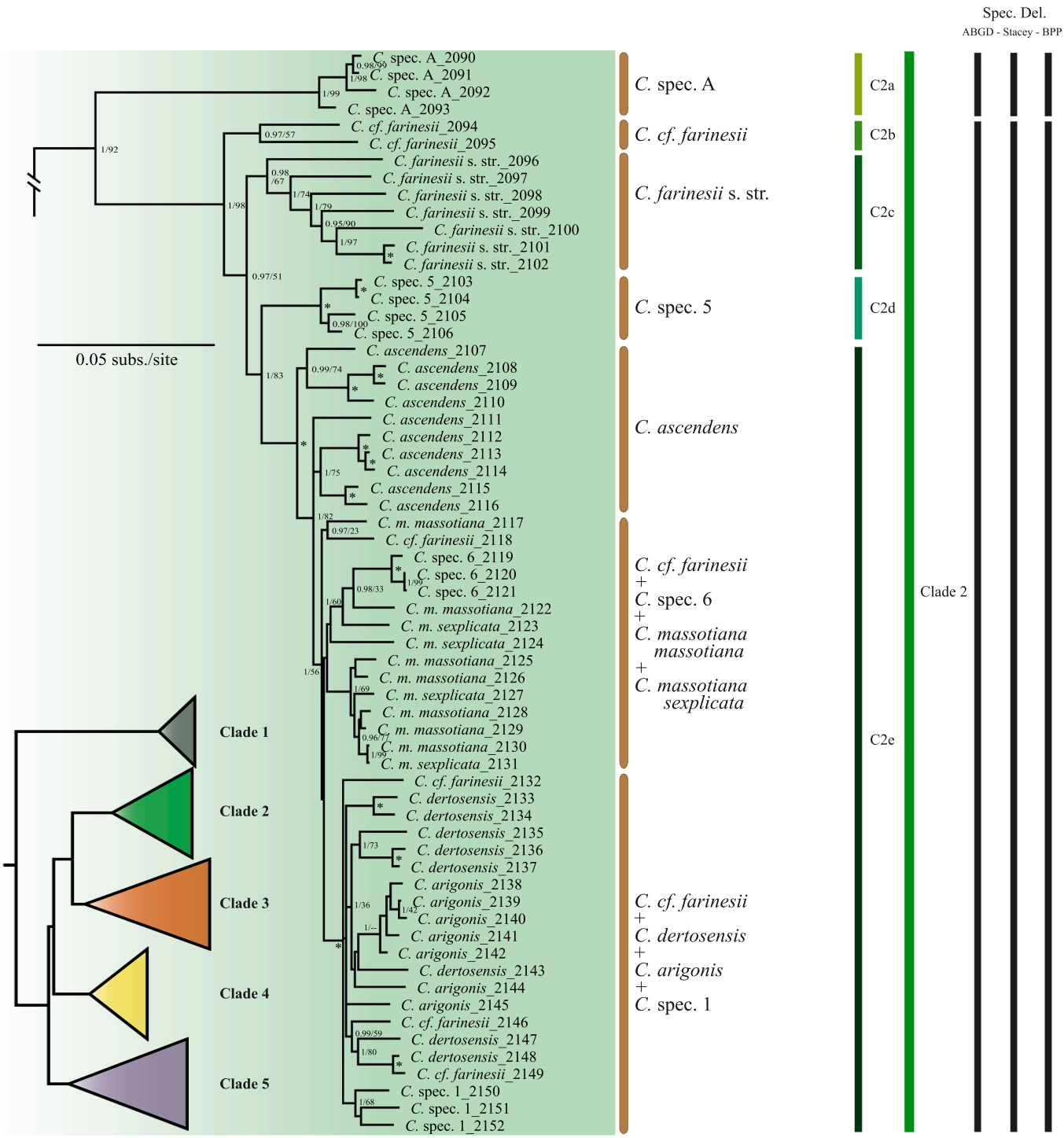

Fig. 2. Phylogenetic reconstruction obtained from the multilocus dataset by Bayesian inference for C2. Numbers on the nodes correspond to BI posterior probabilities and ML bootstrap values, respectively. Fully supported nodes are marked with an asterisk. The collapsed tree is coloured to distinguish the five main phylogroups. Brown bars correspond to specimens' assignation to nominal species and new putative species according to morphology. Green bars represent the subclades. Black bars on the right summarize species delimitation results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Chondrina spec. 3 and *Chondrina* non-spec. 4 (specimens which were collected at the same locality as *Chondrina* spec. 4 of Kokshoorn and Gittenberger (2010) but with a very different morphology of the specimen figured on plate 12 Fig. I1-2), all of them supported. Another two supported lineages, here called *Chondrina* spec. B from Alzira in Valencia (sister to *Chondrina* spec. 3 with full support) and *Chondrina* spec. C from Sierra Espuña in Murcia (sister to *C. guiraoensis* + *Chondrina* non-spec. 4 with full support) were also recovered within C3bi. High genetic divergences were observed within *C. granatensis*. Subclade C3bii grouped with full support *C. arigonoides*, *C. marjae*, *C. ingae*, *C. pseudavenacea* and *C. gasulli*, together with another four separate lineages here called *Chondrina* spec. D, E, F and G, all of them living in the South of Valencia and Alicante provinces. *Chondrina* spec. D was sister to *C. marjae* (PP = 1.00; BS = 81%). *Chondrina* spec. E, *Chondrina* spec. F and

