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Origin and Biogeography of the Colourful Sap-Sucking Sea Slugs Genus *Thuridilla* Bergh, 1872 (Mollusca, Gastropoda, Heterobranchia)

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Keywords: ancestral area reconstruction | dispersal | historical biogeography | phylogeny | Sacoglossa | speciation | vicariance

ABSTRACT

Aim: *Thuridilla* Bergh, 1872, is a lineage of herbivorous sea slugs externally distinguished by bright colours and distinctive patterns of lines and spots. Recent work revealed an exceptionally rapid, cryptic radiation of 13 species in the Indo-Pacific, raising questions about mechanisms of speciation in this group. Here, we (i) study the diversification and historical biogeography of *Thuridilla* in a phylogenetic context and (ii) assess the role of dispersal and vicariance as the predominant mode of speciation in the genus.

Location: Tropical and temperate regions of the Atlantic and Indo-Pacific.

Major Taxa Studies: Gastropoda, Sacoglossa.

Methods: A nearly complete taxon set with 28 out of 32 recognised species of *Thuridilla* was used, in a total sample of 172 specimens, together with sacoglossan outgroups. Phylogenetic relationships were determined using a multi-locus approach combining two mitochondrial (COI and 16S) and one nuclear gene (H3). Species relationships, diversification times, and ancestral geographical ranges were inferred using relaxed-clock methods together with Bayesian discrete phylogeographic methods under three calibration scenarios using the oldest known fossil of Sacoglossa, *Berthelinia elegans* Crosse, 1875, and tectonic events.

Results: *Thuridilla* species branched off into four major clades in all calibration scenarios: two groups from the Atlantic plus Indo-West Pacific (5 and 6 species) and two clades from the Indo-West Pacific (4 and 17 species). The highest diversity of the genus is in the Western Pacific (14 spp.) with a peak in the East Indies Triangle (18 spp.), whereas the Atlantic is depauperate with only four species occurring in this ocean basin. Divergence between Atlantic and Indo-West Pacific lineages occurred in two main temporal periods: the Miocene and the Pliocene. Speciation events within the 13 cryptic species-complex fell mostly within Plio-Pleistocene times.

Main Conclusions: The best supported hypothesis was an Indo-West Pacific origin of *Thuridilla* between 28 and 18 Mya during the Early Miocene. In the western Pacific, speciation likely occurred during transient allopatry during Plio-Pleistocene sea-level fluctuations. Under the three tested calibration scenarios, the limited diversity of the Atlantic Ocean is hypothesized to be derived from Miocene vicariant events associated with the closure of the Tethys Sea, dispersal across southern Africa, or long-distance dispersal across the East Pacific Barrier prior to the uplift of the Isthmus of Panama. *Thuridilla* is absent in the Eastern Pacific, potentially resulting from the extinction of ancestral lineages following the uplift of the Isthmus of Panama. Near-complete sampling of diversity and reconstruction of historical biogeography thus yielded new insight into the relative contributions of dispersal versus vicariance during speciation over the history of this widely distributed, colourful genus.

1 | Introduction

Historical speciation and extinction processes determine the current geographic distribution of biodiversity, but the origin of marine species has long remained a topic of debate. The Darwinian view favours speciation by natural selection in sympatry resulting from ecological or sexual pressures, with subsequent long-distance dispersal from a central point of origin (Darwin 1859). Nearly a century later, the prevailing view changed, favouring the idea that reproductive isolation predominantly evolves between geographically isolated populations (Mayr 1954, 1963), conferring a dominant role to allopatric speciation. However, the processes generating the staggering diversity of the Indo-West Pacific and, in particular, of the East Indies Triangle (EIT), a region encompassing the waters and islands between Indonesia, the Philippines, East Timor, Papua New Guinea and the Solomon Islands (Briggs 2000; Halas and Winterbottom 2009), have been a long-standing matter of debate. Most studies agree that this high diversity is the result of a plurality of processes, with species originating both in the central region of the EIT and peripheral areas over different time scales, with the high proportion of suitable habitat being critical for the survivorship of species in this area (e.g., Briggs 1999, 2005; Williams and Reid 2004; Barber et al. 2006; Williams and Duda 2008; Bellwood and Meyer 2009; Drew and Barber 2009; Halas and Winterbottom 2009; Bowen et al. 2013; Copus et al. 2022).

Patterns of marine speciation certainly depend on complex interactions between geography, life history and ecology (Krug 2011). For example, the planktonic larval development of many marine species, together with the limited geographic barriers to gene flow, enhances the dispersal potential of benthic species, creating opportunities for isolation (Palumbi 1994; Rocha et al. 2007; Krug 2011; Miglietta et al. 2011; Goetze et al. 2017). Species may also disperse far from their centre of origin, resulting in wide distributions for taxa with limited movement as adults.

The morphological diversity of molluscs and their ecological disparities make them an ideal group for evolutionary comparative studies (Krug 2011; Haszprunar and Wanninger 2012; Vinther 2015; Wanninger and Wollesen 2015). Among the Mollusca, gastropods are one of the most heterogeneous taxa, yet across several distinct groups, it was observed that sister species tend to have allopatric distributions (Williams and Reid 2004; Malaquias and Reid 2009; Claremont et al. 2011; Eilertsen and Malaquias 2015; Ekimova et al. 2019). The Sacoglossa constitute a group of small and mainly greenish, herbivorous sea slugs that are often associated with species-specific host algae (Jensen 1989; Poore et al. 2008; Trowbridge et al. 2008; Baumgartner et al. 2009). Given their level of habitat specialisation, reproductive strategies, and shifts in larval dispersal ability, sacoglossans are an emerging model system for the study of speciation mechanisms in the sea (e.g., Krug et al. 2015, 2018; Rodriguez and Krug 2022; Moreno et al. 2023).

Species in the sacoglossan genus *Thuridilla* Bergh, 1872, stand out because of their external bright colours (Gosliner 1995; Martín-Hervás et al. 2021) instead of the more cryptic, greenish colouration of their relatives. Unlike most sacoglossans, *Thuridilla* spp. are not typically associated with a host alga, which may limit opportunities for ecological speciation. A recent systematic revision, based on morphological and molecular analyses, recognised 32 species worldwide in temperate and tropical waters (Figure 1; Martín-Hervás et al. 2021), but the genus is notably absent in the eastern Pacific (Cobb and Willan 2006; Valdés et al. 2006; Gosliner et al. 2018). A striking finding was the rapid radiation of 13 pseudocryptic species that form the so-called *T. gracilis* complex (Martín-Hervás et al. 2021). Species in this clade were distinguished by subtle external and internal anatomical differences following molecular species delimitation and phylogenetic analyses, but a full analysis of their distributions was not performed (Martín-Hervás et al. 2021).

The recognition of a cryptic radiation comprising >40% of species diversity in *Thuridilla* warranted further attention to mechanisms of speciation in this group. The broad distribution of *Thuridilla* also makes it a good model to study global processes of marine biogeography. The higher diversity in

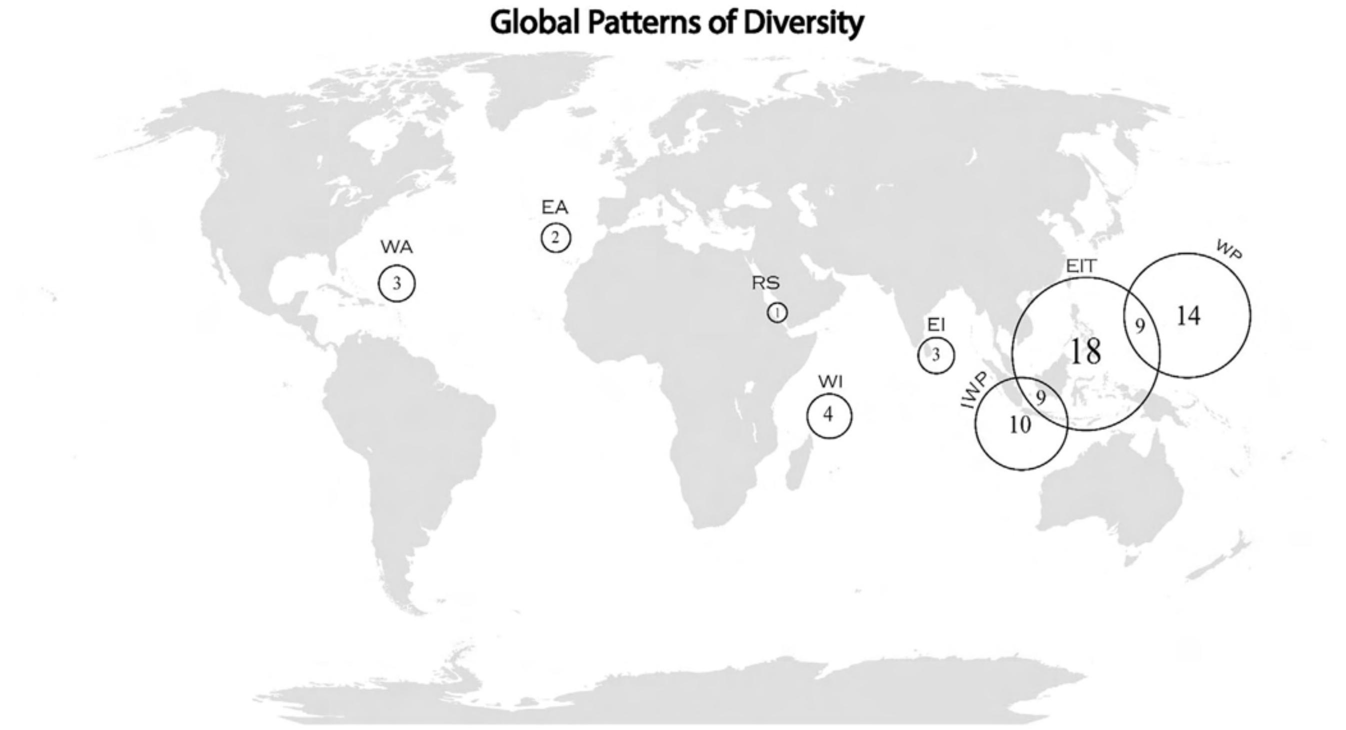

FIGURE 1 | Global patterns of *Thuridilla* diversity. Species richness is indicated numerically for each considered biogeographic unit (see Table 2). Shared species numbers between the IWP and EIT and between the EIT and WP are depicted in the overlapping areas of the correspondent circles. EA, Eastern Atlantic including the Mediterranean Sea; EI, Eastern Indian Ocean; EIT, East Indies Triangle; IWP, Indo-West Pacific; RS, Red Sea; WA, Western Atlantic; WI, Western Indian Ocean; WP, Western Pacific.

the Indo-West Pacific Ocean suggests a possible origin of the group in this marine realm; however, the lack of fossil record and data on larval development makes it difficult to date and locate the origin of *Thuridilla*. The main goal of this paper is to study the diversification and historical biogeography of *Thuridilla* in a phylogenetic context and assess the role of dispersal and vicariance as the predominant mode of speciation in the genus.

2 | Materials and Methods

2.1 | Taxon Sampling

The taxon set used for this study was taken from Martín-Hervás et al. (2021) based on 172 specimens representing nearly the entire diversity of the genus *Thuridilla*: 32 nominal species including 10 unnamed lineages considered candidate species based on species delimitation analyses. The full dataset comprised 155 sequences of the mitochondrial genes cytochrome oxidase *c* subunit I (COI) and large ribosomal subunit rRNA (16S), and 139 of the nuclear gene histone H3 (H3). Only three accepted species were not sampled: *T. coerulea* (Kelaart 1858), *T. haingsisiana* (Bergh 1905) and *T. multimarginata* Gosliner 1995. In addition, 12 species of *Elysia* Risso, 1818, and 10 species of *Plakobranchus* van Hasselt, 1824, were used as outgroup taxa, and one representative of *Bosellia* Trinchese, 1891, was included to root the tree. For details of the taxon set (voucher numbers, collection sites and GenBank accession numbers), see Martín-Hervás et al. (2021, Table 1).

