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DNA BARCODING OF ENDANGERED GIANT CLAMS IN ISLANDS OFF THE EAST COAST OF PENINSULAR MALAYSIA

NUR SYAHIRAH MAMAT[1], YUSRI YUSUF[2], SITI AZIZAH MD NOR[3], SHAHREZA MD SHERIFF [4], MD NIZAM ISMAIL[5] AND NORAINY MOHD HUSIN[1]*

[1]Institute of Tropical Aquaculture and Fisheries (AKUATROP), [2]Faculty of Marine Science and Environment, [3]Institute of Marine Biotechnology, [4]Faculty of Fishery and Food Sciences, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia. [5]Fisheries Research Institute (FRI) Batu Maung, Department of Fisheries Malaysia, Ministry of Agriculture & Food Industry, 11960 Batu Maung, Penang.

**Corresponding author: norainyhusin@umt.edu.my*
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Abstract: This study reports on the taxonomic verification and distribution of three presumed morphologically identical *Tridacna* species (giant clams), namely *T. crocea, T. maxima* and *T. squamosa*, from nine localities in islands off Terengganu, Pahang and Johor in the east coast of Peninsular Malaysia. A 467-bp partial sequence of mitochondrial DNA cytochrome c oxidase 1 (MT-CO1), which serves as the DNA barcoding gene, was analysed for species identification of 247 samples. The MT-CO1 gene was successfully used to identify all the giant clam samples to species level based on GenBank BLAST and BOLD databases. Three highly-supported clusters were obtained, which supported the morphological species determination into *T. crocea, T. maxima* and *T. squamosa*. However, a few discrepancies were observed, which could be attributed to misidentification of juveniles. *T. squamosa* and *T. crocea* were more closely related to each other compared to *T. maxima*. *T. maxima* and *T. squamosa* were found to be ubiquitous in all the islands, while *T. crocea* was restricted to the southeastern islands of Pahang and Johor. The precise identification of samples through the MT-CO1 gene and information on their distributions are useful in strategising the conservation and management of giant clams in this region.

Keywords: DNA barcoding, mtDNA, giant clams, *Tridacna*, conservation.

Introduction

Correct identification of species is a prerequisite for conserving and protecting endangered organisms. The giant clam is the largest group of bivalve molluscs belonging to the genera *Tridacna* and *Hippopus*. Like other marine bivalves, they have a photosynthetic symbiosis with the zooxanthellae algae of the Symbiodiniaceae family for nutritional requirements (Morishima *et al*., 2019). The size of adult clams ranges from 15 cm in the smallest *T. crocea*, to 1.5 m in *T. gigas*, the largest species (Hui *et al*., 2016). However, its striking features have exposed it to varied forms of exploitation. The clams have been overharvested for food, tile manufacturing (shells), the aquarium trade and production of souvenirs (Kochzius & Nuryanto, 2008; Heslinga, 2013). As such, its opulation has reduced throughout its geographic range (Mohammed *et al*., 2019). Most giant clam species, including *T. crocea*, *T. maxima* and *T. squamosa*, are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Appendix II), where sales of the species and its products are supposed to be strictly regulated. The three giant clams may be found in the three major groups of islands off Johor, Pahang and Terengganu in the east coast of Peninsular Malaysia (Neo *et al*., 2017).

The classification of giant clams has traditionally been based on shell morphology and mantle characteristics. While the morphological approach is the basis of taxonomic identification, it has limitations and sometimes may lead to erroneous conclusions (Patwardhan *et al*., 2014). With the cost of genetic analysis becomes more affordable, species verification has become more rapid and precise through this complementary approach (Ali *et al*., 2014).

Owing to its high evolutionary rate, maternal inheritance, lack of recombination, and fast rates of base substitutions, the mitochondrial genome (mtDNA) has been widely used in various aspects of molecular studies, including phylogeography and molecular identification (Caballero *et al*., 2013; Tran *et al*., 2015; Gu *et al*., 2016; Kawamura *et al*., 2017). Specifically, the mitochondrial gene cytochrome c oxidase subunit 1 (MT-CO1) is acknowledged as the global bio-identification gene for animals (Hebert *et al*., 2003), and has been extensively used in DNA barcoding. The success of using MT-CO1 gene as an identification marker has been reported in various marine organisms, such as crabs (Ma *et al*., 2012), fishes (Ward *et al*., 2005; Hanner *et al*., 2011; Yi *et al*., 2017) and bivalves (Ni *et al*., 2012; Su *et al*., 2014; Lizano & Santos, 2014). Thus, although morphological identification is the cornerstone for taxonomic delimitation, genetics has become a necessary complementary tool.

