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Molecular Genetic Analysis of Giant Clam (*Tridacna* sp.) Populations in the Northern Red Sea

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Abstract: Morphologically, two species of giant clams *Tridacna* have been recognized in the Red Sea, *T. squamosa* and *T. maxima*. Similar morphology among species makes classification difficult. For proper classification, we sequenced an approximately 450-nucleotide fragment of the mitochondrial 16S rDNA gene from the tissues of *Tridacna* from eight locations in the Red Sea. Our results suggest that there are three novel species of *Tridacna* in the Red Sea. Two of these species are related to *T. maxima* and one is related to *T. squamosa*. Of the two species related to *T. maxima*, one species was found in Hurghada, Marsa Ghaleb and Safaga while the other was found in Abu Zenima, Abou Galum, Dahab and Nuweiba. All three novel species were found in Ras Mohamed. Our results do not support the morphological classification that suggests the existence of only two tridacnid species, *T. maxima* and *T. squamosa* in the Red Sea.

Key words: *Tridacna*, Mitochondrial 16S rDNA gene, Red Sea, Phylogenetic tree, Novel species

Introduction

Among the different species of invertebrates colonizing the coral reefs in the Indo-Pacific and Red Sea areas are the giant clams of the genus *Tridacna*, the largest and fastest growing living members of the class *Bivalvia* (Yonge, 1980). They are highly morphologically derived cardiids that have evolved an obligate symbiotic association with photosynthetic dinoflagellate algae of the genus *Symbodium*, living in their enlarged siphonal mantle (Schneider, 1998).

Rosewater (1965) recognized six living species of giant clams: *Hippopus hippopus*, *Tridacna gigas*, *T. derasa*, *T. maxima*, *T. squamosa* and *T. crocea*. Later, Rosewater (1982) recognized *Hippopus porcellanus* and Benzie and Williams (1998), using electrophoretically detectable allozyme variation, confirmed that the two *Hippopus* species were genetically differentiated. Lucas *et al*. (1990) subsequently described *T. tevoroa* and suggested that it may represent a transitional species between *Hippopus* and *Tridacna* (Lucas *et al*., 1991). Therefore, the number of known giant clam species has increased from six to eight.

Tridacnins, the major protein isolated from *Tridacna*, has been found to react with various cell types, including red blood cells, lymphocytes, spermatozoa and tumor cells of different origins (Uhlenbruck *et al*., 1975, 1978). Furthermore, Tridacnin from *T. maxima* and *T. squamosa* showed

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stronger effects than tridacnin from *T. derasa* and *T. gigas* (Uhlenbruck *et al*., 1978). Recently, 24-methylenecholesterol was isolated from the Red Sea *Tridacna* mantle and was found to possess modest cytotoxic activity (Kilada, 2001).

Along the coasts of the Red Sea, two living tridacnid species have been recognized morphologically: *T. maxima* and *T. squamosa* (Mansour,1946a-c, 1949; Hughes, 1977; Mergner and Mastaller, 1980; Kilada *et al*., 1998). However, classification based on morphological structure makes it difficult to recognize proper species. Therefore, molecular classification based on comparing the sequence of mitochondrial DNA may offer new insights for a more precise classification of the genus *Tridacna* in the Red Sea.

We sequenced a 450 nucleotide fragment of mitochondrial, large subunit ribosomal gene (16S) from the tissues of the two species of *Tridacna* from different locations in the northern part of the Red Sea.

Materials and Methods

Collection of Clam Specimens

Tridacnid clams were collected from eight locations in the Egyptian Red Sea during the spring of 2002; four in the Gulf of Aqaba-Dahab (DH), Nuweiba (NU), Abou Galum (AG) and Ras Mohamed (RM); three in the main basin of the Red Sea-Hurghada (HU), Safaga (SF) and Marsa Ghaleb (MG) and one location in the Gulf of Suez-Abu Zenima (AZ) (Fig. 1). The specimens were brought alive to the Department of Biochemistry, Faculty of Pharmacy, Suez Canal University where the animals were removed from their shells and dissected. For each specimen, a portion of the adductor muscle tissue was excised with a razor blade and frozen at -80°C. The rest of the organs were separated and frozen at -80°C and the shells were saved.