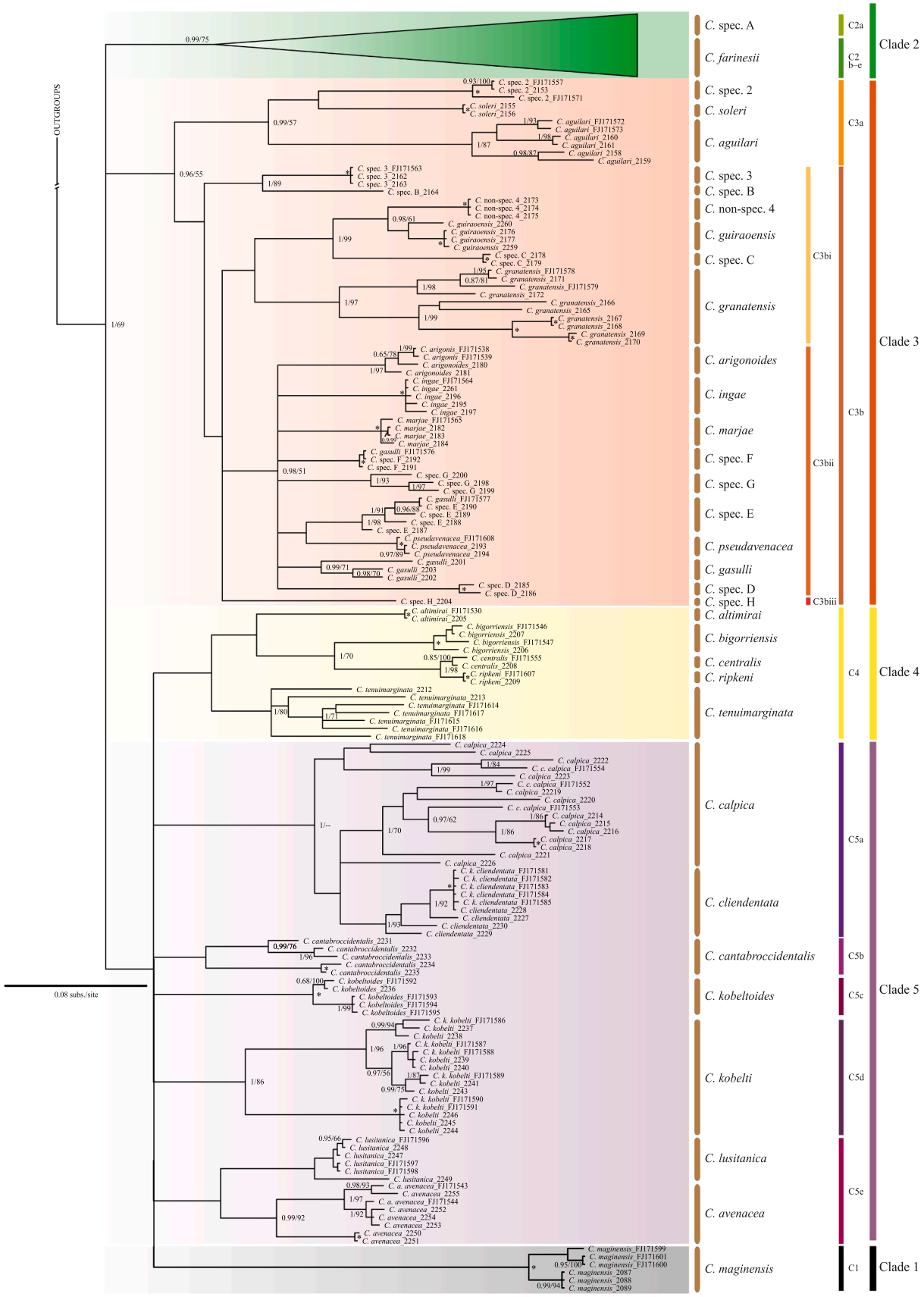

Fig. 3. Phylogenetic reconstruction obtained from the COI dataset by Bayesian inference with clade C2 collapsed. Numbers on the nodes correspond to BI posterior probabilities and ML bootstrap values, respectively. Fully supported nodes are marked with an asterisk. The tree is coloured to distinguish the five main phylogroups. Brown bars correspond to specimens' assignation to nominal species and new putative species according to morphology. Coloured bars represent the main clades and their subclades.

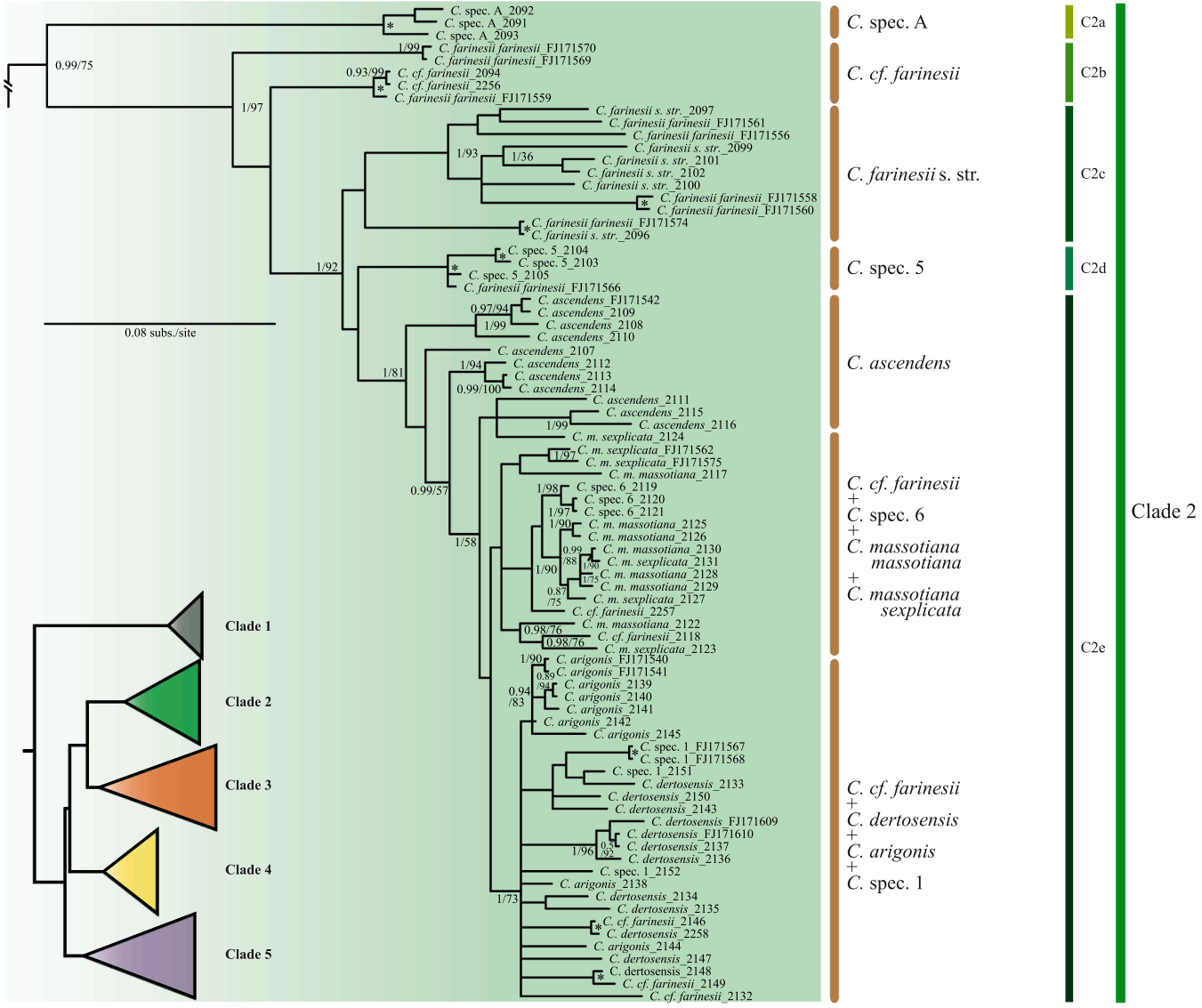

Fig. 4. Phylogenetic reconstruction obtained from the COI dataset by Bayesian inference for C2. Numbers on the nodes correspond to BI posterior probabilities and ML bootstrap values, respectively. Fully supported nodes are marked with an asterisk. The collapsed tree is coloured to distinguish the five main phylogroups. Brown bars correspond to specimens' assignation to nominal species and new putative species according to morphology. Coloured bars represent the main clades and their subclades.

