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RESEARCH ARTICLE

DNA AND EDNA-BASED TECHNIQUES FOR CHARACTERIZING THREE UNIONID BIVALVE SPECIES IN THE NORTHERN RIVER NILE, EGYPT

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ABSTRACT

Bivalve mollusks are avid filter feeders. They filter significant amounts of algae, bacteria, sediment, nutrients, and other particles from the waters, playing a key benthopelagic regulatory role on the freshwater ecosystems. To provide DNA-based approach and environmental DNA (eDNA) based-techniques for accurate species identification and tracking, three different unionid bivalves were collected from North of the River Nile, belonging to the genera *Coelatura*, *Mutela*, and *Chambardia*. They were subjected to DNA barcoding, through sequencing the hypervariable barcode region of the mitochondrial cytochrome oxidase subunit I gene (*COI*), and phylogenetically analyzed. Also, the resulting barcodes were used to design species-specific PCR assays that were tested over waters where the three species were co-incubated. The results revealed that the collected species were mainly *Mutela rostrata* and *Chambardia rubens*. The third species showed barcoding and phylogenetic proximity to *Coelatura aegyptiaca*, yet mixed identity was found with two other Northeastern African *Coelatura* species. eDNA-based PCRs provided specific, clear amplicons for the three species. Based on these results, it is strongly recommended to extend the application for molecular techniques for further investigation of hidden diversity in the world of River Nile bivalves in Egypt to detect their exact species, abundance, and status of conservation.

INTRODUCTION

Freshwater bivalves are one of the most important aquatic resources, providing food, income, and ecological services to humans across the world. This group of animals encompasses 1178 species, belonging to eight families, six of which under order Unioindia, i.e. Unionidae, Margaritiferidae, Hyriidae, Mycetopodidae, Iridinidae, and Etheriidae[1]. Unionid bivalves are strictly freshwater, with moderate- to large-sized bodies[2]. Although they are distributed all

over the world, except Antarctica, only two major diversity hotspots are well-studied, that are in the Southeast of Asia and North America[2]. Most published research about this order came from these parts, more specifically in North America, Europe, and Russia. Therefore, the presence of other, yet poorly studied, hotspots of diversity for this order, like Africa and Southeast Asia, cannot be ruled out[2].

Relatively recently, it was elucidated that the co-existence of non-native fish species with the reproducing unionid bivalve caused that bivalves' parasitic glochidia larvae failed to attach to the gills of their fish hosts in sufficient numbers, which lead to that they were not transformed into juveniles (i.e. excysted) in ecologically viable numbers, in comparison to the same bivalve species upon incubation with native species[3]. This could be explained in part due to the capability of the glochidia to adapt to the immune system of the native hosts, the process that precludes larvae sloughing off" before transformation[3,4]. Hence, impacting the completion of transformation of glochidia into juveniles may be considered as a way by which non-native species can disrupt native ecosystems, owing to its consequences in reducing the numbers of available unionid bivalves. Bivalve persistence is indeed crucial for the natural balance in the aquatic ecosystem. Most bivalves are filter feeders, which partly makes them responsible for a major part of nutrient cycling and habitat modification in aquatic ecosystem[5]. Furthermore, they affect the food webs there both directly (i.e. through prey consumption) and indirectly (i.e. through movement of nutrients and energy)[5]. Accumulation of materials in bivalve shells and soft tissue is used as environmental monitors[5]. Their aggregations also in freshwater and estuarine ecosystems represent hot spots for biodiversity and biogeochemical transformations[5].

Identifying bivalves to species level based on morphological features alone is often difficult or impossible, creating serious challenges for their conservation and management. Indistinct inter- and intra-specific phenotypic variability within some unionid and venerid species for example rendered morphology-based species level definition a difficult task[6,7]. Loss of accuracy in shellfish species identification can have serious implications for conservation and management[8]. Application of molecular markers based on sequencing of short, inter-specific, hypervariable DNA stretches, i.e. DNA barcoding offers a way to overcome these problems by providing a simple, reliable, and objective method for identifying organisms based on their genetic code[9]. Use of this methodology for species identification achieved success in several issues, including the identification of different freshwater bivalves in some areas[10], detection of species collapse[11], monitoring early biological invasions[12], among many other objectives. The current study aimed to fill a major gap in knowledge about Egyptian Nile bivalve mollusks, through mitochondrial DNA (mtDNA)-based barcoding. Being fundamental filter feeders, engineers of aquatic ecosystems, collections points for different aquatic fauna, and main members in the river Nile food webs, we also aimed to design specific molecular techniques for some Nile bivalve species identification. This work had the final goal to aid future efforts aiming to clarify the exact stocks density, species, taxonomic placement, and conservation status of some River Nile bivalves in Egypt.

MATERIAL AND METHODS

Ethical considerations

Sampling, preservation, and dissection were all approved (code: S-GE-2-22) by animal ethics committee of the Zoology Department, Faculty of Science, Menoufia University.

Sampling of freshwater bivalves

Nile bivalve samples were collected from Bahr Shebin irrigation canal in Shebin El-Kom City, Menoufia governate, Egypt (Figure 1). The samples were brought fresh on the same day to Molecular Biology

Figure 1: Map for collection site from Shebin El-Kom inland irrigation waterway, Menoufia Governorate, Nile Delta, Egypt. Photo Credits: GoogleEarth Version 9.189.0.0, May 28, 2023, Coordinates: 30º34´46´´N 31º00´58´´EogleMaps®2019 are shown below the photo.

Laboratory in the Zoology Department, Faculty of Science, Menoufia University. They were euthanized by immersion in ice-cold, dechlorinated tab waters. Samples were initially morphologically identified by Prof. Sherin Sheir and Dr. Abd El-Hafez Ragab (Invertebrates Division in the Zoology Department, Faculty of Science, Menoufia University) and also by using *mussel-project* website of the University of Wisconsin-Stevens Point (https://mussel-project.uwsp.edu). The initial bivalves' identification was as a *Coelatura* sp., an *Chambardia* sp., and a *Mutela* sp. (n = 5-10/species) (Figure 2). The bivalves were dissected, and 100 mg biopsies from adductor muscles were removed from each individual bivalve and preserved in a 1000 µL of 70% alcohol.

