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

Nancy A. Radwan[1]; Mohammed I. Ahmed[2], Nesreen K. Ibrahim[2], Saad Z. Mohammed[2]and Zakaria S. Morsy[1]

1- Department of Zoology, Faculty of Science, El Arish, Suez Canal University, Egypt.
2- Department of Marine Science, Faculty of Science, Ismailia, Suez Canal University, Egypt.

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 "Lessepaisn 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 iswidely 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, threating 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 (*CO1*) 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 areaandSample collection

Brachidonte spharaonis mussels were collected from five locations along the Egyptian costal 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 scrapping the rocky surface on the beaches.

Fig. 1: Map showing the sites of collection of*Brachidontespharaonis*1: Lake Bardawil; 2: Port Said; 3: Lake Timsah; 4: Gulf of Suez; 5: MarsaAlam.

Morphological examinations

The *B. pharaonis* specimens were chosen randomly from each site andthen identified according to Sharabati (1984) for morphological examination (shell characters &colour).

Molecular studies 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 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 1: *Brachidontes sp.* individuals included in analysis, with their collection localities, ID and GenBank accession number.

Species	Locality	ID	Accession number
Brachidontes pharaonis	Mediterranean Sea Lake Bardawil	1	KP164519
		2	KP164520
		3	KP164521
	Mediterranean Sea Port Said	4	KP164522
		5	KP164523
		6	KP164524
	Suez Canal Lake Timsah	7	KP164525
		8	KP164526
		9	KP164527
	Suez Gulf of Suez	10	KP164528
		11	KP164529
		12	KP164530
	Red Sea Marsa Alam	13	KP164531
		14	KP164532
		15	KP164533

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 rminimus* (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 GenBankwas 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 studyand 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.

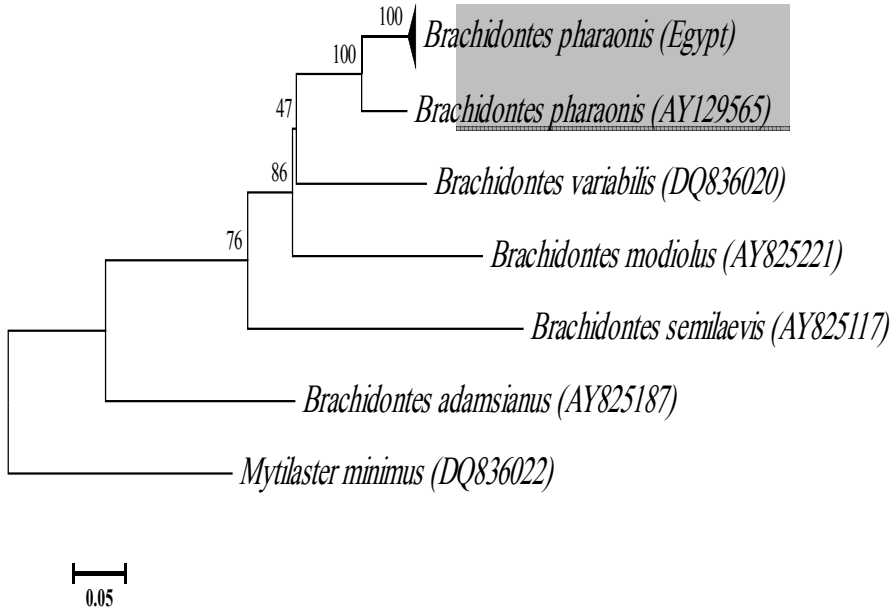

Fig. 2: A Maximum Likelihood tree for *Co1* sequences of individuals of *B. pharaonis* collected from Egypt and different species of *Brachidontes* obtained from GenBank. Internal branches within species from Egypt were compressed. *Mytilaster minimus* was used as outgroup with bootstrap value (100%).

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.

