ARTICLE

Updates on the status of giant clams *Tridacna* spp. and *Hippopus hippopus* in the Philippines using mitochondrial CO1 and 16S rRNA genes

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The CO1 and 16S rRNA genes of six of the possible eight species of giant clams (*Hippopus hippopus*, *Tridacna gigas, T. crocea*, *T. squamosa*, *T. derasa*, and *Tridacna* sp.YCT-2005) under the Tridacnidae family found in the Philippines were sequenced for molecular approach-based species identification. We first reported here the CO1 sequence of *H. hippopus* and made it available online through GenBank. We also reported the first sighting of *Tridacna* sp. YCT-2005 in Philippine waters, an undescribed species of giant clam, which has initially been reported to be a potentially new species that was thought to be found only in Taiwan. Phylogenetic trees of CO1 and 16S rRNA gene sequences of giant clam samples from the Philippines were constructed using both the Neighbor-Joining approach and the Maximum-Likelihood approach. Both trees showed similar topology in which *Tridacna* and *Hippopus* formed two distinct clades. *T. crocea, T. squamosa*, *T. maxima, T. costata*, and *Tridacna* sp. YCT-2005 showed monophyletic grouping under subgenus Chametrachea confirming the recognized groupings of giant clams based on morphology. On the other hand, restriction site mapping based on the 16S rRNA gene showed a unique recognition site at 367-370 bp (5'AGCT3') for the species of *T. maxima* as opposed to the species of *Tridacna* sp. YCT-2005. Alu I restriction endonuclease was identified as a candidate diagnostic enzyme to differentiate between these species. This study confirmed the identity of giant clams found in the Philippines using molecular techniques. The use of DNA barcoding can be a useful tool to identify different species of giant clams which is needed for their proper management and conservation in the Philippines, since they are all declared as endangered.

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INTRODUCTION

Giant clams are one of the world's largest bivalves, ranging from 15 cm for *Tridacna crocea* Lammarck 1819 to 150 cm for *Tridacna gigas* Linnaeus 1758 (Juinio et al. 1989, Lucas 1988). These clams belong to the subfamily Tridacninae, which has two genera namely: *Hippopus* and *Tridacna* (Othman et al. 2010). Two species belong to Genus *Hipopopus*: *Hippopus hippopus* Linnaeus 1758 and *Hippopus porcellanus* Rosewater 1982. Three subgenera belong to Genus *Tridacna*: *Tridacna* sensu *stricto* which is composed of one species (*T. gigas*); Chametrachea which is composed of four species (*Tridacna maxima* Roding 1798, *Tridacna squamosa* Lamarck 1819, *Tridacna crocea* Lamarck 1819, and *Tridacna costata* Roa-Quiaoit, Kochzius,

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Jantzen, Zibdah and Richter 2008); and Persikima which is composed of two species (*Tridacna derasa* Roding 1798 and *Tridacna tevoroa* Lucas, Ledua and Braley (Lucas et al. 1991)). The present status of *Tridacna rosewateri* Sirenko and Scarlato 1991 is still ambiguous (Benzie and Williams 1998).

Past researches on giant clam taxonomy have previously been reported and have focused on molecular phylogeny. Benzie and Williams (1998) studied allozyme variation at 26 loci and found support for the prevailing taxonomy of giant clams. *Hippopus* and *Tridacna* were monophyletic sister taxa, and three monophyletic subgenera could be discriminated within Tridacna: Tridacna s.s. (*T. gigas*); Chametrachea (*T. squamosa, T. maxima* and *T. crocea*); and Persikima (*T. derasa* and *T. tevoroa*). Maruyama et al. (1998) studied the phylogeny of giant clams using partial 18S rDNA and obtained three different phylogenetic trees for subgenus Chametrachea: *T. maxima* (*T. crocea* + *T. squamosa*), *T. crocea* (*T. squamosa* + *T. maxima*), and *T. squamosa* (*T. crocea* + *T. maxima*), all with high bootstrap support. Schneider and O'Foighil (1999) studied tridacnine relationships by analyzing partial mitochondrial 16S rRNA genes and concluded that genera *Hippopus* and *Tridacna* are monophyletic sister taxa and subgenus Chametrachea, which has the same topology with the second tree proposed by Maruyama et al. (1998), is a sister taxon to *T. trevoroa* (*T. derasa* + *T. gigas*), with these three latter taxa all being placed in a single subgenus, *Tridacna (Tridacna)*. In contrast, however, Nuryanto et al. (2007) conducted a phylogenetic analysis of four species of giant clams (*T. maxima, T. squamosa, T. crocea*, and *T. gigas*) using the CO1 genetic marker and showed these giant clams to be monophyletic (*T. crocea* + *T. squamosa)* and (*T. maxima* + *T. gigas)*. Indeed, there is still a need to do genetic profiling to identify giant clams existing in Philippine waters to obtain information that can be used to properly manage and conserve them in the future.

The mtDNA cytochrome *c* oxidase subunit 1 gene (CO1) is being widely used for molecular species identification worldwide (Hebert et al. 2003). This molecular method is also known as DNA barcoding. DNA barcoding overcomes the problem caused by comparable morphologically similar species leading to incorrect species identifications. The CO1 gene also appears to be among the most conservative protein-coding genes in the mitochondrial genome of animals and contains a great range of phylogenetic signals which helps discriminate closely related species and phylogeographic groups within a single species (Brown et al. 1986, Folmer et al. 1994, Hebert et al. 2003, Cox and Hebert 2001, Wares and Cunningham 2001). Another useful marker for supporting species identification is the mitochondrial 16S rRNA gene, which has also been widely used for species identification and for delineating phylogenetic relationships among marine organisms (Guo et al. 2011).

A vast diversity of giant clams is found in the central Indo-Pacific region (Spalding et al. 2007). Unfortunately, anthropogenic and environmental factors all contribute to the decline of their population. Recent surveys on their distribution and density in 15 countries showed that their population density typically ranges from 10^{-3} to 10^{-5} individuals per square meter; however, some populations reached numbers of more than 100 individuals per square meter (Othman et al. 2010). In the Philippines, seven species of giant clams (*H. hippopus, H. porcellanus, T. gigas*, *T. derasa, T. maxima, T. squamosa* and *T. crocea*) are currently reported (Convention on international trade in endangered species (CITES) 2012). They are locally known as "kabibe", "kima", "taklobo", "manglut", or "saliot" and are important food source and substrate for reef-associated marine organisms (Alcazar 1986, Mingoa-Licuanan and Gomez 2002). Several sites in the Philippines have densities of less than 10^{-6} giant clam individuals per square meter (Othman et al. 2010) and some sites have exhibited local extinctions (Lucas 1994).