2.2 | Phylogenetic Analyses and Estimate of Divergence Times

Sequences of *Thuridilla* and outgroups were aligned by gene with the multiple sequence alignment programme MAFFT v.7.402 server (Katoh and Standley 2013), using the L-INS-i iterative refinement algorithm via the CIPRES Portal Science Gateway (Miller et al. 2010). Hypervariable regions of the 16S gene were removed using the least stringent criteria in Gblocks v.0.91b (Castresana 2000). The COI and H3 protein-coding genes were translated into amino acids to test for the presence of stop-codons, and the level of saturation for the first, second, and third codon positions of these genes was investigated using MEGA v.7.0.18 (Kumar et al. 2016) by plotting the observed transitions (Ts) and transversions (Tv) against the inferred uncorrected *p*-distances. Best-fit models of evolution for each gene were selected with JModelTest 2.1.10 (Darriba et al. 2012) based on the Akaike criterion (AIC) (Akaike 1998), rendering the $GTR + \Gamma + I$ for both COI and 16S genes and the $GTR + \Gamma$ for the H3 gene. Final alignments contained 658 base pairs for the COI gene, 435 for the 16S, 328 for H3 and a total of 1421 for the concatenated all-genes combined dataset (H3 + COI + 16S).

Thuridilla is not represented in the fossil record to the best of our knowledge. Given the lack of direct information about its region and timing of origin, we used an indirect method to estimate the age of the most recent common ancestor of the genus. We used a subset of taxa from a phylogeny of Sacoglossa (Krug et al. 2015) and the same three-gene dataset (see Table 1). The

TABLE 1 | List of Sacoglossa specimens used in our analyses from dataset of Krug et al. (2015).

Species	Sample code	COI	16S	H3
Alderiopsis nigra	Al_nig_09Rus01	KM086342	KM204183	KM040788
Aplysiopsis enteromorphae	Ap_ent_04SD01	KM086344	GU191053	KM040789
Ascobulla fragilis	As_fra_NCBI	AY345022	AY345022	—
Berthelinia caribbea	Ber_car_10Swe01	KM086349	KM204188	KM040794
Bosellia mimetica	Bmim_06Ber01	KM086351	KM204190	KM040796
Caliphylla mediterranea	Ca_med_09Cur01	KM086352	GU191041	KM040798
Costasiella formicaria	Cfor_08Jap03	KJ610068	KJ610028	KJ610055
'*Costasiella*' *nonatoi*	Cnon_04Pan01	KJ610069	KJ610029	KJ610056
Costasiella ocellifera (P)	Coce_06Ber05P	KF438672	KF438670	KF438674
Cyerce antillensis	Cy_ant_04Pan01	GU191072	KM204192	KM040800
Cyerce nigra	Cy_nig_09Gua01	KM086354	KM204194	KM040802
Cylindrobulla beauii	Cylin_bea_09FL01	KM086358	KM204198	KM040806
Elysia chlorotica	Echl_06Mas01	KM086377	KM204226	JN819183
Elysia crispata	Ecri_06FL01	JN819090	JN819139	KM040828
Elysia papillosa	Epap_06Jam06	JQ914617	JQ914620	KM040841
Elysia rufescens	Eruf_08Jap01	KC573688	KM204247	KC597152
Ercolania coerulea	Er_cae_USVI_01	KM086389	KM204238	KM040856
Ercolania subviridis	Er_sub_10Jap01	KM086395	KM204244	KM040865
Gascoignella aprica	Ga_apr_11HK01	KM086404	KM204255	KM040868
Hermaea bifida	Hbif_05Ire01	KM086407	GU191043	KM040871
Julia zebra	Jzeb_10Mor01	KM086410	KM204259	KM040874
Limapontia capitata	Lcap_03UK01	KM086411	KM204260	KM040875
Lobiger viridis	Lvir_11How01	KM086415	KM204264	KM040879
Oxynoe azuropunctata	Oxy_sp4_07Gei01	KM086425	KM204274	KM040888
Placida cremoniana	Pl_cre_03Jap01	KM086427	KM204286	KM040896
Placida verticilata	Pl_ver_07SSal01	GU191064	GU191045	KM040900
Plakobranchus sp. 1	Pk_sp1_07Sul01	KC573732	KM204281	KC597163
Platyhedyle denudata	Plty_den_NCBI	JF828035	—	—
Polybranchia viridis	Po_vir_04Cur01	KM086434	GU191052	KM040901
Siphonaria pectinata	Siph_pectinata	HQ386633	HQ386656	AY377780
'*Stiliger*' *smaragdinus*	St_sma_11Syd01	KM086441	KM204300	KM040914
Thuridilla gracilis	Tgra_09Gua01	KM086444	KM204304	KM040917
Thuridilla hopei	Thop_07Ity01	KC573743	KM204305	KC597170
Thuridilla livida	Tliv_07Mal01	KC573745	KM204307	KC597172
Thuridilla picta	Tpic_10Nex01	KC573748	KM204309	KM040919
Volvatella viridis	Vo_vir_NCBI	HQ168451	HQ168413	—

Note: Underlined species belong to the Oxynooidea superfamily.

tree was calibrated with the oldest fossil assigned to Sacoglossa, *Berthelinia elegans* Crosse, 1875 from the Middle Eocene (37.8–47.8 Mya) of the Paris Basin (Keen and Smith 1961), belonging to the family Oxynooidea. The calibration node chosen was the one defining the clade containing representatives of Oxynooidea (underlined in Table 1).

The divergence times of genera were estimated in the software package BEAST v1.10.4 (Suchard et al. 2018). BEAUti v.1.10.4 was used to import the concatenated dataset partitioned by gene, using linked substitution models, clock models and tree priors. The best-fitting models of evolution (inferred with the AIC in JModelTest 2.1.10) were specified as GTR + Γ + I for COI and 16S partitions and GTR + Γ for the H3 gene. We implemented an uncorrelated relaxed lognormal molecular clock and a Yule speciation-process tree prior, which is considered statistically sound for species-level phylogenies (Drummond et al. 2006). The species from Oxynooidea were set as a monophyletic group and time-calibrated with the fossil *B. elegans*, applying an untruncated lognormal distribution [initial value = 1; mean = 42.8; stdev = 3.17; quantiles = 5.0%–95.0%]. Two independent analyses were run for 15 million generations, with a sampling frequency set to 1000.

Tracer v1.7.1 (Rambaut et al. 2018) was used to inspect the effective sample size (ESS, > 200) for each parameter and assess the convergence of runs by plotting the likelihood against the number of steps in the chain. The log tree files from each analysis were combined in LogCombiner v1.10.4 (Suchard et al. 2018) with a burn-in set for each run of 20%. TreeAnnotator v1.10.4 (Suchard et al. 2018) was then used to summarise the trees onto a single target tree, posterior probability limit set to 0.5, maximum clade credibility tree and mean node heights. The resulting tree was converted to graphics in FigTree v1.4.4 (Rambaut 2018) and final adjustments were made in Adobe Illustrator CC 2018 (Figure 2).

The time of speciation and rates of evolution of *Thuridilla* lineages were tested for three possible scenarios of evolution. In scenario 1 (S1), the age range of the most recent common ancestor of *Thuridilla* (estimated using the method described above) was used to calibrate the root of the *Thuridilla* tree resulting from our own concatenated dataset. The dataset was partitioned by gene in BEAUti v.1.10.4 and set up using unlinked substitution models and linked clock models and tree priors. Nucleotide substitution models previously obtained with JModelTest 2.1.10 were specified for each gene.

The ingroup (all species of *Thuridilla*) was defined to be monophyletic and calibrated with a uniform prior distribution and minimum (12.4 Mya) and maximum (24.46 Mya) ages ('hard bounds') estimated for the origin of *Thuridilla* in our first analysis (Figure 2). Markov Chain Monte Carlo (MCMC) analyses consisted of two independent runs of 100 million generations each sampled every 10,000 generations. Because the initial random tree generated by BEAST v.1.10.4 was repeatedly in conflict with the set calibration priors, it was necessary to specify a starting tree that satisfied the calibration constraints. This was achieved by replacing in BEAUti v.1.10.4 the original uniform calibration prior with a normal prior with mean = 18 and standard deviation = 0.1, thus forcing the age of the *Thuridilla* node to be close to 18 My, which is close to the mean age estimated for the origin of *Thuridilla* (Figure 2). This analysis was performed in BEAST for 100,000 MCMC steps sampling every 1000 generations. The last tree of the output tree files was then selected and pasted in the original xml file generated with BEAUti with a uniform prior distribution and the hard bounds defined above.

To validate the divergence times inferred in scenario 1 (S1), we repeated the analysis considering two additional calibration hypotheses. In scenario S2, we assumed the origin of *Thuridilla* to be related to processes resulting from the closure of the Tethys Sea (18–12 Mya)—a significant geotectonic event that separated the Atlantic from the Indo-Pacific Ocean. In scenario S3, we calibrated node 2, which includes splits between Atlantic (*T. mazda*, *T. malaquita*) and Indo-Western Pacific taxa (*T. flavomaculata*, *T. kathae*, *T. carlsoni*, *T. hoffae*), to coincide with the closure of the Tethys Sea.

As described in detail above, convergence was assessed using the software Tracer, log tree files were combined in LogCombiner, TreeAnnotator was used to summarise the trees onto a single target tree, and the resulting tree was converted to graphics in FigTree v1.4.4 with final adjustments made in Adobe Illustrator CC 2018.

2.3 | Historical Biogeographical Analysis

Based on the geographical distributions known for the species of *Thuridilla* (Table 2), each lineage was coded as belonging to one of the following nine areas: Western Atlantic (WA); Eastern Atlantic including the Mediterranean Sea (EA); Atlantic Ocean (ATL), for species present on both sides of the Atlantic Ocean; Red Sea endemic (RS); Western Indian Ocean (WIO); Eastern Indian Ocean (EIO); Indian Ocean (IO), for species present on both sides of the Indian Ocean; or IWP and Western Pacific Ocean (WP). Ancestral geographical ranges were reconstructed using the symmetrical substitution phylogeographic model implemented in BEAST v.1.10.4, treating distributions as discrete traits and reconstructing states at all ancestral nodes (Lemey et al. 2009). The concatenated dataset was partitioned by gene in BEAUti v.1.10.4, and an additional partition was included referring to the geographical distribution trait (Table 2). Parameters of gene partitions were configured following the settings of the calibration scenarios described in the previous section, and the geographical distribution trait was evaluated under a strict clock model (i.e., no rate shifts).

3 | Results

3.1 | Phylogenetic Relationships

The topology and node support of the maximum clade credibility trees recovered for the three evolutionary scenarios all supported the monophyly of *Thuridilla* (PP = 1) and were largely congruent with the Bayesian tree obtained by Martín-Hervás et al. (2021). The exceptions were the position of some lineages within the *T. gracilis* complex that vary slightly between calibration scenarios, probably due to relatively low genetic divergence among lineages in this complex.

