Egyptian Journal of Aquatic Research 47 (2021) 93–99

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Egyptian Journal of Aquatic Research

journal homepage: www.sciencedirect.com/locate/ejar

DNA barcoding of seven cone snail species from Red Sea coast of Egypt

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ARTICLE INFO

Article history:
Received 25 June 2020
Revised 16 October 2020
Accepted 25 October 2020
Available online 23 January 2021

Keywords:
Conidae
Conus
DNA barcoding
COI
Red Sea
Egypt

ABSTRACT

Cone snails are venomous predators comprising ~950 species widely distributed in different marine habitats. The production of bioactive molecules (known as conopeptides or conotoxins) marks the *Conus* as an utmost promising animal source for medicinal applications. This reflects the need for quick and reliable proof of the studied species' identity. However, identification based on morphological characters has limitations and necessitates complementation with molecular techniques. DNA barcoding based on the mitochondrial *cytochrome oxidase* subunit I (COI) is currently used as a quick and reliable tool for species identification throughout the globe. The primary objective of this study is to establish reference sequences for *Conus* species from the Egyptian Red Sea coast, and to evaluate the capacity of DNA barcodes for specimen' identification. The results of the present study revealed that COI sequences were matched for their maximum identity with those available in the GenBank and BOLD engine and gave matches to *Conus* species for all studied species (100% match rate). In all cases, DNA analyses were consistent with species classification based on shell characters. According to the phylogenetic tree, all *Conus* species were recovered as monophyletic and the seven studied species of *Conus* were well nested in seven separate clades/subclades with closely related species. In conclusion, our study successfully confirms the efficiency of DNA barcoding for specimen identification of different cone snails. Such analytical tool offers great chances for exploring *Conus* species to better evaluate their status in the Egyptian Red Sea coast, and more generally along the whole Red Sea coast.

Introduction

Cone snails, members of the genus *Conus* Linnaeus, 1758, are predatory marine gastropods that prey on worms, molluscs, and fish. The genus *Conus* shows unusual high species diversity with more than 800 recognized specie (Kohn, 2018). It is probably the most species-rich taxon of marine invertebrates and one of the largest known genera of venomous animals (Kohn, 1990; Röckel et al., 1995). From 30 species identified by Linnaeus (1758), more than 950 cone snail species are currently accepted, among them 748 are *Conus* species (WoRMS, 2020). Cone snails are diverse, with some species having broad distribution over a wide geographic range but others are endemic to certain areas (Röckel et al., 1995; Tenorio et al., 2004). The importance of the genus *Conus* rises from its species abundance, which is an evidence of morphological diversity, and in the production of powerful peptides (Röckel et al., 1995). These conotoxins are used for prey immobilization, that makes cone snails the favourable source for neurobiologic and therapeutic applications of animals (Olivera et al., 2017). Conotoxins have also been characterized by various therapeutic potentials in pre-clinical or clinical trials, such as antiepileptic, neuroprotective, cardioprotective, and antinociceptive activities (Han et al., 2008; Miljanich, 2012). Furthermore, many peptides with analgesic potency such as Ziconotide (Prialt®) from *C. magus*, Contulakin-G from *C. geographus*, and α-RgIA from *C. regius* have been identified (Safavi-Hemami et al., 2019). In addition, a short insulin-like bioactive peptide was discovered from cone snail venom (Safavi-Hemami et al., 2015).

The molecular phylogeny of gastropod molluscs is extensively studied using morphometric characteristics and DNA sequences (Phuong et al., 2019). DNA barcoding turned out to be a worldwide popular and effective tool for specimen identification and species discrimination of marine organisms. Moreover, the DNA barcoding concept is an accurate, rapid, and globally accessible procedure for species delineation and identification (Hebert et al., 2003). The efficiency of this method relies on the existence of ~655 bp rela-

Peer review under responsibility of National Institute of Oceanography and Fisheries.

