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Phylogenetic analysis and identification of *Charybdis natator* (Herbst, 1794) from the Egyptian Coast of the Red Sea

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ABSTRACT

Charybdis natator is a portunid edible crab that is found in the Indo-Pacific, Indian Ocean, East Africa and the Red Sea. The present study aimed to confirm the molecular identification of *C. natator* from the Red Sea using the DNA barcoding gene Cytochrome Oxidase Subunit I (COI). The molecular analysis was performed using genomic isolation from *C. natator* tissues followed by polymerase chain reaction (PCR) for the COI gene and sequencing. Data analysis was carried out using the Basic Local Alignment Search Tool (BLAST) and phylogenetic analysis conducted using the MEGA 6 program. The molecular identification of *C. natator* was confirmed and agreed with the morphological description. The phylogenetic analysis of genus *Charybdis* revealed that *C. variegata* (KJ168053 and KX018513) should be redefined as *C. natator*. The study supports promoting *Goniosupradens* to the generic level and negates its old morphological classification as a subgenus. It also suggests the re-evaluation of *C. acuta* taxonomical classification and emphasizes the significance of performing further molecular studies on other *Charybdis* species in order to resolve the mis-identifications and controversies.

INTRODUCTION

The genus *Charybdis* does not receive enough attention from researchers due to the difficulty of identification as a result of the great morphological similarity and the key characters overlapping (Smith *et al.*, 2003). With regard to the identification of organisms, the use of morphological characterization is inefficient and misleading (Hebert *et al*., 2003). Recently, molecular techniques are considered the most precise and quick methods for identification (Marshall, 2005). A mitochondrial genome is a common tool in studying species identification, molecular phylogeny and population genetic diversity (Ma *et al*., 2013; Baek *et al*., 2014). One of the most important mitochondrial genomes is the Cytochrome Oxidase subunit I (COI) gene due to its effort in various genetic studies (Buhay, 2009). COI is a barcoding region of species identification,

particularly for crustaceans such as crabs and shrimps (Raupach *et al*., 2015; Van der Meij *et al*., 2015).

The genus *Charybdis* was re-classified into subfamily Portuninae instead of subfamily Thalamitinae by virtue of phylogenetic analysis (Ma *et al.*, 2015). The phylogenetic analysis showed that the juvenile of genus *Charybdis* that was identified as *C. variegata* is essentially *C. hellerii* (Negri and Mantelatto, 2017). De Silva and Munasinghe (2016) found a strong relation between *C. natator* and *C. japonica*, and recommended further phylogenetic studies. Evans (2018) reported that Spiridonov et al. (2014) misidentified *C. natator* which matches the COI sequence of *C. granulata* (KT365713), and instead it should be re-identified as *C. granulata* .

Charybdis natator is a portunid edible crab that is common in the Indo-Pacific, Indian Ocean, East Africa and the Red Sea (Dai and Yang, 1991; Webber, 2001). Although this species is unpopular and hardly consumed by locals, its shell can be used for chitin and chitosan production (Kanagaraj, 2007; Abo-Hashesh *et al*., 2017). In Egypt, studies regarding this species are scarce; Sallam and Gaballa (2009, 2010) studied some aspects of its biology as well as assessing its edibility. On the other hand, Abbas *et al*. (2016) identified *C. natator* morphologically and genetically among other crabs from the Gulf of Suez.

The present study aimed to confirm the morphological and molecular identification of *C. natator* inhabiting the Egyptian coast of the Red Sea using molecular technique and morphological characterization. In addition, phylogenetic analysis of genus *Charybdis* was conducted in order to study the evolutionary relationship within this genus.

MATERIALS AND METHODS

Sample collection and morphological identification

Fresh specimens of *Charybdis natator* (Herbst, 1794) were collected from two localities Hurghada and the Port of Suez, Red Sea during 2017 (Fig. 1). Samples were kept in ice then transferred to the laboratory to be preserved in -80°C until molecular processes were undertaken. Specimens were characterized morphologically and identified according to Wee and Ng (1995).