Despite its threatened status, studies on the giant clam in Malaysian waters have been limited in scope and coverage. Past researches in the country have focused mainly on their distributions based on in situ visual observations and morphological identification of specimens (Mohamed-Pauzi *et al*., 1994; Tan & Zulfigar, 2003; Montagne *et al*., 2013). In the waters of Pahang, Terengganu and Johor islands, four species have been reported, namely *T. squamosa*, *T. crocea*, *T. maxima* and *Hippopus hippopus* (UNEP, 2007). However, *H. hippopus* was not documented in another study in Tioman island (Pahang) (Tan *et al*., 1998) and Redang island (Terengganu) (Mohamed-Pauzi *et al*., 1994). In 2017, Neo *et al*. reported that *H. hippopus* was rare and could be found in Johor islands only. Thus, the occurrence and distribution of this species is still contentious. A survey conducted by the Fisheries Department in Redang island found a single individual of *T. derasa*, while *T.gigas* is extinct when only its fossil was reported (Mohamed-Pauzi *et al*., 1994). There is little knowledge about the present distributions of the giant clams with limited data on both morphological and molecular approaches in Malaysia.

Based on literature, only two genetic-based studies have been documented in Malaysia; population genetics of *T. crocea* from Tioman island (Waheed, 2016) and genetic variability studies of *T. maxima* and *T. squamosa* from Perhentian island (Lim *et al*., 2018). Both studies were conducted in the east coast of Peninsular Malaysia but with limited geographical coverage. There is no comprehensive data yet on their genetic variability, while species identification of giant clams in Peninsular Malaysian waters have been largely based on morphological data, which should be substantiated with a complementary tool. Therefore, the aim of this study is to update the present occurrence and distribution of endangered giant clams inhabiting the east coast of Peninsular Malaysia through precise identification by applying a DNA barcoding approach.

Materials and Methods

Sample Collections

Samples of giant clams were collected during field trips in July, September and October from 2016 to 2019 to groups of islands in three states along the east coast of Peninsular Malaysia; 1. Terengganu islands (northeast), 2. a Pahang island and 3. Johor islands (both southeast) (Figure 1). These groups of islands comprised o Redang and Bidong (Terengganu), Tioman (Pahang), Babi Besar, Pemanggil, Metinggi, Tinggi, Lima Besar and Lima Kechil (Johor) (Table 2). The samples were collected with permits granted by Marine Park Section, Department of Fisheries Malaysia (Prk.ML.630-7(45) Jld.4 and JTLM 630-7 Jld. 7 (22)) during scuba diving using non-destructive method.

The samples were identified to species level based on morphological characteristics using the classification keys of Copland and Lucas (1988), and Norton and Jones (1992). Pictures of giant clams were taken on site and labelled to verify identification or for future reference. Specimens varied in size from juveniles to adults

within the range of 4.0 cm to 34.8 cm. Mantle tissue clippings of 1 cm^3 from 247 samples were placed in 1.5 mL microcentrifuge tubes in 95 % ethanol for preservation. Then, all samples were brought back to the Institute of Tropical Aquaculture and Fisheries (AKUATROP) in Universiti Malaysia Terengganu and stored at -4 °C for further genetic studies.

DNA Extraction and PCR Amplification

DNA from the preserved mantle tissue samples were extracted using the Nucleospin Tissue Kit (Macherey-Nagel, Duren, Germany). A total of 25 mg tissues was cut into small pieces and processed with the kit according to the manufacturer's instructions. Since all samples were morphologically identified as either *T. crocea*, *T. maxima* and *T. squamosa*, primer selection and molecular procedures were based on these species. Sequences of the MT-CO1 gene were PCR-amplified using the MT-CO1 tridacnid-specific primers for *T. crocea* and *T. maxima* (forward: LCO: 5'-GGGTGATAATTCG-AACAGAA-3' and reverse: RCO: 5'-TAGTTAAAGCCCCAG-CTAAA-3') (Nuryanto *et al*., 2007) and *T. squamosa* (forward: SQUA-F3: 5'-CATCGTTTAGAGTAATAATTCG-3' and reverse: SQUA-RI: 5'-ATGTATAA ACAAAACAGGATC-3') (Deboer *et al*., 2008).

