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ORIGINAL PAPER

Intertidal or subtidal/circalittoral species: which appeared first? A phylogenetic approach to the evolution of non-planktotrophic species in Atlantic Archipelagos

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Abstract

Volcanic oceanic archipelagos are fascinating natural laboratories of evolutionary patterns and processes in remote, unique conditions. In the insular marine realm, deepwaters and sea-surface circulation hamper dispersal and, for marine invertebrates, this ability is linked to larval development: planktotrophic organisms disperse easily, whereas non-planktotrophic species usually have restricted ranges. Similarly, bathymetric zonation also influences dispersal: intertidal species are more prone to engage in the process than subtidal/circalittoral species. Therefore, the presence of endemic congeneric non-planktotrophic marine gastropods in two Atlantic archipelagos, hundreds of kilometers apart, inspired a biogeographical hypothesis. It predicts that when two congeneric non-planktotrophic gastropod species, with different bathymetric specific ranges, simultaneously occur and are restricted to two remote archipelagos, the subtidal/circalittoral species is expected to be evolutionarily older than the intertidal species. The present study aims to test this theoretical prediction from a multidisciplinary perspective, with a molecular, Bayesian, fossil-calibrated, phylogenetic analysis of selected Rissoidae species to test the theoretical predictions. We hereby corroborate the earlier speciation of the subtidal/circalittoral *Alvania sleursi*, compared to the congeneric intertidal *Alvania mediolittoralis*. Supported by ecological and palaeontological observations in the Azores and Madeira archipelagos, our study provides the first phylogenetic approach to this biogeographical hypothesis, unveiling the evolution of Rissoids in two insular Atlantic systems. In a broader perspective, combining molecular and palaeontological data contributes to better understand past processes that shaped current diversity in Atlantic Archipelagos. This approach can be further replicated in other related non-planktotrophic invertebrates in remote Archipelagos, to corroborate the biogeographical hypothesis in other marine taxa.

Introduction

Remote volcanic oceanic archipelagos gather ideal conditions to study evolution in ecosystems formed de novo, as life appeared and thrived in unique conditions of isolation

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(Paulay 1994). The exceptional contexts in which organisms have reached such islands, often located far from potential colonization sources, have intrigued generations of researchers devoted to understand the biogeographic patterns that emerge from marine and terrestrial taxa inhabiting remote insular areas (see Patiño et al. 2017 for a review; Ávila et al. 2018, 2019). Unlike continental taxa that benefit from physical continuity of landmasses for stepwise migration, for marine organisms, the deep waters and the distance between continental landmasses and oceanic islands usually act as barriers for the dispersal of insular young and adult specimens (Ávila 2013).

Dispersal of marine invertebrates

Dispersal ability of marine invertebrates, and consequently its geographical distribution, is directly linked to the duration and mode of larval development, with major consequences at evolutionary and biogeographical levels (Scheltema and Williams 1983; Scheltema 1986a, 1989, 1995; Scheltema et al. 1996). Benthic marine gastropods usually disperse (1) by means of pelagic planktotrophic larvae; (2) by phoresy/attached to bird feathers (common in some intertidal molluscs); or (3) by rafting of egg masses, juveniles or adults of small-sized species, attached to suitable floating materials (Ávila 2006, 2013 and references therein). Accordingly, gastropods with planktotrophic (p) larval development can survive and feed in the water column for extended periods of time, facilitating their dispersal and arrival to remote islands, often maintaining a gene flow rate high enough to prevent speciation of remote, insular populations (García-Talavera 1983; Scheltema and Williams 1983; Scheltema 1986b; Scheltema et al. 1996). Contrastingly, non-planktotrophic (np) gastropods are expected to be geographically confined to smaller areas when compared to planktotrophic ones, as the absence of a free-swimming feeding stage greatly reduces their dispersal capabilities (Scheltema 1986a, 1989, 1995). Therefore, the presence of benthic, non-planktotrophic, shallow-water gastropod species, with a geographical distribution restricted to two remote, oceanic archipelagos (e.g., Azores/Madeira, 840 km apart; see Fig. 1) is not a common feature. One of the authors of this work (R. Cordeiro) compiled and reviewed a database of the 5284 shallow-water (< 50 m depth) gastropod species reported for the entire North Atlantic and adjacent seas (see Supplementary Table S1). Rissoidae (Mollusca: Gastropoda) is one of the most diverse families, with a total of 266 shallow-water species, most of them (~ 77.0%) being np-developers. However, only ten np-rissoid species (3.8%) occur simultaneously and exclusively in two of the Macaronesian Archipelagos (one species in the Azores and Madeira; one in the Azores and Canaries; four in Madeira

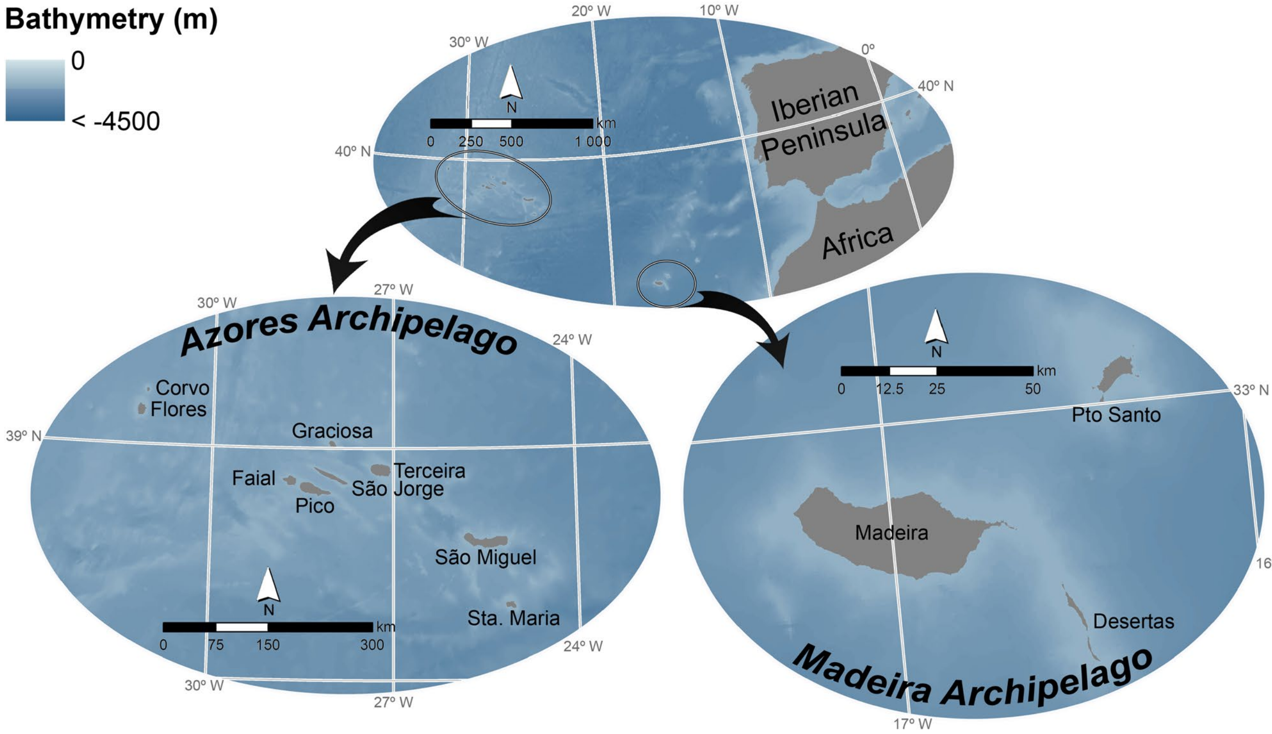

Fig. 1 Geographical location of Azores and Madeira Archipelagos in the Northeast Atlantic Ocean. Coastline from the Portuguese Hydrographic Institute (2014) and bathymetry from GEBCO (2008)

and Selvagens; one in Madeira and Canaries; and three in Selvagens and Canaries).