Genomic isolation and polymerase chain reaction (PCR)

Total genomic DNA was isolated from the crabs' muscles using QIAamp mini kit (Qiagen, GmbH, Germany) and the manufacturer's protocol was followed. The partial coding region of the COI gene was amplified by PCR. The PCR reaction mixture consisted of 12.5 µl (1X) colorless Master Mix Go Taq® G2 (Promega Corporation-Madison, WI, USA), 1 µl (10 pmol/ µl) of each primer pair and 2 µl (10 ng/ µl) of DNA template then complete to 25 µl using nuclease-free double distilled water. The primer pair used were forward primer CrustF1 (5'-TTTTCTACAAATCATAAAGACATTGG-3') and reverse primer HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') as mentioned by Costa *et al*. (2007). Primers were synthetized by Metabion international AG, Germany. The amplification reaction was performed using Mastercycler gradient PCR (eppendorf, Germany). The thermal cycle conditions of PCR was beginning with initial denaturation of 60s at 94 °C then five cycles of 30s at 94 °C, 90s at 45 °C, and 60s at 72 °C; 35 cycles of 30s at 94 °C, 90s at 51 °C, and 60s at 72 °C; followed by a final extension of 5 min at 72 °C. The amplified products were separated according to base

pair (bp) size using 2 % agarose gel electrophoresis stained by ethidium bromide. The gel was then visualized, imaged and analyzed using the Gel Documentation system with UV light box and GeneSys software (Syngene, Synoptics Ltd, England).

Fig. 1. Sampling locations on the Egyptian Red Sea at Hurghada and port of Suez.

Nucleotide sequencing

In preparation for the nucleotide sequencing process, the amplified product was purified using the QIAquick PCR purification kit protocol (Qiagen, Germany). The nucleotide sequencing processes were as follows: The second PCR (cycling sequence) was performed using the Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, USA). The 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 adjusted 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 10s at 96 °C, 5s 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 obtained in DNA sequencer. The DNA sequencing was applied by 3500 genetic analyzer (Applied Biosystems, USA).

Data analysis

Two partial COI sequences obtained from the nucleotide sequencer were checked, edited using the BioEdit-Sequence Alignment Editor software package. The partial COI sequences of the studied crab species were submitted to the National Center for Biotechnology Information (NCBI) GenBank official database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were available with accession numbers MH447070 and MH447071. Both submitted sequences were compared with the maximum compatibility and similarity of pre-published sequences using the Basic Local Alignment Search Tool (BLAST) of the NCBI official database.

Phylogenetic analysis

The partial COI sequences was conducted for 46 organisms downloaded from the NCBI GenBank database that selected from the BLAST result in addition to two present study sequences and one species as an outgroup for the phylogenetic tree construction (Table 1). The COI sequences were first translated into amino acids and then aligned by MUSCLE (Multiple Sequence Comparison by Log- Expectation) (Edgar, 2004). The Maximum likelihood (ML) was generated to estimate the strength of the phylogenetic relationship by taking 10000 as the bootstrap replicates. The ML was based on the General Reversible Mitochondrial model with invariant sites (Adachi and Hasegawa, 1996). The evolutionary divergences were estimated by Pairwise distances calculation between species of the constructed ML tree using the p-distance model. The present phylogenetic analyses were performed using MEGA 6 (Tamura *et al*., 2013).

Table 1. Organisms selected from the BLAST result and used in the phylogenetic tree construction for *C. natator* (MH447070 and MH447071).