PCR amplification was performed in of 25 *µL* of reaction mixture containing approximately 0.50 µL DNA template, 0.10 µM of forward and reverse primers, 0.50 µM 10X Easy Taq® *Buffer, 0.1 µM of 2.5mM dNTP, 1U Easy* Taq® *DNA* polymerase (500 U/*µl*) (Nanogene, Kuala

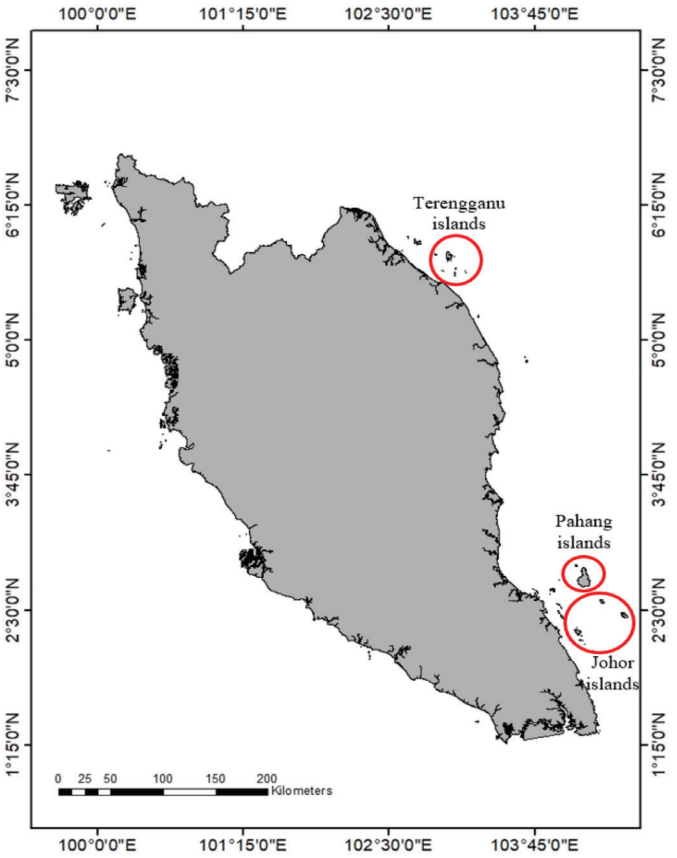

Figure 1: Sampling sites of giant clams in island groups of three states in the east coast of Peninsular Malaysia

Lumpur, Malaysia) and 18.8 µL distilled water (ddH2O). The amplification was carried out in the Master Cycler EP (Eppendorf, Hamburg, Germany). Thermocycling profile for *T. crocea* and *T. maxima* was set to follow Nuryanto *et al.* (2007) while *T. squamosa* was set according to Deboer *et al.* (2008). Successfully amplified products were sent to a third party (Apical Scientific Sdn Bhd, Kuala Lumpur, Malaysia) for purification and sequencing.

DNA Sequencing and Analysis

The sequences were aligned using MEGA 7.0 software (Kumar *et al*., 2016) and the final alignment was screened for stop codons and insertion-deletion mutations, which were absent, to ensure that only the targeted sequences were analysed. Haplotypes were identified using DnaSP software version 5.0.1.1 (Rozas *et al*., 2017). The genetic inter-specific and intra-specific distances were estimated in MEGA 7.0 (Kumar *et al*., 2016) using our haplotype data (Accession No. MT499022 to MT499030) with the outgroup *Cerastoderma edule* (Accession No. EU523670.1).

Sequence searches were conducted for species identification in online databases, which were the Basic Local Alignment Search Tool (BLAST) of the National Centre of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and Barcode of Life Data System (BOLD) (www.barcodinglife.org) (Ratnasingham & Hebert, 2007). Phylogenetic trees were constructed based on two approaches: (1) using current haplotype data of all three putative species and (2) inclusion of representative haplotypes of other giant clam sequences from GenBank (Table 1) to depict the