Evolutionary history of the family Rissoidae

For decades, the family Rissoidae has been studied to clarify its evolutionary history and relationships among its members. The earlier taxonomic reviews of the family were based mainly on the similarity of conchological characters (Thiele 1929; Wenz 1938; Coan 1964). The classification was subsequently revised by Ponder (1967), with the proposal of four distinct subfamilies. More recently, Ponder (1984) refined the taxonomic position of Rissoidae genera, becoming the most consensual classification scheme for several years by recognizing the subfamilies Rissoinae and Rissoininae. Finally, the phylogenetic status of the superfamily Rissooidea was investigated based on molecular data (16S and 28S; Criscione and Ponder 2013), and the authors proposed the elevation of Rissoininae (sensu Ponder 1984) to the family level. Subsequent work led to the most comprehensive molecular phylogeny of the family Rissoidae to date, based on 16S and 28S markers (Criscione et al. 2017), with a thorough revision of Rissoidae as the former subfamily Rissoinae, excluding the genera *Merelina* Iredale, 1915 and *Lironoba* Iredale, 1915. This work provided a new broadly accepted classification scheme for this peculiar family, based on genetic data.

Congeneric non-planktotrophs in remote archipelagos: a theoretical hypothesis

In the Northeast Atlantic Ocean, the evolutionary and biogeographical relationships of shallow-water marine molluscs from volcanic oceanic islands and seamounts are well studied and, particularly, the Azores Archipelago has been considered a model to study the patterns and processes of arrival, colonization, and potential speciation of these species (see Ávila 2005, 2013; Ávila et al. 2008, 2009). Although most np-species are geographically limited to small areas (e.g., the rissoids *Alvania angioyi* van Aartsen, 1982 endemic to the Azores, and *Alvania freitasi* Segers, Swinnen & de Prins, 2009 endemic to the Selvagens), some species can occur in more than one archipelago, as mentioned before, or be distributed over large areas as the Northeast Atlantic Ocean [e.g. the intertidal np-rissoid *Cingula trifasciata* (J. Adams, 1800) has its northern distribution limit in Scandinavia and the southern in Madeira archipelago; Ávila (2005)].

A plausible explanation for these observations would be to consider the speciation of a np-developer in a remote archipelago, where its ancestral first established and later speciated, occupying a suitable, free ecological niche. Through time, the “new” species so far restricted to one remote archipelago would potentially engage in dispersive processes to remote locations (e.g.: another remote archipelago, located far from the area in which the species first appeared) by mediated (e.g., birds) or non-mediated dispersal events. If it successfully established in the new area, a np-species would then have extended its geographical range to more than one remote archipelago. Ávila (2005), and later Ávila et al. (2012), studied in detail the marine benthic microgastropod family Rissoidae in the North Atlantic Ocean, clarifying possible mechanisms for the dispersal of its shallow-water species. A total of 124 rissoids are presently reported from the archipelagos of the Azores, Madeira, Selvagens, Canaries, and Cabo Verde. Of these insular rissoids, the majority (78.9%) possess np-larvae, and their small size (≤ 5 mm) and presence amongst algae, from which they can suspend themselves, make them potential candidates for dispersal by rafting. Thus, when algae are pulled out from the substratum by wave action, intertidal and shallow-water rissoids can be rafted along. However, the initiation of a rafting process does not guarantee that dispersal will be well succeeded, especially in remote volcanic oceanic islands where the distance between the source and sink areas makes the dispersal of young and adult rissoids difficult (Ávila et al. 2012; Ávila 2013).

Ávila (2006, 2013) suggested the addition of a vertical dimension—the bathymetric/ecological zonation—to the considerations of Scheltema on the relationships between modes of larval development and geographical range distribution (Scheltema 1986b, 1989, 1995). Considering rafting as a key mechanism for the dispersal of epibenthic, shallow-water (< 50 m depth), np-species in Atlantic waters, three main assumptions were pointed out by Ávila (2006): (1) intertidal insular species are more prone to be rafted than subtidal/circalittoral species, due to the increased likelihood of being disturbed by natural events that trigger their passive dispersal (see Fig. 2a); (2) bathymetry/ecological zonation and geographical distribution of species are directly correlated, with intertidal species generally having wider ranges than sublittoral species (e.g., 3–6 m depth) and these, in turn, having wider ranges than subtidal/circalittoral species (e.g., 20–30 m depth) (see Fig. 2b); (3) the chances of a successful dispersal are higher for micromolluscs in which the juveniles/adults are the rafting stage, as they are usually associated with algae and remain attached even after the algal substrate is ripped by wave action. Therefore, the smaller the species, the higher the likelihood of dispersal and range expansion, as rafting is more likely to be well succeeded if the species remains attached to the substrate (Ávila 2006).

This relation was later completed by Ávila (2013) and resulted in the formulation of a new biogeographical hypothesis, with the addition of evolutionary time as the fourth and last variable influencing the dispersal and evolution of shallow-water marine molluscs. Based on the compilation

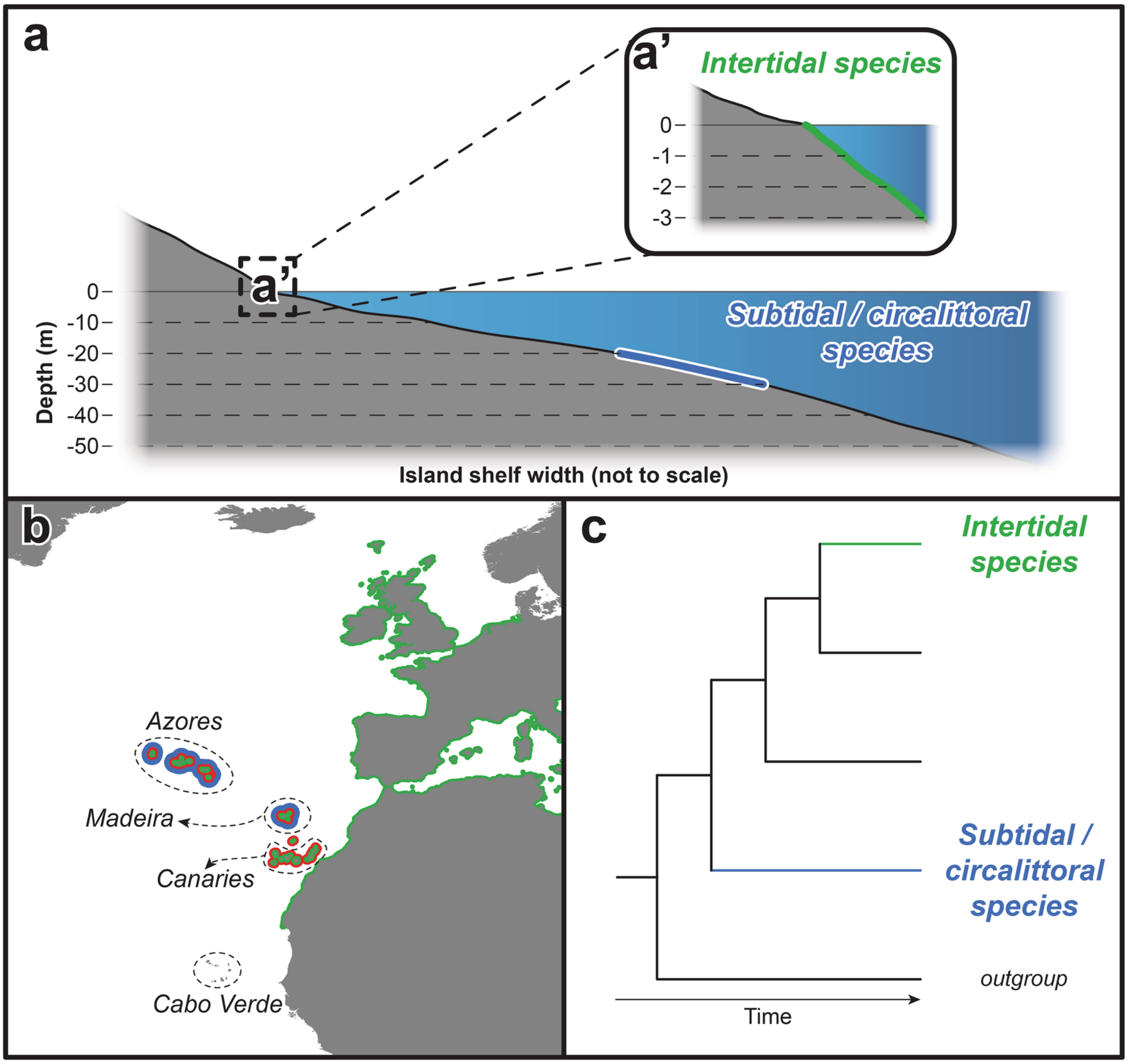

Fig. 2 Graphical illustration of Ávila's (2013) biogeographical hypothesis. **a** Bathymetric range/ecological zonation of intertidal (in green) and subtidal/circalittoral species (in blue); sublittoral species (not represented) have an intermediate bathymetric zonation (3–6 m). Intertidal species are more exposed to disturbing agents (e.g., waves); **b** intertidal species are expected to have a wider geographical distribution (in green) than sublittoral species (in red), which in turn have a wider range than subtidal/circalittoral species (in blue), due to the likelihood of initiating rafting; **c** when the premises of Ávila's (2013) theory are granted, the subtidal/circalittoral species is expected to be evolutionarily older than the intertidal species, conceding enough time for both to disperse successfully