Extraction of DNA from the Clams

DNA was extracted exclusively from adductor muscle tissue from the specimens of all tridacnids. Extraction was limited to the adductor muscle tissue because symbiotic dinoflagellate zooxanthellae are found within the mantle and internal organ tissues. Roughly 200 to 300 mg of tissue was diced finally with a razor blade and the DNA was extracted using a QIAGEN DNeasy™ tissue extraction kit according to the manufacturer's instructions (QIAGEN Gmbh, Germany).

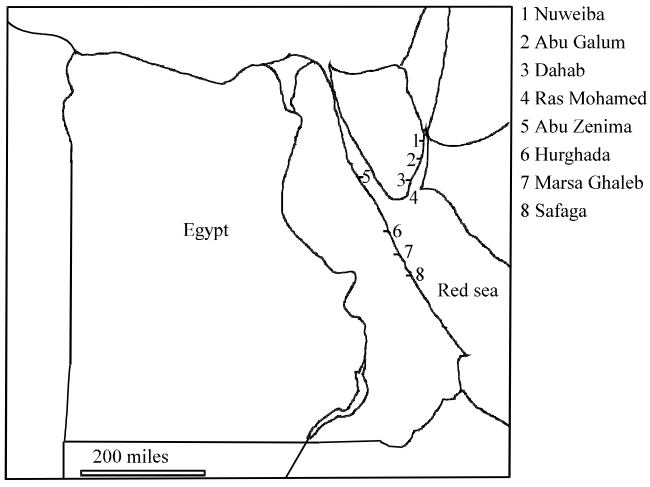

Fig. 1: Map of Egypt indicating sample collection sites of *Tridacna* used in this study

Amplification of 16S rDNA and Sequencing

An approximately 450 nucleotide portion of mitochondrial large subunit (16S) rRNA gene was amplified from the genomic DNA using the primers 16Sar 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr 5'-CCGGTCTGAACTCAGATCACGT-3' (Kessing *et al*., 1989). Polymerase chain reaction (PCR) amplification was performed using an Eppendorf Master Cycler® gradient and a reaction mixture of 100 µL containing 60-120 ng of genomic DNA, 2 units of *Taq* polymerase enzyme, 0.5 µM of each primer, 25 mM of each dNTP, 2.5 mM $MgCl_2$ and 1 X *Taq* buffer (100 mM Tris HCl and 500 mM Kcl). Cycle parameters were 1 min 94°C initial denaturation, 35 cycles of the pattern (30 sec 94°C denaturation, 30 sec 47°C annealing, 1 min 72°C extension) and 10 min 75°C final extension. Double-stranded products were isolated on 2% agarose gels, excised using a QIAquick gel extraction kit (QIAGEN, Germany). A single strand of the amplified partial 16S rDNA gene fragments was directly sequenced with the forward (16Sar) amplification primer using the protocols and reagents for cycle sequencing in the ABI Prism Dye Terminator Cycle sequencing ready reaction kit version 3 (PE Applied Biosystems). Extension products were sequenced on an ABI Prism 3700 automated DNA sequencer (PE Applied Biosystems).

Phylogenetic Analysis

The 16S rDNA sequences were aligned with available *Tridacna* 16S rDNA sequence data (Schneider and Foighil, 1999) from the GenBank database using PHYDIT 3.1 phylogenetic software (Chun, 1995). The computer-generated sequence alignments were adjusted manually and 387 bp was used in the final analysis. Phylogenetic reconstruction was done using Neighbor Joining (NJ) (Saitou and Nei, 1987), Maximum Parsimony (MP) and Fitch-Margoliash (F) (Fitch and Margoliash, 1967) methods. NJ analysis was performed using the tree construction algorithms available with PHYDIT V. 3.1 (Chun, 1995). Evolutionary distance matrices for the NJ and F methods were generated as described by Jukes and Cantor (1969). Tree topologies were evaluated by 1000 bootstrap re-samplings of the NJ data (Felsenstein, 1985). Phylogenetic trees were drawn with Tree View V. 1.6.6 (Page, 1996) and Canvas™ V. 5 on a Macintosh computer in Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland, USA.