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https://doi.org/10.1016/j.ejar.2020.10.012
1687-4285/

Fig. 1. A map showing the collection sites of *Conus* samples included in the study.

tively conserved sequence in the COI gene. Based on these sequences, precise identification of organisms at the species level is relatively easy and has been applied to various animal taxa (Frézal & Leblois, 2008; Smith et al., 2008). Many studies have proven that DNA barcoding was efficient to identify gastropod larvae (Puillandre et al., 2009), also different specimens of Neogastropoda (Zou et al., 2011) and *Conus* species (Barco et al., 2016).

The Red Sea is a body of warm tropical water, that represents the most north-western part of the Indo-Pacific Ocean, and its closest point to Europe. Although there are several studies on the ecology and species diversity of *Conus* from various Indo-Pacific areas (Kohn, 2015; Vallejo, 2005), little information is available on *Conus* in the northern Red Sea (e.g. Abdel-Rahman et al., 2011; Abdel-Wahab et al., 2016, 2017). Despite that several studies listed the then-known existing cone snails (Fainzilber et al., 1992; Fishelson, 1971), studies on their distribution pattern on hard substrata did not distinguish between species (Zuschin & Stachowitsch, 2007; Zuschin et al., 2001). Currently, no DNA barcoding information is available on the cone snails from the Red Sea coast. We have applied DNA barcoding and phylogenetic analyses for specimen identifications of cone snails from the Egyptian Red Sea. Indeed, the sequences generated from this study would be accessible as exclusive for cone snails from the Egyptian Red Sea through other geographical locations.

Materials and methods

Biological material

A total of 30 specimens of *Conus* species were collected from different geographical locations within the Egyptian Red Sea coast during the period of March-October 2019 (Fig. 1). Morphological identification was possible for the thirty studied specimens com-

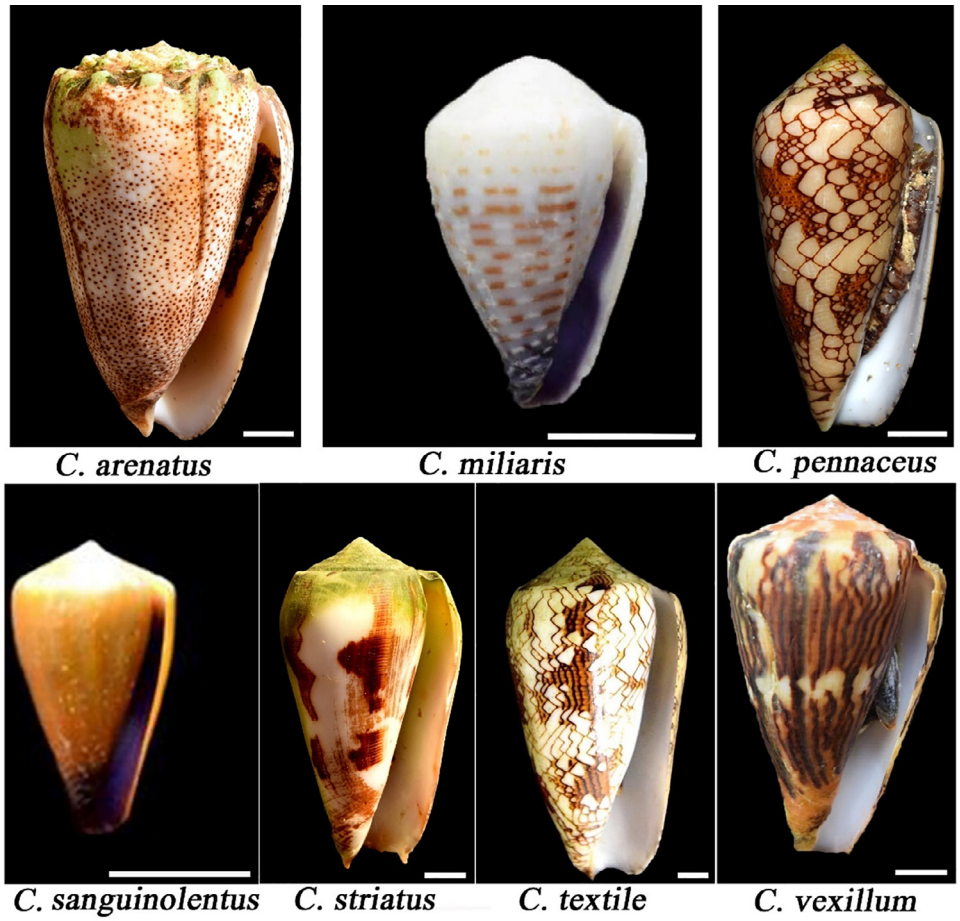

Fig. 2. General morphology of the seven *Conus* species included in the study. Scale bar-1 cm.

