Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 28 (2): 51 – 66 (2024)
www.ejabf.journals.ekb.eg

Morphological Characters and Molecular Phylogeny of the Invasive Spiny File Clam, *Lima lima* (Bivalvia: Limidae) from the Expansion Channel of the Suez Canal

Nesreen K. Ibrahim*, Mahmoud Sami, Amira A. Ali
Department of Marine Science, Faculty of Science, Suez Canal University, Egypt

*Corresponding Author: nesreen_ibrahim@science.suez.edu.eg

ARTICLE INFO

Article History:
Received: Jan. 20, 2024
Accepted: Feb. 3, 2024
Online: march. 6, 2024

Keywords:
Limidae,
Lima lima,
Morphological characters,
DNA barcoding,
Expansion channel of Suez Canal

ABSTRACT

Studying the morphological features of the invasive spiny file clam *Lima lima* was conducted for the first time in the expansion channel of the Suez Canal. Morphological descriptions were based on their exterior morphology and number of radial ribs (mean = 18). Various measurements were recorded, and biometric relationships were examined, particularly the correlations between the independent parameter shell length (SL) and various dependent parameters. Notably, the relationships with shell height and shell inflation demonstrated negative allometric growth. Moreover, regression that describes the relationship between shell length (SL) and soft body weight had a coefficient of determination R^2= 0.709, showing a high significant correlation and a negative growth. Molecular analysis showed that the mantle had the highest quantity and optimum quality of gDNA, while the muscles had low value of DNA concentration. Integration of the morphological characteristics by molecular examination using DNA barcoding was designed to confirm the identification of the anti-lessepsian migrant clam *L. lima* collected from the expansion channel of the Suez Canal.

INTRODUCTION

Molluscs comprise the second-largest phylum in the animal kingdom after Arthropoda **(Carpenter & De Angelis, 2016)**. Many molluscs have an economic important role where some industries rely on their aquaculture and harvest. Moreover, their populations define the construction of benthic assemblages, contribute in the nutrients recycling, and conserve the qualification of water by reducing some heavy metals in the environments **(Coen & Bishop, 2015)**. Furthermore, many mollscan species are an important source of food for human and higher marine organisms, such as fishes and crabs. Bivalves comprise about 23% of all named marine organisms.

File shells or file clams are common names for marine bivalves of family Limidae. Members of this family are filter feeders and can be found in seas worldwide and at various depths. The spiny file clam *Lima lima* inhabits the Mediterranean Sea, the Eastern Atlantic and the Caribbean waters, ranging from southern Florida to the West

Indies and Bermuda **(Mikkelsen & Bieler, 2000)**. Occasionally, it can be found on rocky bottoms and coral, but it usually lives in the seagrass beds dominated by *Posidonia oceanica* at depths of 0 to 50m **(Mikkelsen & Bieler, 2003)**.

Shell morphology is typically significant for taxonomy and has traditionally been used to distinguish most bivalve species. Their characteristic features often include shell outline, size, ornamentation, internal ridges and/or folds and the number of axial ribs. Ribs number has been commonly detected as a species character in several Limidae genera **(Mikkelsen & Bieler, 2003)**. However, species taxonomy based solely on shell diagnoses could be confusing, and many researches depend on it. **Hebert *et al*. (2003)** argued that the most reliable method for a justified identification is a system that utilizes DNA sequences as taxon barcodes. The mitochondrial gene cytochrome c oxidase I (COI) was recognized as the principal of a global bio-identification system for animals. COI identification system provides an accessible solution to the taxonomic collapsing of species.

The expansion channel of the Suez Canal represents a new path for the migration of many biota from the Red Sea to the Mediterranean and vice-versa, and it may offer a new inhabiting environment. The current study is considered the first study of *Lima lima* in the Suez Canal, aiming to identify *L. lima* from the Suez Canal based on its morphological features, and genetically confirming their identification using DNA barcoding.

MATERIALS AND METHODS

1. Study site

The Suez Canal expansion channel (SCEC) is located in Ismailia (30.441385°N 32.355423°E) (Fig. 1). It was opened for navigation in 2015, and extends 72km long and 24m depth, serving a parallel shipping lane for a specific section in the existing 164km long canal **(Suez Canal Authority, 2022)**. Due to the passage of ships and tankers, as well as the dredging of the main navigational channel, the Suez Canal water becomes turbid **(Madkour *et al.*, 2006)**. The sediment of the channel consists of coarse, fine sand, and muddy-sand with gravel and rocks **(Suez Canal Authority, 2022)**.

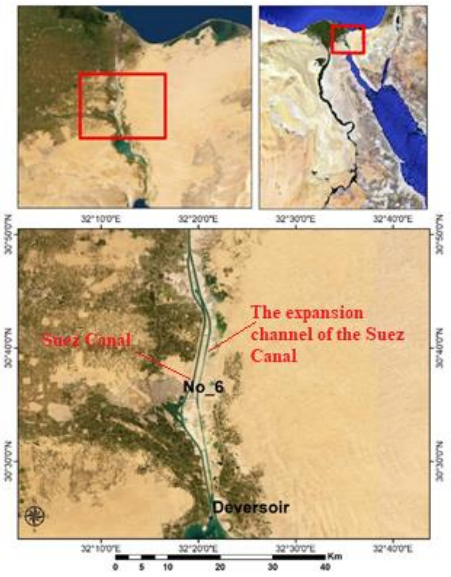

Fig. 1. Map of the Suez Canal showing the sampling site, and the expansion channel of the Suez Canal

2. Samples collection and preservation

Samples were collected during autumn and winter 2022 by fishermen using free diving to depths ranging from five to six meters. Specimens of the spiny file clam *L. lima* were preserved frozen until transporting them to the laboratory for study. The soft body parts (muscle, mantle and gonads) of the clams were separated from the shells, and directly stored in -80°C for DNA analysis. Shells were cleaned from the different bio-fouling organisms by scraping them using a scalpel at the bottom of the covering epibionts and lifting them up.

