

## The Utilization of Molecular Identification for the Validation the Morphological Identification of the Cuttlefish (*Sepia pharaonis* Ehrenberg, 1831) from the Suez Canal, Egypt

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### ABSTRACT

The Cuttlefish *Sepia pharaonis*, is an Indo–Pacific organism and is one of the most economic species in the Suez Canal fisheries. Despite its economic value, there is a shortage of taxonomical information on the species. A total of 50 specimens of cuttlefish were collected from the Suez Canal. The sampling was during the period from winter to autumn 2021. Samples were identified morphologically and genetically by using *COI* gene. The species showed a genetic variation between the different populations of the same species and a genetic variation from the other species of cephalopods. The molecular identification method showed a great reliability in the identification of the cuttlefish.

### INTRODUCTION

The cuttlefish *Sepia pharaonis* is Indo – Pacific organism so it is found in the water of Red Sea in the north to the water of Japan and Australia in the south. With high economic value, it is on the top of Suez Canal fisheries and also, in the northern Indian Ocean, and it is the most profitable cephalopods species (Gabr *et al.*, 1998; Roper *et al.*, 2005).

From fisheries aspect, Cuttle fish have, broadly, high economic value. The lack of taxonomical and fisheries data with the presence of threatened species affect their economic value (Reid *et al.*, 2005; IUCN 2014).

The Suez Canal is an artificial water channel constructed in 1869, connecting the Red Sea and the Mediterranean Basin. The canal consists of Lake Timsah, Bitter Lakes and navigational channel (SCA 2023).

The process of analyzing a uniform gene in order to identify the species through DNA sequencing is referred to as DNA barcoding (Hebert *et al.*, 2003). For the differentiation between one species and another, the used short DNA sequence should

carry sufficient information to distinguish one species from another. The *cytochrome c oxidase subunit 1 (COI)* gene has been widely used as a barcoding gene. One benefit of using the *COI* gene is that its universal primers are quite reliable (Folmer *et al.*, 1994; Zhang and Hewitt, 1997).

DNA barcoding technique showed promising results in the identification of sepia and other cephalopod groups. The genetic variation of *Sepia officinalis* samples from the Bay of Biscay, the English Channel and the southern North Sea by seven microsatellite loci (Wolfram *et al.*, 2006). In 2009, mitochondrial genes were used for the primary identification of *Sepia* (Jacob and Huxley, 2009). Also, the genetic variations among 30 species represent families Octopoidae, Sepiidae and Serpiolidae were studied (Yuan *et al.*, 2012). Microsatellite locus were used as genetic marker for studying the genetic diversity of *Octopus vulgaris* (Casu *et al.*, 2002).

The recent work aims to confirm the identification of the cuttlefish (*Sepia pharaonis*) collected from Suez Canal with the aid of DNA barcoding technique.

## MATERIALS AND METHODS

A total of 50 individuals of *Sepia pharaonis* were collected during the period from winter to autumn 2021. The samples were collected by fishermen representing the three sampling sites (Figure 1). Samples for molecular identification were iced as soon as possible in situ then transferred to a  $-80^{\circ}\text{C}$  freezer in the laboratory.

