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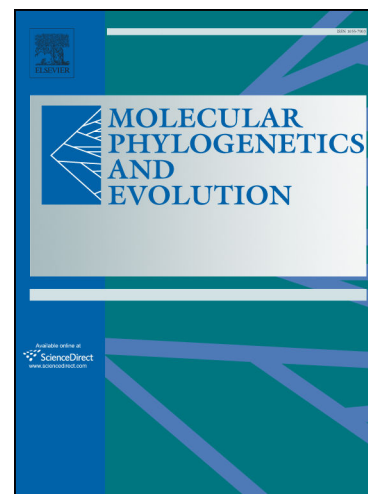
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Underestimated diversity: cryptic species and phylogenetic relationships in the subgenus *Cobalius* (Coleoptera: Hydraenidae) from marine rockpools

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Abstract

Marine rockpools are isolated patches of habitat in the supratidal environment (the so-called splash zone), at the transition between sea and land, found along the rocky shores worldwide and characterized by harsh conditions for life. Nonetheless, few specialized invertebrates successfully colonized this peculiar environment. Among them several members of the water beetles *Ochthebius* Leach, 1815, subgenus *Cobalius* Rey (1886), which are found almost exclusively in supratidal and upper-most intertidal marine rockpools from the eastern Atlantic Ocean (Cape Verde, Canary Islands and Morocco, Madeira, Azores) throughout the whole Mediterranean basin. The subgenus *Cobalius* before 2020 was considered to include ten valid species, based on morphological differences. In late 2020, four additional new species were described. However, recent molecular phylogenetic studies have uncovered further cryptic diversity suggesting the presence of multiple undetected species within this group, highlighting that the species boundaries remain unclear and systematics and taxonomy are in need of revision. In this study we provide a molecular phylogeny based on DNA sequence data from mitochondrial and nuclear genes obtained from ten described species belonging to the subgenus *Cobalius*, and extensive taxon sampling, in order to better understand the phylogenetic relationships within this genus and to infer the biogeographic processes behind its diversification. We also used a molecular clock to define a time window for diversification of distinct clades within the subgenus, and explore aspects of its evolutionary history. Finally, we used three species delimitation methods (PTP, GMYC and ABGD) to clarify taxonomy and validate species boundaries. Our phylogenetic and biogeographic results identified sixteen independent lineages grouped in four main clades and the possible origin of *Cobalius* was estimated to be in the Early Miocene (~22 Mya) in W Mediterranean area. Moreover, species delimitation methods suggest there are between 16 and 24 putative species, most of them diverged during the Late Miocene, Pliocene and Pleistocene (6.0–0.11 Mya).

Keywords: Marine rockpools; cryptic species; species delimitation; *Ochthebius*; *Cobalius*.

1. Introduction

Marine rockpools are patches of habitat in marine ecosystems that are commonly found on richly structured and sloping rocky shores in the supratidal and the upper-most intertidal zones (the so called splash zone) worldwide (Antonini et al., 2010; von der Heyden et al., 2013; Firth et al., 2014), that clearly differ from the surrounding coast and are characterized by harsh conditions for life. In fact, these habitats are influenced by both terrestrial and marine dynamics, are ephemeral and their persistence is closely related to the splashing of sea water from waves and high tides, to the frequency and intensity of sea storms, the exposure to the sunlight, evaporation, to the strength of the wind, the intensity of the rainfalls and are consequently affected by drastic fluctuations of salinity, temperature, oxygen content, pH and concentration of chemical compounds (Ganning, 1967, 1970; Underwood and Skilleter, 1996; Antonini et al., 2010). Despite these unfavorable conditions, few specialized invertebrates, such as the copepod *Tigriopus fulvus* (Vecchioni et al., 2019), mosquitoes of the family Culicidae (Yavasoglu et al., 2016; Rosenfeld et al., 2018, 2019) and species of the water beetle family Hydraenidae, belonging to the genus *Ochthebius* Leach 1815 (Antonini et al., 2010; Audisio et al., 2010; Sabatelli et al., 2013, 2016, 2018, 2021), are known to live in this peculiar environment.

Within the genus *Ochthebius*, members of two lineages are known to inhabit E Atlantic, W Palaearctic and Macaronesian coastal rockpools: the lineage “*Calobius*” (Sabatelli et al., 2016) and the subgenus *Cobalius*. Their distribution is contiguous around the Mediterranean coasts with many sympatric and syntopic areas (Sabatelli et al., unpublished data).

Cobalius species complex was described as subgenus of *Ochthebius* by Rey (1886) for the species *Ochthebius lejolisii* Mulsant and Rey, 1861 and *Ochthebius subinteger* Mulsant and Rey, 1861. Before 2020, ten species were recognized, based on morphological differences: *O. adriaticus* Reitter, 1886 (Central and E Mediterranean coastal areas), *O. celatus* Jäch, 1989 (Central and E Mediterranean coastal areas), *O. lejolisii* Mulsant and Rey, 1861 (Coastal Atlantic areas of NW and W continental Europe and of N Morocco), *O. subinteger* Mulsant and Rey, 1861 (Central and W Mediterranean coastal areas), *O. biltoni* Jäch and Delgado, 2017 (Sicily), *O. algicola* Wollaston, 1871 (Madeira), *O. balfourbrownei* Jäch, 1989 (Cape Verde Islands), *O. freyi* d'Orchymont, 1938 (Azores), *O. lanthanus* Ribera and Foster, 2018 (Gran Canaria) and *O. serratus* Rosenhauer, 1856 (salt streams in S Spain and N Morocco). Recently four additional new species have been described (Villastrigo et al., 2020) from western Mediterranean and eastern Atlantic coastal areas, based on morphological and/or molecular data: *O. anzar* (Lanzarote Island and SW Morocco), *O. cortomaltese* (Malta), *O. evae* (Atlantic coast of Morocco and Mediterranean coast of south Spain), and *O. gorgadensis* (Cape Verde Archipelago). In the same paper, the authors also resurrected from synonymy *Ochthebius asper*

Sahlberg, 1900 (Central Greece), so bringing to 15 the total number of formally known species. All members of *Cobalius* are strictly associated, for their whole life cycle, to the supralittoral marine rockpools with the only exception of the morphologically quite distinct *O. serratus*, associated with saline streams (Millán et al., 2011; Abellán et al., 2013). Adult beetles are able to fly away from drying pools, switching to nearby filled pools. *Cobalius* are widely distributed from the eastern Atlantic Ocean (Azores, Madeira, Cape Verde and Canary Islands), throughout the whole Mediterranean basin (Jäch 1989; Jäch and Delgado, 2017; Sabatelli et al., 2016; Ribera and Foster, 2018; Sabatelli et al., 2018; Villastrigo et al., 2019, 2020; Sabatelli et al., 2021).

A recent molecular phylogeny of the genus *Ochthebius* s.l. (Sabatelli et al., 2016) based on multiple loci, strongly supported the monophyly of *Cobalius* as well as its subgeneric status, as later confirmed by Villastrigo et al. (2019, 2020). Moreover, the phylogenetic reconstruction made by Sabatelli et al. (2016) revealed a significant degree of genetic differentiation in some *Cobalius* species, in particular in *O. subinteger* and *O. celatus*. Specifically, purported specimens of *O. subinteger* from southern Spain and from Sicily, which markedly differ genetically from specimens certainly belonging to the ‘true’ *O. subinteger* from Liguria, and SE Spain. The former group of specimens, surprisingly, shows closer genetic relationships with specimens of *O. lejolisii* from the Atlantic coasts of NW Europe (Swansea, Gales), although external morphology and aedeagal characters do not apparently differ from those of the typical Mediterranean *O. subinteger*.

This complex taxonomic and phylogenetic scenario seems to imply a significant underestimation of the *Cobalius* actual differentiation and genetic diversity, suggesting the presence of multiple undescribed taxa, many of them being morphologically indistinguishable from other recognized and formally accepted *Cobalius* species, as it happened within the “*Calobius*” lineage (Urbanelli et al., 1996; Audisio et al., 2010; Antonini et al., 2010). Hence, understanding the phylogenetic relationships among these species within the genus, based on extensive taxon sampling, is required to assess their taxonomy and classification.

In this study, we provide a molecular phylogeny of the subgenus *Cobalius*, based on mitochondrial and nuclear genes, including all the thus far recognized species, with the only exception of the recently described new taxa, *O. anzar*, *O. cortomaltese*, *O. evae*, *O. gorgadensis*, and of *O. asper*, resurrected to a specific rank (Ribera and Foster, 2018; Villastrigo et al., 2020). The main aim of the present article is to disentangle the phylogenetic relationships within this subgenus and to infer the biogeographic processes behind its diversification. We also use a molecular clock to define a time window for diversification of distinct clades within the subgenus (species-groups and species-complexes), and explore aspects of its evolutionary history. Finally, following the results of

genetic diversity, we used three sequence-based species delimitation methods aimed to clarify the problematic taxonomy of this subgenus and to validate species boundaries in our *Cobalius* dataset.

2. Material and methods

2.1. Taxon sampling

Specimens were collected in coastal rockpools from 43 locations in Italy, France, Spain, Malta, Azores, Madeira, Cape Verde Islands, Canary Islands, Morocco, Wales and Israel (Fig. 1, Fig. S1, Table 1), preserved in absolute ethanol and stored at -20°C . In Table S1, the geographic details for the species are listed. When possible, only males were used for the analysis, and the male genitalia (used for the identification of the described species) dissected and mounted before the extraction, to ensure correct identification. Tissue vouchers and DNA has been deposited at Sapienza University of Rome (Italy), Department of Biology and Biotechnologies “C. Darwin”, under the responsibility of the joint first authors. When the present paper completed, no material was available of the four additional species very recently described by Villastrigo et al. (2020).

2.2. DNA extraction and sequencing

Genomic DNA was extracted from whole specimens following the salting out procedure described by Aljanabi and Martinez (1997). We amplified and sequenced two mitochondrial fragments (3' end of cytochrome c oxidase subunit 1 [COI] and 16S RNA plus tRNA-Leu and the 5' end of NADH dehydrogenase subunit I [16S]) and one nuclear fragment, a portion of the rudimentary gene (CAD). Used primers are listed in Table S2.

Amplifications of the mitochondrial genes were performed with the following general cycle conditions: initial denaturation at 95°C for five minutes, followed by 33–38 cycles of denaturation at 94°C for one minute, annealing at $53^{\circ}\text{--}57^{\circ}\text{C}$ for 30 s, 1-min. extension at 72°C and a last 7-min elongation step at 72°C . Reactions were performed in a $25\mu\text{l}$ volume containing $(\text{NH}_4)_2\text{SO}_4$ 16 mM, Tris–HCl 67 mM (pH 8.8 at 25°C), MgCl_2 3 mM, 1 mM of each dNTP, 0.8 pmol of each primer and 1.25 units of Taq DNA polymerase. A touchdown PCR protocol was used to amplify the CAD marker with the following thermal cycling conditions: 94°C for 3.5 min, followed by 20 cycles of 94°C , 30 s, annealing temperatures step-downs every cycle of 0.4°C (from 58 to 50°C), 35 s, 72°C , 2.5 min and additional 20 cycles of 94°C , 30 s, 55°C , 35 s, 72°C , 2.5 min. We used an MJ MINI Personal Thermal Cycler (BIO-RAD Laboratories, US) and LifeECO Thermal Cycler to perform PCR amplifications. The PCR products were purified with a GENE AID- Gel/PCR DNA Fragments Extraction Kit and sent to an external sequencing service (Macrogen Inc.: www.macrogen.com).

