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Research Article

Molecular identification and genetic variation studies in economically important cephalopods at Beypore Fishing Harbour (Kozhikode), South West coast of India

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Abstract

Cephalopods are ecologically and economically important marine groups in the world. Biodiversity description is essential for sustainable utilization of natural resources and to characterize biological entities for conservation. DNA barcoding is an effective tool used for identification of organisms at species level and is been widely used for delineate several ambiguity species. In this study, partial sequence of mitochondrial cytochrome c oxidase 1 (CO1) gene with a mean size of 680 bp was amplified by universal primers. Totally 13 individuals of Cephalopods comprising of three species, were barcoded and genetic variation was analysed. The maximum A+T content (67.60%) was recorded in *Cistopus indicus* and minimum (63.70%) in *Sepioteuthis lessoniana*. The maximum K2P distance (0.268) was found between the genus *Cistopus* and *Sepioteuthis* whereas the minimum distance (0.188) was observed between *Uroteuthis* and *Sepia.* The neighbour joining tree revealed three distinct clades represents Loligonidae, Sepiidae and Octopodidae with high boot strap values. However, *Sepioteuthis lessoniana* is showing a bifurcated branch and it may due to the co-occurring of cryptic species and till date this species is treated as *Sepioteuthis lessoniana* complex.

Keywords: cephalopods; cytochrome c oxidase 1; DNA barcoding; phylogenetic analysis

Introduction

Cephalopods are coming under the phylum Mollusca, which is having two major groups, the Nautiloids and Coleoids. Nautiloids are covered by an external shell and the Coleoids are lacking this external shell. Coleoids comprises of four orders: Sepioidea, Teuthida, Vampyromorphida and Octopodida. (Yalla and Mohanraju, 2019). They inhabit mainly in coral reef ecosystems taking advantages of this ecosystem as spawning ground, egg placement site, and shelter for the young ones (Pratasik *et al.*, 2019). Cephalopods are known to have diverse body patterns that can immediately change their colour, being controlled by its chromatophore system (Hanlon *et al.*, 2009). The major cephalopod groups are easily distinguishable by

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morphological and anatomical features. But morphological features can facilitate in identification only up to sub family or generic level (Voight, 1993). It is very difficult to discriminate at species level based on traditional morphological characters (Herke and Foltz, 2002). In Indian seas about 210 species were recorded (Appukuttan, 1996), of which 80 species are of commercial importance (Yalla and Mohanraju, 2019).

The present study focuses on genetic variations using DNA sequence of mitochondrial genome as DNA barcode. DNA barcoding is the latest molecular tool that uses a short genetic marker of an organism's genome to identify them at species level (Yalla and Mohanraju, 2019). Cytochrome c oxidase 1 gene (CO1) is the most common mitochondrial gene used for species identification (Folmer *et al.*, 1994) due to its conserved nature among the protein coding genes. It has become a standard tool of molecular taxonomy and species identification (Ratnasingham and Hebert, 2007). A CO1 gene sequence determines relationships within the coleoid cephalopods at higher level (Carlini and Graves, 1999). Molecular techniques have been well applied for stock discrimination studies in fisheries (Murphy *et al.*, 2002) and can provide the basis for better management of whole populations and sustainable fisheries (Pratasik *et al.*, 2019). This study aims to examine the genetic characteristics of some cephalopod species collected from the Beypore Fishing Harbour, Kozhikode, South west coastal waters of India.

Materials and Methods

Sample collection

Cephalopod samples such as *Sepia pharaonis*, *Uroteuthis duvauceli* and *Sepioteuthis lessoniana* were collected from the Beypore Fishing Harbour, Kozhikode (Latitude 11° 09′N; Longitude 75° 48′N). The collected samples were transported to the laboratory in ice boxes. All the specimens were identified morphologically and anatomically using FAO identification keys (FAO, 2006). Tissue samples of all individuals were stored in 95% ethanol for further molecular studies.