Thuridilla species branched off into four major clades in all calibration scenarios (S1 is shown in Figure 3; S2 and S3 are shown in Figures S1.1 and S1.2, respectively). Clade A is weakly supported in S1 (PP = 0.85) and S2 (PP = 0.88) but received maximum support in S3 (PP = 1). This clade includes the amphi-Atlantic (*T. mazda*) and western Atlantic (*T. malaquita* Ortea and Buske 2014) plus a clade of Indo-West Pacific species with *T. hoffae*, *T. flavomaculata* (Gosliner 1995), *T. carlsoni*

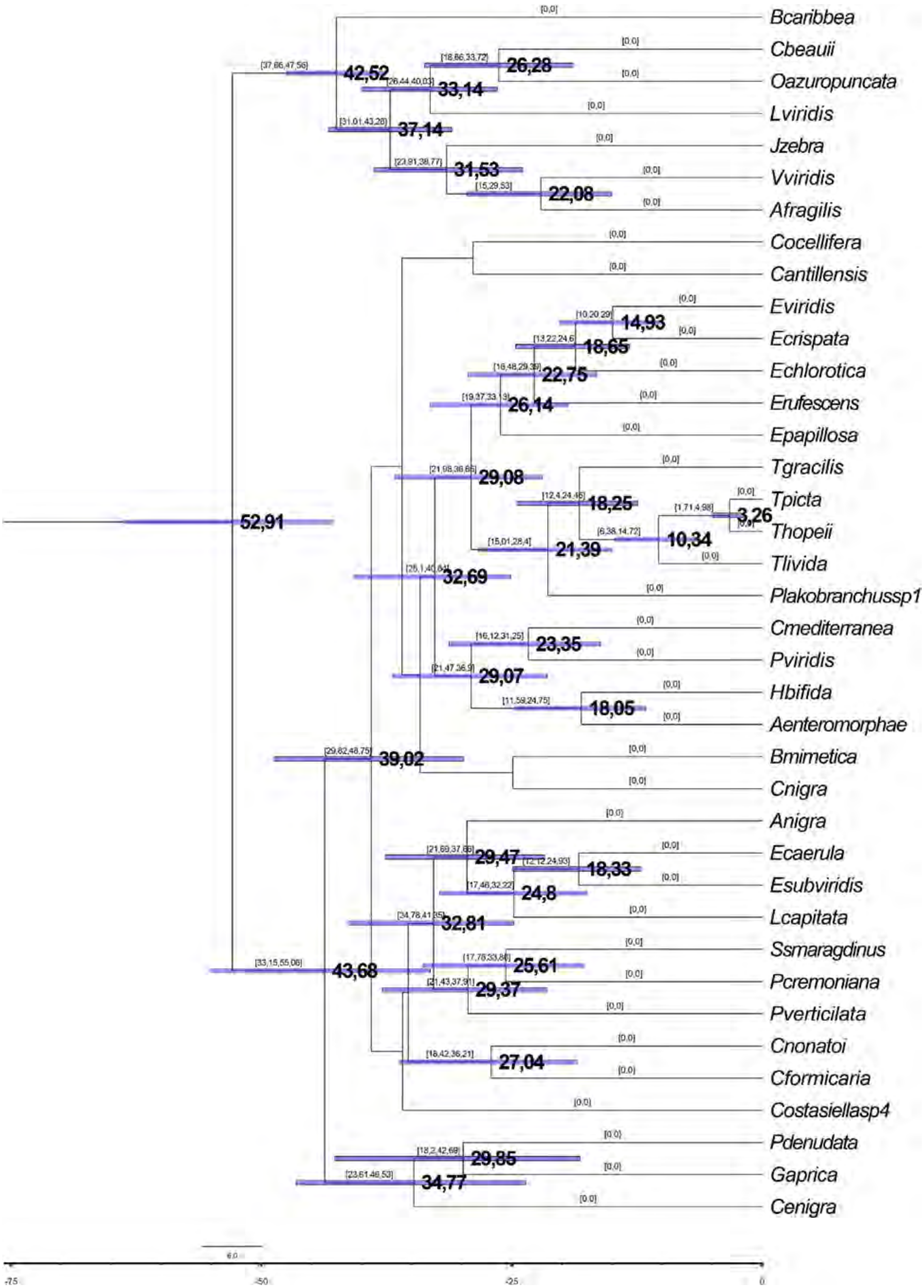

FIGURE 2 | Chronogram representing the ages (in million years) of the nodes estimated in BEAST from a subset of taxa from Krug et al. (2015) calibrated with the oldest fossil assigned to the Sacoglossa (*Berthelinia elegans*) consisting of the concatenated dataset H3, COI and16S rRNA genes under the relaxed-clock analysis using a GTR + Γ + I substitution model. Values in bold show node ages, and blue bars represent 95% highest posterior density (HPD) intervals.

TABLE 2 | Global distributions of *Thuridilla* species sensu Martín-Hervás et al. (2021).

Species	Distribution	Biogeographical code
Thuridilla albopustulosa	Eastern coast of Australia (Cobb and Willan 2006), South Africa (Gosliner 1987 as *Elysia* sp.2), Aldabra Atoll (Gosliner 1995), Papua New Guinea (Gosliner 1995), Indonesia (Gosliner 1995; Eisenbarth et al. 2018; Papu et al. 2020; Martín-Hervás et al. 2021), The Philippines (Gosliner 1995; Martín-Hervás et al. 2021), Japan (Gosliner et al. 2008; Nakano 2018), Taiwan (Martín-Hervás et al. 2021)	IWP
Thuridilla bayeri	Guam (Carlson and Hoff 1978; Jensen 1992; Bass 2006; Krug et al. 2015 as *T. gracilis*; Carlson and Hoff 2000; Burdick 2018; Martín-Hervás et al. 2021), Northern Mariana Is. (Carlson and Hoff 1978), Saipan (Northern Mariana Is.) (Fraser 2010; Martín-Hervás et al. 2021), Marshall Is. (Marcus 1965; Johnson 2007), Maldives (Yonow 1994), Baa Atoll, Maldives (J. Anderson 2016), Madang, Papua New Guinea (Gosliner 1995), Maug and Sarigan Is., Palau (Carlson and Hoff 1978), Mahe Is., Seychelles (Holley 1992), Raroia Atoll, French Polynesia (Levy 2012) and Flinders Reef, Moreton Is., Queensland, Australia (Mullins and Cobb 2009)	WP
Thuridilla carlsoni	Eastern coast of Australia (Cobb and Willan 2006), Western coast of Australia (Wells and Bryce 1993 as *Thuridilla* sp. 2), Lizard Is., Australia (Händeler et al. 2009), Lord Howe Is., Australia (Krug et al. 2013), Papua New Guinea (Gosliner 1995), Indonesia (Gosliner 1995), The Philippines (Martín-Hervás et al. 2021), Malaysia (Krug et al. 2013), Vanuatu (Krug et al. 2013), Saipan, Northern Mariana Is. (Krug et al. 2013), Japan (Ono 2004; Nakano 2018), Guam (Gosliner 1995), New Caledonia (Rudman 2000), Hawai'ian Is (Gosliner 1995; Bass 2006), Marshall Is. (Gosliner et al. 2008), Penghu Is., Taiwan (Martín-Hervás et al. 2021)	IWP
*Thuridilla coerulea**	Sri Lanka (Kelaart 1858 as *Elysia caerulea*), southern coast of India (Eliot 1906 as *Elysia caerulea*; Edward et al. 2022), Andaman and Nicobar Is., India (Shaktivel et al. 2014)	EI
Thuridilla decorata	Red Sea (Heller and Thompson 1983 as *Elysia decorata*; Yonow 2008; Gosliner et al. 2008, 2015, 2018; Martín-Hervás et al. 2021)	RS
Thuridilla flavomaculata	Luzon I., The Philippines (Gosliner 1995; Martín-Hervás et al. 2021), Tingloy Is., The Philippines (Martín-Hervás et al. 2021), Marshall Is. (Gosliner 1995), Indonesia (Gosliner 1995; Eisenbarth et al. 2018; Papu et al. 2020; Martín-Hervás et al. 2021), Japan (Ono 2004; Nakano 2018), Taiwan (Martín-Hervás et al. 2021), Guam (Gosliner 1995; Gosliner et al. 2008), Saipan, Northern Mariana Is. (Urasawa 2006), Lord Howe Is., Australia (Nimbs and Smith 2018), Reunion Is. (Cadet 2010a)	IWP
Thuridilla gracilis	New Caledonia (Risbec 1928; Durbano 2015), Guam, Pagan and Maug, Mariana Is. (Carlson and Hoff 1978), Lizard Is., Australia (Wollscheid-Lengeling et al. 2001 as *T. ratna*; Händeler and Wägele 2007; Händeler et al. 2009; Wägele et al. 2011; Martín-Hervás et al. 2021), North Sulawesi, Indonesia (Eisenbarth et al. 2018), Tulamben, Bali, Indonesia (Yonow and Jensen 2018), Luzon I., The Philippines (Femia 2016)	WP
Thuridilla hoffae	Papua New Guinea (Gosliner 1995; Debelius 2001), Vanuatu (Stenhouse 2000), Japan (Gosliner 1995; Ono 2004; Nakano 2018), Guam (Debelius 2001; Bass 2006), Marshall Is. (Debelius 2001; Gosliner et al. 2008), Indonesia (Yonow and Jensen 2018), Luzon I., The Philippines (Martín-Hervás et al. 2021), Western Australia (Debelius 2001), Samoa (Händeler and Wägele 2007), Mayotte (Poddubetskaia 2003), Reunion Is. (Cadet 2010b), Madagascar (Rassat 2016)	IWP

(Continues)

TABLE 2 | (Continued)

Species	Distribution	Biogeographical code
Thuridilla hopei	Eastern coast of Spain (Händeler and Wägele 2007; Carmona et al. 2011), Menorca, Spain (Carmona et al. 2011), Western Andalucia, Spain (Carmona et al. 2011), Southern coast of France (Vérany 1853 as *Actaeon hopei*; Carmona et al. 2011), Yugoslavia (Jensen 1992), Italy (Jensen 1992; Krug et al. 2013; Furfaro et al. 2014), Croatia (Martín-Hervás et al. 2021), Greece (Manousis 2021); Azores (Carmona et al. 2011), Madeira (Carmona et al. 2011), Cape Verde (Martín-Hervás et al. 2021), Canary Is. (Carmona et al. 2011)	EA
Thuridilla indopacifica	Mozambique (Martín-Hervás et al. 2021), South Africa (Gosliner 1987 as *Elysia* sp.1; Gosliner 1995; Debelius 2001), Aldabra Atoll, Seychelles (Gosliner 1995), Western Australia (Wells and Bryce 1993; Debelius 2001; Gosliner et al. 2008; Martín-Hervás et al. 2021), Kenya (Amar 2015), Madagascar (Rassat, Rassat, n.d.), Mayotte (Bidgrain 2010a), Reunion Is. (Bidgrain 2010a), Omán (Mayes 2008), Tanzania (Rudman 2005)	IO
Thuridilla kathae	Indonesia (Gosliner 1995), The Philippines (Gosliner 1995), Japan (Ono 2004; Nakano 2018), Hawai'ian Is (Gosliner et al. 2008; Krug et al. 2013), Taiwan (Martín-Hervás et al. 2021), Lizard Is., Australia (Händeler and Wägele 2007), Madagascar (Gosliner 1995; Gosliner et al. 2008), Reunion Is. (Cadet 2009a)	IWP
Thuridilla lineolata	Indonesia (Bergh 1905; Gosliner 1995; Debelius 2001; Händeler and Wägele 2007; Gosliner et al. 2008, 2015, 2018; Eisenbarth et al. 2018; Papu et al. 2020; Martín-Hervás et al. 2021), The Philippines (Gosliner et al. 2008, 2015, 2018; Martín-Hervás et al. 2021), Japan (Nakano 2018)	WP
Thuridilla livida	Aldabra Atoll (Gosliner 1987 as *Elysia livida*; Gosliner et al. 2008), Japan (Ono 2004; Gosliner et al. 2008; Nakano 2018), Guam (Bass 2006; Gosliner et al. 2008), Indonesia (Gosliner et al. 2008; Eisenbarth et al. 2018; Papu et al. 2020), Mariana Is. (Urasawa 2005), Malaysia (Krug et al. 2013), The Philippines (Gosliner et al. 2008), Marshall Is. (Gosliner et al. 2008), Taiwan (Martín-Hervás et al. 2021), Eastern Australia (Cobb 2007a), South Africa (Gosliner et al. 2008), Mayotte (Bidgrain 2010b), Reunion Is. (Cadet 2010c), Seychelles (Gosliner et al. 2008)	IWP
Thuridilla malaquita	Costa Rica (Camacho-García et al. 2014 as *Thuridilla* sp.), Colombia (Valdés et al. 2006 as *Thuridilla* sp.), Grenada (Valdés et al. 2006 as *Thuridilla* sp.), Florida (Valdés et al. 2006 as *Thuridilla* sp.; Martín-Hervás et al. 2021), Martinique (Lesser Antilles) (Ortea and Buske 2014), Venezuela (Delgado et al. 2022), Brazil (Delgado et al. 2022)	WA
*Thuridilla multimarginata**	Maui and Midway Atoll, Hawai'ian Is. (Gosliner 1995), South Africa (Gosliner 1987 as *Elysia* sp.3; Gosliner et al. 2008, 2015, 2018), Reunion Is. (Cadet 2009b), Mauritius (Summers 2015), Lizard Is. (Anthes 2004)	IWP
Thuridilla mazda	Bahamas (Redfern 2001 as *T. picta*; Valdés et al. 2006), Guadeloupe (Ortea et al. 2012), Costa Rica (Ortea and Espinosa 2000; Valdés et al. 2006; Rosenberg et al. 2009; Miloslavich et al. 2010), Cuba (Ortea and Espinosa 2000; Valdés et al. 2006; Rosenberg et al. 2009; Miloslavich et al. 2010), Mexico (Carmona et al. 2011), Florida (Martín-Hervás et al. 2021), São Miguel Is., Azores (Malaquias et al. 2012), Tenerife, Canary Is. (Ortea et al. 2015), Granada, Spain (Orfanidis et al. 2021), Sicily, Italy (Lombardo 2023)	ATL