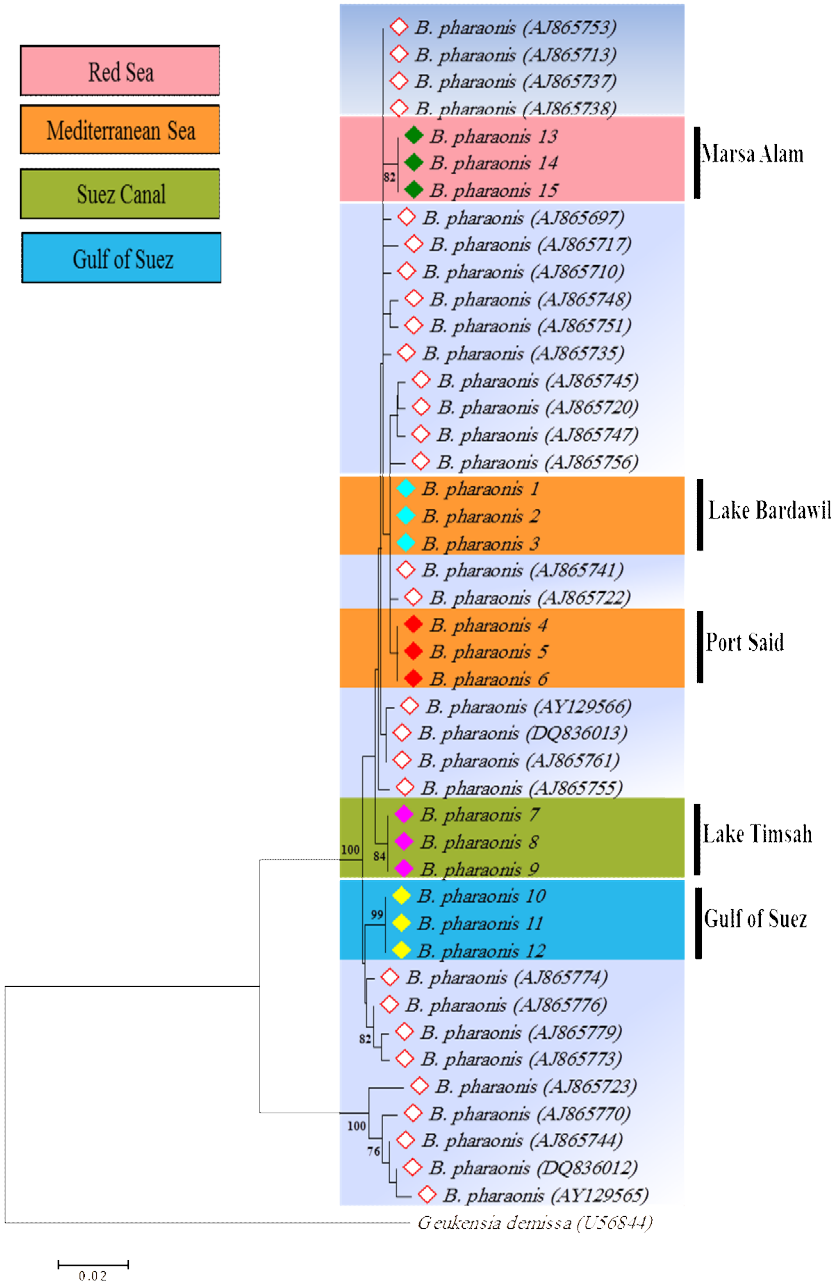

Fig. 3: Neighbor-Joining Tree for*Co1* sequences of *B. pharaonis*in the current study and sequences of *Brachidontes* individuals obtained from GenBank. *Geukensia demissa* was used as outgroup. Only bootstrap value >75% are shown.

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 spharaonis* in Egypt might form a combination of cryptic species complex rather than one species.

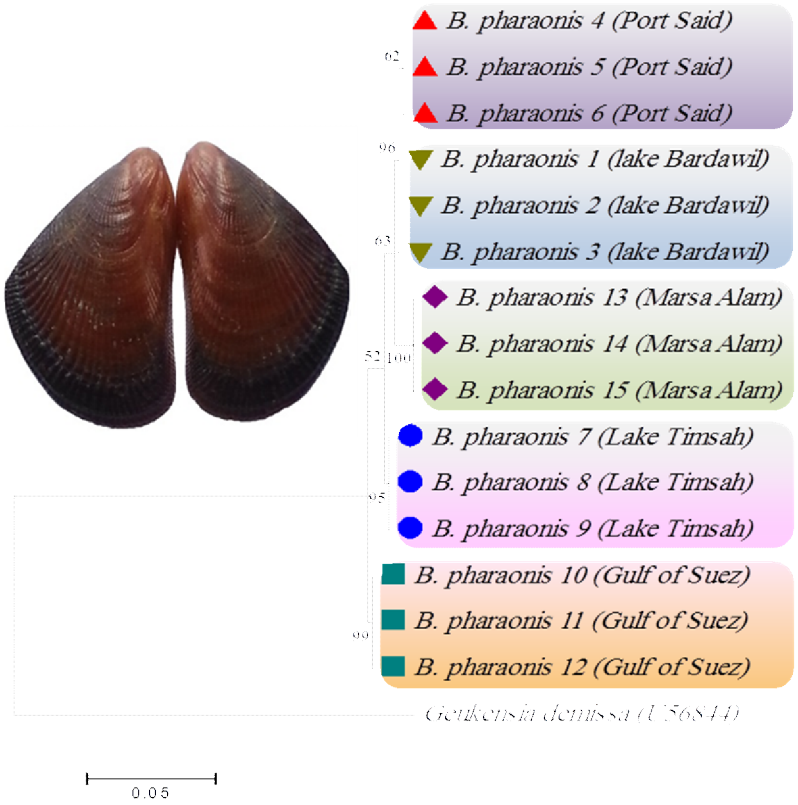

Fig. 4: Maximum Likelihood Tree for *CO1* sequences of *B. pharaonis* collected from 5 different habitats along the Egyptian costal shores. *Geukensia demissa* was used as outgroup with bootstrap value (100%).

