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Molecular Identification of Selected Ascidians from Egypt using COI

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ABSTRACT

Ascidians are one of the most significant individuals of benthic organisms. Their exaggerating recruitment all over the world via their attachments to several natural and artificial substrata has substantially boosted the biodiversity of the marine ecosystem. Morphological identification of ascidian demands highly specialized experience to eliminate them since frequent misidentification. MT-COI gene sequence has been used as a suitable marker for the invertebrate distinctness. In the present study, the Phenol-chloroform method, PCR, and COI sequencing were selected to elucidate the molecular identification of selected ascidians from Egypt and display their phylogenetic relationships with other COIs of ascidian species in the world obtained from GenBank. Constructed phylogenetic trees show that COI could identify the three species: *Microcosmus* sp. from Ismailia, *Symplegma rubra*, and *Botryllus* sp. from Port Said to the closest taxonomic rank.

INTRODUCTION

Ascidians (Class: Ascidiacea, subphylum: Urochordata) are exclusively marine invertebrates. Adult ascidians are sessile found attached to natural or artificial substrates, or fixed in sediments. Morphologically, ascidians are categorized into three types: solitary, colonial and synascidians **(Ali & Tamilselvi, 2016)**. Based on the analysis of the previous studies and the species registered in the online World Register of Marine Species, **Shenkar and Swalla (2011)** concluded that, the species richness of ascidians is the "highest in tropical regions, where colonial species predominate. In higher latitudes solitary species gradually contribute more to the total species richness".

In some countries, such as Chile, Japan, Korea and France, several ascidians have been fished and cultured for food. For a long-term period, the edible solitary ascidian, *Halocynthia roretzi*, has popularly been utilized as seafood in Japan and Korea, with a market value reaching up to $18 million in 2006 **(Nguyen *et al*., 2007)**. Moreover, a unique infectious agent which is kinetoplastid protist has been identified as the causative agent of mass mortality of these cultured ascidians **(Kumagai *et al*., 2010)**.

Natural and derived compounds of ascidians have antimicrobial, antineoplastic, anti-HIV antioxidant, and antihypertensive characteristics, thus they can be used in medical and nutraceutical applications, in addition to the bioactivity of the unique chemical classes discovered in ascidians that make them promising species for more studies to develop novel drugs to cure different diseases **(Arumugam *et al*., 2018)**.

One-to two-thirds of an evaluated half a million marine species may still be undescribed (**Appeltans *et al*., 2012)**, in addition to our rudimentary awareness of the exact number of each extant species, and the new species which have not yet been identified or discovered. Furthermore, anthropogenic impacts and the the extent of the significant alterations of global environment and composition of marine biodiversity are so far unpredictable. Remarkably, all these obstacles cause arduous tasks in biodiversity assessment. To study biodiversity and propose a plan for ocean conservation fundamentally demand accurate species inventories and continuous monitoring **(DeSalle & Amato, 2004)**.

Morphological identification is an essential pillar in the determination of species composition. Significantly, this identification requires specialized expertise in taxonomy. Owing to the scarcity of such professional taxonomists, the taxonomic determination is aligned to higher units as order or class and rarely to family level due to obtaining insufficient knowledge about biodiversity **(Mohrbeck *et al*., 2015)**. Other impediments manifest through the morphological recognition of cryptic species and determination of phenotypic plasticity exhibited by some individuals within a species **(Raupach & Wägele, 2006)**. Furthermore, the morphological identification of the planktonic larvae and the juveniles of fish and marine invertebrates to the true species or genus level, and the determination of damaged samples form a major problem in ecological studies (**Ko *et al.*, 2013)**.

Quick and accurate molecular techniques have been increasingly tested, established and applied to cope with such obstacles that appeared in morphological determination of species **(Hebert *et al*., 2003a; Mohrbeck *et al*., 2015)**. Launching websites included databases of sequences, as GenBank, www.ncbi.nlm.nih.gov (**Benson *et al*., 2012)** and the most specific Barcode of Life Data System, www.barcodinglife.org, **(Ratnasingham & Hebert, 2007)**, which have been considered as a stock or library for

formerly identified species, and served as identification system based on DNA sequences (**Blaxter, 2004)**.