Table 1
Classical measurements (in cm) represented by mean ± SD. Total shell length (SL), shell width (SW), length–width ratio (LW = SL/ SW), aperture width (AW), aperture length (AL), relative diameter (RD = AW/AL) of different cone snail species from the Egyptian Red Sea.

Species	Samples no.	SL	SW	LW	AW	AL	RD
C. arenatus	5	4.92 ± 0.48	2.92 ± 0.41	1.7 ± 0.09	0.68 ± 0.07	4.72 ± 0.62	0.15 ± 0.01
C. miliaris	5	2.36 ± 0.53	1.43 ± 0.16	1.64 ± 0.21	0.35 ± 0.09	2.15 ± 0.52	0.16 ± 0.02
C. pennaceus	5	4.45 ± 0.29	2.10 ± 0.07	2.12 ± 0.07	0.73 ± 0.06	3.69 ± 0.22	0.20 ± 0.01
C. sanguinolentus	5	3.46 ± 0.56	1.93 ± 0.28	1.79 ± 0.06	0.51 ± 0.08	3.05 ± 0.58	0.17 ± 0.01
*C. striatus**	1	9.10	4.55	2.00	1.45	7.30	0.20
C. textile	4	8.15 ± 0.4	4.15 ± 0.05	1.97 ± 0.08	1.16 ± 0.1	7.08 ± 0.14	0.16 ± 0.01
C. vexillum	5	6.76 ± 1.12	4.02 ± 0.56	1.68 ± 0.07	0.99 ± 0.11	6.10 ± 0.96	0.16 ± 0.01

*represented only by one specimen.

prising seven morpho-species (Kohn & Riggs, 1975) (some are shown in Fig. 2). The samples were then preserved in ethanol (96% ethanol) or frozen at − 20 °C until DNA extraction. Number of samples and their morphometrics are listed in Table 1.

DNA extraction, amplification, and sequencing

Total genomic DNA from foot tissue was extracted using BioVision's Mollusca Genomic DNA Kit (Cat. #: K1413 BioVision, Inc. California, USA) following manufacturer's instructions. Fragments of the mitochondrial COI gene were amplified using the invertebrate universal primers as described by Folmer et al. (1994); HCO2198 (R): 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′, LCO1490 (F): 5′-GGTCAACAAATCATAAAGATATTGG-3′. Each PCR mixture consists of 12.5 μl 2x PCR master mix solution 1 ml (iNTRON biotechnology, Korea, cat no-25028). 1 μl of each primer (10 pmol/μl), 5 μl DNA template and the volume was brought to 25 μl by deionized sterile water, all in Thermowell® GOLD 0.2 ml PCR tubes with flat cap. PCR conditions comprised initial denaturation on 94 °C for 5 min, followed by 30 cycles. Each one consists of a denaturation step (1 min at 94 °C), annealing step (1 min at 40 °C) an extension step (1 min at 72 °C), and an extra final extension step was performed for 7 min at 72 °C. PCR products were kept in 4 °C until being electrophoresed and analyzed. Amplified products were purified and cleaned using QIAquick PCR Purification Kit Protocol. Fluorescence-based cycle sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Qiagen Inc., Valencia, CA, USA), with electrophoresis on an ABI 3500 automated sequencer (Applied Biosystems Inc., USA). Obtained sequences were deposited in GenBank database (Table 2). Moreover, sequences were submitted to Barcode Of Life Data system (BOLD) under the project "DNA barcoding of *Conus* species from Red Sea coast, Egypt" for getting BIN IDs.