of the geographical distribution of rissoids in the North Atlantic Ocean by Ávila et al. (2012), this relationship was formulated to address the simultaneous existence of two congeneric np-species—*Alvania sleursi* (Amati, 1987) and *A. mediolittoralis* Gofas, 1989—in two nearby archipelagos (Azores and Madeira), which differ only in their bathymetric range (subtidal/circalittoral and intertidal, respectively). Following this premise, as both species presently occur in two nearby archipelagos, it is expected that the one inhabiting deeper water is evolutionarily older than the shallow-water/intertidal species (Ávila 2013). Both species may successfully colonize and establish in the same far-away location under this scenario, but the subtidal/circalittoral species inhabits less disturbed environments being less prone to engage dispersal, whereas the intertidal is fustigated by wave action, inducing rafting more often. Thus, we expect the subtidal/circalittoral lineage to have appeared earlier in the geological scale, granting it more time to successfully expand its distributional range to the same extent as the intertidal congeneric (see Fig. 2c).

This biogeographical hypothesis was later supported by ecological and palaeontological studies in Madeira archipelago and in the Azores, where Santa Maria is the oldest (~6.01 Ma; Ramalho et al. 2017) and the only island having known exposed marine fossiliferous outcrops. In Santa Maria Island, fossils of the subtidal/circalittoral rissoid *A. sleursi* were found in Pliocene (4.78 ± 0.13 to 4.13 ± 0.19 Ma; Ramalho et al. 2017) and Late Pleistocene (last interglacial Marine Isotopic Stage 5e—MIS 5e; 130–116 ka; Ávila et al. 2015) outcrops. For the intertidal species *A. mediolittoralis*, fossilized remains are only observed in Azorean MIS 5e sediments (Ávila et al. 2015), suggesting it is evolutionarily younger. Despite the earlier emergence of Madeira Islands in the Northeast Atlantic Ocean—Porto Santo ~ 18.8 Ma (Mata et al. 2013) and Madeira's re-emergence ~ 7 Ma (Ramalho et al. 2015)—the fossiliferous outcrops in this archipelago are assigned to only two geological epochs. Reef limestones from the Pliocene are found in Madeira Island, and bioclastic or bio-edified limestones from the Middle Miocene as well as Pleistocene biogenic carbonate sandstone are present in Porto Santo Island (Mata et al. 2013). Nevertheless, specimens of *A. mediolittoralis* and *A. sleursi* are not reported for Madeiran fossiliferous outcrops (Gerber et al. 1989; Ávila et al. 2015).

Notwithstanding its relevance, Ávila's (2013) hypothesis remains untested from a phylogenetic perspective. Therefore, the main aim of our work is to address this evolutionary/biogeographical link between non-planktotrophic larval development, geographic range, bathymetric/ecological zonation and evolutionary time with molecular phylogenetic tools, targeting *A. mediolittoralis, A. sleursi* and other rissoids presently occurring in the Azores and/or Madeira archipelagos. The family Rissoidae was chosen as a study model because of the current broad knowledge at the geological and ecological levels, but also due to the high number of representatives, susceptibility to rafting and distributional range in Atlantic islands (Ávila et al. 2012). We hereby propose a multidisciplinary, combined approach of molecular phylogenetics and palaeontological data to unveil evolutionary relationships of insular rissoids and to assess the prediction of an earlier evolutionary divergence of the subtidal/circalittoral lineage.

Materials and methods

Taxon sampling and laboratory analyses

Samples of rissoids naturally occurring in the Azores Archipelago—*Alvania angioyi, A. formicarum* Gofas, 1989*, A. mediolittoralis, A. sleursi, Cingula trifasciata, Crisilla postrema* (Gofas, 1990)*, Rissoa guernei* Dautzenberg, 1889 and *Setia subvaricosa* Gofas, 1990—were retrieved from the marine molluscs reference collection of the Department of Biology of the University of the Azores (DBUA), stored in 96% ethanol and collected in several coastal sites of the archipelago. We have also used samples of *Pisinna glabrata* (von Mühlfeld, 1824) from DBUA as the outgroup in the phylogenetic reconstructions. DNA was extracted from whole individuals with the commercial kit PureLink® Genomic DNA Mini Kit (Invitrogen™), following the manufacturer's instructions.

Fragments of the mitochondrial gene cytochrome oxidase subunit I (COI) and the nuclear 28S ribosomal RNA (28S rRNA) were amplified by PCR, performed in 25 µl volumes, entailing 3 µl of DNA, − 10 × buffer $MgCl_2$ free, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 10 µM of each primer, 0.1 µg μl^{-1} bovine serum albumin (BSA, Promega) and 0.3 U platinum Taq DNA polymerase. PCR conditions and primers are provided as Supplementary Material (Table S2). Electrophoretic runs in 2% (w/v) agarose gel with GelRed (DNA fluorescent dye, BioTarget™) were performed to assess the efficiency of the amplification reactions and the quality of the DNA products. PCR product purification and bi-directional Sanger sequencing with amplification primers were realized by a commercial facility—Genewiz, London, UK.

Sequence data

A manual check of misreads in COI and 28S chromatograms was performed with BioEdit v.7.0.5.3 for Windows (Hall 1999). Sequences of the mitochondrial coding COI gene were translated into amino acids with ExPASy Translate Tool (https://web.expasy.org/translate/), allowing to inspect the existence of stop codons and thus the presence of pseudogenes. For the phylogenetic analyses of COI and 28S genes, we have also included sequences of species reported for the Northeast Atlantic Ocean, deposited in GenBank. All the sequences analyzed for the reconstruction of the gene trees are provided in Supplementary Table S3. Sequences of the mitochondrial COI marker were aligned using Clustal Omega algorithm via Web Services by EMBL-EBI (McWilliam et al. 2013), whereas the alignment of 28S sequences was performed using the MAFFT program (Katoh et al. 2005). Poorly aligned positions and divergent regions that often obscure the phylogenetic signal (Xia and Lemey 2009) were removed from the alignment of the nuclear 28S marker resorting to GBlocks Server v0.91b (Castresana 2000).

Phylogenetic analyses of COI and 28S

The software PartitionFinder v1.1.1 (Lanfear et al. 2012) was used to determine the best-fit partitioning schemes and models of molecular evolution to each dataset, according to the Akaike's Information Criterion (Akaike 1973). Data partitioning by codon was applied to COI gene to minimize saturation effects of codon positions on phylogenetic reconstructions (Salemi 2009) and to account for different rates of evolution of each codon position (Pond et al. 2009). The following models of molecular evolution were applied to COI partitions: HKY + G (1st partition), GTR + G (2nd partition), HKY + I (3rd partition). Bayesian inference (BI) and maximum likelihood (ML) methods were used to reconstruct the Rissoidae phylogeny. For BI analyses, performed in MrBayes v3.2.6 software (Ronquist et al. 2012), two independent runs (each with four chains for 2×10^7 generations) were performed. Trees and parameters were sampled every 1000 generations, with the heating parameter set to 0.25. Stationarity was considered to be reached when the average standard deviation of split frequencies was lower than 0.01. Majority-rule consensus trees were estimated from both analyses, after discarding 25% of the total samples as burn-in. ML analyses in Garli v2.0.1 (Zwickl 2006) were performed with a ten independent search replicates and 1000 bootstrap replicates. The evaluation of log-likelihood values across searches allowed to check the convergence between the topologies of the trees generated. SumTrees program of the DendroPy package (Sukumaran and Holder 2010) was used to summarize non-parametric bootstrap support (BS) values for the best tree, after generating a majority-rule consensus tree. Consensus trees inferred for each molecular marker were visualized and rooted—setting *P. glabrata* as the outgroup for the COI dataset and *Anabathron contabulatum* (Frauenfeld, 1867) as the outgroup for the 28S dataset—resorting to FigTree v1.4.3 software.