The technique of DNA barcoding is used by scholars in biological studies proving how the data of DNA sequence are affordable. It enabled many researchers to get taxonomic names of species without much reference to morphological taxonomy that are extremely based on sophisticated characters of organisms **(Wilson *et al*., 2017)**.

Different sections of gene are used for the identification of different organisms, such as CO1 or COX1 cytochrome c oxidase 1 for animals and some protists , matK and rbcL for plants **(CBOL Plant Working Group, 2009)** and Internal Transcribed Spacer (ITS) for fungus **(Schoch *et al.*, 2012)**. These diverse loci of gene have less variation within species (intraspecific) than variation between different species (interspecific) that is known as the "Barcoding Gap", and hence they were selected as markers **(Meyer & Paulay, 2005)**.

A chunk of nearly 650 base-pairs(bp) from the mitochondrial CO1 gene are set as a global standard or a DNA barcode for molecular species identification of animals **(Hebert *et al*., 2003b, 2003c)**. The sequence of MT-CO1 gene has sufficiently fast mutation rate that resulted in its variation among closely related species, which is higher than its variation within species, besides its sequence that is conserved among conspecifics. Hence, MT-CO1 gene sequence is used as a suitable marker for the species distinction.

Ascidians are difficult to be identified with morphological methods **(Stefaniak *et al*., 2012)**. Due to the frequent misidentification, taxonomy of tunicates based on morphological basis are highly demanded and requires specialized experience **(Lambert, 2009; Geller *et al*., 2010)**. The COI gene is utilized to assign species on molecular basis and display patterns of diversity among the individual of species and other related species **(Muirhead *et al*., 2008)**. The feasibility of COI as barcoding technique for identification of ascidian was underlined in several studies. In the Palk Bay, India, the first report of molecular identification of *Didemnum candidum*, *Styela clava* and *Ascidia ahodori* was based on using sequence of COI gene **(Iyappan *et al*., 2015)**.

Mastrototaro *et al*. (2019) confirmed their morphological identification of *Symplegma brakenhielmi* through phylogenetic and species delimitation analyses based on the COI-DNA barcode, which also confirmed the red and yellow colonies with identical COI sequences obtained from the same species *S. brakenhielmi*, hence these varied colors belong to the same species. A mtDNA is a powerful tool for the investigation of the evolutionary relationships among species. The study of **Kumaran *et al*. (2017),** using the newly sequenced COI of colonial *Eudistoma* species, in addition to other COIs extracted from NCBI, highlights “the strong differences in mtDNA

evolutionary dynamics between ascidians and remaining chordates. Comparative analysis of mitochondrial COI sequences of colonial ascidian, *E. viride*, revealed contrasting patterns of genetic structure".

In the present study, Phenol-chloroform method, PCR, and CO1 sequencing were selected to elucidate the molecular identification of selected ascidians from Egypt and display their phylogenetic relationships.

MATERIALS AND METHODS

Solitary and colonial ascidians were collected from two coastal sites in Egypt: Ismailia and Port Said. Morphological identification was performed at marine science department, Faculty of Science, Suez Canal University. Samples were preserved at -20°C. For molecular identification, a phenol- chloroform method was used in DNA extraction of selected ascidians, a grinded tissue with liquid nitrogen of each species, taken via fine point scissor was put in 1.5 ml tube. An amount of 600 µl 2XCTAB buffer was added to each tissue, followed by 10µl of proteinase K (20mg/ml), then tubes were inverted several times to mix well. Incubation of all tubes was conducted at 65ºC for 120 minutes. They were shaken every 10 minutes during incubation. An amount of 600µl of CIA (chloroform-isoamyl alcohol) was added to each mixture, each of which was then shaken on for 5 minutes or buzzed on a vortex machine for 10 sec.

