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Molecular identification and phylogenetic relationship of *Erugosquilla massavensis* (Kossmann, 1880) from the Mediterranean Sea, Egypt

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ABSTRACT

The Erythraean species of mantis shrimp, *Erugosquilla massavensis*, has been reported as the first Lessepsian migrant stomatopod to the Mediterranean Sea in 1933, off Alexandria, Egypt via the Suez Canal. Currently, it extended to the middle and western Mediterranean competing with the native spot-tail mantis shrimp *Squilla mantis* (Linnaeus, 1758). The continuous increase of invasive species to the Mediterranean claimed the necessity to update and revise the identification of those early migrants. The present study aimed to confirm the morphological identification of *E. massavensis* from the coast of the Egyptian Mediterranean Sea using the DNA barcoding gene cytochrome oxidase subunit I (COI) and conducting a phylogenetic analysis. Genomic DNA of samples collected from the Mediterranean Sea at Port Said in 2016 was extracted and COI was amplified and sequenced. Our phylogenetic analysis revealed that *E. massavensis* (MH447072 and MH447073) constitutes a single monophyletic clade and appeared more linked with *E. woodmasoni* than the Japanese mantis shrimp *Oratosquilla oratoria* (divergence value < 3%). The phylogenetic relationship between *E. massavensis*, *E. woodmasoni*, and *O. oratoria* was confirmed by the Neighbor-Joining tree. The present study confirmed the morphological identification and document, for the first time in GenBank/EMBL/ DDBJ genetic databases, the genetic status of *E. massavensis* from the Mediterranean of Egypt using COI.

INTRODUCTION

The mantis shrimp *Erugosquilla massavensis* **(Kossmann, 1880)** is a stomatopod species belonging to Superfamily Squilloidea **(Muzzarelli, 1985)**. The previous scientific names of this species were *Squilla masawensis* **(Por, 1971)** and *Oratosquilla massavensis* **Holthuis (1984)**. Geographically, *E. massavensis* is native to the Persian Gulf and the Red Sea **(Froglia and Manning, 1989)**. It has been reported as the first Lessepsian

migrant stomatopod to the Mediterranean Sea in 1933, off Alexandria, Egypt via the Suez Canal (**Steuer, 1936**, as *Squilla africana* Calman, 1917). Subsequently, it was recorded from different locations in the eastern Mediterranean: Israel, Lebanon, Cyprus, Crete, Rhodes Island and Turkey **(Holthuis, 1961; Lewinsohn and Manning, 1980; Galil and Kevrekidis, 2002; Katağan *et al*., 2004; Özcan *et al*., 2008; Bakir and Çevirgen, 2012)**. Its distribution extended to the middle and western Mediterranean reaching Tunis **(Ounifi Ben Amor *et al*., 2015)**, Italy **(Gianguzza *et al*., 2019)** and Malta **(Stern *et al*., 2019)**. *Erugosquilla massavensis* is the only species of *Erugosquilla* found in the Mediterranean Sea and is currently established as a minor fishery resource in Sicily, Italy causing a competitive displacement of native Mediterranean spot-tail mantis shrimp, *Squilla mantis* **(Gianguzza *et al*., 2019)**. It became one of the most edible marine crustaceans in the Mediterranean countries and represents a rich source of protein **(Sallam, 2000)** as well as chitin and chitosan **(Abouzeed *et al*., 2015; Abo-Hashesh *et al*., 2017)**.

In the last century, many species of Indo-pacific origin were transported from the Red Sea to Mediterranean Sea via the Suez Canal and shipping activity **(Hulme, 2015)**. The invasion of the non-native species to the Mediterranean is continuously increasing with time especially with climate change **(Carlton, 1996; Galil *et al*., 2002)**. Recently, a total of 821 invasive marine species has been recorded in the Mediterranean **(Zenetos *et al*., 2017)**. It seems therefore necessary to update and revise the identification of these newly established organisms. Identification of organisms by means of their morphological characterization is ineffective and misleading **(Hebert *et al*., 2003)**. Nowadays, molecular techniques are used as a rapid and accurate method for identification. The mitochondrial genome is common genetic information in studying molecular phylogeny, species identification and population genetic diversity **(Ma *et al*., 2013; Baek *et al*., 2014; Ismail *et al*., 2017; Sadek *et al*., 2018)**. Due to its effort in various genetic studies, the cytochrome oxidase subunit I (COI) gene is one of the most important mitochondrial genomes **(Buhay, 2009; Abdelmeneam *et al*., 2018)**. It is considered as a barcode region of species identification, especially for crustaceans such as mantis shrimps, crabs and shrimps **(Podsiadlowski and Bartolomaeus, 2005; Raupach *et al*., 2015; Kundu *et al*., 2018; Abo-Hashesh *et al*., 2020)**.