Species tree reconstruction and estimation of divergence times

Species tree reconstruction can be achieved by distinct phylogenetic methodologies. Maximum likelihood methods with concatenated sequences estimate a single species tree that best fits the combined alignment, according to the phylogenetic likelihood function (Felsenstein 1981; Ogilvie et al. 2017). However, inferences drawn from concatenated datasets have been associated with inconsistent results and low reliability, including high bootstrap support even in incorrectly estimated branches (Gadagkar et al. 2005; Kubatko and Degnan 2007), due to the influence of model misspecification or long-branch attraction (Liu et al. 2015; Ogilvie et al. 2017) and the incomplete lineage sorting phenomenon that causes systematic errors on the estimation of branch lengths and divergence times (Mendes and Hahn 2016; Ogilvie et al. 2016, 2017). Therefore, a fully Bayesian multispecies coalescent method—StarBEAST2 framework (Ogilvie et al. 2017)—was applied to the dataset comprising both COI and 28S genes, as implemented in BEAST v2.4.5 software (Bouckaert et al. 2014), for the inference of the evolution of eight rissoid species and their divergence time. This approach, based on the co-estimation of multi-individual multi-locus sequence data, provides a reliable estimation of the species tree by searching and embedding each gene tree within a single shared species tree that likely best reflects the evolutionary history of the taxa (Ogilvie et al. 2017), thus avoiding the issues associated with concatenated datasets (Mendes and Hahn 2016; Ogilvie et al. 2016). Due to the requirements of the chosen phylogeny reconstruction framework, we have only considered rissoid species represented by two or more individuals (Heled and Drummond 2010), for which sequences of both COI and 28S were available (Table 1): *Alvania angioyi*, *A. formicarum*, *A. mediolittoralis, A. sleursi, Cingula trifasciata*, *Crisilla postrema*, *Rissoa guernei,* and *Setia subvaricosa* Gofas, 1990. According to the currently accepted phylogeny of the superfamily Rissooidea, based

Table 1 Sequences of mitochondrial COI and nuclear 28S genes of Rissoidae species and of the outgroup *Pisinna glabrata*, included in the species tree reconstruction following StarBEAST2 framework

	COI AN	28S AN
A. angioyi van Aartsen, 1982	MG652373-5	MG663173-4
	MG652378	MG663176 MG663178
A. formicarum Gofas, 1989	MG652379-80	MG663179
A. mediolittoralis Gofas, 1989	MG652382-3	MG663180-1
A. sleursi (Amati, 1987)	MG652386-7	MG663182-3
Cingula trifasciata (J. Adams, 1800)	MG652396-7 MG652401 MG652403-4 MG652406	MG663191-6
Crisilla postrema (Gofas, 1990)	MG652392 MG652394	MG663185-6
Rissoa guernei Dautzenberg, 1889	MG652408-9 MG652410-13 MG652419-21	MG663197-203 MG663207 MG663209
Setia subvaricosa Gofas, 1990	MG652429-30	MG663215-6
Pisinna glabrata (von Mühlfeld, 1824)	MG652432-33 MG652435	MH047302-4

Accession numbers (AN) are provided; all the sequences used were obtained in this study on molecular data [sensu Criscione and Ponder (2013)], *Pisinna glabrata* was selected as an outgroup in the species reconstruction for the same reasons.

The software jModelTest v2.1.10 (Darriba et al. 2012) yielded the following substitution models and parameters as the best fitted to the data analyzed, according to the Akaike's Information Criterion (Akaike 1973): HKY + I + G model, with gamma shape = 0.6100, proportion of invariants = 0.5500 and kappa = 18.7407 for the COI marker; GTR + I + G model, with gamma shape = 0.4710, proportion of invariants = 0.508, rate AC = 0.3118, rate AG = 2.1462, rate AT = 0.7970, rate CG = 0.7967, rate CT = 6.8066 and rate GT = 1.0000 for the 28S marker.

Four fossil calibration points were assigned to estimate the timing of the split events, according to the presence/absence of the species analyzed in fossiliferous outcrops in Santa Maria Island (Azores), dated from the Pliocene and Pleistocene (MIS 5e). Their attribution followed the recommendations of Forest (2009) and Parham et al. (2012) for species tree reconstructions analyses, being defined as the minimum age of the clades to which they were assigned and according to the most recent and valid literature available for the fossil taxa in question (Meireles et al. 2013; Cordeiro et al. 2015; Ávila et al. 2015, 2016; Ramalho et al. 2017), to ensure the accurate temporal placement of the lineage in the phylogenetic analyses. *Alvania sleursi* is an extant species, also found in fossiliferous sediments dated back to the Pliocene, meaning that it was already inhabiting Santa Maria shores during those periods. One must assume its speciation prior to the formation of the Pliocene palaeo-environment, otherwise, fossil specimens of *A. sleursi* would not be present there. In Santa Maria, the ancient habitat where *A. sleursi* existed was covered by lava during a period of transgressive volcanism, promoting the preservation of the Pliocene fossiliferous outcrop. The lava formation on top of the Pliocene sediments was dated as 4.13 ± 0.19 Ma (Ramalho et al. 2017), which was designated as the minimum age for the divergence among *A. sleursi, A. mediolittoralis* and *A. formicarum* as, based on the fossil record, *A. sleursi* already existed but none of its conspecific-related taxa. On the other hand, considering their presence only in more recent fossil sediments from Pleistocene (MIS 5e) (Cordeiro and Ávila 2015; Ávila et al. 2015, 2016), a minimum age of 0.12 Ma (the estimated age of 116 ka was rounded up to 0.12 Ma) was set up for (1) the split between *A. formicarum* and *A. mediolittoralis*; (2) the divergence among *C. trifasciata* and the endemic species *A. angioyi* and *C. postrema*; and (3) the split between *R. guernei* and *S. subvaricosa.* Although the low number of fossil calibration points used ($n = 4$) and their inference from fragmented fossil record from a single island are not considered ideal practices on a species tree reconstruction (Parham et al. 2012), the information to calibrate this inference is only available from the fossiliferous outcrops of Santa Maria Island in the Azores Archipelago.

All this information was manually included in the input file for StarBEAST2 (see Supplementary Material File S4), created using BEAUti package manager. We have tested the use of Uncorrelated Lognormal molecular clocks, as well as using the Calibrated Yule model of speciation, but the analyses did not converge in any of the attempts, reaching ESS (effective sample size) values under 50 in most parameters. For that reason, and after evaluating convergence, we have defined a strict clock for both genes, unlinking the molecular clock and tree priors between the markers, and defined Yule model of speciation for 10^8 generations, with parameters sampled every 10,000 generations. Burn-in was set for 10%, after graphically checking the achievement of stationarity in Tracer v1.6 software (http://beast.bio.ed.ac.uk/Tracer). The convergence and ESS were also assessed with Tracer v1.6. After discarding the burn-in samples, the final species tree with node ages was plotted with TreeAnnotator v2.4.5 (http://tree.bio.ed.ac.uk/software/beast) and visualized with the FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/). Posterior modifications of the phylogeny were performed resorting to Inkscape v0.92 software (http://www.inkscape.org).

Results

Sequenced data and phylogenetic analyses

All the rissoid sequences obtained in this study were deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/), under the accession numbers: MG652373-87, MG652392-422, and MG652429-31 for the COI sequences; MG663173-83, MG663185-209 for the 28S rRNA marker. Sequences were also obtained from *Pisinna glabrata*: MG652432-36 (COI) and MH047302-4 (28S rRNA). The alignment of COI marker had a total length of 658 bp, whereas the alignment of 28S marker had a length of 1015 bp after the removal of uninformative gaps (total length of 1074 bp prior to the GBlocks analysis).