Organism	Accession no.	Country
Charybdis (Charybdis) natator (Herbst, 1794)	MH447070	Egypt
Charybdis (Charybdis) natator (Herbst, 1794)	MH447071	Egypt
Charybdis (Charybdis) natator (Herbst, 1794)	KF793328	Egypt
Charybdis (Charybdis) natator (Herbst, 1794)	KM528124	Sri Lanka
Charybdis (Charybdis) natator (Herbst, 1794)	KX060204	Australia
Charybdis (Charybdis) natator (Herbst, 1794)	KX060205	Oman
Charybdis (Charybdis) natator (Herbst, 1794)	KT365719	Taiwan
Charybdis (Charybdis) variegata (Fabricius, 1798)	KJ168053	Philippines
Charybdis (Charybdis) variegata (Fabricius, 1798)	KX018513	India
Charybdis (Charybdis) hellerii (A. Milne-Edwards, 1867)	KF574085	India
Charybdis (Charybdis) hellerii (A. Milne-Edwards, 1867)	KX060315	Indonesia
Charybdis (Charybdis) hellerii (A. Milne-Edwards, 1867)	KX060317	Indonesia
Charybdis (Charybdis) hellerii (A. Milne-Edwards, 1867)	KX060318	Indonesia
Charybdis (Charybdis) hellerii (A. Milne-Edwards, 1867)	KX060355	Australia
Charybdis (Charybdis) lucifera (Fabricius, 1798)	KX381795	India
Charybdis (Charybdis) lucifera (Fabricius, 1798)	KX381796	India
Charybdis (Charybdis) lucifera (Fabricius, 1798)	KP317980	India
Charybdis (Charybdis) feriata (Linnaeus, 1758)	EU284140	India
Charybdis (Charybdis) feriata (Linnaeus, 1758)	MK091830	India
Charybdis (Charybdis) feriata (Linnaeus, 1758)	KP976184	China
Charybdis (Charybdis) feriata (Linnaeus, 1758)	MF594622	Bangladesh
Charybdis (Charybdis) feriata (Linnaeus, 1758)	KF604888	Philippines
Charybdis (Charybdis) japonica (A. Milne-Edwards, 1861)	KM987387	Sri Lanka
Charybdis (Charybdis) japonica (A. Milne-Edwards, 1867)	KM377975	China
Charybdis (Charybdis) japonica (A. Milne-Edwards, 1867)	HM237597	China
Charybdis (Charybdis) japonica (A. Milne-Edwards, 1867)	HM237600	China
Charybdis (Charybdis) japonica (A. Milne-Edwards, 1867)	HM237599	China

Charybdis (Charybdis) japonica (A. Milne-Edwards, 1867)	HM237601	China
Charybdis (Charybdis) miles (De Haan, 1835)	KX060183	Papua New Guinea
Charybdis (Charybdis) crosnieri (Spiridonov & Türkay, 2001)	KX060185	Madagascar
Charybdis (Charybdis) acuta (A. Milne-Edwards, 1869)	EU284143	China
Charybdis (Charybdis) acuta (A. Milne-Edwards, 1869)	KX060203	Taiwan
Charybdis (Gonioneptunus) bimaculata (Miers, 1886)	KF570008	China
Charybdis (Gonioneptunus) bimaculata (Miers, 1886)	KF570009	China
Charybdis (Gonioneptunus) bimaculata (Miers, 1886)	KF570010	China
Charybdis (Goniohellenus) longicollis (Leene, 1938)	KX060194	Israel
Charybdis (Goniohellenus) longicollis (Leene, 1938)	KX060195	USA
Charybdis (Goniohellenus) longicollis (Leene, 1938)	KX060196	Madagascar
Charybdis (Goniohellenus) longicollis (Leene, 1938)	KT365717	Israel
Charybdis (Goniohellenus) truncata (Fabricius, 1798)	KX060218	Papua New Guinea
Charybdis (Goniohellenus) truncata (Fabricius, 1798)	KX060219	Thailand
Charybdis (Goniohellenus) vadorum (Alcock, 1899)	EU284141	China
Charybdis (Goniohellenus) vadorum (Alcock, 1899)	KX060220	New Caledonia
Charybdis (Goniohellenus) vadorum (Alcock, 1899)	MF043864	India
Charybdis (Goniohellenus) smithii (MacLeay, 1838)	KX060191	Oman
Charybdis (Goniohellenus) omanensis septentrionalis (Leene, 1938)	KY651228	India
Charybdis (Goniosupradens) obtusifrons (Leene, 1936)	KX060179	French Polynesia
Charybdis (Goniosupradens) acutifrons (de Man, 1879)	KX060178	Madagascar
Portunus pelagicus (Linnaeus, 1758)	KP976342	China

