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Molecular phylogeny and divergence time estimates for native giant clams (Cardiidae: Tridacninae) in the Asia-Pacific: Evidence from mitochondrial genomes and nuclear 18S rRNA genes

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Giant clams are conspicuous bivalves that inhabit in coral reefs. Among the giant clams, eight species of subfamily Tridacninae are the most common in the Asia-Pacific. However, very little is known about their evolutionary history. Here, we determined the complete mitochondria genome of *Hippopus porcellanus*, which was 29,434 bp in size and contained 13 protein-coding genes, 2 rRNAs and 23 tRNAs. The A+T composition of protein-coding regions was 57.99%, and the AT composition of the 3rd codon position was 59.33%, of which in agreement with the invertebrate bias favoring codons ending in A or T. Analysis of phylogenetic relationships according to the concatenated nucleotide data set containing 18S rRNA gene and 13 protein-coding genes, the phylogenetic relationship was analyzed by Maximum likelihood and Bayesian inference methods. The results showed that *T. maxima* was placed with the clade comprising *T. noae*, *T. squamosa*, and *T. crocea*, in which *T. squamosa* was highly similar to *T. crocea* and is consistent with the results of the previous studies using15 mitochondrial markers and nuclear 18S rRNA. Moreover, the inferred divergence time of Tridacnidae species is generally consistent with the fossil record of Tridacnidae. The divergence time of *H. porcellanus* and *H. hippopus* was about 10.64 Mya, this result is in agreement with the speculation that *H. porcellanus* also originated in Miocene. The availability of molecular phylogeny and divergence time estimation provides

information genetic relationship of Tridacninae, which could be helpful to the ecological research and conservation of giant clams.

KEYWORDS

Tridacninae, mitochondrial genome, 18S, gene arrangement, phylogeny, molecular clock

Introduction

Molecular phylogenetic analysis is an ideal method to study the relationship among species (Sigwart and Sutton, 2007; Smith et al., 2011; Kocot et al., 2011). Mitochondrial genomes are characterized by high evolution rate, conserved gene components (Curole and Kocher, 1999), rare recombination (Boore, 1999), and maternal inheritance (Barr et al., 2005). Now mitochondrial genomes have been extensively used for studying phylogenetic relationships at various taxonomic levels (Yamanoue et al., 2007; Yokobori et al., 2007).

In the animal kingdom, mollusca is the second most species-rich phylum, and there are great variations in their mitochondrial genomes. Six bivalve families (Veneridae, Mytilidae, Donacidae, Unionidae, Hyriidae and Margaritiferidae) are known to possess an unusual mtDNA inheritance mode (doubly uniparental inheritance) (Theologidis et al., 2008). Similar to mitochondrial tRNAs of nematode, tRNAs of some pulmonate gastropods also lacks T-stem or D-stem (Yamazaki et al., 1997). Unlike metazoans, the mitochondrial genomes of most molluscs, especially Scaphopoda and Bivalves, contain a large number of rearrangements (Serb and Lydeard, 2003). Several specific mitochondrial genomic features have also been found in the published mitochondria genomes of Tridacninae species. The mitogenome gene order of *Tridacna squamosa* is different from *Fulvia mutica* and *Acanthocardia tuberculate*, the other species of the Cardioidea (Gan et al., 2016). It is worth noting that the mitochondrial gene order of *Tridacna crocea* and *Tridacna squamosa* is different from that of other Tridacninae species (Tan et al., 2021).

Giant clams (Cardiidae: Tridacninae) are conspicuous bivalves that inhabit in coral reefs (Yonge, 1975). The adults of the smallest giant clam species, *Tridacna crocea* are about 15 cm long, while the largest giant clam species, *Tridacna gigas*, can grow longer than 1 m and weigh more than 300 kg (Rosewater, 1965). Giant clams not only play an important ecological role in coral reef ecosystems, but also become an important food source for coastal communities in Asia and the South Pacific, and make a significantly contribution to shell and aquarium trade (Keys and Healy, 1999). Overfishing is a major threat to giant clams and caused the extinction of a few larger giant clam species (Juinio et al., 1989). Giant clams are currently listed as vulnerable in the International Union for Conservation of Nature (IUCN) list. According to Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (Wells et al., 1983; Tisdell and Menz, 1992; Tisdell et al., 1994), the trade of giant clams is strictly regulated, in which only the trade of certified aquaculture products is allowed, and the trade of wildlife products is not allowed (Bell et al., 1997).

