



Diversity of Three Small Type's Giant Clams and Their Associated Endosymbiotic Symbiodiniaceae at Hainan and Xisha Islands, South China Sea

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Giant clams are found in a mutualistic association with Symbiodiniaceae dinoflagellates, however, the diversity of the giant clams, as well as the diversity and distribution of Symbiodiniaceae in different Tridacninae species remain relatively poorly studied in the South China Sea. In this study, a total of 100 giant clams belonging to small type's giant clams, *Tridacna maxima*, *T. crocea*, and *T. noae*, were collected from Hainan and Xisha Islands. Based on mtDNA cytochrome c oxidase subunit 1 gene (COI) and 16S rRNA fragments, *T. maxima* and *T. crocea* showed a closer phylogenetic relationship than *T. noae*. All the three species of giant clams hosted Symbiodiniaceae including genera *Symbiodinium* (formerly Clade A) and *Cladocopium* (formerly Clade C). Geographically, symbionts in *Cladocopium* are restricted to Xisha Islands, probably because *Cladocopium* prefers to inhabit in waters with higher mean temperatures. The endosymbiont specificity among the three giant clam species was also detected. *T. noae* and *T. crocea* are found to harbor *Symbiodinium* preferentially, compared with *Cladocopium*. These results could provide important information to understand various endosymbionts occurring in giant clams in the South China Sea.

Keywords: Tridacna, Symbiodiniaceae, symbiont, COI, 16S rRNA, ITS1

INTRODUCTION

Giant clams (Tridacninae) inhabit tropical coral reefs throughout the Indo-Pacific oceans (Lucas, 2014). In the world, the Tridacninae subfamily includes two extant genera, *Hippopus* (two species) and *Tridacna* (10 extant species), and all these species host symbiotic zooxanthellae in the mantle tissues that plays important ecological roles in the coral reef ecosystem (Liu et al., 2021b). Recently, the natural resources of giant clams sharply decreased in many countries, including Australia, Indonesia, Singapore, Philippines, and Japan because of overfishing, habitat destruction, and global climate change (Copland and Lucas, 1988; Pringgenies et al., 1995; Neo and Todd, 2012; Neo et al., 2019). Therefore, all the giant clam species are listed in Appendix II of the Convention on International Trade in Endangered Species [United Nations Environment Programme-World Conservation Monitoring Center (UNEP-WCMC), 2007] and International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Wells, 1997).

In China, giant clams are distributed mainly in the South China Sea, which can be divided into five geographical populations, Hainan Islands, Xisha Islands, Zhongsha Islands, Dongsha Islands, and Nansha Islands (Zhang et al., 2020). Eight giant clams have been reported in the South China Sea, including two large species *Tridacna gigas* and *T. derasa*; three middle species *T. squamosa*, *H. hippopus*, and *H. porcellanus*; and three small species *T. maxima*, *T. crocea*, and *T. noae* (Neo et al., 2017; Liu J. et al., 2020). Recent surveys found that large and middle type's giant clams were in extremely low numbers, and small type's giant clams became the dominant giant clam species (Liu et al., 2021a). However, the genetic and phenotypic variation in several giant clam species has been reported in many geographical locations (Nuryanto and Kochzius, 2009; Hui et al., 2016; Pappas et al., 2017), while there were only a few reports on the diversity of giant clams in the South China Sea (Lim et al., 2019; Liu J. et al., 2020).

Symbiodinium is found in endosymbiosis with marine invertebrates such as giant clams, corals, Porifera, and Foraminifera, which could provide their hosts with up to 100% of energy requirements (Stat et al., 2006; Venn et al., 2008; Reich et al., 2017). Since pediveliger larva stage, the symbiont between the giant clam and Symbiodinium is established (Liu C. et al., 2020; Zhang et al., 2020; Wang et al., 2021). The diversity and community structure of Symbiodiniaceae in the giant clam could hence likely affect the growth, reproduction, and photosynthetic efficiency of the host (DeBoer et al., 2012). According to the previous molecular taxonomic research, nine distinctive Symbiodinium clades (A to I) have been identified (Pochon and Gates, 2010). Each major clade could be further resolved into diverse genetic subclades through the use of highly variable DNA markers such as the nuclear ribosomal internal transcribed spacer (ITS) (Arif et al., 2014; Ikeda et al., 2017). Previous studies have shown that the diversity of endosymbiotic Symbiodiniaceae in host is influenced by numerous factors, such as host species, physiology of the hosts, and environmental factors (LaJeunesse et al., 2010, 2018; Hume et al., 2016; Pappas et al., 2017; Lim et al., 2019).