Molecular identification and phylogenetic analysis

Sequences were screened and analyzed by Finch TV 1.4.0 (Geospiza, Inc., USA; http://www. geospiza.com). The sequence similarity search for species identification was done in two public databases, GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and BOLD (version 2.5 http://www.barcodinglife.org). We recorded the species representing the top BLAST hit for each query. Following this, we built a dataset of 85 COI barcode sequences for 56 different *Conus* species, among which 7 sequences generated in the current study, and 29 sequences representing same species from wide geographic localities. In addition, 48 sequences in which all species of the 7 subgenera were represented, each by 1 individual (when available). Finally, *Californiconus californicus*: a closely related outgroup sequence from family Conidae (GenBank accession no. KX263249), were downloaded from GenBank database. The obtained sequences were aligned using ClustalW extension in MEGA X (Kumar et al., 2018), retaining the default settings. Nucleotide composition was computed from the ingroup sequences only. Tamura and Nei (1993) and Kimura 2-parameter model (K2P) (Kimura, 1980) were used to estimate the sequence divergence for the entire data set, using MEGA X (Kumar et al., 2018). For the phylogenetic analysis, COI sequence data (n = 85) was analyzed using Maximum Likelihood (ML) and Neighbor Joining (NJ) analyses, and a consensus tree was constructed. The in-node confidence was evaluated by 1000 Bootstrap replicates (Felsenstein, 1985).

Table 2
GenBank accession numbers for the seven *Conus* species included in the study.

Species	GenBank Accession numbers
C. arenatus	MT483233
C. miliaris	MT483235
C. pennaceus	MT483230
C. sanguinolentus	MT483232
C. striatus	MT483236
C. textile	MT483231
C. vexillum	MT483234

Results

Based on shell morphology, 30 collected specimens were identified as *Conus* species. The number of individuals per species ranged from one to five, with five species represented by 5 individuals (*C. arenatus, C. miliaris, C. pennaceus, C. sanguinolentus, C. vexillum*), one species represented by 4 individuals (*C. textile*) and one species (*C. striatus*) represented by one specimen (Table 1).

GenBank and BOLD matching

The COI gene was successfully sequenced for the seven examined species, with an average length of 658 bp. Sequences were matched for their maximum identity with those available in the GenBank and BOLD and gave matches to *Conus* species with 100% match rate (Table 3). In all cases, DNA analyses were consistent with species classification based on shell characters. Within the same family Conidae, genus *Conus*, our samples were found to belong to seven different subgenera. Subgenus *Conus* (*Puncticulis*) was represented by *C. arenatus*. The sequence from specimen *C. arenatus* gave a maximum identity of 99.66% to the subspecies *C. arenatus aequipunctatus* when matched with the GenBank database. However, the best similarity for this species using BOLD identification engine was 97.91% to a form *C. arenatus f. aequipunctatus* (not accepted in WoRMS and still counted as a species) from Red Sea, Gulf Aden, Yemen. Only one out of the seven specimens (identified as *C. pennaceus*) belonging to subgenus *Darioconus,* did not match any previous records in BOLD engine. However, GenBank

Table 3
Summary of identification based on each species consensus barcoded sequence using BLASTN search from GenBank and BOLD Identification System (BOLD-IDs). Similarity description used in the study- 97%–100%– significant, 92%–96%– moderate.

No.	Studied species	Species' match by name			Similarity %		
		GenBank (BLASTN)	BOLD-IDS	EMBL-EBI FASTA	GenBank (BLASTN)	BOLD-IDS	EMBL-EBI FASTA
1	*C. arenatus*	*C. arenatus aequipunctatus*	*C. arenatus f. aequipunctatus*	*C. arenatus aequipunctatus*	99.66	97.91	99.3
2	*C. miliaris*	*C. miliaris*	*C. miliaris*	*C. miliaris*	99.53	99.68	98.9
3	*C. pennaceus*	*C. pennaceus*	*C. pennaceus*	*C. pennaceus*	97.19	96.39	96.8
4	*C. sanguinolentus*	*C. sanguinolentus*	*C. sanguinolentus*	*C. sanguinolentus*	97.12	98.56	96.4
5	*C. striatus*	*C. striatus*	*C. striatus*	*C. striatus*	98.66	98.98	97.8
6	*C. textile*	*C. textile neovicarius*	*C. textile neovicarius*	*C. textile neovicarius*	97	97.29	95.9
7	*C. vexillum*	*C. vexillum*	*C. vexillum*	*C. vexillum*	98.61	98.9	98.2