All tubes were centrifuged for 5-15 minutes at 13000 rpm. Appeared aqueous phase (top) was transferred to new labeled tubes. Tubes with bottom organic layer was discarded in CIA waste container. An amount of 600µl of phenol-CIA was added to aqueous layer, then was shaken for 5 minutes and centrifuged for 5-15 minutes at 13000 rpm. Aqueous phase was transferred to final 1.5 tubes. An amount of 1 ml of cold 95% EtOH was added to the aqueous layer, shaken well by hand and stored at -20ºC for precipitation overnight.

Centrifugation of all tubes was conducted for 30 minutes at 13000 rpm. EtOH was removed. For washing, an amount of 0.5 ml of 70 % EtOH was added to each tube, overturned to mix and centrifuged at 13000 rpm for five minutes; this stage was repeated once. The dried pellets were dissolved in 25 µl double distilled sterilized H**2**O and stored at – 20ºC. Samples were visualized on 1.5 % SB agarose gel to detect DNA bands. Sodium borate buffer was used for DNA electrophoresis **(Brody & Kern, 2004)**. PCR amplification of a 700 bp fragment of the COI gene was performed in the thermocycler (Major Science Thermocycler). PCR was conducted using the GeneDirex PCR Master Mix solution (Germany) stored at -20ºC and the universal invertebrate primers (**LCO1490**: GGTCAACAAATCATAAAGATATTGG, **HC02198**:

TAAACTTCAGGGTGACCA AAAAATCA) of **Folmer *et al.* (1994)** were employed to amplify the mitochondrial gene cytochrome oxidase subunit I (COI).

The cycling conditions (PCR protocol for the universal invertebrate primers LCO1490 and HCO2198 consisted of 5 minutes at 94°C, followed by 35 cycles of denaturation step at 94°C for 30 seconds; annealing step at 53° C for 30 seconds, extension step at 72°C for 1 minutes, and a final extension of 10 minutes at 72 °C. Total PCR volume was in some examinations 30µL with 15 mastermix, 2 from each primer 10 M, 3 from DNA sample, 8 ddH_2o, while in others it was 25µL with 12.5 mastermix; 1 µL from each primer; 1 µL from DNA sample; 9.5 µL ddH_2o.

The PCR products were purified and sequenced using 3500 genetic analyzer-applied biosystem. The construction of phylogenetic trees using COI gene sequencing was performed via Mega version 10 to confirm morphological identification. Approximately, all COI sequences of ascidian species were collected from NCBI in Nov 2019 for phylogenetic analysis. The taxonomic information of ascidian species was supplied from two websites: Ascidiacea World Database and World Register of Marine Species, " http://www.marinespecies.org/ascidiacea/aphia.php?p=search" and "http://www.marine species.org/aphia.php?p=taxlist", respectively.

Sequence alignment was performed by using align by muscle; the phylogenetic trees were constructed based on this alignment by using Maximum Likelihood with 1000 bootstrap replicates. "The average genetic divergence values among taxa were calculated based on the Kimura-2-parameter model K2P" **(Kimura, 1980)**.

RESULTS

1. DNA electrophoresis of ascidian samples

1.1. In Fig. (1) shows the DNA extraction proceeded without using liquid nitrogen. The only two DNA extraction samples appearing in gel photo belong to *Microcosmus* sp. from Ismailia.