In this study, we have identified different species of giant clams sampled in the Philippines using DNA barcoding based on CO1 and 16S rRNA gene regions. We also generated for the first time the CO1 sequence of *H. hippopus* species. We also reported here some evidence of the presence of *Tridacna* sp. YCT-2005 in Philippine waters, a possibly new undescribed species of giant clam that was thought to be found only in Taiwan (YC Tang, unpublished observations).

MATERIALS AND METHODS

Sample Collection

Giant clams were collected from two different sites in the Philippines (Figure 1A). Initial identifications were done in the field using FAO species identification guide volume 1 (Carpenter and Niem 1998) and through the help of Mr. Julio Curiano of the giant clam culture divison of NFRDI in Guiuan, Samar. *H. hippopus*, *T. gigas*, *T. crocea*, *T. squamosa* and *T. derasa* (Figures 1B, C, D, E and F) were collected at Guiuan, Eastern Samar. However, *Tridacna* sp. YCT-2005, which was initially identified as *Tridacna maxima* (Figure 1G), was obtained from Bolinao, Pangasinan but later found to have originated from Sibulan, Negros Occidental. Giant clams were taken out of the water for five minutes and a small amount of mantle tissue was clipped and placed in a 1.5-ml microcentrifuge tube containing 95% ethanol. Voucher specimens of some of the giant clam species were placed in an ice chest and brought back to the laboratory for subsequent analysis. The clams were immediately returned to the water after the sampling was done. Voucher specimens were stored in 95% ethanol at the NFRDI-GFL laboratory.

DNA Extraction

One hundred fifty mg of tissue sample were dissected using sterile disposable razors. DNA extraction was done using the method of Santos et al. (2010) with minor modifications. Briefly, tissue samples were placed in a 1.5-ml micro centrifuge tube containing 600 µL of freshly prepared pre-warmed (65°C) 2% CTAB extraction buffer (pH 8.5) and 30 µL of Protenaise K, and

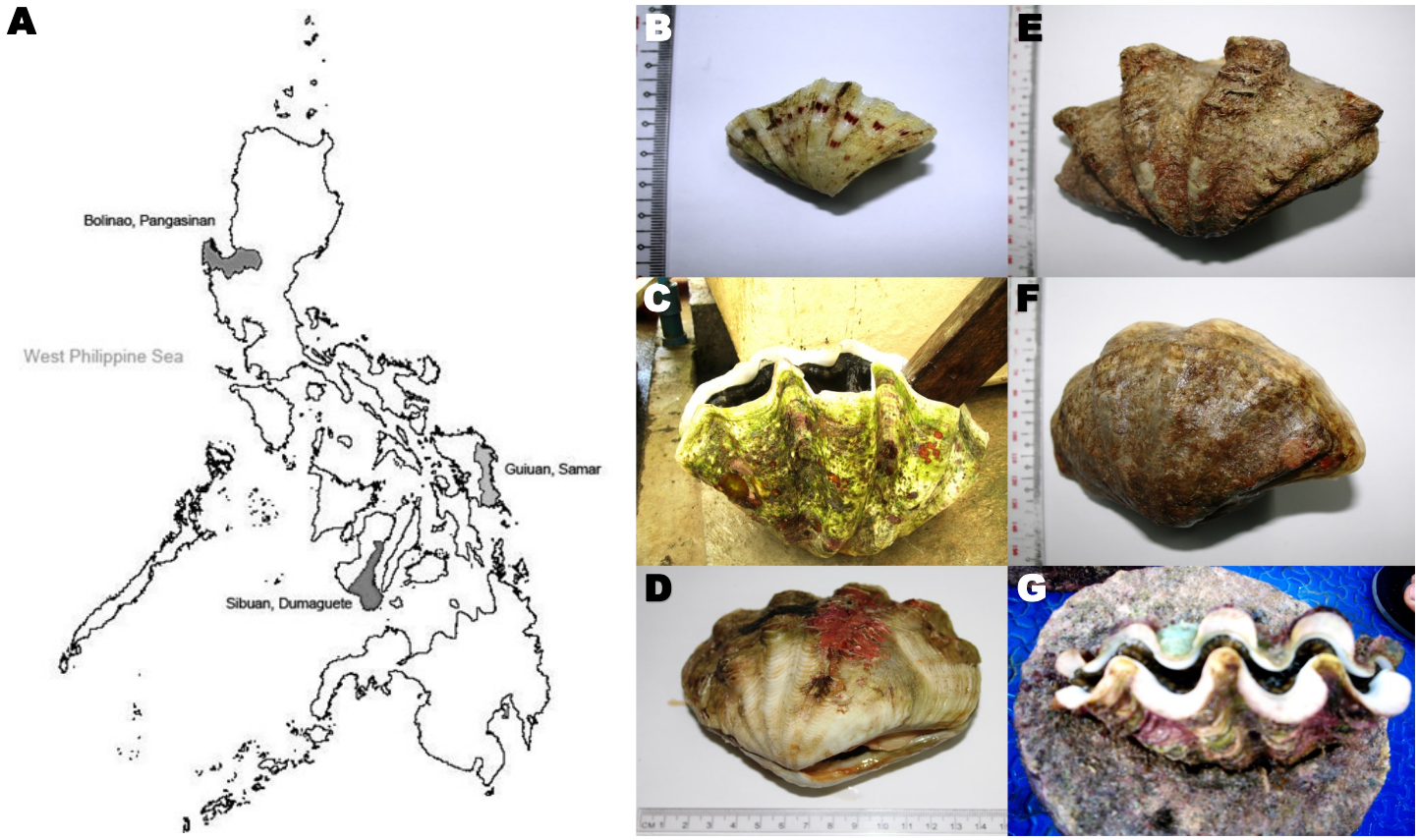

Figure 1. A. Sampling sites: Bolinao, Pangasinan, Guiuan, Samar and Sibulan, Dumaguete; **B**, *Hippopus hippopus* (strawberry clam); **C**, *Tridacna gigas* (true giant clam); **D**, *Tridacna crocea* (boring clam); **E**, *Tridacna squamosa* (fluted clam); **F**, *Tridacna derasa* (Derasa clam); and **G**, *Tridacna* sp. YCT-2005.