Superfamily Squilloidea is considered the largest Superfamily in order Stomatopoda of class Crustacea. The phylogenetic analysis contributed to the confirmation of the position of Stomatopoda among the Eumalacostraca **(Podsiadlowski and Bartolomaeus, 2005)** and was previously performed using only morphological characters. Recently, the morphological and molecular characterization were combined together to indicate the phylogenetic relationship **(Van der Wal *et al*., 2019)**. However, more molecular data is necessary for resolving some of the classifications of stomatopods **(Kundu *et al*., 2018)**. The present study aimed to confirm the morphological identification of *E. massavensis* from the Mediterranean Sea of Egypt using the DNA barcoding technique and conducting a phylogenetic analysis.

MATERIALS AND METHODS

1. Sample collection

A total of 1377 fresh samples of the mantis shrimp, *E. massavensis* (Kossmann, 1880) were obtained from local market at Port Said, Egyptian Mediterranean Sea during 2016 (Fig. 1). Specimens were preserved in ice until transferred to the laboratory. The specimens were sexed and the carapaces' length were measured. The mantis shrimp *E. massavensis* was photographed using digital camera (Canon) and the morphological identification was carried out according to **Manning (1995)**. Samples used in DNA analysis were stored in a freezer at -80 °C.

Fig. 1. Sampling location on the Egyptian Mediterranean coast at Port Said.

2. DNA extraction and PCR

DNA was extracted from specimens' muscle using QIAamp mini kit (Qiagen, GmbH, Germany) following the manufacturer's protocol. Eventually, three samples of COI were amplified by mixing 12.5 µl (1X) colorless Master Mix Go Taq® G2 (Promega Corporation-Madison, WI, USA), 1 µl (10 pmol/ µl) of forward primer CrustF1 (5'-TTTTCTACAAATCATAAAGACATTGG-3') and 1 µl (10 pmol/ µl) of reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') described by Costa *et al*. (2007), and 2 µl (10 ng/ µl) of DNA template. The reaction volume was adjusted to 25 µl

using nuclease-free double distilled water. Primers were synthesized by Metabion international AG, Germany. The amplification reaction was carried out using Mastercycler gradient PCR (eppendorf, Germany). The thermal cycle conditions consisted of initial denaturation of 60 s at 94 °C; five cycles of 30 s at 94 °C, 90 s at 45 °C, and 60 s at 72 °C; 35 cycles of 30 s at 94 °C, 90 s at 51 °C, and 60 s at 72 °C; followed by a final extension of 5 min at 72 °C. PCR product was detected and visualized using 2 % agarose gel electrophoresis stained by 25 µg of ethidium bromide. The gel was then imaged and analyzed (template size detection) using Gel Documentation system with UV light box and GeneSys software (Syngene, Synoptics Ltd, England).

3. DNA sequencing

Two PCR products were purified using QIAquick PCR purification kit protocol (Qiagen, Germany), followed DNA sequencing processes; second PCR (cycling sequence) was carried out using the Big Dye Terminator version. 3.1 Cycle Sequencing kit (Applied Bosystems, USA). Second PCR reaction mixture consisted of 8 µl of Big Dye Terminator, 3.2 µl (1 pmol) of primer (forward or reverse), 2 µl (10 ng/ µl) of PCR product and completed to 20 µl with nuclease-free water. The sequencing PCR reaction was carried out at 96 °C for 2 min, followed by 25 cycles of 10 s at 96 °C, 5 s at 51 °C and 4 min at 60 °C. The product then was purified with CENTRI-SEP columns (Princeton Separation). 10 µl of Hi-Di formamide was added to the purified product before introduced in DNA sequencer. DNA sequencing was applied by 3500 genetic analyzer (Applied Biosystems, USA).

