Journal of
Molluscan Studies

The Malacological Society of London

Journal of Molluscan Studies (2024) **90**: eyae020. https://doi.org/10.1093/mollus/eyae020
Published online 3 July 2024

Featured Article

Mitogenomic phylogeny of Nassariidae (Neogastropoda: Buccinoidea)

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urn:lsid:zoobank.org:pub:F975B39D-75D2-45C9-8CD1-FF3E6CB3A250

(Received 12 October 2023; editorial decision 15 May 2024)

ABSTRACT

Mud snails (family Nassariidae) represent a highly diversified lineage within the superfamily Buccinoidea. Recent molecular phylogenies contradicted in some instances the traditional nassariid classification and revealed important levels of homoplasy in phenotypic characters. In order to clarify the boundaries of the family Nassariidae, as well as to inquire on the diversification of the cosmopolitan Nassariinae, a robust phylogenetic framework is needed. Here, the near-complete mitogenomes of 31 species representing almost all lineages of Nassariidae plus several buccinoid outgroups were sequenced. All mitogenomes of buccinoids shared the same gene order, which is identical to the consensus reported for caenogastropods. The monophyly of Nassariidae as previously defined was not confirmed. The reconstructed phylogeny revealed distant relationships between the genera *Cyllene*, *Anentome*, *Tomlinia*, *Engoniophos*, *Phos* and *Antillophos* and the majority of nassariids, represented by Nassariinae + *Bullia*. Within Nassariinae, a robust phylogeny, which recognized a total of seven regional groups, was reconstructed. The West Atlantic/Mediterranean genus *Tritia* was divided into three clades. The biogeographical analysis together with the inferred chronogram suggested that Nassariinae might have originated during the late Paleocene in the Indo-Pacific region. Subsequent climate change and continental drift events triggered diversification within the subfamily, leading to the worldwide distribution of current genera.

INTRODUCTION

Mud snails or dog whelks (family Nassariidae Iredale, 1916) belong to the superfamily Buccinoidea and consist of more than 600 extant species (MolluscaBase, 2022). Mud snails have fusiform, slender to broad ovate shells, of small to medium size, moderately sculptured from cancellate to smooth surface with a shortened siphonal canal (Cernohorsky, 1984). Distributed worldwide and with a greater diversity of taxa in the Indo-Pacific region (Cernohorsky, 1984), nassariids are mostly found in shallow marine soft bottoms, except few species that live in mesohaline and even in freshwater conditions (Kantor & Kilburn, 2001; Strong *et al.*, 2008; Strong, Galindo & Kantor, 2017; Neiber & Glaubrecht, 2019).

In the past century, classifications of the family Nassariidae were based solely on shell and radula characters. The first thorough classification based on the shape of the shell columella divided Nassariidae into three subfamilies: Dorsaninae, Cylleninae and the cosmopolitan Nassariinae (Cossmann, 1901). This division of Nassariidae into three subfamilies was accepted for many years (Cernohorsky, 1984) until several genera previously included in Dorsaninae were removed to establish the new subfamily Bulliinae (Allmon, 1990). However, due to the putative homoplasy of many morphological characters, the internal classification of Nassariidae and the accepted boundary between Nassariidae and Buccinidae were deemed to need a thorough revision (Haasl, 2000; Kantor, 2003).

More recently, the application of molecular markers helped clarifying the phylogenetic relationships of Nassariidae. Using the DNA sequences of three mitochondrial (*cox1*, *rrnL* and *rrnS*) and two nuclear (*28S rRNA* and *H3*) fragments, Galindo *et al.* (2016) reconstructed the most comprehensive phylogeny of Nassariidae to date (including >200 species) and revisited nassariid systematics. At the family and subfamily levels, several genera (*Antillophos*, *Engoniophos*, *Phos*, *Nassaria*, *Tomlinia* and *Anentome*), which were previously considered as members of the closely related family Buccinidae, were reassigned into Nassariidae, leading to the classification of this later family into seven subfamilies (Photinae, Dorsaninae, Cylleninae, Bulliinae, Buccinanopsinae, Anentominae and Nassariinae; Bouchet *et al.*, 2017). Within the cosmopolitan

Nassariinae, five genera were redefined corresponding to five clades in the phylogeny with distinct geographical distributions (Galindo *et al.*, 2016), including the Indo-Pacific genera *Nassarius* (Yang *et al.*, 2019) and *Reticunassa* (the '*pauper*-complex' *sensu*, Kool & Dekker, 2006, 2007; Galindo, Kool & Dekker, 2017); the mostly East Atlantic-Mediterranean genus *Tritia* (Yang *et al.*, 2021); the (East and West) African genus *Naytia*; and the Caribbean-Panamanian genus *Phrontis*.

Later, Strong *et al.* (2017) revised the systematics of genus *Anentome* (Anentominae) and considered that this freshwater taxon was a complex of at least four molecularly and morphologically distinct species. Moreover, the brackish water genus *Nassodonta* (Anentominae) was found to be polyphyletic (Neiber & Glaubrecht, 2019), with *Nassodonta insignis* (H. Adams, 1867), type species of the genus, from the southwestern coastal region of India (Jayachandran *et al.*, 2018) clustering with *Tritia reticulata* (Linnaeus, 1758), instead of with the congeneric *Nassodonta dorri* (Wattebled, 1886) from Vietnam, which was hence reassigned to the new genus *Oligohalinophila* (Neiber & Glaubrecht, 2019), i.e. *Oligohalinophila dorri* (Wattebled, 1886). Furthermore, the mitogenomic phylogeny of the genus *Tritia* by Yang *et al.* (2021) recovered the Northwest Atlantic species *Ilyanassa obsoleta* (Say, 1822) as sister to the mostly East Atlantic/Mediterranean species within the genus, prompting the reinstatement of the genus *Ilyanassa*. Finally, a phylogeny of Buccinoidea by Kantor *et al.* (2022) revealed a distant relationship between genera *Tomlinia* + *Nassaria* and genus *Cyllene*, and hence the former two genera were removed from Cylleninae to the new subfamily Tomliniinae. In addition, Buccinanopsinae was ranked to Buccinanopsidae, since it was not recovered within Nassariidae (Kantor *et al.*, 2022).

The above-mentioned studies revealed on the one hand that traditional nassariid taxonomy based on morphological traits was rather inconsistent with molecular phylogenies (Pastorino & Simone, 2021), but also on the other hand that mitochondrial and nuclear partial nucleotide sequences failed to resolve with strong statistical support the deeper nodes within Buccinoidea and Nassariidae phylogenies, often rendering contradicting results. For instance, Strong *et al.* (2017) recovered the genus *Anentome* sister to the genus *Nassodonta*, whereas according to Galindo *et al.* (2016) these two genera were two isolated offshoots within Nassariidae (but note that both alternative phylogenetic hypotheses received low support values in each study). Similarly, in the phylogeny of Buccinoidea by Kantor *et al.* (2022), the family Columbellidae clustered within the clade formed by subfamilies Cylleninae and Tomliniinae, thus rendering Nassariidae paraphyletic. In short, the various flaws found in previous molecular studies indicate that phylogenetic relationships of Nassariidae remain rather unresolved and still plagued by uncertainty.