Table 1: Archived GenBank sequences of MT-CO1 gene included in the study

Species	Locality	Accession Numbers
Tridacna noae	Australia	KT865882.1
Tridacna noae	Australia	KT865883.1
Tridacna noae	Australia	KT865884.1
Tridacna noae	Australia	KT865885.1
Tridacna gigas	Philippines	KJ202113.1
Tridacna crocea	Indonesia	EU003606.1
Tridacna crocea	Indonesia	EU003607.1
Tridacna crocea	Indonesia	EU003608.1
Tridacna crocea	Philippines	KJ202111.1
Tridacna maxima	Indonesia	EU003613.1
Tridacna maxima	Indonesia	EU346365.1
Tridacna maxima	Indonesia	EU346366.1
Tridacna maxima	Indonesia	EU346367.1
Tridacna derasa	Philippines	KJ202112.1
Tridacna squamosa	Indonesia	EU346362.1
Tridacna squamosa	Indonesia	EU346363.1
Tridacna squamosa	Singapore	JN392020.1
Tridacna squamosa	Singapore	JN392021.1
Hippopus hippopus	Philippines	KJ202105.1
Hippopus hippopus	Philippines	KJ202106.1
Cerastoderma edule	Spain	EU523670.1

relationships within and between populations and species, with *C. edule* as an outgroup. Phylogenetic trees of Neighbor Joining (NJ) and Maximum Likelihood (ML) were constructed using best fit model (Tamura-three-parameter model) with 1,000 bootstrap replicates in MEGA 7.0 software (Kumar *et al*., 2016) to ensure the robustness of the trees.

Results and Discussion

Species Identification

An approximately 467-bp fragment of the MT-CO1 gene was amplified in all specimens. The sequences generated were compared with GenBank BLAST and BOLD archived sequences. This validation step revealed several discrepancies between the morphological and genetic identification. Five morphologically identified *T. crocea* specimens from Terengganu islands (Redang and Bidong) were genetically identified as *T. maxima*. It should be noted that all of these ambiguous identifications were juvenile individuals. *T. squamosa* and *T. maxima* were more widespread, while *T. crocea* appeared to be restricted to the southeastern islands of Pahang and Johor (Table 2).

Levels of inter- and intra-specific divergences in MT-CO1 were estimated in Table 3. No overlap was detected between inter- and intra-specific divergence i.e. presence of a barcode gap was noted. Pairwise genetic distances were according to those expected at different hierarchical levels. I Inter-specific divergence varied from 10.6 % to 16.2 %, whereas intra-specific divergence varied from 0.6 % to 0.9 %. Inter-generic distance between the outgroup *C. edule* and ingroup members showed values ranging from 37.2 % to 38.5 % (Table 4). *T. squamosa* and *T. crocea* were more closely related to each other (0.106) than with *T. maxima* (0.162 and 0.146, respectively) (Table 3).

Table 2: Comparative identification of giant clams based on morphological features and MT-CO1 gene analysis.

Islands Group	Island (N)	Species Identification		Average Percentage Similarities	
		Morphological (N)	MT-CO1 (N)	BLAST	BOLD
Terengganu	Redang (60)	*T. maxima* (41)	*T. maxima* (41)	99.08	98.46
		T. squamosa (17)	T. squamosa (17)	97.98	99.24
		T. crocea (2) #	*T. maxima* (2)	99.28	98.61
	Bidong (47)	*T. maxima* (25)	*T. maxima* (25)	99.16	98.27
		T. squamosa (19)	*T. squamosa* (19)	97.92	99.18
		T. crocea (3) #	*T. maxima* (3)	99.17	97.93
Pahang	Tioman (46)	*T. crocea* (9)	*T. crocea* (9)	99.45	99.10
		T. squamosa (14)	*T. squamosa* (14)	98.10	97.79
		T. maxima (23)	*T. maxima* (23)	99.10	99.58
Johor	Babi Besar (28)	*T. crocea* (28)	*T. crocea* (28)	99.07	98.96
	Pemanggil (28)	*T. crocea* (28)	*T. crocea* (28)	99.38	99.05
	Tinggi (6)	*T. crocea* (6)	*T. crocea* (6)	99.34	99.08
	Metinggi (11)	*T. crocea* (11)	*T. crocea* (11)	99.02	99.05
	Lima Besar (18)	*T. squamosa* (15)	*T. squamosa* (15)	98.13	98.95
		T. maxima (3)	*T. maxima* (3)	99.28	99.44
	Lima Kechil (3)	*T. squamosa* (3)	*T. squamosa* (3)	98.12	99.37

*N: No of individuals; #: Juveniles

Table 3: Inter- and intra-specific divergence of MT-CO1 sequences using Tamura-3-parameter (T92) with 1,000 bootstrap replications.