DNA extraction

DNA extraction was carried out using the method described in Mohammed-Geba *et al*.[13]. In brief, each individual tissue sample was lysed in 200 µL of Tris-NaCl-EDTA-sodium dodecyl sulfate (TNES)-urea buffer with 2.4 U/mL proteinase K solution (ThermoFischer Scientific, Waltham, MA, USA). The samples were incubated at 55ºC for 1.5 hours. Next, 54 µL of 6 mol NaCl were added, then the tubes were centrifuged at 4000 ×*g* for 10 minutes. The aqueous supernatant was received in a new, sterile 1.5 mL tube, to which 200 µL of cold isopropanol were added with shacking to precipitate the DNA. The tubes were centrifuged at 11000 ×*g* for 10 minutes, then the supernatant was fully decanted, and the DNA pellet was washed using 400 µL of 70% ethanol. The tubes were centrifuged for 5 minutes at 11000 ×*g*, the ethanol was completely removed, and the tubes were dried at room temperature for 10 minutes. Finally, 40 µL of Tris EDTA buffer (10 mmol TRIS.HCl at pH 8, 2 mmol EDTA at pH8) were added for resuspending the DNA pellet. DNA quality was checked by electrophoresis in a 0.8% agarose gel stained with 0.5 µg/mL ethidium bromide (ThermoFisher Scientific) and visualized using UV transilluminator (Biometra, Göttingen, Germany). The DNA was used for subsequent polymerase chain reaction (PCR)-based amplification of the barcode region of the mitochondrial cytochrome oxidase subunit 1 gene (*COI*).

Figure 2: The three Nile bivalve species collected in the current study. A: *Chambardia* sp., B: *Mutela* sp., and C: *Coelatura* sp. A centimeters-graded ruler below bivalves' shells for length demonstration.

PCR amplification of *COI* gene barcode region

Amplification of *COI* gene barcoding region proceeded using the protocol and primers described by Folmer *et al*.[14]; LCO1490: 5'-GGTCAACAAATCATAAAG ATATTGG-3' and LCO2198: 5'-TAAA CTTCAGGGTGACCAAAAAATCA-3'. In 0.2 mL sterile PCR tubes the following components were assembled: 12.5 µL of COSMO PCR Master Mix (Willowfort, UK), 2 µL from each DNA extract, 0.5 µL from each of the forward and reverse primers (300 nmol), 1.25 µL bovine serum albumin (BSA: 0.3 mg/mL), and completed to 25 µL using deionized, PCR-grade waters. PCR amplification started with 10 minutes at 95ºC for initial denaturation and polymerase activation. Then, the program proceeded through 35 cycles of 95ºC for 1.0 minute, 46ºC for 1.0 minute, 72ºC for 0.5 minutes, followed by final extension step at 72ºC for 7 minutes. To check for success of the PCR, the amplicons were electrophoresed in a 1% agarose gel stained with 0.5 µg/mL ethidium bromide, besides O'GeneRuler 1.0 kb DNA ladder (Catalogue number: #SM116, ThermoFisher Scientific), and visualized using UV transilluminator. The positive PCR products for each species were sent to Macrogen Inc. (Seol, South Korea) for performing chain termination Sanger sequencing.

Sequences and phylogenetic analyses

COI sequences were received and opened using the freeware Chromas Lite version 2.6.2 (Technelysium Pty Ltd, South Brisbane, Australia). The sequences were reviewed and manually trimmed for removal

of non-informative nucleotides at the 5' and the 3' extremities, then checked for absence of any base call error. Edited sequences were compared to archived reference sequences in GenBank database using BLAST algorithm. A cut-off identity value of >99% was set to accept species-level delineation of the sequences.

For identifying phylogenetic relationships among the identified samples and other related species/genera, *COI* sequences for related unionid species were retrieved from GenBank database. The sequences were aligned using CLUSTALW integrated with the program Mega 7.0.14 software [15]), after calculating the percentage of each nitrogenous base within the obtained *COI* sequences for each species. The alignment generated for these species was uploaded as a nexus format to MrBayes 3.2.1 software[16]. The best nucleotide substitution model was identified using JModelTest software V. 2.1.10[17]. Later, in the MrBayes 3.2.1 platform, four Markov Chains Monte Carlo (MCMC) chains were analyzed for 10 million (ngen=10000000) generations, saving a tree each 1000 generations. The analysis was stopped when the average standard deviation of split frequencies reached below 0.001. The number of burn-ins was identified using Tracer 1.7[18]. After removal of 25% of the trees as burn-ins, the tree was completed using MrBayes 3.2.1, and viewed using the interactive tree of life tool (iTOL)[19].

Design and eDNA-based testing species-specific unionid primers

The obtained, corrected *COI* sequences from the three bivalve species were aligned using Mega 7.0.14 software. Each sequence was individually uploaded into the online primer designing tool Primer3Plus[20] (available in https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). This tool was used for designing species-specific reverse primers and a common forward primer, all with annealing temperature of 55-60ºC, less than 3 repeated same bases, and the least self and inter-primers complementation. The primers that fulfilled the selection criteria were chosen and ordered from Macrogen Inc.

Environmental DNA (eDNA) extraction and primers testing

The three bivalve species (Figure 3) were collected in a 14-L plastic aquarium filled with dechlorinated tab water and left for 24 hours. After this, one liter of aquarium waters was collected, filtered through Cellulose Nitrate Filter, with mesh size 0.45 µm (11406-47-ACN, Sartorious Stedim Biotech GmbH, Goettingen, Germany), placed in a standard three-port aluminum manifold (Sartorious AG, Type 16612, Goettingen, Germany). The filter was then cut into fine pieces and subjected to DNA extraction using QIAamp DNA Mini Kit (Catalogue number: 51304, QIAGEN, Germany) according to the manufacturer's instructions. DNA quantity and quality were assessed through spectrophotometric measurement at A260/A280 and running a 3% agarose gel electrophoresis. DNA concentration was adjusted to 50 ng μL^{-1}. Then, this DNA was used to amplify *COI* gene of each bivalve separately using the species-specific primers. PCR components were the same as mentioned above, but using species-specific designed primers instead of Folmer *et al.*[14] primers. PCR amplification included 10 minutes of incubation at 95ºC; then 35 cycles of denaturation (95ºC for 1.0 minute), annealing (55ºC for 1.0 minute), and extension (72ºC for 1.0 minute); then concluded by a final extension step at 72ºC for 7 minutes.