shaken vigorously by inversion. The tubes were incubated at 55° C in a water bath overnight with intermittent shaking and swirled every 30 minutes for the first three hours. After incubation, samples were centrifuged at 6,000 rpm for 30 seconds. 600 µL of chloroform:isoamylalcohol (3:1) were added and mixed properly by inversion for three minutes, and centrifuged at 8,000 rpm for 5 minutes to separate the phases. The supernatant was transferred to a new tube and the procedures done from and following the addition of chloroform:isoamyalchol were done twice. Samples were precipitated with 50 µL of 3M sodium acetate (NaOAc) and 900 µL of cold 95% ethanol; the solution was gently mixed to produce fibrous DNA and incubated at –20°C overnight. After precipitation, the tubes were centrifuged at 12,000 rpm for 10 min. Lastly, DNA pellets were washed with 900 µl 70% ethanol twice, air dried and resuspended in 500 µL of 1X TE buffer, and stored in a refrigerator.

PCR Amplification

mtDNA CO1 gene. A fragment of the CO1 gene for the six species of giant clams was amplified using three types of primer. A general primer from Folmer et al. (1994) (forward: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse: HCO2198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') only amplified *H. hippopus* and *T. derasa.* Thus, we used another pair of primers specific for *Tridacna* based on the study of Nuryanto et al. (2007) (forward: LCO: 5'-GGGTGATAATTCG-AACAGAA-3' and reverse: RCO: 5'-TAGTTAAAGCCCCAG-CTAAA-3') to amplify CO1 genes from the remaining giant clam species. However, the tridacnid-specific primer only amplified *T. crocea*, *Tridacna* sp. YCT-2005, *H. hippopus* and *T. derasa*. Therefore, we designed a new tridacnid-specific primer for the remaining tridacnid species (forward: TF1: 5'-GAACAGAA -TTAGCATGGCCTG-3' and reverse: RF1: 5'-AGCTAACACA -GGCATTGCCAC-3'), which successfully amplified the CO1 gene in *T. squamosa* and *T. gigas*. The list of primers used in this study is shown in Table 1. Polymerase Chain Reaction (PCR) reactions were carried out in a total volume of 25 µL containing approximately: 1 µL DNA template, 2.5 µL 10x PCR Buffer with 1.5mM $MgCl_2$, 2.5 µL dNTPs (2mM), 2.5 µL $MgCl_2$ (10mM), 2.0 µL of each primers (10µM), 0.2 µL 5 units Taq Polymerase (Kappa Taq), and 12.3 µL ddH_20. PCR was performed under the following conditions: initial denaturation temperature at 94°C for 5 minutes, 35 cycles of denaturation temperature at 94°C for 1 min., 1.5 minutes annealing temperature (at 47°C for *T. crocea,* 48°C for *H. Hippopus* and *T. derasa*, and 45° C for *T. squamosa*, *T. gigas* and *Tridacna* sp. YCT-2005) and one minute extension at 72°C. Final DNA extension was carried

Table 1. Information on giant clam species collected in the Philippines.

Species Name	Sample ID	Place of Collection	Accession No. (GenBank database system)		Collector
			CO1	16S rRNA	
Tridacna crocea	Tc1	Guiuan Samar, Philippines	KJ202107	N.A	Apollo Lizano & Nonita Cabacaba
	Tc2		KJ202108	KJ508352	
	Tc4		KJ202111	KJ508351	
	Tc5		KJ202109	KJ508350	
	Tc7		KJ202110	KJ508349	
Tridacna squamosa	Ts1	Guiuan Samar, Philippines	KJ202117	KJ508358	Apollo Lizano & Nonita Cabacaba
Tridacna sp.YCT-2005	Tm1	Bolinao, Pangasinan	KJ202114	KJ508356	Apollo Lizano & UP MSI staff
	Tm2		KJ202115	KJ508355	
	Tm3		KJ202116	KJ508357	
Tridacna derasa	Td1	Samar, Philippines	KJ202112	KJ508353	Apollo Lizano & Nonita Cabacaba
Tridacna gigas	Tg	Samar, Philippines	KJ202113	KJ508354	Apollo Lizano & Nonita Cabacaba
Hippopus hippopus	Hh1	Samar, Philippines	KJ202105	KJ508348	Apollo Lizano & Nonita Cabacaba
	Hh2		KJ202106	KJ508347	

Partial sequences of mitochondrial DNA CO1 gene were submitted to GenBank database.

out at 72°C for 5 minutes. PCR products were then run on a 1% agarose gel and viewed under UV light.

16S rRNA. The large 16S ribosomal RNA genes of the six species of giant clams were amplified using the following primers from Kessing et al. (1989): 16Sar (5'-CGCCTGTTTATC-AAAAACAT-3') and 16Sbr (5'CCGGTCTGAACTCAGATC-ACGT-3'). PCR reactions were done using the protocol for mtDNA CO1 gene amplification except that the annealing temperature was adjusted to 45°C for all giant clam species.

DNA Sequencing and Analysis

PCR products were sent to Macrogen Inc., Korea for sequencing. All mtDNA generated sequences including 13 CO1 sequences and 12 16S rRNA sequences (five *T. crocea,* one *T. squamosa,* three *Tridacna* sp. YCT-2005, one *T. gigas,* one *T. derasa* and two *H. hippopus* for both mtDNA markers) were initially aligned and edited using Geneious software (Drummond and Rambaut 2007). A total of 60 CO1 sequences from five species of giant clams (*T. crocea, T. squamosa, Tridacna* sp. YCT-2005, *T. maxima*, and *T. derasa*) and 71 16S rRNA sequences from 10 species of giant clams (*T. crocea, T. squamosa*, *Tridacna* sp. YCT-2005, *Tridacna costata*, *T. maxima, T. tevoroa, T. gigas, T.* derasa, *Hippopus porcellanus,* and *Hippopus hippopus*) were downloaded from GenBank and were included in the phylogenetic analysis (Table 2). All sequences were collapsed to 75 unique haplotypes for CO1 and 53 unique haplotypes for 16S rRNA using the online software FaBox (Villesen 2007). Multiple sequence alignment for all unique haplotypes was performed using Clustal W (Thompson et al. 1994). Molecular identification of the different giant clams in the Philippines using DNA barcoding was based on the initial six species of *Tridacna*, and one species of *Hippopus*. *Cerastoderma edule* from GenBank (Accession Numbers EU733081 for 16S rRNA and EU523670 for CO1) was used as an outgroup taxon. Identification and discrimination of giant clam samples were based on CO1 and 16S rRNA gene sequences. Phylogenetic trees for both loci were constructed using the Neighbor-Joining (NJ) approach and the Maximum-Likelihood (ML) approach with the model Tamura 3-parameter with Gamma correction (T92+G) for both genes based on model selection implemented in MEGA software (Ver. 5.02). Sequence alignment of *Tridacna* sp. YCT-2005 and *T. maxima* for restriction enzyme site mapping was performed using the program Geneious software ver. 6.0.1 (Drummond and Rambaut 2007).