Complete mitochondrial (mt) genomes have proven to be useful molecular markers for reconstructing relatively highly resolved phylogenies at the family and superfamily levels, and have been already applied to different buccinoid groups within Nassariidae (Yang *et al.*, 2019, 2021) and Buccinidae (Vaux *et al.*, 2017). At present, the complete or near-complete mitogenomes of 39 nassariid species, restricted to subfamilies Nassariinae and Anentominae, are available in GenBank. Here, we sequenced the nearly complete mitochondrial (mt) genomes of another 31 species, representing six out of the seven subfamilies of Nassariidae proposed by Kantor *et al.* (2022) (except for Dorsaninae, today restricted to the single species *Dorsanum miran* (Bruguière, 1789) from West Africa), as well as other buccinoid outgroups. We aimed at (1) delimiting Nassariidae within Buccinoidea and confirming the phylogenetic placements of major lineages within this family; (2) improving the resolution of phylogenetic relationships within the cosmopolitan Nassariinae; and (3) dating major cladogenetic events involving geographical structuring of genera within Nassariinae.

MATERIAL AND METHODS

Samples and DNA extraction

The complete list of specimens analysed in the present study, including their sampling sites and museum vouchers, is shown in Supplementary Material Table S1. All samples were stored in 100% ethanol and total genomic DNA was isolated from about 25 mg of foot tissue using the DNeasy Blood & Tissue Kit (Qiagen, Germany).

PCR amplification and sequencing of mt genomes

Nearly complete (without the control region) mt genomes were amplified through long PCRs Supplementary Material Table S2), which were designed in a previous study (Yang *et al.*, 2021). The long PCR reactions contained 2.5 µl of 10 × buffer (Mg^{2+} plus), 3 µl of dNTPs (2.5 mM), 0.5 µl of each primer (10 µM), 0.8 µl of template DNA (25–40 ng/µl), 0.2 µl of TaKaRa LA Taq DNA polymerase (5 U/µl), 0.2 µl of BSA (10 mg/ml) and DEPC (diethyl-pyrocarbonate) water up to 25 µl. The following PCR conditions were used: initial denaturing step at 94 °C for 60 s; 45 cycles of denaturing at 98 °C for 10 s, annealing at 53–56 °C for 30 s and extension at 68 °C for 60 s per kb; and a final extension step at 68 °C for 10 min. Long PCR products were purified by ethanol precipitation, and fragments from the same mitogenome were pooled together in equimolar concentrations for high-throughput sequencing. For each mitogenome, a separate indexed library was constructed using the NEXTERA XT DNA library prep kit (Illumina, San Diego, CA, USA) and run (together with indexed libraries from other sequencing projects of unrelated species) in an Illumina MiSeq platform (2 × 150 paired-end) at AllGenetics (A Coruña, Spain) and NIMGenetics (Madrid, Spain).

Sequence gaps in some mitogenomes were filled out through standard PCRs, which were carried out in a total volume of 25 µl with 1 µl of template DNA (25–40 ng/µl), 2.5 µl of 10 × buffer (Mg^{2+} plus), 0.5 µl of dNTPs (2.5 mM), 0.5 µl of each primer (10 µM), 0.2 µl of Taq DNA polymerase (5 U/µl), 0.25 µl of BSA (10 mg/ml) and 19.55 µl of DEPC water. The PCR conditions were as follows: an initial denaturing step at 94 °C for 5 min; 40 cycles of denaturing at 94 °C for 60 s, annealing at 39–48 °C for 60 s and extension at 72 °C for 90 s; and a final extension step at 72 °C for 10 min. Standard PCR products were purified by ethanol precipitation, and Sanger sequenced at Macrogen (Seoul, Korea). Information on standard PCR primers is shown in Supplementary Material Table S2.

Mitogenome assembly and annotation

The Sanger sequences were assembled using Sequencher 5.0.1. For some of the specimens, the already reported *cox1* sequences were downloaded from GenBank (Supplementary Material Table S2). For Illumina sequence data, the reads corresponding to different individuals were sorted by the corresponding library indices. The reads corresponding to each mt genome were cleaned following Abalde *et al.* (2017) and assembled using Geneious Prime 2019.0.3 (Kearse *et al.*, 2012). The mitogenomes were constructed by repeatedly mapping the clean reads (setting a minimum identity of 99%) to contigs that elongated from the Sanger fragments of the same mitogenome.

The newly determined mt genomes were annotated using Geneious Prime 2019.0.3. Annotations of the 13 protein-coding genes (PCGs) were defined by setting a limit of 75% nucleotide identity to previously published nassariid mitogenomes (Supplementary Material Table S1), and further corroborated using the MITOS Webserver (Bernt *et al.*, 2013) with the invertebrate

mitochondrial genetic code. The transfer RNA (tRNA) genes were identified using tRNA scan-SE 1.21 (Schattner, Brooks & LOWE, 2005) and ARWEN (Laslett & Canbäck, 2008). The ribosomal RNA (rRNA) genes were identified by comparison with other nassariid mt genomes, and their boundaries were assumed to be between the adjacent genes.

Sequence alignment

The newly sequenced mitogenomes were aligned along with those of other buccinoids available in GenBank (Supplementary Material Table S1). Two datasets were constructed and analysed following Uribe, Puillandre & Zardoya (2017). The first dataset (hereafter referred to as the Nassariidae dataset) was aimed at testing the monophyly of Nassariidae and included main lineages within the family as well as closely related members of buccinoid families. The species *Crassicantharus noumeensis* (Crosse, 1870) (family Dolicholatiridae after Kantor *et al.*, 2022) and *Belomitra viridis* (Okutani, 1966) (family Belomitridae) were used as an outgroup, following Kantor *et al.* (2022). The Nassariidae dataset was comprised of the deduced amino acid sequences of the 13 mt PCGs and the nucleotide sequences of the 2 rRNA genes. The second dataset (hereafter referred to as the Nassariinae dataset) was aimed at reconstructing the internal phylogenetic relationships within Nassariinae, using five species of the genus *Bullia* (subfamily Bulliinae) as outgroup taxa. This dataset included the nucleotide sequences of the 13 mt PCGs and the 2 rRNA genes.

For each dataset, the deduced amino acid sequences of the 13 PCGs were aligned separately and used to guide the alignment of the corresponding nucleotide sequences with Translator X (Abascal, Zardoya & Telford, 2010), using the invertebrate mitochondrial genetic code. The nucleotide sequences of the rRNA genes were aligned separately using MAFFT v7 (Katoh & Standley, 2013) with default parameters. Ambiguously aligned positions were removed using Gblocks v. 0.91b (Castresana, 2000) with the following settings: minimum sequence for flanking positions: 85%; maximum contiguous nonconserved positions: 8; minimum block length: 10; gaps in final blocks: no. Finally, the different single-gene alignments were concatenated into a single dataset in Geneious Prime 2019.0.3. Sequences were converted into different formats for further analyses using DAMBE5 (Xia, 2013).

Phylogenetic analyses

Phylogenetic trees were reconstructed under maximum likelihood (ML; Felsenstein, 1981) and Bayesian inference (BI; Huelsenbeck & Ronquist, 2001) using the CIPRES Science Gateway v.3.3 (Miller, Pfeiffer & Schwartz, 2010). ML analyses were carried out using RAxML-HPC2 on XSEDE (Stamatakis, 2006) with the rapid bootstrap algorithm and 1000 replicates. BI analyses were conducted with MrBayes v. 3.2.6 on XSEDE (Ronquist & Huelsenbeck, 2003), running four simultaneous Monte Carlo Markov chains for 10,000,000 generations, sampling every 1,000 generations, and discarding the first 25% generations as burn-in. Two independent BI runs were performed to increase the chance of adequate mixing of the Markov chains and of detecting failure to converge, as determined by using Tracer v. 1.6. The effective sample size of all parameters was more than 200. The resulting phylogenetic trees were visualized in FigTree v. 1.4.2.

