Molecular analysis of *Brachidonte spharaonis* (Fischer P., 1870) in Egypt reveals cryptic species complex.

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### ABSTRACT

Species and genetic diversity of the migrant *Brachidonte spharaonis* bivalve were studied along the Egyptian waters in order to determine the phylogenetic status of the species and to detect speciation with geographical isolation. Samples were collected from the Red Sea, Mediterranean Sea and Suez Canal. Genetic diversity was estimated using the DNA barcoding technique for mitochondrial *CO1* gene. The DNA barcoding results showed that *B. pharaonis* collected from different localities were clustered in different clades indicating that the *B. pharaonis* population in the Egyptian coasts might form a cryptic species complex rather than a population of one species. These results may have a great impact on the conservation and fisheries status of *B. pharaonis* in the Egyptian waters.

**Keywords:** *Brachidontespharaonis*, DNA barcoding, Cryptic species, *CO1*.

### INTRODUCTION

Suez Canal is considered to be the shortest link between the east and the west due to its unique geographic location between the Mediterranean Sea at Port Said and the Red sea at Suez, Egypt. The opening of Suez Canal in 1869, initiated the invasion of marine species, usually from the Red Sea to the Mediterranean Sea "Lessepsian migration" (Por, 1978) and more rarely in the opposite direction. Biological invasion have recently become an important issue both in conservation as well as in theoretical ecology (Holland, 2000), that invasion pose a great threat to the integrity of natural communities; alter the ecosystem dynamics and world-wide community structure (Doğan et al., 2007). *Brachidonte spharaonis* (Fischer, 1870) is one of these invasion species that able to migrate and colonies through the Suez Canal.

*B. pharaonis* is a lessepsian mussel and one of the earliest Erythrean invaders to the Mediterranean Sea (Rilov and Galil, 2009). It originally from the Indo-Pacific area mainly South-Eastern Asia, that colonized the Mediterranean Sea via the Suez Canal,settles in dense clusters on midlittoral rocky habitats (Terranova et al., 2006), and competes for space and food with its Mediterranean ecological equivalent *Mytilaster minimus* (Safriel et al., 1980). It is widely distributed; the first collection from the Mediterranean Sea was from Port Said, Egypt in 1876 (Pallary, 1912), and was not recorded in the Mediterranean before opening the Suez Canal (Issel, 1869); along the Red Sea coasts of Egypt (Shefer et al., 2004); successively found in Lebanon; Israel (Sara' et al., 2008); Italy; Greece; Syria; Southern Turkey; northern Cyprus; Croatia (Barash & Danin, 1992); Eritrea and Sri Lanka (Shefer et al., 2004). In the recent warming trend of the Mediterranean Sea, (in the future), *B. pharaonis* may actively invade more habitats, threatening indigenous bivalve species which may
unable to compete with it in terms of reproductive effort and density (Sara’ et al., 2008).

Primarily identification of *Brachidontes* depends on morphological characters but the high degree of morphological variation makes identification and systematic studies more difficult, and potentially hides cryptic species, compelling a search for systematic characters not influenced by environmental variations (Terranova et al., 2007). Recently, the mtDNA variation is used to describe the population structure of *B. pharaonis* and to make inference about its invasion of the Mediterranean Sea. DNA barcode is the use of short, standardized gene for species identification (Hebert et al., 2004). In this study the mitochondrial DNA gene cytochrome c oxidase subunit 1 (*COI*) was used as a universal barcoding marker for identifying and describing the population structure of *B. pharaonis* in Egypt.

The main objective in this study is to investigate the molecular diversity of *B. pharaonis* in different locations from the Mediterranean Sea to the Red Sea and study the effect of environmental and ecological differences between the two seas on the formation of cryptic species.

**MATERIALS AND METHODS**

**Study area and Sample collection**

*Brachidonte spharaonis* mussels were collected from five locations along the Egyptian coastal shores: Mediterranean Sea (Port Said), North Sinai (Lake Bardawil), Suez Canal (Lake Timsah), Suez (Gulf of Suez), and Red Sea (Marsa Alam) during 2012-2013 (Fig. 1). Samples were collected by scraping the rocky surface on the beaches.

Fig. 1: Map showing the sites of collection of *Brachidontes pharaonis*: 1: Lake Bardawil; 2: Port Said; 3: Lake Timsah; 4: Gulf of Suez; 5: Marsa Alam.

**Morphological examinations**

The *B. pharaonis* specimens were chosen randomly from each site and then identified according to Sharabati (1984) for morphological examination (shell characters & colour).
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Molecular studies: gDNA Extraction, PCR amplification and sequencing

The samples were frozen at -70°C and the gDNA was extracted from a 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 ng template 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 photo documentation 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 15 specimens of B. pharaonis in the present study with their accession number on GenBank are described in Table (1).