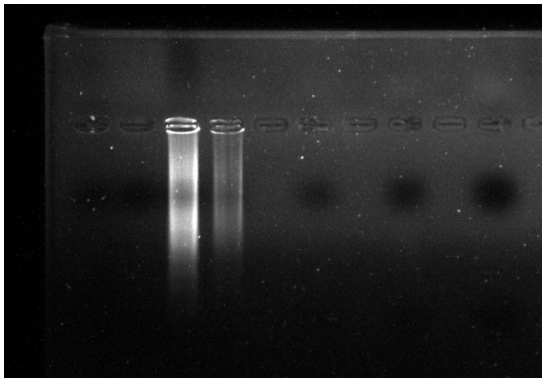

Fig. 1. 1.5 % Agarose gel stained with ethidium bromide of DNA extracted from ascidian species *Microcosmus* sp. from Ismailia

1.2. In Fig. (2), DNA extraction was proceeded using liquid nitrogen in the grinding of every tissue sample; this gel photograph shows the success of phenol-chloroform method with grinding using liquid nitrogen in extraction of ascidian DNA.

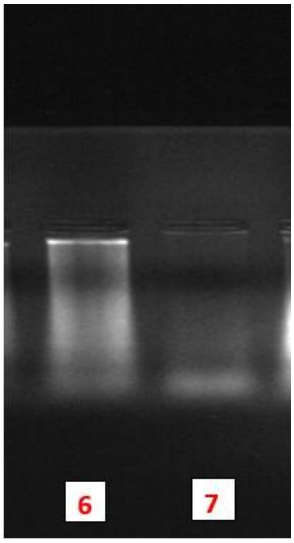

Fig. 2. Photograph of agarose gel 1.5% stained with ethidium bromide showing DNA extracted from two ascidian species. **6:** *Botrylus* sp. and **7:** *Symplegma rubra* from Port Said.

2. Gel electrophoresis of PCR samples

Extracted DNA samples exhibited in gel photos were subjected to PCR reactions with the conditions of the universal primers.

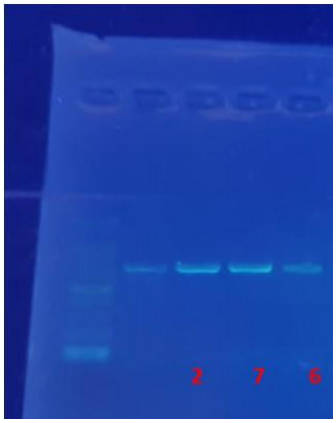

Fig. 3. Photograph of 1.5% agarose gel of PCR final products of selected ascidian species. Ascidian species showed in gel image: **2:** *Microcosmus* sp. Ismailia, **7:** *Symplegma rubra*, Port Said, **6:** *Botryllus* sp., Port Said

3. Identity and maximum score of the submitted sequences in the NCBI

The three sequences of *Microcosmus* sp. from Ismailia, *Symplegma rubra* and *Botryllus sp*. from Port Said were blasted in April 2021 and submitted on GenBank with accession numbers of MT361974, MT605851, and MT985155.1, respectively. *Botryllus* sp. from Port Said., 621 bp after editing, was blasted with maximum identity **99.35%**, 0.0 E value and query cover of 99% with *Botryllus eilatensis*, 856 bp (MT873574.1). Besides, *Microcosmus* sp., 643 bp after editing, from Ismailia was blasted with maximum identity **84.09%**, 2e-139 E value and query cover of 81% with *Microcosmus exasperates*, 580 bp (KX138506.1.)**.**

Whereas, *Symplegma rubra*, 635 bp after editing, from Port Said was blasted with maximum identity of **87.80%,** 0.0 E value and query cover of 91% with *Symplegma rubra*, 620 bp (FJ528648.1). Moreover, *Symplegma rubra*, 635 bp after editing, from Port Said, was blasted with maximum identity **87.18%**, 0.0 E value and query cover of 99% with *Symplegma brakenhielmi*, 834 bp (LS992554.1).

4. NCBI-ascidian species

Although class of Ascidiacea has almost three thousand described species that inhabit all marine environments whether shallow, deep or hadal parts **(Cameron *et al.*, 2000;Kott, 2005)**, less than 1000 COIs of ascidian species were only recorded in NCBI databases, with several replicates for each species COI, according to a survey performed in 2019.