4. Data analysis

Before conducting the phylogenetic analysis, two sequences electropherograms were checked and COI were edited with the aid of the BioEdit-Sequence Alignment Editor software package. Both partial COI sequences of the studied species were submitted to National Center for Biotechnology Information (NCBI) GenBank official database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were available with accession numbers MH447072 and MH447073. The submission was carried out by Sequin application software version 15.5. From one hand, edited sequences were compared with the maximum compatibility and similarity of pre-published sequences using the BLAST option (Basic Local Alignment Search Tool) of NCBI under the default algorithms. The organisms selected from the BLAST result which used in the phylogenetic tree construction were presented in Table (1). On the other hand, whether downloaded sequences from NCBI GenBank or introduced by the current study, COI sequences translated into amino acids then aligned by muscle. The phylogenetic relationship was determined using MEGA6 **(Tamura *et al.*, 2013)**. The evolutionary history was inferred using the Maximum Likelihood (ML) and the Neighbor-Joining method based on the Jones-Taylor-Thornton (JTT) model with Gamma Distributed (G) **(Jones *et al.*, 1992)**. They were generated with 10000 bootstrap replicates. The Neighbor-Joining tree was constructed according to **Saitou and Nei (1987)** and **Felsenstein (1985)**. The Pairwise distances between species of constructed ML tree were calculated to evaluate the evolutionary divergences using the JTT model **(Jones *et al.*, 1992)**.

Table 1. Organisms selected from the BLAST result and used in the phylogenetic tree construction for *E. massavensis* (MH447072 and MH447073).

Organisms	Country	Accession no.
Erugosquilla massavensis (Kossmann, 1880)	Egypt	MH447072
Erugosquilla massavensis (Kossmann, 1880)	Egypt	MH447073
Erugosquilla woodmasoni (Kemp, 1911)	China	MF173600
Erugosquilla woodmasoni (Kemp, 1911)	China	MF173601
Oratosquilla oratoria (de Haan, 1844)	Korea	HM180738
Oratosquilla oratoria (de Haan, 1844)	South Korea	JX503007
Oratosquilla oratoria (de Haan, 1844)	South Korea	JX503008
Oratosquilla oratoria (de Haan, 1844)	China	KM197040
Oratosquilla oratoria (de Haan, 1844)	China	KM197042
Oratosquilla oratoria (de Haan, 1844)	China	KP976312
Oratosquilla oratoria (de Haan, 1844)	China	KP976326
Oratosquillina interrupta (Kemp, 1911)	China	FJ229793
Oratosquillina interrupta (Kemp, 1911)	China	FJ229795
Harpiosquilla raphidea (Fabricius, 1798)	Indonesia	KF697110
Harpiosquilla raphidea (Fabricius, 1798)	Indonesia	KF697111
Harpiosquilla raphidea (Fabricius, 1798)	Indonesia	KF697112
Harpiosquilla harpax (de Haan, 1844)	China	FJ229770
Harpiosquilla harpax (de Haan, 1844)	China	FJ229773
Harpiosquilla harpax (de Haan, 1844)	Australia	MH168261
Harpiosquilla melanoura (Manning, 1968)	Australia	MH168260
Harpiosquilla sp. (Holthuis, 1964)	Australia	KJ828811
Alima orientalis (Manning, 1978)	Australia	KF205335
Alima orientalis (Manning, 1978)	Australia	KF205337
Alima orientalis (Manning, 1978)	Australia	KF214292
Dictyosquilla foveolata (Wood-Mason, 1895)	China	FJ229764
Dictyosquilla foveolata (Wood-Mason, 1895)	China	FJ229765
Dictyosquilla foveolata (Wood-Mason, 1895)	China	FJ229767
Miyakella nepa (Latreille, 1828)	China	FJ229776
Miyakella nepa (Latreille, 1828)	China	FJ229779
Miyakella nepa (Latreille, 1828)	China	FJ229780
Squilla mantis (Linnaeus, 1758)	Germany	GQ328967
Squilla mantis (Linnaeus, 1758)	Turkey	JQ624005
Squilla chydaea (Manning, 1962)	Australia	MH168257
Squilla empusa (Say, 1818)	USA	KU905833
Squilla empusa (Say, 1818)	USA	HM138809
Squilla empusa (Say, 1818)	USA	MH087673
Clorida decorata (Wood-Mason, 1875)	China	FJ229762
Clorida decorate (Wood-Mason, 1875)	China	FJ229763
Clorida decorate (Wood-Mason, 1875)	Australia	MH168256
Rissoides desmaresti (Risso, 1816)	USA	KT208805
Rissoides barnardi (Manning, 1975)	Australia	MH168250
Parvulobathynella distincta (Bandari & Totakura, 2011)	India	MF443333