<table>
<thead>
<tr>
<th>Species</th>
<th>Locality</th>
<th>ID</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Mediterranean Sea</td>
<td>1</td>
<td>KP164519</td>
</tr>
<tr>
<td></td>
<td>Lake Bardawil</td>
<td>2</td>
<td>KP164520</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>KP164521</td>
</tr>
<tr>
<td></td>
<td>Mediterranean Sea</td>
<td>4</td>
<td>KP164522</td>
</tr>
<tr>
<td></td>
<td>Port Said</td>
<td>5</td>
<td>KP164523</td>
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<tr>
<td></td>
<td></td>
<td>6</td>
<td>KP164524</td>
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<td></td>
<td>Suez Canal</td>
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</tr>
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<td></td>
<td>Marsa Alam</td>
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<td></td>
<td>15</td>
<td>KP164533</td>
</tr>
</tbody>
</table>

Using Blast, CO1 sequences of the most related species of B. pharaonis were screened on the NCBI GenBank data base and then added to the present analysis to construct phylogenetic tree using MEGA 6 (Tamura et al., 2013) to test the presence of cryptic sibling species. Mytilaste ruminimus (DQ836022) and Geukensia demissa (U56844) were used as outgroups with bootstrap value 100%.
The phylogenetic relationship between *B. pharaonis* from Egypt and other *Brachidontes* species on GenBank was constructed according to the lowest Bayesian Information Criterion (BIC) using the substitution model (HKY+G+I). Neighbor-Joining tree based on p-distance was constructed for the phylogenetic relationship between *B. pharaonis* in the present study and 29 members of *B. pharaonis* obtained from GenBank (collected along the Mediterranean and Red Sea). To construct a phylogenetic relationship between *B. pharaonis* individuals collected from the five populations, Maximum likelihood tree was constructed with the (HKY+I) model.

### RESULTS

#### Morphological examinations

Using of morphological characters to identify *B. pharaonis* from different locations revealed that no significant differences were found between the individuals in all populations.

#### Phylogenetic analysis

As shown in Fig. (2), DNA barcoding was successfully able to distinguish between *Brachidontes* species in the current study and different species of *Brachidontes* obtained from GenBank. The phylogenetic tree shows that *B. pharaonis* from all sites in Egypt were clustered together with *B. pharaonis* from GenBank (AY129565) with high bootstrap value (100%) and diverged from *B. variabilis* with low bootstrap value.

![Fig. 2: A Maximum Likelihood tree for CoI sequences of individuals of *B. pharaonis* collected from Egypt and different species of *Brachidontes* obtained from GenBank. Internal branches within species from Egypt were compressed. *Mytilaster minimus* was used as outgroup with bootstrap value (100%).](image)

Neighbor-Joining tree (Fig. 3) illustrates the phylogenetic relationships between 15 individuals in the present study and 29 individuals obtained from GenBank. The tree revealed that individuals from Egypt are clustered together with other *B. pharaonies* obtained from GenBank, which represented *Brachidontes*
collected along the Mediterranean and Red Sea, showing closely phylogenetic relationship between the all individuals supported by low bootstrap values at the internal nodes.

Fig. 3: Neighbor-Joining Tree for CoI sequences of *B. pharaonis* in the current study and sequences of *Brachidontes* individuals obtained from GenBank. *Geukensia demissa* was used as outgroup. Only bootstrap value >75% are shown.

Maximum likelihood tree (Fig. 4) represented the phylogenetic relationship between all individuals of *B. pharaonis* in the current study. The phylogenetic analysis revealed that *B. pharaonis* from different sites formed clusters with no samples falling on other sites revealing that *Brachidonte pharaonis* in Egypt might form a combination of cryptic species complex rather than one species.
DISCUSSION

Using of morphological examinations in identification of *Brachidontes* individuals showing that there is no significant differences between all samples collected from the five sites along the Egyptian coasts (Lake Bardawil, Port said, Lake Timsah, Gulf of Suez, Marsa Alam), where the morphological characters were not obvious enough to distinguish between individuals from the different populations. The main problem in using morphological characteristics in species identification is the difficulty to measure the point at which the similarity/difference is taken to indicate taxa (Baker and Bradley, 2006).

Due to different interpretations of the high variability of the shell characters, many authors (Arcidiacono and Di Geronimo, 1976; Chemello and Oliverio, 1995; Gianguzza *et al*., 1997; Rilov *et al*., 2002) have used *B. pharaonis* as a synonym of *B. variabilis* (Krauss, 1848), and this causes a big ambiguity for the Mediterranean Sea and the Red sea regions.