5. Phylogenetic tree of *Microcosmus* sp.

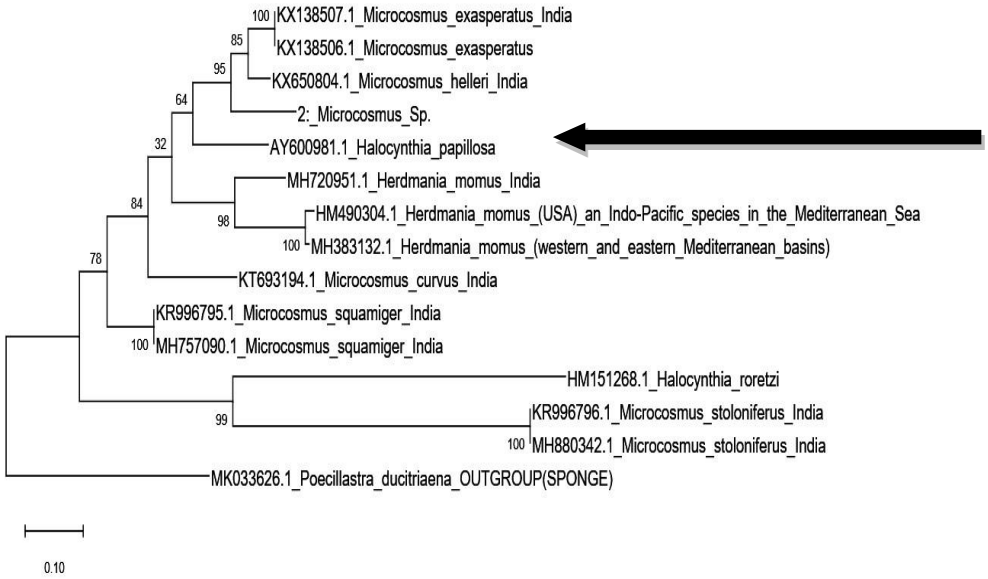

Fig. 4. Phylogenetic tree for COI sequences of *Microcosmus* sp. in the present study and different related species of family Pyuridae with 1000 bootstrap value.

The phylogenetic analysis between the present *Microcosmus* sp. and other species obtained from GenBank in their family Pyuridae was shown in Fig. (4). *Poecillastra ducitriaena* was used for rooting the tree. All related species were clustered together within the same clade. The present *Microcosmus* sp. was closely clustered with the two *M. exasperates* (KX138506) and *M. helleri* (KX650804.1) from India.

6. Phylogenetic tree of *Symplegma rubra* & *Botryllus* sp.

Phylogenetic tree, the phylogenetic analysis between *Botryllus* sp. and *Symplegma rubra* collected from Egypt compared to other related species in GenBank in their family Styelidae is shown in Fig. (5). *Poecillastra ducitriaena* is used for rooting the tree. All related species were clustered together within the same clade.

Botryllus sp. (the Egyptian isolate) was clustered with all *Botryllus eilatensis* (MT873574.1), with bootstrap value of 100%. The present isolate (*Symplegma rubra*) was closely grouped with all species of its genus *S. rubra* (FJ528648.1) from Kenya and *S. brakenhielmi* (KX138510.1, KT276224.1 & LS992554.1) from India and Italy, with bootstrap value of 90%.