	T. crocea	*T. maxima*	*T. squamosa*	*C. edule*
T. crocea	**0.009**			
T. maxima	0.146	**0.006**		
T. squamosa	0.106	0.162	**0.007**	
C. edule	0.372	0.385	0.385	**n/c**

*Texts in bold indicate intra-specific divergences

The present study successfully verified the taxonomy and distribution of giant clams in the east coast of Peninsular Malaysia using the DNA barcoding technique. The findings indicated that MT-CO1 gene was efficient in species delineation of giant clams as evidenced by the distinct barcoding gap in line with a comprehensive study by Mikkelsen *et al*., (2007) of marine bivalves, which did not detect overlapping values of inter- and intra-specific divergence. In spite of the intensive sampling, only three species of giant clams were found in the surveyed areas, namely *T. crocea, T. maxima* and *T. squamosa*, although an additional species (*H. hippopus*) had been previously documented based on morphological characteristics (Neo *et al*., 2017).

In addition, based on the underwater observation during sampling, all samples had the general characteristics of the genus Tridacna and no sample could be identified to the genus *Hippopus*. Morphologically, the appearance of mantles differentiated between the two genera of giant clams, in which the *Hippopus* mantle did not extend over the margin of the shell. However, mantles from *Tridacna* did extend over the margin of the shells (Figure 2). This taxonomic identification was confirmed through the DNA barcoding approach. The current study confirmed the presence of only three giant clam species: *T. crocea*, *T. maxima* and *T. squamosa* with high identity (> 98%) when compared with database sequences (Table 2).

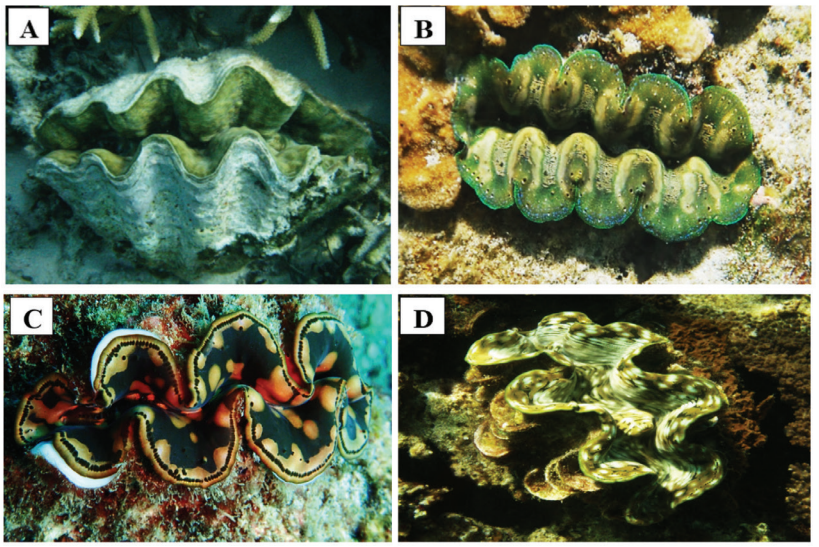

Figure 2: Mantles of giant clam species (A) *Hippopus hippopus* of Layang-layang Island (Courtesy of Kee Alfian Abdul Adzis), (B) *Tridacna crocea* from Metinggi island, (C) *Tridacna maxima* from Bidong island (Courtesy of Muhammad Haris Hanafi Mohd Habali) and (D) *Tridacna squamosa* from Bidong island

Previous studies had noted the rarity of *H. hippopus*, which was only limited to the islands off Johor and in Sabah waters (UNEP, 2007; Neo *et al*., 2017). However, this study had failed to detect the species in the Johor islands. Since the current study was fairly extensive in its geographical coverage, we believe that the species might be on the verge of local extinction, if not already. It had been reported to be locally extinct in Singapore (Othman *et al*., 2010).