matching significantly identified the sample, establishing a maximum identity of 97.19% to *C. pennaceus*. Subgenus *Virroconus* is represented by *C. miliaris* and was appropriately identified with a maximum GenBank identity of 99.53%, and similarity of 99.68% at BOLD engine. Furthermore, both GenBank and BOLD databases significantly identified *C. sanguinolentus* (subgenus*: Lividoconus*) by 97.12% and 98.56%, *C. striatus* (subgenus: *Pionoconus*) 98.66% and 98.98%, while *C. vexillum* was identified by 98.61% and 98.9% respectively. The specimen of *C. textile* (subgenus*: Cylinder*) gave a maximum identity to a subspecies *C. textile neovicarius* by a maximum identity of 97% in GenBank and 97.29% BOLD similarity.

Phylogenetic analysis

This study comprised 84 sequences of *Conus* and one outgroup. The COI data set consisted of 564 aligned nucleotides. Within the in-group, 228 (40.4%) were variable and 214 (38.1%) were parsimony informative. In total 200 (35.5%) bases were variable and 114 (20.2%) were parsimony informative within the sequences of the seven studied species. The tree topologies recovered based on the COI data set using the NJ and ML analyses were almost similar in defining the main clades. According to the NJ phylogenetic tree, all *Conus* species were recovered as monophyletic and exhibited a long branch, which separated them from the outgroup (Fig. 3). Moreover, the seven species of *Conus* were well nested in seven separate clades/subclades. *C. sanguinolentus* species from different localities were nested to a clade including *C. diadema, C. lividus, C. eximius, C. quercinus, C. floridulus and C. lischkeanus,* belonging to subgenus *Lividoconus* (all preying on worms). This latter clade was sister to all the remaining represented *Conidae*, with (99%) Bootstrap value. To a clade of subgenus *Virroconus* (also preying on worms), including *C. aristophanes*, *C. abbreviatus*, *C. dorreensis*, *C. judaeus*, *C. coronatus*, *C. ebraeus*, *C. taeniatus*, *C. chaldaeus*, and *C. miliaris* species were all clustered. This clade in turn was sister to the remaining analysed *Conidae* with (100%) Bootstrap value. The next clade was divided into two sister subclades including *Puncticulis* and *Rhizoconus* subgenera. It is a sister to the remaining studied subgenera. In the subclade containing subgenus *Puncticulis*, different *C. arenatus* species were clustered with *C. pulicarius*, *C. caracteristicus* and *C. zeylanicus* species forming two subclades. In the other close sister *Rhizoconus* clade, *C. vexillum* from different international localities together with *C. pertosus, C. miles*, *C. rattus*, *C. namocanus*, *C. mustelinus and C. capitaneus* were clustered. Furthermore, *C. striatus* is nested to the clade of subgenus *Pionoconus* including *C. gubernator, C. achatinus, C. consors, C. magus metcalfii*, *C. catus*, *C. striolatus*, *C. floccatus*, *C. barthelemyi*, *C. stercusmuscarum*, *C. circumcisus*, *C. gauguini* and *C. aurisiacus*. Moreover, a major clade contained two close sister subclades of food related subgenera, *Darioconus* and *Cylinder*. *C. pennaceus* species in addition to *C. crocatus*, *C. omaria*, *C. magnificus* and *C. aulicus* were clustered together under subgenus *Darioconus.* Finally, *C. textile* and related species *C. dalli*, *C. canonicus*, *C. ammiralis*, *C. retifer*, *C. legatus*, *C. aureus*, *C. gloriamaris* and *C. victoriae* were clustered together with subgenus *Cylinder*.