(Continues)

TABLE 2 | (Continued)

Species	Distribution	Biogeographical code
Thuridilla moebii	Andaman and Nicobar Is., India (Sreeraj 2010), Mauritius (Bergh 1888), Reunion Is. (Gosliner 1987 as *Elysia moebii*), Comoro Ids., Mayotte (Gosliner et al. 2008), Madagascar (Bini, Bini, n.d.), South Africa (Gosliner 1987 as *Elysia moebii*; Gosliner et al. 2008), Abrolhos Ids., Western Australia (Wells and Bryce 1993)	IO
Thuridilla neona	Eastern coast of Australia (Cobb and Willan 2006), Lizard Is., Australia (Burghardt 2005), Lord Howe Is., Australia (Krug et al. 2013), Midway Atoll (Gosliner 1995), Northwest and main Hawai'ian Is (Debelius 2001; Gosliner et al. 2008, 2015, 2018)	WP
Thuridilla picta	Gulf of Mexico (De la Cruz-Francisco et al. 2017), Florida (Valdés et al. 2006), Bermuda (Valdés et al. 2006; Rosenberg et al. 2009; Carmona et al. 2011), Cuba (Miloslavich et al. 2010; Carmona et al. 2011), Bahamas (Valdés et al. 2006; Krug et al. 2013), Curaçao (Valdés et al. 2006), Jamaica (Valdés et al. 2006; Miloslavich et al. 2010), Lesser Antilles (Rosenberg et al. 2009), Costa Rica (Camacho-García et al. 2014), Colombia (Carmona et al. 2011), Brazil (Martín-Hervás et al. 2021)	WA
Thuridilla ratna	The Philippines (Bass 2006; Martín-Hervás et al. 2021), Guam (Carlson and Hoff 1978; Bass 2006; Martín-Hervás et al. 2021), Saipan, Northern Mariana Is. (Martín-Hervás et al. 2021), Taiwan (Martín-Hervás et al. 2021), Palau (Marcus 1965; Carlson and Hoff 1978; Medina et al. 2011 as *T. gracilis*; Martín-Hervás et al. 2021), Pohnpei, Eastern Caroline Islands (Carlson and Hoff 1978), Bil Bil Is. and Sek Is., Papua New Guinea (Martín-Hervás et al. 2021), Maldives (Martín-Hervás et al. 2021), Pulau Hantu, Singapore (Jensen 2009), Okinawa, Japan (Takano et al. 2013 as *T. gracilis*; Imagawa 2015; Martín-Hervás et al. 2021), Bangka Archipelago, North Sulawesi, Indonesia (Papu et al. 2020 as *T. gracilis*). Based on online images, see references in Martín-Hervás et al. 2021	WP
Thuridilla splendens	Japan (Baba 1949; Gosliner 1995; Gosliner et al. 2015, 2018; Takano et al. 2013; Krug et al. 2015; Nakano 2018; Martín-Hervás et al. 2021), New Taipei City, Taiwan (Martín-Hervás et al. 2021)	WP
Thuridilla undula	Maldives (Yonow 1994; Debelius 2001), Salomon Is. (Gosliner et al. 2008), Papua New Guinea (Gosliner 1995), The Philippines (Gosliner 1995; Bass 2006), Guam (Gosliner 1995), Palau (Debelius 2001; Gosliner 1995; Gosliner et al. 2008), Indonesia (Eisenbarth et al. 2018; Papu et al. 2020), Japan (Ono 2004; Nakano 2018)	IWP
Thuridilla vataae	Eastern Australia (Cobb 2007b), New Caledonia (Risbec 1928 as *Elysia vataae*), Guam (Carlson and Hoff 1978; Bass 2006; Gosliner et al. 2008), Vanuatu (Gosliner et al. 2008), Papua New Guinea (Gosliner 1995), Indonesia (Gosliner 1995; Eisenbarth et al. 2018; Papu et al. 2020), The Philippines (Gosliner 1995), Japan (Ono 2004; Gosliner et al. 2008; Nakano 2018), Palau (Carlson and Hoff 1978; Gosliner et al. 2008), Marshall Islands (Gosliner et al. 2008), French Polynesia (Krug et al. 2015), Maug (Carlson and Hoff 1978), Western Australia (Jensen 1992), Taiwan (Martín-Hervás et al. 2021), Samoa (Händeler and Wägele 2007), Maldives (Yonow 2012), Mayotte (Bidgrain 2010c), South Africa (Gosliner 1987 as *Elysia vatae*), Aldabra Atoll (Gosliner 1995), Reunion Is. (Gosliner 1995), Laccadive Is. (Apte 2009)	IWP
Thuridilla virgata	Mauritius (Bergh 1888), Madagascar (Martín-Hervás et al. 2021), South Africa (Gosliner 1987 as *Elysia virgata*), Tanzania (Gosliner et al. 2008), Reunion Is. (Eby 2005), Mayotte (Fontaine 2008), Nuarro, Mozambique (Martín-Hervás et al. 2021)	WI

(Continues)

TABLE 2 | (Continued)

Species	Distribution	Biogeographical code
Thuridilla sp. 1	Guam (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 2	Browse Is. and Ashmore Reef, Australia (Martín-Hervás et al. 2021), Cobrador Is. and Lubang Is, The Philippines (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 3	Indonesia (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 4	Kananam, Madang Province (Martín-Hervás et al. 2021), Tab Is., Papua New Guinea (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 5	Adele Is. and Long Reef, Western Australia (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 6	Pemba Bay, Nuarro, Vamizi Is. and Memba, Mozambique (Martín-Hervás et al. 2021), Mangapwani, Tanzania (Martín-Hervás et al. 2021), Lubang Is., The Philippines (Martín-Hervás et al. 2021)	IWP
Thuridilla sp. 7	Siar Is., Papua New Guinea (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 8	Lubang Is., The Philippines (Martín-Hervás et al. 2021), Pingtung County, Taiwan (Martín-Hervás et al. 2021)	WP
Thuridilla sp. 9	Memba, Mozambique (Martín-Hervás et al. 2021)	WI
Thuridilla sp. 10	Arkwright Shoal, Queensland, Australia (Martín-Hervás et al. 2021)	WP

Note: Asterisks indicate species that have not been included in the BEAST analysis.
Abbreviations: ATL, Atlantic Ocean; EA, Eastern Atlantic including the Mediterranean Sea; EI, Eastern Indian Ocean; IO, Indian Ocean; IWP, Indo-West Pacific; RS, Red Sea; WA, Western Atlantic; WI, Western Indian Ocean; WP, Western Pacific.

(Gosliner 1995), and *T. kathae* (Gosliner 1995). Clade B (PP = 1 in all three calibration scenarios) includes the Indo-West Pacific species *T. albopustulosa* (Gosliner 1995), *T. virgata* (Bergh 1888), *Thuridilla* sp. 1 and *T. vataae* (Risbec 1928). Clade C (PP = 1 in all three calibration scenarios) comprised of a clade with the Atlantic species *T. picta* (A. E. Verrill 1901) and *T. hopei* and the Indo-West Pacific species *T. livida* (Baba 1955), *T. indopacifica* (Gosliner 1995), and *T. neona* (Gosliner 1995). Finally, clade D (PP = 1 in S1 and S3; PP = 0.99 in S2) contained the Indo-West Pacific species *T. decorata* (Heller and Thompson 1983), *T. moebii* (Bergh 1888), *T. undula* (Gosliner 1995), *T. lineolata* (Bergh 1905) and the *T. gracilis* species-complex (Figure 3).

3.2 | Divergence Times and Rates of Evolution

All estimates for the origin of *Thuridilla* across the three scenarios were similar (±3 My): mean divergence time estimates since the last common ancestor were 16.62 Mya (S1), 14.38 Mya (S2) and 17.08 Mya (S3), all during the Burdigalian and Langhian stages of the Miocene (Figure 3, Table 3). By all three methods, clade A was estimated to be older than clades B–D, which were all estimated to originate 8–10 Mya (Table 4). In the three proposed scenarios, the most recent divergence times estimated were obtained in S2, followed by S1 and S3, respectively.

Divergence between Atlantic and Indo-West Pacific lineages occurred in two main temporal periods. Mean height estimates were centred in the Miocene for *T. mazda* (ATL) and its sister group, consisting of five taxa from the IWP, ranging between 20.19–8.18 Mya (S1) (Table 4: node 2); as well as the group consisting of *T. neona* (WP) and the sister pair *T. picta* (WA) and *T. hopei* (EA) between 10.35–3.31 Mya (S1) (Table 4: node 11). The mean height was centred in the Pliocene for the sister pair *T. carlsoni* (IWP) and *T. malaquita* (WA), between 7.66–2.02 Mya (S1) (Table 4: node 4). Under the two other scenarios tested, the results were largely consistent, varying only by ±1 My and ±2 My; except in the split between *T. mazda* and its sister group with higher differences in the maximum value of the range to ±5 My.

Speciation events within the *T. gracilis* species-complex, with its 13 Indo-West Pacific lineages, fell mostly within Plio-Pleistocene times ranging between 0.83 and 7.15 Mya (S1), 0.75 and 5.81 Mya (S2) and 0.85 and 7.23 Mya (S3) (Table 4: nodes 16, 19).

The average rates of evolution for the COI gene in *Thuridilla* across the three scenarios were 1.22% Myr^{-1} (S1), 1.39% Myr^{-1} (S2) and 1.42% Myr^{-1} (S3). This is in line with rates found by several authors for other gastropods using similar calibration approaches (Marko 2002; Frey and Vermeij 2008; Wilke et al. 2009; Marko et al. 2014).