Furthermore, while Neo *et al*. (2017) noted a wide occurrence of *T. crocea* in Malaysian waters, our study did not succeed in obtaining the species at Bidong and Redang islands in Terengganu. However, they did not state whether this species was also recorded at the two sites surveyed in the current study. A genetic diversity study of *T. maxima* and *T. squamosa* in Perhentian island, Terengganu, also did not make any mention of *T. crocea* (Lim *et al*., 2018). The author reported an intra-specific genetic variation ranging from 8.1 % to 8.3 % and inter-specific variation from 12.9 % to 17.2- %. The inter-specific variation of 16.2 % in the current study is concordant with the earlier study, but our intra-specific variation was markedly different. The current study recorded intra-specific variation of 0.6 % to 0.9 %, which was consistent with a study on Malaysian oysters (Suzana *et al*., 2011) and deep-sea clams (Liu and Zhang, 2018), which observed values ranging from 0.1 % to 1.1 % and 0.0 % to 2.66 %, respectively. Intra-specific variation in this present study was also in concordance with a comprehensive study of four marine bivalve genera that recorded an intra-species variation of 0.0 % to 3.2 % (Mikkelsen et al., 2007). Thus, while the giant clam genetic variability was low, there was still some level of variability that should be considered in their management strategies.

Inconsistencies of morphologically identified juveniles, initially presumed to be *T. crocea* specimens, were observed. They were then re-classified as *T. maxima* based on genetic data. During the juvenile stage, shells of *T. maxima* were highly similar to *T. crocea* (Knop, 1996). Thus, it was not surprising that the immature specimens of the former might have been mistaken for the latter. This was in agreement with the study by Waheed (2016), who found presumed *T. crocea* collected from Redang island to be juvenile *T. maxima*. Both species characteristically embedded into the substrate (Neo *et al*., 2017). Using phenotypic characters alone could be tricky in identifying the species as the patterns were highly variable due to environmental effects (Colgan *et al*., 2007).

Phylogenetic Relationships

The Neighbor-Joining (NJ) and Maximum Likelihood (ML) trees based on 57 unique haplotypes from three species of giant clams based on a Tamura three-parameter model showed similar topology. Each giant clam species formed a monophyletic group with high support as depicted in Figure 3. A re-analysis of combined GenBank reference sequences with other giant clam species yielded similar tree topology in both trees with monophyletic clustering into their own respective groups. Our study showed a close relationship between *T. squamosa* and *T. crocea* with *Tridacna noae,* while *T. maxima* was a sister to this cluster. *T. derasa* was basal to these four species. *H. hippopus* was more closely related to *T. gigas* than to other *Tridacna* species analysed (Figure 4).

In parallel with previous research, our study reported the genetic affinity of *T. squamosa* with *T. crocea* compared to *T. maxima* (Hui *et al*., 2016; Lizano & Santos, 2014) although the first pair was well delineated morphologically. However, *T. crocea* and *T. maxima* had higher morphological similarities, such as mantle coloration (blue, green and brown) and both were found embedded into the substrate. This characteristic often led to misidentification, particularly in juveniles as observed by Waheed (2016), and similar in this study. Classification of many bivalve groups based on morphology alone (e.g: shell characters) was often very challenging, even among experienced

