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SHORT COMMUNICATION

Molecular Phylogeny of Giant Clams Based on Mitochondrial DNA Cytochrome C Oxidase I Gene

AGUS NURYANTO[1‡*], DEDY DURYADI[1], DEDI SOEDHARMA[2], DIETMAR BLOHM[3]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, [2]Faculty of Fisheries and Marine Sciences, Bogor Agriculture University, Darmaga Campus, Bogor 16680, Indonesia
[3]Department of Biotechnology and Molecular Genetics, FB2-UFT, University of Bremen, Bremen, German

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There is an uncertainty for the relationships among giant clam species of Tridacninae, in particular among species belongs to subgenus *Chametrachea* i.e. *Tridacna crocea, T. maxima,* and *T. squamosa* based on different genetic markers. This study examined the relationships among three species within subgenus *Chametrachea* compared to the previous studies. Neighbour Joining, Maximum Parsimony and Maximum Likelihood tree were constructed based on 455 bp of the mitochondrial DNA cytochrome c oxidase I gene from *T. crocea, T. squamosa, T. maxima, T. gigas,* and several sequences derived from Genbank for the outgroups. The results showed that giant clams formed a monophyletic group. Within *Tridacna* group, *T. crocea* was more closely related to *T. squamosa* than to *T. maxima* and they formed a monophyletic group. *T. crocea* and *T. squamosa* were sister taxa and sister group to *T. maxima* and *T. gigas*. Close affinity between *T. crocea* and *T. squamosa* was also supported by high similarity on nucleotide level (94.30%) and concordant with the results of the previous studies using mitochondrial 16S rRNA and nuclear 18S rRNA.

Key words: phylogenetic relationships, *Chametrachea*, cytochrome c oxidase I

The subfamily of Tridacninae is conspicuous bivalves inhabitanting coral reef across the Indo-Pacific regions (Lucas 1988). Total length of the adult individuals range from 15 cm (*Tridacna crocea*) up to 150 cm (*T. gigas*). There is an increasing interest to the clams, not only for their beautiful colouration but also tremendous decline on natural populations throughout their geographic range due to over-harvesting (Lucas 1988) and environmental deterioration (Pandolfi *et al*. 2003). Development of mariculture method and conservation technology of the clams has led a paramount research on clam biology (Copland & Lucas 1988). However, their systematic and phylogeny showed inconsistent results (Schneider & O'Foighil 1999). Resolved phylogeny is vital for marine resources conservation and management.

Eight extant species of giant clams are recognized. Two species belong to *Hippopus*: *Hippopus hippopus* Linnaeus 1759 and *H. porcellanus* Rosewater 1982 (Lucas *et al*. 1991). *Tridacna* is consisted of three subgenera i.e. (i) *Tridacna sensu stricto* with one species (*Tridacna gigas* Linnaeus 1758); (ii) *Chametrachea* with three species (*T. maxima* Röding 1798, *T. squamosa* Lamarck 1819 and *T. crocea* Lamarck 1819); and (iii) *Persikima* consists of *T. derasa* Röding 1798 and *T. trevoroa* Lucas, Ledua and Braley 1991. The present status of *T. rosewateri* Sirenko and Scarlato 1991 is still ambiguous. Benzie and Williams (1998) suggested that this species is a synonym of *T. squamosa*.

Tridacna (*Chametrachea*) is characterized by having wide byssal orifice and scales or corrugations on their shell. Adult individuals of *Chametrachea* are usually sessile and attached to hard surfaces throughout their lifespan (Lucas 1988). *Tridacna maxima* is often attached to coral rubbles. *Tridacna crocea* bores into coral head and only their mantle tissue is visible (personal observation). On the other hand, *T. squamosa* may attach to any hard surface and large individuals may lose their byssal attachment (Lucas *et al*. 1991).

Several studies were carried out on the phylogeny of giant clams. However, inconsistent results were produced about their relationships, especially within subgenus *Chametrachea*. For example, based on the partial sequences of 18S rDNA gene, Maruyama *et al*. (1998) obtained three phylogenetic trees i.e. (*T. maxima* (*T. crocea* + *T. squamosa*)), (*T. crocea* (*T. squamosa* + *T. maxima*)) and (*T. squamosa* (*T. crocea* + *T. maxima*). Each tree had a similar bootstrap value on the branching node of the *Chametrachea*. Based on partial sequences of mitochondrial 16S rDNA gene, Schneider and O'Foighil (1999) showed the same topology as the second tree of Maruyama *et al*. (1998). By using allozyme variations for 26 loci, Benzie and Williams (1998) confirmed that the major group currently recognized within Tridacninae based on morphological characters i.e. *Hippopus* and *Tridacna* and subgenera within *Tridacna*. Within the subgenus *Chametrachea* the relationship was (*T. squamosa* (*T. crocea* + *T. maxima*)).