3.3 | Biogeographical Patterns and Geographical Distribution

Phylogeographic analyses of *Thuridilla* conducted on the three distinct evolutionary scenarios yielded similar results. The geographical distributions of relevant *Thuridilla* clades are shown together with their phylogenetic relationships inferred under S1 (Figure 4). The Indo-Pacific holds the highest diversity of *Thuridilla* with 24 species. Ten are distributed across the Indo-West Pacific, five are restricted to the Indian Ocean, and two species are only known from the Western Indian Ocean (*T. virgata*, *Thuridilla* sp. 9); one species is endemic to the Red Sea (*T. decorata*), and *T. coerulea* occurs only in the Eastern Indian Ocean. Fourteen species are present in the Western Pacific, while the most specious region is the EIT with 18 species,

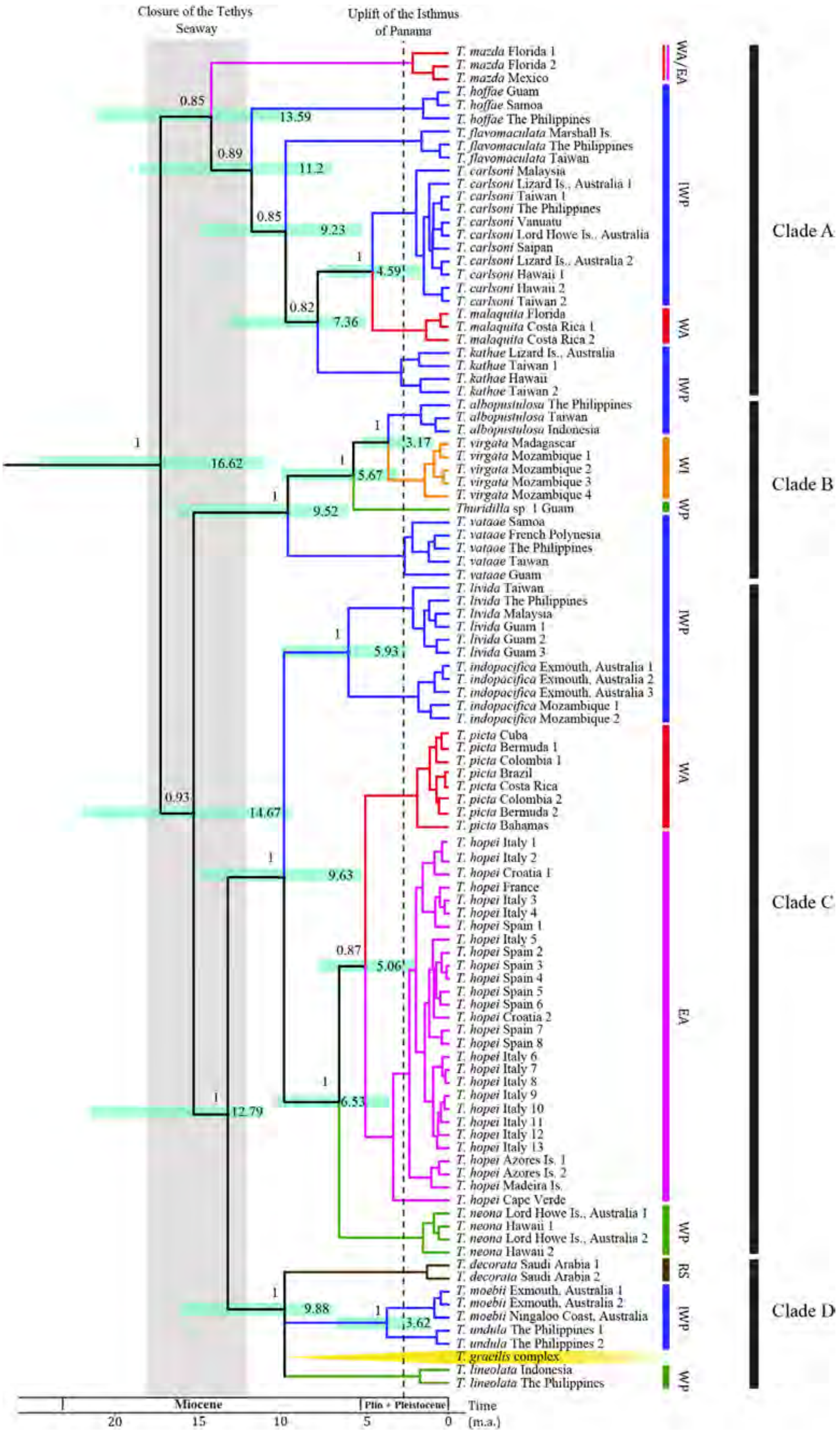

FIGURE 3 | Time-calibrated worldwide phylogenetic tree of *Thuridilla* produced by divergence times using a relaxed uncorrelated lognormal molecular clock in BEAST for the concatenated dataset under the calibration scenario S1. Values above branch labels show posterior probabilities, blue bars represent 95% highest posterior density (HPD) intervals and values inside the blue bars show median ages of supported nodes. The outgroup was pruned from the tree for clarity. Colours of clades are based on geographical distributions. EA, Eastern Atlantic; IWP, Indo-West Pacific; RS, Red Sea; WA, Western Atlantic; WI, Western Indian; WP, Western Pacific.

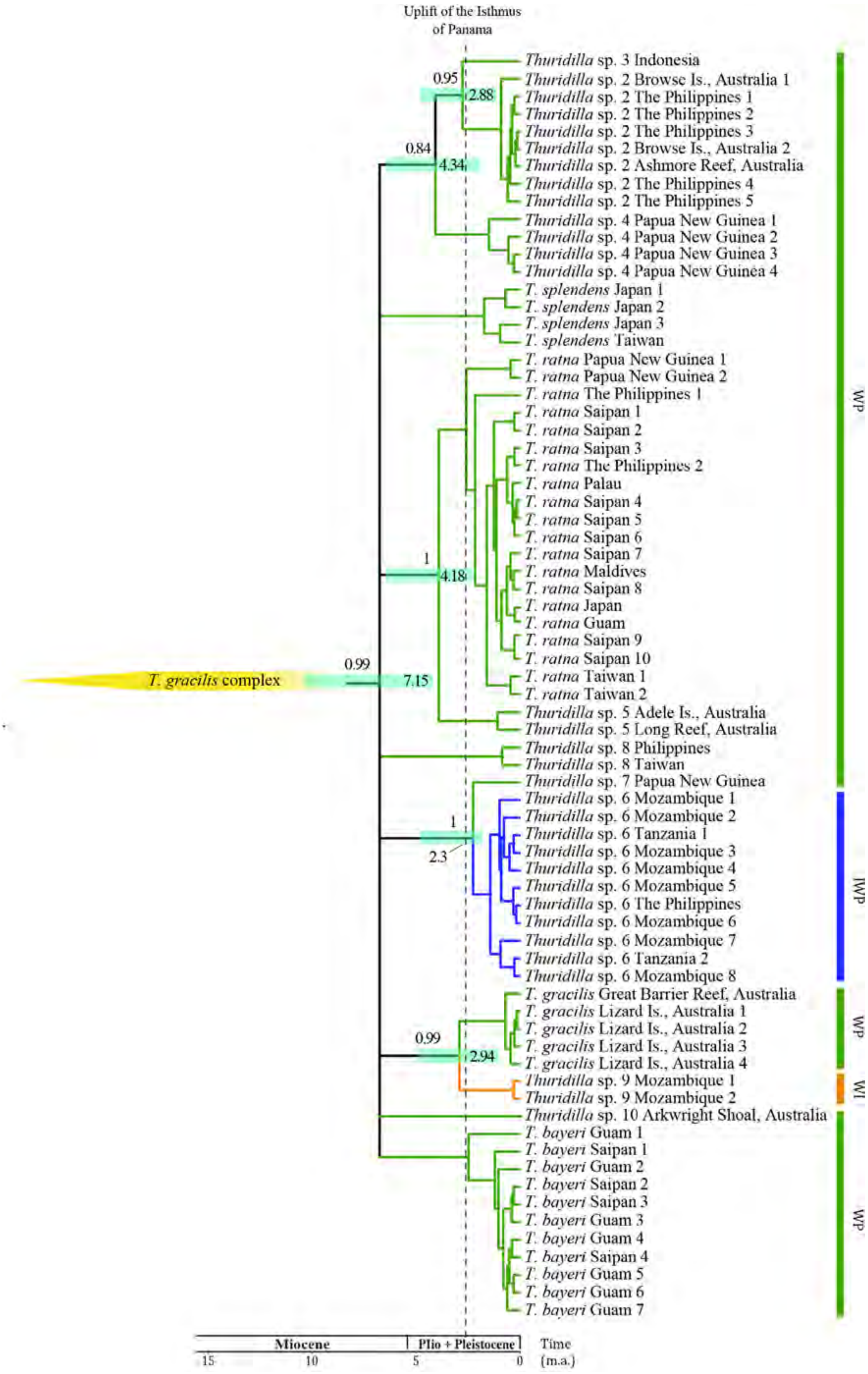

FIGURE 3 | (Continued)

TABLE 3 | List of mean divergence times for sister species pairs in millions of years ago (Mya) with upper and lower limits of 95% HPD obtained in the analyses with BEAST of the genus *Thuridilla* following the three different calibration scenarios specified in Section 2.2 *Phylogenetic analyses and estimate of divergence times.*

Sister pair	Scenario	Mean height (Mya)	HPD 95% (Mya)	
			Min	Max
T. carlsoni–T. malaquita	1	4.59	2.07	7.66
	2	4.03	1.82	6.61
	3	4.72	2.25	7.55
T. livida–T. indopacifica	1	5.93	2.70	9.73
	2	5.18	2.62	7.99
	3	6.12	2.90	9.91
T. hopei–T. picta	1	5.06	2.40	8.09
	2	4.36	2.34	6.68
	3	5.18	2.63	8.26
T. gracilis–Thuridilla sp. 9	1	2.94	1.01	5.29
	2	2.59	0.96	4.52
	3	3.08	1.09	5.42
Thuridilla sp. 7–*Thuridilla* sp. 6	1	2.30	0.83	4.14
	2	2.23	0.80	3.95
	3	2.61	0.91	4.63
Thuridilla sp. 3–*Thuridilla* sp. 2	1	2.88	1.01	5.14
	2	—	—	—
	3	—	—	—
T. ratna–Thuridilla sp. 5	1	4.18	1.91	6.88
	2	3.55	1.86	5.51
	3	4.23	2.01	6.78
T. moebii–T. undula	1	3.62	1.11	6.61
	2	3.13	1.07	5.58
	3	3.69	1.27	6.70
T. albopustulosa–T. virgata	1	3.17	1.21	5.58
	2	2.75	1.13	4.61
	3	3.27	1.27	5.64

resulting from the overlapping of species restricted to the WP with species broadly distributed across the IWP (Table 2). The Atlantic Ocean is comparatively poorer with only four species: *T. picta* and *T. malaquita* from the Western Atlantic, *T. hopei* from the Eastern Atlantic including the Mediterranean Sea, and *T. mazda* with an amphi-Atlantic distribution. There are no species known from the eastern Pacific (Figure 1).

Well supported pairs of sister species (PP ≥0.90) are all allopatric or nearly allopatric, including those in the *T. gracilis* species-complex (Figure 3, Table 2). *Thuridilla* is hypothesized to have originated in the IWP (S1; probability [Pr] = 0.72; Table 5; probabilities varied by ±0.02 or less under scenarios S2 and S3).