RESULTS AND DISCUSSION

1. Morphological characterization of C. natator

The studied crab has orange-red body color and deep reddish brown legs. Body weight range 107.7- 655g for males and 107.1-401.2g for females. Carapace width ranges 77-130 mm for males and 79-120 mm for females. Whole carapace coated with tiny pubescence except in anterior part of numerous wide granulated ridges. Width of carapace wider than anterior-orbital border. Curve formation of carapace posterior with posterolateral border. Carapace behind last pair point of anterolateral teeth has granular ridges. eight teeth found on anterior-orbital edge, 6 teeth on anterolateral edge and first anterolateral tooth truncated. According to cheliped, three spines found on frontal border of merus while posterior border has only one distal spinule; carpus holds 3 spinules on external angle and sturdy spine on inner angle (Fig. 2).

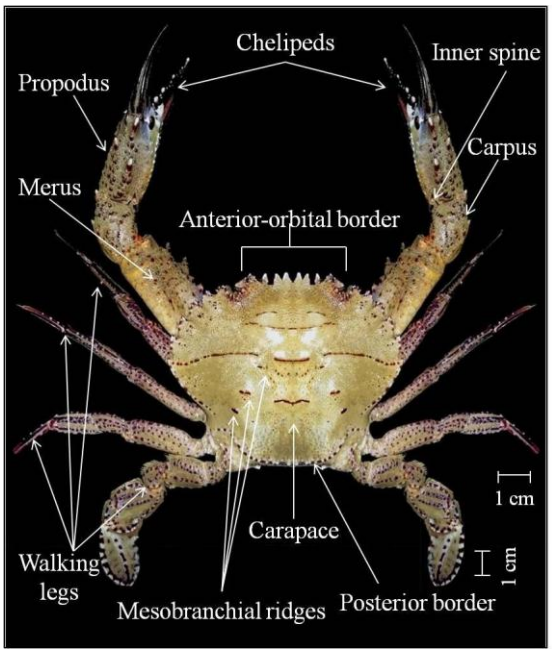

Fig. 2. *Charybdis natator* from the Egyptian coast of Red Sea.

2. Molecular identification

The PCR products of COI gene region were 710 base pair (Fig. 3) which agree with the expected amplicon size of De Silva and Munasinghe (2016). The length of resulted partial COI sequences was 648 bases for the two specimens of *C. natator* (MH447070 and MH447071). The length of COI sequences is close to those previously recorded for *C. natator* from the Gulf of Suez, Egypt (Abbas *et al.*, 2016).

The blast analysis revealed that the partial COI sequence of studied sequences (MH447070 and MH447071) shows blast retrieves sequences similar to the COI gene of *C. natator* (KX060205) from Oman with the identity 100% and 99.85%; 0 e-value; query cover of 98% and 100%; with 1182 and 1192 scores, respectively. Accordingly, this result proves the morphological identification of the studied sequence as *C. natator* and confirms the previous identification by Wee and Ng (1995) and Abbas *et al.* (2016).

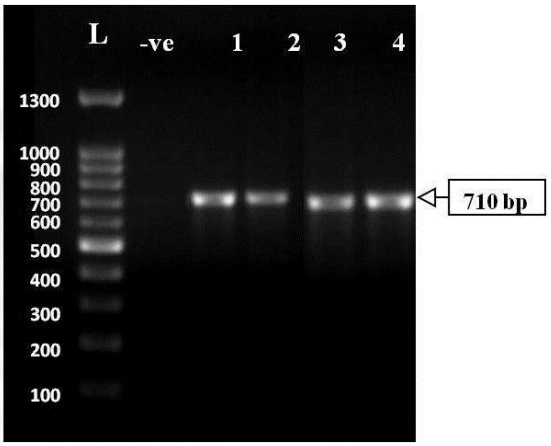

Fig. 3. Gel electrophoresis showing the length of the amplicons of COI gene (710 bp) of *C. natator* (1-4) and 100bp DNA ladder.