All CO1 and 16S rRNA gene sequences generated in this study were submitted to GenBank and were assigned accession numbers.

RESULTS

Table 1 shows the list and GenBank Accession Numbers of the 16S rRNA and CO1 sequences from the giant clam species collected from Philippine waters. Tables 2 and 3 show the 16S rRNA and CO1 pairwise genetic distance estimates between groups of giant clams with respect to the outgroup species of the most basal living member of the Lymnocardiinae family (*C. edule*).

Table 2. Pairwise genetic distance estimates between ten species of giant clams and one species of the outgroup taxon *Cerastoderma edule* using 413 bp of large 16s ribosomal RNA gene based on Tamura 3-parameter; 1000 bootstrap value implemented in MEGA ver. 4.0

		1	2	3	4	5	6	7	8	9	10	11
1	*Cerastroderma edule*	-										
2	*Tridacna crocea*	0.446	-									
3	*Tridacna squamosa*	0.443	0. 024	-								
4	*Tridacna* sp. YCT-2005	0.450	0. 046	0.034	-							
5	*Tridacna gigas*	0.487	0.114	0.110	0.098	-						
6	*Tridacna derasa*	0.492	0.070	0.066	0.039	0.083	-					
7	*Hippopus hippopus*	0.402	0.164	0.162	0.142	0.181	0.168	-				
8	*Hippopus porcellanus*	0.407	0.175	0.169	0.152	0.184	0.180	0.079	-			
9	*Tridacna maxima*	0.419	0.064	0.049	0.044	0.097	0.077	0.152	0.165	-		
10	*Tridacna tevoroa*	0.478	0.099	0.102	0.91	0.109	0.088	0.180	0.160	0.111	-	
11	*Tridacna costata*	0.425	0.064	0.059	0.032	0.101	0.062	0.153	0.149	0.035	0.095	-

Figure 2B shows the NJ tree of 75 sequences (75 unique haplotypes) from seven species of giant clams and one outgroup taxon (*C. edule*) based on 417 bp of the mitochondrial DNA CO1 gene using Tamura 3-parameter with a bootstrap support (N=1000 replicates). CO1 and 16S rRNA gene sequences were used for species discrimination and identification of giant clams collected from the Philippines in relation to reference sequences mined from GenBank. The tree showed a monophyletic grouping under the genera *Hippopus* and *Tridacna*, and majority of collected giant clams clustered with the reference sequences. Interestingly, samples Tm1, Tm2 and Tm3 (Table 1), initially identified as *T. maxima*, grouped with the new undescribed species of giant clam, *Tridacna* sp. YCT-2005 (GenBank Accession Number DQ168140). In addition, a BLAST search for samples Tm1, Tm2 and Tm3 were done in BOLD and the GenBank database, and yielded a 98.5% similarity with *Tridacna* sp. YCT-2005 (uploaded by YC Tang, unpublished observations). Furthermore, *Tridacna* sp. YCT-2005 grouped with the species under the subgenus *Chametrachea* and showed a closer affiliation with *T. squamosa* than to other species under the same subgenus. Consequently, the sequence of *T. gigas* (GenBank Accession Number EU003616) clustered with other reference *T. maxima* sequences based on our CO1 analysis, which might indicate possible misidentification.

Figure 2A shows a similar analysis using the 16S rRNA gene from 83 sequences (53 unique haplotypes) of 10 species of giant clams and the results show a similar topology with the NJ tree constructed using the mtDNA CO1 gene. In the analysis, the 16S rRNA sequence of the Tm1, Tm2 and Tm3 samples also showed monophyletic grouping with the existing *Tridacna* sp. YCT-2005 16S rRNA reference sequence from GenBank. Our

Table 3. Pairwise genetic distance estimates between giant clam species based on cytochrome oxidase I gene (CO1) using Tamura 3-parameter; 1000 bootstrap value implemented in MEGA ver. 4.0

		1	2	3	4	5	6	7
1	*Cerastoderma edule*	-						
2	*Tridacna crocea*	0.385	-					
3	*Tridacna squamosa*	0.408	0.132	-				
4	*Tridacna* sp. YCT-2005	0.368	0.197	0.178	-			
6	*Tridacna derasa*	0.392	0.208	0.204	0.223	-		
7	*Hippopus hippopus*	0.405	0.270	0.260	0.244	0.225	-	
8	*Tridacna gigas*	0.418	0.225	0.228	0.287	0.251	0.254	-
9	*Tridacna maxima*	0.400	0.181	0.178	0.201	0.185	0.231	0.174