The aim of this study is to illustrate the diversity of three small type's giant clams (*T. maxima*, *T. crocea*, and *T. noae*) using the mtDNA cytochrome c oxidase subunit I gene (COI) and 16S rRNA gene in Hainan and Xisha Islands. Furthermore, the diversity of their endosymbiotic Symbiodiniaceae is also identified using DNA barcoding based on ITS1 region of rDNA.

MATERIALS AND METHODS

Ethics Statement

All giant clams and experimental protocols used in this study were reviewed and approved by the committee and laboratory animal department of the Hainan University.

Samples and DNA Extraction

The small type's giant clam samples (*T. maxima*, *T. crocea*, and *T. noae*) were collected from the two different sites, the lagoon of Zhaoshu, Xisha (112° 12'–112° 19' E, 16° 57'–16° 59' N) and

the coastal waters of Sanya, Hainan (including Dongmao-Ximao Island, Luhuitou Peninsula, Yalong Bay and Wuzhizhou Island, 109° 21'–109° 46' E, 18° 11'–18° 19' N) in the South China Sea in June and July, and coded as Xisha01-Xisha77 and Sanya01-Sanya23, respectively. Briefly, about 1 cm² mantle tissue was removed from each individual using sterile disposable razors and stored in 100% ethanol at 4°C.

A subsample of mantle tissue of size about 2 mm² was used for the DNA extraction that was conducted using Tiangen DNA kit (Tiangen Biotech, Beijing, China) according to the instructions of the manufacturer.

PCR Amplification and Sequencing

The COI, 16S, and ITS1 fragments of the giant clams and Symbiodiniaceae, respectively, were amplified by PCR, which were carried out in a total volume of 20 µl with 10 µl of 2 × Rapid Taq Master Mix (with Taq DNA polymerase, dNTP mix, MgCl₂, and PCR buffer), 0.6 µl each of forward and reverse primers (10 µM), 1 µl of DNA diluted 1:5, and 7.8 µl of water. The primers were shown in **Table 1**. PCR conditions were set as follows: an initial denaturing step at 94°C for 5 min; 40 cycles of denaturing at 94°C for 30 s, annealing at 43–53°C for 30 s, and extension at 72°C for 90 s; and a final extension step at 72°C for 10 min. PCR products were sequenced at BGI (China).