3. Phylogenetic analysis

Figure 4 represents the phylogenetic tree that was built from 48 organisms' amino acid sequences belonging to genus *Charybdis* (Crustacea, Decapoda, Portunidae) and one sequence of *Portunus pelagicus* (Linnaeus, 1758) as an outgroup. The ML tree differentiated genus *Charybdis* into four subgenera; *Charybdis*, *Gonioneptunus*, *Goniohellenus* and *Goniosupradens* and various monophyletic clades of 10 species, with high bootstrap values at each node (>70%). These species are *C. acuta*, *C. feriata*, *C. natator*, *C. longicollis*, *C. truncata*, *C. japonica*, *C. lucifera*, *C. bimaculata*, *C. hellerii* and *C. vadorum*. The two specimens of the present study (MH447070 and MH447071) were clustered with *C. natator* obtained from genetic database, confirming the morphological identification. The evolutionary divergence estimated that the pairwise distance between *C. natator* individuals was 0% followed by *C. truncata* (0.8%); *C. hellerii*, *C. longicollis*, *C. smithii*, *C. omanensis septentrionalis* (1.6%); *C. feriata*, *C. vadorum*, *C. japonica*, *C. miles*, *C.crosnieri* (2.3%); *C. lucifera*, *C. acutifrons*, *C. obtusifrons* (3.1%); *C. bimaculata* (4.7%); *C. acuta* (7%).

The present tree showed that *C. variegata* (KJ168053 and KX018513) were clustered in the same clade of *C. natator* as a sister species, with the lowest pairwise distance (0%). However, *C. natator* and *C. variegata* have approximately close morphological characters as described by Apel and Spiridonov (1998). It is worth mentioning that there is difficulty in identifying *C. variegate* especially when the specimen is in a juvenile stage. Negri and Mantelatto (2017) redefined *C. variegate*, that was misidentified, to be *C. hellerii* which appear in the same cluster with *C. variegata* with the lowest evolutionary divergence values (0-3.6%). Furthermore, According to the present phylogenetic analysis, it is suggested to redefine *C. variegata* (KJ168053 and KX018513) to *C. natator*.

C. acuta was separated into a paraphyletic clade while other *Charybdis* species were grouped into another branch which means that those species have great similarities in COI sequences other than *C. acuta*. This observation was found in the phylogenetic tree conducted by Negri *et al*. (2018). Moreover, the divergence values were high between *C. acuta* and the other *Charybdis* species. It was 3.9% with *C. acutifrons*; 4.7% with *C. feriata*; 5.4% with *C. hellerii*, *C. lucifera*, *C. longicollis*, *C. japonica*, *C. obtusifrons*; 6.2% with *C. bimaculata, C. vadorum*, *C.crosnieri*; 6.3% with *C. truncate*, *C. miles*; 7% with *C. natator*, *C. variegate*, *C. smithii* and *C. omanensis septentrionalis*. This finding suggests that *C. acuta* could be promoted to the generic level and more genetic data and further studies are needed to confirm or negate this suggestion.

The phylogenetic tree showed that *C. obtusifrons* and *C. acutifrons* which belong to subgenus *Goniosupradens* was grouped into a separate branch as a sister species. The divergence values were almost high (≥ 3.1%) between each of *C. obtusifrons* and *C. acutifrons* with the other *Charybdis* species, supporting the promotion of subgenus *Goniosupradens* to the generic level as recommended by Evans (2018). This contradict the assumption of Leene (1938) and Ng *et al*. (2008) who considered *Goniosupradens* as a subgenus under genus *Charybdis* using a morphological description. On the other hand, the clustering of *C. miles* with *C. crosnieri* and *C. smithii* with *C. omanensis septentrionalis* as sister species indicate the paucity of genetic information on some species of genus *Charybdis* and more studies are needed.