Genomic DNA isolation, amplification and sequencing

Total genomic DNA was isolated from the tentacle muscle tissue according to method of Sambrook *et al.* (2001). The isolated DNA was quantified by an UV spectrophotometer. The cytochrome c oxidase subunit I (COI) gene was amplified in a 50 µl volume with 100 ng template DNA, 10 µmol of each specific primer, 200 µM of each dNTPs, 1.0 units of Taq DNA polymerase and 1× Taq buffer containing 1.5 mM $MgCl_2$. The universal primers *LCOI 1490F1*-5'GGTCAACAAATCATAAAGATATTGG3' and *HCOI 2198* - 5'TAAACTTCAGGGTGACCAAAAAATCA3' (Folmer *et al.*, 1994), were used to amplify the COI gene. The PCR conditions were initial denaturation at 95 °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 gels. The amplified product was sequenced by a commercial sequencing facility (Eurofins, Bangalore).

Sequence analysis

The thirteen sequences of the present study have been submitted to the NCBI GenBank through BankIt portal and accession number will be assigned soon. The size of each sequence was 684 bp. The amplified sequences belonging to COI gene were confirmed by percent similarity in the NCBI's BLASTn program. Higher percentage similarity (95-100%) against the reference sequence was used to confirm the identity of the species. Thirteen nucleotide sequences of three taxa from the present study along with six reference sequences from GenBank (Badhe *et al.*, 2013) were incorporated for the phylogenetic study analysis. Nucleotide composition, genetic variation and pairwise evolutionary distance among sequences were estimated by Kimura 2-parameter (K2P) method (Kimura, 1980) using the software program MEGA 5 (Kumar *et al.*, 2011). The

maximum likelihood (ML) tree was constructed and to verify the robustness of the internal nodes of these trees, bootstrap analysis was carried out using 1000 pseudo replications.

Results and Discussion

Nucleotide composition

All the sequences were conceptually translated into proteins using invertebrate mitochondrial genetic code translation pattern. There were no insertions, deletions or stop codons in any of the obtained sequences. The nucleotide frequency in five cephalopod species is given in Figure 1. Thiamine was more (0.372) and guanine was less (0.152) frequency in *C. indicus*. Whereas, thiamine was less (0.354) and guanine frequency was more (0.168) in *U. edulis* when compared with *C. indicus*. The fours nucleotides *i.e.* A, T, G and C were divided into two groups as A+T and C+G. The maximum A+T content (67.60%) was recorded in *C. indicus* and minimum (63.70%) in *S. lessoniana*. Similarly, the maximum C+G value of 36.30% was observed in *S. lessoniana* and least content of 32.40% was estimated in *C. indicus*.

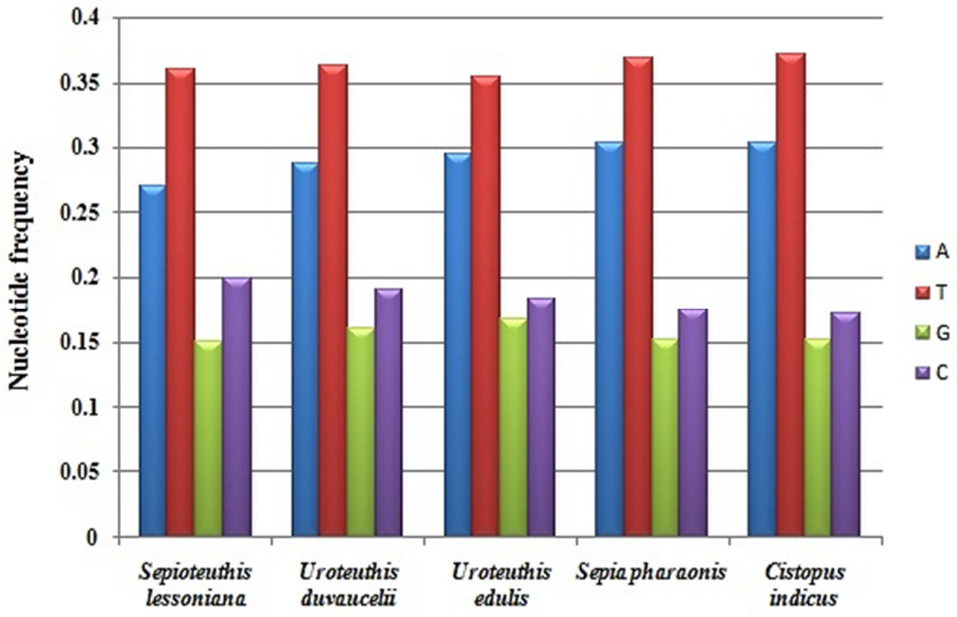

Figure 1. Nucleotide frequency of CO1 gene in cephalopod species

Genetic distance between families

The genetic distance was measured by K2P parameter in three cephalopod families in this study and Lower level of value was observed among the families. The maximum K2P distance (0.262) was found between Octopodidae and Loligonidae. Whereas, the minimum K2P distance of 0.194 was observed between Loligonidae and Sepiidae.