The phylogenetic trees obtained for COI (Fig. 3) and 28S (Fig. 4) displayed similar topologies and acceptable resolution in clarifying systematic positions among Rissoidae taxa. Within each marker, BI and ML analyses produced phylogenetic trees with similar topologies for most relationships, thus only the topology inferred with ML methods is shown (Bayesian Inference gene trees of COI and 28S markers are provided as Supplementary Figures S5 and S6, respectively). Posterior probabilities (PP) tended to be higher than BS values at most nodes, but it is known that PP are typically higher than BS values (Suzuki et al. 2002). The major relationships were concordant between the mtDNA

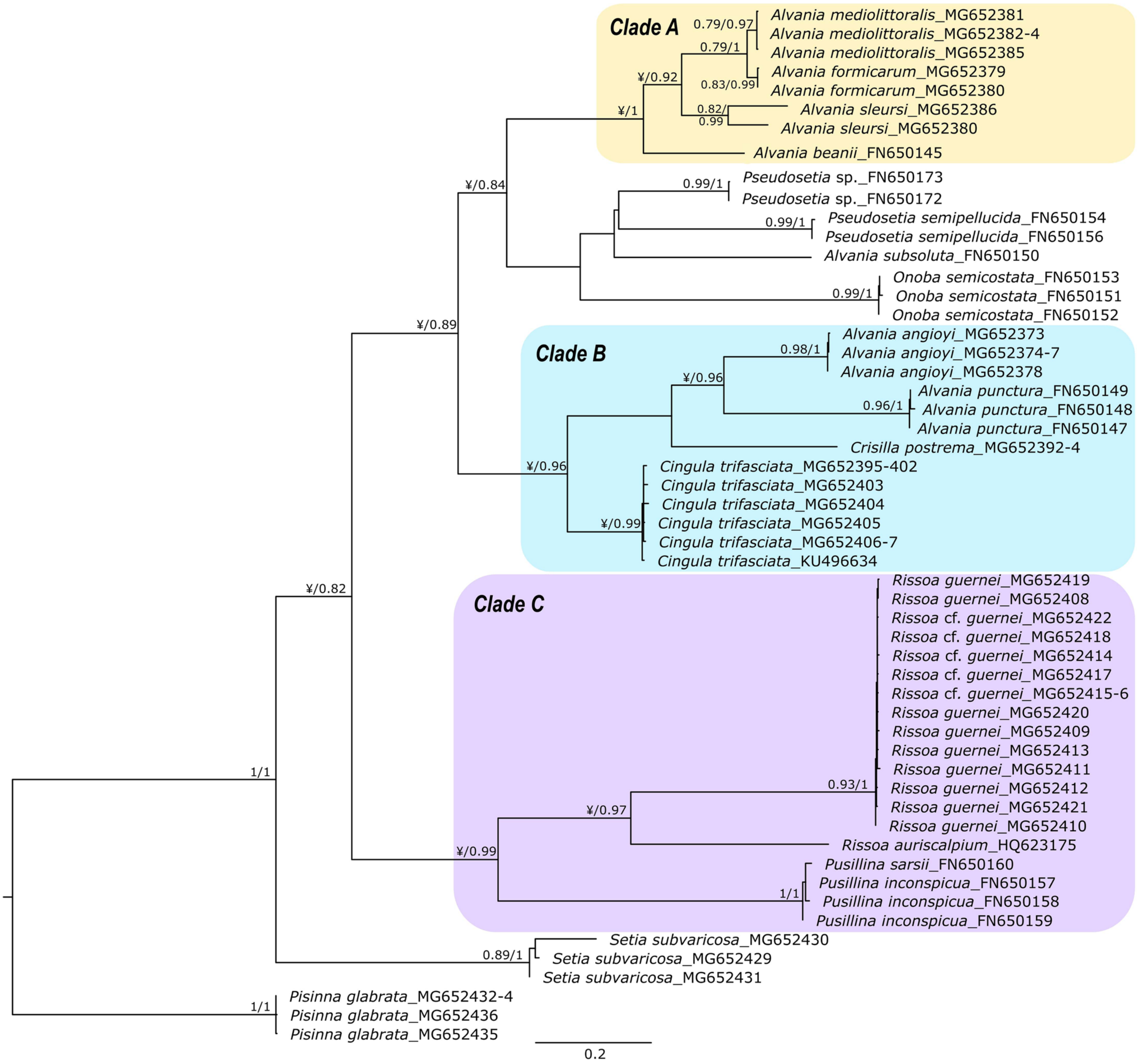

Fig. 3 Maximum likelihood tree obtained for mtDNA COI gene. Values at the nodes correspond to bootstrap support (BS) values and posterior probability (PP). Clades A–C are identified. Accession numbers of sequences are provided after the species designation. Yen (¥) symbol is used to represent support values inferior to 0.7. Colors and names assigned to the clades match between gene trees and species tree

COI (Fig. 3) and nDNA 28S (Fig. 4) phylogenies, as three clades were supported by PP values. Clade A comprised several *Alvania* spp. in both gene trees (PP 99–100%), whereas in Clade B (PP 96% in the COI phylogeny; PP 88% in the 28S phylogeny) *Cingula trifasciata* occupied a basal position, closely related to *Crisilla postrema* and some *Alvania* spp. Clade C (PP 99% in COI and 28S gene trees) comprised taxa assigned to *Pusillina* and *Rissoa* genera, as well as *Setia ambigua* in the 28S phylogeny. Moreover, the basal phylogenetic position of *Setia subvaricosa* in relation to the remaining rissoids was supported in the COI gene tree (BS and PP 100%), but for the 28S, its position within Clade C was not supported. In the COI phylogeny, another group comprising *Pseudosetia* and *Onoba* genera was also retrieved, but no further considerations were made regarding its phylogenetic position as it was not supported by the analyses. The polyphyly of the genus *Alvania* was depicted and well-supported in the two markers analyzed.

In both phylogenies, *A. formicarum, A. mediolittoralis,* and *A. sleursi* were depicted as closely related, with an