C. pseudavenacea were closely related (PP = 1.00; BS = 89%), but the relationships among them were not resolved. *Chondrina ingae*, *C. gasulli and Chondrina* spec. G were also grouped together, although without support. Finally, lineage C3biii consisted only of one specimen, collected from Cabo Cope in Murcia, that we called *Chondrina* spec. H. With the exception of *Chondrina* non-spec. 4, which is restricted to Cuenca (middle-east Iberian Peninsula), all the species grouped within the C3b clade are distributed in the Southeast Iberian Peninsula, from Serra de Corbera in Valencia to Cerro Juan in Málaga. In the COI tree, all sequences published by Kokshoorn et al. (2010) for *C. arigonoides*, *C. ingae*, *C. marjae* and *C. pseudavenacea* joined with our new sequences for the same species. Nevertheless, the two COI sequences ascribed by Kokshoorn et al. (2010) to *C. gasulli* did not group with our *C. gasulli* clade containing topotypes of this species. Instead, one of them grouped with *Chondrina* spec. E and the other with *Chondrina* spec. F (Fig. 2).

Clade C4 was recovered as the sister group of the C2 + C3 clade, although supported only by Bayesian analysis (PP = 0.99; BS = 68%). This clade grouped the species *C. altimirai*, *C. bigorriensis*, *C. centralis*, *C. ripkeni* and *C. tenuimarginata*, all of them fully supported. *Chondrina ripkeni* and *C. centralis* were recovered as sister species (PP = 1.00; BS = 100%) closely related to *C. bigorriensis* with strong support (PP = 1.00; BS = 97%). However, the remaining relationships within this clade were not resolved. Sequences published by Kokshoorn et al. (2010) for each of these species joined with our new sequences for the same species in the COI tree.

Clade C5 was recovered as the sister group of clades C2–C4 but this relationship was only supported by Bayesian analysis (PP = 1.00; BS = 64%). The C5 phylogroup was divided into five main lineages, groups C5a–e. Subclade C5a (PP = 1.00; BS = 97%) joined the supported *C. calpica* from the South Iberian Peninsula with *C. cliendentata* living in the southern slope of the Cantabrian Mountains, including specimens from the type locality of the latter. Subclade C5b (PP = 1.00; BS = 100%) grouped several populations of the newly described *C. cantabroccidentalis* Somoza-Valdeolmillos & Vázquez-Sanz, 2021, living also in the southern slope of the Cantabrian Mountains. *Chondrina kobeltoides* (lineage C5c) was recovered sister to *C. kobelti* (PP = 0.99; ML = 70%). *Chondrina kobelti,* living in the northern slope of the Cantabrian Mountains, constituted subclade C5d (PP = 1.00; BS = 100%)

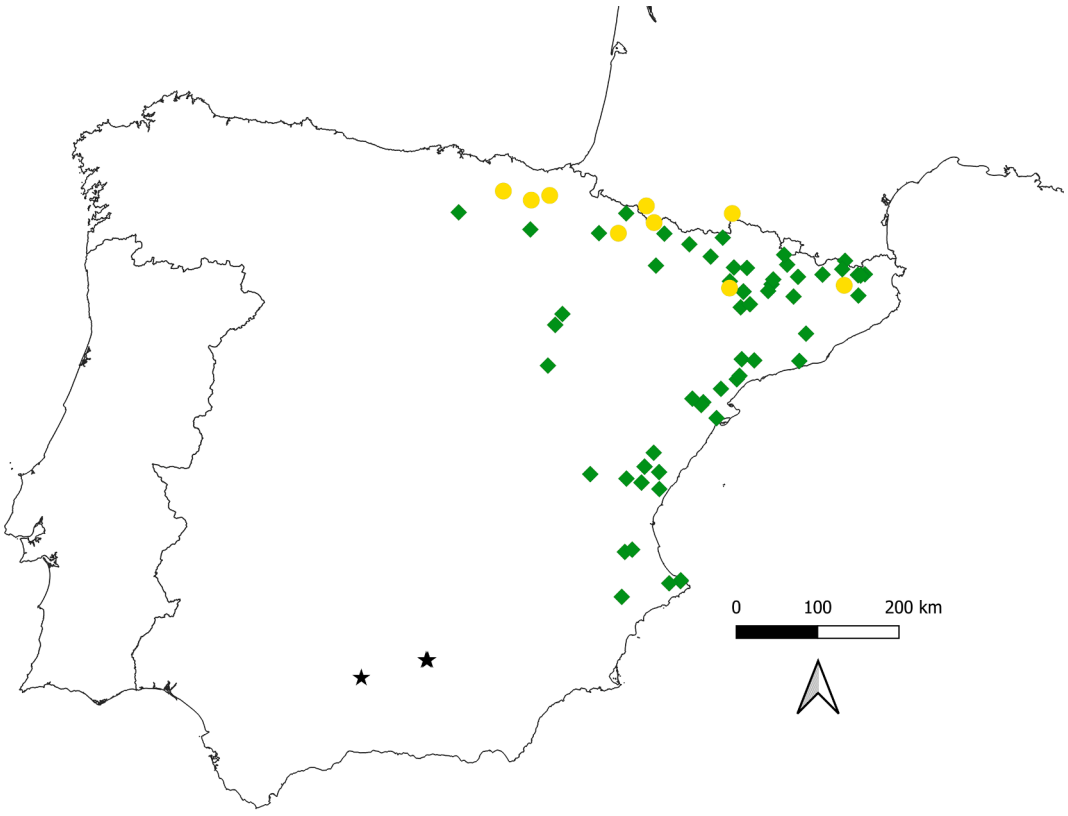

Fig. 5. Distribution map of the samples assigned to the main phylogenetic clades C1 (black stars), C2 (green diamonds) and C4 (yellow circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and included two very differentiated lineages. Finally, subclade C5e (PP = 1.00; BS = 83%) joined the supported *C. avenacea* and *C. lusitanica* as sister groups. In the COI dataset tree, there was a full correspondence between the new sequences and those published by Kokshoorn et al. (2010) for *C. avenacea*, *C. calpica*, *C. cliendentata* (=*C. kobelti cliendentata*), *C. kobelti* (=*C. kobelti kobelti*), *C. kobeltoides* and *C. lusitanica*.

3.3. Species delimitation

The results of the three species delimitation methods used to explore species limits are summarised in the three black columns within Figs. 1 and 2. For ABGD, different prior maximal distances yielded different numbers of groups (Supplementary Material S8). We used geographical distribution and the inferred phylogenetic relationships as independent data to choose among the different partitions recovered with the ABGD method and we considered the result with 49 groupings (P = 0.0215, K80 kimura Distance MinSlope = 0.4) as the most plausible. Table 3 lists the five best clusterings obtained with STACEY for each of the main clades excluding C1, not included in these analyses nor in the BPP analyses as stated above (in Supplementary Material S4 are listed the groups tested and 10 best clusterings obtained with STACEY). For BPP, species posterior probabilities for the groupings tested in each main clade (excluding C1) are provided in Table 4. These two multispecies coalescent species delimitation methods recovered the same 36 species within clades C2–C5.