Geneious v9.1.6 (Kearse et al., 2012) was used for assembling and editing the chromatographs manually. Multiple sequence alignments were performed with the online application of MAFFT v7 (Kato and Standley, 2013) applying the Q-INS-I algorithm. A total of 314 new sequences have been deposited in GenBank (accession numbers: see Table S1).

2.3. *Molecular phylogeny and divergence time estimation*

Phylogenetic analyses (BI) were first performed using single-fragment alignments and then with Bayesian Inference (BI) and Maximum Likelihood (ML) methods on the concatenated (mtDNA + nucDNA) dataset using, respectively, MRBAYES v3.2.2 (Huelsenbeck and Ronquist, 2001) and IQ-TREE (Nguyen et al., 2015) as implemented in W-IQ-TREE (Trifinopoulos et al., 2016). Our final dataset was completed with the addition of COI and 16S sequences of four *Cobalium* species (see Table S1) deposited in GenBank by Abellán et al. (2013), and Villastrigo et al. (2019), plus thirty sequences by Sabatelli et al. (2016). *Ochthebius marinus* (Paykull, 1798) was used as outgroup to root the tree. For all the three fragments (two mitochondrial and one nuclear), the Generalized Time-Reversible model with a proportion of invariable sites and heterogeneous substitution rates following a gamma distribution (GTR + I + G: Rodríguez et al., 1990) was selected as the best substitution model with JMODELTEST v2.1.6 (Posada, 2008) according to the Akaike Information Criterion (AIC). The BI analysis was performed by running 5,000,000 generations, with Markov chains sampled every 1,000 generations. A 10% burn-in was applied and the remaining trees were used to compute a 50% majority rule consensus tree and posterior probabilities. We assessed convergence of the runs by investigating the average standard deviation of split frequencies and effective sample size (ESS) of all parameters in TRACER 1.6 (Rambaut et al., 2014). A value of ESS >200 was acknowledged as a good indicator of convergence. A ML phylogenetic reconstruction was performed running 1,000 ultrafast bootstrap replications (Minh et al., 2013) followed by 1,000 replications of assessment of branch supports with single branch tests with SH-like approximate likelihood ratio test. The best fitting model to analyze each partition was selected as for BI.

We used MEGA 6 (Tamura et al., 2013) to calculate pairwise K2P and *p*-distance, as well as intra and interspecific K2P and *p*-distance for each putative species (clade).

To estimate the relative age of lineage divergences we used the Bayesian relaxed phylogenetic approach, on the mtDNA dataset, implemented in BEAST v.1.8.2 (Drummond et al., 2012). The input file was built using BEAUTi with the following settings: unlinked substitution models, unlinked molecular clock model (Uncorrelated relaxed lognormal), linked tree model and the Yule process of speciation as the tree prior. The GTR model was transferred to the HKY model because there are low ESS values for some parameters in the analyses when applying the GTR model. Given the lack of

fossil record to calibrate trees, we used the COI substitution rate of 0.0115 substitutions per site per million years (Myr) (Brower, 1994). The latter has been extensively used to date divergences in insects (Ribera and Vogler 2004; Ribera et al., 2010a; Salgueiro et al., 2019) including Hydraenidae and *Ochthebius* (Ribera et al., 2010b; Abellán et al., 2013; Sabatelli et al., 2016; Villastrigo et al., 2020). Moreover, we used substitution rate of 0.0016 substitutions per site per million years (Myr) for the 16S fragment used for different groups of Coleoptera (Andújar et al., 2012, Arribas et al., 2013, Hidalgo-Galiana et al., 2014, Villastrigo et al., 2018). The analysis was independently performed three times, with 100 million generations and sampling of trees every 10,000 steps. Effective Sample Size (ESS) was evaluated in TRACER v1.6 (Rambaut et al., 2014), considering runs with ESS values above 200. Output trees were generated in TREE ANNOTATOR v1.8.2 (BEAST package), using maximum clade credibility (MCC) after a 10% burn-in and median heights.

2.4. *Haplotype networks*

Haplotype networks were constructed for each gene partition using the TCS algorithm (Clement et al., 2002) implemented in the software PopART v.1.7 (Leigh and Bryant, 2015). The nuclear gene alignment was phased using the PHASE algorithm (Stephens, Smith & Donnelly, 2001) as implemented in DNAsp v6.10 (Rozas et al., 2017).

2.5. *Species delimitation*

Following the results of genetic diversity, we used three sequence-based species delimitation methods to validate species limits in our dataset and match them with described species: the Poisson Tree Processes method (PTP; Zhang et al., 2013), the General Mixed Yule Coalescent method (GMYC; Pons et al., 2006; Fujisawa and Barraclough, 2013) and the Automatic Barcode Gap Discovery method (ABGD; Puillandre et al., 2012). We used each of these methods with the concatenated three-fragment dataset, the COI dataset, the 16S dataset. The PTP and GMYC methods were also performed using the CAD fragment on its own. The PTP is a tree-based approach; it requires a simple phylogenetic tree as input. This method models speciation or branching events in terms of number of mutations. We used MRBAYES v3.2.2 (Huelsenbeck and Ronquist, 2001) to obtain the trees, setting the same parameters of the phylogenetic reconstruction. The PTP analysis was executed on the web server provided by the Exelixis Lab (<https://species.h-its.org>). We ran the bPTP analysis for 500000 MCMC generations, with a thinning value of 100; we applied a burn in of 25% and we checked the convergence of the MCMC chain. GMYC is also a tree-based approach; it requires an ultrametric tree in order to detect the transition in the tree where the branching pattern switches from being attributed to speciation to when it can be attributed to intra-specific coalescent process. We

used BEAST v.1.8.2 (Drummond et al., 2012) to estimate the ultrametric trees, setting the same parameters and time calibration used for the phylogenetic reconstruction. The GMYC analysis was performed on a web server provided by the Exelixis Lab (<https://species.h-its.org/gmyc/>) using single threshold. The ABGD method is based on genetic distance distributions; it sorts sequences into hypothetical species based on barcode gaps: the main assumption of the method is that the divergence among organisms of the same species is smaller than the divergence among organisms of different species. We used the ABGD method on the web server (<https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html>) under the default parameters of $P_{\min} = 0.001$, $P_{\max} = 0.1$, X (relative gap width) = 1.5, Steps = 10, number of bins = 20, distance method = Kimura (K80). In each analysis the selected outgroup species (*Ochthebius marinus*) was removed from the dataset.

2.6. Biogeographic reconstruction

Biogeographic reconstructions were conducted using Reconstruct Ancestral State in Phylogenies by RASP v.4.2 (Yu et al., 2015) and BioGeoBEARS (Matzke, 2013a) on BEAST MCC tree. We performed three models on RASP: S-DIVA (Yu, et al., 2010), DEC (Ree and Smith, 2008) and S-DEC (Beaulieu, et al., 2013) and three models on BioGeoBEARS: DIVALIKE, DIVALIKE+J and DEC+J (Matzke, 2013a). Four areas were defined in the distribution range of *Cobalius*: A) West Mediterranean; B) East Mediterranean; C) Coastal NE Atlantic areas of continental Europe, N Morocco and archipelagos of the Macaronesian region -Azores, Madeira, Cape Verde and Canary Islands; D) salt streams areas in S Spain and N Morocco. All models were tested using 1,000 trees randomly sampled from the input trees. The six models were compared in BioGeoBEARS ready-to-use version in RASP to choose the best fitting model given the data, based on likelihood scores and the Akaike Information Criterion (AICc and AICw) (Matzke, 2013a,b, 2014; Yu et al., 2015; R Core Team; 2017).

3. Results

3.1. Molecular phylogeny and divergence time estimation

Our final dataset consisted of 2344 bp (798bp for COI, 762bp for 16S and 784bp for CAD) obtained from 127 specimens including sequences downloaded from GenBank (see Materials and methods). Phylogenetic trees resulting from the BI and ML analyses using the concatenated dataset of mitochondrial and nuclear genes showed a concordant topology (Fig. 2). In both analyses, the

subgenus *Cobalius* was recovered as monophyletic. Sixteen clades were recovered, grouped in four main clades with a recognizable geographical pattern (Figs. 1 and S2): i) *O. subinteger*- *O. lejolisii* complex clade (1- 7); ii) *O. celatus*- *O. adriaticus* complex clade (8- 10); iii) *O. algicola*+ *O. serratus* complex clade (11- 15) and iv) *O. biltoni* clade (16). The *O. subinteger*- *O. lejolisii* complex (BI = 1, ML = 100) includes individuals currently ascribed to the species *Ochthebius (Cobalius) subinteger* from Mediterranean coast of Spain (clade 1), Corsica, Sardinia, Liguria (clade 2) and Sicily (clades 3, 4, 6, 7), together with individuals of *Ochthebius (Cobalius) lejolisii* from the Atlantic coast of Wales and Spain (clade 5). The *O. celatus*- *O. adriaticus* complex (BI = 1, ML = 100) includes individuals, of *Ochthebius (Cobalius) celatus*, all from the central Mediterranean coast of Sicily, Malta, Pontine Islands and Apulia (clade 8) and individuals of *Ochthebius (Cobalius) adriaticus* from the central and eastern Mediterranean coast of Sicily, Apulia and Israel (clade 9). Furthermore, in this complex we recovered a single individual from Israel (clade 10), sister to the *O. adriaticus* and *O. celatus* clade. Within *O. algicola*+ *O. serratus* complex (BI = 0,86, ML = 78), the Macaronesian species (clades 11-14) were recovered as sister to *O. serratus* (clade 15). The most basal clade (BI = 1, ML = 100) includes *Ochthebius (Cobalius) biltoni* from a single site of Sicily (clade 16).

The BI trees based on mitochondrial genes presented topological differences among them and with the multilocus tree, nevertheless the monophyly of clades recovered in the multilocus phylogram was supported (Fig. S3, S4). The BI tree based on nuclear gene recovered all clades reported in the multilocus phylogram, but the relationships between clades showed lower phylogenetic resolution and support (Fig. S5).

We detected a distinct gap between intra- and interspecific genetic distances (*p*-distance/K2P) for the sixteen clades recovered in the phylogenetic analysis, with only two exceptions (Tables S3- 9). The COI mean *p*- distance within clades ranged between 0% and 2.01%. The highest value was for the *O. adriaticus* clade, while the lowest was for the *O. biltoni* clade (Table S7). The COI mean *p*- distance between clades ranged from 1.4% and 14.8%. The highest distance value was observed between *O. serratus* and the *O. subinteger* clade 1. The lowest distance was observed between the *O. lejolisii* clade 5, from the Atlantic coast of Wales and Spain, and the *O. subinteger* clade 6 from western Sicily (Table S7). Both 16S and CAD mean *p*-distances suggested a significant differentiation among clades (Tables S4, 6, 8 and 9).