‡Current address: Faculty of Biology, Jenderal Soedirman University, Jalan Dr. Suparno 63, Karangwangkal, Purwokerto 53123, Indonesia
*Corresponding author. Phone: +62-281-638794, Fax: +62-281-637100, E-mail: aghnia_unsoed@yahoo.com

Although the previous studies used different genetic markers, nevertheless, there is still a problem with the relationship of *T. crocea, T. maxima,* and *T. squamosa* that needs to be solved. Given that all giant clams are listed as vulnerable species (Wells 1997), therefore, it is imperative to understand their systematic and phylogeny so that appropriate decisions can be made concerning their conservation.

Here we studied the phylogeny of tridacnid clams using partial sequences of mtDNA cytochrome c oxidase I (COI) gene. This gene was chosen due to its high mutation rate (Hebert *et al*. 2003a), highly divergent among populations (Bucklin *et al*. 2003), and its broader phylogenetic sign (Hebert *et al*. 2003b). Therefore, it is suggested that COI gene fragment is suitable for studies the relationships among close related species and population genetic study. Clear discrimination among close related species was observed on various organisms (Bucklin *et al*. 1999; Bucklin *et al*. 2003; Klinbunga *et al*. 2005; Yosida *et al*. 2006). The aim of this study was to verify and provide better understanding of the relationships among giant clams species based on COI gene, especially for the species under *Chametrachea* subgenus.

Mantle tissues of seven species i.e. *T. crocea, T. maxima, T. squamosa, T. derasa, T. gigas, Hippopus hippopus,* and *H. porcellanus* were collected from several locations in the Indonesian Archipelago during the field trips in 2004 and 2005, i.e. from Padang, Pulau Seribu, Spermonde, Togian Islands, and Biak. Tissue samples of *T. squamosa* were also collected from the Red Sea in 2004 (Table 1). The determination of giant clams species were following Lucas (1988).

A small piece of mantel tissues was cut off from seven species carried out under water in order to minimise the sampling impact. Tissue samples were preserved in 96% of ethanol and stored at 4 °C. Total genomic DNA was isolated using Chelex® method following the protocols from Walsh *et al*. (1991). The extraction procedures were as follow: approximately 0.5 mg of chopped tissue was incubated in 100 µl 5% Chelex, 5 µl 100 mM dithiothreitol (DTT), and 4 µl of 9.85 or 10.3 mg/ml proteinase-K. The mixtures were then incubated at 54 °C and centrifuged at 1,000 rpm for minimum four hours. Afterwards, lysate was centrifuged at 13,000 rpm for three minutes to remove cell debris and the rest of Chelex granule. Subsequently, the supernatant was transferred into new tubes and incubated at 95 °C and centrifuged at 1,000 rpm for five minutes to inactivate the proteinase-K.

A fragment of COI gene was amplified using a pair of primers from Folmer *et al*. (1994) (forwards: LCO 1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and reverse: HCO 2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3'). However, this primer could only amplify seven specimens of *T. crocea*. Hence, we designed new tridacnid specific primer (forwards: LCO: 5'-GGG TGA TAA TTC GAA CAG AA-3' and reverse: RCO: 5'-TAG TTA AAG CCC CAG CTA AA-3') based on *T. crocea* sequences obtained in a preliminary analysis. PCR reactions were carried out in a total volume of 50 µl contained approximately 10 pg of DNA template, 1 x PCR buffer, 2 mM of $MgCl_2$, 0.02 µM of each primer, 0.2 mM of each dNTPs and 1 unit Taq polymerase (*MolTaq,* Molzym GmBh & Co.KG, Germany). Thermal cycling was as follow: one cycle at 94 °C for 5 minutes, follows by 35 cycles of 1 minute at 94 °C, 1.5 minutes annealing temperature (at 50, 43, and 47 °C for *T. maxima* and *T. gigas*, *T. crocea,* and for *T. squamosa*, respectively) and one minute at 72 °C for extension. Final extension was carried out at 72 °C for five minutes.