The resulting PCR amplicons were electrophoresed, besides SiZer^{TM}-1000 DNA marker (iNtRON Biotechnology, Seongnam, South Korea), in a 1.0% agarose gel stained with ethidium bromide with the concentration 0.5 µg/mL and visualized using UV transilluminator. Positive PCR products were sent to Macrogen Inc. for further confirmation of specificity of amplification, using conventional Sanger Sequencing.

Figure 3: Collective bivalves' tanks where different, arbitrary collected River Nile bivalve species were placed, before collecting the waters for eDNA extraction.

RESULTS

Amplification, sequencing, and identification of sampled Nile bivalves

The size of PCR amplicons for the three bivalves was a band of about 650 base pairs (bp) in length (Figure 4). Sequencing of these amplicons resulted in good quality chromatograms (Figure 5). Manual trimming of the PCR amplicons for removal of non-informative and non-integrated nucleotide background resulted in keeping about 600 bp from all sequences. All sequences did not show stop codons, which excludes the possibility of having amplified pseudogenes or NuMTs (nuclear copies of mitochondrial genes).

For the first bivalve species, i.e. *Coelatura*, the produced *COI* sequences could be aligned with similar sequences in GenBank database with 100% coverage and e-values of 0.0. The highest identities found were with *Coelatura aegyptiaca* (100% with the GenBank sequence with accession number: KJ081163.1; 99.81% with accession number: JN243892.1), *C. bakeri* (100% with accession number: MT216491.1; 99.81% with accession number: MT216489.1), *C. stuhlmanni* (100.00% with accession number: MT216373.1; 99.62% with accession number: MT216357.1), and *C. hauttecoeuri* (100.00% with accession number: MT216369.1; 99.81% with accession number: MT216378.1). Lower identity levels were found with other Nile bivalves like *Nitia acuminata* (97.32% identity with accession number: MT216375.1), *C. horei* (93.87% with accession number: MT216453.1), and *C. kunenensis* (93.49% with accession number: KJ081166.1).

The other two bivalve species, i.e. *Mutela* sp. and *Chambardia* sp. showed more consistent relationships with their GenBank references than *C. aegyptiaca. Mutela* showed 99.6-100% identity with several *Mutela rostrata* references (accession numbers: U56849.1, DQ241804.1, JN243884.1, and AY785387.1). Less identities were found with other species belonging to the genus *Mutela,* like 88.75% identity with *M. dubia* (accession number: NC_034844.1), and 88.16% with *M. hargeri* (accession number: KX713482.1).

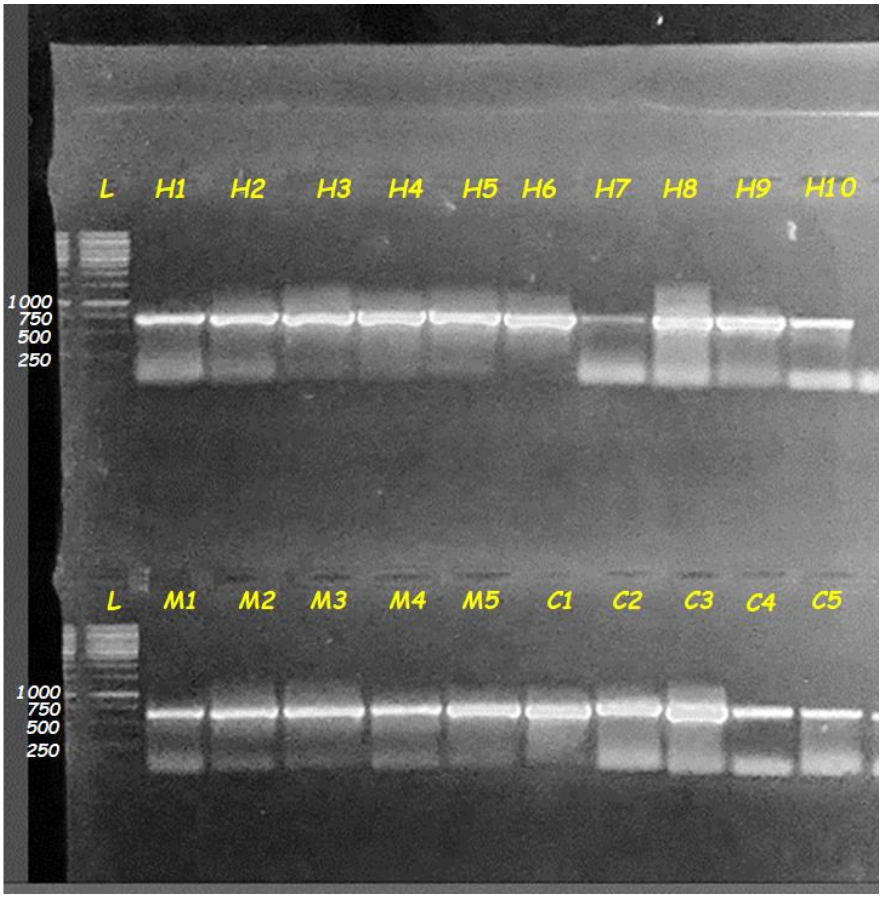

Figure 4: Agarose gel (1%) stained with ethidium bromide (0.5 µg/mL) for Folmer *et al.* [14]-based barcoding PCR. 5-10 samples were amplified for each species, yet only 5 samples with the highest amplicon concentration were used for the next Sanger sequencing procedure. Ladder bands' sizes are shown left-hand side to the ladder (L). H: *Chambardia*, M: *Mutela* sp., and C: *Coelatura* sp.