3. Biometric measurements

Exterior morphological characters were recognized and described according to **Sharabati (1984)**. Different measurements were recorded. Shell length (SL) was the maximum distance on the anterior to posterior axis. Shell height (SH) was the maximum distance from the dorsal to the ventral axis. Shell inflation (SI) was the measure of the maximum distance on the lateral axis (Fig. 2). All the measurements were taken to the nearest 0.01mm using the Vernier caliper according to method of **Gallois (1976)**. The soft part was removed from the shell using a spatula and weighed (SBW) to the nearest 0.01g. The number of shell rib of each individual was recorded near the growth edge on the shell.

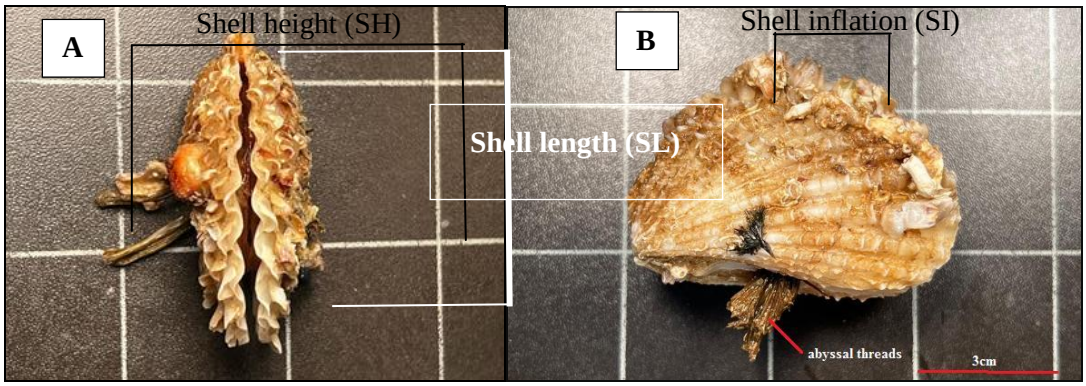

Fig. 2. Measurements of the spiny file clam *L. lima* showing: **(A)** Shell length (SL), Shell height (SH) and **(B)** Shell inflation (SI)

3. Analysis of morphometric relationships

The length - weight relationship between soft body weight (SBW) in g and the shell length (SL) in mm was defined by the equation $W = a L^b$ **(Ricker, 1975)**, where "W" is the dependent variable; "L" is the independent variable; "a" is the initial growth coefficient, and "*b*" is the relative growth rate of variables. Soft body weight and shell length were converted to natural logs, and linear regression equation was determined as:

$\log W = \log a + b \log L$. Student's *t*-test was used to determine if *"b"* which is the slope value was significantly different from the hypothetical isometric value (H_o: $b = 3$) at a confidence level of $P \leq 0.05$. For the relative growth rate of the shell length, shell height and shell inflation, regression equations were calculated by the allometric equation: $Y = a + b X$, where X= shell length, and Y= shell height or shell inflation. H_o: $b = 1$ was used to determine whether the slope differs from isometric growth or not. All statistical analyses were performed using SPSS (version 22) and Microsoft Excel. Significant levels for all analyses were set at $P < 0.05$.

4. Molecular analysis

DNA of *L. lima* was extracted from different organs (muscle, mantle and gonads) to choose the best organ. DNA was extracted with Genomic DNA - Solution Kits (Jena Bioscience), using the Tissue Protocol as mentioned by the manufacturer. The Polymerase Chain Reaction (PCR) was performed using the universal COI primers HCO 2198 (5 ′ -TAA ACTTCA GGG TGA CCA AAA AAT CA-3 ′) and LCO 1490(5 ′ -GGT CAA ATC ATA AAG ATA TTG G-3 ′) **(Folmer *et al.*, 1994)**. The primers were chosen according to the guidelines of **Mikkelsen, *et al*. (2007)**. PCR test was achieved for each sample in 25µl reaction volume containing 12.5µl of Master Mix (Qiagen), 2µl of DNA template (20ng/ µl)"DNA measured by ND1000 Nanodrop" and 2µl of the primer (20 pmole) and 8.5µl deionized H_2O. Thermal profile for the PCR was: preliminary denaturation for 3 minutes at 94°C, thirty cycles of denaturation for 40 seconds at 94°C, 30 cycles of annealing for 40 seconds at 53°C, 30 cycles of extension for 1 minute at

72°C, and 10 minutes of final elongation at 72°C. 1.5% of agarose gel in 1X TAE buffer stained with ethidium bromide was used in gel electrophorese at 130V.

Gel photos were taken using the system of gel documentation (Camera, Synoptics 3.8MP) and then examined by Gel Docs advanced (GeneSys Version, 1.4.1.0 and Database Version 1.84) software. PCR products were sanitized using QIA quick PCR Purification Kit (QIAGEN), and then PCR was attained using Big Dye Terminator cycle sequencer version 3.1 Cycle Sequencing Kit.

To complete the volume to 20µl, each reaction had 8µl terminator ready reaction mix, 3.2 pmol primer, DNA template (template quantity was intended by using the PCR product size) and deionized water.