Although we used tridacnid-specific primers, the COI gene could only be amplified for *T. crocea*, *T. maxima*, *T. squamosa*, and *T. gigas*. Those COI gene from remaining species of giant clams (*T. derasa, H. hippopus, H. porcellanus*) were tried to be amplified using new-designed universal COI primers (COIb-F: 5' ATC AYA WAG AYA TTG GHA S 3' and COIb-R: TGM CCA AAA AAY CAA AAYARR3'). These efforts were still unsuccessful eventhough the experiments were carried out in different annealing temperatures ranging from 40 °C up to 65 °C. Finally, we considered to continue our analysis only for *T. crocea, T. maxima, T. squamosa,* and *T. gigas*.

The PCR products were purified using the PeqGOLD cycle-pure kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) following the protocol from the manufacturer. Both strands were sequenced using the DyeDeoxy terminator chemistry (PE Biosystem, Foster City) and an automated sequencer (ABI prism 310; Applied Biosystem, Weiterstadt).

All sequences were initially aligned and edited manually using Sequences Navigator (version 1.0.1; Applied

Table 1. List of species used in this study

Group	Species name	Sample number	Abbreviation	Location	Accession number	Collector
Ingroup	*Tridacna crocea*	Sample 1	TcPS8301	Pulau Seribu	EU003608	Nuryanto A
		Sample 2	TcSp0301	Spermonde	EU003606	
		Sample 3	TcSp0302	Spermonde	EU003607	
		Sample 4	TcBk8804	Biak	EU003609	
	Tridacna maxima	Sample 1	TmPa4601	Padang	EU003610	
		Sample 2	TmPS8301	Pulau Seribu	EU003614	
		Sample 3	TmSp0301	Spermonde	EU003611	
		Sample 4	TmTI2901	Togian Islands	EU003612	
		Sample 5	TmBk9006	Biak	EU003613	
	Tridacna gigas	Sample 1	T gigas	Togian Islands	EU003616	
	Tridacna squamosa	Sample 1	TsRS9501	Red Sea	EU003615	Roa-Quiaoit HAF
	Tridacna crocea			Taiwan	DQ269479	Tang TC
Outgroup	*Parvicardium*	-	-	European	AF120664	Giribet G
	Ruditapes	-	-	Korea	AY874536	Kim JJ *et al.*
	Mytilus	-	-	-	AY484747	Hoffmann *et al.*

Biosystem). Multiple sequences alignment was performed using ClustalW (Thompson *et al.* 1994) as implemented in Bioedit (ver. 7.0.4.1; Hall 1999).

Molecular phylogenetic analysis was based on four species of *Tridacna* (Table 1) collected by Nuryanto and Ron -Quiaoit, with the sequences of *T. crocea* DQ269479 from Genbank as the reference. *Parvicardium exiguum* (AF120664), *Ruditapes variegata* (AY874536), and *Mytilus edulis* (AY484747) were used as the outgroups (Table 1). Phylogenetic trees implemented in MEGA programme version 4 (Tamura *et al*. 2007) and PAUP version 4.0 (Swofford 1998) were constructed by using Neighbour-Joining (NJ) and Maximum Parsimony (MP) methods. Maximum Likelihood (ML) tree was constructed by using a gamma shape parameter of 0.24 and Tamura-Nei substitution model based on the result obtained with the programmes PAUP (ver. 4.0b10, Swofford 1998) and Modeltest (ver. 3.7, Posada & Crandall 1998). Support for tree branching was based on 1,000 non-parametric bootstrap replicates.

The mtDNA COI gene from *T. crocea*, *T. maxima*, *T. squamosa*, and *T. gigas* were aligned to analyze the number of shared nucleotide by species pairwise. In this analysis the sequences from GenBank were excluded.

A giant clam phylogenetic tree was constructed based on Kimura 2-parameter genetic distances by NJ method and the same topology was resulted from the MP approach (Figure 1). All giant clams species formed a monophyletic group and this was well supported with 100 bootstraps value. The tree also showed that *T. crocea* and *T. squamosa* constitud monophyletic group (subgroup 1), whereas *T. maxima* and *T. gigas* formed another monophyletic group (subgroup 2) (Figure 1). In ML tree, *T. crocea* and *T. squamosa* were mixed and not clearly separated (Figure 2).

Analysis of shared nucleotide by all possible pairwise of giant clams species and sequence divergences showed that among 11 sequences from four species and 455 bp of the mtDNA COI gene, 79 polymorphic sites were observed. Sequences divergences between species ranges from 5.49 up to 17.36% (Table 2). Within *T. crocea*, we found five polymorphic sites with five mutations events. Sequence divergences for this species was 1.1% (Table 3). On *T. maxima* sequences, we observed 13 polymorphic sites and 13 mutations, whereas sequences divergences was 2.86% (Table 2).