The best partition schemes and best-fit substitution models for the two datasets were determined using PartitionFinder 2 (Lanfear *et al.*, 2017), under the Bayesian information criterion (Schwarz, 1978). For PCGs of the Nassariidae dataset (analysed at the amino acid level) and of the Nassariinae dataset (analysed at the nucleotide level), the partitions tested were: all genes combined; all genes separated (except *atp6-atp8* and *nad4-nad4L*); and genes grouped by enzymatic complexes (*atp*, *cob*, *cox* and *nad*). Additionally, the three partition schemes were tested considering separately the three codon positions. The rRNA genes were analysed with two different schemes (genes grouped or separated). The best best-fit substitution models of the two datasets are provided in Supplementary Material Table S3.

Reconstruction of ancestral geographical distributions

Biogeographical inferences were performed with an ancestral character state reconstruction in Mesquite v. 3.6.1 (Maddison & Maddison, 2018) using the tracing character history option under ML and mapping onto the topology inferred by the ML analysis. The distribution range of each species was derived from previous reviews (Cernohorsky, 1975, 1984; Allmon, 1990; Galindo *et al.*, 2016; Gofas *et al.*, 2017, among others).

Estimation of divergence times

BEAST v. 1.10.4 (Drummond & Rambaut, 2007) was used to perform a Bayesian estimation of divergence times only among genera within Nassariinae (based on the Nassariinae dataset) given that the other lineages previously considered as part of the Nassariidae were distantly recovered in the buccinoid tree (see Results). An uncorrelated relaxed molecular clock was used to infer branch lengths and node ages. The tree topology was fixed using the one recovered by the BI analysis. For the clock model, the lognormal relaxed-clock model was selected, which allows rates to vary among branches without any *a priori* assumption of autocorrelation between adjacent branches. For the tree prior, a Yule process of speciation was employed. The partitions selected by PartitionFinder 2 (see above) were applied. The Markov chain was run twice for 100 million generations, sampling every 10,000 generations and discarding the first 10 million as burn-in, according to the convergence of chains checked with Tracer. The effective sample size of all the parameters was above 200.

The posterior distribution of the estimated divergence times was obtained by specifying two calibration points, which were based on fossil records as priors for divergence times of the corresponding splits. The first calibration point was set at the origin of *Tritia*. A lognormal distribution was applied, with the minimum of 23 Ma and a 95% upper limit of 28.4 Ma (offset: 23; mean: 1.9; standard deviation: 1.9) based on the species *Nassarius (Hinia) pygmaea* (von Schlothein, 1820) from Germany (Albright *et al.*, 2019) and *Nassarius (Hinia) schlotheimi* (Beyrich, 1854) from Hungary (Báldi, 1973), both from the Upper Oligocene (Chattian; 28.4–23.0 Ma), the oldest Nassariinae fossils in Europe. A second calibration point was set at the root of the tree. A lognormal distribution was applied, with the minimum of 55.8 Ma and a 95% upper limit of 58.7 Ma (offset: 55.8; mean: 1.0; standard deviation: 1.0) based on the oldest known Nassariinae fossil, which is *Buccitriton sagenum* (Conrad, 1833) from the Paleocene (Thanetian; 58.7–55.8 Ma) of Lousiana (Glawe, Anderson, & Bell, 2014).

RESULTS

Sequencing, assembly and genome organization

The number of reads, mean coverage, length and accession number in GenBank of each newly sequenced mitogenome are provided in Supplementary Material Table S1. The mt genomes of *Bullia* sp. and *Cyllene parvula* (Bozzetti, 2014) (family Nassariidae) received the minimum (23,590) and maximum (2,142,544) reads, respectively. Those of *Peristernia marquesana* (A. Adams, 1855) (family Fasciolariidae) and *Cyllene parvula* received the minimum (20×) and maximum (16,095×) coverage, respectively (Supplementary Material Table S1).

All mitogenomes had 13 PCGs, 2 rRNA and 22 tRNA genes (but note that all lacked the sequences of the *trnF* gene, the control region and the start of the *cox3* gene because the corresponding fragment could not be PCR amplified; see annotation of each mt genome in Supplementary Material Table S4). They all shared the same consensus genome organization described for Caenogastropoda (Osca *et al.*, 2015), with most genes encoded by the major strand and a cluster of tRNA genes (*trnM*, *trnY*, *trnC*, *trnW*, *trnQ*, *trnG* and *trnE*) and the *trnT* gene encoded by the minor strand.

Phylogenetic relationships of Nassariidae within Buccinoidea

The monophyly and phylogenetic relationships of Nassariidae within Buccinoidea (Fig. 1) were analysed based on the Nassariidae dataset using probabilistic methods and *Crassicantharus noumeensis* (Dolicholatiridae), sister to *Belomitra viridis* (Belomitridae) as an outgroup. The final matrix was 5,120 positions in length. The best partition scheme for the amino acid sequences of PCGs was combining genes by subunits, whereas that for the nucleotide sequences of rRNA genes was combining *rrnL* and *rrnS* genes. Both ML ($-lnL$ = 86,830.23) and BI ($-lnL$ = 87,375.33 for run 1; $-lnL$ = 87,376.87 for run 2) arrived at similar topologies that only differed in some internal relationships within Buccinidae and Nassariinae (Fig. 1). In the reconstructed phylogeny, the family Nassariidae was not monophyletic because some of the taxa previously considered as nassariids were recovered in five distant lineages in the tree: (1) the freshwater *Anentome* sp. was recovered sister to a clade including (2) *Cyllene* plus the remaining buccinoids to the exclusion of the family Belomitridae (the earliest offshoot in the ingroup), with maximum statistical support; (3) *Tomlinia frausseni* Thach, 2014, was recovered as sister to *Nassaria* sp. with maximum support, and both grouped together with the family Columbellidae (only strongly supported by BI; Fig. 1); and (4) the highly supported group formed by *Engoniophos unicinctus* (Say, 1826) + (*Phos hirasei* G. B. Sowerby III, 1913 + (*Phos* cf. *roseatus* Hinds, 1844, and *Antillophos chazaliei* (Dautzenberg, 1900))) clustered with a clade including (5) *Buccinanops* sister to the family Buccinidae with a maximum Bayesian posterior probability (BPP) value and moderately high bootstrap proportion value of 77%, and both groups with the cosmopolitan Nassariinae but this later node received low statistical support (Fig. 1).

Phylogenetic relationships of Nassariinae

In order to further clarify phylogenetic relationships within Nassariinae, the Nassariinae dataset was reconstructed and analysed with probabilistic methods using the genus *Bullia* as an outgroup (Fig. 2). The final matrix was 12,587 positions in length. The best partition schemes for the PCGs and rRNA genes analysed at the nucleotide level were combining genes by subunits but analysing each codon position separately and combining *rrnL* and *rrnS* genes, respectively (Supplementary Material Table S3). Both ML ($-lnL$ = 175,595.22) and BI ($-lnL$ = 172,291.41 for run 1; $-lnL$ = 172,295.27 for run 2) arrived at almost identical topologies, only differing in the internal relationships within *Nassarius* (Fig. 2). The reconstructed phylogeny divided Nassariinae into seven lineages: (1) *Nassarius jasckso-nianus* (Quoy & Gaimard, 1833) and *Nassarius* sp.; (2) *Reticunassa*; (3) *Naytia* (including *Tritia vaucheri* (Pallary, 1906)); (4) *Phrontis*; (5) *Nassarius*; (6) *Ilyanassa*; and (7) *Tritia* (Fig. 2).