ABGD supported the validity of *C. maginensis*, the only species belonging to clade C1, which was not tested with BPP and STACEY precisely because it was monospecific. All the three species delimitation methods recovered two species within Clade C2. *Chondrina* spec. A (subclade C2a) was strongly supported as a valid species and the remaining taxa, joined in subclades C2b–C2e, constituted a second species.

Within clade C3, STACEY and BPP yielded 21 species while ABGD recovered 23. The three species delimitation methods recovered as valid taxa the nominal species *C. soleri*, *C. aguilari*, *C. guiraoensis*, *C. arigonoides*, *C. marjae*, *C. pseudavenacea*, *C. ingae* and *C. gasulli*. *Chondrina* spec. B, C, D, E, F, G and H, as well as *Chondrina* spec. 2, *Chondrina* spec. 3 and *Chondrina* non-spec. 4 were also recovered as valid species by the three analyses. Regarding the nominal species *C. granatensis*, both the STACEY and BBP analysis supported three species within the taxon, while ABGD suggested five.

The BPP and STACEY analyses recovered *C. altimirai*, *C. bigorriensis* and *C. tenuimarginata* from clade C4 as valid species. ABGD supported *C. altimirai* and *C. bigorriensis,* but split *C. tenuimarginata* into two taxa. All the three analyses supported the species *C. centralis* and C. *ripkeni* from clade C4 as a single species.

Within clade C5, all the species delimitation methods recovered *C. cliendentata*, *C. kobeltoides* and *C. lusitanica* as valid species. The species status of the newly described *C. cantabroccidentalis* was also validated by the three analyses. *Chondrina avenacea* was recovered as one species by the STACEY and BPP analyses, but ABGD divided it in two groups. *Chondrina kobelti* was split into two species by all the delimitation methods. Finally, the results concerning *C. calpica* showed the greatest discrepancies between the analyses. ABGD yielded 10 groupings within this nominal species, while STACEY and BPP recovered two species. However, it must be highlighted that some of the BPP analyses supported the species status of two populations of *C. calpica* (*C. calpica*-a and *C. calpica*-b) (see Table 4), but the other groupings were not supported as species (*C. calpica*-c, *C. calpica*-d and *C. calpica*-e) and, according to the phylogeny obtained, is was not possible to create monophyletic groups leaving aside the two supported populations (i.e. *C. calpica*-a and *C. calpica*-b). So that, we finally tested the entire clade collapsed (*C. calpica*-a/b/c/d/e) and it was fully supported.

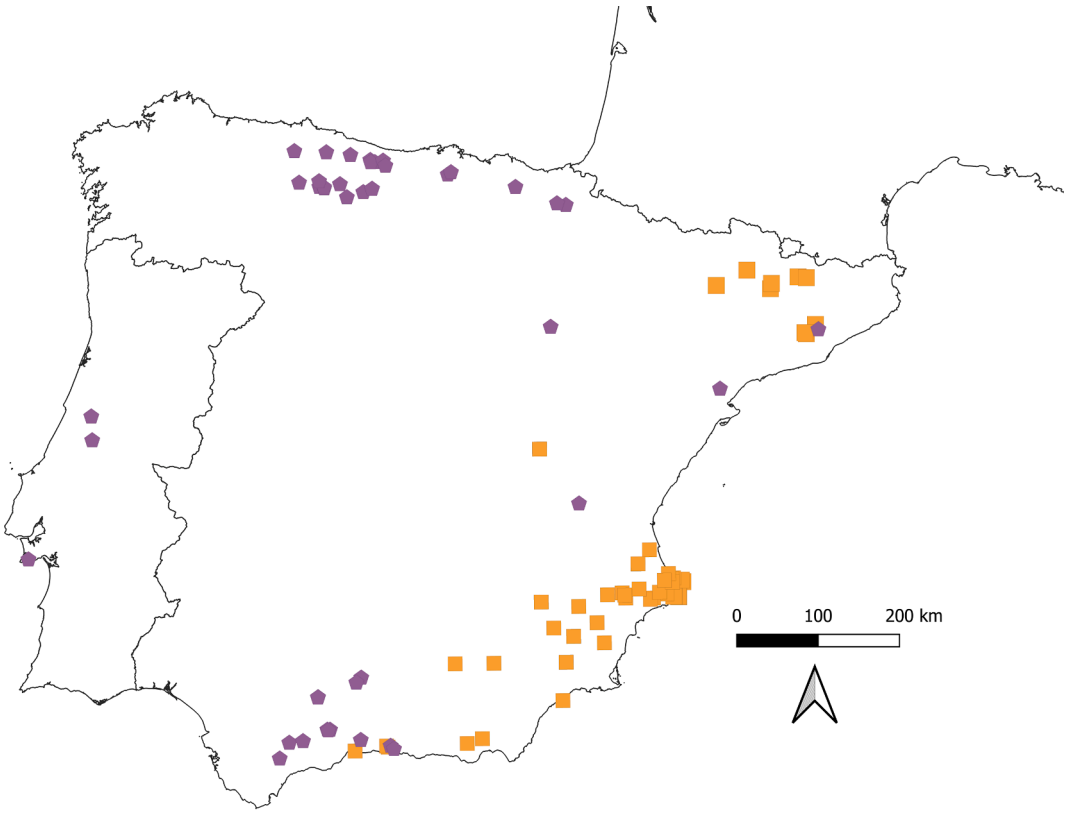

Fig. 6. Distribution map of the samples assigned to the main phylogenetic clades C3 (orange squares) and C5 (purple pentagons). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

It is widely agreed that species are the fundamental units in biology (Birky et al., 2010) and the base of many scientific studies (Agapow et al., 2004; Fraser and Bernatchez, 2001). Consequently, identifying all the taxa within a genus is essential to estimate biodiversity in conservation biology (Terlizzi et al., 2003). Here, we have addressed the systematics of the genus *Chondrina* in the Iberian Peninsula (western Mediterranean) by carrying out the most extensive molecular phylogeny for the genus to date and then employing automated molecular delimitation methods. Considering species as separately evolving metapopulation lineages (de Queiroz, 2007), the use of different character types (e.g. morphology or molecular markers) has been increasingly recognized as an indispensable approach to correctly resolve taxonomies, since each of them may constitute alternative lines of evidence to determine lineage boundaries. Indeed, it has been widely acknowledge that relying on a single kind of data may result in incorrect taxonomies (e.g. Padial et al., 2010; Sauer and Hausdorf, 2012). Despite the promising utility of automated molecular delimitation methods, they also have limitations, specifically that they are prone to confuse population genetic structure with species boundaries (Lohse, 2009; Sukumaran and Knowles, 2017). Therefore, it is important that other lines of evidence are also taken into account when interpreting the results of these methods.