It was difficult to amplify COI gene from all giant clam mantle tissue using universal primer from Folmer *et al*. (1994). It could be due to the primer has limited capability to amplify the COI fragments from wide range of organisms. Another explanation would be that a high proportion of polysaccharides in giant clams tissues that could inhibit the activity of polymerase (Skolov 2000).

The NJ and MP trees showed similar topologies, except for the outgroup. Both analyses based on COI gene fragment confirmed the grouping between *T. crocea* and *T. squamosa* as shown in the analysis based on the mitochondrial 16S rDNA and nuclear 18S rDNA (Maruyama *et al*. 1998; Schneider & O'Foighil 1999; Roa-Quiaoit 2005).

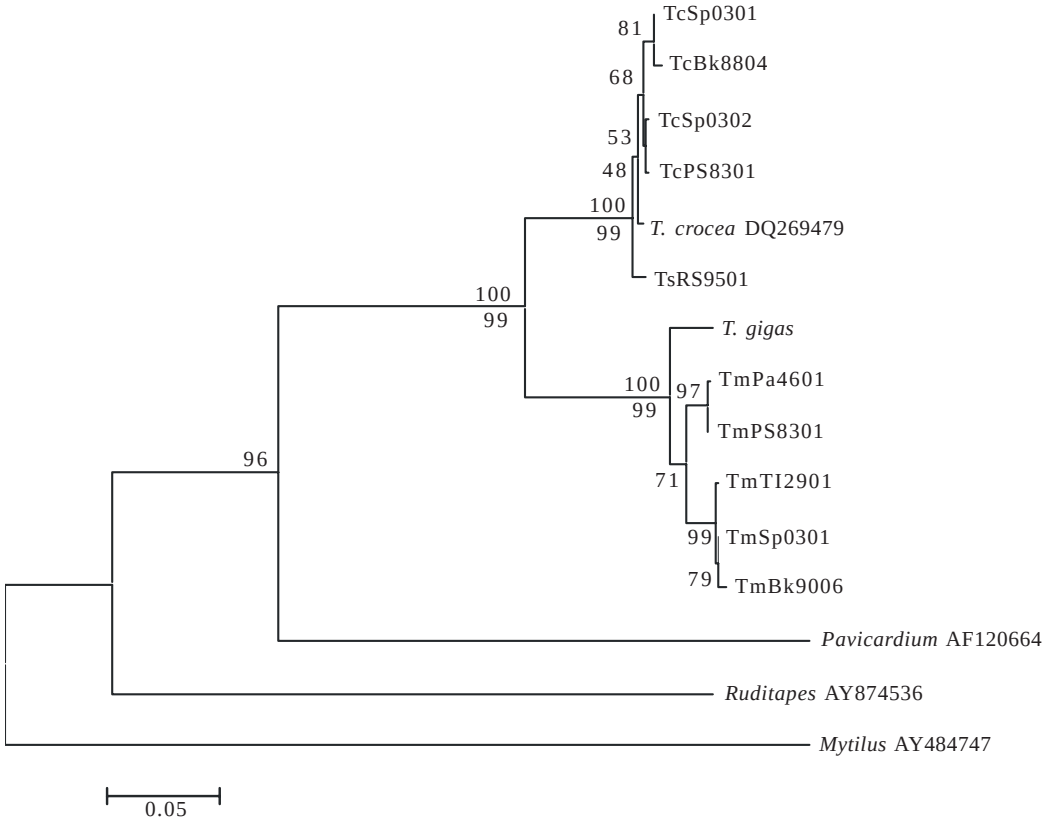

Figure 1. Phylogenetic tree of giant clams based on 455 bp of the mitochondrial DNA COI gene using genetic distances Kimura 2-parameter on NJ approach; bootstrap analysis with 1,000 replicates. The same topology resulted from MP analysis. Bootstrap values for NJ are above the branches and MP are shown below branches. Abbreviation refer to Table 1.