The thermal profile for cycle sequencing involved an initial denaturation step of one minute at 96°C, followed by 25 cycles consisting of ten seconds at 96°C, five seconds at 50°C, and four minutes at 60°C. Following amplification, CENTRI-SEP Columns from PRINCETON SEPARATIONS were used for additional purification steps. DNA sequencing was performed using the 3500 Genetic Analyzer from Applied Biosystems.

The sequences of COI were surveyed to ensure and edit sequences on MEGA- X software. Multiple sequence alignments of COI regions in the current study and that downloaded from the National Center of Biotechnology and Information (NCBI) were performed by Clustal- W in MEGA- X **(Tamura *et al*., 2013)**. Sequences were blasted on NCBI GenBank after editing (https://blast.ncbi.nlm.nih.gov/).

Whereas, the sequences of the COI gene of the studied species were matched with a sequence of the reference in the GenBank for identity confirmation using BLAST search. A Clustal- W software was applied to support the sequences **(Thompson *et al*., 1994)**. Sequences of the COI gene for the spiny file clam *L. lima* were uploaded to the database of the GenBank, and phylogenetic tree was constructed by using MEGA-X software.

RESULTS

1. Morphological characters of the spiny file shell *L. lima*

The color of shells is white to bright brown; shells have equal valves with long anterior slope, higher than long (Fig. 3A). The shells are trigonal and intensely radially ribbed with rounded lamellae like the finger-nails, these ribs are scabrous to spinose (Fig. 3B). The shell surface shows 14- 22 tough ribs with thorns especially near the margin. The umbo is separated from the hinge line by a small triangular area. The hinge line is straight and typically jointed by a non-calcified ligament without any teeth. The soft parts are bright red, and many tentacles extend from the open valves (Fig. 4).

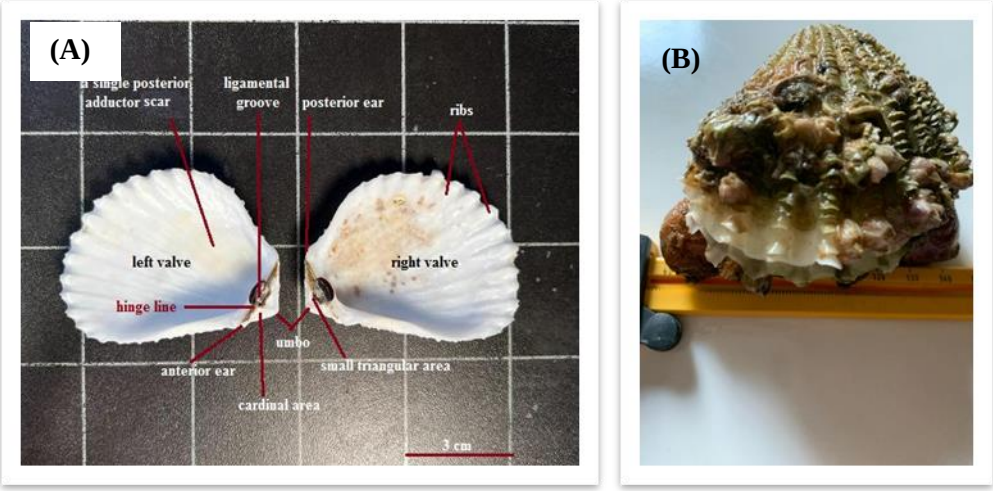

Fig. 3. Shell of *L. lima* showing: **(A)** Internal view and **(B)** External view with different characteristic features

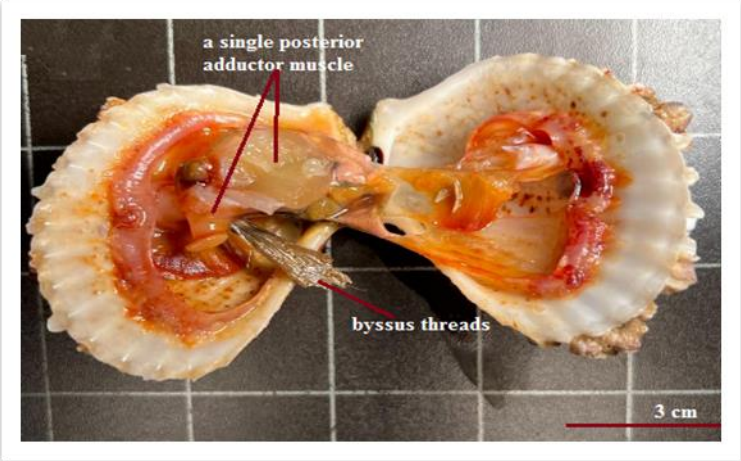

Fig. 4. Anatomy of *L. lima* showing adductor muscle, mantle, gut and byssus

Three main groups of epibiont assemblages were recorded on the shell of *Lima*. All epibionts were completely attached to both valves. Barnacles showed the highly percentage cover in all collected individuals followed by ascidians and tube worms (Fig. 5).