All eight species of Tridacninae giant clams [two *Hippopus* (*H. porcellanus* and *H. hippopus*) and six *Tridacna* (*T. crocea*, *T. derasa*, *T. maxima*, *T. gigas*, *T. noae* and *T. squamosa*)] are distributed along the Asia-Pacific coast (Neo et al., 2017; Zhang et al., 2020). These giant clams can be classified according to their adult size: large clams (*T. derasa* and *T. gigas*), medium clams (*H. porcellanus*, *H. hippopus*, and *T. squamosa*) and small clams (*T. crocea*, *T. noae* and *T. maxima*) (Zhou et al., 2020). Due to morphological similarity, *T. noae* was previously misidentified as a variant of *T. maxima* (McLean, 1947; Rosewater, 1965). However, recent genetic analysis found that *T. maxima* and *T. noae* are belong to different species (Su et al., 2014). Therefore, identification based on morphological characteristics sometimes caused misidentification. Recently, although molecular methods have resolved some confusion in the identification of giant clams (Schneider and Foighil, 1999; Su et al., 2014), very little is known about the evolutionary history of the Tridacninae species. Moreover, most available phylogenetic analyses were based on short DNA fragments or different genetic markers, yielding different results (Benzie and Willianm, 1998; Schneider and Foighil, 1999; Nuryanto et al., 2007; Herrera et al., 2015). Recently, although Tan et al. (2021) performed a comprehensive phylogenetic analysis of 12 giant clam species using mitochondrial genome and nuclear 18S rRNA data, this study did not deeply analyze the divergence time between Tridacninae species and lacked the mitochondrial genome of *H. porcellanus*.

In this study, we characterized the complete mitochondrial genome of *H. porcellanus*, including its structural characteristics and nucleotide composition. We also studied the gene order of giant clam mitochondrial and speculated the scenario of gene order rearrangement. Based on complete mitochondrial genomes, the divergence time among Tridacninae species was

estimated using a relaxed molecular clock calibrated with fossil evidence. Moreover, the molecular phylogeny of giant clams in the Asia-Pacific was reconstructed by using mitochondrial genome and nuclear 18S rRNA gene.

Materials and methods

Sample collection and DNA extraction

Individual of *H. porcellanus* was sampled from the South China Sea. The total genomic DNA was extracted following a modified CTAB DNA extraction protocol (Attitalla, 2011), and then stored at -80°C.

Mitochondrial DNA sequencing and genome assembly

A total amount of 1μg DNA per sample was used as input material for the DNA library preparations. Sequencing library was generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to the sample. Briefly, genomic DNA sample was fragmented by sonication to a size of 350 bp. The short fragments were then sent to the Total Genomics Solution (TGS) Institute in Shenzhen, China, and sequenced using the Illumina Novaseq 6000 sequencing system (Borgström et al., 2011).

Prior to assembly, raw reads were filtered by using NGS QC Tool Kit v2.3.3 (Patel and Jain, 2012) and 9.29GB clean data were obtained. The GC content of the clean data, Q20-value and Q30 value were 45.36%, 97.55%, and 92.95%, respectively, resulting in 232-fold depth of coverage of the mitochondrial genome, indicating that the quality of the mitochondrial genome sequencing and assembly results was very high. Complete circular assembly graph was checked and further extracted by visualization (e.g., Bandage) of the GFA graph files that were assembled from SPAdes v3.11.0 (Bankevich et al., 2012).

