Molecular Phylogeny of four bivalve species collected from Egyptian Mediterranean and Red Sea based on mitochondrial *CO1* gene sequences

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ABSTRACT

The marine bivalve (family Veneridae and Mytilidae) are dominant groups in terms of species numbers, diversity of habitat and economical importance. The aim of the current study is to gain new insights onto the phylogeny of four different species of bivalvein Egypt. Partial sequence of the mitochondrialcytochrome oxidase subunit 1 (*CO1*) is sequenced to assess whether different species of bivalve could be distinguished by DNA barcoding technique. The obtained molecular results revealed the efficacy of DNA barcoding in species identification and differentiation between the different species.

Keywords: Phylogeny, DNA barcoding, mitochondrial cytochrome oxidase subunit 1 (*CO1*).

INTRODUCTION

The phylogeny of Mollusca is a controversial subject; in fact some of the traditionally classified molluscs may be redefined as distinct but related (Goloboff *et al*., 2009). More than 99% of living molluscan species belong to two classes (Gastropoda and Bivalvia).

Bivalves are species that probably need common tools and methods in order to have a clear picture about their relationships where different approaches are using genetic markers to understand phylogenetic, population structure, taxonomy, ecology…..etc (David and Savini, 2011).

Many marine taxa represent an ideal target for DNA barcoding due to a lack of reliable morphological characters for easy diagnosis. One of the most frequently used regions of the mitochondrial (mt) DNA is cytochrome c oxidase subunit 1 (*CO1*) (Feral, 2002). It is the most used marker in molecular studies and barcoding (Hebert *et al*., 2003). This marker is a phylogenetical signal stronger than any other mitochondrial markers, where it can discriminate not only among strongly related species but also among phylogroups belonging to the same species (Hebert *et al*., 2003a). Also, *CO1* shows distinct divergence and provides valuable information in species identification to complete taxonomic data and validation of systemic position and phylogeny (Machordom *et al*., 2003; Smith *et al*., 2004; Donald *et al*., 2005).

Despite the importance of bivalve in marine biota, their deeply phylogenetic relationships were scarcely investigated from a molecular perspective, whereas much valuable work has been done on taxonomy, as well as physiology, of the lower taxa (Plazzi *et al*., 2011).

Due to the limitation of the information about the molecular studies on species of family veneridae and mytilidae in Egypt, the present study focused onidentification the species of family veneridae (*Paphia sp.* and *Polititapes sp.)* and family mytilidae *(Modiolus sp*. and *Brachidontes sp.*) in Egypt based on DNA barcoding. The General

aim is to develop a fast and easy method for identification by using the molecular examination of nucleotide sequence for *CO1* gene.

MATERIALS AND METHODS

Study areaand Sample collection

Fifteen individual of bivalve samples were collected for molecular examination during the summer 2012-2013.They were *Paphia sp.* and *Polititapes sp.* from Lake Timsah; *Modiolus sp.* from Marsa Alam and *Brachidontes sp.* from Lake Bardawil (Fig.1).

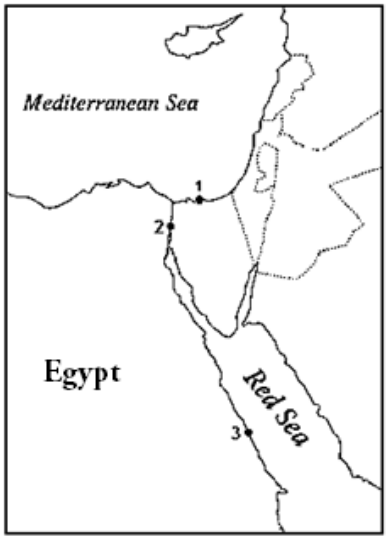

Fig. 1: Map showing the sites of collection of different bivalve species1: Lake Bardawil; 2: Lake Timsah; 3: MarsaAlam.

gDNA Extraction, PCR amplification and sequencing

The samples were frozen at -70◦c and the gDNA was extracted froma very small piece of foot using the phenol-chloroform (CTAB) procedure as described by Coffroth *et al*. (1992), and then the DNA was stored at -20◦c. A small region (~ 600-700 bps) of the mitochondrial CO1 gene was amplified in the thermocycler (Major Science Thermocycler) using the universal primers described by Folmer *et al*. (1994):

LCO1490 (F): 5'-GGTCAACAAATCATAAAGATATTGG-3'.

HCO2198 (R): 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'.

PCR reaction was performed in 25 µl volume containing 12.5 µl Master Mix, 0.5 µl of each primer (10 pmol), 6 µl of template DNA (about 100 ngtemplate DNA) and sterile distilled water to final volume of 25 µl. To optimize PCR products, annealing temperature and times were varied. PCR conditions were as follows: an initial denaturation for 3 min at 94◦C, followed by 45 sec at 94◦C, 1 min at annealing temperature 52◦c and 2 min at 72◦c for 35 cycles, and a final extension of 5 min at 72◦C.