Using a calibration of 0.0115 substitutions/site per Myr for COI fragment and 0.0016 substitutions/site per Myr for the 16S fragment (see Material and methods), the possible origin of *Cobalius* was estimated to be in the Early Miocene, approximately 22 million years ago (Mya) (95% HPD: 27–16.25 Mya) (Fig. 3). The split between *O. algicola* + *O. serratus* assemblage (N Macaronesia, N Morocco and S Spain) and the remaining clades can be traced back to the Early-

Middle Miocene (~18 Mya; 95% HPD: 22.70–13.07 Mya). Divergence between the *O. subinteger-O. lejolisii* complex (central-West Mediterranean and Atlantic coasts of W Europe) and the *O. celatus-O. adriaticus* complex (central-East Mediterranean) appears to have occurred in the Middle Miocene, ca. 11 Mya (95% HPD: 16.87–5.23 Mya). Within the *O. subinteger-O. lejolisii* complex (clades 1- 7), most of the diversification occurred between 9 and 2 Mya. The separation between clade 7 and the other clades was estimated in the Late Miocene (~9 Mya; 95% HPD: 14.50–4 Mya). Clades 1, 2 and 3 diverged from clades 4, 5 and 6 in the Late Miocene (~7 Mya; 95% HPD: 12–3 Mya). Looking at the three other clades of the *O. celatus- O. adriaticus* complex (clades 8- 10), the diversification started in the Late Miocene ~6 Mya.

3.2. Haplotype networks

Networks showing phylogenetic relationships among COI and 16S haplotypes in the *O. subinteger - O. lejolisii* complex, *O. celatus* and *O. adriaticus*, are depicted in Fig. 4. The haplotype networks of COI and 16S (Fig.4, I, left-right) showed a phylogeographical structure within the *O. subinteger- O. lejolisii* complex. A clear separation between haplogroups corresponding to the seven clades recovered by the phylogenetic analysis was evidenced. Amongst the two data sets, only one haplotype was shared among sites. The COI haplotype network of *O. celatus* (Fig.4, II, left) was geographically structured and showed fifteen haplotypes, only one shared among sites, divided into four haplogroups, while the 16S haplotype network (Fig.4, II, right) was less structured and showed three haplotypes, two of them shared among sites. The COI haplotype network of *O. adriaticus* (Fig.4, III, left) showed nine haplotypes, divided into four haplogroups. Absence of shared haplotypes was observed in this dataset. The 16S haplotype network of *O. adriaticus* (Fig.4, III, right) showed five haplotypes.

Results of nuclear gene networks based on CAD showed a clear segregation between all seven recognized clades of the *O. subinteger- O. lejolisii* complex (Fig. 5, I), as in the mitochondrial haplotype network. In this complex, absence of shared haplotypes was observed. Such separation was far less marked for *O. celatus* and *O. adriaticus* (Fig. 5, II and III). The structure of the networks resembled the phylogenetic tree with the concatenated dataset.

3.3. Species delimitation

The bPTP analysis identified in the *O. subinteger-O. lejolisii* complex (clades 1–7) eight different putative species for the concatenated data, eight for the COI data and five for the 16S data. In the *O. celatus-O. adriaticus* complex (clades 8 and 9), the analysis found five putative species for the concatenated data, six for the COI and two for the 16S dataset. The bPTP delimitation analysis

recognized “O_COB_ISRA”, *O. balfourbrownei*, *O. lanthanus*, *O. algicola*, *O. freyi*, *O. serratus* and *O. biltoni* (clades 10–16) as single and isolated entities in all three datasets (Fig. 1).

In all GMYC analyses, the maximum likelihood of GMYC model was higher than that of the null model. The single-threshold GMYC analysis found in the *O. subinteger*-*O. lejolisii* complex (clades 1–7) eight putative species for the concatenated data, eleven for COI and ten for 16S datasets. The analysis yielded five putative species in the *O. celatus*-*O. adriaticus* complex (clades 8 and 9) for the concatenated data, eight for the COI data and seven for the 16S dataset. The GMYC model identified respectively “O_COB_ISRA”, *O. balfourbrownei*, *O. lanthanus*, *O. algicola*, *O. freyi*, *O. serratus* and *O. biltoni* (clades 10–16) as separate entities in all three datasets, as reported in Fig. 1.

The results of the ABGD analysis on the *O. subinteger*-*O. lejolisii* complex (clades 1–7) are somehow consistent with the results of the bPTP analysis, finding eight putative species for both the concatenated and the COI dataset and five for the 16S dataset. Differently, the analysis of the *O. celatus*-*O. adriaticus* complex (clades 8 and 9) identified two, eight and five putative species for the concatenated, the COI and the 16S dataset, respectively. The ABGD analysis, according to the other delimitation method, recognized respectively “O_COB_ISRA1_1”, *O. balfourbrownei*, *O. lanthanus*, *O. algicola*, *O. freyi*, *O. serratus* and *O. biltoni* (clades 10–16) as separate entities in all three datasets, as reported in Figure 1.

Finally, the CAD single locus analyses, showed a low resolution as reported in figs. S6 and S7. In fact, both bPTP and GMYC methods, recognized only five entities within our dataset.

3.4. Biogeographic reconstructions

Among the six different models used in RASP for the biogeographic reconstruction, the DIVALIKE + J received the highest AICc wt value (Table S10). The biogeographic analyses results suggested the West Mediterranean area (A) as the most probable ancestral area for the subgenus *Cobalius*, with probabilities of 0.60 (Fig. 6, node 1 in Fig. S8). The diversification of the subgenus started with an event that split *Cobalius* into two main groups: one limited to W Mediterranean coasts (A) including *O. biltoni* and a second one distributed across W and E Mediterranean coasts, E Atlantic areas of continental Europe, N Morocco, archipelagos of Azores, Madeira, Cape Verde Canary Islands and salt streams areas in S Spain and N Morocco (ABCD), including all the others species. The ancestor of the E Atlantic/Mediterranean clade (*O. algicola* + *O. serratus* assemblage and the remaining clades) was present in the Mediterranean area and experienced dispersal into E Atlantic areas and salt streams areas in S Spain and N Morocco, immediately followed by a vicariant event with probability of dispersal + vicariance of 0.60 (Node 2 in Fig. S8). Differentiation between *O. serratus* and *O. algicola* Macaronesian species complex is also associated with one dispersal and one

vicariant event. The analysis suggested that the most probable ancestral area for the *O. algicola* + *O. serratus* clade, combines E Atlantic areas, Macaronesian region and salt streams areas in S Spain and N Morocco (CD) (probability of dispersal + vicariance of 0.35; node 3 in Fig. S8). The Mediterranean remains the main area in which the diversification between and within the *O. subinteger*-*O. lejolisii* complex and *O. celatus*-*O. adriaticus* complex occurred (probabilities of 0.99, 0.98, 1; nodes 7, 8, 12 respectively in Fig. S8). In the clade constituted by *O. lejolisii* and *O. subinteger* (4, 5, 6), the model recovered a re-colonization of E Atlantic areas (C) and posterior diversification involving: one dispersal and one vicariant event (Node 18 in Fig. S8).

4. Discussion

Previous phylogenetic studies of the genus *Ochthebius* s.l. (Sabatelli et al., 2016) and of the tribe Ochthebiini (Villastrigo et al., 2019), strongly supported the monophyly of *Cobalius* as well as its (at least) subgeneric status. Moreover, our previous results have highlighted the existence of a significant degree of genetic differentiation in some *Cobalius* species, in particular in *O. subinteger* and *O. celatus*, suggesting the possible presence of multiple cryptic species within the Atlantic and W Palearctic marine rockpools beetles (Sabatelli et al., 2016). Our study, by an extensive taxon sampling, provides the most comprehensive approach on the systematics, biogeography, and evolution of the subgenus *Cobalius*. Overall, mitochondrial and nuclear data are congruent with the current taxonomy, and supports the presence of undescribed biodiversity in the Mediterranean area.

Phylogenetic results consistently showed *Cobalius* as monophyletic (BI and ML analyses), and recovered sixteen well supported clades, grouped in four main clades with a recognizable geographical pattern: i) *O. subinteger*- *O. lejolisii* complex clade (1- 7); ii) *O. celatus*- *O. adriaticus* complex clade (8- 10); iii) *O. algicola*+ *O. serratus* complex clade (11- 15) and iv) *O. biltoni* clade (16) (Fig. 2, Fig S2). Our results are congruent with Sabatelli et al. (2016), confirmed the ten species currently included in the subgenus, used in our dataset, and led us to detect at least four new lineages (1, 3, 7, 10) morphologically indistinguishable from the present-day formally accepted *Cobalius* species (Fig.1), highlighting the importance of taxon sampling, both at an interspecific and intraspecific level. The topology and phylogenetic relationships recovered by our analysis were mainly driven by the more variable mitochondrial markers despite nuclear inferences also supported the most of the clades (Fig. 2; Figs. S3–5). Within the *O. subinteger*- *O. lejolisii* complex, the taxonomic splits of the Mediterranean *O. subinteger* (clades 1-4 and 6-7), suggested a previously undescribed diversity along the geographic range of this putative species. The levels of mitochondrial genetic divergence between putative species “hidden” in these clades are in line with the level of

interspecific variability found within the genus (Tables S3–8) (Audisio et al., 2010; Sabatelli et al., 2016). Specimens belonging to *O. lejolisii* (clade 5), although morphologically distinct from *O. subinteger*, showed to be sister group of clade 4 with a very low genetic distance towards both clades 4 and 6 (Tables S3–8). Despite the geographical distribution of clades 4, 5 and 6 and the results of the species delimitation analyses (see below) suggested the presence of three distinct taxa, the shallow genetic distance between them, compatible with intraspecific genetic variability, requires careful consideration of outcomes. An evolutionary process relevant in this scenario is the recent divergence of the putative species. In this case, hypothesized cryptic species are sister taxa with short divergence times, which are likely too recent to have accumulated substantial morphological differences (Gustafsson et al., 2014). In addition, clade 7, identified for the first time in this study, showed to be endemic to a narrow area in SW Sicily (Fig. 1). Interestingly, even though specimens belonging to clade 7 inhabit a geographical area which is enclosed within the distribution area of clade 6 (Fig. S2), no syntopy were observed between members of these two clades, in association with significant genetic distances (Tables S3-9). A possible explanation for the considerable mtDNA divergence and genealogical structuring found in populations separated by relatively small geographical distances could be the scattered and discontinuous occurrence of suitable habitats (Abellàn et al., 2007, Antonini et al., 2010). Instead, taking into consideration the *O. celatus*-*O. adriaticus* complex (clades 8 and 9), strongly differentiated morphologically from each other, our results are congruent with the current taxonomy and the geographic distribution detected by Jäch (1989). Within clade 9, our results also showed higher intra-group genetic distances than in the other clades (Tables S3–8), results that agree with the morphological variability described within this species by Reitter (1886), Pretner (1929) and by Jäch (1989). Moreover we found high genetic distances (Tables S3–8) in line with the level of interspecific variability found within the genus, between samples from Sicily (site16), morphologically assigned to *O. adriaticus* and the others belonging to clade 9. This degree of differentiation suggests the possible presence of a cryptic species within *O. adriaticus*.