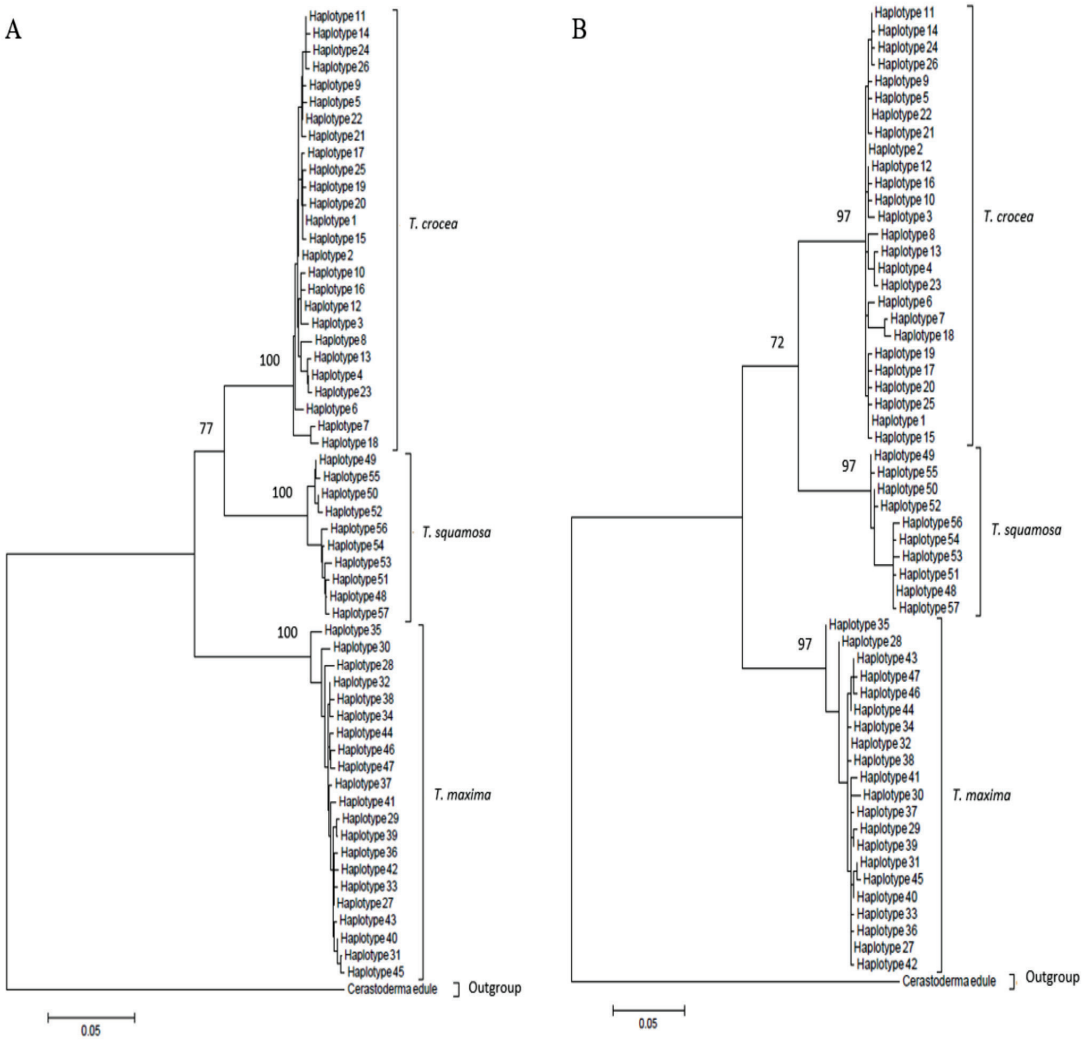

Figure 3: Phylogenetics relationships of three *Tridacna* spp. from east coast of Peninsular Malaysia based on (A) Neighbour-Joining (B) Maximum Likelihood (Haplotype 1-26 is *T. crocea*, Haplotype 48-57 is *T. squamosa* and Haplotype 27-47 is *T. maxima*)

taxonomists, as highlighted in this case when shells in the juvenile stage of giant clam species closely resembled each other (Knop, 1996). This study affirmed that the application of DNA barcoding in identification of species would enable differentiating species that shared similar morphology during juvenile stages.

Implications to Conservation

In the past decades, giant clam populations had been over-exploited due to high economic demand as a food source and in the ornamental aquarium trade (Van Wynsberge *et al*., 2015; Mies *et al*., 2017). Considering the global declination of giant clam species worldwide, it had become a great urgency to protect and conserve these marine bivalves. This was because giant clams were keystone species that