Genome annotation and sequence analysis

Based on the annotated *H. hippopus* mitochondrial genome with minor revisions, the exact boundary of each gene was determined. The annotation of protein-coding genes (PCGs) was conducted using MITOS (http://mitos.bioinf.uni-leipzig.de/index.py) with invertebrate mitochondrial genetic code. tRNA scan-SE 1.21 was used to identify tRNA genes (Lowe and Eddy, 1997), employing cove only search mode and invertebrate mitochondrial genetic code. The graphical map of *H. porcellanus* mitogenome was drawn using the online software of mitochondrial visualization tool OGDRAW (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html). The mitogenome sequences of *H. porcellanus* were submitted to GenBank (ON009026).

Ka_Ks calculator was used to estimate the ratio of nonsynonymous to synonymous substitution rates (Ka/Ks) of all 13 protein genes in eight giant clams (subfamily Tridacninae).

Phylogenetic analysis

Eight Tridacninae species mitochondrial genomes (including one obtained in this study) and ribosomal gene sequences were used for phylogenetic analysis (Table 1). Three Cardiidae species were used as outgroups: *Acanthocardia tuberculata* (NC_008452.1), *Cerastoderma edule* (NC_035728.1), *Fulvia mutica* (NC_022194.1). The aligned gene matrices were concatenated for phylogenetic reconstruction using Maximum Likelihood (ML) and Bayesian analysis.

MAFFT algorithm was used for codon-based nucleotide alignment (Katoh and Standley, 2013). Then, under the GTR+R6+F nucleotide substitution model, the maximum likelihood (ML) method in IQ-TREE v1.6.12 was used to reconstruct the phylogenetic tree (Nguyen et al., 2015). A bootstrapping of 1000 replicates was used to support inferred ML tree. ML analyses of nuclear 18S rRNA genes and mitochondrial PCGs were performed separately using the same parameters as above to compare the concatenated-data phylogenetic tree with mitochondrial and nuclear trees. For Bayesian analysis, based on the Bayesian information criterion (BIC), jModelTest v2.1.10 was used to select the most appropriate alternative model for each gene partition (Darriba et al., 2012). A posterior support for phylogenetic relationships among taxa was obtained using MrBayes v3.2.6 (Ronquist et al., 2012). Subsequently, two independent Markov chain Monte Carlo (MCMC) runs were performed using two cold chains and two heated chains, with a total of 10,000,000 generations. Each run initiated with random tree, default priors and sampling tree every 1000 generations, and the first 25% was discarded. FigTree v1.4.2 was used to view the resulting phylogenetic trees (Rambaut, 2014).

Estimate of divergence time

A Bayesian analysis based on concatenation data (13 PCGs) was used to estimate the divergence time in Tridacninae using software BEAST v 1.7.5 (Drummond and Rambaut, 2007). We selected one node as the calibration point: 20.4-23.0 Mya was used as prior divergence time for *Hippopus* and *Tridacna* based on the fossil record, with a normal prior distribution, mean 21.7 and standard deviation 0.5 (Schneider and Ó Foighil, 1999). The general time-reversible model with discrete gamma distribution

TABLE 1 Overview of the Tridacninae species, including three outgroup species, examined in this study.

Tridacninae species	GenBank accession no.	Tissue	Sample origin	References
Hippopus hippopus	MG722975	Adductor muscle	Hainan province, China	Ma et al. (2019a)
Hippopus porcellanus	ON009026	Adductor muscle	Hainan province, China	Ma (unpublished)
Tridacna gigas	MT755623	mantle	Sanya, Hainan province, China	Ma et al. (2020)
Tridacna derasa	MG755811	Adductor muscle	Huangsha fishery market, Guangzhou city, Guangdong province, China	Ma et al. (2018)
Tridacna squamosa	KP205428	Adductor muscle	Northern Territory, Australia	Gan et al. (2016)
Tridacna maxima	MK105973	Adductor muscle	Sanya, Hainan province, China	Ma et al. (2019b)
Tridacna noae	MT755624	Adductor muscle	Sanya, Hainan province, China	Ma and Yu (unpublished)
Tridacna crocea	MT902179	Adductor muscle	Sanya, Hainan province, China	Ma (unpublished)
Outgroup species				
Cerastoderma edule	MF374632	Adductor muscle	Spain	Quinteiro and Rey-Mendez (unpublished)
Acanthocardia tuberculata	DQ632743	N.A.	Adriatic Sea, Croatia	Dreyer and Steiner (2006)
Fulvia mutica	AB809077	Adductor muscle	Miyazu Bay, Japan	Imanishi et al. (2013)