In addition, the Indo-West Pacific was supported as the biogeographic region where the ancestral lineages that led to sister relationships between Atlantic and Indo-West Pacific species originated (nodes 2, 4, 9, 11; Figure 5). The exception was the sister pair *T. hopei* and *T. picta* (node 12) since the analysis suggests that these two species most likely shared an Atlantic ancestor either from the EA (Pr = 0.36) or the WA (Pr = 0.27) according to S1 (probabilities were the same in S2 and varied by ±0.01 in S3).

Otherwise, node 13 representing the ancestor of 17 species (*T. decorata*, *T. moebii*, *T. undula*, *T. lineonata* and *T. gracilis* complex) is inferred to have a WP origin (Pr = 0.72 in S1, and ± 0.01 in the other two methods). The same origin was inferred for the ancestor of the *T. gracilis* complex (node 15) and sister lineages within all three evolutionary scenarios (see probabilities in Table 5).

4 | Discussion

4.1 | Divergence Times on the Origin and Evolution of *Thuridilla*

During recent years, several molecular phylogenetic studies have suggested a mostly Cretaceous or Upper Jurassic origin of the Sacoglossa (84.8 Mya by Dinapoli and Klussmann-Kolb 2010; 192.79 Mya by Jörger et al. 2010; 123.26 Mya by Jörger et al. 2014; 147 Mya by Ayyagari and Sreerama 2019). In our study, the origin of Sacoglossa is estimated between 70 and 45 Mya, with a mean age of 52.91 Mya (Figure 2) more or less coinciding with the transition between the Mesozoic and Cenozoic eras, a period marked by significant extinction and cladogenesis processes on Earth (e.g., Signor 1990; Bambach et al. 2004; Stanley 2007; Benton 2009; Neubauer 2024). Nevertheless, this estimate is almost twice as old as the oldest fossil known of Sacoglossa (*Berthelinia elegans* from the Middle Eocene of the Paris Basin between 37.8–47.8 Mya; Keen and Smith 1961), but this is not surprising since fossils only provide minimum ages, and the fossil record of sacoglossans is most certainly a misrepresentation of their ancestral diversity because of the soft-bodied nature of these animals and, when present, fragile shells of difficult fossilisation.

Jörger et al. (2010) suggested an age approximately between 80 and 25 Mya for the split between the genera *Elysia* and *Thuridilla*, with a mean age estimate of 27.02 Mya. Our estimates for the origin of *Thuridilla* across the three evaluated scenarios are younger ranging between 23.65 and 10.80 Mya with mean age estimates between 17.08 and 14.38 Mya during the Early Miocene epoch, a period of tectonic rearrangements and warming climate known to have created opportunities for diversification (Steinthorsdottir et al. 2021; see below for discussion).

Potential limitations in our methods include dating old splits using a phylogeny based on three genes, which makes it hard to recover with precision the age of older speciation events due to limited data and possible saturation. The use of a single

TABLE 4 | List of mean divergence times for outstanding nodes 1, 2, 11, 15 and sister species pairs in millions of years ago (Mya) with upper and lower limits of 95% HPD and posterior probability based on the three different calibration scenarios specified in Section 2.2 *Phylogenetic analyses and estimate of divergence times*.

Node number (see Figure 4)	Sister pair	Scenario	Posterior probability	Mean height (Mya)	HPD 95% (Mya)	
					Min	Max
1	Origin of *Thuridilla*	1	1.00	16.62	12.40	22.90
		2	1.00	14.38	10.80	17.91
		3	1.00	17.08	11.34	23.65
2	------	1	0.85	13.59	8.18	20.19
		2	0.88	11.46	7.17	15.63
		3	1.00	14.11	10.40	17.68
4	*T. carlsoni–T. malaquita*	1	1.00	4.59	2.02	7.66
		2	0.99	4.03	1.82	6.61
		3	1.00	4.72	2.25	7.55
5	------	1	1.00	9.52	4.94	14.83
		2	1.00	8.06	4.55	13.73
		3	1.00	9.85	4.94	15.13
7	*T. albopustulosa–T. virgata*	1	1.00	3.17	1.21	5.58
		2	0.99	2.75	1.13	4.61
		3	1.00	3.27	1.27	5.64
9	------	1	1.00	9.63	5.51	14.52
		2	1.00	8.39	5.26	11.57
		3	1.00	10.04	5.54	14.68
10	*T. livida–T. indopacifica*	1	1.00	5.93	2.70	9.73
		2	1.00	5.18	2.62	7.99
		3	1.00	6.12	2.90	9.91
11	------	1	1.00	6.53	3.31	10.35
		2	1.00	5.64	3.14	8.36
		3	1.00	6.74	3.40	10.43
12	*T. hopei–T. picta*	1	0.87	5.06	2.40	8.09
		2	0.87	4.36	2.34	6.68
		3	0.89	5.18	2.63	8.26
13	------	1	1.00	9.88	5.91	14.70
		2	0.99	8.51	5.53	11.67
		3	1.00	9.98	5.92	14.38
14	*T. moebii–T. undula*	1	1.00	3.62	1.11	6.61
		2	1.00	3.13	1.07	5.58
		3	1.00	3.69	1.27	6.70

(Continues)

TABLE 4 | (Continued)

Node number (see Figure 4)	Sister pair	Scenario	Posterior probability	Mean height (Mya)	HPD 95% (Mya)	
					Min	Max
15	------	1	1.00	7.15	4.07	10.90
		2	0.99	6.15	3.86	8.66
		3	1.00	7.28	4.15	10.70
16	------	1	0.84	4.34	1.95	7.15
		2	0.90	3.70	1.83	5.81
		3	0.88	4.43	2.01	7.23
17	*Thuridilla* sp. 3–*Thuridilla* sp. 2	1	0.95	2.88	1.01	5.14
		2	—	—	—	—
		3	—	—	—	—
18	*T. ratna*–*Thuridilla* sp. 5	1	1.00	4.18	1.91	6.88
		2	1.00	3.55	1.86	5.51
		3	1.00	4.23	2.01	6.78
19	*Thuridilla* sp. 7–*Thuridilla* sp. 6	1	1.00	2.30	0.83	4.14
		2	1.00	1.99	0.75	3.42
		3	1.00	2.31	0.85	4.04
20	*T. gracilis*–*Thuridilla* sp. 9	1	0.99	2.94	1.01	5.29
		2	0.99	2.59	0.96	4.52
		3	0.99	3.08	1.09	5.42

calibration point may also lead to misestimates of rates and ages, as well as outgroup selection. Nonetheless, the estimates for the ages of clades appear reasonable and consistent with expectations. The fact that the various methods used yield similar estimates suggests robustness and reliability in our results.

4.2 | Global Patterns of Distribution and Diversity in *Thuridilla*

Thuridilla slugs are distributed in tropical and temperate waters around the globe but are notably absent in the eastern Pacific coastlines of the American continents. The lack of species of the genus in the eastern Pacific is difficult to explain because the biotic characteristics of the coastal areas are not so distinct from other world temperate and tropical regions with availability of algal habitats where it would be expected to find *Thuridilla*. Likely this is the result of extinction of ancestral lineages in connection with processes related to the closure of the Panamanian Seaway. Significant faunal extinction associated with the uplift of the Panamanian isthmus has been documented in several groups of marine invertebrates through fossil records (Lessios 2008; Leigh et al. 2013; Medrano et al. 2019).

The East Pacific is in general depauperate in sacoglossans, and the lineages that inhabit the area are recently derived from Western Pacific or Hawaiian lineages (Krug et al. 2016; Medrano et al. 2019). There are several other groups of heterobranch slugs with a 'global' distribution but absent in the eastern Pacific. A few examples are the sacoglossan genera *Volvatella* and *Costasiella* (Jensen 2007), the headshield slugs of the genera *Aglaja*, *Chelidonura*, *Mariaglaja* (Zamora-Silva and Malaquias 2018) and the nudibranch genus *Hexabranchus* (Tibiriçá et al. 2023).

The Western Pacific is home to the greatest diversity of *Thuridilla* species, over three times higher than other worldwide marine realms, such as the Atlantic Ocean, where only four species occur.

4.3 | Tethyan Vicariance and Dispersal Across Oceans

Neither Atlantic (ATL) nor Indo-West Pacific species assemblages were recovered as monophyletic radiations; instead, there were three dispersal events between these regions. A similar evolutionary pattern was documented for other heterobranch taxa (Johnson and Gosliner 2012 for *Hypselodoris*, *Glossodoris*, *Mexichromis* and *Chromodoris*; Krug et al. 2018 for *Placida*; Medrano et al. 2019 for *Polybranchia*).

Our reconstruction of ancestral distributions suggests not only an IWP origin of *Thuridilla* between 22.90 and 12.40 Mya around the closure of the Tethyan Sea, but also an IWP origin for two ancestral lineages that led to sister relationships between

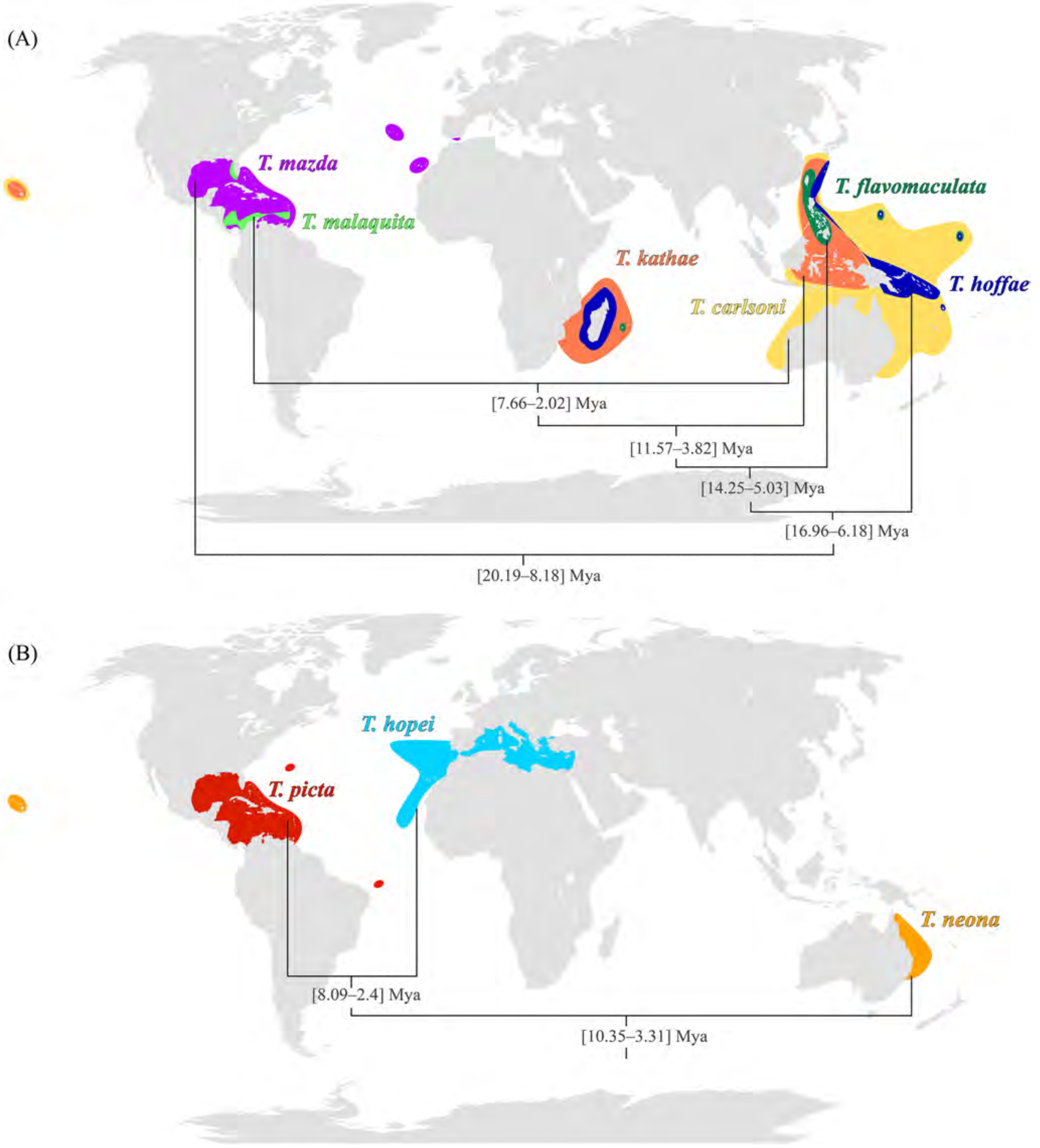

FIGURE 4 | Geographical distributions and phylogenetic relationships of Atlantic-Indo-Pacific *Thuridilla* species. (A) Clade with species *T. mazda*, *T. malaquita, T. kathae, T. carlsoni, T. flavomaculata* and *T. hoffae*; (B) clade with species *T. picta*, *T. hopei* and *T. neona*; and (C) clade with species of *T. gracilis* complex.