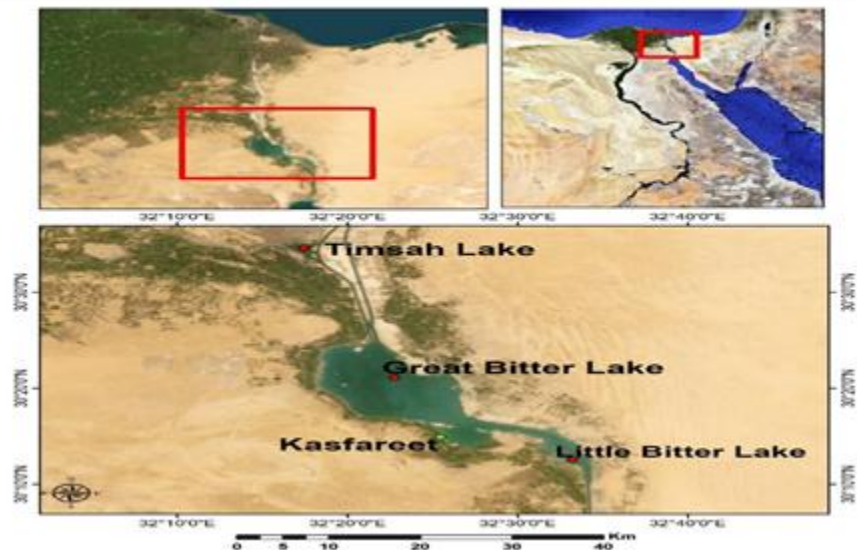


Figure 1. Sampling sites of *Sepia pharaonis*

Total genomic DNA was segregated from the tentacle muscle tissue of the samples by the DNeasy Blood & Tissue Kit DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) (cat. nos. 69504 and 69506) following manufacturer's protocol. The universal primers LCOI 1490F1-5'GGTCAACAAATCATAAAGATATTGG3' and HCOI 2198 - 5'TAAACTTCAGGGTGACCAAAAATCA3' (Folmer *et al.*, 1994), were used to amplify the target region of *COI* gene. Polymerase Chain Reaction was carried on under the following conditions: initial denaturation at  $95^{\circ}\text{C}$  for 5 min followed by 35 cycles of

45 sec at 94 °C, 45 sec at 54 °C, 60 sec at 72 °C and final extension at 72 °C for 10 min. The PCR products were visualized on 1.5% agarose gel.

The obtained sequences from the recent study have been submitted to the NCBI GenBank through BankIt portal and accession number are assigned (OR398860, OR398861 and OR398862). The sequences were proofread, aligned and edited using Mega Software (Tamura *et al.*, 2013). To confirm the identity of the species, the obtained sequences have been compared as a reference with those in the NCBI GenBank (similarity 95-100%) by the NCBI's BLASTn program. The phylogenetic relationship was obtained by constructing Maximum-likelihood tree between the sequences of *Sepia* species in the present study and that obtained from NCBI GenBank using the software program MEGA X (Tamura *et al.*, 2013). Bootstrap analysis was carried out using 500 pseudo replications. The genetic similarity matrix index was determined through pairwise comparison of three collected samples with NCBI GenBank samples using the MEGA X software program.

## RESULTS

DNA was extracted from a total of 50 samples of *S. pharaonis* collected from three distinct sampling locations. A total of nine PCR products that yielded successful amplification were subsequently subjected to sequencing in order to get the gene sequence of COI. PCR products of 650 – 700 bp were acquired from the samples under investigation (Figure 2).

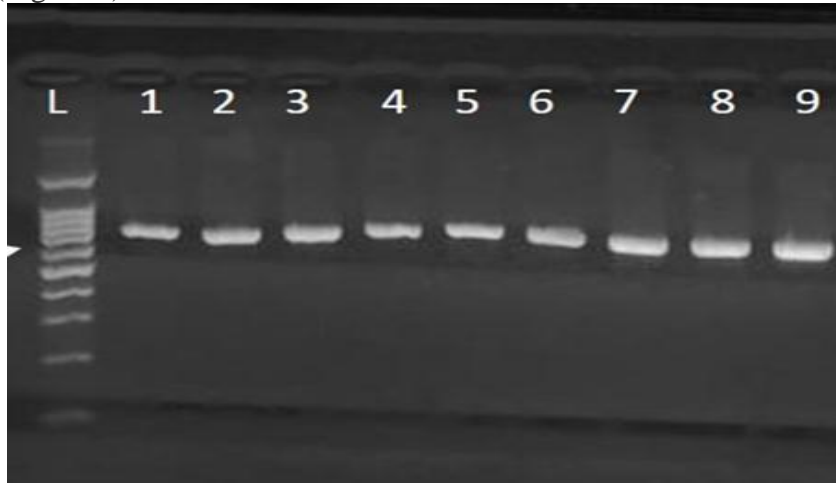


Figure 2. Electrophoresis of 1.5% agarose gel for PCR final product

The sequences obtained from the sampling sites were subjected to a phylogenetic analysis (Figure 3) which involved the construction of a Maximum-likelihood tree. The tree is rooted by the sequence of *Liza carinata* as out group. The sequence of Timsah Lake's sample was observed to be concentrated at a singular branch inside a subclade, distinct from the remaining samples.