Genetic distance between genus

Genetic distance between genus and the result are shown in Table 1. The maximum K2P distance (0.268) was found between the genus *Cistopus* and *Sepioteuthis* whereas the minimum distance (0.188) was observed between *Uroteuthis* and *Sepia.* The maximum K2P distance within genus was observed in *Sepioteuthis* (0.057). The intra genus K2P genetic distance in *Sepia* was zero.

Table 1. K2P genetic distance between cephalopod species based on CO1 gene sequence

Species	*S. pharaonis*	*U. duvaucelii*	*S. lessoniana*	*U. edulis*	*C. indicus*
Sepia pharaonis	**0.000**				
Uroteuthis duvaucelii	0.188	**0.004**			
Sepioteuthis lessoniana	0.201	0.211	**0.057**		
Uroteuthis edulis	0.188	0.185	0.225	**0.003**	
Cistopus indicus	0.236	0.265	0.268	0.248	**0.030**

Phylogenetic relationship

The evolutionary history was inferred by using the Maximum Likelihood method based on the K2P model. Initial tree(s) for the heuristic search were obtained automatically, when the number of common sites was < 100 or less than one fourth of the total number of sites in the maximum parsimony method. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The neighbour joining tree clearly demonstrates the efficacy of COI gene in discriminating cephalopod individuals both at conspecific and congeneric levels (Figure 2). The genus and species level discrimination can be ascertained by strong bootstrap support of over 99%. All the cephalopod families formed a distinct clade. Topologically two major clades were formed; one major clade comprising two families with four species (*Sepia pharaonis*, *Uroteuthis duvauceli,* S*epioteuthis lessoniana* and *Uroteuthis edulis*). The next clade consisting of only Octopodidae. From this NJ tree it can be also visualized that the clade entailing Sepiidae, Loliginidae and Octopodidae family showed perfect distinction between the genus within the family by high bootstrap values (67-100).

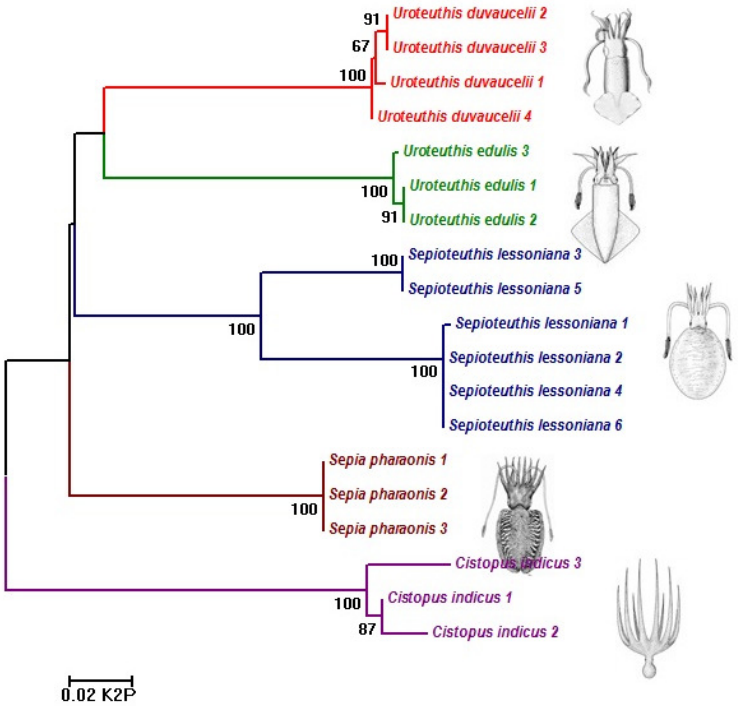

Figure 2. NJ Phylogeny of cephalopod species based on CO1 gene sequence with bootstrap values