N.A. information not available

and invariant sites (GTR+G+I) was selected as the best-fit model of nucleotide substitution. The Bayesian analyses were performed using a GTR model with four gamma categories, a Yule process of speciation, and a strict clock model of rate as the tree priors, as well as other default parameters. The Markov Chain Monte Carlo (MCMC), sampling and effective sample size of each parameter were 100 million gene rations, every 10,000 generations and above 200, respectively. Posterior distributions for parameter estimates and likelihood scores to approximate convergence were visualized with the Tracer v1.7.1 (Rambaut et al., 2018). Visual inspection of traces within and across runs, as well as the effective sample sizes (EES) of each parameter (>200), allowed us to confirm that the analyses were adequately sampled. A maximum clade credibility (MCC) tree was chosen by TreeAnnotator v1.7.5 from the output of the MCMC run using the LogCombiner program after the removal of the initial trees (25%) as burn-in (Drummond et al., 2012). The MCC tree was visualized with the program FigTree v1.4.2.

Results and discussion

The complete mitochondrial genome size of *H. porcellanus* was 29,434 bp. The gene content of *H. porcellanus* was similar to that of other Tridacninae mitogenomes, including two mitochondrial rRNA genes (rrnL and rrnS), 13 PCGs (cox1-3, nad1-6, nad4L, cob, atp6 and atp8), 23 tRNA genes, and a major non-coding region called Control region (Figure 1). The lengths of genes (including rRNAs, tRNAs and PCGs) and intergenic nucleotides of *H. porcellanus* were 15,392 bp and 14,042 bp, respectively (Table 2). There were 3,887 codons excluding stop codons in the protein-coding genes of *H. porcellanus*. The length of the D-loop of the mitochondrial genome was 3580 bp. This region was difficult to assemble and annotate, and thus may not be precise. Moreover, we also found two long unannotated sections of the mitochondrial genome between trnM-CAT and trnR-TCG, trnL-TAA and rrnS, and their specific functions need further study. The A+T composition of the protein-coding region was 57.99%, and the AT composition of the third codon position elevated by 59.33%, which is in agreement with the tendency of typical invertebrates to prefer codons ending in A or T (Brown, 1985).

The genomes of Molluscs have unexpected/highly gene rearrangement (Ren et al., 2010). The gene order of Tridacninae species is presented and compared with that of three cardiidae species in Figure 2. Compared with cardiidae species, the mitochondrial genome of Tridacninae species has a higher rate of gene rearrangement. Gene rearrangements have also occurred within Tridacninae species. The gene order of genus *Tridacna* was different from that of *Hippopus* in the translocation of rrnS and trna L_2, as well as some tRNA. Comparing the gene arrangement within the genus *Tridacna*,