Discussion

Until Recently, the main contribution to registering cone species that are endemic to the Egyptian Red Sea (based mainly on morphological characters) was due to the work of Rusmore-Villaume (2008). Uncovering the real diversity of a region, without over-/or underestimation of the species number, is of supreme importance, because it might cause consequences in defining the true conservation status of the species. Cone snails have been traditionally identified and described based on characters, mainly from the shell, which are prone to homoplasy. Such morphological approach was historically-misleading in the delimitation of many species that were later uncovered when applying molecular methods (Duda, 2008; Duda et al., 2009). Many of shell characters vary according to environmental factors: they can be thicker (Brookes & Rochette, 2007), the shape may modify in response to the current speed (Baker et al., 2004), biochemical conditions (Chiu et al., 2002), or presence of predators (Palmer, 1990). Hence, the morphology of cone snail shells could be in a number of cases merging ecotypes, sibling, or cryptic species to local environments (Dowle et al., 2015; Hollander & Butlin, 2010). Furthermore, what one specialist explains as geographical or bathymetrical variation can be judged by another as species differences. In consequence, many quite opposing ideas about species delimitations are not unusual in literature (Röckel, 1980; Tenorio et al., 2004) such as in the Cape Verde *Conus*. Moreover, shell characters in the pulmonate freshwater snail genus "*Radix*" are associated with environmental factors, with no constant divergence among the species defined with DNA (Pfenninger et al., 2006). Likewise, in the genus *Conus*, a DNA-based analysis reported complexes in the species diversity of *C. orbignyi* and *C. sponsalis* where shell characters alone were incapable to accurately identify species boundaries (Puillandre et al., 2010). DNA sequences can satisfy this criterion and are now commonly used to delimit species (Wheeler, 2008; Wiens, 2007). Yet, the simplest way to identify anonymous specimens is by matching sequence with online databases. In the present study, COI sequences successfully recovered all the morpho-species analysed. This suggesting that the taxonomy of this species needs to be revised. Besides, the GenBank database was advantageous in giving steadfast matching results. On the other hand, matching sequences to the BOLD engine was specific since it compares sequences with the same gene region in the database. The sequence-based specimen identification (the best close match) turn DNA barcoding into a leading tool in terms of established classification (Collins & Cruickshank, 2013). The Barcode Index Numbers (BINs) analysis tool computed by BOLD engine can use genetic information to generate an estimate of the number of operating taxonomic com-