Previous works on the genus *Chondrina* have already reviewed the number of existing species in the Iberian Peninsula (Gittenberger, 1973; Kokshoorn and Gittenberger, 2010) and their phylogenetic relationships (Kokshoorn et al., 2010; Somoza-Valdeolmillos et al., 2021). Our results are consistent with those reported by Kokshoorn et al. (2010), but the addition of new molecular markers as well as the extended number of populations included in our work, have allowed us to deepen our knowledge of the systematics of this group and gave us a better resolved phylogeny for the genus (Supplementary Material S9 summarized the classifications of *Chondrina* taxa living in the Iberian Peninsula given by Gittenberger (1973) and Kokshoorn and Gittenberger (2010) compared with our proposal).

As in Kokshoorn et al. (2010), our results also recovered *C. maginensis* as the sister group to all remaining *Chondrina* species living in the Iberian Peninsula.

There was a full correspondence between our clade C2 and clade B obtained by Kokshoorn et al. (2010). Kokshoorn et al. (2010) and Kokshoorn and Gittenberger (2010) considered six species within this clade, four of them traditionally classified within *C. farinesii s.l.* (Gittenberger, 1973): *C. arigonis*, *C. dertosensis*, *C. farinesii* and *C. massotiana* (including *C. massotiana massotiana* and *C. m. sexplicata*). Besides, they considered *C. ascendens* and the undescribed *Chondrina* spec. 1 with full species status. Our results indicate that the taxa mentioned by Kokshoorn and Gittenberger (2010) under the names *Chondrina* spec. 5 and *Chondrina* spec. 6, that were not included in their molecular analysis, also belong to this clade. However, the species delimitation approaches used in the present study, upon many more sequences of all these taxa, indicated that only two species should be considered in this clade. One of them, named *Chondrina* spec. A is an undescribed species living in the Iberian System, separated from the rest of species of this group by the whole middle Ebro Valley. Speciation processes on both sides of the Ebro Valley are quite common in land snails living in rocky substrates (Caro et al., 2019; Gomez and Rallo, 1988; Puente et al., 1998). The remaining taxa should be included within *C. farinesii s.l.* Thus, the putative species *Chondrina* spec. 1 and *Chondrina* spec. 6 proposed by Kokshoorn and Gittenberger (2010) should no longer be considered as valid taxa, as they belong to the groups *C. arigonis* + *C. dertosensis*, and *C. massotiana* + *C. sexplicata*, respectively. On the contrary, *Chondrina* spec. 5 really constitute a very different lineage within *C. farinesii s.l.*, and a subspecific name could be created to name this taxon according to genetic

Table 3
Results obtained in the STACEY analysis for each of the main clades: counts, Posterior Probabilities and the nclusters. The tested groups are presented in Supplementary Material S4.

	Groups									
	Clusters results assigned to multilocus dataset tree									
	C2					C3				
count	39,525	24,761	2121	410	270	1671	756	641	572	556
Posterior probability	0.5855	0.3668	0.0314	0.0061	0.0040	0.0265	0.0120	0.0102	0.0091	0.0088
nclusters	2	1	3	2	3	21	18	20	17	20
	C. spec. A	*C.* spec. A + *C. cf. farinesii* +	*C.* spec. A	*C.* spec. A + *C. cf. farinesii*	*C.* spec. A	*C.* spec. 2	*C.* spec. 2	*C.* spec. 2	*C.* spec. 2	*C.* spec. 2
	C. cf. farinesii + *C. farinesii* s. str. + (*C. cf. farinesii*/*C.* spec. 6/*C. m. massotiana*/*C. m. sexplicata*) + (*C. cf. farinesii*/*C. dertosensis*/*C. arigonis*/*C.* spec. 1)	*C. farinesii* s. str. + (*C. cf. farinesii*/*C.* spec. 6/*C. m. massotiana*/*C. m. sexplicata*) + (*C. cf. farinesii*/*C. dertosensis*/*C. arigonis*/*C.* spec. 1)	*C. cf. farinesii* *C. farinesii* s. str. + (*C. cf. farinesii*/*C.* spec. 6/*C. m. massotiana*/*C. m. sexplicata*) + (*C. cf. farinesii*/*C. dertosensis*/*C. arigonis*/*C.* spec. 1)	*C. farinesii* s. str. + (*C. cf. farinesii*/*C.* spec. 6/*C. m. massotiana*/*C. m. sexplicata*) + (*C. cf. farinesii*/*C. dertosensis*/*C. arigonis*/*C.* spec. 1)	*C. cf. farinesii* + *C. farinesii* s. str. (*C. cf. farinesii*/*C.* spec. 6/*C. m. massotiana*/*C. m. sexplicata*) + (*C. cf. farinesii*/*C. dertosensis*/*C. arigonis*/*C.* spec. 1)	*C.* spec. 3 *C. aguilari* *C.* spec. 3 *C.* spec. B *C. granatensis*-1 *C. granatensis*-2	*C.* spec. 3 *C.* spec. 4 *C. aguilari* *C. arigonoides* *C. granatensis*-1 *C. granatensis*-2	*C.* spec. 3 *C. aguilari* *C. arigonoides* *C. gasulli* *C. granatensis*-1 *C. granatensis*-2	*C.* spec. 3 *C.* spec. 4 *C. aguilari* *C. granatensis*-1 *C. granatensis*-2 *C. granatensis*-3	*C.* spec. 3 *C.* spec. 4 *C. aguilari* *C. gasulli* *C. granatensis*-1 *C. granatensis*-2
						C. granatensis-3 *C.* spec. 4 *C. guiraoensis* *C.* spec. C *C. arigonoides* *C. marjae* *C.* spec. D *C.* spec. E *C.* spec. F *C. pseudavenacea* *C. ingae* *C.* spec. G *C. gasulli* *C.* spec. H	*C. granatensis*-3 *C. guiraoensis* *C. ingae* *C. marjae* *C. soleri* *C.* spec. B *C.* spec. C *C.* spec. D *C.* spec. G *C.* spec. H *C. gasulli* + *C. pseudavenacea* + *C.* spec. E + *C.* spec. F	*C. granatensis*-3 *C. ingae* *C. marjae* *C. pseudavenacea* *C. soleri* *C.* spec. B *C.* spec. C *C.* spec. D *C.* spec. E *C.* spec. F *C.* spec. G *C.* spec. H *C.* spec. 4 + *C. guiraoensis*	*C. guiraoensis* *C. ingae* *C. marjae* *C. soleri* *C.* spec. B *C.* spec. C *C.* spec. D *C.* spec. G *C.* spec. H *C. arigonoides* + *C. gasulli* + *C. pseudavenacea* + *C.* spec. E + *C.* spec. F	*C. granatensis*-3 *C. guiraoensis* *C. ingae* *C. marjae* *C. pseudavenacea* *C. soleri* *C.* spec. B *C.* spec. C *C.* spec. D *C.* spec. E *C.* spec. G *C.* spec. H *C. arigonoides* + *C.* spec. F