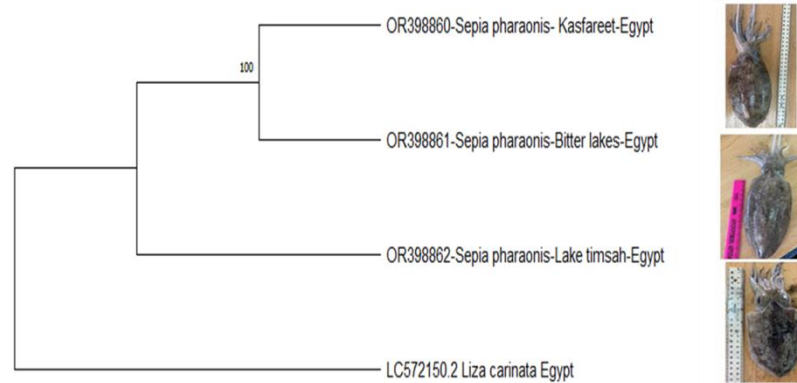


Figure 3. Maximum-Likelihood Phylogeny of *S. pharaonis* based on *COI* gene sequences.

The maximum-likelihood tree was employed to compare the sequences obtained in the study with the sequences available in the GenBank database (Figure 4). The sequences obtained from the investigation were shown to cluster together in a unique subclade, which had a high bootstrap value 87%. The results of the phylogenetic analysis indicate a relationship between these sequences and those originating from China and Japan, as supported by a high bootstrap value 49%.

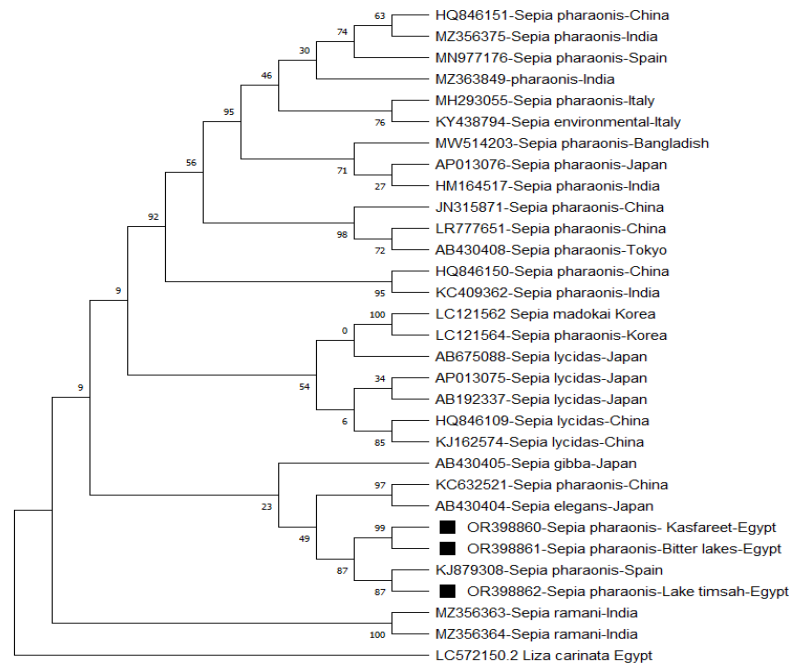


Figure 4. Maximum-Likelihood Phylogeny of *S. pharaonis* *COI* gene sequences of this study and sequences from the Genbank

The determination of the genetic similarity matrix index involved doing pairwise comparisons between three obtained samples and samples from the NCBI GenBank database, utilizing the MEGA X software program (Figure 5). The findings indicated that the sample exhibited a greater resemblance to samples originating from Korea. On the