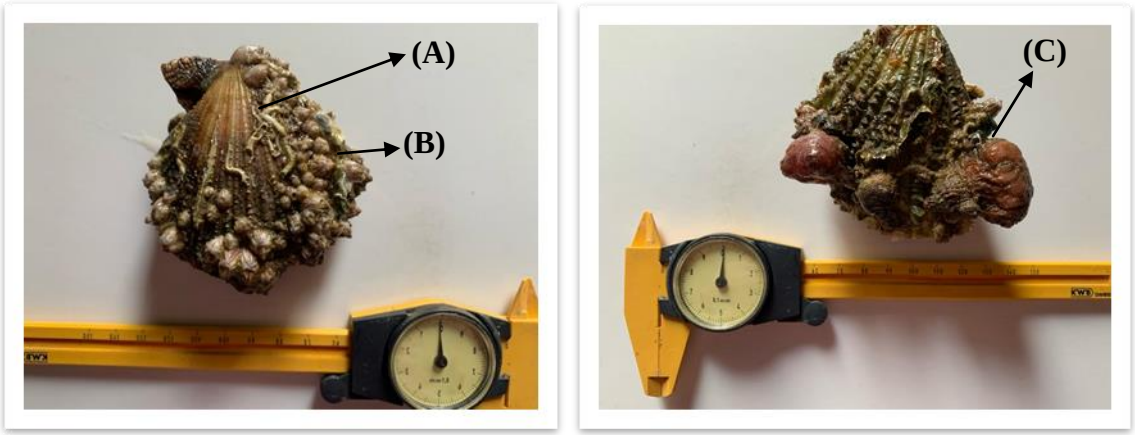

Fig. 5. Shell of the spiny file clam *L. lima* covered with different groups of epibionts showing: **(A)** Tube worms, **(B)** Barnacles, and **(C)** Ascidians

2. Morphometric measurements and biometric relationships

In the present study, 189 individuals were collected during autumn and winter 2022. The measured mean shell length was 49.1mm, while mean shell height and shell inflation were 65.47 and 28.3mm, respectively (Table 1).

Table 1. Minimum, maximum, mean and standard deviation (SD) of morphometric measurements of *L. lima* collected from the expansion channel of the Suez Canal. (Number of individuals = 189)

Variable	Minimum	Maximum	Mean	±SD
Number of ribs	14	22	18	1.83
SL (mm)	30	72.5	49.1	8.50
SH (mm)	42.5	86	65.47	10.63
SI (mm)	18.5	39.2	28.3	10.30
TWt (g)	34.4	76.6	57.3	7.91
SBW (g)	5.14	29.2	17.37	6.05

SL: Shell length, SH: Shell height, SI: Shell inflation, TWt: Total weight, and SBW: Soft body weight.

The size measurement showed that most of the individuals were between 40-59.9mm SL (64%), while only 2 % of samples were larger than 69.9mm (Fig. 6).

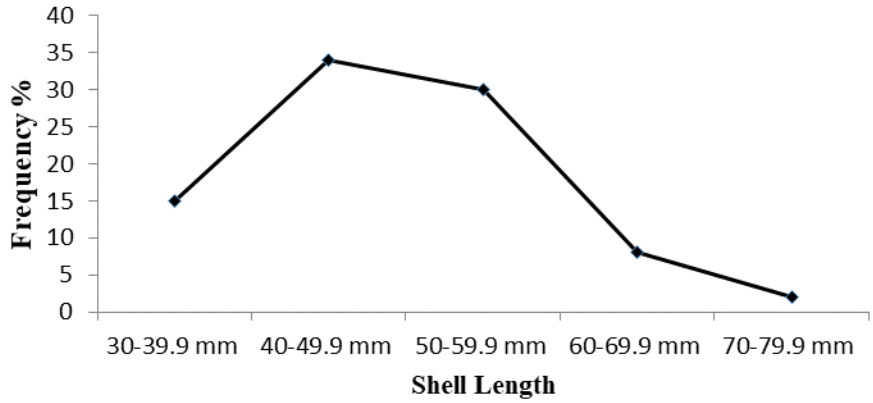

Fig. 6. Percent frequency of the different size classes of *L. lima* from the expansion channel of the Suez Canal

Table (2) shows the relationships between the independent parameter shell length (SL) and the different dependable parameters (shell height and shell inflation). The slope "*b"* of the regression lines fluctuated from 0.996 to 0.394, indicating a negative allometric growth. Moreover, regression that describes the relationship between shell length (SL) and soft body weight had a coefficient of determination R^2= 0.709, showing a high significant correlation at P< 0.05. Slope value "*b*"= 1.916 was significantly deviated from the value of three at P< 0.05, showing a negative growth.

Table 2. Morphometric relationships of *L. lima* from the expansion channel of the Suez Canal

Morphometric relationship	Allometric equation	R^2	Allometry
SH – SL	SH = 0.996 SL + 16.55	0.6351	Negative*
SI – SL	SI = 0.394 SL + 10.041	0.4555	Negative*
SBW – SL	Log SBW = 1.916 log SL - 2.019	0.7088	Negative*

SL: Shell length, SH: Shell height, SI: Shell inflation, SBW: Soft body weight, R^2 = Coefficient of determination. *Significant at P< 0.05. Number of individuals= 189.

3. Molecular analysis

The mantle had the highest quantity and optimum quality of gDNA, while the muscles had a low value of DNA concentration, and the purity index showed contamination with protein (Fig. 6). Although the gonads recoded high concentrations of gDNA, the ratio of 260/280 of purity was 1.83, which means that there were some impurities of RNA (Fig. 6).