	Groups									
	Clusters results assigned to multilocus dataset tree									
	C4					C5				
count	24,338	15,313	6337	5690	2954	2248	1771	1158	799	692
Posterior probability	0.3605	0.2268	0.0939	0.0843	0.0438	0.0357	0.0281	0.0184	0.0127	0.0110
nclusters	4	3	2	3	1	9	8	10	10	10
	C. altimirai	*C. altimirai*	*C. altimirai* + *C. tenuimarginata*	*C. altimirai* + *C. tenuimarginata*	*C. altimirai* + *C. tenuimarginata* + *C. bigorriensis* + *C. centralis* + *C. ripkeni*	*C. calpica*-1	*C. calpica*-1	*C. calpica*-1*	*C. calpica*-1*	*C. calpica*-1*
	C. bigorriensis *C. centralis* + *C. ripkeni* *C. tenuimarginata*	*C. tenuimarginata* *C. bigorriensis* + *C. centralis* + *C. ripkeni*	*C. bigorriensis* + *C. centralis* + *C. ripkeni*	*C. bigorriensis* *C. centralis* + *C. ripkeni*		*C. calpica*-2 *C. cliendentata*	*C. calpica*-2 *C. cliendentata*	*C. calpica*-2* *C. calpica*-3*	*C. calpica*-2* *C. calpica*-3*	*C. calpica*-2* *C. calpica*-3*
						C. cantabroccidentalis *C. kobeltoides* *C. kobelti*-1 *C. kobelti*-2 *C. lusitanica* *C. avenacea*	*C. cantabroccidentalis* *C. kobeltoides* *C. kobelti*-1 *C. kobelti*-2 *C. lusitanica* *C. lusitanica* + *C. avenacea*	*C. cliendentata* *C. cantabroccidentalis* *C. kobeltoides* *C. kobelti*-1 *C. kobelti*-2 *C. lusitanica* *C. avenacea*	*C. cliendentata* *C. cantabroccidentalis* *C. kobeltoides* *C. kobelti*-1 *C. kobelti*-2 *C. lusitanica* *C. avenacea*	*C. cliendentata* *C. cantabroccidentalis* *C. kobeltoides* *C. kobelti*-1 *C. kobelti*-2 *C. lusitanica* *C. avenacea*

*Calpica groups that Stacey delimits but with different internal relationships.

Table 4
BPP results obtained without guide species tree for the candidate species recovered in mtDNA delimitation analyses (ABGD), before and after collapsing unsupported candidate species (supported candidate species PP > 0.97). The previously tested BPP groups of *Chondrina* are presented in Supplementary Material S5.

Candidate species	Posterior probabilities				Candidate species	Posterior probabilities			
	Algorithm 0		Algorithm 1			Algorithm 0		Algorithm 1	
					C2				
	1st run					**2nd run**			
C. spec. A	1.000	1.000	1.000	1.000					
C. farinesii	1.000	1.000	1.000	1.000					
					C3				
	1st run					**2nd run**			
C. spec. 2	0.999	1.000	0.999	1.000	*C.* spec. 2	0.999	0.999	0.999	
C. soleri	0.999	1.000	0.999	1.000	*C. soleri*	0.999	0.999	0.999	0.999
C. aguilari	1.000	1.000	1.000	1.000	*C. aguilari*	1.000	1.000	1.000	1.000
C. spec. 3	0.986	0.986	0.983	0.985	*C.* spec. 3	0.982	0.984	0.984	0.982
C. spec. B	1.000	0.999	1.000	1.000	*C.* spec. B	1.000	1.000	1.000	1.000
C. granatensis-a	0.901	0.895	0.899	0.898	*C. granatensis*-1(a/b)	0.992	0.996	0.997	0.997
C. granatensis-b	0.899	0.894	0.896	0.898					
C. granatensis-c	0.933	0.935	0.933	0.936	*C. granatensis*-2(c/d)	0.976	0.974	0.976	0.982
C. granatensis-d	0.940	0.940	0.940	0.940					
C. granatensis-e	0.996	0.996	0.998	0.997	*C. granatensis*-3(e)	0.981	0.976	0.977	0.983
C. spec. 4	0.999	1.000	1.000	1.000	*C.* spec. 4	0.999	1.000	0.999	0.999
C. guiraoensis	0.997	0.997	0.998	0.998	*C. guiraoensis*	0.997	0.998	0.998	0.997
C. spec. C	0.984	0.984	0.981	0.984	*C.* spec. C	0.980	0.981	0.982	0.980
C. arigonoides	0.996	0.996	0.996	0.996	*C. arigonoides*	0.996	0.995	0.996	0.995
C. marjae	0.999	0.999	0.999	0.999	*C. marjae*	0.999	0.999	0.998	0.999
C. spec. D	0.997	0.996	0.997	0.997	*C.* spec. D	0.997	0.997	0.997	0.997
C. spec. E	0.981	0.982	0.983	0.984	*C.* spec. E	0.982	0.981	0.983	0.982
C. spec. F	0.990	0.989	0.990	0.989	*C.* spec. F	0.988	0.989	0.989	0.987
C. pseudavenacea	0.974	0.974	0.975	0.975	*C. pseudavenacea*	0.973	0.973	0.976	0.972
C. ingae	1.000	1.000	1.000	1.000	*C. ingae*	1.000	1.000	1.000	1.000
C. spec. G	1.000	0.998	0.999	1.000	*C.* spec. G	0.999	0.999	1.000	1.000
C. gasulli	0.999	0.998	0.998	0.999	*C. gasulli*	0.998	0.998	0.999	0.999
C. spec. H	1.000	1.000	1.000	1.000	*C.* spec. H	1.000	0.999	1.000	1.000
					C4				
	1st run					**2nd run**			
C. altimirai	0.998	0.998	0.997	0.998	*C. altimirai*	0.989	0.990	0.990	0.989
C. bigorriensis	1.000	1.000	0.999	1.000	*C. bigorriensis*	0.999	0.999	0.999	0.999
C. centralis & C. ripkeni	1.000	1.000	1.000	1.000	*C. centralis & C. ripkeni*	1.000	1.000	1.000	1.000
C. tenuimarginata-a	0.963	0.963	0.963	0.966	*C. tenuimarginata* (a/b)	0.989	0.990	0.990	0.989
C. tenuimarginata-b	0.964	0.963	0.963	0.966					
					C5				
	1st run					**2nd run**			
C. calpica-a	0.989	0.987	0.987	0.988	*C. calpica*-a	0.984	0.985	0.984	0.984
C. calpica-b	0.987	0.985	0.987	0.986	*C. calpica*-b	0.982	0.985	0.984	0.983
C. calpica-c	0.973	0.972	0.974	0.973	*C. calpica*-c	0.963	0.969	0.969	0.974
C. calpica-d	0.944	0.946	0.946	0.946	*C. calpica*-d/e	0.965	0.971	0.971	0.976
C. calpica-e	0.947	0.945	0.949	0.947					
C. calpica-f	0.707	0.701	0.692	0.698	*C. calpica*-f/g/h/i/j	1.000	1.000	1.000	1.000
C. calpica-g	0.707	0.701	0.692	0.699					
C. calpica-h	0.940	0.938	0.936	0.941					
C. calpica-i	0.898	0.898	0.903	0.902					
C. calpica-j	0.922	0.920	0.927	0.924					
C. cliendentata	0.999	1.000	0.998	0.999	*C. cliendentata*	1.000	0.999	0.999	1.000
C. cantabroccidentalis	1.000	1.000	1.000	1.000	*C. cantabroccidentalis*	1.000	1.000	1.000	1.000
C. kobeltoides	1.000	1.000	0.999	0.999	*C. kobeltoides*	1.000	0.999	0.999	1.000
C. kobelti-a	1.000	0.999	1.000	0.999	*C. kobelti*-a	0.999	0.999	0.999	0.999
C. kobelti-b	1.000	0.999	1.000	0.999	*C. kobelti*-b	0.999	0.999	0.999	0.999
C. lusitanica	1.000	1.000	1.000	1.000	*C. lusitanica*	1.000	1.000	1.000	1.000
C. avenacea-a	0.938	0.935	0.935	0.934	*C. avenacea* (a/b)	1.000	1.000	1.000	1.000
C. avenacea-b	0.938	0.935	0.935	0.934					