Our phylogenetic results also recovered a new cryptic lineage (clade 10) that includes a single female individual exclusive to a single site of Israel, Rosh HaNikra. This specimen, clearly distinct genetically (Tables S3–8), was found in syntopy with two individuals belonging to the clade 9. Our analysis further confirmed the species currently included in the *O. algicola* + *O. serratus* complex (*O. balfourbrowni*, *O. lanthanus*, *O. algicola*, *O. freyi* and *O. serratus*) (clades 11-15) according to the current taxonomy. Finally, clade 16 (*O. biltoni* from W Sicily) appears to be placed as outgroup of all other *Cobalius*. This position is fully consistent with morphological data (Sabatelli et al., 2018), although being discordant from the evidence reported by Villastrigo et al., 2020, but only based on COI gene data.

Species delimitation analyses promote the identification of distinct evolutionary lineages within a sample of individuals (Fujita et al., 2012; Fujisawa and Barraclough, 2013; Zhang et al., 2013). All previous species delimitation studies of Atlantic and W Palaearctic marine rockpools beetles have been predicated on traditional morphology, which is nearly ineffective for cryptic groups. Our study is the first to implement a robust species delimitation analysis using molecular data to elucidate cryptic species boundaries of water beetles belonging to the subgenus *Cobalius*. Here, we used three different approaches (PTP, GMYC, ABGD) to infer putative species boundaries on our dataset.

Species delimitation methods, based on concatenated dataset, the COI dataset, the 16S dataset showed similar outcomes for most putative species clades (Fig. 1) while the CAD single locus analyses showed low resolution in identifying species boundaries, recognizing only 5 entities in our dataset (Figs. S6 and S7). The results on concatenated, COI and 16s data, generally supported the ten recognized *Cobalius* species present in our dataset but, at the same time, highlighted a previously undescribed diversity within the subgenus, with a number of putative species identified ranging between 16 and 24, most of which diverged during the Pliocene and Pleistocene (5.3–0.11 Mya). In the case of *O. subinteger*, both single locus and multilocus analyses supported clades 1, 3 and 7 as separate new taxonomic entities, suggesting the need for a taxonomic reassessment. Clustering disagreement was reported in clades 2, 4, 5 and 6. Within clade 2, bPTP and GMYC tended to subdivide lineages more finely showing 1–2 more entities. The tendency to over-split species in these methodologies is sometimes attributed to confounding effects of intraspecific geographic structuring (Luo et al., 2018). On the other hand, clades 4, 5 and 6 were collapsed as one single putative species by the bPTP and ABGD analysis based on the 16S dataset, likely due to an effect of shallow 16S mitochondrial fragment divergence (Table S4 and S6). Results of bPTP, GMYC, ABGD based on COI fragment and multilocus analyses supported them as separate taxonomic entities. This results are not conclusive and genetic data from more loci are needed.

The splits identified within *O. celatus* (clade 8) by both GMYC and ABGD single locus analyses recognized from 1 up to 4 taxonomic units. Regarding the results of the GMYC method for this clade were likely influenced by large values of N/t that could have led to false positives involving oversplitting, as highlighted in previous simulation and empirical studies (Luo et al., 2018). Evidence obtained from phylogenetic inferences suggested that delimited species could represent in some cases intrapopulation variation.

In the case of *O. adriaticus* (clade 9), Jäch (1989) documented morphological variability between specimens from West, Centre and East Mediterranean, exhibiting a good correspondence to the localities represented in our dataset and the four putative species recognized by our analyses within this clade. Although both unbalanced sampling and mutation rate heterogeneity at different levels

were found to have small effects on the performance of GMYC and bPTP methods (Lim et al., 2012; Luo et al., 2018), additional sampling in Eastern Mediterranean area and genetic data are necessary to confirm the match between genetic and morphological differentiation, to better define the distributions of each putative species within *O. adriaticus*. Moreover, results of bPTP, GMYC, ABGD clearly indicated a putative new species from Israel (clade 10). However, also in this case, additional sampling and genetic data, supported by morphological diagnosis, are needed.

Finally, all methods of species delimitation showed similar outcomes for Macaronesian species (clades 11-14), *O. serratus* (clade 15) and *O. biltoni* (clade 16). These results are congruent with documented distinct morphology and the current taxonomy (Sabatelli et al., 2016; Villastrigo et al., 2020).

The clustering agreement of ten clades (1, 3, 7, 10-15 and 16), four of them (1, 3, 7 and 10) recognized for the first time in this study, supported the results from phylogenetic analyses. Although we cannot assert that the putative cryptic species evidenced in this study represent in all cases true species, that is, they are not potentially interbreeding, they are evolutionary significant units that should be considered for taxonomic purposes.

Divergence time estimates and historical biogeographic reconstruction analyses indicated an early Miocene origin (about 22 Mya) of subgenus *Cobalius* with an ancestral distribution in the W Mediterranean area (Figs. 3, 6 and S6). This first cladogenetic event probably involved an ancestral W Mediterranean species closely related to the present-day rare and peculiarly shaped *Ochthebius* (*Cobalius*) *biltoni* from Sicily (and to its vicariant taxa *O. evae* and *O. cortomaltese*).

According to our results *O. algicola* + *O. serratus* assemblage (Macaronesia, N Morocco and S Spain) and the remaining clades diverged in the W Mediterranean area during Early-Middle Miocene approximately 18 Mya (Figs. 3, 6 and S6). Origin of these taxa probably followed the dispersal into E Atlantic areas and salt streams areas in S Spain and N Morocco, as well as the subsequent vicariant events, likely mediated by the changes in the Atlantic–Mediterranean connections. During this period, the link between the Mediterranean Sea and the Atlantic Ocean through the Betic Cordillera (southern Spain) was, in fact, progressively reduced to a few seaways (Martín et al., 2014; Achalhi et al., 2016). This split was later followed (~15 Mya) by an ecological shift from marine rock pools to subcoastal salt rivers, involving the separation, in the areas between E Atlantic and W Mediterranean, of the markedly isolated *O. serratus* (Millán et al., 2011) (Figs. 3, 6 and S6).

The onset of the diversification between the *O. subinteger*-*O. lejolisii* complex (central-West Mediterranean and Atlantic coasts of W Europe) and the *O. celatus*-*O. adriaticus* complex (central-East Mediterranean) occurred in W Mediterranean during Middle Miocene ~11 Mya and continued during the Plio-Pleistocene (Figs. 3, 6 and S6). The *O. subinteger*-*O. lejolisii* complex showed a

complicated pattern of diversification. Phylogenetic analyses, combined with integrative species delimitation suggested that *O. subinteger* is probably not a single species and more likely represents a complex of at least four cryptic species (clades 1, 2, 3, 7), originated in W Mediterranean between 9 and 2 Mya. Moreover the nested position of *O. lejolisii*, to date exclusively reported along Atlantic coast from South Morocco to the British Islands, within this Mediterranean clade, suggested the W Mediterranean origin of this lineage (clade 5, Fig. 2, Fig. 3). The timing and biogeographic reconstructions of this event suggested a dispersal pathway, on the one hand along the coasts of southern Spain through up to the Strait of Gibraltar, on the other through a southern passage along rocky coasts of N. Africa, associated with sea-level drops during the Pliocene–Pleistocene and followed by a vicariant event (Figs. 6 and S6).

The extreme geographic fragmentation of Mediterranean clades (1-10) found especially in SW Sicily, could be probably explained by a parallel fragmentation and prolonged isolation of several rocky coastal areas in W Mediterranean Islands during the Late Miocene, chiefly in the Middle and Late Messinian times (ca. 6-5.5 Mya). During this period, the W Mediterranean has been affected by striking lowering of the sea level occurred in a time frame fully compatible with the scenario estimated by our molecular and biogeographic analyses (Gargani and Rigollet, 2007; Gargani et al., 2014). In this situation, ecologically suitable rocky areas at the land/sea interface were, in fact, likely available only some hundreds meters below the present-day sea level, around more or less isolated tranches and paleo-islands. These areas were located rather far from the present-day coastal lines of Sicily, E Spain, and surrounding islands, probably allowing divergence in allopatry of different groups of populations, which subsequently re-established a partial contact in their present-day locations. Similarly, further episodes of expansion and fragmentation of geographical range of these species occurred during the Plio-Pleistocene Glacial Cycles (Urbanelli et al., 1996; Audisio et al., 2010). For instance, the coast line advanced and retreated repeatedly during glacial and interglacial phases, which led to the formation and disappearance of land bridges and additional small islands between present-day largest Mediterranean islands and the mainland (e.g., between Liguria, Sardinia, Corsica, and Tuscan Archipelago Islands; Orszag-Sperber et al., 1993; Médail and Diadema, 2009; Ketmaier and Caccone, 2013). Moreover, W Sicily experienced particularly drastic migrations of the shorelines in response to sea-level rise and lowering during the numerous Pleistocene glacial cycles, due to the complex bathymetric condition of the area (Shackleton et al., 1984; Lambeck et al., 2004; Lodolo et al., 2020), which certainly contributed to shape the present-day complex genetical structure of coastal *Ochthebius*. Additionally, the whole area of the NW Sicily Channel in the Pleistocene was repeatedly involved in the formation and disappearing of a series of small islands and archipelagos (other than the present-day Lampedusa, Linosa, Pantelleria, Malta and Gozo Islands), which

disappeared the last time only some 9.500 Ya, certainly allowing for several thousand years the *in situ* partial isolation and differentiation of local *Cobalius* populations. Other recent phylogeographic studies on Western Mediterranean island endemics (amphibian and reptiles) revealed how past climatic oscillations have greatly affected the pattern of distribution, genetic differentiation and evolutionary histories of many animal species in the western Mediterranean region (Canestrelli and Nascetti, 2008; Salvi et al., 2010, 2017; Senczuk et al., 2018). Likewise, these drastic geo-climatic conditions, ephemeral nature of rock pool habitats, together with the moderate vagility of these beetles, would have determined episodes of bottleneck and local extinctions, or drastic reduction in size of several populations that led to shape the current genetic pattern of *Cobalius*.

5. Conclusion

In this study we have identified sixteen evolutionary lineages within the subgenus *Cobalius*, supporting previous recognized species, and evidenced the presence of at least four unknown taxa suggesting a taxonomic reassessment of *O. subinteger*- *O. lejolisii* and *O. adriaticus-celatus* complexes. Our results recovered a robust phylogeny with a calibrated timeframe that confirmed the monophyly of the subgenus *Cobalius*, and, in conjunction with biogeographic reconstruction analyses, suggested the W Mediterranean as the ancestral geographic area of this lineage and its Early Miocene origin (about 22 Mya).

CRedit authorship contribution statement

Simone Sabatelli: Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Software, Investigation, Resources, Project administration, Writing - original draft, Writing - review & editing. **Paola Ruspantini** Investigation, Resources. **Paolo Cardoli:** Formal analysis, Software, Writing - original draft, Writing- review & editing. **Paolo Audisio:** Conceptualization, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

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CAPTIONS

Tables, Figures and Supplementary material

Table 1 List of sampling sites reported in Fig. 1

Table S1 List of specimens used for the molecular phylogeny, localities, collector and accession numbers. The sequences downloaded from GenBank are showed in gray (Abellán et al., 2013; Villastrigo et al., 2019) and green (Sabatelli et al., 2016).