Meanwhile, the simplicity and clarity of gDNA extraction; PCR amplification and sequencing techniques used in DNA barcoding were found to overcome the morphological examination problems that can lead to incorrect identification.

*COI* proving highly effective in identifying large groups of animals (Hebert *et al*., 2003), successfully applied to a variety of taxa (e.g. birds, Hebert *et al*., 2004; crustaceans, Lefebure *et al*., 2006; fungi, Seifert *et al*., 2007; mammals, Hajibabaei *et al*., 2007; amphibians, Smith *et al*., 2008; fish, Zhang and Hanner, 2011; mollusks, Feng *et al*., 2011) in the past few years.
In the present study, our molecular results showed that *Brachidonte spharaonis* and *Brachidontes variabilis* formed different distinct clades. Adding to that, using of DNA barcoding revealed that the *Brachidontes* species found in Egypt is *B. pharaonis* and that is contrasted with Kandeel (1992) who identified *Brachidontes* as *Brachidontes variabilis*, and matched the identification of (Shefer et al., 2004) who identified the Egyptian population as *Brachidonte spharaonis*. Likewise, Terranova et al. (2007) stated that the systematic revision of the taxon *B. variabilis* is needed and the name of *B. pharaonis* is most appropriate for the species in the Mediterranean Sea and the Red Sea.

In the marine realm, climatic changes have shifted the chemical and biological properties of many marine systems and the geographical distances are associated with the temperature and salinity gradients (Lo Brutto et al., 2011). The ecological plasticity of *Brachidontes* has likely played an important role in the persistence of *B. pharaonis* in the Mediterranean coasts (Apte et al., 2000).

In the current study, *B. pharaonies* collected from different localities formed distinct clades; therefore they are not a one species complex but rather a cryptic species complex. The formation of cryptic species complex in Egypt might be related to the geographical distance between the sites and different ecological habitats from which samples were collected. This result reported a similar result: the taxon previously recognized as *B. exustus* is composed of four cryptic species (Lee and O’Foighil, 2004). As like, Shefer et al. (2004) revealed two well-differentiated clades within *B. pharaonis* on the Mediterranean coast of Israel, in the Gulf of Suez, and in the northern Red sea using the mitochondrial CO1 gene. Terranova et al. (2007) by using Genetic analysis on *Brachidontes* samples in the Caribbean revealed three well-differentiated clades identifying three cryptic species.

All these findings of phylogenetic analysis provides the formation of cryptic species but not support the formation of separated species, this could be attributed to that the time from the invasion of the indo-Pacific *B. pharaonis* from Red Sea to Mediterranean Sea is no longer enough for the changing in the genetic structure and reproduction to form a new species. This is agreed with Shefer et al. (2004) who stated that the time frame for the Mediterranean invasion by *B. pharaonis* is < 150 years (Por, 1978; Safriel et al., 1980), while the average rate of its expansion eastwards from the Canal's Mediterranean end was ~10 km / year and it's only in the past 30 years that a dramatic increase in population size has been reported (Safriel et al., 1980; Rilov et al., 2001). This time frame is undoubtedly not long enough for reproductive isolation and a high proportion of uniqueness (up to 80%) to evolve.

Finally this study showed that the use of morphological characters in the identification of marine bivalve was not accurate enough in species identification and was not able to discover cryptic or hybrid species complex. The use of DNA barcoding in the identification of *Brachidontes* in Egypt revealed that the Egyptian population of *Brachidontes* is formed by the species *B. pharaonis* not *B. variabilis* as previously thought. The results also revealed a cryptic species complex of five cryptic species located in different areas. Our results may have a great impact on the conservation and fisheries management, in addition to species diversity of the Egyptian waters, and highlight the need for further research on species complexes and migrated species.
REFERENCES


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دراسات جزيئية على براكينونتز فارونيز في مصر تكشف مجموعة من الأنواع المحتملة

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لقد تم دراسة الأنواع والتنوع الجيني لثنائي المصريين "براكينونتز فارونيز" على طول السواحل المصرية لتحديد التركيب الجيني لهذه الأنواع ولاكتشاف التنوع مع العزل الجيولوجي. تم تجميع العينات من البحر الأحمر - البحر المتوسط - قناة السويس. تم تعيين التنوع الجيني باستخدام تقنية تعرف الحمض النووي لجين السيركتوروم التأكسدي رقم 1 الموجود بالميوكوندريوم. وأظهر النتائج أن براكينونتزارونيزا يتحملا تنوع جيني مع مختلف الأنواع. وقد يكون لهذه النتائج تأثير كبير في الحفاظ على براكينونتزارونيز في المياه المصرية.