RESULTS AND DISCUSSION

1. Morphological characterization of *E. massavensis*

Abdomen characterized by greenish-gray color. Carapace length ranges between 72-164 mm for males and 70-170 mm for females. Lateral process of 5th thoracic segment (first free segment behind carapace) terminated by 2 lobes, anterior lobe longer and curved forwards while posterior one straight in a narrow triangle shape, thoracic somites 5–7 lateral process bilobed (Fig. 2A). Presence of eye stalks (Fig. 2B). Dactylus of raptorial claw with 6 or 7 teethes (Fig. 2C). Telson lacks large black spots in middle anterior-thoracic area (Fig. 2D). This morphological characterization agrees with the description of **Manning (1995)**; revision of **Ahyong (2001)** and re-description of **Abdelsalam (2014)**.

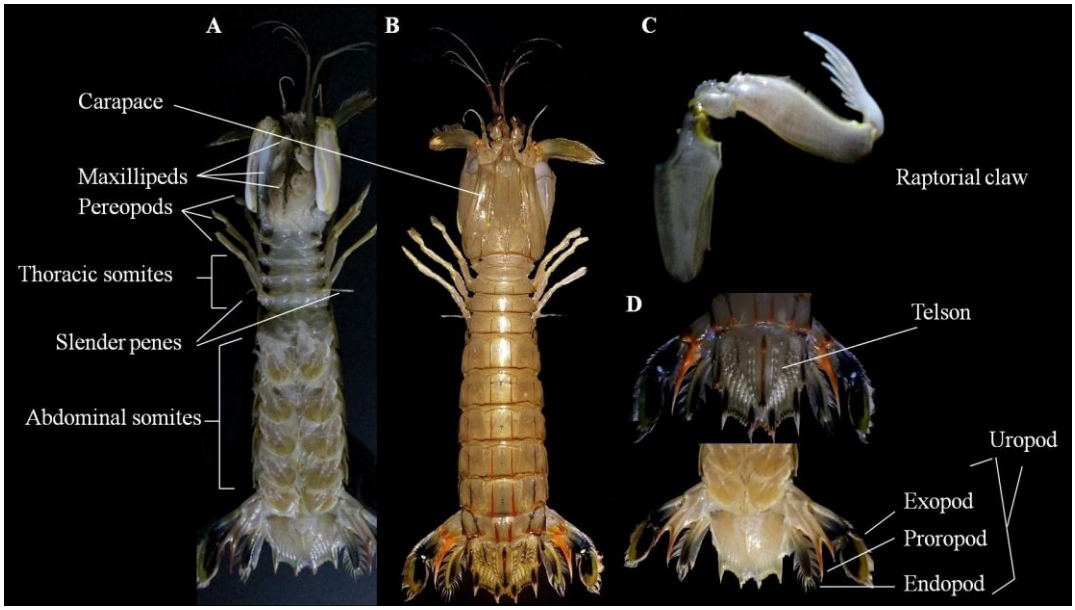

Fig. 2. *Erugosquilla massavensis*; A) Ventral view shows the greenish-gray color and slender penis (in males only), B) Dorsal view shows the eye stalks, C) Right lateral raptorial claw with 6 or 7 teeth, D) Telson lacks large black spots in the middle anterior-thoracic.

2. Molecular identification

PCR products of amplified mitochondrial COI gene region from *E. massavensis* were 710 base pairs (Fig. 3) in agreement with the expected amplicon size ≥ 500 bp by **Ratnasingham and Hebert (2007)**. Resulted partial sequences of the COI gene from *E. massavensis* were 621 and 609 bases. Sequences were Blast using BLAST analysis of the GenBank database.

Sequence analysis of the COI gene from *E. massavensis* obtained in the present study was deposited in the GenBank database (Accession numbers: MH447072 and MH447073). It was noticed that no previous submitted sequence data was recorded for *E.*

massavensis in the GenBank/EMBL/DDBJ genetic database. The result of Blast analysis showed that the partial sequence of COI gene from *Oratosquilla oratoria* (KY197203) is the closest one to the COI gene of the studied *E. massavensis* (MH447072 and MH447073) with identity of 89%, 0 e-value, query cover of 97% and with 741 and 760 scores, respectively.