Table S2. Oligonucleotide primers used for PCR amplification and sequencing reactions.

Table S3. COI genetic distances (% uncorrected p-distances) between the examined species estimated using MEGA 6 (Tamura et al., 2013).

Table S4. 16S genetic distances (% uncorrected p-distances) between the examined species estimated using MEGA 6 (Tamura et al., 2013).

Table S5. COI genetic distances (Kimura two-parameter pairwise distances) between the examined species estimated using MEGA 6 (Tamura et al., 2013).

Table S6. 16S genetic distances (Kimura two-parameter pairwise distances) between the examined species estimated using MEGA 6 (Tamura et al., 2013).

Table S7. COI genetic distances (% uncorrected p-distances) between and within clades estimated using MEGA 6 (Tamura et al., 2013).

Table S8. 16S genetic distances (% uncorrected p-distances) between and within clades estimated using MEGA 6 (Tamura et al., 2013).

Table S9. CAD genetic distances (% uncorrected p-distances) between the examined species estimated using MEGA 6 (Tamura et al., 2013).

Table S10. Results of model evaluation for biogeographic reconstruction analyses. d = dispersal; e = extinction; j = founding event.

Fig. 1 Map showing the geographic distribution of sampling sites

Fig. 2 Phylogenetic interrelationships of representative members of the subgenus *Cobalius* based on the concatenated molecular dataset (COI, 16S, CAD) using Bayesian inference (BI) performed by MRBAYES and Maximum likelihood (ML) analyses performed by IQ-TREE. See Table S1, for details on the examined specimens. Only BI posterior probability (black) values ≥ 0.70 and ML bootstrap (red) values ≥ 70 are shown. Colored dots next to the specimens, indicates the sampling site area as reported on the left side of phylogeny. Colored bars on the right indicate clades identified by phylogenetic analyses (Consensus) and candidate species using three sequence-based species delimitation methods (PTP, GMYC, ABGD) with the concatenated and single mitochondrial data, respectively. The same color on the bars indicates an agreement across methods of delimitation, while different colors indicate disagreement across methods. The color gray indicates the possible presence of different entities not assigned to any specific cluster.

Fig. 3 Time-calibrated BEAST phylogeny of representative members of the subgenus *Cobalius*, inferred from combined mitochondrial sequences (COI, 16S). Numbers at nodes correspond to estimated age (Mya); bars represent highest posterior densities (95%) around mean date estimates. Nodes with black dots were supported with high posterior support (>95). The main geological periods and epochs are indicated in the time scale at the bottom of the tree.

Fig. 4 Haplotype networks showing phylogenetic relationships among COI (left) and 16S (right) haplotypes in: (I) *O. subinteger*- *O. lejolisii* complex, (II) *O. celatus*, (III) - *O. adriaticus*. The size of each circle is proportional to the number of samples that share that haplotype; hatch marks between

black dots represent one mutational step. Colors assigned to each circle refers to the sampling geographic areas, as reported in the legend (top right). In section (I), numbers from 1 to 7 refers to clades identified by phylogenetic analyses, and H in bold (1-7) corresponds to main haplo-groups.

Fig. 5 Haplotype networks showing phylogenetic relationships based on nuclear haplotypes for the CAD gene fragment: (I) *O. subinteger*- *O. lejolisii* complex, (II) *O. celatus*, (III) - *O. adriaticus*. The size of each circle is proportional to the number of samples that share that haplotype; hatch marks between black dots represent one mutational step. Colors assigned to each circle refers to the sampling geographic areas, as reported in the legend (right). In section (I), numbers from 1 to 7 refers to clades identified by phylogenetic analyses, and H in bold (1-7) corresponds to main haplo-groups.

Fig. 6 Biogeographic reconstructions for the subgenus *Cobalius* based on RASP analyses and the species tree inferred with BEAST. Small black dots represent support values with high posterior support (>95). The main geological periods and epochs are indicated in the time scale at the bottom of the tree. Biogeographic reconstructions are based on four areas: West Mediterranean (A-light red); East Mediterranean (B-yellow); Coastal Atlantic areas of continental Europe, N Morocco and archipelagos of the Macaronesian region -Azores, Madeira, Cape Verde and Canary Islands (C-blue); salt streams areas in S Spain and N Morocco (D-olive green). The pie at each node represent the result of the geographic reconstruction with DIVALIKE + J in BioGeoBEARS, with coloured slices representing relative probability of ancestral areas. In correspondence of the nodes, Di and V identifies Dispersion and Vicariance events, respectively. Circle colours at the tip represent current distribution of lineages. For more details about probabilities and dates, see Fig.S4.

Fig. S1 Habitat of the species of *Ochthebius* (*Cobalius*): **a)** Italy, Sicily, Riserva N.O. dello Zingaro; **b)** Italy, Sicily, Terrasini; **c)** Italy, Sicily, Torretta Granitola; **d)** Malta, Sliema, Tigné point; **e)** France, Corsica, Ile Rousse; **f)** France, Corsica, Marina di Brando; **g)** Spain, Calp; **h)** Cabo Verde, Island of Sal, Murdeira.

Fig. S2 Geographical distribution of sixteen clades recovered in the phylogenetic analysis. Colored circles indicate the geographic distribution of sampling sites; each colors refers to the clade showed in the phylogenetic tree.

Fig. S3. Bayesian tree based on COI sequences. The posterior probabilities ≥ 0.70 are shown at nodes.

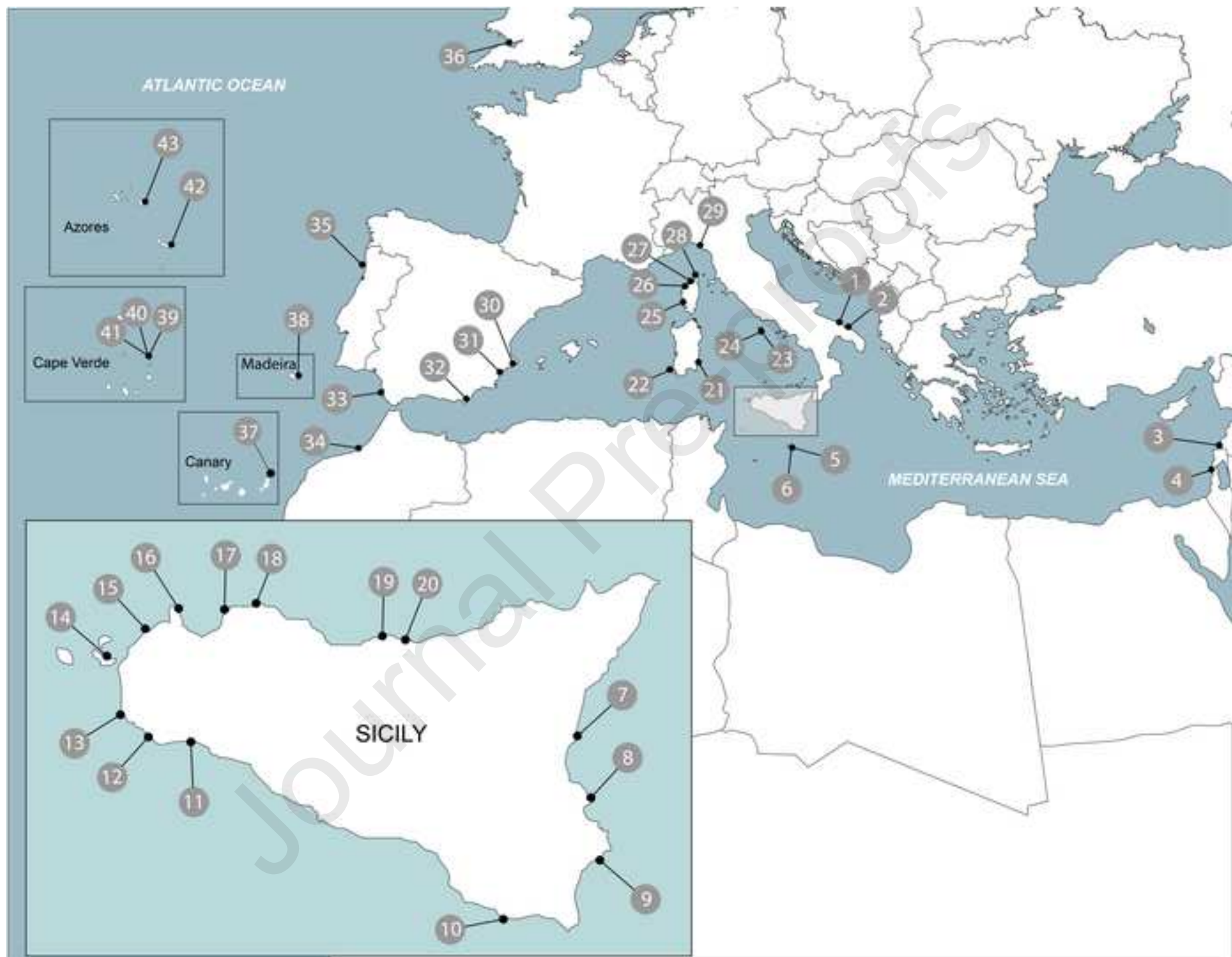
Fig. S4. Bayesian tree based on 16S sequences. The posterior probabilities ≥ 0.70 are shown at nodes.

Fig. S5. Bayesian tree based on CAD sequences. The posterior probabilities ≥ 0.70 are shown at nodes.

Fig. S6. Results of PTP species delimitation analysis performed using the CAD fragment.

Fig. S7. Results of GMYC species delimitation analysis performed the CAD fragment.