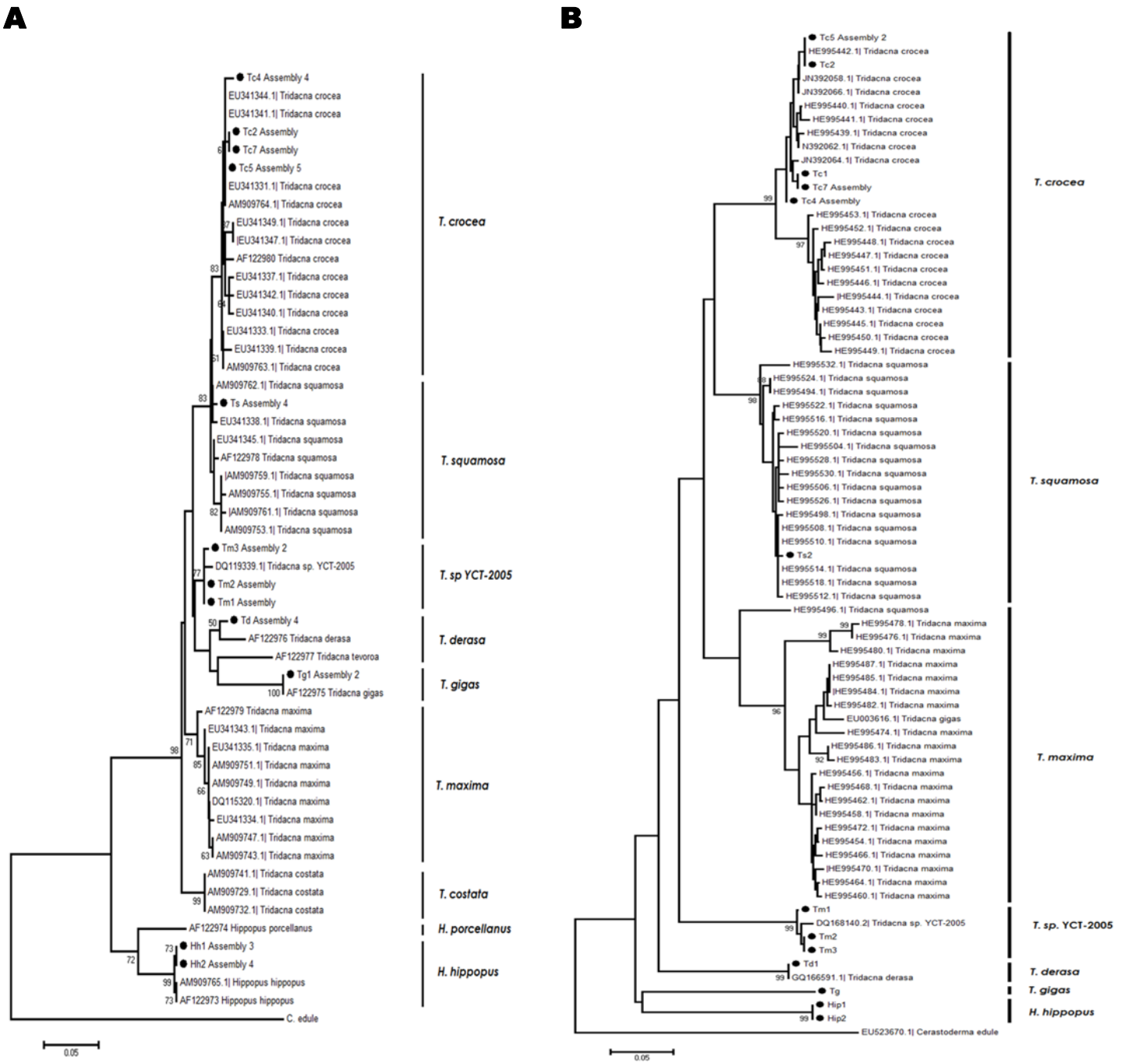

Figure 2. Neighbor-Joining tree of giant clams. A, 16S rRNA and **B**, mtDNA CO1. Giant clams sampled in the Philippines (with black circle) grouped with reference giant clams mined from GenBank except for pre-identified *T. maxima*, Tm1, Tm2, and Tm3 which grouped with *Tridacna* sp. YCT-2005. Node labels refers to bootstrap support (N=1000 replicates).

Philippine sample of *T. gigas* grouped with the only available 16S rRNA gene for *T. gigas* (GenBank Accession Number AF122977) with a bootstrap support of 100.

We also performed phylogenetic analysis using the ML approach for both mtDNA CO1 and 16S rRNA, and the tree yielded a similar result with that of the NJ approach (Figure 3).

Figure 4 shows the 16S rRNA sequence alignment of *Tridacna* sp. YCT-2005, *Tridacna maxima,* and samples Tm1, Tm2 and Tm3. After doing restriction site mapping we found that there is a unique restriction recognition site for the species of *T. maxima* at 367-370 bp (5'AGCT3') as opposed to the *Tridacna* sp. YCT-2005 species. Alu I was identified as a candidate diagnostic enzyme to differentiate between these species