Due to the non distinguishable morphological characters we can use molecular tools to detect cryptic species via the phylogenetic species concept (Bickford *et al.*, 2007). CO1 gene got more popular due to the universal primers potential to represent a broad range of phylogenetic signal than other mitochondrial genes (Hebert *et al.*, 2003). Using this molecular technique, many marine species that were once considered as complex are actually comprised of many genetically distinct species (Cheng *et al.*, 2014). The data collected in this study allowed for a robust and extensive phylogenetic assessment of five cephalopod species and the COI gene clearly delineate the five species. The divergences found between lineages exceed values commonly observed for congeneric species (Avise, 2000) and similar very deep divergences in mitochondrial gene regions have been reported in cephalopods (Strugnell and Lindgren, 2007) including the *Sepia pharoanis* (Anderson *et al.*, 2011) and *Sepioteuthis lessoniana* (Allcock *et al.*, 2011) complexes. The average transversional pairs (Tv= 51) were ten times lesser than transitional pairs (Ti=523) with an average ratio of 10.25. This value was relatively more when comparing with earlier study by Badhe *et al.* (2013) in cephalopods. The mean sequence length in this study was 680bp and each of this sequence clustered tightly with conspecific sequences. Same kind of result was also reported by Badhe *et al.* (2013). Hajibabaei *et al.* (2005) stated that at least 100-200bp of CO1 gene sequence is enough to discriminate a species. The average GC content in cephalopod CO1 gene was 34.8% which is lower than teleost's CG content where it was 47.10% as per Ward *et al.* (2005). The barcoded sequences clearly demonstrated the taxonomic status of all the five species studied.

Hebert (2004) insisted a standard barcode sequence threshold level of 10X the mean intraspecific genetic variation for differentiate species. This barcoding gap is sufficient to discriminate the cephalopods to their taxonomic position (Badhe *et al.*, 2013). The COI genetic variation in cephalopods increased from lower taxonomic level to higher levels and supported and demonstrated the genetic divergence at the species level. This observation supports the previous findings of Hubert *et al.* (2008). The Neighbour Joining tree revealed distinct clades where closely related species were clustered under same nodes while distantly related species were clustered under separate nodes with high boot strap values (Figure 2). But, *S. lessoniana* is showing a bifurcated branch and it may due to the co-occurring of cryptic species and till date this species is treated as *S. lessoniana* complex.

Conclusions

The results from our data clearly discriminated all the species collected from Beypore Fishing Harbour, Kozhikode and were congruent with the taxonomic divisions of the cephalopods. However, for defining new species some more molecular markers might be necessary for accurate description.

Authors' Contributions

Work designed by: LA, MKA. Animals collected and identified by: LA, JJJ. Experiments performed by: LA, JJJ, MT. Data analyzed by: LA, JJJ, MT. Reagents/materials/analysis tools provided by: MKA, MT. Paper written by: LA, JJJ, MT. The final draft edited by: MKA. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Allcock AL, Barratt I, Eleaume M, Linse K, Norman MD, Smith PJ, ... Strugnell JM (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 FE, Engelke R, Jarrett K, Valinassab T, Mohamed KS, Asokan PK, ... Dunning M (2011). Phylogeny of the *Sepia pharaonis* species complex (Cephalopoda: Sepiida) based on analyses of mitochondrial and nuclear DNA sequence data. Journal of Molluscan Studies 77:65-75. *http://dx.doi.org/10.1093/mollus/eyq034*

Appukuttan K (1996). Marine molluscs and their conservation. In: Menon NG, Pillai CSG (Eds). Marine Biodiversity, Conservation and Management CMFRI pp 66-80. *http://eprints.cmfri.org.in/id/eprint/4097*

Avise JC (2000). Phylogeography: The history and formation of species. Harvard University Press, Cambridge, MA, pp 447.

Badhe MR, Pavan-Kumar A, Gireesh-Babu P, Nandanpapwar P, Chaudhari A, Jaiswar AK, ... Lakra WS (2013). DNA barcoding of selected cephalopods from Indian coast. Indian Journal of Animal Science 83(8):862-866.

Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, ... Das I (2007). Cryptic species as a window on diversity and conservation. Trends in Ecology and Evolution 22:148-155. *https://doi.org/10.1016/j.tree.2006.11.004*

Carlini DB, Graves JE (1999). Phylogenetic analysis of cytochrome c oxidase I sequences to determine higher-level relationships within the coleoid cephalopods. Bulletin of Marine Science 64:57-76.