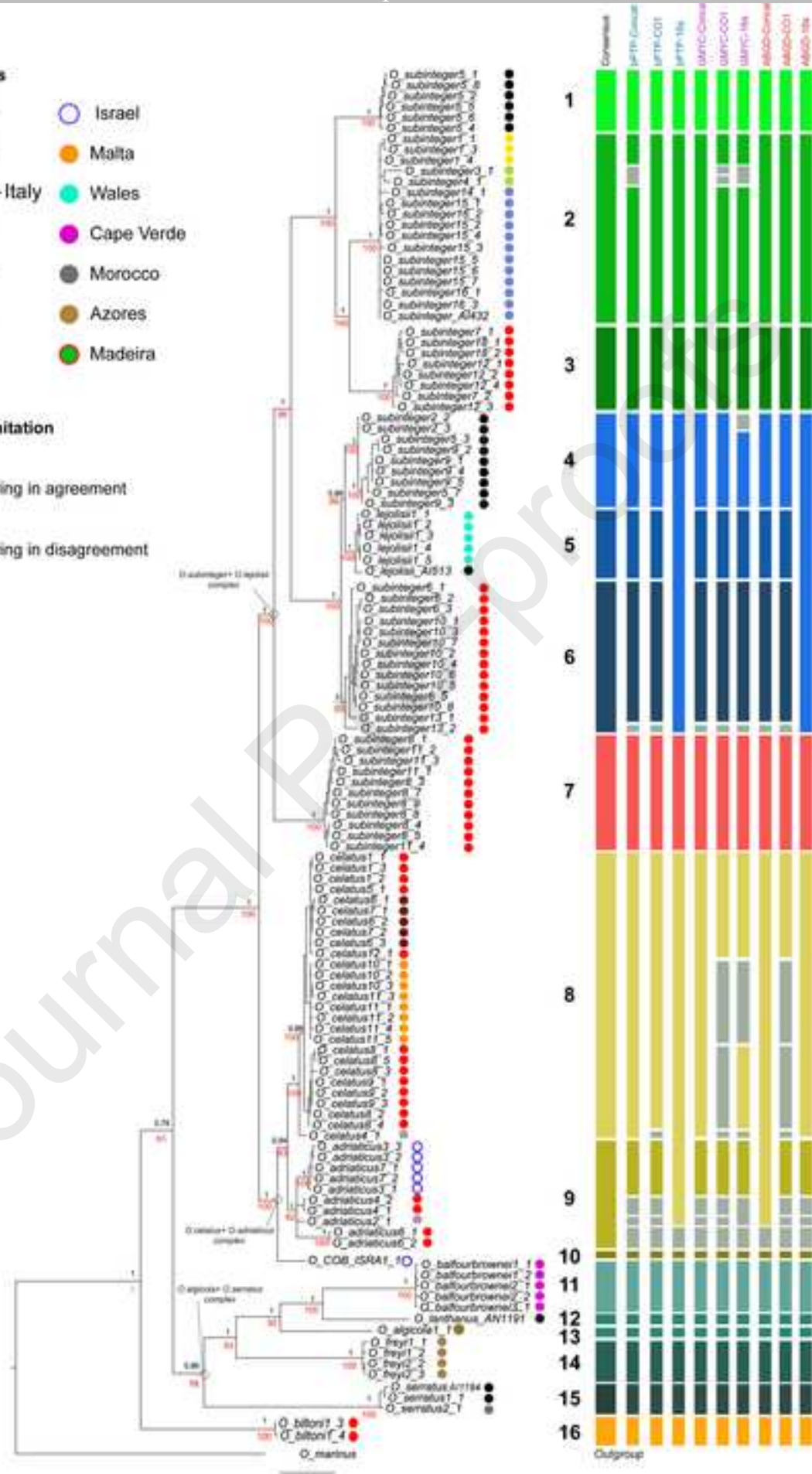
Fig. S8: Results of the Biogeographic reconstruction analyses. Biogeographic reconstructions are based on four areas: West Mediterranean (A-light red); East Mediterranean (B-yellow) Coastal NE Atlantic areas of continental Europe, N Morocco and archipelagos of the Macaronesian region - Azores, Madeira, Cape Verde and Canary Islands (C-blue) salt streams areas in S Spain and N Morocco (D-olive green). In the Statistical Summary for each node (numbers as in the tree) are reported the inferred ancestral areas and associated probability as well as inferred dispersal and vicariant events.

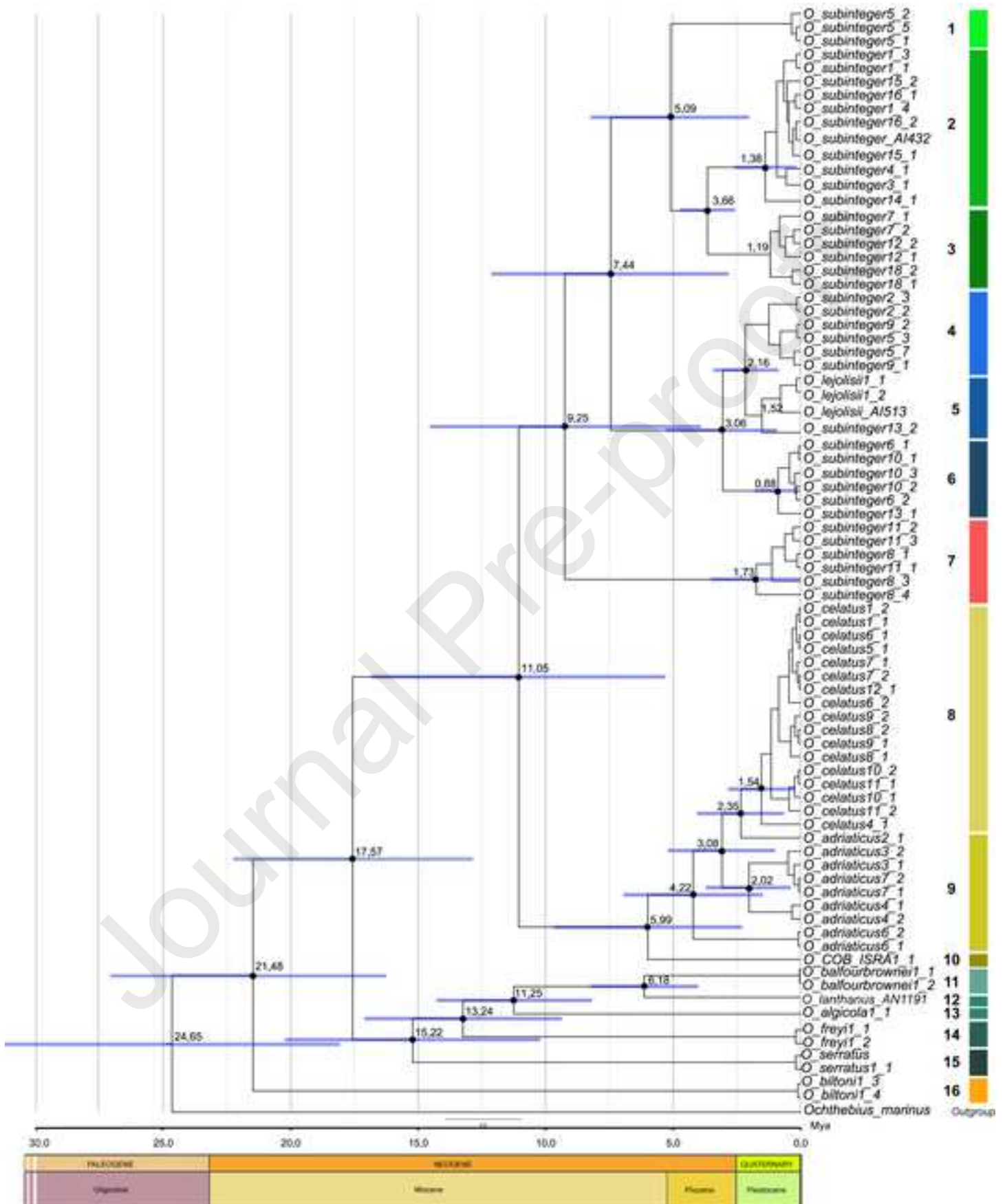


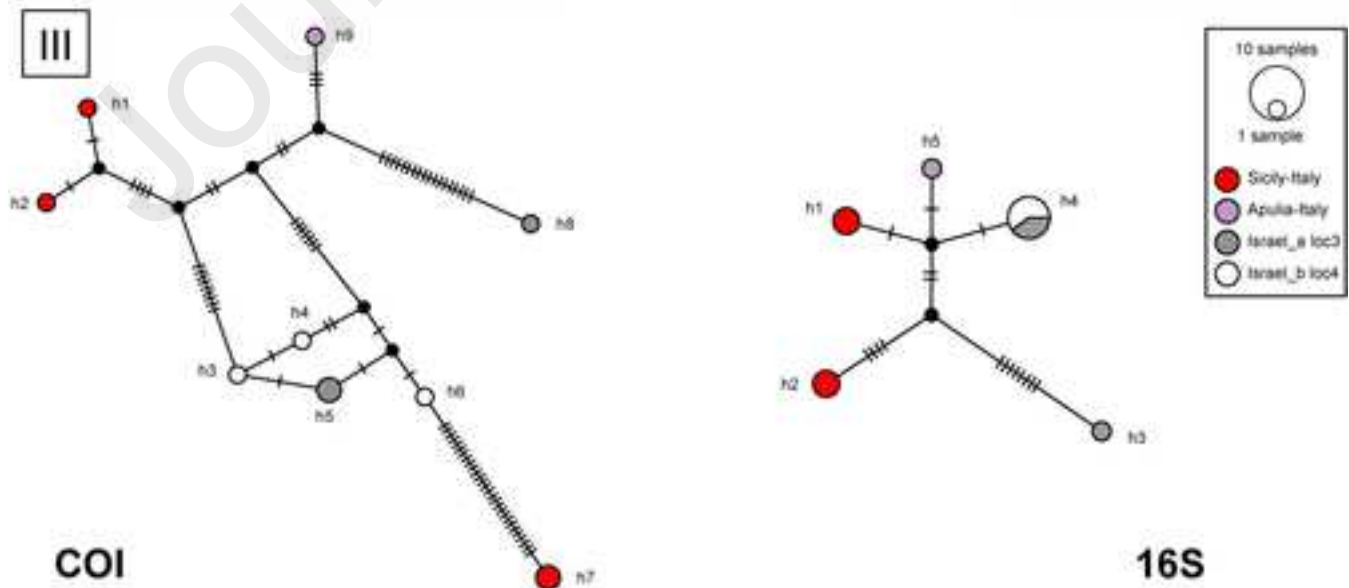
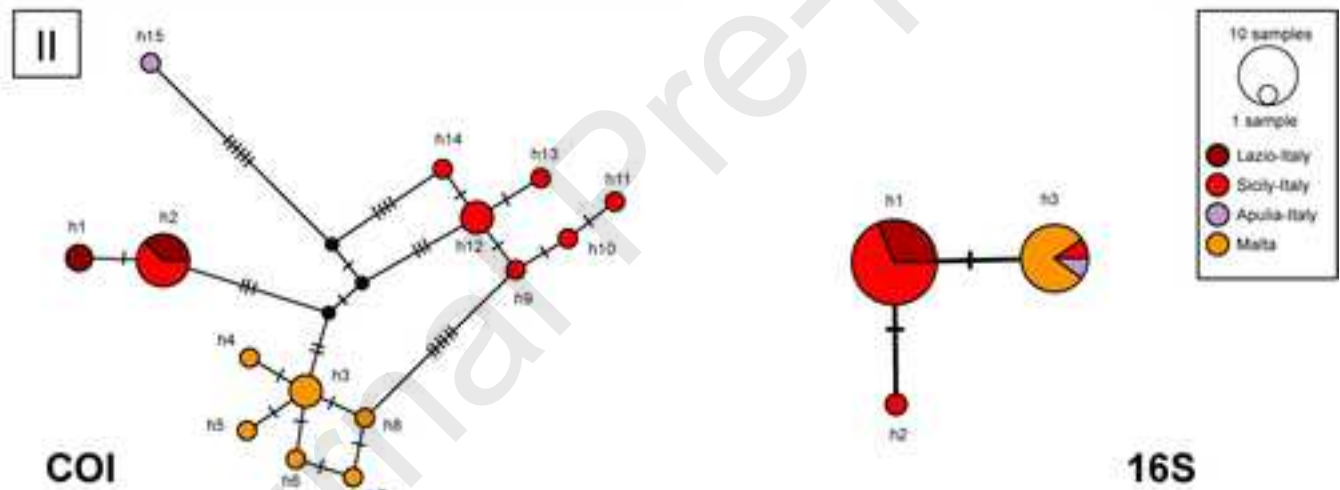
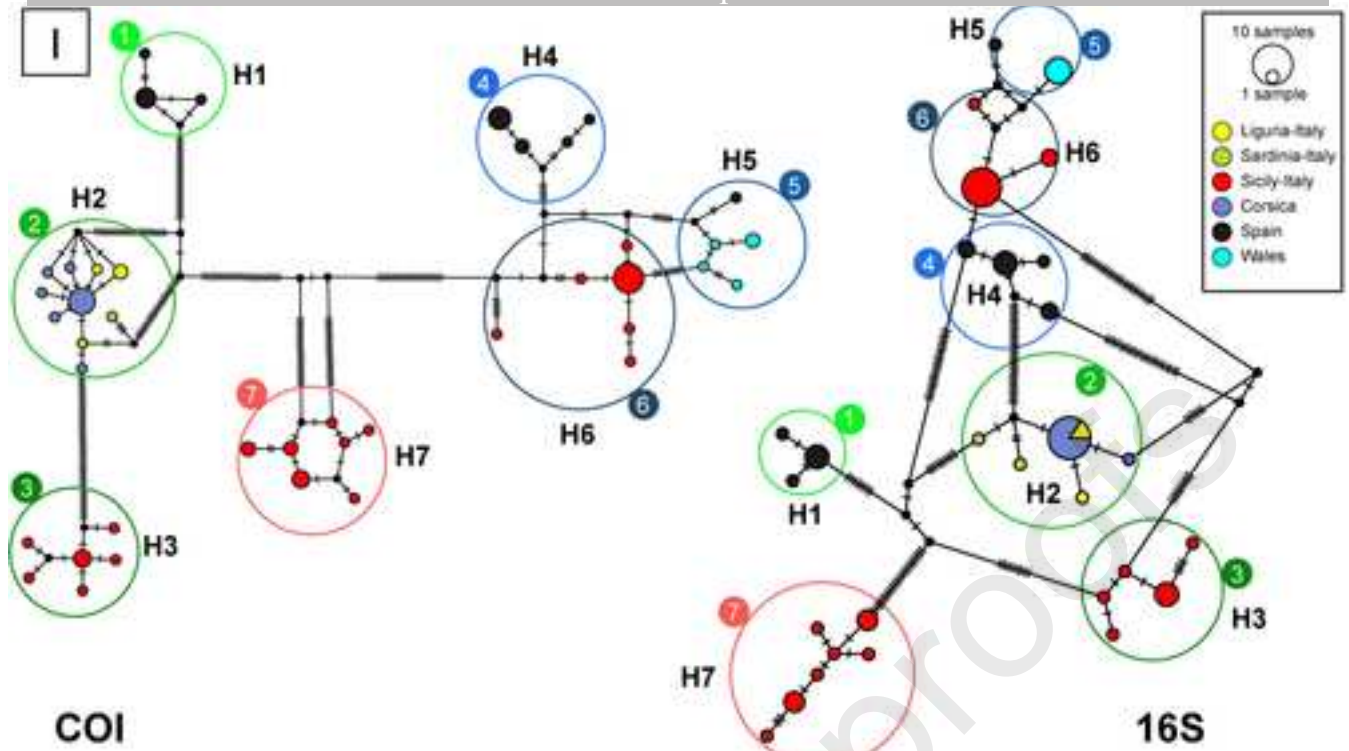
Sampling sites