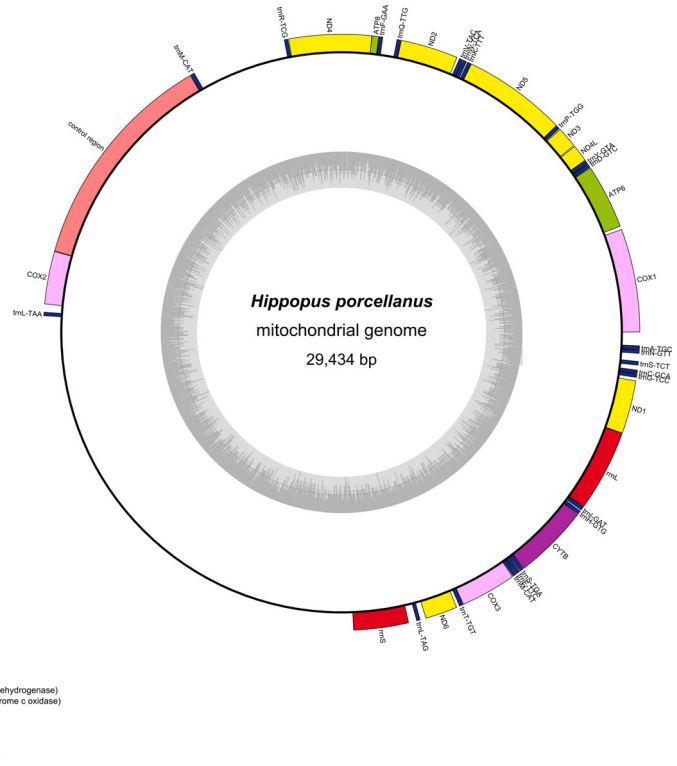

FIGURE 1
The mitochondrial genome map of *H. porcellanus*.

only *T. gigas* and *T. derasa* were completely identical to *T. maxima*. The gene arrangement of *T. squamosa* was similar to that of *T. crocea*, but their gene order was completely different from that of the other four giant clams by the translocation of atp8 and nad4, which is in agreement with the previous studies (Tan et al., 2021).

The thirteen PCGs found in the mitochondrial DNA of most other animal (cob, nad1-nad6, cox1-cox3, nad4L, atp6 and atp8) were also determined in all giant clam genomes. All PCGs were encoded on and transcribed from the same strand. Atp8 deletion has been found in the mitochondrial genomes of bivalve molluscs (Gissi et al., 2008), but it was annotated in all giant clams in our study. Thus, the deleted atp8 gene is not unique characteristics of the mitochondrial genome of marine bivalves.

In order to understand the evolutionary dynamics of biological protein-coding sequences, it is important to estimate the nonsynonymous (Ka) and synonymous (Ks) substitution rates of closely related species (Ohta, 1995; Fay and Wu, 2003). The ratio of Ka and Ks is widely accepted as an indicator of the evolution rate of PCG sequence, where Ka/Ks>1, Ka/Ks<1 and Ka/Ks not significantly different from 1indicate positive selection, purifying selection and neutral evolution, respectively (Yang and Bielawski, 2000; Zhang et al., 2006). In this study, the Ka and Ks of eight Tridacninae species, were calculated and illustrated in Figure 3. The Ka/Ks ratios of 13 PCGs ranged from 0.0021 for cox2 in *T. derasa _H. hippopus* to 0.4101 for atp8 in *T. gigas _T.derasa*, suggesting the existence of purifying selection among genes. Most amino acid substitutions occur in the atp6, atp8, nad2 and nad6, indicating that the purification selection of these genes is relaxed compared with conservative genes such as cox1 and cob.

In this study, phylogenetic analyses were conducted based on a concatenated nucleotide data set containing 13 PCGs and nuclear 18S rRNA from 8 Tridacninae species and 3 Cardiidae species. The trees inferred from ML and BI methods have same topology, and all nodes have strong support (Figure 4). Based on the previous phylogenetic analysis that using different genetic markers, the relationships among Tridacninae (especially *T.squamosa*, *T. crocea* and *T. maxima)* was uncertain (Benzie and Williams, 1998; Maruyama et al., 1998; Schneider and Ó Foighil, 1999; Nuryanto et al., 2007; Herrera et al., 2015). Among them, the three recent phylogenies based on concatenated multi-gene and mitochondrial genome datasets were the most consistent with the present study (Huelsken et al., 2013; Fauvelot et al., 2020; Tan et al., 2021). In our study, we found that the obvious close relationship between *T. squamosa* and *T.*

10.3389/fmars.2022.964202

TABLE 2 Organization of the mitochondrial genome of *H. porcellanus* (29,434bp).