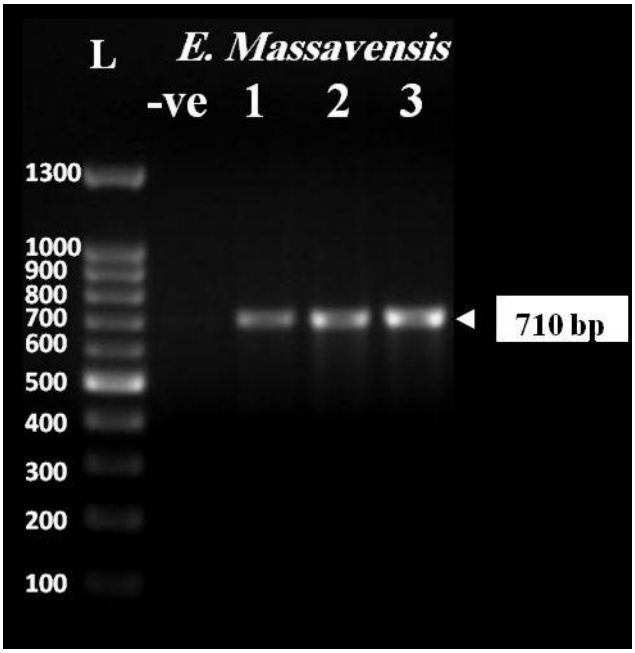

Fig. 3. Gel electrophoresis shows length of amplicons of COI gene (710 bp) of *E. massavensis* and 100 bp DNA ladder.

The phylogenetic tree was built from 41 organisms' amino acid sequences belonging to Family Squillidae (Crustacea, Stomatopoda) and one sequence of *Parvulobathynella distincta* (Suborder: Syncarida) as an outgroup (Fig. 4). The maximum likelihood (ML) tree showed several monophyletic clades of various genera of family squillidae. The present tree differentiated species of the same genus in separated branches of its clade as shown with *E. massavensis* and *E. woodmasoni*; *O. oratoria* and *O. interrupta*; *H. raphidea*, *H. harpax* and *H. melanoura*; *S. mantis*, *A. orientalis*, *D. foveolata*, *M. nepa* and *C. decorata* with high bootstrap values at each node (≥70%). *Harpiosquilla* sp., *Erugosquilla* sp., *Oratosquilla* sp., *Dictyosquilla* sp., *Alima* sp., *Squilla* sp., *Oratosquillina* sp., *Clorida* sp. and *Miyakella* sp. clustered from the same branch which means that those species have great similarities in COI sequences indicating that they have the same ancestor. The same observation was made for *E. massavensis* and *E. woodmasoni*; *H. raphidea* and *H. harpax*; *D. foveolata*, *H. melanoura*, *Harpiosquilla* sp. and *A. orientalis*; *S. empusa*, *S. mantis* and *S. chydaea*; *O. interputa*, *C. decorata* and *M. nepa*; *R. desmaresti* and *R. barnardi*. The grouping of the presented genera in monophyletic clades gives an indication for the possibility of classifying Family Squillidae into subfamilies.