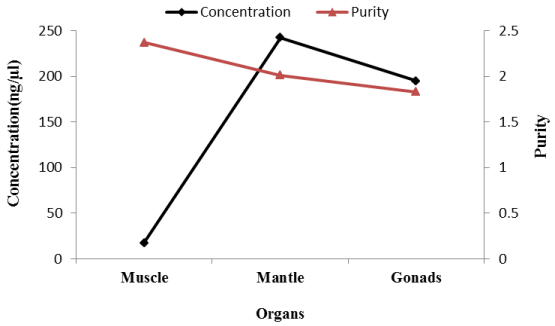

Fig. 6. gDNAs concentration and purity extracted by genomic tissue kit from different organs of *L. lima*

The COI datasets for the studied species *L. lima* were 352bp long. Sequences were submitted on NCBI (GenBank database) with the accession numbers of OQ299540, OQ299541 and OQ299542. The analyses of software tool evolutionary were showed in MEGA- X **(Kumar *et al.*, 2018)**. The uploaded and downloaded sequences were aligned by using Clustral- W, and the best model were used for COI datasets of nucleotide composition relied on the TN93: Tamura-Nei model (**Nei & Kumar, 2000)**. The model of TN93 with the lowest Bayesian information criterion (BIC) scores was applied to define the best replacement design.

Phylogeny analyses were showed according to the maximum likelihood (ML) method, and evaluation of the statistical confidence for ML bootstrap (confidence limit) was employed for testing interior branches, and applicable to all tree-building methods. All the constructed trees rooted by *Conus textile* (EU812758) as an out group.

Two ML trees were constructed for different scenarios as the following:

Tree (1)

The maximum likelihood (ML) phylogeny tree was constructed for the most available references for different families of the bivalves on NCBI including the submitted studied species *L. lima* (three replicates). The purpose of constructing the tree was to demonstrate how DNA barcoding technique is successful in separating different families genetically for each other (Fig. 7). There are two main clades: The first clade included *Pilsbryoconcha* sp. (OP589123) collected from Thailand and *Lampsilis teres* (MK391768) from USA, while the second clade included the rest of families with different species.

Tree (2)

L. lima species were collected from the expansion channel of the Suez Canal in Egypt, and references species of their relatives obtained from NCBI for partial fragments of the COI mitochondrial gene were analyzed (Fig. 8). The tree was divided into two major clades: The first clade comprised the present study species with a confidence value of 100%, with the other affiliated *L. lima* species AF120649 and KC429101 from USA.

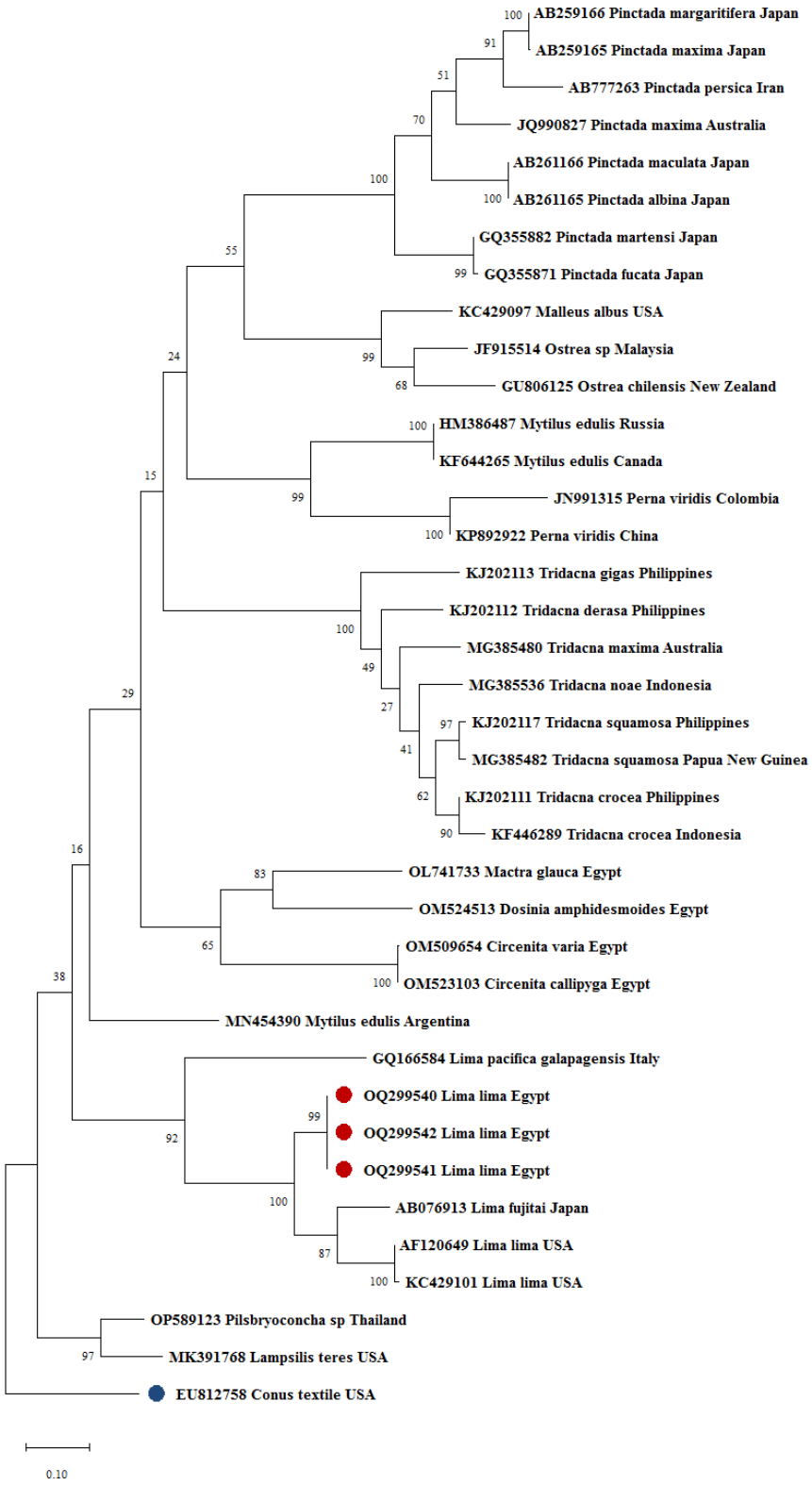

Fig. 7. Maximum likelihood tree (ML) for COI sequences of the different families of the bivalves and the studied species *L. lima* collected from the expansion channel of the Suez Canal, Egypt. *Conus textile* was utilized as an out group with the bootstrap value of 100%