The PCR products were run on a 1.5 % horizontal agarose gel stained with ethidium bromide. The bands were visualized and photographed in UV photodocumentation unit. Purification was carried out by using QIAquick PCR Purification Kit (QIAGEN). The purified PCR product was sequenced in Macrogen Ltd (Korea) and Biotechnology Research Center (Suez Canal University, Egypt) by (3500 Genetic Analyzer, Applied Biosystems).

Phylogenetic analysis

Sequence chromatograms of *CO1* sequences were edited for all taxa using MEGA V6.06 software and aligned using the Clustal W program then adjusted manually. The dataset for 27 specimens of speciesused in the present study with their accession number on GenBank are described in Table (1).

Table 1: Species included in analysis, with their collection localities and GenBank accession number.

Species	Family	Locality	Accession number
Modiolus sp.	**Mytilidae**	**Red Sea** MarsaAlam	KP164534
Brachidontespharaonis	**Mytilidae**	**Mediterranean Sea** Lake Bardawil	KP164519 KP164520 KP164521
		Mediterranean Sea Port Said	KP164522 KP164523 KP164524
		Suez Canal Lake Timsah	KP164525 KP164526 KP164527
		Suez Gulf of Suez	KP164528 KP164529 KP164530
		Red Sea MarsaAlam	KP164531 KP164532 KP164533
Polititapesaureus	**Veneridae**	**Suez Canal** Lake Timsah	KP164535 KP164536 KP164537 KP164538 KP164539 KP164540 KP164541 KP164542 KP164543 KP164544
Paphia textile	**Veneridae**	**Suez Canal** Lake Timsah	KP164545

Using Blast, *CO1* sequences of the most related species of bivalves were screened on the NCBI GenBank data base. They were added to the present analysis to construct phylogenetic tree using MEGA 6 (Tamura *et al*., 2013).To generate the phylogenetic relationship between *CO1* sequences for all bivalves in the current study and sequences from GenBank, Maximum likelihood tree was constructed with the Hasegawa-Kishino-Yano substitution model (Hasegawa *et al*., 1985) and Gamma distributed among sites (HKY+G) according to the lowest Bayesian Information Criterion (BIC) with bootstrap value 100%,the tree was rooted by *Laternula sp.* (GU227114). Also, Neighbor-Joining tree based on p-distance (Saitou and Nei, 1987) was constructed for the phylogenetic relationship between all bivalve species collected from Egypt, the tree was rooted by *Mactra veneriformis* (JN674624). Each species of bivalve was represented by one individual in the phylogenetic tree.

RESULTS

The Maximum likelihood tree between *CO1* sequence for all bivalves in the current study and other bivalves collected from GenBank (Fig. 2) shows two major

clads. The first comprises all species of family veneridae and the second clade includes the different species of family mytilidae.

Within each clade, the species from each subfamily were clustered together with their resemble species from GenBank with high bootstrap at each node. In the clade of family veneridae, *P. aureus* from Egypt and *P. aureus* (JX051549) were robustly grouped together; also *P. textile* was grouped with *P. textile* (JF969277). In clade of family mytilidae, *Modiolus sp.* from Egypt was closely related to *Modiolus sp.* (AB972412); and *B. pharaonis* was clustered with *B. pharaonis* (DQ836013). Also, the phylogenetic relationship between the four different species of bivalve in Egypt was confirmed by the Neighbor joining tree as shown in Fig. 3.