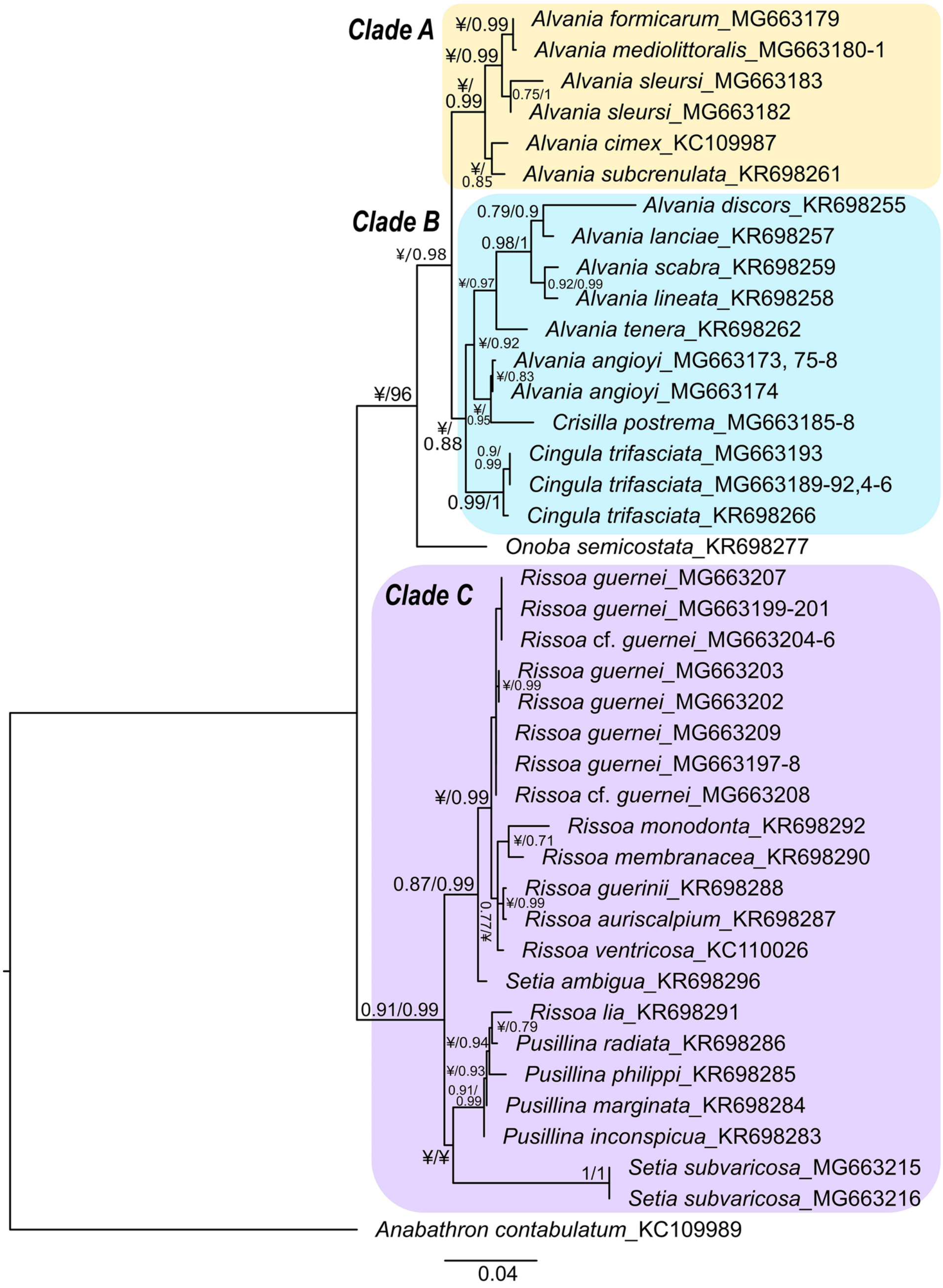

Fig. 4 Maximum likelihood tree obtained for nDNA 28S rRNA gene. Values at the nodes correspond to bootstrap support (BS) values and posterior probability (PP). Clades A–C are identified. Accession numbers of sequences are provided after the species designation. Yen (¥) symbol is used to represent support values inferior to 0.7. Colors and names assigned to the clades match between gene trees and species tree

earlier divergence of the subtidal/circalittoral *A. sleursi*. In the COI gene tree, the split between *A. mediolittoralis* and *A. formicarum* was supported by ML and BI analyses (BS 79%, PP 100%), whereas the older splits were only supported by PP (> 90%). In the 28S phylogeny, splits within Clade A were only supported by PP values (> 85%).

Species tree reconstruction

In the species tree reconstruction using StarBEAST2 framework, the effective sample sizes of every parameter reached values higher than 200, and assessment of parameter statistics in Tracer v1.6 suggested the convergence of the analysis. Posterior probabilities higher than 99% were recovered for all but one node (PP 56.8%, broad credibility interval ranging from 12.82 to 23.17 Ma) (Fig. 5). Confidence intervals were calculated for the age of all the splits inferred and, the older the split event, the lower the associated confidence level.

The species tree reconstruction (Fig. 5) showed early diversification of the studied species during the Early Miocene, ca. 17.52 Ma, resulting in the separation of the lineage that originated *Rissoa* and *Setia* genera and the one that leads to the speciation of *Alvania*, *Cingula* and *Crisilla* genera.

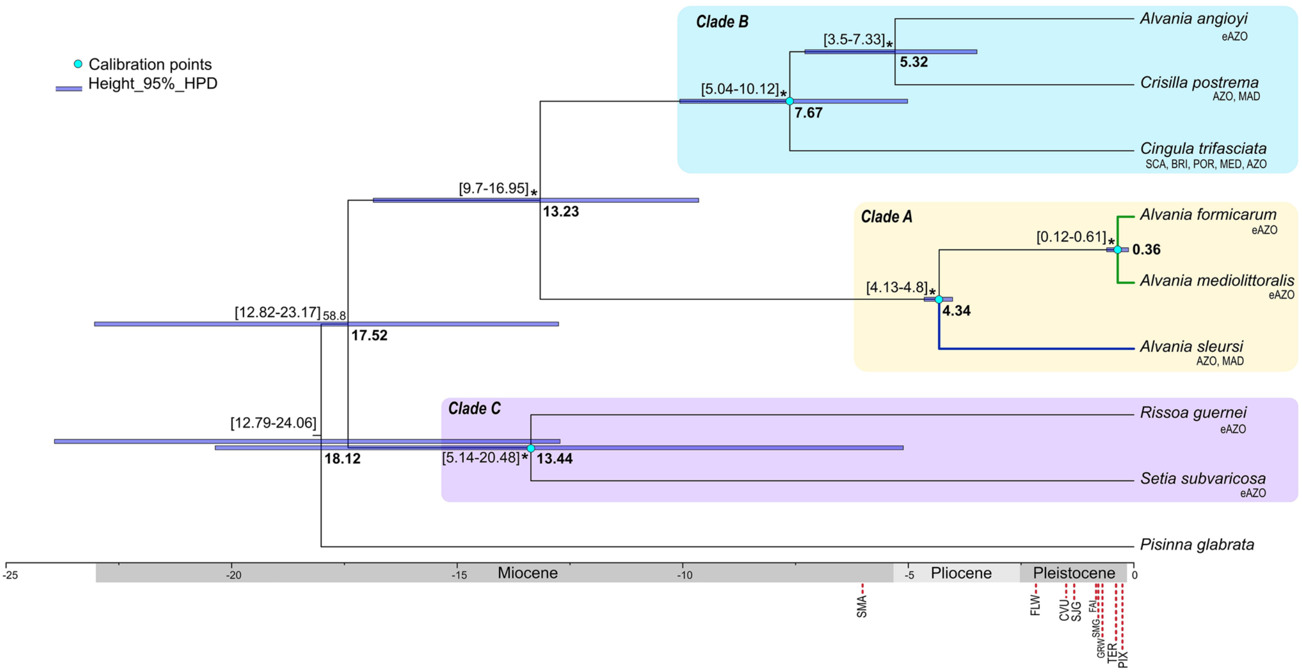

Fig. 5 Rissoidae species tree, estimated according to a fully Bayesian multispecies coalescent method implemented in StarBEAST2 software with datasets of mtDNA COI gene and nDNA 28S rRNA gene. Four calibrations were added to some nodes and represented by a light blue circle. Clades A–C are depicted by different colors; colors and names assigned match between gene trees and species tree. Within Clade A, the colored branches follow the same color scheme as Fig. 2 to represent the ecological zonation of the species: intertidal (green) or subtidal/circalittoral (blue). Posterior probability values are displayed at the nodes and here represented by an asterisk (*) when higher than 99%. Values at the nodes indicate the estimated age of the split event and blue node bars represent 95% of the highest posterior density interval. Numbers within brackets represent the 95% credibility interval associated with the node bars and, thus, split events. The reported geographical distribution of the species is provided under each species name, at the tip of the species tree: *eAZO* endemic to the Azores, *AZO* Azores, *BRI* British Isles, *MAD* Madeira, *POR* Portuguese shores, *SCA* Scandinavia. The appearance of the Azorean Islands is depicted chronologically: *SMA* Santa Maria, 6.01 Ma (Ramalho et al. 2017); *FLW* Flores, 2.16 Ma (Azevedo 1998); *CVU* Corvo, 1.50 Ma (França et al. 2002); *SJG* São Jorge, 1.32 Ma (Hildenbrand et al. 2014); *FAI* Faial, 0.85 Ma (Hildenbrand et al. 2014); *SMG* São Miguel, 0.79 Ma (Sibrant et al. 2015); *GRW* Graciosa, 0.70 Ma (Sibrant et al. 2014); *TER* Terceira, 0.40 Ma (Hildenbrand et al. 2014); *PIX* Pico, 0.27 Ma (Demand et al. 1982)

Curiously, *A. angioyi* was depicted as more closely related to *Crisilla postrema* and *Cingula trifasciata* than to other *Alvania* species. Three major clades can be distinguished in the species tree: (1) Clade A estimated the first diversification of three species during the Middle Pliocene (ca. 4.34 Ma), by splitting *Alvania sleursi* and the lineage that later (ca. 360 ka, during the Pleistocene) originated *A. formicarum* and *A. mediolittoralis*. As both splits were calibrated, narrow Highest Posterior Density (HPD) intervals were estimated for both split events providing high confidence in this inference: credibility interval of 0.73 Ma for the oldest event and ranging from 0.12 to 0.61 Ma in the youngest; (2) Clade B comprised a first split event (calibrated) during the Late Miocene (ca. 7.67 Ma) between *C. trifasciata* and the ancestor of *A. angioyi* and *C. postrema,* and a divergence between these two taxa around 2.5 Ma later. Both estimations were associated with a low degree of uncertainty, with credibility intervals ranging from 5.04 to 10.12 Ma for the older split, and between 3.5 and 7.33 Ma for the most recent event; and (3) Clade C showed the split between *Rissoa guernei* and *Setia subvaricosa* sometime between 20.48 and 5.14 Ma, which decreased the credibility of this inference.