Biogeographical origin and divergence times

The main lineages (i.e. genera) within Nassariinae showed distinct geographical distributions. An ancestral reconstruction analysis under ML inferred an Indo-Pacific origin for the subfamily (Fig. 3). Major cladogenetic events within Nassariinae were dated using an uncorrelated relaxed molecular clock, which was calibrated using two fossils. The origin of the subfamily Nassariinae was dated in the Paleocene at a mean of 56.5 (58.2–55.8, credible interval) Ma. Within Nassariinae, the first divergence was dated in the Eocene/Oligocene transition at 37.3 (43.0–32.8) Ma, separating the Indo-Pacific groups (*Nassarius jacksonianus* and *Nassarius* sp.) + *Reticunassa* from the remaining nassariids. The divergences separating the western African genus *Naytia*, the Caribbean-Panamic *Phrontis* and the Indo-Pacific group *Nassarius* plus *Tritia* and *Ilyanassa* were dated at 32.0 (36.3–28.6), 29.8 (33.7–26.5) and 24.2 (26.8–23.2) Ma, respectively. Further, the divergence between the Northwest Atlantic *Ilyanassa* and the Northeast Atlantic/Mediterranean genus *Tritia* was dated at 22.1 (24.8–20.0) Ma. Finally, the main diversification events within the above-mentioned genera occurred during the Miocene (Fig. 4).

DISCUSSION

Phylogenetic relationships of Nassariidae within Buccinoidea

The recent classification of Buccinoidea by Kantor *et al.* (2022) confirmed the monophyly of most families except for Buccinidae, which was completely revised and redefined, and for Nassariidae, which remained open to further revision. Even though some buccinid-like species were reassigned to Nassariidae following the phylogenies of Galindo *et al.* (2016) and Kantor *et al.* (2022), there is still no morphological synapomorphy to define the boundary of this family.

Among the seven subfamilies recently assigned to Nassariidae (Kantor *et al.*, 2022), all but Dorsaninae were included in our phylogeny. In contrast to the most comprehensive phylogenetic study to date based on partial gene sequences (Galindo *et al.*, 2016), the monophyly of Nassariidae as currently proposed (Kantor *et al.*, 2022; WoRMS, 2024) was not recovered here, as species of the subfamilies Photinae, Cylleninae, Anentominae and Tomliniinae fell outside the majority of nassariids, made up by subfamilies Nassariinae and Bulliinae. It should be noted that Kantor *et al.* (2022) recovered Nassariinae + Dorsaninae with strong support. Bulliinae and Anentominae were sister to that clade but without statistical support in the ML analysis. The next sister clade included Cylleninae, Tomliniinae and Columbellinae, but had no statistical support and rendered Nassariidae, as currently understood, paraphyletic (unless Columbellinae are considered one more subfamily of Nassariidae). Finally, the subfamily Photinae was recovered as the earliest offshoot of the Nassariidae.

In the phylogeny here reconstructed, the freshwater species *Anentome* sp. (Anentominae) was recovered as an early offshoot of the superfamily Buccinoidea (Fig. 1), contradicting previous studies that supported *Anentome* to be classified as part of Buccinidae (Strong *et al.*, 2008) or Nassariidae (Galindo *et al.*, 2016). The assassin snail *Anentome helena* (von dem Busch, 1847) was found to comprise a group of molecularly and morphologically distinct taxonomic species (including the *Anentome* sp. of our study) that were sister to the estuarine *Oligohalinophila dorri* (Strong *et al.*, 2017). The latter was previously classified in the genus *Nassodonta*, whereas according to Neiber & Glaubrecht (2019), the type species *Nassodonta insignis* (H. Adams, 1867) is more closely related to the Nassariinae than to the Anentominae, despite the low support values.

Represented by *Cyllene parvula* and *Cyllene lamarcki* (Cernohorsky, 1975) in our phylogenetic analysis, Cylleninae showed a distant relationship with Nassariinae + *Bullia* with moderate support values, and was placed in a second offshoot of the superfamily Buccinoidea after Anemontinae. The genus *Cyllene* is distributed in Western Africa and the Indo-Pacific Ocean, and was considered as the only genus of Cylleninae (Cernohorsky, 1984; Allmon, 1990) until Galindo *et al.* (2016) included *Trajana*, *Nassaria* and *Tomlinia* in this subfamily. However, the affinity of *Tomlinia* and *Cyllene* was not recovered here, hence contradicting both Galindo *et al.* (2016) and Strong *et al.* (2017), who recovered *Cyllene* as sister to

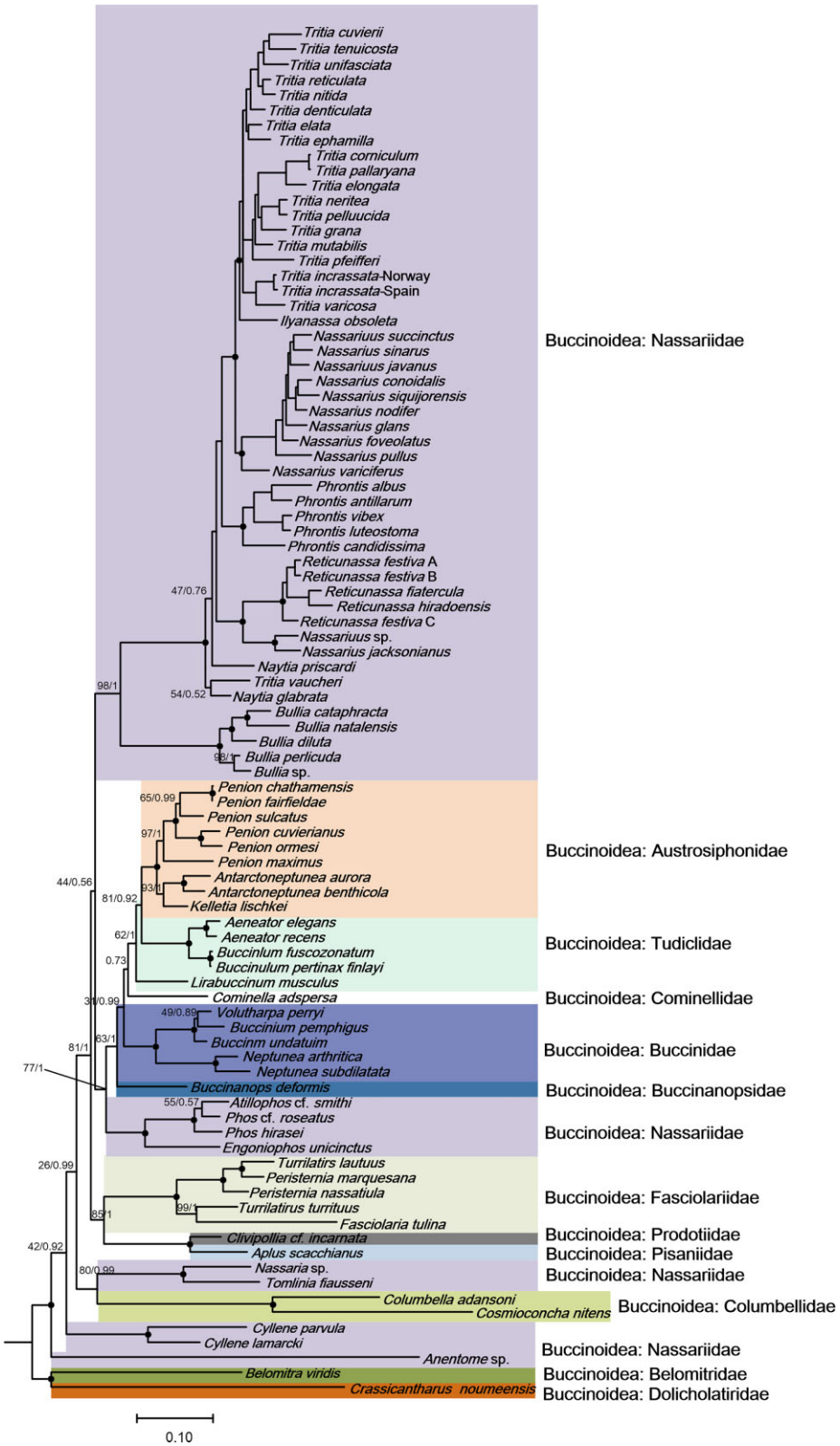

Figure 1. Phylogenetic relationships of Nassariidae within Buccinoidea based on the amino acid sequences of 13 mitochondrial protein-coding genes and the nucleotide sequences of 2 rRNAs. The reconstructed Bayesian inference phylogram using *Crassicantharus noumeensis* (family Dolicholatiridae) and *Belomitra viridis* (family Belomitridae) as an outgroup is shown. The names of the families are indicated. Numbers at nodes are statistical support values for maximum likelihood (bootstrap proportions)/Bayesian inference (Bayesian posterior probabilities) analyses. The black circle indicates nodes with maximal maximum likelihood and Bayesian inference support. The scale bar indicates substitutions/site.