	Algorithm 0		Algorithm 1	
	3rd run			
C. calpica-1(a/b/c/d/e)	1.000	1.000	1.000	1.000
C. calpica-2(f/g/h/i/j)	1.000	1.000	0.999	1.000
C. cliendentata	1.000	1.000	0.999	1.000
C. cantabroccidentalis	1.000	1.000	1.000	1.000
C. kobeltoides	1.000	1.000	1.000	1.000
C. kobelti-1(a)	0.999	0.999	0.999	0.999
C. kobelti-2(b)	0.999	0.999	0.999	0.999
C. lusitanica	1.000	1.000	1.000	1.000
C. avenacea (a/b)	1.000	1.000	1.000	1.000

differences, morphology and geographic distribution. *Chondrina* species have traditionally been recognized on the basis of shell characters and particularly on the number of apertural teeth (Gittenberger, 1973; Gómez and Angulo, 1982). Nevertheless, molecular results indicated that shell morphology can be very different in populations phylogenetically very closely related. Surprisingly, within subclade C2e, for which the molecular data and species delimitation methods found no big genetic discontinuities, there is a high polymorphism in shell characters. Within subclade C2e populations of *C. arigonis* morphotype, characterised by a thickened peristome and two conspicuous palatal teeth (Kokshoorn and Gittenberger, 2010), grouped within *C. dertosensis* with very weak or without teeth on the palatal wall (Cadevall and Orozco, 2016). Some populations of the morphotype *C. farinesii*, without palatal teeth and with a tiny peristome, are also grouped within this same subclade. The incorporation of topotypes of *Chondrina jumillensis unidentata* described by Altimira (1960) from Llabería, Cueva del Ramé (Tarragona), and considered by Kokshoorn and Gittenberger (2010) within *C. farinesii*, are also grouped within subclade C2e, despite it is a form of *C. dertosensis* with extreme shell teeth reduction. The same can be said regarding the morphospecies *C. sexplicata* characterised by six strong teeth in the aperture of the shell and *C. massotiana* with reduced apertural teeth (Cadevall and Orozco, 2016), joined also in the same subclade C2e and constituting no monophyletic entities.

The clade C3 of the present work corresponded to clade C of Kokshoorn et al. (2010). In this work, the species status of *C. aguilari* and *C. soleri*, included in the synonymy of *C. farinesii* and *C. guiraoensis* respectively by Gittenberger (1973), as well as *C. gasulli,* are validated within this clade. Our results also confirmed the validity of four new species described by Kokshoorn and Gittenberger (2010), named *C. arigonoides*, *C. marjae*, *C. ingae*, and *C. pseudavenacea*. Besides, the putative new species provisionally named by Kokshoorn and Gittenberger (2010) under the names *Chondrina* spec. 2 and *Chondrina* spec. 3 were also confirmed. Finally, another eight monophyletic and very divergent lineages have been identified (here named *Chondrina* spec. B to *Chondrina* spec. H and *Chondrina* non-spec. 4) and constitute eight undescribed species as indicated by ABGD, STACEY and BPP analyses. These results indicate that the genus has extensively radiated within Alicante province. The addition of more *Chondrina* populations in the molecular analyses allowed us to identify several microendemic taxa within clade C3. This is just the opposite situation to clade C2, where the incorporation of more populations shortened branch length between lineages, grouping within the same species several morphospecies currently considered with full species status (Kokshoorn and Gittenberger, 2010). *Chondrina granatensis*, included also in this clade, showed high genetic divergences, and probably as a consequence, ABGD recovers 5 species while STACEY and BPP recover 3. Prioritising multilocus methods, it seems that 3 species may be involved, however, not published sequence information indicate a more complicate scenario; so more studies are needed within this species that occupies a widespread distribution range throughout the South Iberian Peninsula (Arrébola and Gómez, 1998).

Our clade C4 fully corresponded with clade D of Kokshoorn et al. (2010). This clade is mainly a Pyrenean group, with most species living in the Pyrenean region, although *C. tenuimarginata* also extends to Castellón province and *C. ripkeni* is endemic to the Basque Country Mountains (Gittenberger, 1973). According to Kokshoorn et al. (2010) all these species are characterised by having a more or less reflexed apertural lip of the shell. Within this clade, BPP and STACEY supported the validity of *C. tenuimarginata* as a single species, but ABGD split it into two. ABGD analysis is known to be specially sensitive to population structuring and to consider population structuring as different species when structuring is present even if it is not very pronounced (Dellicour and Flot, 2018; Puillandre et al., 2012a). In addition, our ABGD dataset do not fit any of the two evolutionary models implemented in the method and it is been reported that this can hinder delimitation with this analysis (Fregin et al., 2012). Consequently, we prioritised BPP and STACEY results, considering *C. tenuimarginata* as monospecific taxa. On the contrary, *C. altimirai* and *C. bigorriensis* were recovered with full species status by all the analyses. The phylogenetic relationships of *C. altimirai* and *C. tenuimarginata* were not resolved, but the high similarity of the shell morphology of both species (Gittenberger, 1973) indicate that they could constitute sister species. *Chondrina centralis* and *C. ripkeni* are two taxa very closely related, and all the three species delimitation analyses joined them in a single species.