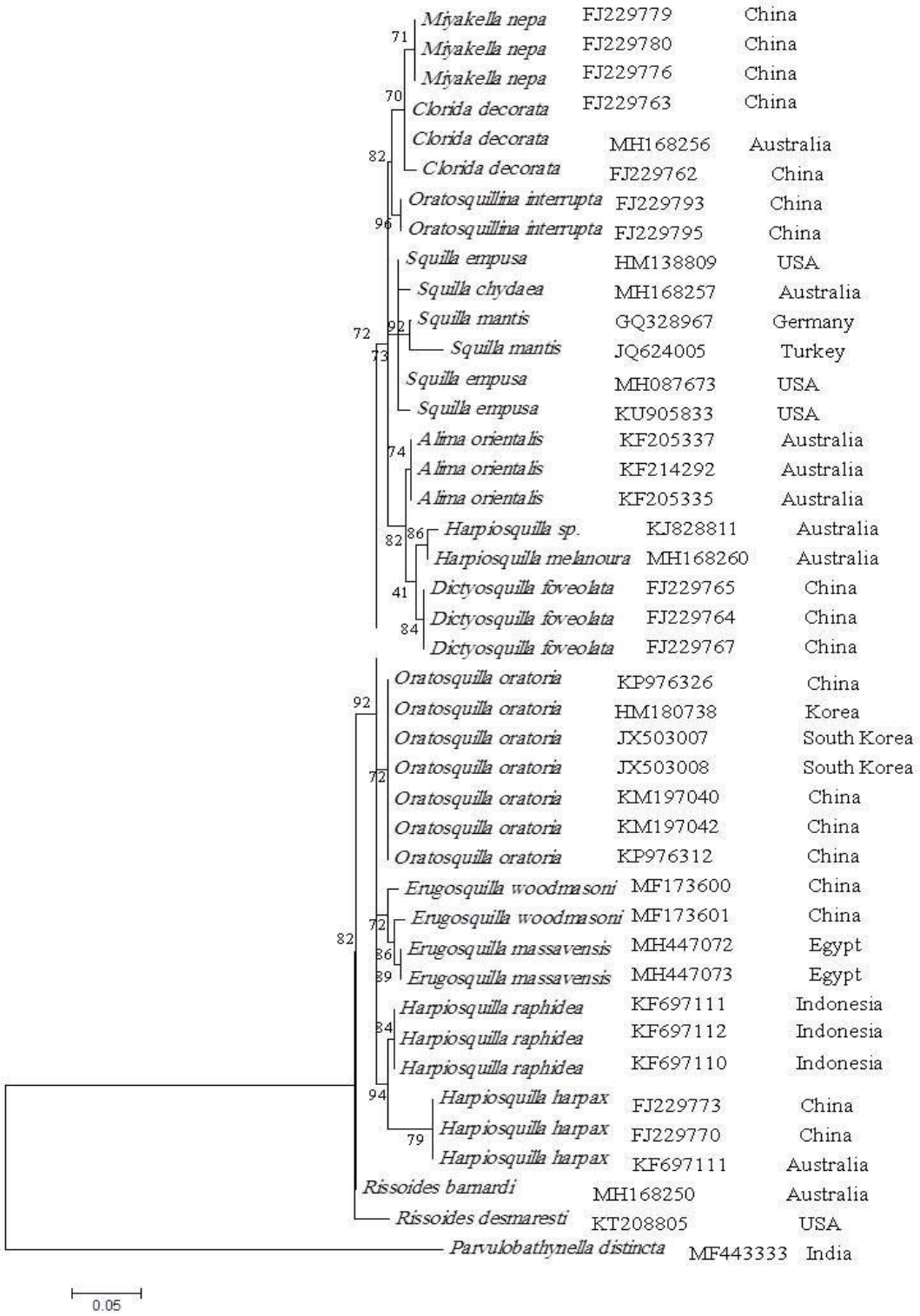

Fig. 4. Phylogenetic tree of coding COI amino acid sequence of *E. massavensis* was compared with other sequences of family Squillidae from gene bank using MEGA 6 program using *Parvulobathynella distincta* as an outgroup. Numbers in below the nodes are bootstrap values (10000 replicates).

The evolutionary divergence estimates between *E. massavensis* and both *E. woodmasoni* and *O. oratoria* were < 3% while the divergence value was > 3% with the other genera. Although the pairwise analysis in the current study showed that *E. massavensis* is closely related to *E. woodmasoni* and *O. oratoria* (divergence value <3%), the phylogenetic tree showed that *E. massavensis* is more linked with *E. woodmasoni* than *O. oratoria* (Fig. 4). The phylogenetic relationship between *E. massavensis*, *E. woodmasoni* and *O. oratoria* was confirmed by the Neighbor-Joining tree as shown in Figure (5). This evidence proves the classification of the studied mantis shrimp as *E. massavensis* and confirms the morphological identification. This finding agree with **Abdelsalam (2014)** who re-descripted the mantis shrimp *E. massavensis* morphologicaly from Baltim sector, Eastern Mediterranean coast of Egypt. It is worth mentioning that the previous misidentification of *Erugosquilla massavensis* as *Oratosquilla massavensis* was due to the close morphological characters between the two genera as described by **Manning (1968, 1995)**. On the other hand, a close relationship between *E. woodmasoni* and *O. oratoria* existed in the phylogenetic tree as reported by **Tang *et al*. (2010)**.