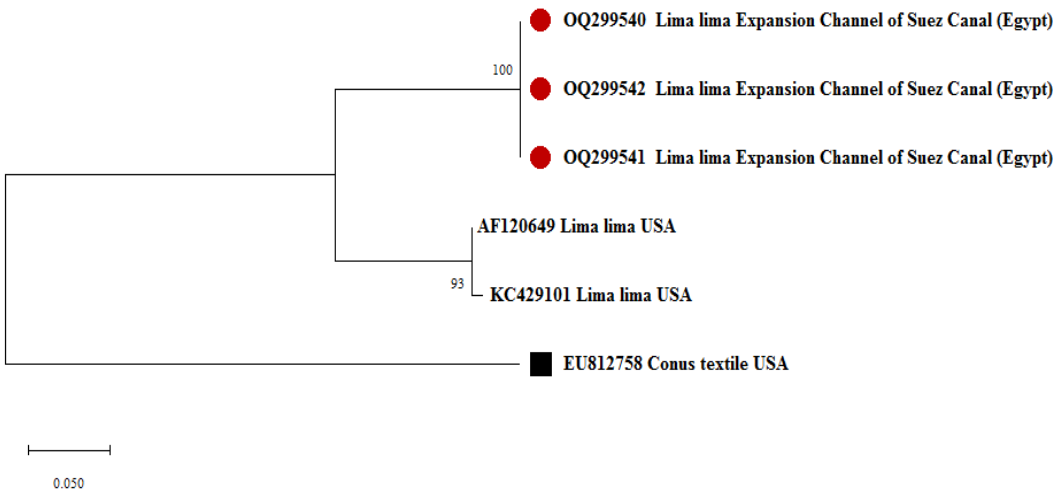

Fig. 8. Maximum likelihood tree for COI sequences of the spiny file clam *L. lima* collected from the expansion channel of the Suez Canal, Egypt and their relatives in the world. *Conus textile* was utilized as an out group with the bootstrap value of 100%

DISCUSSION

This work is the first study of the spiny file clam *L. lima* from the expansion channel of the Suez Canal. The description of external shell characters is almost restricted due to the number of radial ribs in addition to their different form over diverse areas of the shell.

Mikkelsen and Bieler (2003) provided a fully description of the western Atlantic file clams, *Lima* and *Ctenoides* (Bivalvia: Limoida: Limidae). They approved that species differences depend on external shell characters, such as color and ornamentation. Moreover, the identification to the genus level relies on the internal anatomy of tentacles, gut and mantle. Limids have a specific character among all other clams where the shell hinge teeth are absent, and the foot is twisted by 180º comparative to visceral mass **(Gilmour, 1990)**. Individuals of *L. lima* were recognized as a distinct species according to total number of ribs that ranged from 14 to 22 in the present study. This result is in agreement with the study of **Poppe and Goto (1993)** from the Mediterranean.

A few studies have analyzed morphological measurements and biometric relationships of limids. The different morphological measurements and the ratios between them attained in our study commonly show significant correlation between shell height, shell inflation and soft body weight with shell length. **Marshall (2001)** and **Gagnon *et al.* (2015)** compared shell height, length and inflation, along with ratios, between *Acesta* spp. from the southwestern Pacific and the Northwest Atlantic, respectively.

Also, **Gagnon and Haedrich (2003)** studied the ratio between the shell height and width of the European giant file clam, *Acesta excavata* from the northwest Atlantic.

Integration of the morphological characteristics by molecular examination using DNA barcoding was designed to confirm the identification of *L. lima* collected from the expansion channel of the Suez Canal.

Hebert *et al.* (2004), **Ward *et al.* (2005)** and **Hajibabaei *et al.* (2006)** stated that in 'DNA barcoding' a small portion of DNA sequence is used to identify species. Neither the technology nor the idea behind DNA barcoding is novel. The controversial and debatable issues are the idea of using just a short section of a single gene to identify species from a wide taxonomic range, including animals such as birds, fish and insects. This recent usage and its subsequent successes has induced criticism and taxonomic debate. **Radwan *et al.* (2014a, b)** revealed the efficiency of DNA barcoding in the identification of four species of bivalve and differentiation among them based on mitochondrial *CO1* gene sequence

Since the early DNA- barcoding papers in 2003 **(Hebert *et al.*, 2003; Blaxter, 2004)**, Hebert and co-workers have touted the achievements of barcoding in a series of publications. From this single short section of sequence of the COI gene, individuals have been identified down to species level with a success percentage ranging from 98 to 100% in North American birds **(Hebert *et al.*, 2004)**, Australian fish **(Ward *et al*, 2005)** and most recently in tropical Lepidoptera **(Hajibabaei *et al.*, 2006)**, in addition to correctly identifying species, a number of cryptic species have been discovered by using DNA barcoding technique. This obvious success has operated speculation that an accurate species identification is now allowed by anyone with access to DNA sequencing even though they lack taxonomic expertise.