Discussion

Phylogenetic analyses of COI and 28S markers

The phylogenetic analyses performed for Rissoidae taxa supported the monophyly of the family Rissoidae. The genus *Alvania* was shown to be polyphyletic in both phylogenies, with species assigned clades A and B and in accordance with previous work by Criscione et al. (2017). Within Clade A, we highlight the relationship established between the intertidal *A. mediolittoralis* and the subtidal/circalittoral *A. sleursi,* highly supported in both ML and BI analyses (BS and PP > 75% in COI gene tree; PP 99% in 28S gene tree). The systematic position of *A. sleursi*, currently reported for the Azores and Madeira archipelagos, in a molecular phylogeny of the family Rissoidae had already been hypothesized by Ávila (2013) as expected to occupy a basal position in relation to *A. mediolittoralis*. This hypothetical systematic position of *A. sleursi* was corroborated by both COI and 28S phylogenies.

Within Clade B, *A. punctura* and the Azorean endemic *A. angioyi* were positioned as sister species in the COI dataset, whereas the endemic taxon was depicted as closely related to the subclade containing *A. discors, A. lanciae, A. lineata* and *A. scabra* in the 28S phylogeny. Some controversy is attributed to the classification of the latter four species as *Alvania*, as they form a lineage distantly related to other *Alvania* spp. Although no formal attempt has been made to reclassify it, we support Criscione et al.'s (2017) suggestion of revision as *Alvaniella* Sacco, 1895 or *Alvanolira* Nordsieck, 1972. On the other hand, *Crisilla postrema*, currently found in relatively shallow water of the Azores and Madeira (Cordeiro et al. 2015), was positioned as sister species to the Azorean endemic *A. angioyi*. The type species of *Crisilla*—*C. semistriata* (Montagu, 1808)—was considered a subgenus of *Alvania* for decades due to morphological similarities (Ponder 1984; Gofas 1990). Although poorly supported in the phylogenetic analyses, there are affinities between *Crisilla postrema* and *Alvania angioyi* that might call for the need to review their classification. Clade C comprises *Pusillina* spp., *Rissoa* spp. and *Setia* spp., which is concordant with the relationships within Clade E retrieved by Criscione et al. (2017). The phylogenetic position of Set*ia ambigua* suggests it to be more closely related to *Rissoa* spp. than to the congeneric Azorean endemic *S. subvaricosa*. Morphological and molecular distinction of *S. ambigua* relatively to other *Setia* spp., displaying accentuated differences in the shell features, suggests the need of a taxonomic revision of this taxon, as already stated by Criscione et al. (2017).

Evolutionary history of the Azorean Rissoidae species

The species tree obtained in this study revealed that the lineage that later originated *Alvania angioyi, A. formicarum, A. mediolittoralis, A. sleursi, Cingula trifasciata, Crisilla postrema, Rissoa guernei,* and *Setia subvaricosa,* first started to diversify during the Early Miocene, ca. 17.52 Ma. However, this estimate is not reliable as the low support for this node (PP 56.8%) broadens the credibility interval to ages ranging from 12.82 to 23.17 Ma. Rissoidae-like organisms are known since the Lower Jurassic (Conti et al. 1993; Criscione and Ponder 2013; Criscione et al. 2017) and some genera were already diversified in the Central Paratethys during the Miocene (Kowalke and Harzhauser 2004), thus, despite not being able to assertively define an age for this divergence event, we believe it is possible that the divergence of these lineages occurred sometime within the estimated range, as it agrees with the fossil record of rissoids worldwide. The concordance in the typology of the species tree inferred (Fig. 5) and the gene trees (Figs. 3, 4), all revealing a clade comprising *A. sleursi*, *A. mediolittoralis* and *A. formicarum* with an earlier divergence of the subtidal/circalittoral *A. sleursi*, increases the confidence in its phylogenetic position among the rissoids.

Different confidence intervals were retrieved for the several splits inferred. Large credibility intervals (e.g., Clade C) obscure the estimation of a trustworthy age for the split event analyzed, turning inferences regarding the taxonomic position and temporal range of existence of the species involved quite dubious. Even when calibrated, older splits were associated with lower confidence levels. This scenario might reflect a discrepancy between the placement of the calibration point and its true position in the phylogeny, as the further the calibration point is assigned relative to the node of interest, the greater the uncertainty associated with the estimations (Forest 2009). As this work constitutes a phylogenetic study, split events reflect the timing of divergence of the lineages that later originated modern assemblages and not the time of arrival of rissoids or their ancestors to the Azores Archipelago's vicinity.

Nevertheless, the divergence dates and relationships proposed by the species' tree obtained seem reasonable based on the worldwide fossil record of the family Rissoidae, the geological history of the Azores Archipelago, and the evolutionary history revealed by the main patterns retrieved from mtDNA COI and nDNA 28S phylogenies (see Figs. 3, 4). As expected, the relationships inferred in the gene trees are, sometimes, not concordant among phylogenies (e.g., in the COI phylogeny *Setia subvaricosa* is not included in the clade comprising *Rissoa guernei*, but it is in the 28S gene tree). Nuclear and mitochondrial molecules have different evolutionary rates, which influence the evolutionary history inferred by a gene tree (Degnan and Rosenberg 2006). Particularly, due to its faster evolutionary rate, mitochondrial markers are expected to show higher saturation levels than the nuclear genes and will, therefore, obscure the estimations of older relationships by overestimating them. In salamanders, the overestimation of the divergence events by mitochondrial markers has reached levels of 3–10 times for recent events and 2–3 times for ancient ones, when compared with the nuclear markers (Zheng et al. 2011), whereas in a study of ray-fishes it ranged from 1 to 1.5 times (Hurley et al. 2007). Overestimation is not exclusive to mitochondrial markers, as it also occurs in nuclear genes, which require longer times to coalesce. Nevertheless, these biases are particularly misleading in cases of few calibration points and studies of deep phylogenies in higher taxonomic levels, where the time span is wide and the lineages are considerably old (Zheng et al. 2011).

Due to stochastic events during speciation, discordance between the topologies of the gene trees and the species tree is expected under the coalescent model, especially when assuming the most frequently observed gene tree typology as an estimate of the species tree (Degnan and Rosenberg 2006; Kubatko and Degnan 2007). Reasons pointed out as responsible for the discordance between gene trees and species trees include horizontal transfer, incomplete lineage sorting, and gene duplication and extinction (Maddison 1997). The larger the time between speciation events, associated with longer internal branches in the species tree, and the older the species origins reduce the chances for gene species tree discord. However, in the case of recent diversifications, the time elapsed is insufficient to grant the fixation of gene lineages by genetic drift, and discordance between gene and species tree will arise due to deep coalescence of gene lineages from non-closely related species (Knowles 2009). Nevertheless, when comparing the major patterns of the species tree with the ones inferred in the gene trees, the Clades A–C are found in the three phylogenies despite some differences in the relationships within. To avoid the incorrect use of gene trees as a proxy for species phylogeny and to obtain a more reliable estimation of the "true" topology of the rissoid's phylogeny, we opted to apply StarBEAST2 framework, which allows an integrated analysis of each marker's evolution and embeds each tree within a most likely single shared species tree. With this approach, we avoided the interference of biasing phenomena such as overestimation by mitochondrial markers and incomplete lineage sorting (Ogilvie et al. 2017). This constitutes the first time that a species tree is obtained for rissoids in the Azores, as well as the first approach to its evolution in an integrated perspective that includes clues about past environments, given by the palaeontological record.