Cheng SH, Anderson FE, Bergman A, Mahardika GN, Muchlisin ZA, Dang BT, ... Barber PH (2014). Molecular evidence for co-occurring cryptic lineages within the *Sepioteuthis* cf. *lessoniana* species complex in the Indian and Indo-West Pacific Oceans. Hydrobiologia 725:165-188. *https://doi.org/10.1007/s10750-013-1778-0*

FAO (2006). Western Indian Ocean. Fishing area 51. Volumes I-VI. FAO Species Identification Sheets for Fishery Purposes. Rome, FAO. 2006. CD-ROM.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3:294-299.

Hajibabaei M, Smith AA, Janzen DH, Rodriguez JJ, Whitfield JB, Hebert PDN (2005). A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes 6:959-964. *https://doi.org/10.1111/j.1471-8286.2006.01470.x*

Hanlon RT, Chiao CC, Mäthger LM, Barbosa A, Buresch KC, Chubb C (2009). Cephalopod dynamic camouflage: bridging the continuum between background matching and disruptive coloration. Philosophical Transactions of the Royal Society of London B: Biological Sciences 364:429-437. *Https://doi.org/10.1098/rstb.2008.0270*

Hebert PD, Stoeckle MY, Zemlak TS, Francis CM (2004). Identification of birds through DNA barcodes. Plos Biology 2(10):e312. *https://doi.org/10.1371/journal.pbio.0020312*

Hebert PDN, Ratnasingham S, Waard JR (2003). Barcoding animal life: cytochrome C oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society of London B: Biological Sciences 270:S96-99. *https://doi.org/10.1098/rsbl.2003.0025*

Herke SW, Foltz DW (2002). Phylogeography of two squid (*Loligo pealei* and *L. plei*) in the Gulf of Mexico and Northwestern Atlantic Ocean. Marine Biology 140:103-115. *https://doi.org/10.1007/s002270100680*

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, ... Bernatchez L (2008). Identifying Canadian freshwater fishes through DNA barcodes. PLoS One 3:e2490. *https://doi.org/10.1371/journal.pone.0002490*

Kimura M (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120. *https://doi.org/10.1007/BF01731581*

Kumar S, Tamura K, Peterson D, Peterson N, Stecher G, Nei M (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731-2739. *https://doi.org/10.1093/molbev/msr121*

Murphy JM, Balguerias E, Key LN, Boyle PR (2002). Microsatellite DNA markers discriminate between two *Octopus vulgaris* (Cephalopoda: Octopoda) fisheries along the Northwest African Coast. Bulletin of Marine Science 71:545-553.

Pratasik SB, Sambali H, Manoppo L, Tilaar FF, Salaki MS (2019). Genetic variations of cuttlefish *Sepia latimanus* (Cephalopoda, Sepiidae) in North Sulawesi waters, Indonesia. Aquaculture, Aquarium, Conservation & Legislation 12(3):792-803.

Ratnasingham S, Hebert PDN (2007). BOLD: the barcode of life data system. Molecular Ecology Notes 7:355-364. *https://doi.org/10.1111/j.1471-8286.2007.01678.x*

Sambrook SJ, Russel DW, Janssen KA, Irwuin NJ (2001). Molecular cloning: a laboratory manual. 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

Strugnell JM, Lindgren AR (2007). A barcode of life database for the Cephalopoda? Considerations and concerns. Reviews in Fish Biology and Fisheries 17:337-344. *https://doi.org/10.1007/s11160-007-9043-0*

Voight JR (1993). A cladistic reassessment of Octopodid classification. Malacologia 35:343-349.

Ward RD, Zemlak TS, Innes BH, Last PA, Hebert PDN (2005). DNA barcoding Australia fish species. Philosophical Transactions Royal Society B: Biological Sciences 360(1462):1847-1857. *https://doi.org/10.1098/rstb.2005.1716*

Yalla SK, Mohanraju R (2019). DNA barcoding of commercially important cephalopods from Andaman Islands, India. Regional Studies in Marine Science 25:100479. *https://doi.org/10.1016/j.rsma.2018.100479*