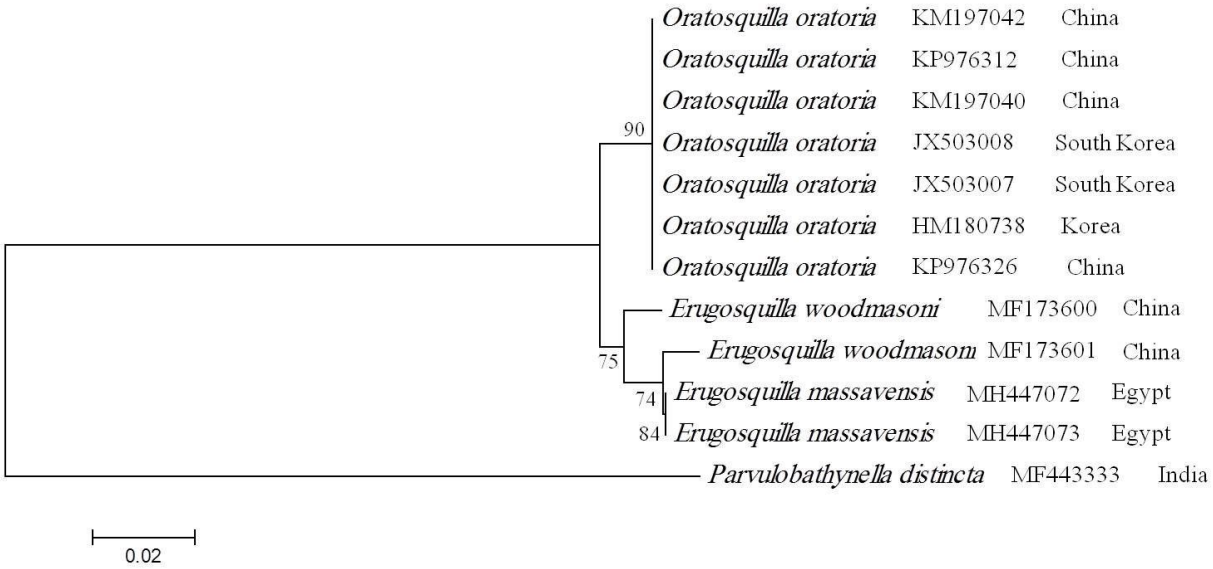

Fig. 5: Neighbor-Joining tree for coding COI amino acid sequences of *E. massavensis* from Egypt was compared with neighbor sequences of family Squillidae from gene bank using MEGA 6 program using *Parvulobathynella distincta* as an outgroup.

On the other hand, the phylogenetic tree shows that the *Harpiosquilla* sp. (KJ828811) which has not been classified to the species level appears to be clustering with *H. melanoura* (MH168260) as a sister species resulting in the probability of being classified under the same species "*melanoura*" particularly because the divergence value between *Harpiosquilla* sp. (KJ828811) and *H. melanoura* (MH168260) was the lowest (0.6%) compared to *H. raphidea* (4%) and *H. harpax* (3%). *Harpiosquilla* sp. (KJ828811) was collected by **Feller and Cronin (2014)** from the Lizard Island, Australia. The specimen was not identified since it was at a larval stage and no published descriptions of the larval stages were available. Furthermore, no morphological or

molecular barcode record of any adult *Harpiosquilla* species at Lizard Island was carried out at that time. The presence of *H. melanoura* (MH168260) from Australia in the genetic database and the present phylogenetic analysis contributed to the classification of that specimen. The phylogenetic relationship shows that *H. raphidea* is grouped with *O. oratoria* with low bootstrap (14%) at the internal node, therefore cannot be taken into consideration. The tree topology revealed that *S. mantis* was at first grouped together with its sister species *S. empusa* and then formed a monophyletic group with *S. chydaea*. The molecular evidence presented here supports the species traditional taxonomy.

CONCLUSION

In conclusion, the present study confirmed that the studied mantis shrimp *E. massavensis*is belongs to family Squillidae and introduced the sequence of this species for the first time to the genetic database. It also confirmed the morphological identification of *E. massavensis* using the mitochondrial COI gene as a successful molecular technique for identification. Further studies using multiple genetic markers and suitable phylogenetic and morphological analysis would be needed on this species from different sites along with the Mediterranean Sea. In this study, the phylogenetic relationship was determined between *E. massavensis* and other genera of family Squillidae in the world obtained from the GenBank. The present study indicates the probability of classifying family Squillidae into subfamilies, further studies are required in order to confirm this idea.

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