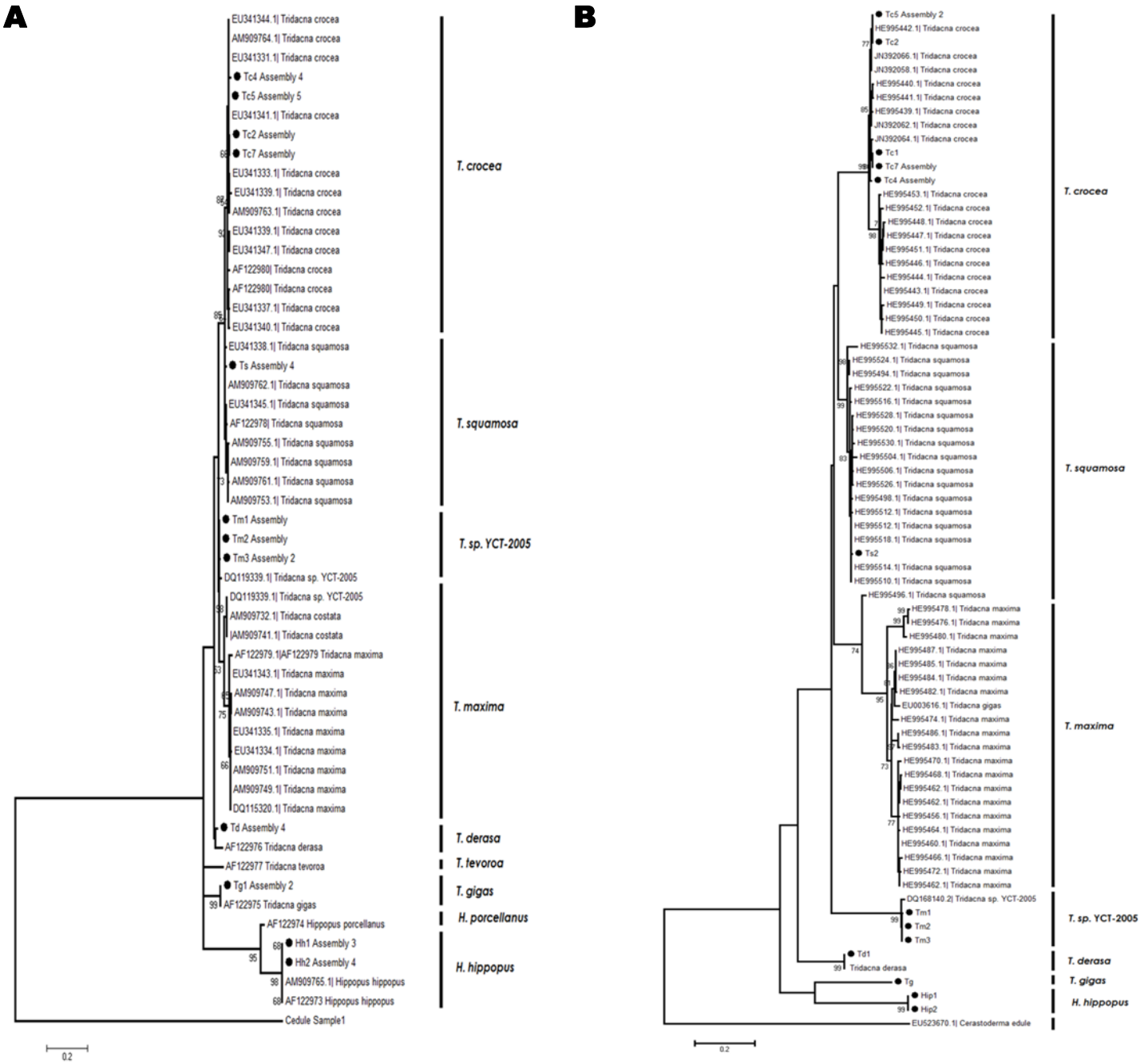

Figure 3. Maximum-Likelihood tree of giant clams. **A**, 16S rRNA and **B**, mtDNA CO1 showed similar topology with the NJ tree. Taxon with black circles represents giant clams sampled in the Philippines. Node labels refers to bootstrap support (N=1000 replicates).

and that could possibly be used for restriction fragment length polymorphism (RFLP) analysis.

DISCUSSION

This study was mainly referenced with the studies of Nuryanto et al. (2007) and Schneider and O'Foighil (1999), which also used the mitochondrial DNA cytochrome *c* oxidase I gene and the 16s rRNA gene for constructing phylogenetic trees of giant clam species.

Six species of giant clams from different areas in the Philippines were collected namely: *H. hippopus, T. crocea, T. squamosa, T. gigas, T. derasa* and *Tridacna* sp. YCT-2005 (initially identified as *T. maxima*). Three different primers in amplifying the CO1 gene of giant clams were used: the general primer by Folmer et al. (1994), the tridacnid-specific primer used by Nuryanto et al. (2007) and our newly designed tridacnid-specific primer. On the other hand, only the primer from the study of Kessing et al. (1989) was used to amplify the 16s rRNA gene. The CO1 and 16S rRNA genes of all collected giant clam spe-

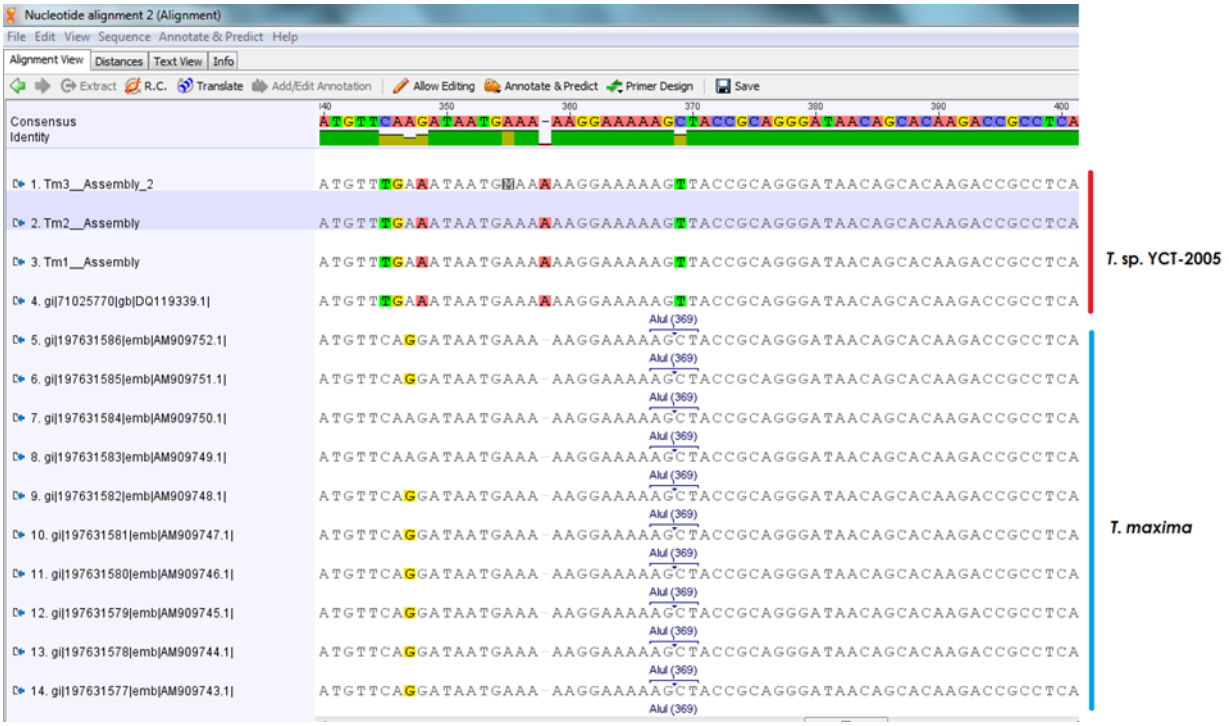

Figure 4. Alignment of four *Tridacna* sp. YCT-2005 (red line) and ten *Tridacna maxima* (blue line) haplotypes showing recognition site for Alu I restriction endonuclease at 367-370 bp (5'AGCT3') specific for *T. maxima* species. Sequence analysis and image generation performed using Geneious software (Drummond and Rambaut 2007).

cies from the Philippines were successfully amplified. Nuryanto et al. (2007) amplified the CO1 genes of four species of giant clams (*T. crocea, T. maxima, T. squamosa and T. gigas*) using the general CO1 primer and a tridacnid-specific primer, but not for *H. hippopus* and *T. derasa.* The reason for non-amplification was not elaborated. In this study, however, we amplified the CO1 gene of *H. hippopus* and *T. derasa* using the general primer by Folmer et al. (1994), but with different annealing temperatures as opposed to the study of Nuryanto et al. (2007). The results may be attributed to the varied annealing temperature specific for every species of giant clams.