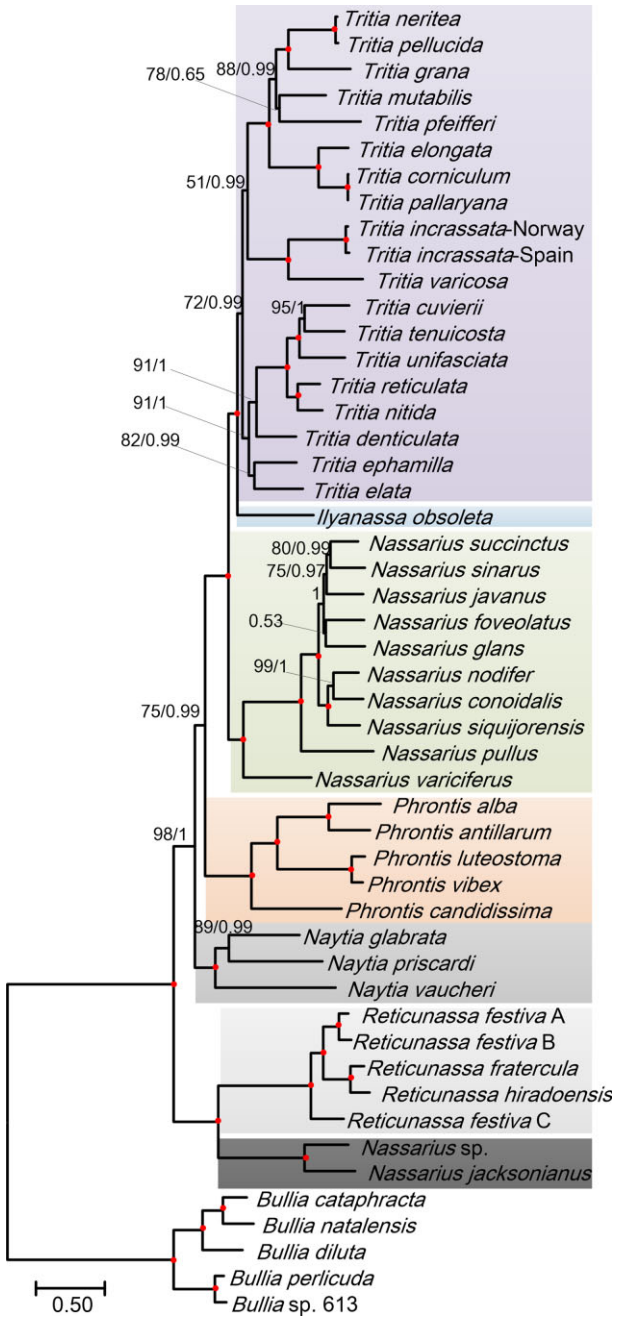

Figure 2. Phylogenetic relationships within Nassariinae based on the nucleotide sequences of 13 mitochondrial protein-coding genes and 2 rRNAs. The reconstructed Bayesian inference phylogram using genus *Bullia* (subfamily Bulliinae) as an outgroup is shown. The names of the genera are indicated. Numbers at nodes are statistical support values for maximum likelihood (bootstrap proportions)/Bayesian inference (Bayesian posterior probabilities). The circle indicates nodes with maximal maximum likelihood and Bayesian inference support. The scale bar indicates substitutions/site.

Photinae; Kantor *et al.* (2022), who found *Cyllene* to be sister to Columbellidae, and both these taxa to be sister to *Tomlinia* but without support; and deMaintenon & Strong (2022), who recovered Cylleninae as sister to Columbellidae, and both to be sister to Photinae but without support. Therefore, the position of Cylleninae varies in different molecular phylogenies (even in those using the same five partial gene sequences) and its systematic assignment remains uncertain.

The brackish water species *Tomlinia frausseni* (Thach, 2014), which is only found in estuaries in Vietnam (Thach, 2014), was recovered as sister to a representative of *Nassaria* (Fig. 1), which is a diversified marine genus distributed in the Indo-Pacific region (Cernohorsky, 1981). In recent phylogenies (Galindo *et al.*, 2016; Strong *et al.*, 2017; Kantor *et al.*, 2022), *Tomlinia frausseni* also clustered with *Nassaria*. However, while both genera were recovered sister to *Cyllene* by Galindo *et al.* (2016) and Strong *et al.* (2017), and hence included in Cylleninae by them, Kantor *et al.* (2022) removed *Nassaria*, *Trajana* and *Tomlinia* from Cylleninae to a newly established subfamily Tomliniinae. In our phylogenetic analysis, *Tomlinia* plus *Nassaria* were recovered sister to Columbellidae with strong support only in the BI analysis. The distant phylogenetic positions of *Anentome* sp. and *Tomlinia frausseni* indicate that at least these freshwater invasions happened independently within Buccinoidea, and pending elucidation of the phylogenetic position of other freshwater taxa from Southeast Asia (*Clea*, *Nassodonta* and *Oligohalinophila*) that were not included here.

The subfamily Photinae, represented by the monospecific genus *Engoniophos* (from the Caribbean), *Phos* (diversified in the Indo-Pacific) and *Antillophos* (present on both sides of the Atlantic but mostly diversified in the Western side) in our phylogenetic analysis, was grouped with Buccinidae (with maximum support value of BI and moderate support value of ML). This result contradicted the affinity of Photinae to Nassariidae obtained by Galindo *et al.* (2016) and Kantor *et al.* (2022), as well as the anatomy-based argument provided by Abbate *et al.* (2018) for *Engoniophos uncinatus* (Say, 1826), despite their noting some disparities with typical nassariids. In any case, Photinae was recovered by Galindo *et al.* (2016), Strong *et al.* (2017) and Kantor *et al.* (2022) as a first offshoot and sister to the remaining taxa they considered Nassariidae. Moreover, the phylogenetic position of Photinae within Nassariidae was already questioned by other phylogenetic studies. For example, in the phylogeny of Zou, Li & Kong (2011) based on five partial gene sequences, the genus *Phos* was recovered in a distant position from Nassariidae (there represented by the Indo-Pacific genus *Nassarius*) with well-supported values. Likewise, Waters (2009) included *Antillophos* within the subfamily Pisaniinae (Buccinidae). Furthermore, most members in Photinae (except for *Engoniophos*) are characterized by paucicuspid rachidian teeth, a character state shared with most species of Buccinidae, what is in agreement with the close relationship of Photinae and Buccinidae in our phylogeny.