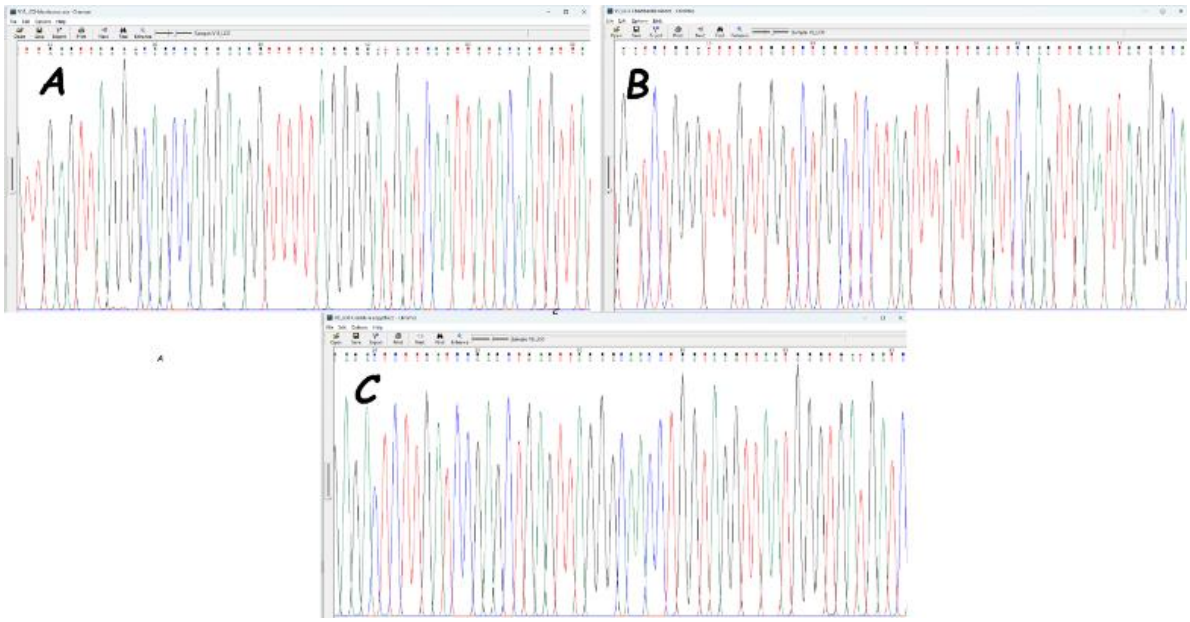

Figure 5: Examples for *COI* sequence chromatograms for *A. Mutela*, B: *Chambardia*, and C: *Coelatura* collected in the current study.

Likewise, *Chambardia* sp. showed 99-100% sequences' identities with *Chambardia rubens*, accession numbers: DQ241807.1, DQ241808.1, and AY785389.1. Only 93-94% *COI* sequences identity was found with another species of the same genus, which was *C. wahlbergi* (JN243886.1, KX713448.1). Sequences identities dropped much upon comparing them to members of genus *Anodonta*, the early nomenclature for the genus *Chambardia*. For example, the current study's *COI* sequences for *Chambardia* showed only 81-83% identities with *Anodonta arcaeformis* (accession number: GQ451869) and *A. impure* (accession number: KF672902.1), respectively.

Phylogenetic analysis of sequence

The frequency of nitrogenous bases (nucleotides) was as follows in each species: *Coelatura* sp. (T: 43%, C: 13.7%, A: 20.6%, and G: 22.7%); *Chambardia* sp. (T: 42.3%, C: 14.6%, A: 16.4%, and G: 26.7%); and *Mutela* sp. (T: 42.5%, C: 14.7%, A: 19.8%, and G: 22.9%). The constructed Bayesian inference (BI) showed similar pattern to the barcoding results, with high bootstrap support (Figure 6). *C. rubens* collected in the current study occupied the same subclade encompassing different references for the same species. This sub-clade was directly related to another one encompassing other *Chambardia* species, i.e. *C. wahlbergi* (Figure 6). *Chambardia* clade was directly related to *Mutela* clade, where the collected *M. rostrata* in the current study was found. *Chamardia* clade was completely separated from *Anodonta* clade, being *Chambardia* more related to *Mutela* than to *Anodonta* bivalves (Figure 6). *C. aegyptiaca*, however, was present in the most variable subclade of all analyzed Nile bivalve species in the current study. In one subclade, *C. hauttecoeuri*, *C. allaudi*, and the collected *C. aegyptiaca* sample were all together in the same subclade (Figure 6). Finally, and based on the results of DNA barcoding and phylogenetic analysis, the obtained *COI* sequences for unionid mussels were compressed into haplotypes using DnaSP6 (http://www.ub.edu/dnasp). As only one haplotype was found in each species, the three haplotypes characterizing the three mussel species were uploaded to GenBank database. They were assigned the accession numbers MK764009.1 for *C. aegyptiaca*, MK764010.1 for *C. rubens*, and MK764011.1 for *M. rostrata*.

Design of specific eDNA-based PCR assay for bivalves' detection

The PCR primers that fulfilled the selection criteria achieved success in producing PCR amplicons with exact sizes as expected for each species. Sites of the primers are shown for each species in Figure "7". A common forward, degenerate primer could be designed, with the sequence 5'-GTWATTGTWACCGCTCATGC-3' (W: adenine or thymine bases). *M. rostrata* reverse primer had the sequence 5`-GGCAGCATTACCAGATAAAGGA-3`, and it produced an almost 300 bp (the expected amplicon size was 351 bp). *C. rubens* specific reverse primer had the sequence of 5´-GCAACGCAGCAATTAAC AAA-3´. It was set to produce an amplicon of 400 bp in length. Its gel band appeared slightly below the ladder band 400 bp). *C. aegyptiaca* reverse primer had the sequence 5'- ACAGGTAAAGCTGCCAC TAA-3'. It was set to produce a 419 bp band, and the result was slightly above the ladder band of 400 bp in size (Figure 8). A trial was carried out for cross-amplifying the DNA extracted from each species using the other species' primers, and it resulted in zero cross-amplification.