ATL and IWP extant species. The first divergence between ATL and IWP lineages took place between 20.19 and 8.18 Mya and therefore is partially compatible with vicariance associated with the closure of the Tethys seaway around 18–12 Mya (Hou and Li 2018) that isolated populations in each realm leading to speciation (Steeman et al. 2009; Cowman 2014; Schiffer and Herbig 2016).

The divergence of the ancestral lineage between *T. carlsoni* and *T. malaquita* occurred more recently after the closure of the Tethys seaway between 7.66 and 2.02 Mya. Since ancestral geographic reconstruction supported an origin in the IWP of the most recent common ancestor of these radiations, one hypothesis for the observed phylogenetic pattern is dispersal around South Africa, with establishment of lineages in the Atlantic

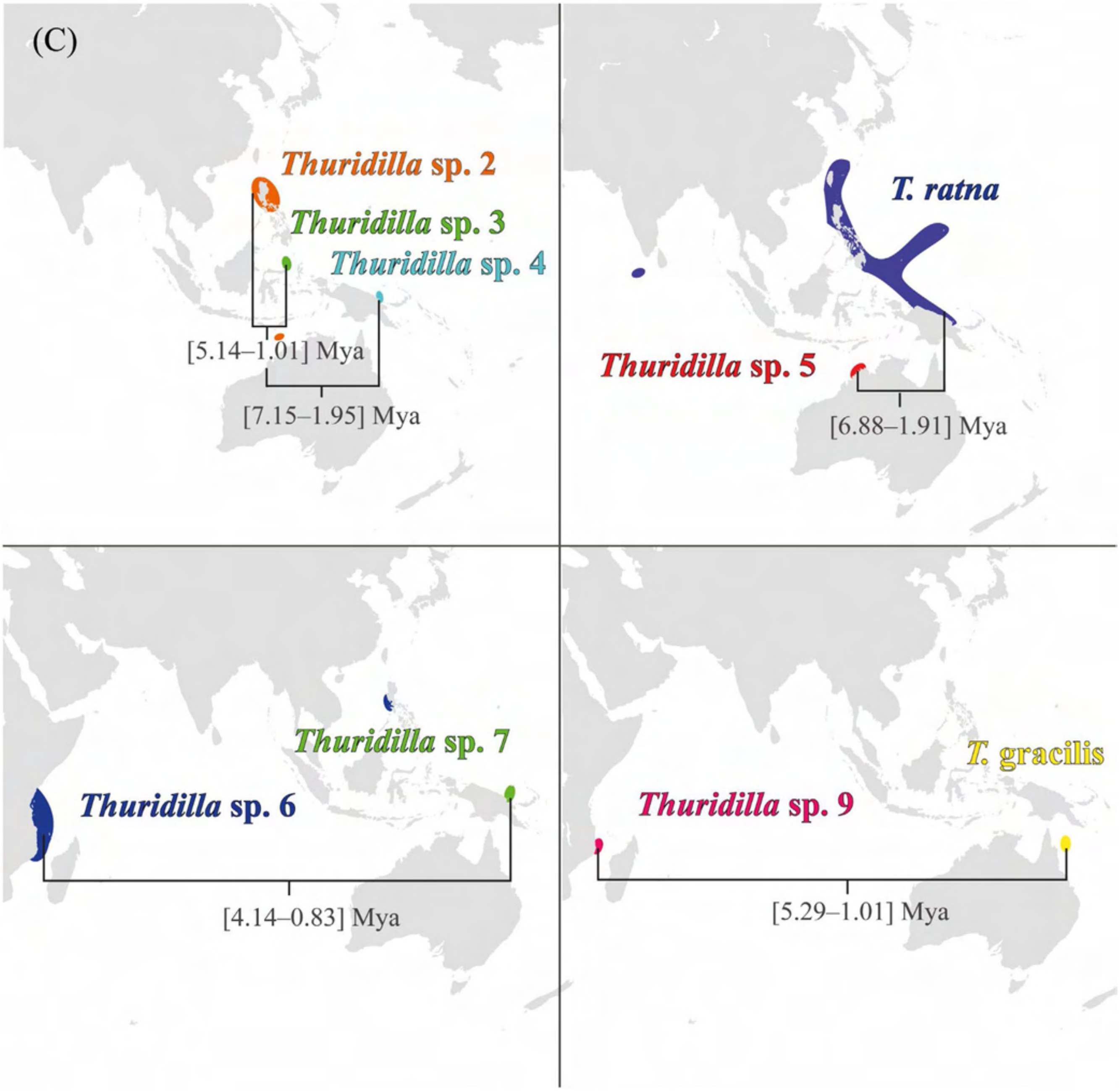

FIGURE 4 | (Continued)

Ocean. Alternatively, long-distance dispersal across the eastern Pacific Ocean and Central America may have occurred before the closure of the Isthmus of Panama, which remained open and permitted larval transport until about 3 Mya (O'Dea et al. 2016). These hypotheses of dispersal followed by allopatric speciation are supported by planktotrophic larval development in some *Thuridilla* species (Jensen 2001; Krug et al. 2015; Krug, pers. comm.), which would allow larvae to travel long distances and successfully establish in remote locations. The Benguela current off Namibia and South Africa was established around 10 Mya (Siesser 1980), isolating the tropical fauna of the IWP and the Atlantic by creating a thermal barrier for larval dispersal. Yet, the Benguela current was disrupted several times during warmer periods of the late Pliocene and Pleistocene, creating opportunities for inter-oceanic dispersal as documented for several molluscs based on the fossil record and morphology (Vermeij and Rosenberg 1993) and for fish based on molecular data (Rocha et al. 2005; Floeter et al. 2008).

Speciation between *T. neona* (WP) and the sister pair *T. hopei*–*T. picta* (ATL) between 10.35–3.31 Mya mostly predates the closure of the Isthmus of Panama and the Plio-Pleistocene epochs; this split is therefore more compatible with long-distance dispersal of larvae across the Eastern Pacific Barrier and invasion of the Atlantic with subsequent speciation. The Eastern Pacific Barrier, the vast span of water between the islands of French Polynesia, Hawaii and the American continent, is considered a nearly insurmountable barrier for dispersal of marine

TABLE 5 | Summary of node data probabilities in defined regions estimated with BEAST following the three different calibration scenarios specified in Section 2.2. *Phylogenetic analyses and estimate of divergence times*.

		Node data probabilities							
Node number (see Figure 4)	**Scenario**	**IWP**	**WP**	**WA**	**EA**	**ATL**	**WI**	**IO**	**RS**
1	1	0.72	0.28	0	0	0	0	0	0
	2	0.73	0.27	0	0	0	0	0	0
	3	0.74	0.26	0	0	0	0	0	0
2	1	0.87	0.12	0	0	0.01	0	0	0
	2	0.88	0.11	0	0	0.01	0	0	0
	3	0.87	0.12	0	0	0.01	0	0	0
3	1	0.99	0.01	0	0	0	0	0	0
	2	0.99	0.01	0	0	0	0	0	0
	3	0.99	0.01	0	0	0	0	0	0
4	1	0.99	0	0.01	0	0	0	0	0
	2	0.99	0	0.01	0	0	0	0	0
	3	0.99	0	0.01	0	0	0	0	0
5	1	0.85	0.15	0	0	0	0	0	0
	2	0.86	0.14	0	0	0	0	0	0
	3	0.86	0.14	0	0	0	0	0	0
6	1	0.82	0.17	0	0	0	0.01	0	0
	2	0.83	0.16	0	0	0	0.01	0	0
	3	0.83	0.16	0	0	0	0.01	0	0
7	1	0.89	0.08	0	0	0	0.03	0	0
	2	0.89	0.08	0	0	0	0.03	0	0
	3	0.89	0.08	0	0	0	0.03	0	0
8	1	0.57	0.43	0	0	0	0	0	0
	2	0.60	0.40	0	0	0	0	0	0
	3	0.61	0.39	0	0	0	0	0	0
9	1	0.58	0.41	0	0	0	0	0.01	0
	2	0.60	0.39	0	0	0	0	0.01	0
	3	0.61	0.38	0	0	0	0	0.01	0
10	1	0.75	0.18	0	0	0	0	0.07	0
	2	0.76	0.17	0	0	0	0	0.07	0
	3	0.76	0.17	0	0	0	0	0.07	0
11	1	0.34	0.54	0.06	0.06	0	0	0	0
	2	0.36	0.52	0.06	0.06	0	0	0	0
	3	0.36	0.51	0.06	0.06	0	0	0.01	0
12	1	0.16	0.21	0.27	0.36	0	0	0	0
	2	0.17	0.20	0.27	0.36	0	0	0	0
	3	0.17	0.20	0.27	0.35	0	0	0.01	0

(Continues)

TABLE 5 | (Continued)

Node number (see Figure 4)	Scenario	Node data probabilities							
		IWP	WP	WA	EA	ATL	WI	IO	RS
13	1	0.28	0.72	0	0	0	0	0	0
	2	0.29	0.71	0	0	0	0	0	0
	3	0.27	0.73	0	0	0	0	0	0
14	1	0.65	0.20	0	0	0	0	0.14	0.01
	2	0.65	0.20	0	0	0	0	0.14	0.01
	3	0.64	0.20	0	0	0	0	0.15	0.01
15	1	0	1.00	0	0	0	0	0	0
	2	0	1.00	0	0	0	0	0	0
	3	0	1.00	0	0	0	0	0	0
16	1	0	1.00	0	0	0	0	0	0
	2	0	1.00	0	0	0	0	0	0
	3	0	1.00	0	0	0	0	0	0
17	1	0	1.00	0	0	0	0	0	0
	2	0	1.00	0	0	0	0	0	0
	3	0	1.00	0	0	0	0	0	0
18	1	0	1.00	0	0	0	0	0	0
	2	0	1.00	0	0	0	0	0	0
	3	0	1.00	0	0	0	0	0	0
19	1	0.05	0.95	0	0	0	0	0	0
	2	0.04	0.96	0	0	0	0	0	0
	3	0.04	0.96	0	0	0	0	0	0
20	1	0	0.99	0	0	0	0.01	0	0
	2	0	0.99	0	0	0	0.01	0	0
	3	0	0.99	0	0	0	0.01	0	0

Abbreviations: ATL, Atlantic Ocean; EA, Eastern Atlantic including the Mediterranean Sea; IO, Indian Ocean; IWP, Indo-West Pacific; RS, Red Sea; WA, Western Atlantic; WI, Western Indian; WP, Western Pacific.

benthic invertebrates (Grigg and Hey 1992), but, under special circumstances, such as climatic phenomena like El Niño, stochastic dispersal is more prone to take place and has been documented, for example, in some crustaceans (Hickman and Zimmerman 2000), echinoderms (Lessios et al. 1998) and molluscs (Kay 1991; Emerson and Chaney 1995).