The sister group relationship of Nassariinae and *Bullia* (Bulliinae) was highly supported in our phylogeny, and represents another inconsistency with previous studies that placed *Bullia* in a more distant position with respect to Nassariinae (Haasl, 2000; Galindo *et al.*, 2016; Kantor *et al.*, 2022). Based on morphological characteristics, the southern hemisphere *Bullia*, together with the South American *Buccinanops* and the West African *Dorsanum*, were traditionally included in the subfamily Dorsaninae (Cernohorsky, 1984). Moreover, recently, the new genus *Buccinastrum* was split from *Buccinanops* and also added to this group based on anatomical studies (Pastorino & Simone, 2021). However, according to reported molecular phylogenies (Galindo *et al.*, 2016; Kantor *et al.*, 2022; present study), the synapomorphies that define the group should be considered as a case of convergence (and thus homoplastic characters for phylogenetic reconstruction). Previous molecular phylogenies prompted the exclusion of *Buccinanops* and *Bullia* from Dorsaninae. Galindo *et al.* (2016) supported *Buccinanops* as a monogeneric subfamily (Buccinanopsinae) within Nassariidae, whereas Kantor *et al.* (2022) proposed *Buccinanops* as a separate family, Buccinanopsidae, only distantly related to Nassariidae. In our phylogeny, *Buccinanops* was recovered sister to Buccinidae (with moderate and maximal support in ML and BI analyses, respectively), supporting Buccinanopsidae as a separate family (Kantor *et al.*, 2022).

As a conclusion, we agree with Kantor *et al.* (2022) in considering Buccinanopsidae as a separate family. On the other hand, our

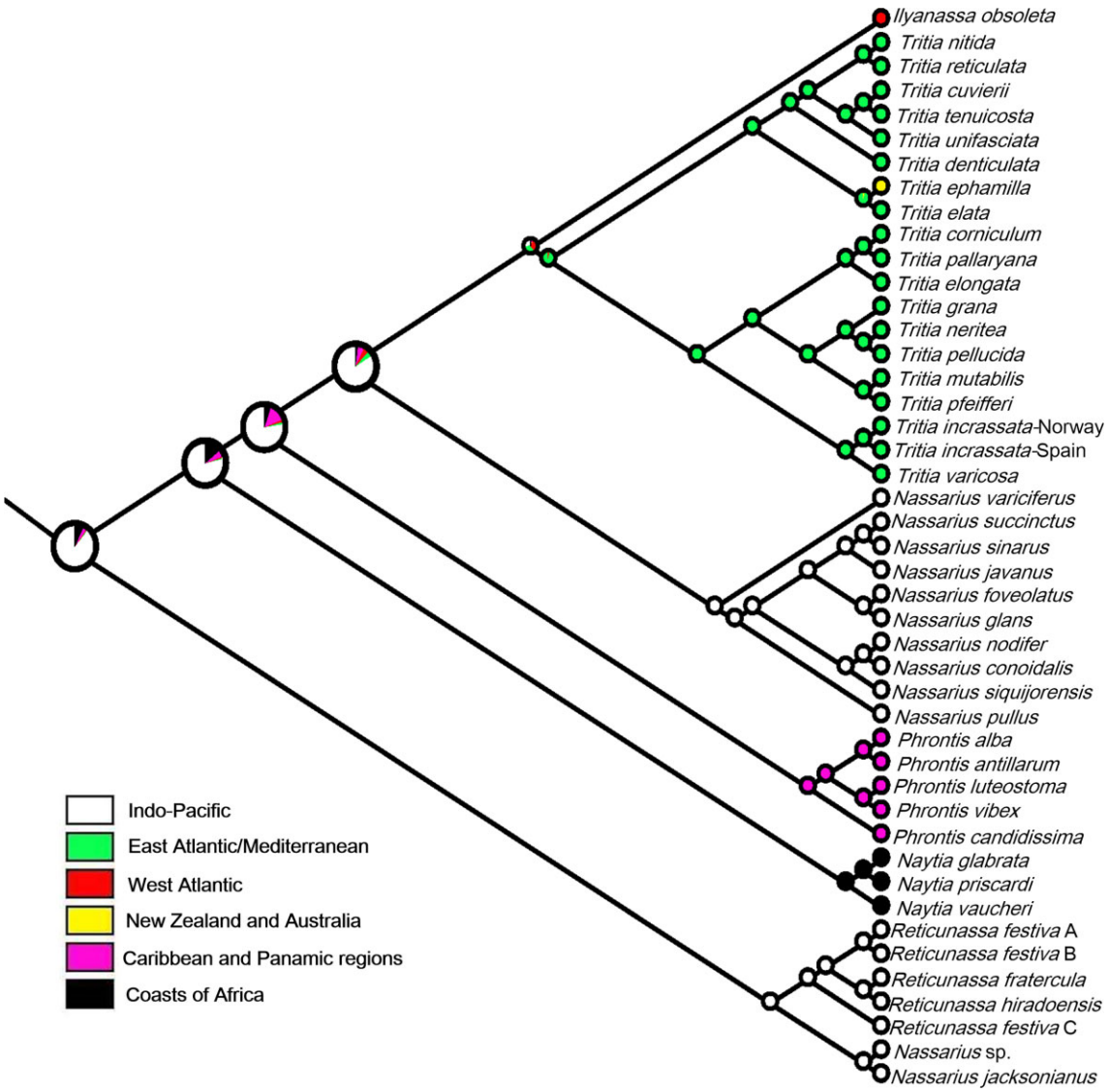

Figure 3. Biogeographic ancestral reconstruction of the subfamily Nassariinae under maximum likelihood. Pie charts represent the statistical support at every node.

phylogeny recovers the subfamilies Photinae, Cylleninae, Anentominae and Tomliniinae as distantly related to subfamilies Nassariinae and Bulliinae. Although we have not included any species of Dorsaninae in our analysis, this subfamily has been always placed with strong support within the Nassariidae in previous phylogenies. Although several key nodes in the phylogeny here reconstructed (node recovering Collumbellidae sister to Tomliniinae; node recovering Photinae sister to Buccinanopsidae plus Buccinidae, Cominellidae, Tudiclidae and Austrosiphonidae; node connecting the latter to Bullia + Nassariinae to the exclusion of several families and in particular, subfamilies Cylleninae and Anentominae) received relatively strong support, other nodes did not, and the long branch of Collumbellidae may be introducing serious bias in the final reconstructed topology. This prevents us from suggesting further nomenclatural changes, at least until new studies based on nuclear genomic data are performed.

Phylogenetic relationships of Nassariinae

The phylogenetic relationships of Nassariinae were well resolved, with seven main lineages recognized. The first offshoot of the tree showed the Indo-Pacific '*Nassarius*' *jascksonianus* and '*Nassarius*' sp. (incorrectly identified as *Nassarius acuticostus* (Montrouzier, 1864) in Yang *et al.*, 2019; see also Yang *et al.*, 2021) sister to the sympatric *Reticunassa*, as previously suggested (Yang *et al.*, 2019, 2021). Both morphological and molecular differences between *Reticunassa* and '*Nassarius*' *jascksonianus* + '*Nassarius*' sp. support the establishment of a new genus for the latter clade, which relies on a larger taxon sampling and thorough morphological revision beyond the scope of this study. *Reticunassa* was elevated to full genus rank by Galindo *et al.* (2017) to include the species of the so-called '*Nassarius pauper* complex'. The type species of the genus is *Reticunassa paupera* (A. Gould, 1850), which was recovered in the same clade as *Nassarius festivus* (Powys, 1835) (see Galindo *et al.*, 2016; Liu & Yang, 2021), which prompts its inclusion in the genus *Reticunassa* along with *Nassarius fraterculus* (Dunker, 1860) and *Nassarius hiradoensis* (Pilsbry, 1904), within the first offshoot of the Nassariinae. This genus has experienced high diversification in the Western Pacific (Galindo *et al.*, 2017; Zhang, Zhang & Lee, 2022).