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.

CO1 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 attributedto 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

Apte, S.; Holland, B. S.; Godwin, L. S. and Gardner, J. P. A. (2000). Jumping ship: a stepping stone event mediating transfer of a nonindogenous species via a potentially unsuitable environment. Biol. Invasions, 2: 75-79.

Arcidiacono, A. and Di Geronimo, I. (1976). Studio biometrico di alcunicampioni di Brachidontesvariabilis (Krauss). Conchiglie, 12:61–74.

Baker, R. J. and Bradley, R. D. (2006): Speciation in Mammals and the Genetic species concept. J. Mammal., 87(4):643–662.

Barash A. and Danin, Z. (1992). Fauna Palaestina: Mollusca I. Annotated list of Mediterranean molluscs of Israel and Sinai. Israel Acad. Sci. Human., Jerusalem.

Chemello, R. and Oliverio, M. (1995). Lessepsian migrations: a theoretical island-jumping- model. Biol. Mar. Medit., 3: 444–446.

Coffroth, M. A.; Lasker, H. R.; Diamond, M. E.; Bruenn, J. A. and Bermingham, E. (1992). DNA fingerprints of a gorgonian coral: a method for detecting clonal structure in a vegetative species. Mar. Biol., 114 (2): 317-325.

Doğan, A.; Önen, M. and Öztürk, B. (2007). A new record of the invasive Red Sea mussel *Brachidontes pharaonis* (Fischer P., 1870) (Bivalvia: Mytilidae) from the Turkish coasts. Aquat. Invasions, 2 (4): 461-463.

Feng, Y.; Li, Q.; Kong, L. and Zheng, X. (2011). CO1-based DNA barcoding of *Arcoida species* (Bivalvia: Pteriomorphia) along the coast of China. Mol. Ecol. Res., 11: 435-441.

Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol., 3: 294 - 229.

Gianguzza, P.; Chemello, R. and Riggio, S. (1997). Segnalazione di *Brachidontes pharaonis* (P. Fischer, 1870) (Bivalvia, Mytilidae) nellasalina di Marsala e considerazioni sulla distribuzione della specie in Mediterraneo. Boll Malac 33:169–172.

Hajibabaei, M.; Singer, G. A. C.; Hebert, P. D. N. and Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. TrendsGenet., 23 (4): 167-172.

Hebert, P.D.N.; Cywinska, A.; Ball, S. L. and de Waard, J. R. (2003), Biological identifications through DNA barcodes. Proc. R. Soc. Lond. (B) Biol. Sci., 270:313–321.

Hebert, P. D. N.; Stoeckle, M. Y.; Zemlak, T. S. and Francis, C. M. (2004). Identification of birds through DNA barcodes, Plos. Biol. 2 (10) 1657–1663.

Holland, B. S. (2000). Genetics of marine bioinvasions. Hydrobiologia 420: 63-71.

Issel, A. (1869). Malacologiadel Mar Rosso, Pisa pp 387.

Kandeel, S. K. (1992). Biological studies on the reproduction of some bivalves in Lake Timsah. M.Sc. Thesis, Fac. Sci. Suez Canal Univ., Ismailia, Egypt.

Lee, T. and O´ Foighil, D. (2004). Hidden Floridian biodiversity: mitochondrial and nuclear gene trees reveal four cryptic species within the scorched mussel, *Brachidontes exustus*, species complex. Mol. Ecol., 13:3527–3542.

Lefebure, T.; Douady, C.J.; Gouy, M. and Gibert, J. (2006). Relationship between morphological taxonomy and molecular divergence within crustacea: proposal of a molecular threshold to help species delimitation. Mol. Phylogenet. Evol., 40: 435-447.

Lo Brutto, S.; Arculeo, M. and Grant, W. S. (2011). Climate change and population genetic structure of marine species. Chem. Ecol., 27 (2): 107-119.

Pallary, P. (1912). Catalogue des mollusques du littoral méditerranéen de l'Egypte. Mémoires de l'Institutd'Egypte, 7: 69-207, pl. 15-18.

Por, F.D. (1978). Lessepsian migrations: The influx of Red Sea biota in to the Mediterranean by way of the Suez Canal. Heidelberg: Springer. 228p.