Gene	From	To	Size(nts)	Size(aa)	Intergenic nucleotides	Start codon	Termination codon
COX1	1	1656	1656	551		ATT	TAG
ATP6	1702	2745	1042	348	45	ATG	missing
trnD-GTC	2746	2812	67		0		
trnY-GTA	2813	2878	66		0		
ND4L	2880	3161	282	93	1	ATG	TAA
ND3	3170	3514	345	114	8	ATT	TAA
trnP-TGG	3523	3586	64		8		
ND5	3587	5254	1668	555	0	ATG	TAG
trnK-TTT	5254	5317	64		-1		
trnW-TCA	5328	5394	67		10		
trnV-TAC	5396	5459	64		1		
ND2	5493	6417	925	308	33	TTG	T–
trnQ-TTG	6418	6486	69		0		
trnF-GAA	6704	6769	66		217		
ATP8	6771	6884	114	37	1	ATG	TAG
ND4	6878	8200	1323	440	-7	ATT	TAG
trnR-TCG	8205	8269	65		4		
trnM-CAT	9775	9845	71		1505		
D-loop	9846	13425	3580		0		
COX2	13426	14274	849	282	0	ATA	TAG
trnL-TAA	14396	14461	66		121		
rrnS	22259	23143	885		7797		
trnL-TAG	23264	23328	65		120		
ND6	23414	23935	522	173	85	GTG	TAG
trnT-TGT	23977	24042	66		41		
COX3	24043	24939	897	298	0	TTG	TAA
trnM-CAT	24944	25007	64		4		
trnE-TTC	25010	25076	67		2		
trnS-TGA	25081	25149	69		4		
CYTB	25152	26361	1210	403	2	TTG	T–
trnH-GTG	26364	26425	62		2		
trnI-GAT	26437	26503	67		11		
rrnL	26504	27814	1311		0		
ND1	27815	28672	858	285	0	ATG	TAG
trnG-TCC	28718	28780	63		45		
trnC-GCA	28781	28843	63		0		
trnS-TCT	28912	28980	69		68		
trnN-GTT	29098	29159	62		117		
trnA-TGC	29160	29226	67		0		

crocea is also agreed with the first tree of Maruyama et al. (1998). Reciprocal hybrids of *T. squamosa* and *T. crocea* have been successfully produced in the South China Sea, which also indicated that there is an obvious close relationship between them (Zhou et al., 2020). Based on genetic and morphological description, *T. noae* was a new species recently rediscovered from *T. maxima* (Su et al., 2014). This study found that *T. maxima* was placed with the clade comprising *T. noae*, *T. squamosa*, and *T. crocea*, in which *T. squamosa* was highly similar to *T. crocea*. The phylogenetic relationship of four giant clams was 100% supported.

In the fossil record, the divergence time of *Hippopus* and *Tridacna* is approximately 20.4-23.0 Mya ago (Schneider and Ó Foighil, 1999). The divergence times of Tridacnidae species estimated in this study is summarized in Figure 5. The results showed the same topology as the phylogenetic tree. Our