Sequence Alignment and Phylogenetic Analyses

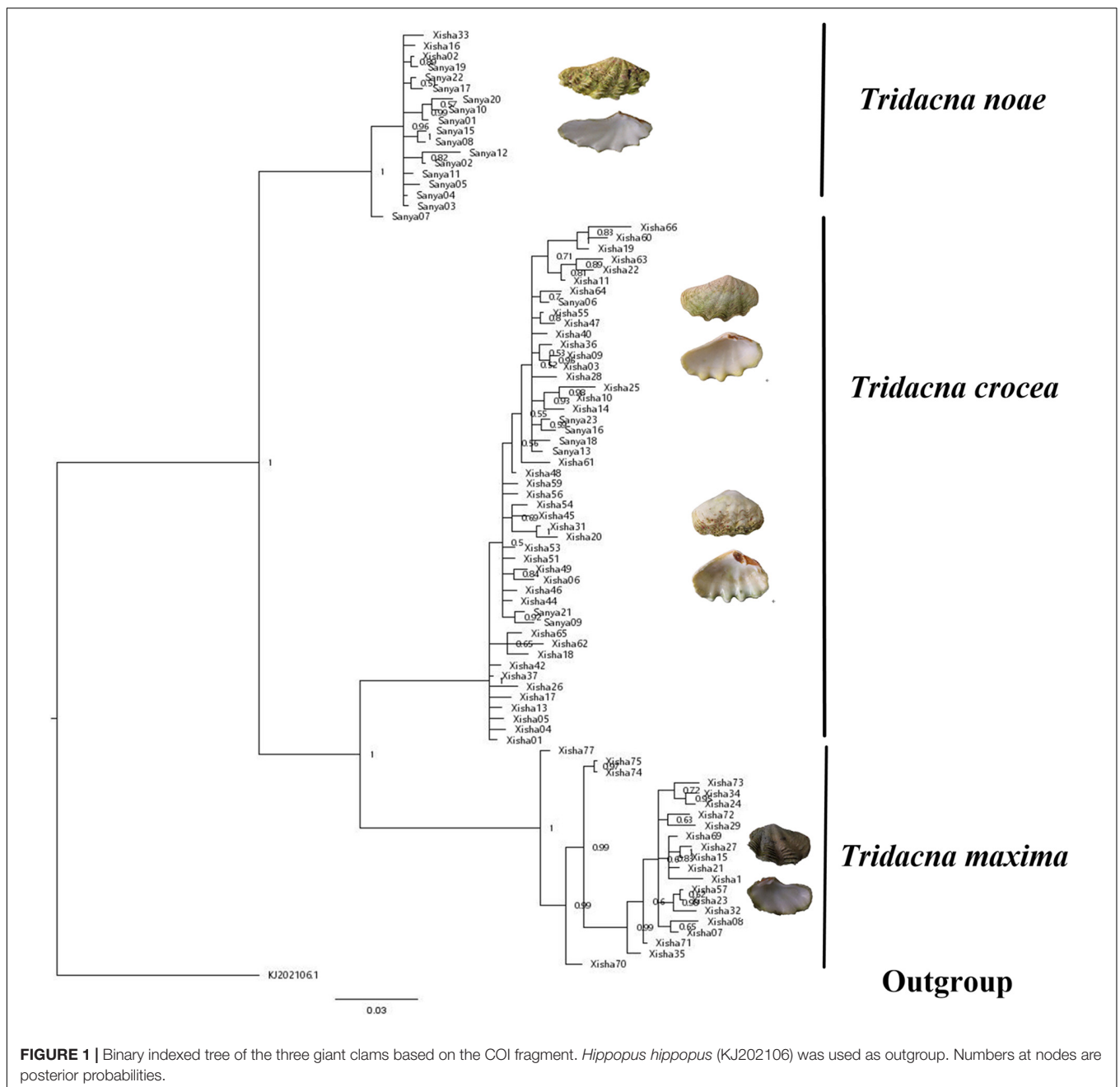
The three fragments (COI, 16S, and ITS1) were aligned separately using Clustal W in MEGA X (Kumar et al., 2018). Sequences were converted into NEXUS format for phylogenetic analysis using DAMBE5 (Xia, 2013). Phylogenetic trees were reconstructed using Bayesian inference (BI; Huelsenbeck and Ronquist, 2001) in the CIPRES gateway (Miller et al., 2010). BI analyses were performed with MrBayes v.3.2.7 (Ronquist and Huelsenbeck, 2003), running four simultaneous Monte Carlo Markov chains (MCMC) for 10,000,000 generations, sampling every 1,000 generations and discarding the first 25% generations as burn-in. Two independent runs were performed to increase the chance of adequate mixing of the Markov chains and to increase the chance of detecting failure to converge, as determined by using Tracer v1.6. The effective sample size (ESS) of all the parameters was higher than 200. The resulting phylogenetic trees were visualized in FigTree v1.4.4.

Genetic Diversity and Population Structure

Population genetic analyses were conducted based on the COI and 16S fragments of three giant clams. Genetic diversity indices, including haplotype diversity (Hd) and nucleotide diversity (π), were calculated in DNASP 5 (Librado and Rozas, 2009). To investigate the genetic relationships among haplotypes, a median-joining network was generated with PopART 1.7 (Clement et al., 2000). To infer if the three giant clams have experienced population expansion, Tajima's D (Tajima, 1989) and Fu's Fs statistics (Fu, 1997) of COI sequences were calculated in Arlequin

TABLE 1 | Information of primers used in this study.

Gene	Primer	Sequences (5'-3')	Length (bp)	Annealing Temperature (°C)	References	
Giant clams	COI	GGGTGATAATTCGAACAGAA	500	43	Nuryanto et al., 2007	
	COI(R)	TAGTTAAAGCCCCAGCTAAA				
	16S	16SarF 16SbrR	CGCCTGTTTATCAAAAACAT CCGGTCTGAACTCAGATCACGT	500	53	Marco et al., 2014
Zooxanthellae	ITS1	ITSF ITS4R	CCGGTGAATTATTCCGACTGACGCAGT TCCTCCGCTTATTGATATGC	750	51	Satoe et al., 2012



3.5 (Excoffier and Lischer, 2010). The significance levels were evaluated under 10,000 permutations.

RESULTS

Phylogenetic Analyses of Giant Clams

A total of 88 COI and 89 16S sequences were obtained and submitted to Genbank (Supplementary Table 1). The phylogenetic relationships of three giant clams were reconstructed based on the aligned nucleotide sequences of COI (681 bp) and 16S (518 bp) fragments, respectively. Both BI analyses arrived at the identical topologies (Figures 1, 2).