contrary, the sample obtained from Lake Timsah exhibited a lesser degree of similarity when compared to the other samples analyzed in the current study.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1. OR398862-S. pharaonis-Lake Timsah-Egypt	0.101																				
2. KC632521-S. pharaonis-China	0.070	0.033																			
3. KJ879308-S. pharaonis-Spain	0.165	0.077	0.077																		
4. AB430405-S. gibba-Japan	0.186	0.089	0.093	0.117																	
5. HQ846130-S. pharaonis-China	0.206	0.097	0.110	0.122	0.047																
6. MW514203-S. pharaonis-Bangladesh	0.218	0.118	0.127	0.143	0.050	0.051															
7. JN15871-S. pharaonis-China	0.223	0.122	0.132	0.139	0.054	0.055	0.003														
8. LR77651-S. pharaonis-China	0.206	0.097	0.110	0.114	0.047	0.006	0.058	0.055													
9. ME93053-S. pharaonis-Italy	0.070	0.033	0.000	0.077	0.093	0.130	0.127	0.132	0.110												
10. OR398860-S. pharaonis-Kasfaree-Egypt	0.052	0.120	0.096	0.188	0.212	0.228	0.246	0.252	0.228	0.096											
11. OR398861-S. pharaonis-Bitter lakes-Egypt	3.311	3.216	3.292	3.406	3.434	3.553	3.349	3.338	3.329	3.292	3.444										
12. LC121562-S. madokai-Korea	0.101	0.000	0.033	0.077	0.089	0.097	0.118	0.122	0.097	0.033	0.120	3.216									
13. AB430404-S. elegans-Japan	0.168	0.099	0.099	0.123	0.130	0.134	0.142	0.146	0.134	0.099	0.192	3.456	0.099								
14. MZ363683-S. namani-India	3.347	3.239	3.327	3.526	3.353	3.539	3.375	3.364	3.316	3.327	3.485	0.177	3.239	3.538							
15. LC121564-S. pharaonis-Korea	0.223	0.122	0.132	0.139	0.054	0.055	0.003	0.000	0.055	0.132	0.252	3.338	0.122	0.146	3.364						
16. AB430408-S. pharaonis-Tokyo	0.207	0.126	0.122	0.145	0.130	0.160	0.149	0.154	0.160	0.122	0.217	3.152	0.126	0.156	3.012	0.154					
17. AP013075-S. lycidas-Japan	0.206	0.132	0.122	0.145	0.141	0.167	0.141	0.165	0.167	0.122	0.216	3.205	0.132	0.156	3.062	0.165	0.006				
18. HQ846109-S. lycidas-China	0.207	0.126	0.122	0.145	0.130	0.160	0.149	0.154	0.160	0.122	0.217	3.152	0.126	0.156	3.012	0.154	0.000	0.006			
19. AB192337-S. lycidas-Japan	0.216	0.097	0.118	0.122	0.061	0.013	0.058	0.055	0.013	0.118	0.239	3.541	0.097	0.143	3.327	0.055	0.169	0.176	0.169		
20. MZ363849-S. pharaonis-India																					

Figure 5. The genetic similarity matrix index of the three samples under study with the samples acquired from the gen bank.

## DISCUSSION

The traditional taxonomy of all samples collected from Suez Canal were executed, according to Roper et al., (1984) and FAO identification sheets. All collected samples were found to be *Sepia pharaonis*.

As a universal primer representing a wide range of phylogenetic characters, *COI* gene are widely used in species molecular identification (Hebert et al., 2003). The neighbor joining tree can clarify the ability of *COI* gene in distinguishing between cephalopod samples in an accurate way (Lincy et al., 2021). The results of the study agreed with previous facts, showing the ability of *COI* gene to distinguish not only between the species but also within the species. The genetic identification results supported the traditional taxonomy data defining the same species.

The results of the study reveal the genetic distances between samples of Suez Canal and each other and discriminate between them and other cephalopod samples around the world. The results agreed with many previous studies used molecular identification to describe and discriminate between cephalopod species (Avisé, 2000; Strugnell and Lindgren, 2007; Anderson et al., 2011; Allcock et al., 2011; Lincy et al., 2021).

According to non-discriminated morphological characters between cryptic species, DNA barcoding is considered as a useful tool for identification (Bickford et al., 2007). Molecular identification was used to discriminate between marine species complexes and reveal genetic distinction (Cheng et al., 2014). The maximum-likelihood tree of Suez Canal samples clustered the sample of Lake Timsah in a single branch of subclade although they are morphologically identical. This genetic difference may be due to the cryptic speciation of the Lake Timsah population.

The genetic similarity matrix index revealed that the species under study exhibited a significant resemblance to Korean samples, confirming their Indo-Pacific origin. This

may be an indicator for a start of Lesbian migration of the species through the Suez Canal.

The study of *COI* gene of *S. pharaonis* samples from Suez Canal reveal the ability of molecular identification between the species and within the species, more studies are needed to get a complete picture of genetic map of the species inhabit Suez Canal and to remain informed on the most recent developments about the migration of *S. pharaonis* from the Red Sea through the Suez Canal.