DISCUSSION

The interest in freshwater bivalves come from both the previous environmental services they provide, such as being key regulators of aquatic ecosystem health and chemistry, and the possibility of applying them in aquaculture activities. Studies about both topics in Egypt are very poor. Therefore, the current study maybe a starting point for more works that aim to shed more

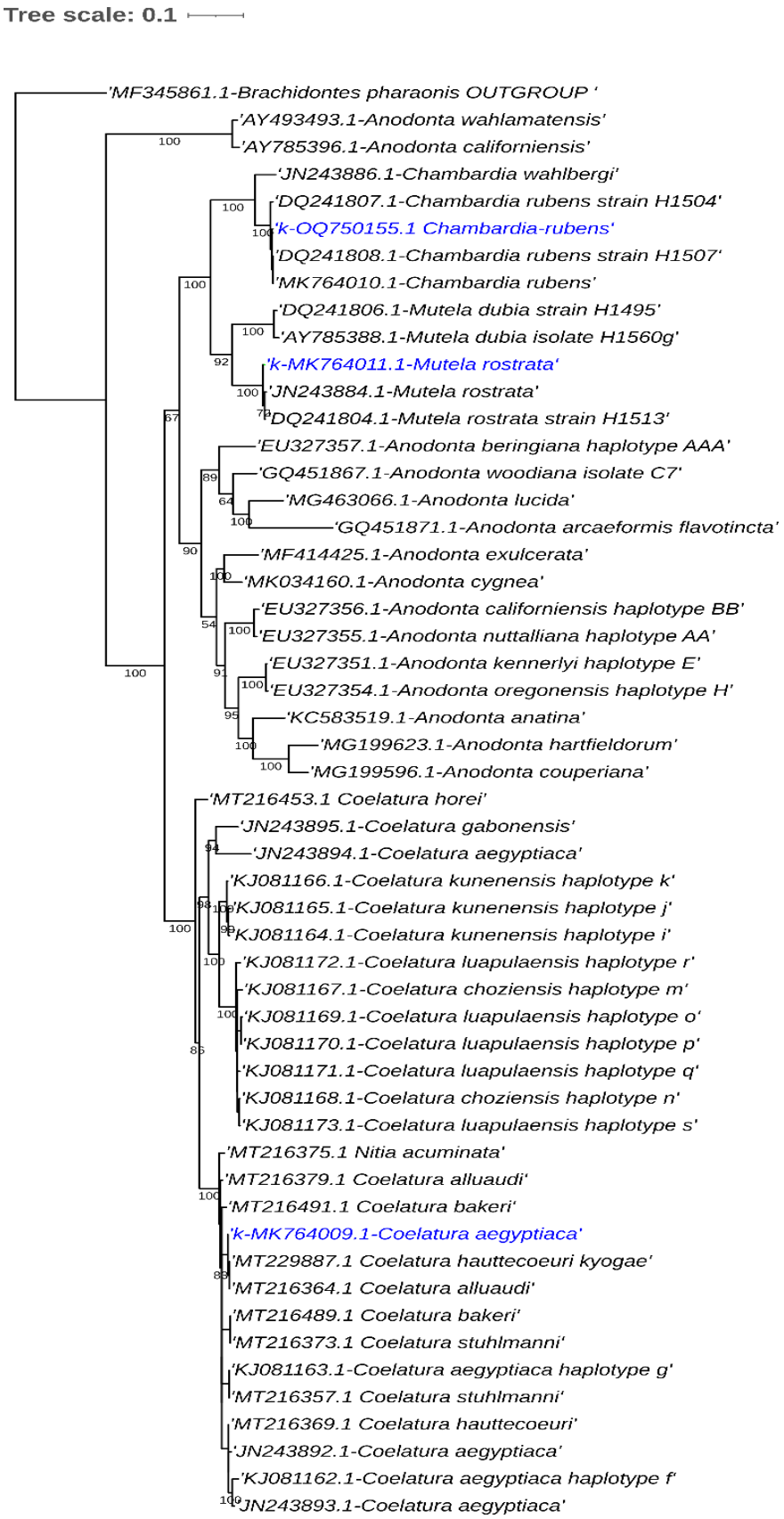

Figure 6: Bayesian phylogenetic analysis for the bivalves collected in the current study. Four Markov Chains Monte Carlo (MCMC) chains were analyzed for 10 million (ngen=10000000) generations. Posterior probability values are shown above the branches. Blue color: example sequences from each species collected in the current study.