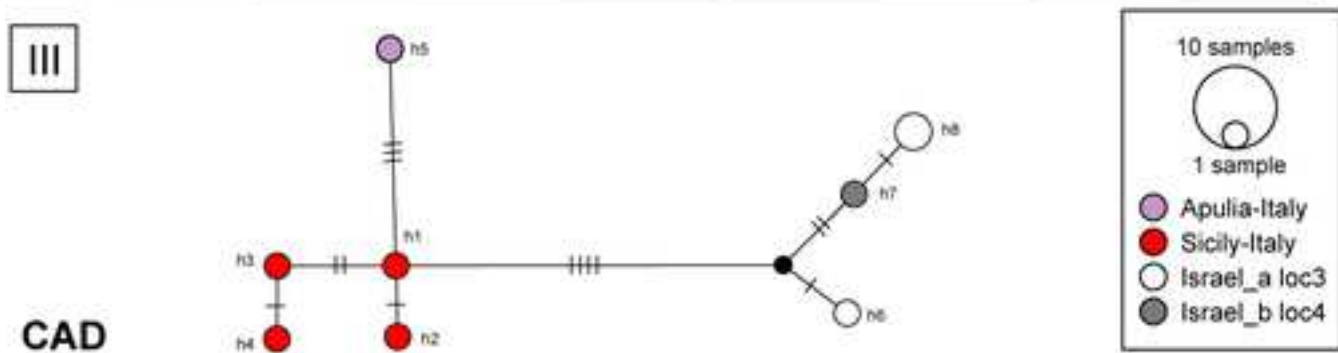
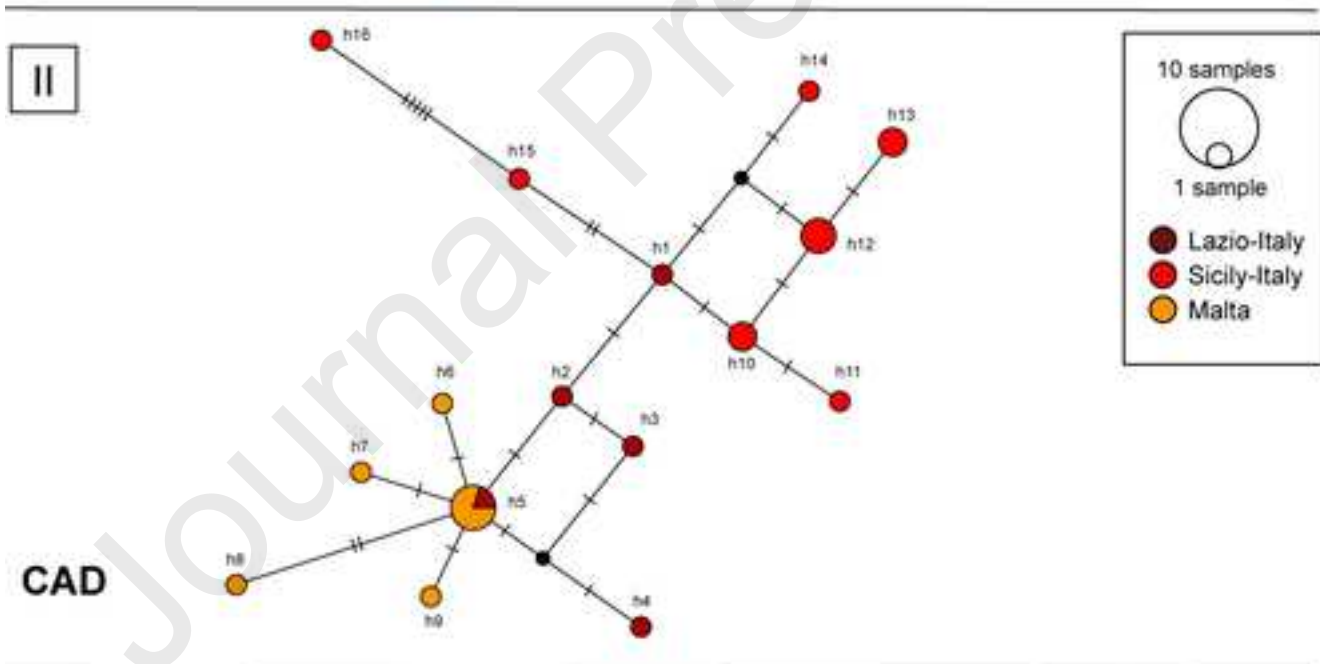
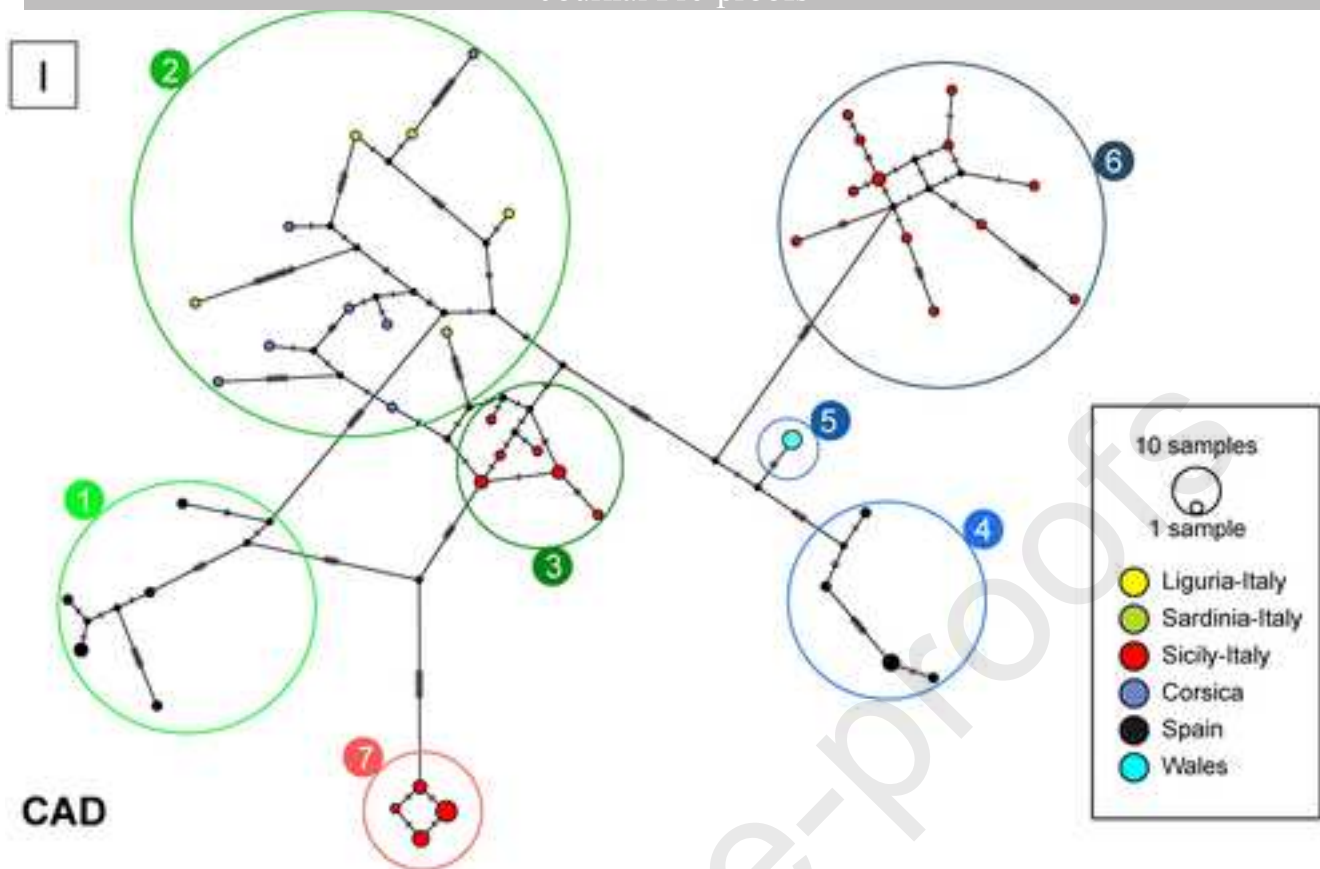