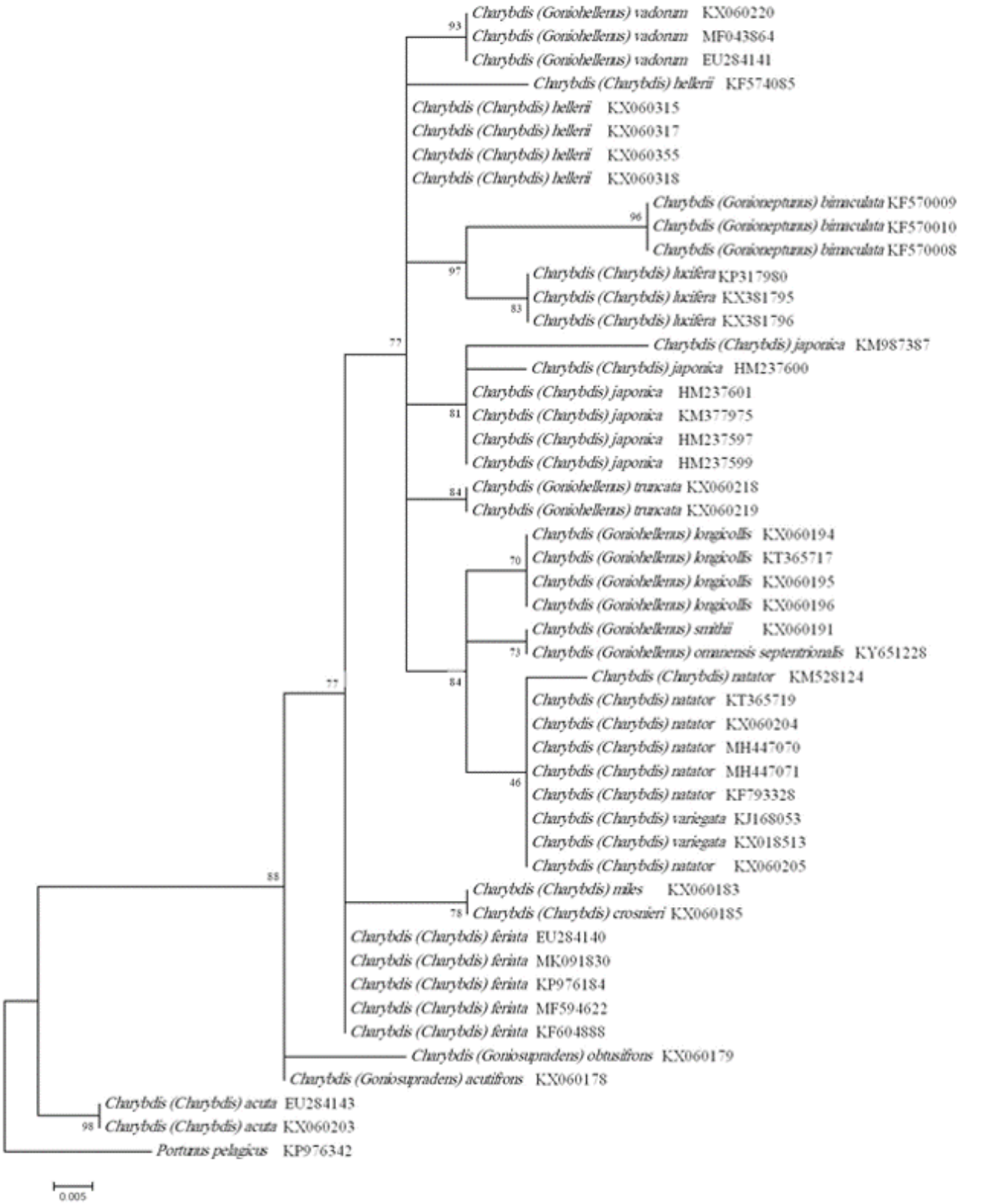

Fig. 4. Molecular phylogenetic tree of coding COI amino acid sequences among 48 species of genus *Charybdis* (including two studied *C. natator* MH447070 and MH447071) and *P. pelagicus* (family: Portunidae) as an outgroup. The evolutionary history was inferred by using the Maximum Likelihood (10000 replicates). Numbers of bootstrap values represented below the nodes.

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