Rilov, G. and Galil, B. (2009). Marine bioinvasions in the Mediterranean Sea-History, distribution and Ecology. Biological invasions in Marine Ecosystems, Ecolo. Stud., 204.

Rilov, G.; Benayahu, Y. and Gasith, A. (2001). Low abundance and skewed population structure of the whelk *Stramonitab haemastoma* alongthe Israeli Mediterranean coast. Mar. Ecol. Prog. Ser., 218, 189–202.

Rilov, G.; Gasith, A. and Benayahu, Y. (2002). Effect of an exotic prey on the feeding pattern of a predatory snail. Mar. Envir. Res., 54:85–98.

Safriel U. N.; Felsenburg, T. and Gilboa, A. (1980). The distribution of Brachidontes variabilis (Krauss) along the Red Sea coasts of Sinai. Argamon, 7(3): 31-43.

Sara`, G.; Romano, c. and Mazzola, A. (2008). Anew Lessepsian species in the western Mediterranean (*Brachidonte spharaonis* Bivalvia: Mytilidae): density, resource allocation and biomass. Mar. Biodiv.Record.,1, (e8).

Seifert, K. A.; Samson, R. A.; Dewaard, J. R.; Houbraken, J.; Lévesque, C. A.; Moncalvo, J. M.; Louis-Seize, G. and Hebert, P. D. N. (2007). Prospects for Fungus identification using CO1 DNA barcodes, with Penicillium as a test case. Proc. Natl. Acad. Sci., U.S.A., 104: 3901-3906.

Sharabati, D. (1984). Red sea Shells. KPI limited, London. 122pp.

Shefer, S.; Abelson, A.; Mokady, O.and Geffen, E. (2004). Red to Mediterranean Sea bioinvasion: natural drift through the Suez Canal, or anthropogenic transport. Mol. Ecol., 13:2333–2343.

Smith, M. A.; Poyarkov, N. A. and Hebert, P. D. N. (2008). DNA barcoding amphibians: take the chance, meet the challenge. Mol. Ecol. Resour., 8: 235-246.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol. Biol. Evol., 30: 2725-2729.

Terranova, S. M.; Brutto, L. S.; Arculeo, M. and Mitton, J. B. (2006). Population structure of *Brachidonte spharaonis* (P. Fischer, 1870) (Bivalvia, Mytilidae) in the Mediterranean Sea, and evolution of a novel mtDNA polymorphism. Mar. Biol., 159:89–101.

Terranova, S. M.; Brutto, L. S.; Arculeo, M.; Mitton, J. B. (2007). A mitochondrial phylogeography of *Brachidontes variabilis* (Bivalvia: Mytilidae) reveals three cryptic species. J. Zool. Syst. Evol. Res., 45(4): 289–298.

Zhang, J. B. and Hanner, R. (2011). DNA barcoding is a useful tool for the identification of marine fishes from Japan. Biochem. Syst. Ecol., 39: 31-42.

ARABIC SUMMARY

دراسات جزيئية على براكيدونتس فاراؤنيز فى مصر تكشف مجموعة من الأنواع المبهمة

نانسى ابوفندود رضوان[١] - محمد اسماعيل أحمد[٢] - نسرين قدرى ابراهيم[٢] - سعد زكريا محمد[٢] – زكريا صبرى مرسى[١]

١- قسم علم الحيوان– كلية العلوم– جامعة قناة السويس – العريش ، مصر

٢- قسم علوم البحار– كلية العلوم – جامعة قناة السويس – الاسماعيلية ، مصر

لقد تمت دراسة الأنواع والتنوع الجينى لثنائى المصرعين "براكيدونتس فاراؤنيز" على طول السواحل المصرية لتحديد التركيب الجينى لهذه الأنواع ولاكتشاف التنوع مع العزل الجيوغرافى. تم تجميع العينات من البحر الأحمر- البحر المتوسط - قناة السويس.وتم تعيينالتنوع الجينى باستخدام تقنية شفره الحمض النووى لجين السيتوكروم التأكسدى رقم ١ الموجود بالميتوكوندرياوأشارت النتائج إلـى أن براكيدونتسفاراؤنيزالتى تم تجميعها من أماكن مختلفة تتعنقد فى منظومةمتفرعة مما يشير إلـى أن مجتمع "البراكيدونتس فاراؤنيز" فى الشواطىء المصرية ربما يكون انواعا خفية (متشابهة ظاهريا ومختلفة جينيا) اكثر منه مجتمع من نوع واحد. وقد يكون لهذه النتائج تأثير كبير فى الحفاظ على براكيدونتس فاراؤنيز فى المياه المصريه.