According to the reconstructed phylogeny, *T. maxima* was recovered closer to *T. crocea* than to *T. noae*. Although the sister relationship between *T. maxima* and *T. crocea* was not highly supported in the 16S analysis (Figure 2), it arrived at a maximum support value in the COI phylogeny (Figure 1). Both the COI and 16S sequences supported the monophyly of *T. crocea*, however, high-intraspecific morphological diversity was found within this group, such as the values of shell width/shell length, byssal opening length/shell length, byssal opening width/shell length and number of hinge tooth (Supplementary Table 2).

Genetic Diversity and Gene Genealogy

The Hd and nucleotide diversity (π) across two sampling sites were presented in Table 2. The genetic diversity indices derived

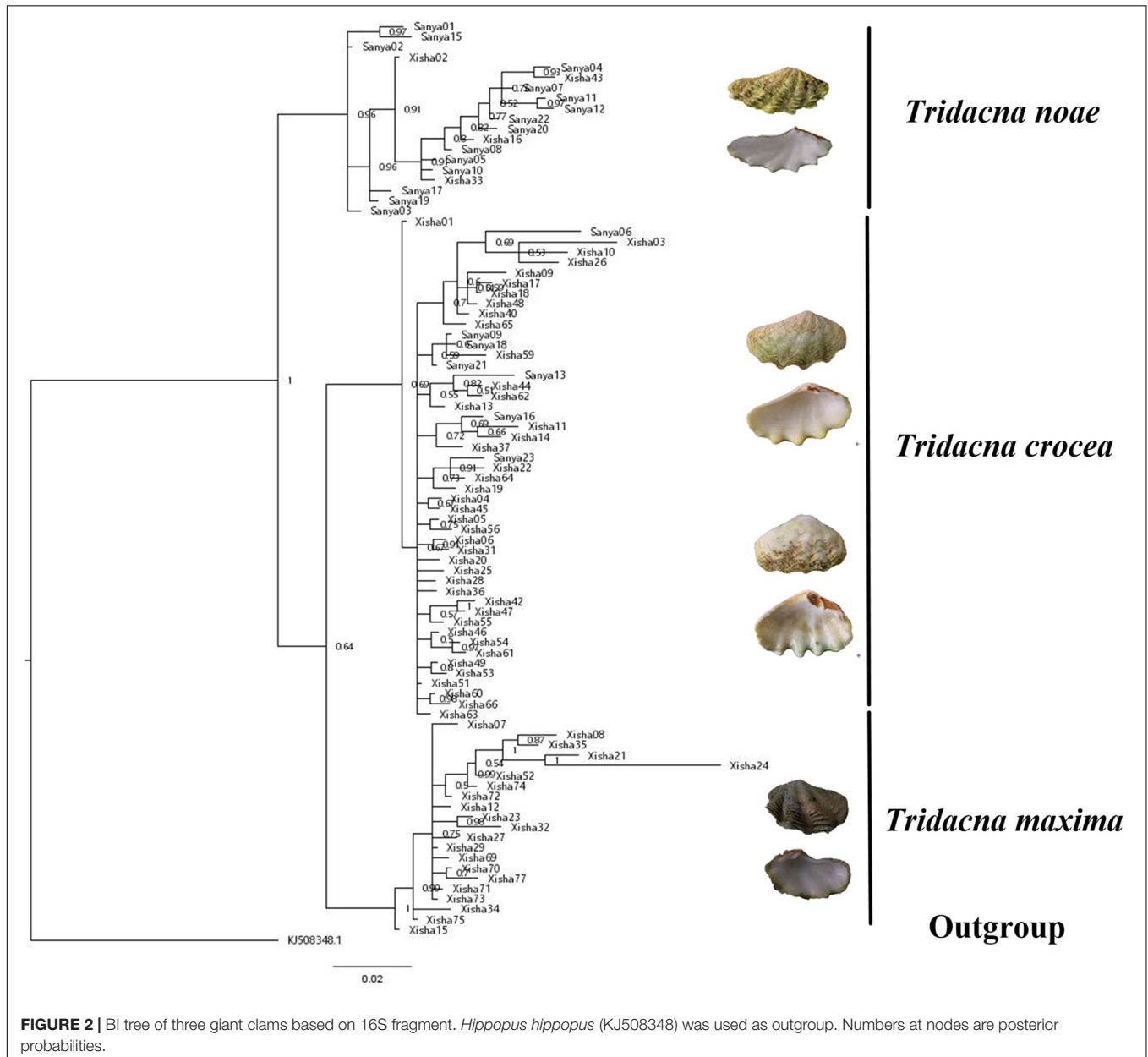


TABLE 2 | Genetic diversity of the COI and 16S among different species and sites, and COI neutrality tests.