## REFERENCES

- Allcock, A.L.; Barratt, I.; Eleaume, M.; Linse, K.; Norman, M.D.; Smith, P.J. and Strugnell, J.M. (2011). Cryptic speciation and the circumpolarity debate: a case study on endemic Southern Ocean octopuses using the CO1 barcode of life. *Deep Sea Research. Part II: Topical Studies in Oceanography* 58:242-249. <https://doi.org/10.1016/j.dsr2.2010.05.016>
- Anderson, F.E.; Engelke, R.; Jarrett, K.; Valinassab, T.; Mohamed, K.S.; Asokan, P.K. and Dunning, M. (2011). Phylogeny of the *Sepia pharaonis* species complex (Cephalopoda: Sepiida) based on analyses of mitochondrial and nuclear DNA sequence data. *J. Molluscan Stud.* 77:65-75. <http://dx.doi.org/10.1093/mollus/eyq034>.
- Avice, J.C. (2000). *Phylogeography: The history and formation of species*. Harvard University Press, Cambridge, MA, pp.447.
- Bickford, D.; Lohman, D.J.; Sodhi, N.S.; Ng, P.K.L.; Meier, R.; Winker, K. and Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends Ecol. Evol.* 22:148-155. <https://doi.org/10.1016/j.tree.2006.11.004>.
- Casu, M.; Ferruccio, M.; Marilena, M.; Daniela, C.; Piero, C. and Alberto, C. (2002). Genetic structure of *Octopus vulgaris* (Mollusca, Cephalopoda) from the Mediterranean Sea as revealed by a microsatellite locus. *Ital. J. Zool.*, 69: 295-300.
- Folmer, O.; Black, M.; Hoeh, W.; Lutz, R. and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.*, 3: 294-299.
- Gabr, H. R.; Hanlon, R. T.; Hanafy, M. H. and El-Etreby, S. G. (1998). Maturation, fecundity and seasonality of reproduction of two commercially valuable cuttlefish, *Sepia pharaonis* and *S. dollfusi*, in the Suez Canal. *Fish. Res.*, 36: 99 – 115.
- Hebert, P. D.N.; Cywinska, A.; Ball, S. L.; and deWaard J. R. (2003). Biological identifications through DNA barcodes. *Proc R Soc Lond B.*, 270: 313-321
- IUCN, 2014 The IUCN Red List of Threatened Species. Version 2014.2. Available at: [www.iucnredlist.org](http://www.iucnredlist.org).
- Jacop, M. and Huxley, A. (2009). Molecular diversity analysis in cuttle fish (*Sepia officinalis*) populations using RAPD markers. *Adv. Biotech.*, 8: 23-26.

- Lincy, A.; Anil, M. K.; Thangaraj, M. and Jose, J.J. (2021). Molecular identification and genetic variation studies in economically important cephalopods at Beypore Fishing Harbour (Kozhikode), South West coast of India. *Not Sci Biol* 13(1):10862.
- Reid, A.; Jerep, P. and Roper, C. F. E. (2005). Family Sepiidae. In: *Cephalopods of the world. An annotated and illustrated catalogue of Cephalopod species known to date. Vol.1. Chambered Nautilus and Sepioids (Nautilidae, Sepiidae, Sepiolidae, Sepiadariidae, Idiosepiidae and Spirulidae)*. Jerep P., Roper C. F. E. (eds), FAO, Rome, pp. 54-152.
- Roper, C.F.E.; Sweeny, M.J. and Nauen, C.E. (1984). *Cephalopods of the world*. FAO Fisheries Synopsis. 3(125). 277 pp.
- Roper, C. F. E.; Sweeney, M. J. and Nauen, C. E. (2005). *Cephalopods of the World*. FAO.
- SCA 2023. New Suez Canal. [SuezCanalgov.eg/English/about/Suezcanal//pages/NewSuezCanal.aspx](http://SuezCanalgov.eg/English/about/Suezcanal//pages/NewSuezCanal.aspx). Accessed: June, 2023.
- Strugnell, J.M. and Lindgren, A.R. (2007). A barcode of life database for the Cephalopoda? Considerations and concerns. *Rev. Fish Biol. Fish.*17:337-344. <https://doi.org/10.1007/s11160-007-9043-0>
- Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A. and Kumar, S. (2013), MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30: 2725 - 2729.
- Wolfram, A.; Mark, F.C; John, U.; Lucassen, M. and Pörtner, H.O. (2006). Microsatellite DNA variation indicates low levels of genetic differentiation among cuttlefish (*Sepia officinalis* L.) populations in the English Channel and the Bay of Biscay. *CBP. Part D*, 1: 375-383.
- Yuan, Y.; Liu, S.; Bai, B.; Liu, H. and Zhimeng, Z. (2012). Isolation and Characterization of New 24 Microsatellite DNA Markers for Golden Cuttlefish (*Sepia esculenta*). *Int. J. Mol. Sci.*, 13: 1154-1160.
- Zhang, D.-X. and Hewitt, G. M. (1997). Assessment of the universality and utility of a set of conserved mitochondrial primers in insects. *Insect Mol. Biol.*, 6:143–150.