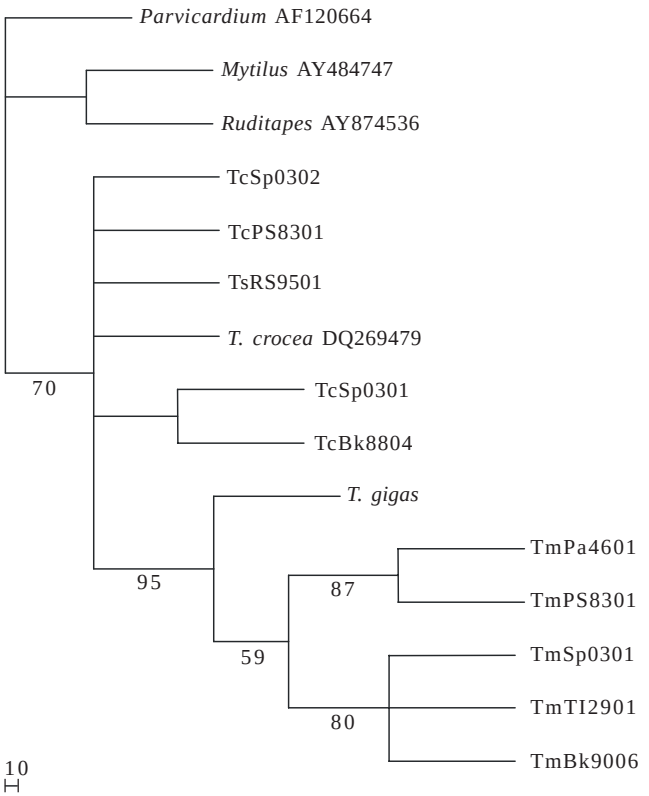

Figure 2. Giant clam maximum likelihood tree construction based on 455 bp of the COI gene. Bootstrap support 1,000 replicates. Abbreviations refer to Table 1.

Table 2. Number of shared nucleotide by all possible pairwise and intraspecies sequence divergences of giant clam

Species	*T. crocea*	*T. maxima*	*T. squamosa*
T. crocea	450 (98.90%)	-	-
T. maxima	380 (83.53%)	442 (97.14%)	-
T. squamosa	430 (94.51%)	386 (84.84%)	-
T. gigas	386 (84.84%)	429 (94.29%)	396 (87.03%)

Table 3. Inter- and intraspecies sequence divergences of giant clam

Species	*T. crocea*	*T. maxima*	*T. squamosa*
T. crocea	1.10%	-	-
T. maxima	16.48%	2.86%	-
T. squamosa	5.49%	15.16%	-
T. gigas	15.16%	5.71%	12.97%

Schneider and O'Foighil (1999) and Maruyama *et al*. (1998) showed that all *Chametrachea* are placed in the same monophyletic group (*T. gigas* (*T. crocea* + *T. squamosa* + *T. maxima*). However, in our NJ and MP trees showed that: 1) *T. crocea*, *T. maxima*, *T. squamosa*, and *T. gigas* was a monophyletic group; 2) within the group, (*T. crocea* + *T. squamosa*) was a monophyletic group and were sister taxa thus formed sister group to *T. maxima* and *T. gigas* although they belonged to different subgenus. This can be explained that COI gene has higher mutation rate compared to 16S rRNA (Hebert *et al*. 2003a). It is suggested that the COI gene was too variable for the separation on the subgenus level, but suitable for species discrimination. Uniting *T. maxima* and *T. gigas* was concordant with the palaeontological data. The appearance of both species was reported for the first time in the Late Miocene (Beets 1986). Uniting *T. crocea* and *T. squamosa* was also fit with the palaeontogical data because both species appeared almost nearly at the same period, i.e. early and late Pleistocene for *T. squamosa* and *T. crocea*, respectively (Beets 1986). Close proximity between *T. crocea* and *T. squamosa* in this study was also supported by the number of shared nucleotide (94.51%, Table 2). The relationship between *T. maxima* and *T. gigas* was supported by 94.29% nucleotide similarity (Table 2).

The clear close affinity between *T. crocea* and *T. squamosa* as shown in this study and supported by the studies from Schneider and O'Foighil (1999) and Maruyama *et al*. (1998) implied that both species can be treated with a similar method regarding for their conservation. For example, if the biological information is only available for *T. crocea*, that information can be used theoretically for *T. squamosa* as well. However, more reliable conservation efforts can only be made if biological information is available for both species.

This study showed that COI gene has low level sequence divergences within species but high divergences among species (Table 3). It is suggested that high level sequence divergences on the COI gene provide a strong, useful and suitable diagnostic character for species identification and discrimination, especially for both molecular systematic and phylogenetic assessment of giant clam species. The low sequence divergences within species and the high sequences divergences of COI gene among species observed in this study were lower to those observed in calanoid copepods (Bucklin *et al*. 1999, 2003).

High level of intraspecific divergences in *T. maxima* (Table 3) was obtained from the comparison between distantly separated or isolated populations. *T. maxima* has the broadest geographic distribution among the other member of giant clams. This species distributes from Eastern Coast of Africa through Southeast Asia up to French Polynesia (Lucas 1988). Therefore, it is reasonable that distantly separated allopatric species showed a high genetic divergence as a result of spatial isolation and local adaptation leading to a different mutation rate and mutation pattern.

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