Results and Discussion

In the present study, the length of the amplified fragment of mitochondrial 16S rDNA gene was approximately 450 bp. The amplified region contained sufficient conserved residues to permit unambiguous alignment to be made among tridacnid species. Of the 387 sites, 115 were variable in our data. The neighbor joining tree in Fig. 2 shows a group supported by a high bootstrap value (97) made up of *T. crocea*, *T. squamosa*, *T. maxima* and tridacnid clams from different locations in the northern Red Sea (Fig. 1). The bootstrap value at the node *T. squamosa* and sample RM1 was 92. This indicates that they are closely related. The rest of the tridacnid samples are related to *T. maxima* (bootstrap value of 51). The genetic distance between the samples collected from Safaga, Hurghada, Marsa Ghaleb and Ras Mohamed (RM2) is negligible. Moreover, the genetic distance between samples colleted from Abu Zenima, Nuweiba, Dahab, Abou Galum and Ras Mohamed (RM3) is also insignificant.

Our phylogenetic analyses of 16S rDNA sequences suggest that there are three novel species of *Tridacna* in the Red Sea. Two of these species are related to *T. maxima* and one is related to *T. squamosa*. Of the two species related to *T. maxima*, one species was found solely in the main basin of the Red Sea (Hurghada, Marsa Ghaleb and Safaga) whereas the other was confined to the Gulfs of Aqaba and Suez (Abu Zenima, Abou Galum, Dahab and Nuweiba). Ras Mohamed, a site at the point where the two gulfs join the main basin, was found to harbor all three novel species. Our results do not support the morphological classification (Mansour, 1946b, c, 1949; Hughes, 1977; Mergner and

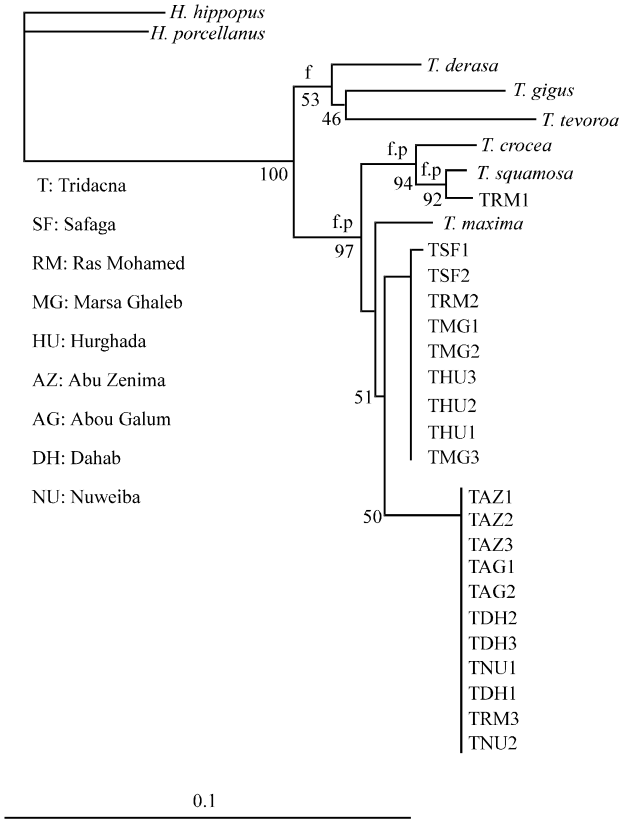

Fig. 2: Neighbor-joining phylogenetic tree from analysis of 387 bp of 16S rDNA gene sequences of *Tridacna* species and Tridacnid clams from the Red Sea. f and p indicate branches which were also found using Fitch-Margoliash and maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on neighbor-joining analysis of 1,000 re-sampled data sets. The scale bar represents 0.1 substitutions per nucleotide position

Mastaller, 1980; Kilada *et al*., 1998) that suggests the existence of only two tridacnid species, *T. maxima* and *T. squamosa* in the northern Red Sea.

In conclusion, this study suggests that the Red Sea *Tridacna* population is not exclusive to the two species *T. maxima* and *T. squamosa* as was previously believed. Three novel species were discovered. More rapidly evolving sequences will be useful for future additional DNA-based genetic studies of these species.

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