The next lineage branching off in the phylogeny was the African genus *Naytia* (Fig. 2), which is characterized by high morphological disparity (Galindo *et al.*, 2016). While the type species, *Naytia glabrata*

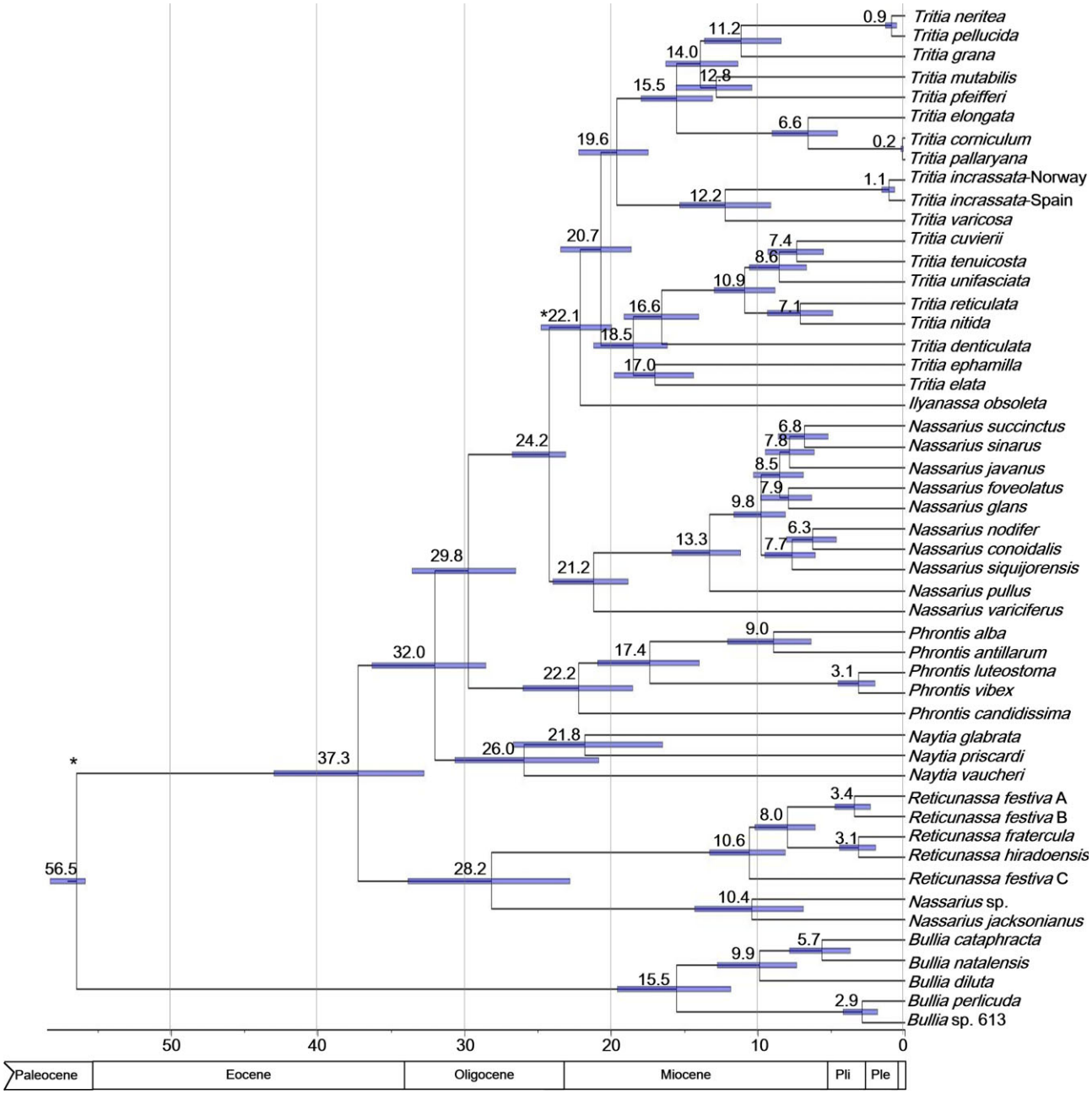

Figure 4. Chronogram with age estimates of major divergence events within Nassariinae based on the concatenated nucleotide sequences of 13 mitochondrial protein-coding genes and 2 rRNAs using Bayesian relaxed dating methods. Horizontal bars represent 95% credible intervals of relevant nodes, and calibration constraints are indicated with an asterisk on the corresponding nodes. Dates (and highest posterior density intervals) are in millions of years. Geological ages are indicated.

(G. B. Sowerby II, 1842) exhibits a smooth shell (also *N. granulosa* (Lamarck, 1822); not included here), *N. priscardi* (Bozzetti, 2006), *N. vaucheri* and *N. johni* (Monterosato, 1889) (not included here) are characterized by a reticulate shell sculpture. Geographically, *N. vaucheri* is found in the southwestern Spanish Mediterranean and the nearby Atlantic coasts (Gofas, 2011). Since the distribution of *N. vaucheri* is partially sympatric with several species of the Northeast Atlantic/Mediterranean *Tritia*, and it is morphologically similar to *Tritia incrassata* (Strøm, 1768), it had been erroneously assigned to *Tritia* until Yang *et al.* (2021) recovered a distant phylogenetic relationship between them. Here, we clearly assign this species to the genus *Naytia*. This result does not reveal any consistency between the molecular phylogeny and shell sculpture as pointed out by Yang *et al.* (2021). Interestingly, *N. vaucheri* is sympatric with *N. johni*. Another species that may belong to this clade is *Nassarius argenteus* (Marrat, 1877) from Mauritania, which is very similar, if not conspecific to *N. vaucheri*. According to Galindo *et al.* (2016), molecular data seem to be the only source of support to group these species within the same genus.

The remaining taxa included in the phylogeny formed a clade in which *Phrontis* was recovered sister to *Nassarius* plus *Tritia* and *Ilyanassa*. According to Galindo *et al.* (2016), the genus *Phrontis*, restricted to the Caribbean-Panamic region, is the result of a reinvasion of nassariids into American coasts after the *Buccinanops* and Photinae radiations. The phylogenetic relationships of *Tritia* + *Ilyanassa* were discussed in a previous study