Species	Sites	COI						16S			
		N	h	Hd	π	Tajima's D	Fu's Fs	N	h	Hd	π
<i>T. crocea</i>	Xisha	42	29	0.9779	0.00917	-1.56699*	-24.14330**	42	7	0.5854	0.00178
	Sanya	7	3	0.6667	0.00475	0.13210	1.41593	7	4	0.7143	0.00335
	Total	49	31	0.9677	0.00882	—	—	49	9	0.5961	0.00200
<i>T. maxima</i>	Xisha	21	15	0.9429	0.01000	-1.67280*	-6.90257*	21	6	0.4286	0.00539
	Sanya	0	—	—	—	—	—	0	—	—	—
	Total	21	15	0.9429	0.01000	—	—	21	6	0.4286	0.00539
<i>T. noae</i>	Xisha	3	2	0.6667	0.00151	—	—	4	1	0.0000	0.00000
	Sanya	15	9	0.8762	0.00637	-1.86906*	-3.06649*	15	4	0.4667	0.00230
	Total	18	10	0.8431	0.00560	—	—	19	4	0.3801	0.00185

Significant values ($P < 0.05$) are marked with an asterisk, while those of great significant values ($P < 0.01$) are indicated with two asterisks.

from COI were higher than those from 16S sequences. Compared with *T. noae*, *T. crocea*, and *T. maxima* showed relatively higher COI Hd and nucleotide diversity. Estimates of neutral tests for *T. crocea* and *T. maxima* of Xisha and *T. noae* of Sanya indicated population expansion by significant negative values of Fu's Fs and Tajima's D statistics (Table 2). The median-joining network analysis based on COI and 16S sequences revealed a star-like haplotype network (Figure 3).

Endosymbiont Genera and Species Diversity and Distribution

A total of 59 ITS1 sequences were amplified and submitted to GenBank (Supplementary Table 1). The final 814 bp length alignment was used for phylogenetic reconstruction by BI inference (Figure 4). The phylogenetic tree was successfully separated into two clades (namely, Clade I and Clade II; Figure 4A) that were identified by blasting in NCBI. The results (not shown here) indicated that they corresponded to *Symbiodinium* and *Cladocopium*, respectively. Since considerable sequence divergence could impede accurate alignments between distantly related lineages (LaJeunesse, 2001), two phylogenetic analyses of Clade I and Clade II were further conducted, respectively. Clade I was not separated while Clade II could be divided into two subclades when they were considered individually (Figures 4B,C).