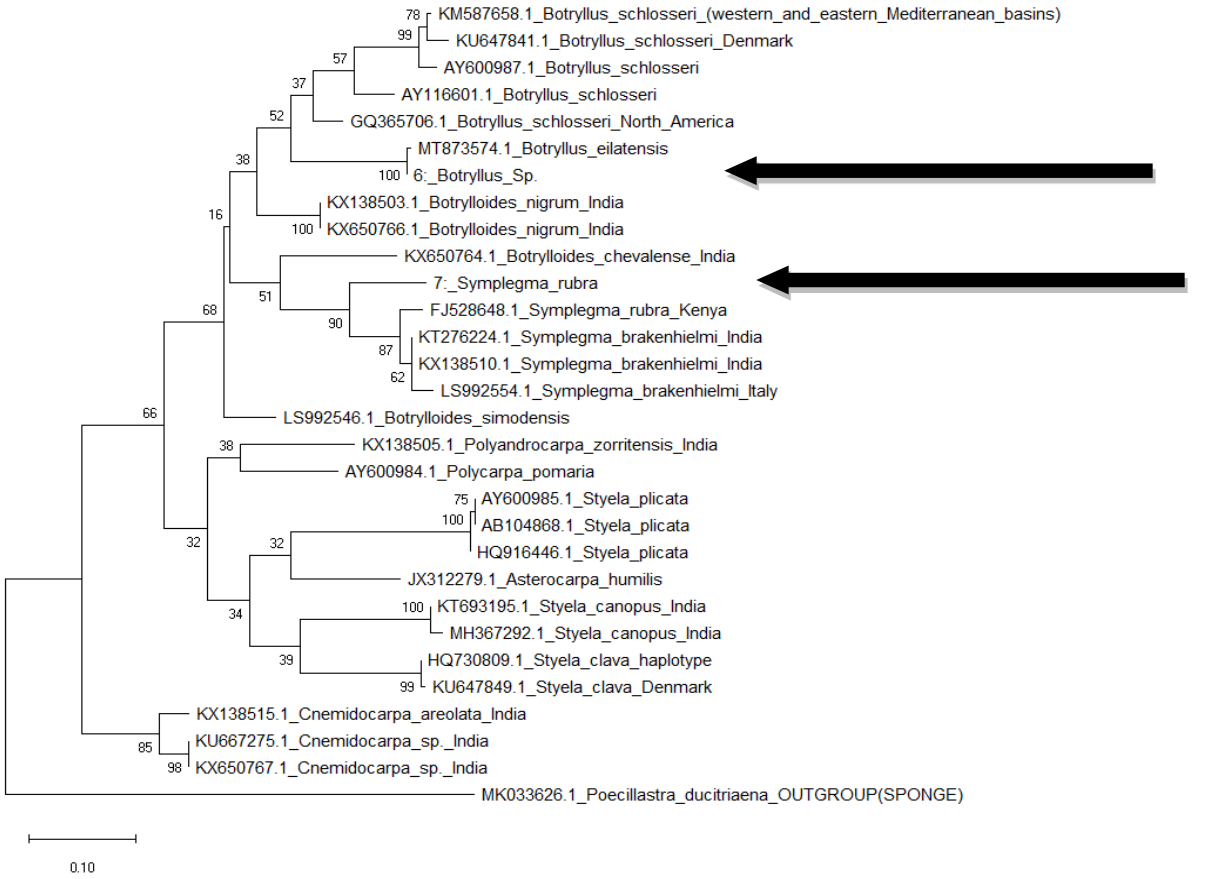

Fig. 5. Phylogenetic tree of different species of family Styelidae with 1000 bootstrap value

DISCUSSION

The identification of species is an objective for scientists or any person who observes nature, and a prerequisite for any biological study**(Galtier, 2019)**. In addition, naming species assists in ecosystem monitoring via detecting species which disappears or remains.

Ascidian species are deemed as economic (in some countries) **(Nguyen *et al*., 2007)**, medical **(Arumugam *et al*., 2018)** and invasive species. DNA barcoding for naming and discriminating of species, which is the cytochrome *c* oxidase subunit 1 gene (COI) for invertebrates, has been introduced by **Hebert *et al*. (2003a, 2003b)**, owing to its clear genetic differentiation between species and exhibiting a better phylogenetic signal in several phyla and orders.

In the present study, morphological identification of ascidian species has confronted intractable problems because of insufficient experts and studies on these species in Egypt. As far as, we investigated molecular databases on ascidian sequences of NCBI in Nov 2019, we did not find any sequences of ascidian COI from Egypt, so this present study may be considered the first molecular identification of ascidians using COI gene sequences in Egypt.