DNA barcoding is considered as an important tool for identifying unknown molluscs in addition to linking life-history stages of the same species. **Martin and Palumbi (1993**) reported that mitochondrial DNA tool (=mtDNA) is broadly used for differentiation between closely related species otherwise mitochondrial sequences are useful for analyzing relationship among distantly related organisms **(Kocher *et al.*, 1989)**. Additionally, the cytochrome *c* oxidase subunit I gene is conservative among Metazoa **(Jacobs *et al.*, 1988)**. For that reason, COI gene was selected to work as a proper molecular marker for analyzing phylogenetic relationships among Pteriomorphia: Limidae, in comparison with corresponding of mt DNA downloaded from NCBI.

The scientists and policy makers **(Walsh *et al.* 2016)** focused their works on the non-indigenous invasive species (NIS); they represented a main cause of economic, ecological and health harm. Decisions concerning management should be made early to enhance the possibility of success though this is largely dependent upon early detection **(Brown *et al.*, 2016; Holden *et al.*, 2016; Xiong *et al.*, 2016; Xia *et al.*, 2018**). Traditionally approaches based on morphological traits were used to identify species. However, these methods have limitations upon dealing with cryptic species **(Heinrichs *et al.*, 2011)** and species that have extremely phenotypic plasticity **(Kekkonen & Hebert, 2014)**.

Classification of different groups of bivalve, depending on the external shell morphology, often makes troubles for taxonomists and phyllo-geneticists due to the non-appearance of some identifiable features. Shells of some bivalve display many significant morphological changes due to the diversity of habitat and changes in ecological conditions **(Baker *et al.*, 2003)**.

Generally, file shells family Limidae is ignored and neglected in the Suez Canal. The exclusivity of this study concerns the first record of a species of the family Limidae in the Suez Canal. More studies are recommended in the Suez Canal and the Mediterranean Sea to improve our faunal diversity due to lessepsian and/ or anti-lessepsian migration, especially after the fluctuation that have occurred in the climate and due to the global warming.

REFERENCES

Baker, A. M.; Bartlett, C.; Bunn,S. E.; Goudkamp, K.; Sheldon, F. and Hughes, J. M.(2003). Cryptic species and morphological plasticity in long-lived bivalves (Unionoida: Hyriidae) from inland Australia. Mol. Ecol. 12: 2707–2717. http://dx.doi: 10.1046/j.1365-294x.2003.01941.x.

Blaxter, M. L. (2004). The Promise of DNA Taxonomy. Philosophical Transactions of the Royal Society of London Series B: Biol. Sci., 359, 669-679. http://dx.doi.org/10.1098/rstb.2003.1447.

Brown, E. A.; Chain, F. J. J.; Zhan, A.; MacIsaac, H. J. and Cristescu, M. E. (2016). Early detection of aquatic invaders using metabarcoding reveals a high number of non-indigenous species in Canadian ports. Divers Distrib. 22:1045–1059. doi: 10.1111/ddi.12465 http://wileyonlinelibrary.com/journal/ddi

Carpenter, K. E. and De Angelis, N. (2016). FAO species identification guide for fishery purposes. The living marine resources of the western central Atlantic. Vol. 5re2: Bivalves, gastropods, hagfishes, sharks, batoid fishes, and chimaeras. FAO, Rome, 665–1509. https://www.fao.org/3/y4160e/y4160e00.htm

Coen, L. D. and Bishop, M. J. (2015). The ecology, evolution, impacts and management of host–parasite interactions of marine molluscs, J. Invertebr. Pathol., 131: 177-211, https://doi.org/10.1016/j.jip.2015.08.005.

Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol. 3(5):294-299.

Gagnon, J.M. and Haedrich, R.L. (2003). First record of the European giant file clam, *Acesta excavata* (Bivalvia: Pectinoidea: Limidae), in the northwest Atlantic. The Canadian Field-Naturalist, 117:440 - 447. http://canadianfieldnaturalist.ca/index.

Gagnon, J., Kenchington, E., Port, A., Anstey, L., and Murillo, F. (2015). Morphological and genetic variation in North Atlantic giant file clams, *Acesta* spp.

(Bivalvia: Limidae), with description of a new cryptic species in the northwest Atlantic. Zootaxa, 4007 2, 151-80. https://doi.org/10.11646/zootaxa.4007.2.1.

Gallois, D. (1976). Biométrie des palourdes" *Venerupis decussate* "(Linné) et des clovisses "*Venerupis aurea* (Gmelin) de L'étangde Thau (Hérault).Bull. Soc. Linn. Bord. 6:11- 24.

Gilmour, T. H. J. (1990). The adaptive significance of foot reversal in the Limoida. In 'Proceedings of a Memorial Symposium in Honor of Sir Charles Maurice Yonge (1899–1986), Edinburgh, 1986'. (Ed. B. S. Morton.) pp. 249–263. (Hong Kong University Press: Hong Kong.)