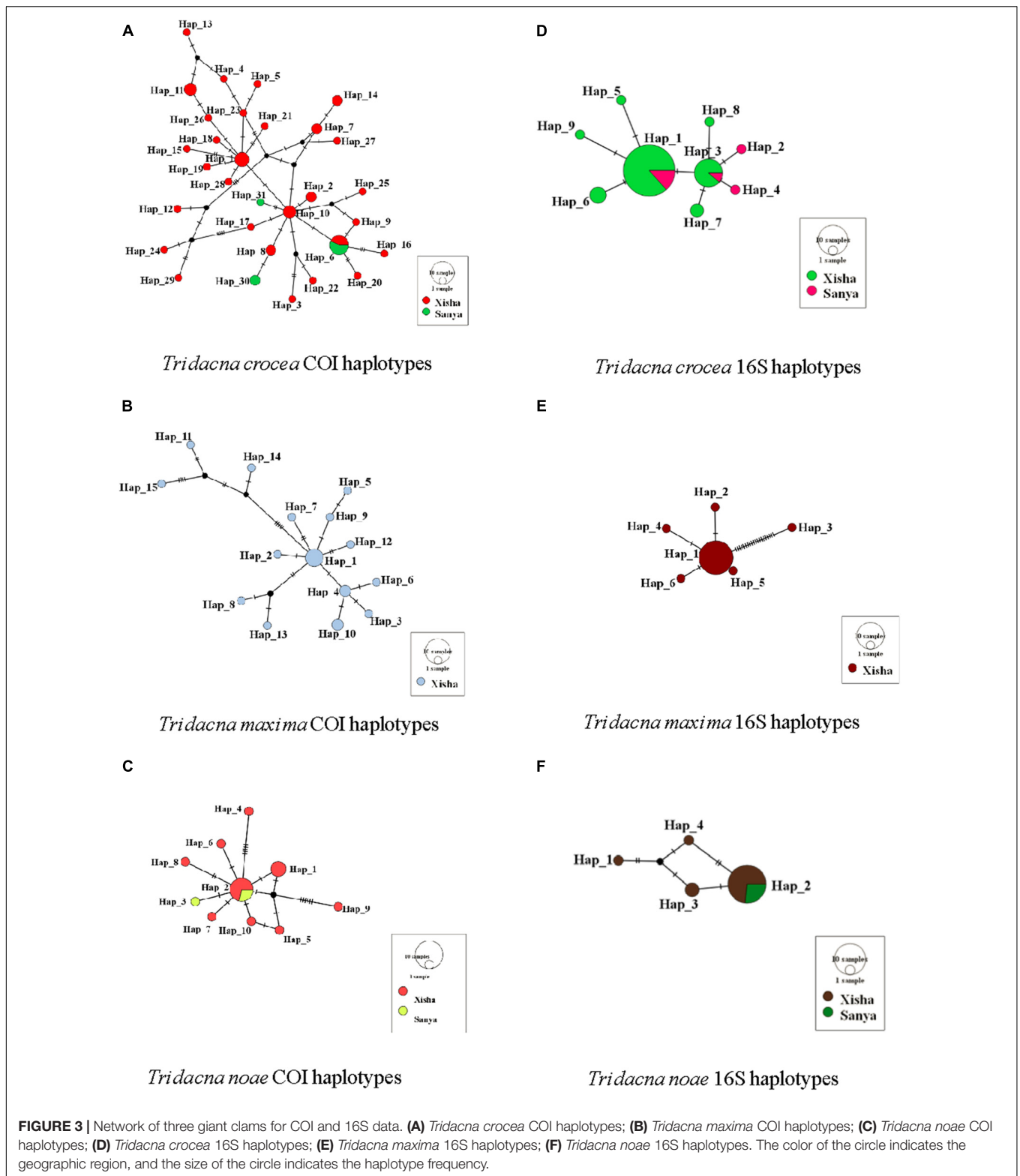
The abundance of endosymbiont genus distribution, and their association to the three giant clam host species are shown in Table 3.

DISCUSSION

The COI and 16S sequences generated identical topologies, supporting the separation of three major clades corresponding to three individual species *T. crocea*, *T. maxima*, and *T. noae* (Figures 1, 2). The latter two species are similar in general appearance, and they were even considered the same species (Rosewater, 1965). However, previous studies found that they could be distinguished by several morphological and ecological characteristics. For example, *T. maxima* was mainly distributed at the edges and crests of reefs, while *T. noae* was often found