Microcosmus sp. from Egypt in the present study clustered with similar species of its genus *M. exasperatus*, and *M. helleri*, COI submitted by **Jaffarali *et al*. (2016)** from India with high bootstrap (95 %). However, identification percentage through BLAST shows less probability to be one of the two species.

Besides, COI of *M. curvus* KT693194, submitted by **Jaffarali *et al.* (2015)**, *M. squamiger* submitted by **Jaffar and Shabeer (2015)**, *M. stoloniferous* submitted by **Ahmed and Jaffar (2015)** from India, doesn't cluster with *Microcosmus* sp. of present study. This indicates that it cannot be one of these species. *Symplegma rubra* from Egypt is clustered with species of *Symplegma* from India KX138510.1 **(Jaffarali *et al*., 2016)**, Italy (**Mastrototaro *et al*., 2019**) and Kenya. The closest species of this genus to our species was the only COI for *Symplegma rubra* from Kenya (FJ528648), which was submitted by **Perez-Portela *et al.* (2009)**. Hence, the results are consistent with morphological examination done by the expert.

According to an investigation performed in Nov 2019, a few number of genus *Symplegma* COIs were found on NCBI. In addition, **Mastrototaro *et al.* (2019) "**suggested a possible synonymy between *S. brakenhielmi* and *S. rubra*, based on their all ABGD species delimitation analyses", which included *S. brakenhielmi* and *S. rubra* sequences in the same OTU. Therefore, they recommended further molecular and morphological analyses for the two species to investigate this possible synonymy. This suggestion could be the explanation of the similarity appeared in BLAST analysis between *S. rubra* of this study and *S. brakenhielmi and S. rubra* in identity percentages.

Botryllus sp. from Egypt is clustered with species of *Botryllus eilatensis* **(Salonna *et al*., 2021)**, with 100% supporting the great molecular similarity between the two COIs. In addition, the maximum percent of identity that appeared through BLAST analysis is 99.35%, which enabled us to assess the identification of the *Botryllus* on species level although identification was morphologically on genus level.

In addition, COI barcode would assist in solving a considerable dispute of the taxonomy of Botryllinae species owing to scarce distinguished features of their small zooids. Furthermore, most of their morphological features show high intraspecific variability **(Manni *et al*., 2019)**. Consequently, a great uncertainty for illustration of new species, cryptic species or synonymies occurred by time **(Bock *et al*., 2012)**. The

phylogenetic analysis in this present study has confirmed CO1 efficacy in the definition and the discrimination of species via showing distinct divergence and assigning each ascidian species collected from Egypt to its closest taxonomic rank. *Symplegma rubra* and *Botryllus eilatensis* are identified at the species level, whereas the identification of the last species is at the genus level. Therefore, it is an effective technique to study and monitor biodiversity.

In various studies, although the feasibility of DNA barcodes in molecular identification of several species was proved, the practicability of NCBI-ascidian databases should be improved via molecular and morphological investigations, using more ascidian species on a large scale across the world and specifically, Egypt. As mentioned earlier, the number of NCBI-ascidian-COI datasets represents a very small percentage of the true number of ascidian species. As a precondition, the DNA barcoding technique for accurate identification is the constant elaboration of the barcode database. Ongoing efforts are demanded to add new ascidian data, especially the unrecorded species in NCBI to more gain an accurate and practical assignation process. Notably, the inadequate data decreased the practicability of DNA barcoding.

CONCLUSION

In this study, three COI sequences were successfully obtained, including *Microcosmus sp*. from Ismailia, *Symplegma rubra* and *Botryllus eilatensis* from Port Said. COI successfully elucidated the molecular identification of selected ascidian from Egypt, displaying their phylogenetic relationships with other COIs of worldwide ascidian species obtained from the GenBank. In each phylogenetic tree, each species clustered with its closest species confirm the morphological identification. Excessive studies must be proceeded in the future to provide molecular databases on ascidian species and monitor any changes in ecosystem.

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