(Yang *et al.*, 2021) that reestablished the validity of the latter genus. The present results corroborate previous ones. The mostly Northeast Atlantic/Mediterranean *Tritia* form a natural group divided into two main clades. The first clade includes *Tritia cuvierii* (Payraudeau, 1826), *T. tenuicosta* (Bucquoy, Dautzenberg & Dollfus, 1882), *T. unifasciata* (Kiener, 1834), *T. nitida* (Jeffreys, 1867), *T. denticulata* (A. Adams, 1852), *T. ephamilla* (R. B. Watson, 1882), *T. elata* (A. Gould, 1845) and *T. reticulata* (Linnaeus, 1758). This latter is the type species of the genus, and thus the species of this clade are those that must be retained within the genus *Tritia*. Other species not studied here but that should be kept in this genus are: *T. lanceolata* (Bucquoy, Dautzenberg & Dollfus, 1882) and *T. djerbaensis* (Aissaoui *et al.*, 2017) (endemic to the Gulf of Gabès), which are close to *T. unifasciata* according to Aissaoui *et al.* (2017); *T. miga* (Bruguière, 1789) and *T. caboverdensis* (Rolán, 1984) (both from West Africa), which are close to *T. cuvierii* (Rolán, 1984; Galindo *et al.*, 2016), and the West African species of the group of *T. denticulata* according to Rolán & Hernández (2005) (i.e. *T. turbinea* (A. Gould, 1845), *T. webbei* (Petit de la Saussaye, 1850), *T. clathrata* (Born, 1778), *T. desmoulioides* (G. B. Sowerby III, 1903) and *T. arcadioi* (Rolán & Hernández, 2005)). The second clade (that could deserve a new putative genus) is composed of two subclades. One subclade has species characterized by a smooth shell, that is, *T. neritea* (Linnaeus, 1758), *T. pellucida* (Risso, 1826), *T. mutabilis* (Linnaeus, 1758), *T. pfeifferi* (R. A. Philippi, 1844), *T. corniculum* (Olivi, 1792), *T. pallaryana* (Aissaoui *et al.*, 2017) and *T. elongata* (Bucquoy, Dautzenberg & Dollfus, 1882); the other subclade includes *T. incrassata* and *T. varicosa* (W. Turton, 1825). Other extant species not studied here that should be included in the first subclade is *T. compersa* (R. A. Philippi, 1849), which according to Segers, Rolán & Swinnen (2008) replaces *T. pfeifferi* (R. A. Philippi, 1844) in the Canary Islands. The second subclade should also include *T. varicosa* (W. Turton, 1825), *T. goreensis* (Maltzan, 1884) and *T. senegalensis* (Maltzan, 1884), since Galindo *et al.* (2016) recovered these last two species within this clade.

The few species included from the species-rich genus *Nassarius* in our phylogeny preclude further discussion, as a separate phylogenetic analysis with a richer taxon sampling is needed. In addition to the above-mentioned genera, three species-poor genera, *Nassodonta* from India, *Caesia* from America and *Demoulia* from Africa, were included within Nassariinae by Kantor *et al.* (2022) based on morphological evidence. In fact, none of these three genera has been included in previous molecular phylogenies. *Nassodonta* was represented by '*Nassodonta*' *dorri* in Galindo *et al.* (2016) and Strong *et al.* (2017), which was later removed to *Oligohalinophila* by Neiber & Glaubrecht (2019). Hence, the placement of *Nassodonta* within Nassariinae has yet to be tested.

Diversification of Nassariinae in the light of phylogeny

A robust phylogeny of Nassariinae could help understanding how the global diversification of this cosmopolitan group occurred. In fact, our reconstructed molecular phylogeny of Nassariinae fits better with biogeography than those reconstructed based on morphology, as has been pointed out by Oyston *et al.* (2022) for other animal groups. The result of the biogeographical reconstruction and the molecular clock dating supports the hypothesis that Nassariinae originated from the Indo-Pacific region (Galindo *et al.*, 2016), back in the Paleocene, *c.* 56 Ma, when one of the most dramatic global warming events of the Cenozoic, the Paleocene-Eocene thermal maximum (PETM) occurred; this might have triggered diversification processes in Nassariidae and the origin of Nassariinae, as has been suggested for other marine organisms (Gingerich, 2006; Speijer *et al.*, 2012). The PETM caused substantial increases in sea surface temperatures over a considerable timescale and has been linked to the emergence of several shallow-water-associated marine species (Zolkaply *et al.*, 2021). This environmental change could also explain the first diversification of the Indo-Pacific lineage formed by *Nassarius jacksonianus* and *Nassarius* sp. + *Reticunassa,* extending back to the late Eocene. Another two diversifications were dated back to the Oligocene, leading to the successive divergences of *Naytia* and *Phrontis*. During the early Oligocene, there was a dramatic cooling event in the northern Pacific. This climatic change event caused a striking turnover of certain marine species (Kelly & Eernisse, 2008), and could have been involved in the divergence of the assumed tropical ancestors of both *Naytia* in Africa and *Phrontis* in the Caribbean-Panamic region. Finally, continental drift may account for the divergence of Indo-Pacific *Nassarius* and North Atlantic/Mediterranean *Tritia* plus North American *Ilyanassa* (Yang *et al.*, 2021). Altogether, these results support a major role of vicariant events in producing disjunct geographical distributions, which ultimately could have led to the astonishing global diversification of mud snails.

CONCLUSIONS

Buccinoideans are an example of group in which convergent processes likely shaped most of the morphological diversity observed today and thus, resolving phylogenetic relationships within this superfamily necessarily requires reconstructing robust molecular phylogenies. The nodes with strong statistical support in the phylogeny of buccinoidean families here reconstructed suggest that the genera *Cyllene*, *Anentome*, *Tomlinia*, *Engoniophos*, *Phos, Antillophos* and *Buccinanops* are only distantly related to Nassariinae and Bulliinae. However, the internal nodes connecting the different families are rather short and their statistical support, particularly for ML inferences, is not high enough to lead to definitive conclusions. This pattern of short branches indicates rapid radiation processes at the origin and early diversification of the Buccionidea, which will require denser taxon sampling and further phylogenomic datasets to be resolved. The robust phylogeny of Nassariinae clarified the placement of *Naytia vaucheri* within the African genus *Naytia* and allowed inferring that the ancestor of Nassariinae had originated from the Indo-Pacific region. The inferred divergence dates suggest an important role for climatic and tectonic changes during the Eocene to Oligocene in promoting vicariant events that promoted diversification and the origin of the different genera within Nassariinae. At the subfamily level, mt genomes provide high levels of resolution and could render similar robust results for other subfamilies such as Nassariinae, provided that an appropriate range of taxa are represented. Moreover, they could be highly useful in reconstructing a robust phylogeny of the entire family, hence providing the long-needed framework to understand the diversification and adaptive processes underpinning the evolutionary success of this family.

ACKNOWLEDGEMENTS

Most of the specimens newly sequenced here originated from various expeditions of the Tropical Deep-Sea Benthos (MNHN, IRD) and the Our Planet Reviewed (MNHN, Pro-Natura International) programmes (more details here: expeditions.mnhn.fr) in Papua New Guinea (BIOPAPUA—dx.doi.org/10.17600/10100040, PAPUA NIUGINI—dx.doi.org/10.17600/18000841), New Caledonia (EXBODI—dx.doi.org/10.17600/11100080), Taiwan and the China Sea (ZhongSha 2015), Madagascar (ATIMO VATAE—dx.doi.org/10.17600/10110040), Mozambique (INHACA 2011), French Guyana (GUYANE 2014) and Guadeloupe (KARUBENTHOS 2012). These expeditions operated under the regulations then in force in the countries in question and satisfy the conditions set by the Nagoya Protocol for access to genetic resources. We are indebted to the cruise leaders of these expeditions, including Sarah Samadi, Laure Corbari and Professor Philippe Bouchet (MNHN). We are also grateful to Barbara Buge and Yuri Kantor for help in curating the samples and in identifying them, respectively. We thank two anonymous reviewers for

insightful suggestions on the manuscript. This work was funded by the Spanish Ministry of Science and Innovation (PID2019-103947GB-C22/AEI/10.13039/501100011033 to R.Z.). Y.Y. was supported by a scholarship from the China Scholarship Council (CSC-201806330034) for studying and living abroad. The project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. 865101) to N.P.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

CONFLICT OF INTEREST

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or nonfinancial interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY

All sequence data newly generated in this study have been deposited at NCBI and the corresponding accession numbers are listed in Supplementary Material Table S1.

AUTHORS' CONTRIBUTIONS

All authors conceptualized the study and contributed to the collection of the data, Y.Y. obtained and analysed the sequence data. All authors participated in writing the manuscript and approved the final version.

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