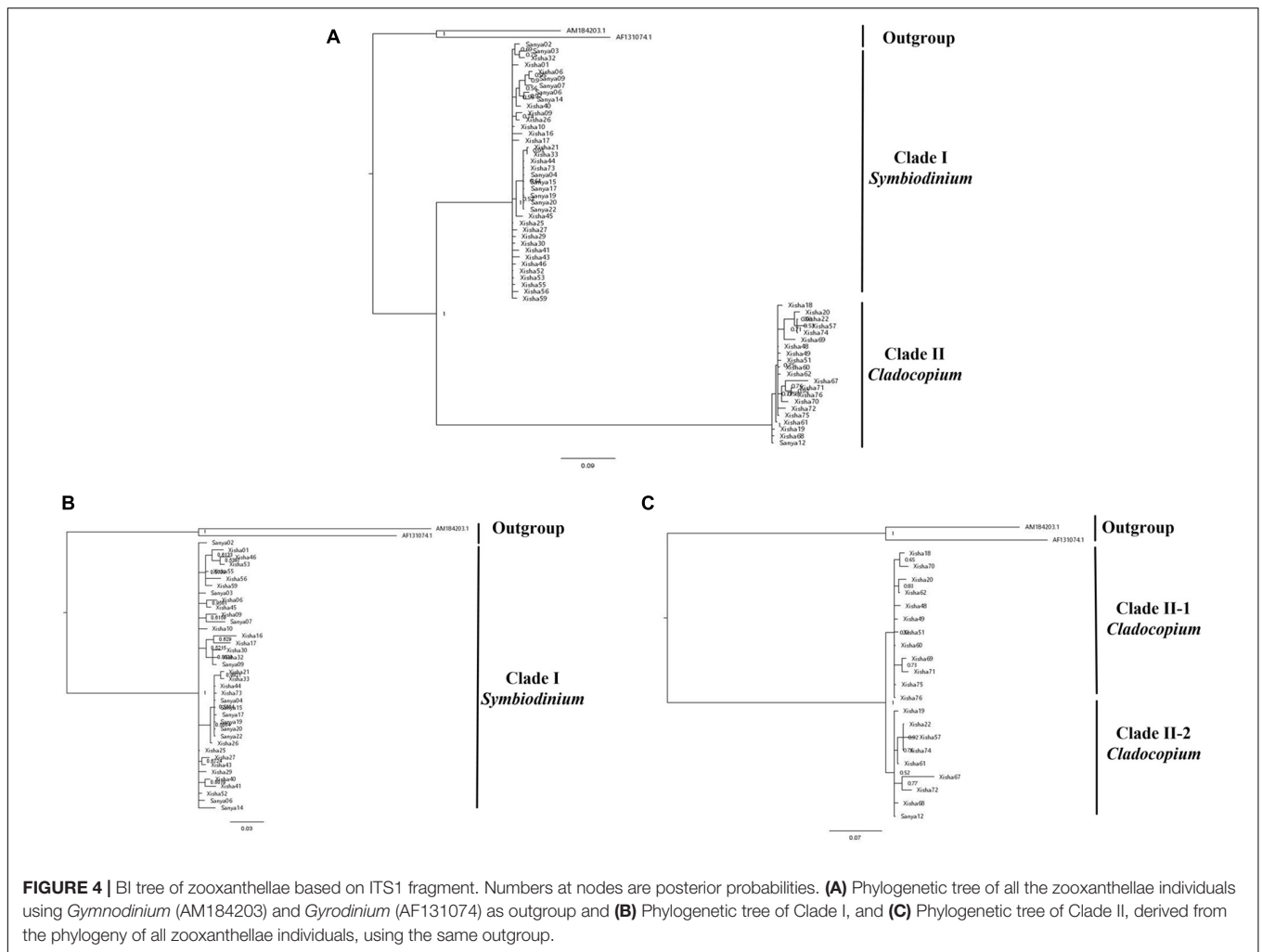
in the reef flat (Kubo and Iwai, 2007). In addition, *T. noae* has well-spaced rib scales, particularly on the upper third of the shell, while the ribs in *T. maxima* were closely crowded together (Su et al., 2014). Our phylogeny derived from COI and 16S fragments indicated a distant relationship between *T. noae* and *T. maxima*, shown as *T. noae* + (*T. maxima* and *T. crocea*), and therefore supported the validity of *T. noae* (Su et al., 2014). Although *T. maxima* has a short planktonic larvae duration (9 days; Lucas, 1988), it is widely distributed from the Red Sea to the central Pacific (Nuryanto and Kochzius, 2009). In our study, however, *T. maxima* was only found in Xisha, and this may probably be due to the low sample size of Sanya. High intraspecific morphological diversity was found within *T. crocea*, even though both the COI and 16S sequences supported the genetic monophyly of this species. Special attention should be paid during *T. crocea* identification since it shows high intraspecific diversity. Geographically, the sampling locations of *T. crocea* (Sanya and Xisha) in our study were restricted to the South China Sea, and corresponded to the western Pacific OTU (Liu J. et al., 2020).

A total of two clades (namely, Clade I and Clade II) were separated based on the ITS1 marker (Figure 4A), and they corresponded to *Symbiodinium* (formerly Clade A) and *Cladocopium* (formerly clade C; LaJeunesse et al., 2018), respectively. Until now, giant clam species have been reported to be associated with 30 Symbiodiniaceae phylotypes, all belonging to genera *Symbiodinium*, *Cladocopium*, and *Durusdinium* (not detected here) (Mies, 2019). In this study, Clade II could be further divided into two closely related lineages (Figure 4B), indicating the diversity of *Cladocopium*. Previous studies have also revealed that the diversity of *Cladocopium* was higher than *Symbiodinium* or *Durusdinium* in Dongsha Atoll (Lim et al., 2019), and this could be explained as the abundance of *Cladocopium* that is also species-rich among hosts in the South China Sea (Wong et al., 2016). Symbionts in *Symbiodinium* are widely distributed in the world (Baker, 2003; Tonk et al., 2013), and most members have been accustomed even to extreme conditions (Venn et al., 2008). For example, only *Symbiodinium* symbionts were found in the giant clams (*Tridacna* spp.; Pappas et al., 2017) in the Red Sea, which



is characteristic of high heat and irradiance conditions. This could also be supported in our study, since the symbionts in *Symbiodinium* were found in both Xisha and Sanya, whereas most of the symbionts in *Cladocopium* were restricted to Xisha