Finally, clade C5 corresponded with clades E (=clade C5a), F (=clades C5c + C5d), and G (=clade C5e) obtained by Kokshoorn et al. (2010). The incorporation of more DNA sequences including additional gene fragments and more populations of the species joined within these clades, allowed us to recover all these groups in a monophyletic clade. Within clade C5, we recovered the populations of *C. avenacea* from East of the Baetic System as the sister group of this species' populations living in the North Iberian Peninsula and Portugal (Altonaga et al., 1994; Gittenberger, 1973). As in *C. tenuimarginata*, ABDG suggested two species for *C. avenacea* matching these groupings, while STACEY and BPP supported it as a single species. This discrepancy is probably due to a certain population structuring between the two groups (Dellicour and Flot, 2018; Puillandre et al., 2012a) and, therefore, we propose to consider *C. avenacea* as monospecific taxon. *Chondrina kobelti* formed two very divergent clades that occupy different geographic ranges, one living in the East and the other in the West Cantabrian Mountains. Somoza-Valdeolmillos et al. (2021) already noted that they hybridize in the contact zone and concluded that they constituted two different taxa of subspecific status according to the biological species concept (Aldhebiani, 2018; de Queiroz, 2005, 2007) and named them as *C. kobelti kobelti* (Westerlund, 1887) and *C. kobelti ordunensis* Pilsbry, 1918. *Chondrina cantabroccidentalis* was recovered as a valid species too, as previously concluded by Somoza-Valdeolmillos et al. (2021). *Chondrina cliendentata*, *C. kobeltoides* and *C. lusitanica* were supported with full species status in all three species delimitation analyses, revalidating the data obtained by Kokshoorn et al. (2010). Finally, in the case of *C. calpica,* which has a wide distribution range, we did not obtain fully conclusive results regarding species delimitation. ABGD results largely dissented with BPP and STACEY results and even within BPP the results were blurred. It seems that at least two species may be involved, but it would be needed to analyse more populations of this species to fully understand its systematics.

The extensive sampling of this study has made it possible to include in our analyses almost all species described in the Iberian Peninsula until now, increasing the number of known localities for many of them. In spite of this, two nominal taxa mentioned in the review published by Kokshoorn and Gittenberger (2010) could not be included in our work. One of them was *C. jumillensis* (Pfeiffer, 1853). Figures of the type of *C. jumillensis* have been published by Haas (1926: pl. 27 fig. 8) and by Kokshoorn and Gittenberger, (2010: pl. 11 Fig. G1-2) and it is characterised by the presence of two palatal folds. The original label of this species indicated two localities, Orihuela in Alicante and Jumilla in Murcia (Gittenberger, 1973; Haas, 1926). However, Kokshoorn and Gittenberger (2010) concluded that this species does not occur near Jumilla and the type locality should be Orihuela. We intensively sampled the Orihuela Mountains looking for this species, but only specimens with reduced dentition were found and, despite they turned out to represent a new species here named as *Chondrina* spec. D, they did not resemble the specimens described for *C. jumillensis*. As a result, *C. jumillensis* is still one enigmatic species and no locality could be ascribed to it.

The second species mentioned in Kokshoorn and Gittenberger (2010) and not included in our study was *Chondrina* spec. 4. These authors did not give any description of this species and no molecular data were included for it. They only figured one shell under the name *Chondrina* spec. 4 coming from Cuenca, Ciudad Encantada, collected by Vilella and Tejedo (Kokshoorn and Gittenberger, 2010: plate 12: I1-2). *Chondrina* spec. 4 resembles a conical *C. farinesii s.l.* with reduced dentition and

without palatal teeth. We extensively sampled the surroundings of Cuenca, Ciudad Encantada, looking for specimens with reduced dentition, but we only found two morphs and they did not match with the original figure of *Chondrina* spec. 4. One of them was the typical *C. avenacea* characterized by four palatal teeth (Gittenberger, 1973). The other specimens found in Ciudad Encantada had as many as five teeth, including always two evident palatal teeth. These individuals were supported as a valid species by our analyses and we provisionally called it *Chondrina* non-spec. 4, to highlight that although the specimens came from the cited locality, they did not correspond morphologically with the figured specimen.

Molecular methods have documented both, under- and over-estimated species diversity within molluscs genus (Caro et al., 2019; Raphalo et al., 2021; Vidigal et al., 2018). The former occurs when several highly supported monophyletic clades are classified within one species and referred to as cryptic species (Bickford et al., 2007). The latter can occur when various shell forms are joined together within a given species-level clade (Horsáková et al., 2019). Underestimation of species diversity in *Chondrina* is especially evident within Clade C3, containing as many as ten undescribed species. On the other hand, Clade C2 represents a good example of over-estimated species diversity, indicating that *C. arigonis*, *C. dertosensis*, *C. farinesii s.str.*, *C. massotiana* and *C. sexplicata* (and also the undescribed *Chondrina* spec. 1, *Chondrina* spec. 5 and *Chondrina* spec. 6) should be included within one species, named *Chondrina farinesii s.l.* As a result, the genus *Chondrina* illustrates very well the limits of morphology alone to delimit species owing to the few diagnostic characters, limited to shell morphology. Kokshoorn et al. (2010) and Kokshoorn and Gittenberger (2010) described four new species and proposed another 6 putative taxa based on their COI phylogenetic results. Somoza-Valdeolmillos et al. (2021), based on a multilocus molecular phylogeny, described another new species and revalidated one subspecies. The present work shows that at least nine species (*Chondrina* spec. A to *Chondrina* spec. H and *Chondrina* non-spec. 4) should be described. Besides, we confirm that another two species named *Chondrina* spec. 2 and *Chondrina* spec. 3 by Kokshoorn and Gittenberger (2010), need a formal description, too. More studies are needed before considering further subdivisions within *C. granatensis* and *C. calpica*, two taxa where more than one species could be involved, as suggested by the species delimitation methods. In short, this makes a total amount of 16 new species delimited by DNA sequencing out of 33 species living in the Iberian Peninsula (considering *C. farinesii* as only one species). Thus, 46% of the species of this genus living in the Iberian Peninsula (MolluscaBase, 2021) were not recognized by previous morphological studies. There are other land snail taxa with similar taxonomic problems regarding the plasticity of the apertural armature and they needed to implement molecular analysis to resolve their taxonomies like the genus *Pupilla* and *Vertigo* (Nekola et al., 2014, 2018).

The new species identified within *Chondrina* highlight the importance of the Iberian Peninsula as the main centre of radiation of this genus. Although the limits of the distribution ranges of many undescribed species of *Chondrina* are not fully known, some of them might show very restricted geographic ranges, as is prevailing in rock-dwelling snails (Hoekstra and Schilthuizen, 2011; Schilthuizen et al., 2005), characterised by high calcium requirements and scarce dispersal abilities. Some *Chondrina* species live in one mountain system or in a few adjacent mountain areas, indicating the role of alluvial deposits in valleys as barriers to gene flow, favouring allopatric speciation. This patchy distribution is particularly evident in the Southeast Iberian Peninsula.

Most cryptic species in *Chondrina* corresponded to species with reduced shell teeth, fine radial shell sculpture and non-thickened apertural lip, previously classified within *C. farinesii s.l.* This makes it difficult to find diagnostic characters to describe them. This is true for *C. ingae* and *C. marjae*, but also for the following undescribed species: *Chondrina* spec. 2, *Chondrina* spec. A, *Chondrina* spec. D, and *Chondrina* spec. G. Some other undescribed species are similar in shell morphology to *C. gasulli* (*Chondrina* spec. E and *Chondrina* spec. F) or to *C. granatensis* (*Chondrina* spec. 3, *Chondrina* non-spec. 4, *Chondrina* spec. B, *Chondrina* spec. C and *Chondrina* spec. H) and many of them have reduced teeth in the aperture, too. All these cryptic and pseudocryptic species, in the sense of (Bickford et al., 2007; Lajus et al., 2015) are under study and a full description will be published in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107480.

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