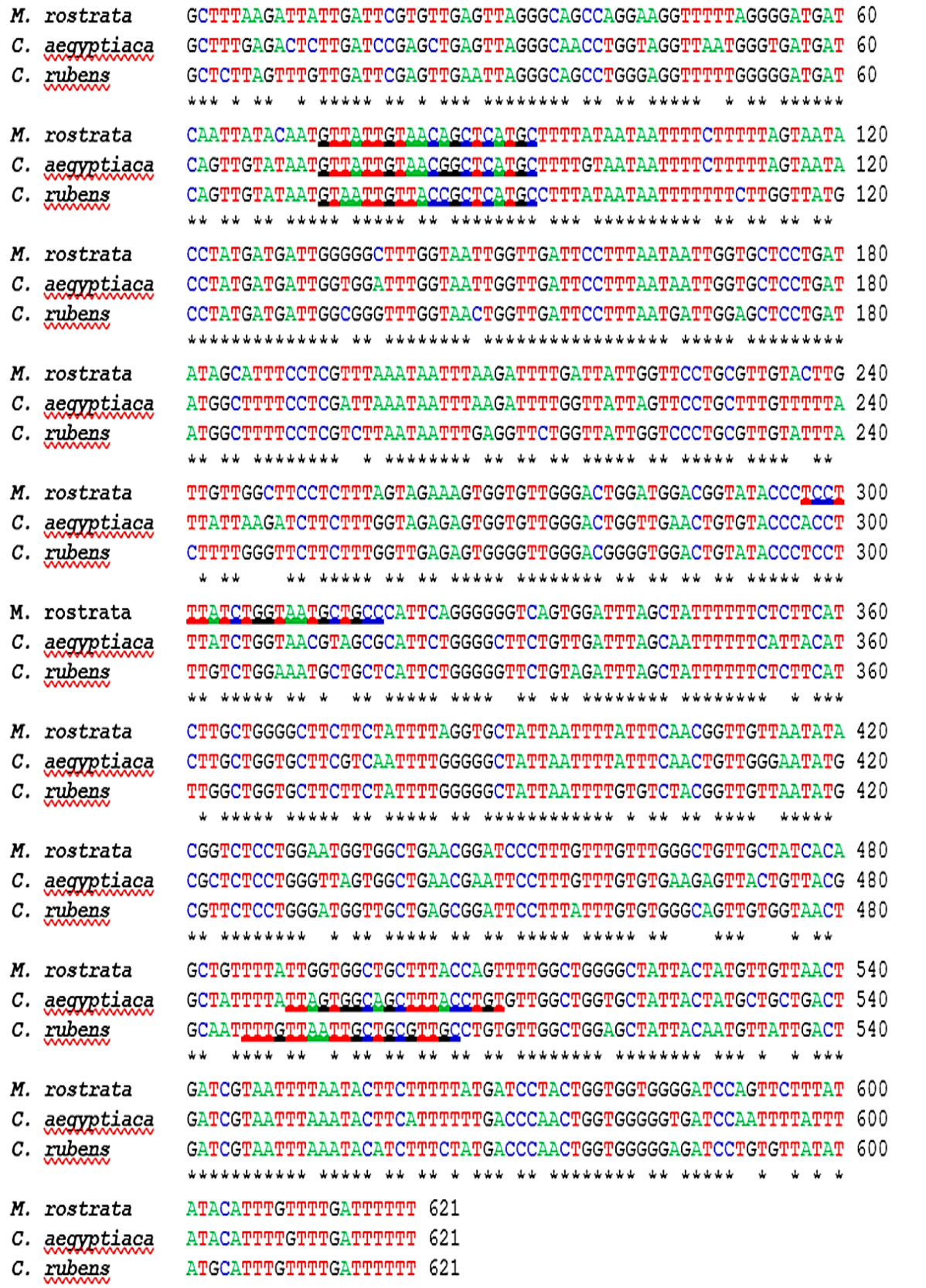

Figure 7: CLUSTAL 2.1 multiple sequence alignment for the analyzed 3 Egyptian Nile bivalves *Mutela rostrata*, *Coelatura aegyptiaca*, and *Chmbardia rubens.* Underlined stretches: sites of primers, bases in bold and italics: degenerate bases, red: thymine, green: adenine, blue: cytosine, and black: guanine.

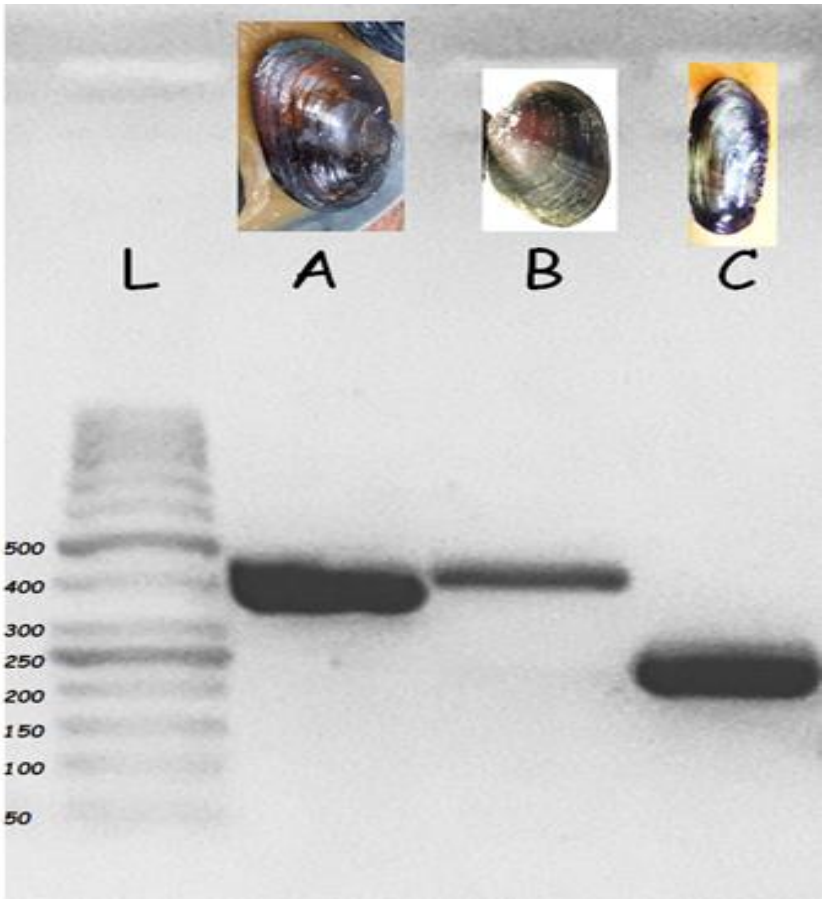

Figure 8: Species-specific amplicons for targeted bivalves from extracted eDNA, electrophoresed in a 3% agarose gel stained with ethidium bromide as 0.5 µg/mL (final concentration). L: 1kb ladder, A: *Chambardia rubens*, B: *Coelatura aegyptiaca*, C: *Mutela rostrata*. Ladder grading (SiZer^{TM} -50 DNA marker, iNtRON Biotechnology) at the left part of the figure.

light on the exact biological capabilities of freshwater Nile bivalves in Egypt and Africa in general.

DNA barcoding results and phylogenetic positions for the identified species varied strongly, especially for the case of *C. rubens* and *M. rostrata* on one hand, and for *Coelatura* sp. on the other hand. *Chambaria* and *Mutela* bivalves showed perfect grouping with their GenBank reference sequences. Also, their phylogenetic proximity in the same clade, but two different subclades, agrees with their common pertinence to the family Iridinidae Swainson, 1840. Similar situation was found before, using different nuclear and mito-chondrial markers genes, for representatives from genera *Chambardia* and *Mutela* in Yangtze River in China[21]. However, results for *Coelatura* sp. were not as clear as they were for both *C. rubens* and *M. rostrata*.

The systematic position of the genus *Coelatura* appeared to be paraphyletic to other unionids, even representing one of the earliest clades in freshwater bivalves of the world (Scholz and Glaubrecht [22]). *C. aegyptiaca* was reported to be the most widely distributed congeneric species for this genus in Africa, and it occurs in the Nile River in Egypt[22]. Species belonging to this genus in Africa are subjected to continued review efforts, leading to various works that produce taxonomic joining or splitting of them. For example, as reviewed by Scholz and Glaubrecht [22], 34 species and subspecies of *Coelatura* were previously reported in rivers and lakes in Africa. The morphological similarity among different

Coelatura species was reported to be not only for genetic factors, but also for some spatial one, like the sizes of the containing water bodies[22]. Very recently, and applying two mitochondrial markers (COI, 16SrDNA) and two nuclear markers (H3, 28SrDNA) sequencing, a close, monophyletic relationship among *C. aegyptiaca*, *C. hauttecoeuri*, and *C. allaudi* was found[23]. Taking together, the phylogenetic patterns for collected *Coelatura* sp., as well as the variable phylogenetic locations of *C. aegyptiaca* GenBank references, can all support Graf and Cummings[24] idea about the presence of unexpected diversity within genus *Coelatura* in Egypt and Africa, which necessitates more descriptive works. Therefore, more morphogenetic works regarding *Coelatura* species and phylogenies in the Nile are strongly recommended. Especially, since more and more studies related to the biology and ecology of this genus are increasingly coming into interest. For example, it was assessed as a heavy metals-toxicity biomarker[25]. Even it is targeted for exploration of bioactive ingredients and characteristics, like its antitumor and antiosteoporosis activities[26,27].