(Figure 4A), indicating that the former could be better adapted to the various conditions. This result may also be explained as that the clams with *Cladocopium* (formerly clade C) and *Durudinium* (formerly clade D) were usually located in areas



with higher mean temperatures as previous studies have revealed (DeBoer et al., 2012) since the climate of Xisha is warmer than Sanya at the same time.

This study reveals the endosymbiont specificity among the three giant clam species. At first, *T. maxima* associates slightly with *Cladocopium* ($n = 7$) over *Symbiodinium* ($n = 5$), but sample sizes are low. In the previous studies, both *T. maxima* host associations with *Cladocopium* and *Symbiodinium* endosymbionts have been reported. For example, in Dongsha (Lim et al., 2019), *T. maxima* associated preferentially with *Cladocopium*. In the Red Sea (Rossbach

et al., 2021), however, strong *T. maxima* host associations with *Symbiodinium* endosymbionts were suggested. In French Polynesia, both *Symbiodinium* and *Cladocopium* were the dominant genera in *T. maxima* (Pochon et al., 2019). In order to better clarify *T. maxima* host associations in the South China Sea, future studies with broad sampling sites around this area are still needed. Different from *T. maxima*, the giant clams *T. noae* and *T. crocea* were found to associate with *Symbiodinium* over *Cladocopium*, and this host-endosymbiont specificity appears more obvious on *T. noae*. This result is supported by the study of Ikeda et al. (2017) showing that *T. crocea* had an apparent dominance of *Symbiodinium* (Clade A), although it is inconsistent with some previous studies which revealed *T. noae* and *T. crocea* host associations with *Durusdinium* (Lim et al., 2019) and *Cladocopium* (DeBoer et al., 2012), respectively. Within the stony coral *Stylophora pistillata*, *Symbiodinium* (Clade A) have been observed to be more related to individuals that inhabited shallow waters, whereas individuals in deeper waters were more associated with *Cladocopium* (Clade C) (Winters et al., 2009). In addition, *Symbiodinium* (Clade A) was found

TABLE 3 | Abundance of *Tridacna* clams with respective endosymbiont genus according to host species and sampling sites.

	<i>T. maxima</i>		<i>T. noae</i>		<i>T. crocea</i>	
	Hainan	Xisha	Hainan	Xisha	Hainan	Xisha
<i>Symbiodinium</i>	0	5	9	2	2	15
<i>Cladocopium</i>	0	7	1	0	0	10

in other coral species inhabiting shallow waters (Rowan et al., 1997), and therefore, it is inferred that *Symbiodinium* (Clade A) tends to be more insensitive to the high irradiance and high temperature stresses of shallow waters (Ikeda et al., 2017). The habits of *Symbiodinium* (Clade A) may attribute to *T. noae* and *T. crocea* that inhabit shallow waters, associating preferentially with *Symbiodinium*.

CONCLUSION

In this study, the reconstructed phylogeny using the COI and 16S fragments indicated a relationship shown as *T. noae* + (*T. maxima* and *T. crocea*) and supported the validity of *T. noae*. A total of two clades of Symbiodiniaceae symbionts that corresponded to *Symbiodinium* (formerly Clade A) and *Cladocopium* (formerly clade C) were separated based on the ITS1 marker. The wide distribution of *Symbiodinium* may indicate that it could be better adapted to various conditions, while the restriction of *Cladocopium* may be explained as a temperature preference. This study also reveals the endosymbiont specificity that is related to the shared living habits between giant clams and dinoflagellates.

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**.

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ETHICS STATEMENT

The animal study was reviewed and approved by The Committee and Laboratory Animal Department of Hainan University.

AUTHOR CONTRIBUTIONS

QC was involved with the data curation, investigation, and writing the original draft. ZG worked with the software and supervision. AW was involved in the supervision and funding acquisition. CL was helped in the conceptualization, writing review and editing, supervision, and funding the acquisition. YY was helped in the data curation, formal analysis, methodology, and writing the original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.774925/full#supplementary-material>

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