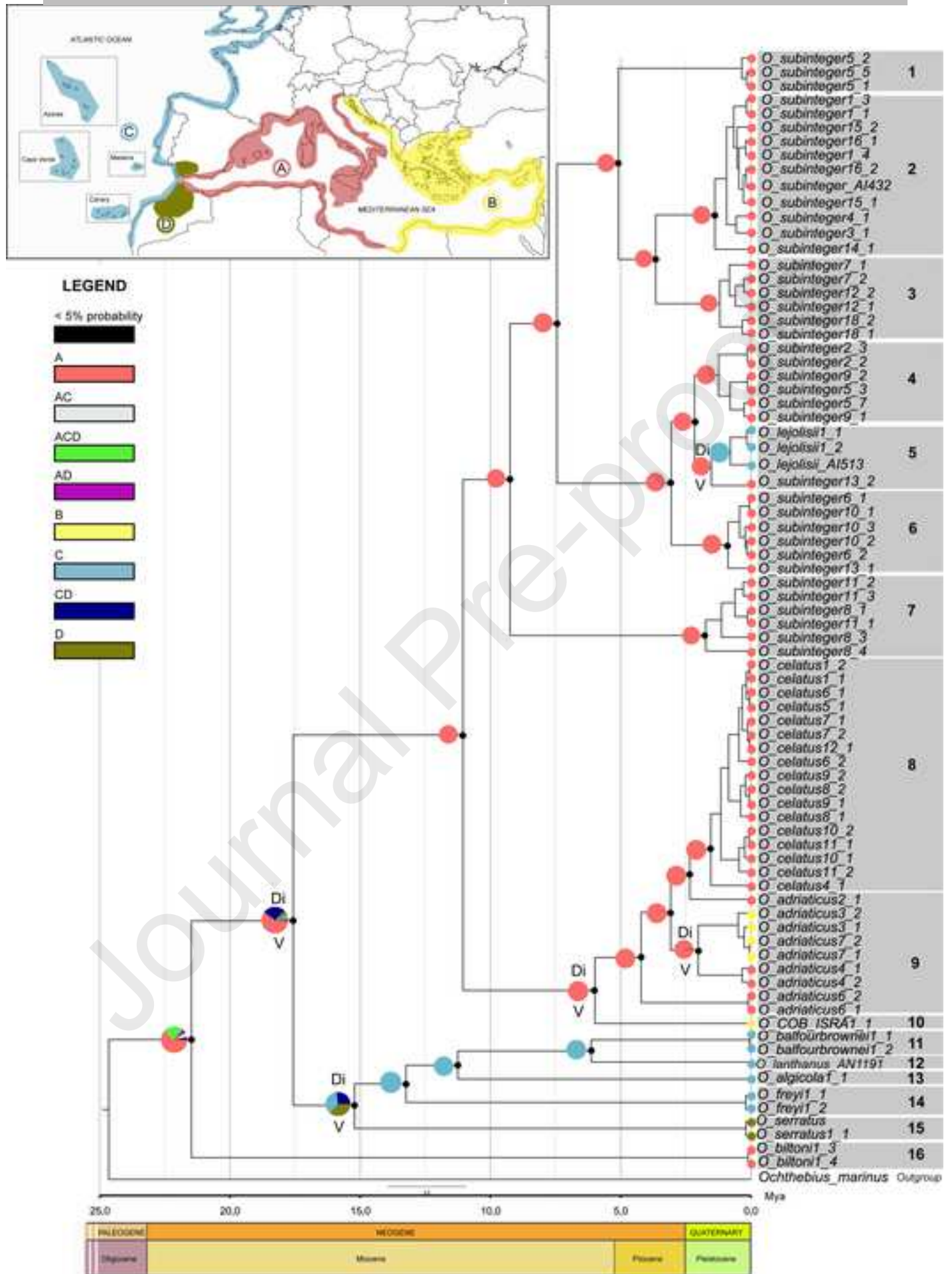
Species delimitation







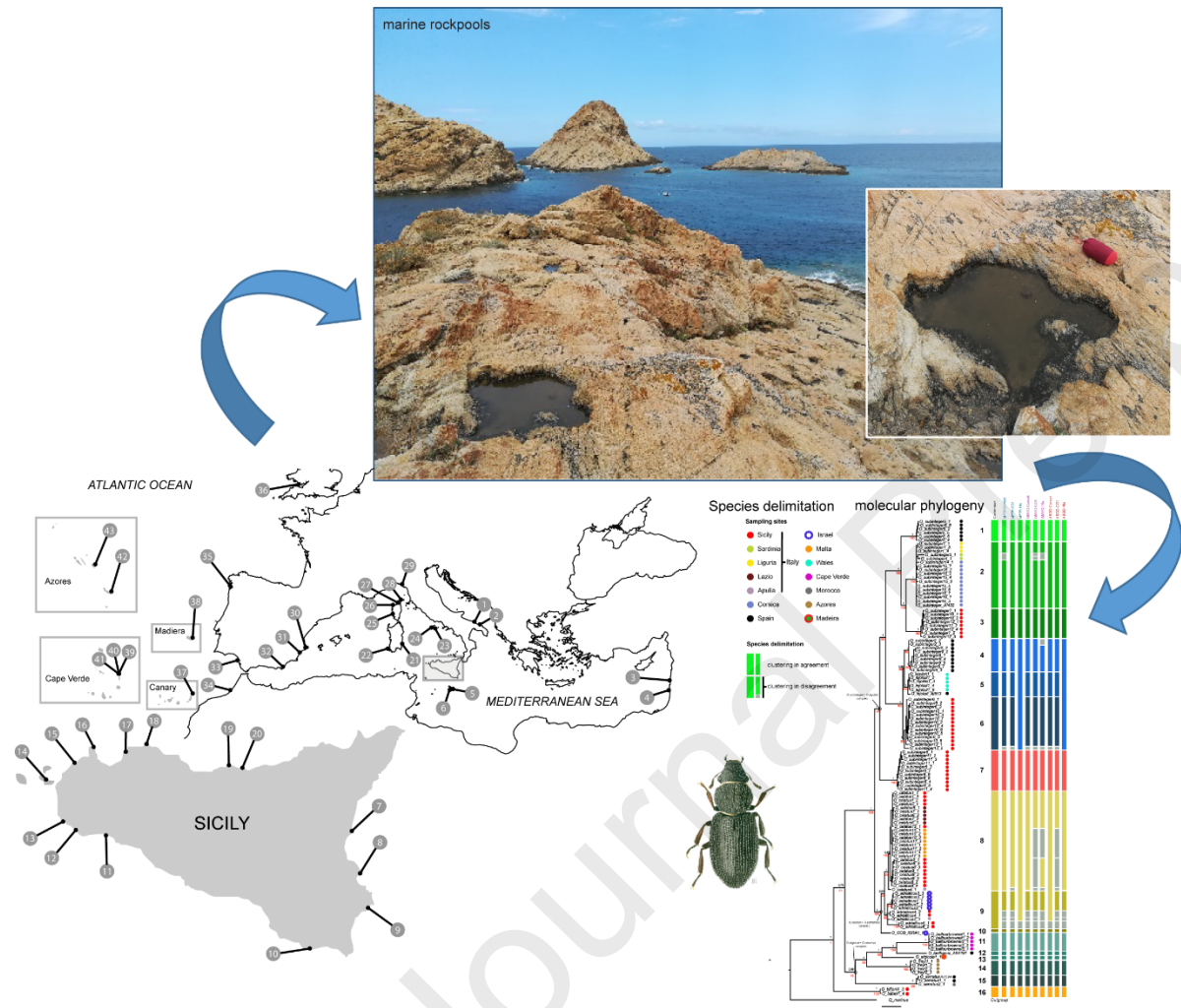




Site	Country	Localities
1	Italy	Apulia- Brindisi- La Forcatella
2	Italy	Apulia- Brindisi- Torre Pozzelle
3	Israel	HaTzafon- Rosh HaNikra- Israel (a)
4	Israel	Haifa- Dor- Israel (b)
5	Malta	S.Giljan- Balluta Bay
6	Malta	Sliema- Tigné point
7	Italy	Sicily- Catania- Aci Castello
8	Italy	Sicily- Siracusa- Augusta
9	Italy	Sicily- Siracusa- Ognina
10	Italy	Sicily- Ragusa- Marina di Modica
11	Italy	Sicily- Agrigento- Porto Palo
12	Italy	Sicily- Trapani- Torretta Granitola
13	Italy	Sicily- Trapani- Mazara del Vallo
14	Italy	Sicily- Trapani- Favignana
15	Italy	Sicily- Trapani- Erice Pizzo Lungo
16	Italy	Sicily- Trapani- Riserva N.O.dello Zingaro
17	Italy	Sicily- Palermo- Terrasini
18	Italy	Sicily- Palermo- Sferracavallo
19	Italy	Sicily- Palermo- Cefalù
20	Italy	Sicily- Palermo- Punta Finale
21	Italy	Sardinia- Nuoro- Barisardo
22	Italy	Sardinia- S. Sardegna- Calasetta

Site	Country	Localities
23	Italy	Lazio- Latina- Ponza- Cala Gaetano
24	Italy	Lazio- Latina- Ponza- Caletta
25	France	Corsica- Ajaccio- Barbicaja
26	France	Corsica- Ile Rouse
27	France	Corsica- Cap Corse- Port de Centuri
28	France	Corsica- Cap Corse- Marina di Brando
29	Italy	Liguria- La Spezia- Deiva Marina
30	Spain	Alicante- Calp
31	Spain	Alicante- Cabo del l'Huerta
32	Spain	Malaga- Fuengirola- Faro de Calaburras
33	Spain	Cadice
34	Morocco	Rabat- Oudaya
35	Spain	Pontevedra- Nigran- Playa Pato
36	UK	Wales- Swansea
37	Spain	Canary Islands- Lanzarote- Punta Gorda
38	Portugal	Madera- Camara de Lobos
39	Cabo Verde	Sal- Murdeira
40	Cabo Verde	Sal- Regona
41	Cabo Verde	Sal- Buracona
42	Azores	Sao Miguel- Mosteiros
43	Azores	Terceira- Sao Pedro

Graphical abstract



Highlights

- Molecular phylogeny of the subgenus *Cobalius* identified sixteen independent lineages
- Molecular clock estimated the origin of the subgenus in Early Miocene (about 22 Mya)
- Species delimitation methods evidenced the presence of four cryptic taxa
- Plio-Pleistocene climatic changes shaped the intraspecific genetic diversity

Journal Pre-proofs

CRedit authorship contribution statement

Simone Sabatelli: Conceptualization, Methodology, Formal analysis, Data curation, Visualization, Software, Investigation, Resources, Project administration, Writing - original draft, Writing - review & editing. **Paola Ruspantini** Investigation, Resources. **Paolo Cardoli:** Formal analysis, Software, Writing - original draft, Writing- review & editing. **Paolo Audisio:** Conceptualization, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

Journal Pre-proofs