Finally, the current study could present new primers pairs that could successfully be used to detect the presence of the targeted three species from their DNA traces in waters. For the best of our knowledge, this is the first time to provide such molecular techniques for eDNA-based detection of Nile bivalves. In general, and looking for works in major scholar databases like https://www.ncbi.nlm.nih.gov and https://scholar.google.com retrieved fewer similar approach for fluvial bivalves than for marine bivalves. This target seems to be crucial for conservation of natural biodiversity, especially in light of being one of the targeted genera in the current study, i.e. *Coelatura*, having some vulnerable species as reported in the IUCN red list of species[28]. This species is *C. alluaudi*, which shared the same subclade with the collected *Coelatura* sp. in the current study. eDNA-based methods serve mainly the objective of achieving conservation. Besides, they provide non-invasive, species-specific, and highly sensitive way for identifying fluvial bivalves whose cryptic diversities and phenotypic plasticity render their conventional sampling and identification methodologies missing some accuracy[29]. Therefore, and considering differential existence, abundance, and seasonal patterns of different River Nile unionid bivalves (for example, see Abd El-Wakeil *et al.*[30]), it is of great importance to use and develop further eDNA-based techniques for other species of mollusk bivalves to understand their exact distribution and diversity in that great fluvial ecosystem.

In conclusion, Nile fluvial bivalves elucidated necessities for further investigation of the actual genetic structures of some species, mainly the ones belonging to the genus *Coelatura*. Application of eDNA-based molecular techniques that were developed in the current study for the first time can provide powerful way for tracking their distribution along River Nile and its tributaries in Egypt, in order to provide more accurate knowledge about their biology, ecology, and taxonomy.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Graf, D. L. (2013). Patterns of freshwater bivalve global diversity and the state of phylogenetic studies on the Unionoida, Sphaeriidae, and Cyrenidae. Am Malacol Bul, 31: 135-153.

[2] Lopes-Lima, M.; Burlakova, L. E.; Karatayev, A. Y. *et al.* (2018). Conservation of freshwater bivalves at the global scale: diversity, threats and research needs. Hydrobiologia, 810*:* 1-14.

[3] Moore, T. P.; Collier, K. J. and Duggan, I. C. (2019). Interactions between Unionida and non-native species: a global meta-analysis. Aquat Conserv: Mar Freshw Ecosyst, 29(9): 1438-1451.

[4] Jansen, W.; Bauer G. and Zahner-Meike, E. (2001). Glochidial Mortality in Freshwater Mussels. In: Ecology and Evolutionary Biology of the Freshwater Mussels Unionoidea (Bauer, G. and Wachtler, K., eds), pp. 185-211. Springer-Verlag, Heidelberg.

[5] Vaughn, C. C. and Hoellein, T. J. (2018). Bivalve Impacts in Freshwater and Marine Ecosystems. Annu Rev Ecol Evol Syst, 49: 183-208.

[6] Chen, J.; Li, Q.; Kong, L. *et al*. (2011). How DNA barcodes complement taxonomy and explore species diversity: the case study of a poorly understood marine fauna. PLoS ONE, 6(6): e21326 (DOI: 10.1371/journal.pone.0021326).

[7] Sano, I.; Saito, T.; Ito, S. *et al.* (2022). Resolving species-level diversity of *Beringiana* and *Sinanodonta mussels* (Bivalvia: Unionidae) in the Japanese archipelago using genome-wide data. Mol Phylogenet Evol, 175: 107563 (DOI: 10.1016/j.ympev.2022.107563).

[8] Jacquet, J. L. and Pauly, D. (2008). Trade secrets: renaming and mislabeling of seafood. Mar Policy, 32(3): 309-318.

[9] Hebert, P. D. N.; Cywinska, A.; Ball, S. L. *et al*. (2003). Biological identifications through DNA barcodes. Proc Biol Sci, 270(1512): 313-321.

[10] Bikashvili, A.; Kachlishvili, N.; Japoshvili, B. *et al*. (2022). Species diversity and DNA barcode library of freshwater Molluscs of South Caucasus. Biodivers Data J, 10: e84887 (DOI: 10.3897/BDJ.10. e84887).

[11] Reunov, A.; Lutaenko, K.; Vekhova, E. *et al.* (2021). In the Asia-Pacific region, the *COI* DNA test revealed the divergence of the bivalve mollusc *Mactra chinensis* into three species; can these species be distinguished using shell coloration and sperm structure? Helgol Mar Res, 75: 7 (DOI: 10.1186/s10152-021-00553-0).

[12] Prié, V.; Danet, A.; Valentini, A. *et al.* (2023). Conservation assessment based on large-scale monitoring of eDNA: application to freshwater mussels. Biol Conserv, 283: 110089 (DOI: 10.1016/j.biocon.2023.110089).

[13] Mohammed-Geba, K.; Hassab El-Nabi, S. E. and El-Desoky, M. S. (2016). Development of cytochrome-c-oxidase 1 specific primers for genetic discrimination of the European eel *Anguilla* (Linnaeus, 1758). JBSAR, 2(4)*: 258-262.*

[14] Folmer, O.; Black, M.; Hoeh, W. *et al.* (1994). DNA primers for amplification of mitochondrial cytochrome C oxidase subunit I from diverse metazoan invertebrates. Mol Mar Biol Biotechnol, 3(5): 294-299.

[15] Kumar, S.; Stecher, G. and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol, 33: 1870-1874.

[16] Ronquist, F.; Teslenko, M.; van der Mark, P. *et al.* (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol, 61(3): 539-542.

[17] Darriba, D.; Taboada, G. L.; Doallo, R. *et al.* (2012). jModelTest 2: more models, new heuristics and parallel computing. Nat Methods, 9(8): 772 (DOI: 10.1038/nmeth.2109).