We also found that the mitochondrial DNA CO1 sequence of *H. hippopus*, which we produced in this study, is the first available DNA sequence online in the GenBank database. Moreover, based on our mtDNA CO1 gene analysis, it is very likely that the CO1 sequence of *T. gigas* in GenBank (Accession Number EU003616) is from a species of *T. maxima* because of its monophyletic grouping with other reference *T. maxima* sequences.

Tables 2 and 3 show the pairwise genetic distance between groups of giant clams including the outgroup taxon *C. edule* using the 16S rRNA and mtDNA CO1 genes. The results indicate that all giant clams species are distantly separated from the outgroup taxon *C. edule* (Lymnocardiinae), which is a sister group of the Tridacnidae family as inferred from the high sequence divergence value (0.385-0.492). The sequence divergence between *T. derasa* and *T. gigas* is at 0.083 for 16S rRNA and 0.251 for mtDNA CO1. *Tridacna* sp. YCT-2005 is more affiliated with *T. squamosa* and *T. costata* than with other species under subgenus *Chametrachea.* Furthermore, the lowest sequence divergence between giant clam groups is between the *T. crocea* and *T. squamosa* clades. This suggests that the *T. crocea* and *T. squamosa* species are the most closely related species of giant clams based on the analyzed nucleotide sequence divergence. In addition, *Tridacna* sp. YCT-2005, with its high sequence divergence values (CO1, 0.034; 16S rRNA 0.178) relative to *T. squamosa*, is possibly a different species under the subgenus *Chametrachea.* The sequence divergence between the species of *Tridacna* sp. YCT-2005 and its morphologically related species, *T. maxima*, is at 0.044 for the 16S rRNA and 0.201 for the CO1 analysis. The specific threshold value for separating giant clam species is not yet known. The universal threshold value for separating species has been suggested at 3%, or 0.03, in the interspecific or between-group genetic-distance matrixes (Hebert et al. 2003). Moreover, the study of Mikkelsen et al. (2007) showed that a 1.9%-14% threshold value is already sufficient to distinguish between the bivalve species investigated in their study, although they suggested limiting the use of a fixed threshold value as a basis for distinguishing between species.

Nuryanto et al. (2007) showed, by using NJ, ML and MP tree analyses, that *T. crocea, T. maxima, T. squamosa* and *T. gigas* were a monophyletic group. Within the monophyletic group, *T. crocea* and *T. squamosa* were monophyletic and were

sister taxa to *T. maxima* and *T. gigas*. However, we also constructed our CO1 NJ trees to compare with the study of Nuryanto et al. (2007) and our results revealed a different tree topology. Based on our results shown in Figure 2b, only the species *T. crocea, T. maxima, T. squamosa and Tridacna* sp. YCT-2005 clustered in one group and excluded the *T. gigas* species. This result can be due to the probable misidentification of the *T. gigas* (GenBank Accession Number EU003616) in their analysis. The monophyletic grouping of *T. gigas* with *H. hippopus* in our CO1 analysis may be attributed to the absence of a *H. porcellanus* reference sequence and the closer sequence divergence (16S rRNA: 0.079) with its closely related taxon, *H. hippopus*. The availability of genetic sequences for the *H. porcellanus* species is still not addressed in this study due to the limited information about its range and because of sampling difficulty.

The results that we obtained with our 16S rRNA NJ and ML trees are similar to the results obtained by Schneider and O'Foighil (1999). The *T. gigas* species sampled in the Philippines grouped with the only *T. gigas* 16S rRNA reference sequence available online (GenBank Accession Number AF122975) supported by a 100 bootstrap value. This is the first attempt to include all 16S rRNA sequences (83 sequences) available online from all 10 species of giant clams, with the addition of *Tridacna* sp. YCT-2005 as a possibly new species (and excluding *T. rosewateri* for which taxonomic information is still ambiguous). Furthermore, the result of our 16S rRNA analysis showed support for the possibility that *T. costata* and *Tridacna* sp. YCT-2005 indeed belong to the subgenus *Chametrachea.*

The study of YC Tang (unpublished observations), showed that *Tridacna* sp. YCT-2005 can potentially be a new species of giant clams, based on a comparison of its shell and mantle patterns with those of its closely related species, *T. maxima.* Further support for the claim that *Tridacna* sp. YCT-2005 is a new species came from showing that it is not a hybrid species using Denaturing Gradient Gel electrophoresis.

In this paper, we aligned the sequences of *Tridacna* sp. YCT -2005 with the sequence of *T. maxima* and found differences in the 367-370 bp region of the 16S rRNA gene. This polymorphic site can possibly be used to distinguish between these two species using a restriction-fragment length-polymorphism method with the use of the Alu I enzyme.

We have first reported the sighting of this species in Philippine waters specifically in the Visayan region. We also showed that this species is under the subgenus *Chametrachea* and has a closer affinity to *T. squamosa* and *T. costata* than to the other giant clam species under the same subgenus. Additional morphological and ecological studies must be done to fully characterize and distinguish *Tridacna* sp. YCT-2005 species from the other giant clam species. This study only showed a general overview of its molecular characterization based on mitochondrial CO1 and 16S ribosomal RNA gene comparisons with other giant clam species.

In summary, we have demonstrated that the use of DNA barcoding can be a powerful tool for the identification of endangered aquatic species, specifically giant clams. The molecular techniques used in this study can also be used to address issues regarding species identification, which might help in the conservation and effective management of giant clams in the Philippines.

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CONFLICTS OF INTEREST

There are no conflicts of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

Sample collection, construction of hypothesis, laboratory work, data analyses and interpretation, and manuscript preparation were done by Apollo Marco D. Lizano and Mudjekeewis D. Santos

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Supporting Information

Supplementary Table 1. List of genetic primers used in this study to amplify mtDNA CO1 and 16S rRNA genes from different giant clam species.