Hajibabaei, M.; Janzen, D. H.; Burns, J. M.; Hallwachs, W. and Hebert P. D. N. (2006). Design and applicability of DNA arrays and DNA barcodes in biodiversity monitoring. BMC Biology, 5: (24).
doi: 10.1186/1741-7007-5-24. http://www.biomedcentral.com/1741-7007/5/24

Hebert, P.D.N., Cywinska, A., Ball, S.L. and deWaard, J.R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society Series B–Biol. Sci., 270: 313–321. http://dx.doi.org/10.1098/rspb.2002.2218

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

Heinrichs, J.; Kreier, H. P.; Feldberg, K.; Schmidt, A. R.; Zhu R. L.; Shaw, B.; Shaw, A. J. and Wissemann, V. (2011). Formalizing morphologically cryptic biological entities: new insights from DNA taxonomy, hybridization, and biogeography in the leafy liverwort *Porella Platyphylla* (Jungermanniopsida, Porellales). Am. J. Bot. 98:1252–1262.

Holden, M. H.; Nyrop J. P. and Ellner, S. P. (2016). The economic benefit of time-varying surveillance effort for invasive species management. J Appl Ecol 53:712–721.

Jacobs, H. T.; Elliott, D. J.; Math, V. B. Farquharson, A. (1988). Nucleotide sequence and gene organization of sea urchin mitochondrial DNA, J.M.B. 202 (2):185-217, https://doi.org/10.1016/0022-2836(88)90452-4.

Kekkonen, M. and Hebert, P. D.N. (2014). DNA barcode-based delineation of putative species: efficient start for taxonomic workflows. Mol. Ecol. Res. 14(4):706-15.
doi: 10.1111/1755-0998.12233.

Kocher, T. D.; Thomas, W. K.; Meyer, A.; Edwards, S. V.; Pääbo, S.; Villablanca, F. X. and Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. PNAS, 86(16), 6196-6200. https://doi.org/10.1073/pnas.86.16.6196.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. biol. Evol., 35(6): 1547-1549. https://doi.org/10.1093/molbev/msy096.

Madkour F.F., Aamer M.A. and El-Sherbiny M.M. (2006). Assessment of eutrophication in Lake Timsah, Suez Canal, Egypt**.** Egyptian J. Aqua. Res., (32): 259-272.

Marshall, B.A. (2001). The genus *Acesta* in the south-west Pacific (Bivalvia: Limidae). In: Bouchet P, Marshall BA (eds) Tropical Deep-Sea Benthos, vol 22. Mém Mus natn Hist nat 185: 97–109.

Martin, A.P. and Palumbi, S.R. (1993). Body size, metabolic rate, generation time, and the molecular clock Proc Natl Acad Sci USA, 90: 4087-4091.

Mikkelsen, P. M. and Bieler, R. (2000). Marine bivalves of the Florida Keys: discovered biodiversity. Geological Society London Special Publications.177(1):367-387. doi: 10.1144/GSL.SP.2000.177.01.25

Mikkelsen, P. M. and Bieler, R. (2003). Systematic revision of the western Atlantic file clams *Lima* and *Ctenoides* (Bivalvia: Limoida: Limidae). Invertebrate Systematics. 17: 667-710. doi:10.1071/IS03007

Mikkelsen, N.T.; Schander, C. and Willassen T. (2007). Local scale DNA barcoding of bivalves (Mollusca): A case study. Zoologica Scripta 36(5):455 – 463. doi:10.1111/j.1463-6409.2006.00289.x.

Nei, M. and Kumar, S. (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Poppe, G. T., and Goto, Y. (1993). 'European Seashells.' Volume II. (Verlag Christa Hemmen: Wiesbaden, Germany.)

Radwan, N.A.; Ahmed, M. I.; Ibrahim, N.K.; Mohammed, S.Z. and Morsy, Z.S. (2014a). Molecular analysis of *Brachidonte spharaonis* (Fischer P., 1870) in Egypt reveals cryptic species complex. Egypt. J. Aquat. Biol. & Fish.18 (4): 37 – 46.

Radwan, N.A.; Ahmed, M. I.; Ibrahim, N.K.; Mohammed, S.Z. and Morsy, Z.S. (2014b). Molecular Phylogeny of four bivalve species collected from Egyptian Mediterranean and Red Sea based on mitochondrial *CO1* gene sequences. Egypt. J. Aquat. Biol. Fish.18 (4): 55-61.

Ricker, W.E. (1975). Computation and interpretation of biological statistics of fish populations. B. Fish. Res. Board Can. (191): 1–382.

Sharabati, D. (1984). Red Sea Shells. KPI Ltd., London, Boston, Melbourne, Henley. 127pp.

Suez Canal Authority, (2022). https://www.suezcanal.gov.eg

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution, 30(12): 2725-2729**.**

Thompson, J.D.; Higgins, D.G. and Gibson, T.J. (1994). Nucleic Acids Res.22: 4673–4680.

Walsh, J. R.; Carpenter, S. R. and Vander Zanden, M. J. (2016). Invasive species triggers a massive loss of ecosystem services through a trophic cascade. Proc Natl Acad Sci USA, 113(15), 4081-4085. https://doi.org/10.1073/pnas.1600366113.

Ward, R. D.; Zemlak, T. S.; Innes, B. H.; Last, P. R. and Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. Philosoph. Trans. R. Soc. B Biol. Sci. 360, 1847–1857.

Xia, Z.; Zhan, A.; Gao, Y. et al., (2018). Early detection of a highly invasive bivalve based on environmental DNA (eDNA). Biol Invasions 20: 437–447. https://doi.org/10.1007/s10530-017-1545-7

Xiong, W.; Xie, D.; Wang, Q.; Wang, H. .; Wang, Z. Sun, H.; Li, T. and Bowler, P. A. (2016). Non-native species in Poyang Lake Basin: status, threats and management. Aquatic invasions.18 (1):119- 134. https://doi. org/10.3391/ai.2023.18.1.103610