[18] Rambaut, A.; Drummond, A. J.; Xie, D. *et al.* (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. Syst Biol, 67(5): 901-904.

[19] Letunic, I. and Bork, P. (2021). Interactive tree of life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49(W1): W293-W296.

[20] Untergasser, A.; Nijveen, H.; Rao, X. *et al.* (2007). Primer3plus, an enhanced web interface to primer3. Nuc Acid Res, 35(Web Server issue): W71-W74.

[21] Huang, X.-C.; Su, J.-H.; Ouyang, J.-X. *et al.* (2019). Towards a global phylogeny of freshwater mussels (Bivalvia: Unionida): species de-limitation of Chinese taxa, mitochondrial phylogenomics, and diversification patterns. Mol Phylogenet Evol, 130: 45-59.

[22] Scholz, H. and Glaubrecht, M. (2004). Evaluating limnic diversity: toward a revision of the unionid bivalve *Coelatura* Conrad, 1853 in the Great Lakes of East Africa and adjacent drainage systems (Mollusca, Bivalvia, Unionidae). Zoosystematics Evol, 80: 89-121.

[23] Ortiz-Sepulveda, C. M; Stelbrink, B; Vekemans, X. *et al.* (2020). Diversification dynamics of freshwater bivalves (Unionidae: Parreysiinae: Coelaturini) indicate historic hydro-graphic connections throughout the East African Rift System. Mol Phylogenet Evol, 148: 106816 (DOI: 10.1016/j.ympev.2020.106816).

[24] Graf, D. L. and Cummings, K. S. (2007). Preliminary review of the freshwater mussels (Mollusca: Bivalvia: Unionoida) of northern Africa, with an emphasis on the Nile. J Egypt Ger Soc Zool (D. Invertebrate Zoology), 53: 89-118.

[25] Fahmy, S. R., & Sayed, D. A. (2017). Toxicological perturbations of zinc oxide nanoparticles in the *Coelatura aegyptiaca* mussel. Toxicol Ind Health, 33(7), 564-575.

[26] Sayed, A. A.; Soliman, A. M.; Fahmy, S. R. *et al.* (2013). Antiosteoporotic effect of *Coelatura aegyptiaca* shell powder on ovariectomized rats. Afr J Pharm Pharmacol, 7(34): 2406-2416.

[27] Soliman, A. M.; Fahmy, S. R. and El-Abied, S. A. (2015). Anti-neoplastic activities of *Sepia officinalis* ink and *Coelatura aegyptiaca* extracts against Ehrlich ascites carcinoma in Swiss albino mice. Int J Clin Exp Pathol, 8(4), 3543-3555.

[28] Seddon, M. B.; Van Damme, D. and Lange, C. (2017). *Coelatura alluaudi* (amended version of 2016 assessment). The IUCN Red List of Threatened Species 2017: e.T44248A110777886 (DOI: 10.2305/IUCN.UK.2017-1.RLTS.T44248A110777886. en).

[29] Gasparini, L.; Crookes, S.; Prosser, R. S. *et al.* (2020). Detection of freshwater mussels (Unionidae) using environmental DNA in riverine systems. Environ DNA, 2(3): 321-329.

[30] Abd El-Wakeil, K. F.; Obuid-Allah, A. H.; Mohamed, A. H. *et al.* (2013). Community structure of molluscans in River Nile and its branches in Assiut governorate, Egypt. Egypt J Aquat Res, 39(3): 193-198.

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تقنيات معتمدة على الحمض النووي الديوكسي ريبوزي والحمض النووي الديوكسي ريبوزي البيئي لتمييز ثلاثة من أنواع المحاريات النيلية في شمال نهر النيل بمصر

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تتميز الرخويات المحارية بكونها مغتذيات مفلترة نهمة، حيث تقوم بتصفية كميات كبيرة من الطحالب والبكتيريا والرواسب والمغذيات والجزيئات الأخرى من المياه، وتلعب من خلال هذه القدرة دورًا تنظيميًا رئيسيًا على المستويات القاعية والسطحية في النظم البيئية للمياه العذبة. لتطوير منهجية قائمة على الحمض النووي وتقنيات تعتمد على الحمض النووي البيئي لتحديد الأنواع وتتبعها بدقة، تم جمع ثلاثة أنواع من المحاريات من شمال نهر النيل، تنتمي إلى الأجناس "*Coelatura* و *Mutela* و *Chambardia*"، وتم إخضاعها للتشفير اللوحي للحمض النووي الديوكسي ريبوزي من خلال تحديد تسلسل منطقة الباركود فائقة الاختلاف لجين السيتوكروم أوكسيديز 1 للميتوكوندريا (*COI*)، وتحليلها نسبيًا. كذلك، تم استخدام التتابعات الناتجة لتصميم اختبارات متخصصة لكل نوع ومعتمدة على تفاعل البلمرة المتسلسل، حيث تم اختبار كفاءتها من خلال تحليل توافر الحمض النووي الديوكسي ريبوزي الخاص بتلك الأنواع في مياه أحد الأحواض، حيث تم جمع الأنواع الثلاثة بشكل اختباري. أظهرت النتائج أن الأنواع التي تم جمعها من مياه شمال النيل بمحافظة المنوفية للمحاريات "*Mutela rostrata* و *Chambardia rubens*" بشكل مؤكد، بينما أظهر النوع الثالث تقاربًا حتى مستوى النوع مع الشفرات اللوحية للنوع "*Coelatura aegyptiaca*"، لكن ذلك بهوية مختلطة مع نوعين آخرين للجنس "*Coelatura*" في شمال شرق إفريقيا. قدمت الاختبارات الجينية-البيئية المستندة إلى تفاعل البلمرة المتسلسل والحمض النووي الديوكسي ريبوزي البيئي الخاص بالأنواع المستكشفة، نتائج تكبير محددة وواضحة ومتمايزة حجمًا لكل نوع من الأنواع الثلاثة. وبناءً على هذه النتائج، توصي الدراسة بشدة بتوسيع نطاق تطبيق التقنييات الجزيئية لمزيد من تمييز التنوع المخفي في عالم محاريات نهر النيل في مصر، للكشف عن أنواعها الدقيقة، ووفرتها، وحالتها من الحفظ أو التهديد.