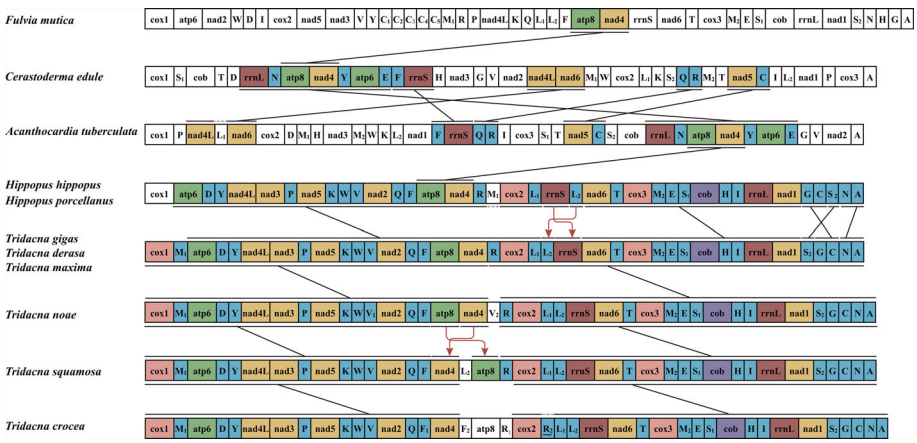

FIGURE 2
Mitochondrial genome rearrangements in Tridacninae and representatives from Veneroida species (All genes are transcribed from left-to-right. The bars show identical gene blocks. The arrows indicate gene translocation. The non-coding regions are not presented and gene segments are not drawn to scale).

molecular estimation showed that the genus *Hippopus* and the genus *Tridacna* diverged about 21.67 Mya (95% HPD: 20.71-22.69). The six *Tridacna* giant clams started their divergence about 18.08 Mya (95% HPD: 16.87-19.28) with the separation of *T. gigas* from other species first. *T. derasa* diverged from *T. maxima*, *T. noae*, *T. squamosa* and *T. crocea* 15.65 Mya (95% HPD: 14.77-16.54). *T.maxima* diverged from *T. noae*, *T. squamosa* and *T. crocea* 10.64 Mya (95% HPD: 9.89-11.47). *T. noae* diverged from *T. crocea* and *T. squamosa* 7.96 Mya (95% HPD: 7.43-8.57). *T. crocea* and *T. squamosa* have the closest relationship in our phylogenetic analysis, and the first split of their most recent common ancestors happened at about 5.01 Mya (95% HPD: 4.64-5.42). In the other clade, *H. porcellanus* and *H. hippopus* diverged about 10.64 Mya (95% HPD: 10.04-11.40).

In this study, the inferred divergence time of Tridacnidae species is generally consistent with the fossil record of Tridacnidae (Harland et al., 1990; Schneider and Ó Foighil, 1999). Genus *Hippopus* and *Tridacna* are believed to evolve independently from *Byssocardium*-like ancestor in the early Miocene (Stasek, 1962), and *Hippopus* and *Tridacna* fossil record was first discovered in the Miocene (Stasek, 1962). The fossil records of *T. maxima*, *T. derasa* and *T. gigas* are in the Late Miocene (about 14 million years later) (Martin, 1880; Beets, 1986). The earliest fossil record of *T. squamosa* was in the Late Pliocene or Early Pleistocene (Nomura and Zinbo, 1935), while the earliest fossil record of *T. crocea* was in the Late Pleistocene or Holocene age (Rosewater, 1965). As for *H. hippopus*, the oldest known occurrence is in the Early Miocene (Cloud et al., 1956), but *H. porcellanus* lacks known fossil records. Since *H. hippopus* occurred in the Early

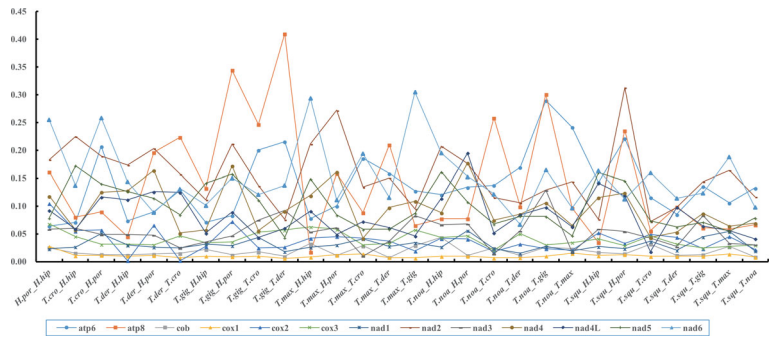

FIGURE 3
The ratio of nonsynonymous and synonymous substitutions (Ka/Ks) estimated in all 13 protein genes of eight giant clam species.