Available data cannot reject either hypothesis. The fact that relationships between IWP and ATL lineages involve always extant species with a WA affinity (*T. mazda*, *T. malaquita*, *T. picta*) could favour the idea of eastern Pacific dispersal with establishment of viable populations in the proto-Caribbean province. Nevertheless, this phylogenetic pattern may be an artefact caused by extinction of some marine lineages in the eastern Atlantic after the closure of the Tethys seaway, as has been documented in corals, molluscs, and foraminiferans (F. E. Anderson 2000; Harzhauser and Piller 2007; Studencka and Jasionowski 2011). Additional possible extinction events are also suggested by *T. mazda*, the only ancient lineage from the Atlantic which survived significant environmental changes. The remaining Atlantic lineages are relatively young, which suggests possibly higher extinction and lower speciation rates in this ocean.

Our reconstruction of ancestral distributions did not resolve the origin of the clade containing the amphi-Atlantic sister species *T. hopei* (EA) and *T. picta* (WA). Speciation is inferred to have occurred at 8.09–2.40 Mya grossly during the Plio-Pleistocene and the Messinian (Late Miocene), when the width of the Atlantic was similar to the present configuration. Likely stochastic dispersal events allowed larvae to travel across the Atlantic with consequent founding of viable populations followed by speciation. The direction of dispersal is difficult to ascertain but based on empirical evidence it is known that trans-Atlantic dispersal in an eastward direction has increased since

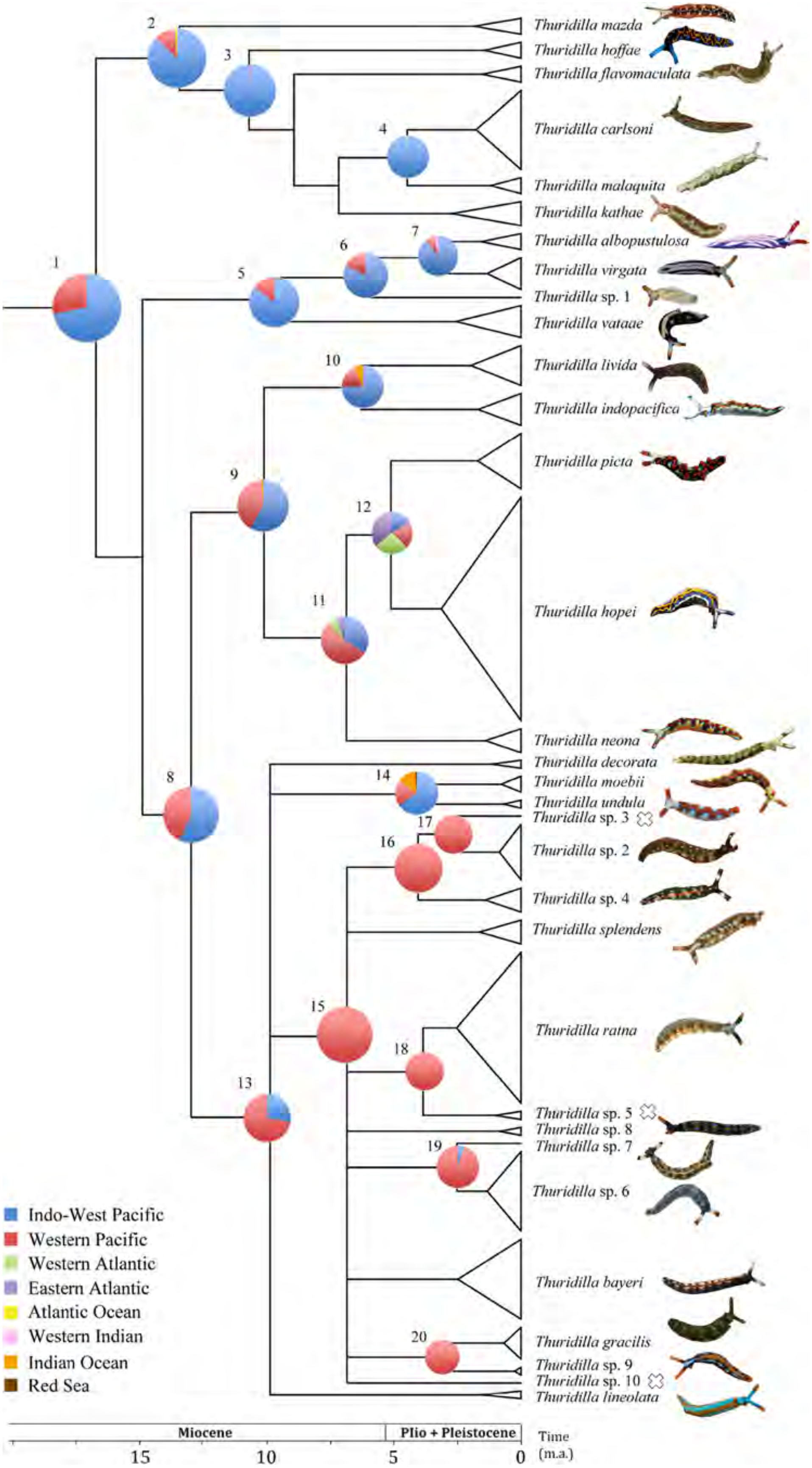

FIGURE 5 | Phylogeographic reconstruction of the ancestral distribution of *Thuridilla* using a Bayesian approach as implemented in BEAST for the concatenated dataset under the scenario 1. Pie charts at nodes represent the most likely distribution areas of the most recent common ancestor (MRCA). Colours of the pie charts for the different geographical regions are provided in the figure. Crosses next to species names indicate no pictures available.

the middle Pliocene, during the final closure of the Panamanian Isthmus and strengthening of the Gulf Stream (Vermeij and Rosenberg 1993; Briggs 2003; Malaquias and Reid 2009).

4.4 | *Thuridilla gracilis Species*-Complex: An Example of a Recent Radiation

The closure of the Tethys seaway not only separated the ATL and IWP marine realms but also the closure of the Indonesian deep-water flow between Southeast Asia and Australia between 25 and 22 Mya (Hall 2009) promoting a rapid speciation in the IWP (Williams 2007; Frey and Vermeij 2008; Tian et al. 2024). Therefore, the spatial pattern of diversity observed in the genus can be attributed to a combination of historical biogeographic events and ecological factors (e.g., variation in environmental conditions such as temperature, salinity, food availability, etc.) which in the Western Pacific region have provided a range of ecological niches for *Thuridilla* species to evolve and diversify over time. Consequently, species have exploited new environments, resources, or ecological roles, resulting in increased diversity. Thus, the influence of sea-level changes associated with Plio-Pleistocene climatic oscillations, particularly in the EIT, contributed to high rates of speciation, making this area the richest marine biodiversity hotspot in the world (e.g., Palumbi 1997; Vermeij 2001; Bellwood and Wainwright 2002; Briggs 2003; Kool et al. 2011; Gaither and Rocha 2013). The Plio-Pleistocene eustatic cycles contributed during colder periods to broaden the continental area and to restrict seaways between the Indian and Pacific basins, noticeably within the EIT itself, promoting opportunities for transient allopatry, isolation and speciation (McManus 1985; Hewitt 2000; Wallace et al. 2000; Williams and Reid 2004; Kool et al. 2011).

The *Thuridilla gracilis* complex provides several examples of the potential role of the Plio-Pleistocene eustatic cycles in diversification within the EIT, supporting this region as a centre of origin. For the radiation formed by *Thuridilla* sp.2, *Thuridilla* sp.3 and *Thuridilla* sp.4, all three species are only known from the EIT. In the sister pair *T. ratna* and *Thuridilla* sp.5, both species extend their ranges slightly beyond the strict boundaries of the EIT. Such extended distributions could result from the southwards Indonesian Throughflow (Sprintall 2009) and northwards Kuroshio Current (Qiu 2009), which facilitate dispersal of larvae to tropical and sub-tropical peripheral regions of the EIT, such as Taiwan and southern Japan (e.g., *T. ratna*) and Adele Island Long Reef in northwestern Australia (e.g., *Thuridilla* sp.5). The divergence between *Thuridilla* sp.6 and *Thuridilla* sp.7 likely took place within the EIT, followed by geographical range expansion through dispersal of larvae of *Thuridilla* sp.6 to the Western Indian Ocean. In contrast, the sister relationship between *T. gracilis* (EIT and several western Pacific localities) and *Thuridilla* sp.9 (Western Indian Ocean) suggests stochastic long-distance dispersal to the Western Indian Ocean, with Indian and Pacific populations speciating allopatrically around the Pliocene–Pleistocene transition.

The remarkable radiation of the *T. gracilis* species-complex seems to be explained by small-scale geographical isolation probably related to transient allopatry caused by Pleistocene climatic oscillations. This micro-allopatry, together with the plethora of habitats and oceanographic conditions within the EIT (currents and productivity regimes) provided the conditions for populations to become isolated, leading to divergent evolutionary paths and the formation of new species. Additionally, repeated vicariant events driven by long-distance dispersal from the EIT to the western Indian Ocean also played an important role. These vicariant events caused populations to become geographically separated, further promoting diversification and contributing to the radiation observed in the complex. This rapid radiation of the *T. gracilis* complex contrasts sharply with the diversification observed in remaining species within the genus *Thuridilla*. Thus, the synergy between micro-allopatric speciation and long-distance dispersal events seemed to have exceptionally accelerated the evolutionary trajectory of this particular complex.

5 | Concluding Remarks

Thuridilla is hypothesized to have an Indo-West Pacific origin between 23.65 and 10.80 Mya during the Early Miocene. Presently, these slugs are distributed across tropical and temperate latitudes of the Atlantic Ocean and tropical areas of the IWP but are absent in the eastern Pacific coastlines of the American continent, likely due to extinction of ancestral lineages related to geological and biological events connected to the uplift of the Isthmus of Panama. The EIT (18 spp.) and the WP (14 spp.) are the regions of highest diversity, whereas the Atlantic is depauperate of species, with only four lineages occurring in this realm.

In the IWP, speciation events are dominated by allopatric processes connected with pulses of transient allopatry associated with Plio-Pleistocene sea-level fluctuations, which have been particularly relevant in the diversification of species within the EIT and peripheral areas, suggesting this region to be a centre of origin for these slugs. Long-distance dispersal from the western Pacific across the Indian Ocean has also been responsible for allopatric speciation in the Western Indian Ocean.

The diversity of the Atlantic Ocean is hypothesized to be derived from Miocene vicariant events associated with the closure of the Tethys Sea that left ancestral lineages isolated in this realm and with dispersal across South Africa or long-distance dispersal across the East Pacific Barrier prior to the uplift of the Isthmus of Panama that has fed the Western Atlantic with lineages of these slugs.

Author Contributions

Manuel António E. Malaquias: conceptualised the work and contributed to the experimental design and writing the manuscript. J. Lucas Cervera: responsible for tissue sample collection and secured funding. Terence Gosliner: coordinated field expeditions, including sample collection and secured funding. Patrick J. Krug: coordinated field expeditions, including sample collection and reviewed the biogeographic analyses. Leila Carmona: conducted laboratory processing and species identification. M. Rosario Martín-Hervás: performed the literature review, molecular work, analyses and writing of the manuscript. All authors participated in revising the manuscript and approved the final version.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.