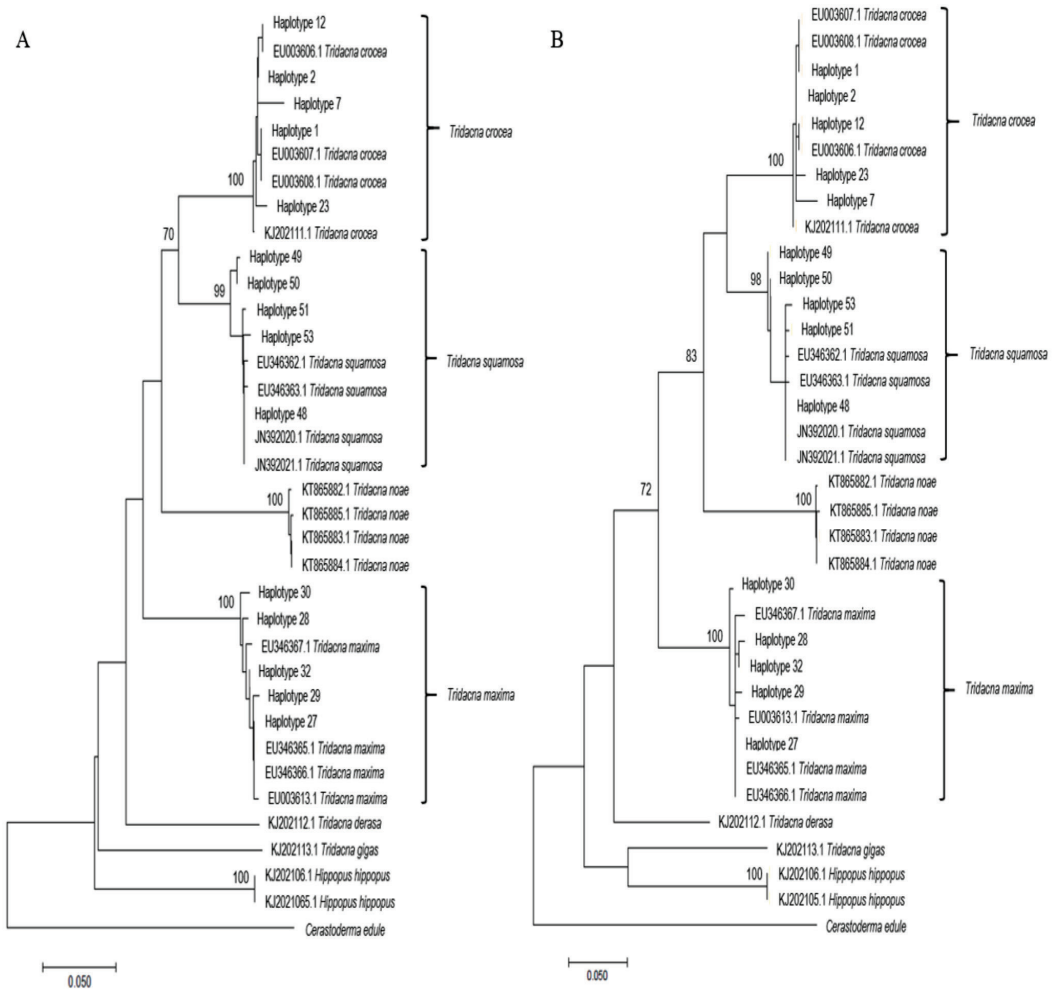

Figure 4: (A) Neighbor-Joining (B) Maximum Likelihood trees of three *Tridacna* spp. with sequences of other giant clam species from GenBank. Note: Only representative haplotypes of *T. crocea*, *T. maxima* and *T. squamosa* are shown in the tree

played significant roles in coral reef ecosystems (Guibert *et al*., 2020). Giant clams lived in a symbiotic-mutualism relationship with the microalgae, which allowed both organisms to benefit from each other. Giant clam provided shelter and enough sunlight to zooxanthellae, while the microalgae provided food and energy requirements via photosynthesis (Ikeda *et al*., 2017; Morishima *et al*., 2019).

In addition, any excess zooxanthellae algae released from giant clams could be taken up by other zooxanthellate-dependent species, including other marine classes like Anthozoa, Scyphozoa, Hydrozoa, Gastropoda and Bivalvia. Hence, this could contribute to a balance in the coral reef ecosystem health and biodiversity (Neo *et al*., 2015; Morishima *et al*., 2019). Apart from that, the calcium carbonate shells of giant clams also provided substrate for epibiont colonization, increasing the topographic features of coral reefs and act as nurseries for fish (Cabaitan *et al*., 2008; Neo *et al.,* 2015).

In summary, maintaining healthy populations of giant clams could provide various benefits to coral reef ecosystems in numerous

underappreciated ways. Therefore, with this knowledge, conservation and rehabilitation of giant clams should be prioritized in future management strategy, and any rehabilitation and restocking programme should take into consideration the distribution of native giant clam species. For example, to avoid disrupting the local ecosystem in Bidong and Redang islands off Terengganu, , *T. crocea* should not be introduced in those places because they were originally absent in the area.

Conclusions

Our findings had identified three species of giant clams: *T. crocea*, *T. maxima* and *T. squamosa* inhabiting the east coast of Peninsular Malaysia. *T. crocea* was restricted to the southeastern islands of Pahang and Johor. *T. maxima* and *T. squamosa* were found to be omnipresent in all the islands, whereas *H. hippopus* was not recorded in all the surveyed islands. The molecular barcoding approach used in this study had proven to be beneficial in understanding the taxonomic status and distribution of giant clam species in the east coast of Peninsular Malaysia. The output from a DNA barcoding study would be useful for conservation and sustainable management of the giant clams.

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