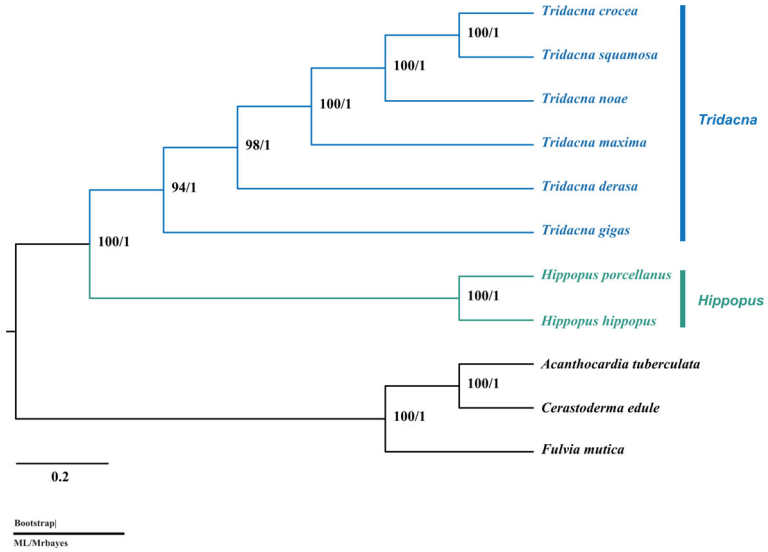

FIGURE 4
Consensus phylogenetic tree of giant clam species and outgroups from Maximum likelihood and Bayesian inference methods based on a concatenated nucleotide data set containing 13protein-coding genes and 18S rRNA gene.

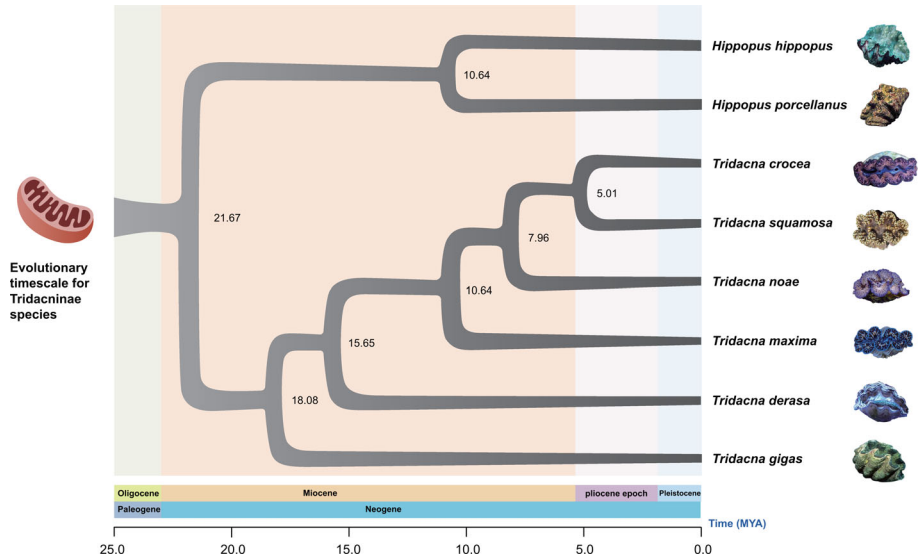

FIGURE 5
Evolutionary timescale for eight giant clam species inferred from a mitochondrial data set comprising 13 protein codons by mtDNA. Numbers at nodes indicate the mean estimated divergence times (in mya).

Miocene (Cloud et al., 1956), it is reasonable to speculate that *H. porcellanus* also originated in the Miocene using genetic distance based on partial 16S rDNA gene sequences (Schneider and Foighil, 1999). The results of this study are in agreement with this speculation. In future studies, in order to better understand the phylogenetic relationship and replacement events, a more representative of Tridacnidae mitochondrial genomes should be used in the phylogenetic analysis.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

YuZ and ZY conceived the study. HM and DY carried out the field and laboratory work, participated in the data analysis, and drafted the manuscript. JL and YQ collected the giant clam specimens. YaZ and ZX contributed to the phylogenetic analysis. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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