Primer Name	Primer motif	Primer sequence (5'-3')	Species amplified	Reference
mtDNA CO1 gene				
LCO1490	Forward	GGTCAACAAATCATAAAGATATTGG	*H. hippopus* and *T. derasa*	Folmer et al. 1994
HCO2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA		
LCO	Forward	GGGTGATAATTCGAACAGAA	*T. crocea, Tridacna* sp. YCT-2005	Nuryanto et al. 2007
RCO	Reverse	TAGTTAAAGCCCCAGCTAAA	*H hippopus and T. derasa*	
TF1	Forward	GAACAGAATTAGCATGGCCTG	*T. squamosa* and *T. gigas*	Lizano et al. 2013
RF1	Reverse	AGCTAACACAGGCATTGCCAC		
16s rRNA gene				
16Sar	Forward	CGCCTGTTTATCAAAAACAT	All giant clam species	Kessing et al. 1989
16Sbr	Reverse	CCGGTCTGAACTCAGATCACGT		

Supplementary Table 2. Summary information of reference giant clam sequences mined from GenBank used in the phylogenetic analysis.

Accession Number (GenBank)	Collection Country	Citation
mtDNA CO1		
Tridacna crocea		
HE995452.1	Malaysia	1
HE995450.1	Malaysia	1
HE995448.1	Malaysia	1
HE995446.1	Malaysia	1
HE995444.1	Malaysia	1
HE995442.1	Malaysia	1
HE995440.1	Malaysia	1
HE995453.1	Malaysia	1
HE995451.1	Malaysia	1
HE995449.1	Malaysia	1
HE995447.1	Malaysia	1
HE995445.1	Malaysia	1
HE995443.1	Malaysia	1
HE995441.1	Malaysia	1
HE995439.1	Malaysia	1
JN392066.1	Singapore	2
JN392064.1	Singapore	2
JN392062.1	Singapore	2
JN392060.1	Singapore	2
JN392058.1	Singapore	2

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Supporting Information

continuation of Supplementary Table 2

Accession Number (GenBank)	Collection Country	Citation
Tridacna squamosa		
HE995532.1	Malaysia	1
HE995530.1	Malaysia	1
HE995528.1	Malaysia	1
HE995526.1	Malaysia	1
HE995524.1	Malaysia	1
HE995522.1	Malaysia	1
HE995520.1	Malaysia	1
HE995518.1	Malaysia	1
HE995516.1	Malaysia	1
HE995514.1	Malaysia	1
HE995512.1	Malaysia	1
HE995510.1	Malaysia	1
HE995508.1	Malaysia	1
HE995506.1	Malaysia	1
HE995504.1	Malaysia	1
HE995502.1	Malaysia	1
HE995500.1	Malaysia	1
HE995498.1	Malaysia	1
HE995496.1	Malaysia	1
HE995494.1	Malaysia	1
Tridacna maxima		
HE995486.1	Malaysia	1
HE995484.1	Malaysia	1
HE995482.1	Malaysia	1
HE995480.1	Malaysia	1
HE995478.1	Malaysia	1
HE995476.1	Malaysia	1
HE995474.1	Malaysia	1
HE995472.1	Malaysia	1
HE995470.1	Malaysia	1
HE995468.1	Malaysia	1
HE995466.1	Malaysia	1
HE995464.1	Malaysia	1
HE995462.1	Malaysia	1
HE995460.1	Malaysia	1
HE995458.1	Malaysia	1
HE995456.1	Malaysia	1

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Supporting Information

continuation of Supplementary Table 2

Accession Number (GenBank)	Collection Country	Citation
HE995454.1	Malaysia	1
HE995487.1	Malaysia	1
HE995485.1	Malaysia	1
HE995483.1	Malaysia	1
Tridacna gigas		
EU003616.1	Indonesia	3
Tridacna derasa		
GQ166591.1	Italy	4
***Tridacna* sp. YCT-2005**		
DQ168140.2	Taiwan	5
***Cerastderma edule* (outgroup)**		
EU523670.1	Spain	6
16s rRNA		
Tridacna crocea		
EU341349.1	Indonesia	7
EU341347.1	Indonesia	7
EU341341.1	Indonesia	7
EU341339.1	Indonesia	7
EU341337.1	Indonesia	7
EU341335.1	Indonesia	7
EU341333.1	Indonesia	7
EU341331.1	Indonesia	7
EU341348.1	Indonesia	7
EU341346.1	Indonesia	7
EU341344.1	Indonesia	7
EU341342.1	Indonesia	7
EU341340.1	Indonesia	7
EU341336.1	Indonesia	7
EU341332.1	Indonesia	7
AM909763.1	Jordan	8
AM909764.1	Jordan	8
AF122980.1	Jordan	8
Tridacna squamosa		
AM909762.1	Jordan	8
EU3435.1	Indonesia	7
AF122978	not specified	9

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Supporting Information

continuation of Supplementary Table 2

Accession Number (GenBank)	Collection Country	Citation
AM909755.1	Jordan	8
AM909759.1	Jordan	8
AM909761.1	Jordan	8
AM909753.1	Jordan	8
***Tridacna* sp. YCT-2005**		
DQ11939.1	Taiwan	5
Tridacna costata		
AM909732.1	Jordan	8
AM909741.1	Jordan	8
Tridacna maxima		
AF122979		9
EU341343.1	Indonesia	7
EU341335.1	Indonesia	7
EU341334.1	Indonesia	7
AM909751.1	Jordan	8
AM909749.1	Jordan	8
DQ115320.1	Taiwan	5
Tridacna derasa		
AF122976	Michigan USA	9
Tridacna tevoroa		
AF122977	Michigan USA	9
Tridacna gigas		
AF122975	Michigan USA	9
Hippopus porcellanus		
AF122974	Michigan USA	9
Hippopus hippopus		
AF122973	Michigan USA	9
AM909765	Jordan	8

[1]Hui et al. 2011
[2]Neo et al. 2013
[3]Nuryanto et al. 2007
[4]Plazzi and Passamonti 2010
[5]YC Tang, unpublished observations
[6] M Fernandez-Moreno, unpublished observations
[7]Deboer et al. 2008
[8]Richter et al. 2008
[9]Scheinder and O'Foighil 1999