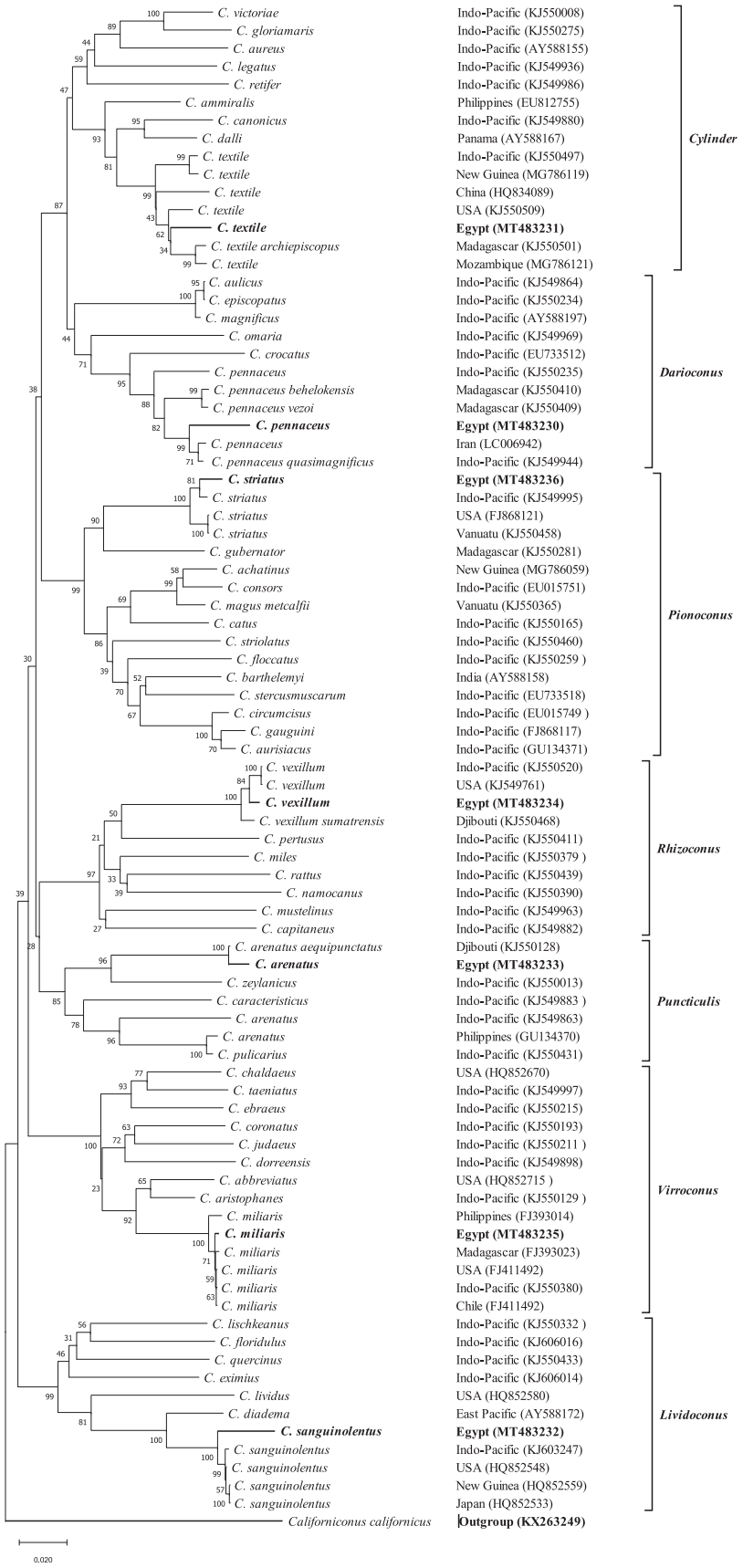

Fig. 3. Neighbor joining (NJ) tree developed using K2P distance among 85 COI sequences. The numbers at the nodes are bootstrap values based on 1000 replications. Species' name, origin, and GenBank accession numbers are shown for each taxon. Bolded species correspond to the sequences from this study.

ponents that diligently relate to species. The reference library of DNA barcode is fast-growing by the contributions of the international community in the BOLD. The standard pairwise distance method such as NJ based on K2P and GC content divergence assessment is currently the primary method used to analyze patterns of diversity with COI barcode region.

Although we intended to only use the phylogenetic signal in the COI to identify *Conus* species, the major branches in the resulting NJ tree represented different subgenera. Additionally, it was possible to compare and validate the results using sequences of similar or related *Conus* species (same genera) downloaded from GenBank. The seven analysed species from that relatively narrow geographic range in the Red Sea have been found to relate to the three different prey taxa known in cone snails. Our data showed that sequence of C. *arenatus* from this study grouped with *C. arenatus* from Djibouti. However, *C. arenatus* from the Philippines grouped with *C. pulicarius* from Indo-Pacific. The same observation was reported previously as *C. arenatus* was found to occur in two clades, one corresponding to the form *aequipunctatus* and the other being mixed with *C. pulicarius* (Puillandre et al., 2014). This suggesting that the taxonomy of *C. arenatus* needs to be revised with more in-depth analyses. Based on the tree, the geographic distributions of species in these clades indicated that cone snails in the Red Sea are probably originated from the Indo-Pacific Ocean, as reported in Puillandre et al. (2014). These results further support the geographic generality of genus *Conus* revealed in earlier studies in regions of the tropical Indian Oceans, where closely related species are co-occurring and partitioning both food and space resources (Schluter, 2000).

Thus, it can be concluded that seven species representing 100% of the studied cone snail species were identified straightforward through DNA barcoding, which restated the efficacy of the technique. Hence, we barcoded and confirmed the presence of the following species: *C. arenatus aequipunctatus*, *C. miliaris*, *C. pennaceus*, *C. sanguinolentus*, *C. striatus*, *C. textile neovicarius* and *C. vexillum*. Future studies with more collected specimens are needed to barcode the cone snail composition in this area. It is necessary to carry out large scale sample barcoding that covers the whole cone snail population of the Red Sea because there is a high possibility to detect new/cryptic species from this under-explored region. Finally, our study contributed the first new barcode data in the global database for seven *Conus* species from the Red Sea.

Conflicts of interest statement

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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