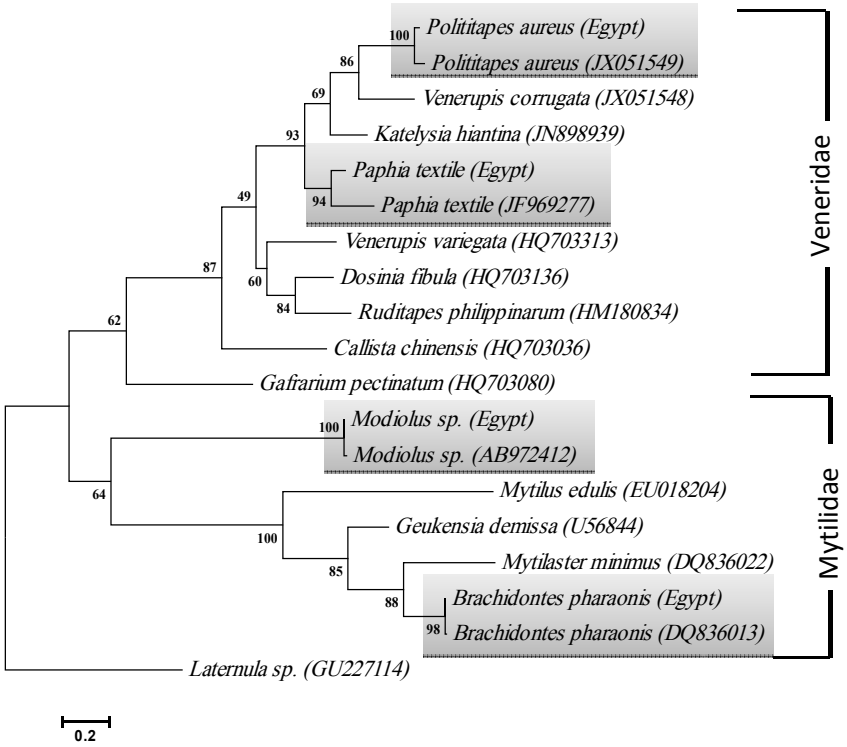

Fig. 2: Maximum likelihood tree for *CO1* sequences of different species of bivalves collected from Egypt. *Laternula sp.* was used as outgroup.

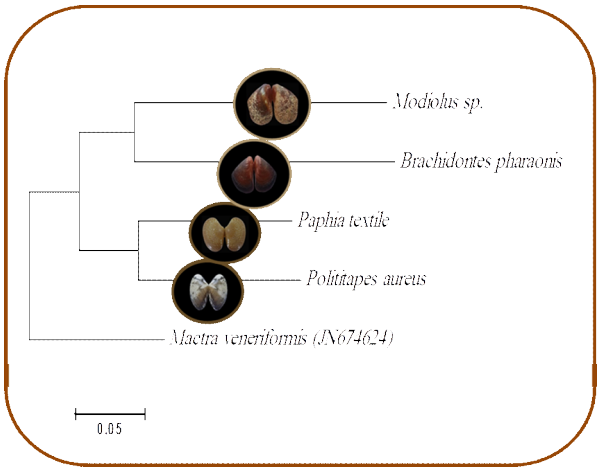

Fig. 3: Neighbor-Joining tree for *CO1* sequences of different species of bivalves collected from Egypt. *Mactra veneriformis* was used as outgroup.

DISCUSSION

In this study, PCR technique and direct sequencing of *CO1* gene were selected to elucidate taxonomy of different bivalves and to understand their phylogenetic relationships with other bivalve species in the world that collected from GenBank.

Many authors used molecular method in many purposes like providing information on relationship between species (Huff *et al*., 2004; Mahidol *et al*., 2007; Espineira *et al*., 2009; Vierna *et al*., 2010), on their evolutionary history (Ahmed, 2010; Cunha *et al*., 2011; Etter *et al*., 2011); on genetic variation within species populations (Luttikhuizen *et al*., 2003; Zardus *et al*., 2006); on population size, migratory events, and biodiversity conservation issues such as hybridization events (Westfall and Gardner, 2010).

The present phylogenetic analysis revealed that using of DNA barcoding was successful and proved to be a good technique. The results were clear enough to identify and taxonomy the different bivalve species collected from Egypt (*Polititapes aureus, Paphia textile, Brachidonte spharaonis, Modiolus sp.*). Hebert *et al*. (2003) stated that DNA barcoding is proposed to be a new tool to achieve accurate, rapid and automatable species identification without morphological knowledge.

Moreover, using of *CO1* sequence has demonstrated its ability to distinguish between the different families of bivalve (Veneridae and Mytilidae) in the current study and other bivalves collected from GenBank. *Modiolus sp., Brachidonte spharaonis, Paphia textile,* and *Polititapes aureus* from Egypt were unambiguously distinguishable from all other species and clustered only with their closely resemble sister species in GenBank with high bootstrap close to 100%. The suitability of *CO1* gene data in taxonomy has been shown in numerous other molecular analyses of bivalves (e.g. Park and Ó Foighil, 2000; Canapa *et al*., 2003; Cooley and Ó Foighil, 2000; Matsumoto, 2003; Roe *et al*., 2001).

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ARABIC SUMMARY

التاريخ التطورى الجزيئ لأربعة أنواع من ذوات المصراعين تم تجميعها من البحر المتوسط والبحر الاحمر داخل مصر وذلك على اساس التتابع النيوكليتيدى لجين السيتوكروم التأكسدى رقم ١ الموجود بالميتوكوندريا

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يعد ذوات المصراعين البحرية (عائلة الفينيريدى والميتيليدى) من المجموعات المهيمنة من حيث عدد الانواع وتنوع المعيشة والأهمية الاقتصادية. الهدف الرئيسى من الدراسة الحالية هو الحصول على مفاهيم جديدة لدراسة التاريخ التطورى لأربعه انواع من ذوات المصراعين الموجودة فى مصر. وحيث أن التتابع النيوكليوتيدى لجين السيتوكروم التأكسدى رقم ١ الموجود بالميتوكوندريا يعد من أحد الوسائل للتمييز بين الأنواع المختلفة من الرخويات، فقد تم تطبيق هذه التقنية، وأسفرت النتائج عن كفاءة تقنية شفرة الحمض النووى فى تحديد الأنواع المختلفة من ذوات المصراعين والتمييز بينها.