Testing Ávila's (2013) biogeographical hypothesis

Throughout this study, Ávila's (2013) theory was tested by the reconstruction of the evolutionary history of eight Rissoidae species (Fig. 5), including the two species which observations encouraged the early formulation of the idea. Within the species tree inferred, Clade A must be emphasized to test this hypothesis, as it comprises *A. mediolittoralis* and *A. sleursi,* two np-species currently found in the Azores and Madeira archipelagos. Nowadays, *A. mediolittoralis* is commonly found in the intertidal algal turf, with much rarer reports from depths of up to 24 m (Ávila 2005, 2013; Cordeiro et al. 2015), whereas *A. sleursi* frequently occurs in deeper waters from 15 to 25 m down to 40 m depth (Ávila 2013; Cordeiro et al. 2015).

The inferred species tree indicates that the divergence between the lineages that originated *A. sleursi* and the shallow-water species occurred 4.8–4.13 Ma ago. By this time, the volcanic edifice of Santa Maria Island (Azores), which first emerged at ~6.01 Ma (Ramalho et al. 2017), is believed to have been under a period of waning volcanism, erosion, and subsidence leading to the formation of a truncated, top-flat seamount (Ramalho et al. 2017). Thus, during this period, Santa Maria's edifice became a shallow-water sandy shoal where volcanic activity was rare, mostly submarine (Ramalho et al. 2017). A wide variety of marine environments existed in this guyot, yielding diverse organisms typical from open-water marine environments (see Uchman et al. 2018 and references therein) and benthic species from shallower waters, including rissoids and other molluscs (Ávila et al. 2015).

According to Ávila's (2013) hypothesis, subtidal/circalittoral species would be less prone to be rafted than intertidal ones, which would be expected to have also wider geographical distributions. Despite these predictions, both species are currently found in the same geographical areas—they are common species in all the islands of the Azores and are reported for the coastal areas of the Madeira Islands (Cordeiro et al. 2015). The palaeontological record of both archipelagos holds valuable clues to interpret the results of the phylogenetic approach used in this study and better understand the current distribution of the non-planktotrophic *A. mediolittoralis* and *A. sleursi.*

The finding of fossilized specimens of *A. sleursi* (Meireles et al. 2013) on Pliocene sediments (4.78 ± 0.135 to 4.13 ± 0.19 Ma; Ramalho et al. 2017) of Santa Maria Island suggests that the divergence of the lineages encompassed within Clade A occurred earlier than the fossilization event, and that, by that time, viable populations of *A. sleursi* were already established in the Azores. As all the species assigned to Clade A are endemic to one (the Azores) or two (the Azores and Madeira) Atlantic Archipelagos and are not found in fossiliferous outcrops outside of the Azores, it is plausible to assume that they do not constitute relicts of a former wider distribution. Therefore, and considering the climatic and oceanographic conditions of the last 5 Ma, we hypothesize that this divergence occurred in the Azores vicinities, where the ancient lineage diversified in, at least, three species—*A. sleursi, A. mediolittoralis* and *A. formicarum*—that established in the Azores and have only recently dispersed to Madeira. The finding of *A. sleursi* representatives also in MIS 5e sediments, with an age of 130–116 ka (Ávila 2005; Cordeiro and Ávila 2015; Ávila et al. 2015, 2016) suggests that this subtidal/circalittoral rissoid has been inhabiting this region for a considerable time, where it supposedly established long before *A. mediolittoralis* speciated. The posterior divergence of lineages that originated *A. formicarum* and *A. mediolittoralis* is estimated to have taken place ca. 360 ka ago, during the Pleistocene. Considering that representatives of both species are found in MIS 5e highstand deposits of Santa Maria Island (Ávila et al. 2009, 2015, 2016), the age estimated for the split event leading to their speciation can be accepted. The divergence is also believed to have happened in the Azores Archipelago, as *A. formicarum* is endemic to this region, with no knowledge of previous wider distribution (Cordeiro et al. 2015).

Despite the older age of Porto Santo Island, its Miocene fossiliferous outcrops, mainly composed by massive coral reefs, rhodoliths, echinoderms and some molluscs (Mata et al. 2013), are similar in taxonomic composition to Santa Maria's Pliocene outcrops (Ávila et al. 2016 and references therein), with the exception of the massive coral reefs, which are only represented at Santa Maria by small, solitary corals. Although efforts to evaluate the palaeontological record of Madeira Archipelago have been made, there are still no reports of fossilized *A. mediolittoralis* and *A. sleursi* in Madeiran region (Gerber et al. 1989; Ávila et al. 2015). This absence suggests that these molluscs were still not present in the latter archipelago during the Pliocene or Pleistocene, when they had already established in the Azores (Ávila et al. 2015, 2016), having reached the Madeira Archipelago later. Therefore, based on the evolutionary history inferred and data provided by ecological and palaeontological observations, we propose early speciation within the Azores Archipelago's vicinity, followed by their dispersal to Madeira.

From an evolutionary perspective, this phylogenetic approach allowed us to test if the subtidal/circalittoral species was indeed longer lived than the intertidal species. Clade A revealed that the oldest split event was the one leading to the divergence of *A. sleursi*, recently followed by the speciation of the intertidal *A. mediolittoralis* during the Pleistocene. The early speciation of the subtidal/circalittoral species provided enough time for individuals to engage dispersal, even though less prone to do so, settle and successfully colonize another archipelago, and thus, to widen its geographical range. For the first time, Ávila's theoretical biogeographical hypothesis, proposed to explain the simultaneous existence of two congeneric np-species with different bathymetric ranges in two nearby archipelagos, is hereby corroborated with genetic data from rissoids, the marine mollusc gastropods that inspired its formulation. Observations in distinct scientific fields—phylogenetics, paleontology and ecology—contribute to increase the robustness of its theory. Further studies involving other marine invertebrates with similar biogeographical patterns should be addressed to further sustain Ávila's (2013) hypothesis.

Conclusions

Out of 266 shallow-water (< 50 m depth) rissoid species reported for the entire North Atlantic and adjacent seas, a total of 12 rissoid species are reported simultaneously and exclusively for three archipelagos (Madeira, Selvagens, and the Canaries). This shows that the dispersal of formerly endemic species to one of the archipelagos, albeit infrequent, is probably a more common event than previously thought. A consequence of this work is the prediction that, for marine species having similar ecological traits (ecological zonation depths, mode of larval development, habitat), those having wider geographical ranges are expected to be older (in an evolutionary sense) in comparison with species that occur in only two, or are restricted to just one, archipelagos.

Extending and testing of this biogeographical hypothesis to other marine phyla will impact our current knowledge of inter-archipelagic patterns and processes of dispersal, as well as the evolutionary times of speciation of shallow-water marine organisms living in remote volcanic oceanic islands, thus providing a more complete and robust understanding for recent marine island biogeography theory (see Ávila et al. 2018, 2019).

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Data availability All the mitochondrial COI and nuclear 28S sequences analyzed during the current study are deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). COI sequences are deposited in GenBank with the accession numbers: MG652373-8 (*Alvania angioyi*), MG652379-80 (*A. formicarum*), MG652381-5 (*A. mediolittoralis*), MG652386-7 (*A. sleursi*), MG652395-407 (*Cingula trifasciata*), MG652392-4 (*Crisilla postrema*), MG652408-13 and MG652419-21 (*Rissoa guernei*), MG652414-8 and MG652422 (*R.* cf. *guernei*), MG652429-31 (*Setia subvaricosa*), and MG652432-36 (*Pisinna glabrata*). Sequences of 28S rRNA gene are available under the following accession numbers: MG663173-8 (*A. angioyi*), MG663179 (*A. formicarum*), MG663180-1 (*A. mediolittoralis*), MG663182-3 (*A. sleursi*), MG663189-96 (*C. trifasciata*), MG663185-8 (*C. postrema*), MG663197-203, MG663207 and MG663209 (*R. guernei*), MG663204-6 and MG663208 (*R.* cf. *guernei*), MG663215-6 (*S. subvaricosa*), and MH047302-4 (*P. glabrata*).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The sequences used in the molecular phylogenies of COI and 28S were mainly retrieved from the GenBank database. The new sequences were obtained from specimens deposited in the Marine Molluscs Reference Collection of the Department of Biology of the University of the Azores (DBUA). Sampling was not performed for this work. All applicable national and/or institutional guidelines for the use of collection material were followed. Only invertebrates were used in this study.

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