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Sam Peter
School of Aquaculture and Biotechnology, Kerala University of Fisheries and Ocean Studies, Panangad, Cochin, 682506, Kerala, India.

B. Manoj Kumar
Associate Professor, School of Fisheries Resource Management and Harvest Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Cochin, 682506, Kerala, India

Fejila Agnes
School of Fisheries Resource Management and Harvest Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Cochin, 682506, Kerala, India

Devika Pillai
Associate Professor, School of Aquaculture and Biotechnology, Kerala University of Fisheries and Ocean Studies, Panangad, Cochin, Kerala, India

Correspondence
Devika Pillai
Associate Professor, School of Aquaculture and Biotechnology, Kerala University of Fisheries and Ocean Studies, Panangad, Cochin, Kerala, India
Email:
devikamanoj.pillai@gmail.com

Molecular Characterization and Phylogenetic Analysis of *Diphyes dispar* (Siphonophora: *Diphyidae*) from the Laccadive Sea, off the south-west coast of Arabian Sea, Indian Ocean

Sam Peter, B. Manoj Kumar, Fejila Agnes and Devika Pillai

Abstract
Diphyes dispar is a hydroid in the family *Diphyidae*. Ecologically, they are insatiable predators of zooplankton and can have significant effects on marine ecosystem formation and functioning. Using morphological characters to accurately assign this species based on taxonomy would require comprehensive anatomical studies. Molecular characterization using DNA barcodes are useful to discover new species, reveal cryptic species and assess taxonomically-significant variation within species with broad or disjunct distributions. In this study, a portion of the mitochondrial Cytochrome Oxidase I (mtCOI) gene obtained from *D. dispar* was used as DNA barcode for molecular taxonomy. Kimura-2-Parameter (K2P) genetic distance between sequence variants within the *dispar* species ranged from 0 to 0.058 (mean 0.033). K2P genetic distances were significantly lower between individuals of the same species than between individuals of different species within the genus, *Diphyes* (mean 0.133; S.D.0.011). A phylogenetic tree generated by Neighbor Joining (NJ) using K2P distances reliably clustered all barcodes of the same species with 100% bootstrap support, ensuring accurate identification of species. Intra- and inter-specific variation of the mtCOI gene is appropriate to be used as a DNA barcode for species-level identification and phylogenetic analysis. This is the first study conducted to characterize *D.dispar* from the Laccadive Sea, off the south-west coast of Arabian Sea in the Indian Ocean using the sequence analysis of mtCOI. This study provides a set of molecular tools that can be used to address questions of speciation, biodiversity, and population boundaries. In light of the crucial position of zooplankton in ocean food webs, their usefulness as rapid responders to environmental alteration, and the increasing scarcity of taxonomists, the use of DNA barcodes is an important and useful approach for rapid analysis of species diversity and distribution.

Keywords: DNA Barcoding; mtCOI; Phylogenetic Analysis; Gelatinous Zooplankton; *Diphyes dispar*

1. Introduction
Gelatinous zooplankton is the least understood of all planktonic animal groups. This is partly due to their fragility, which typically precludes the capture of intact specimens with nets or trawls. These animals include some radiolarians and foraminifera, as well as medusae, pyrosomes, ctenophores, chaetognaths, pteropods, heteropods, appendicularians, salps, doliolids, and siphonophores [1]. The siphonophores are complex polymorphic pelagic cnidarians that are widespread in the marine pelagic realm. Although a siphonophore appears to be a single organism, each specimen is actually a colony composed of many individual animals [2]. Some siphonophores are the longest animals in the world, and specimens as long as 40 meters have been found [3]. There are about 175 described species [4]. *Diphyes dispar*, the organism covered in this study is a hydroid in the family *Diphyidae*. The polyp comes from the seed *Diphyes. D. dispar* was for the first time scientifically described in 1821 by Chamisso & Eysenhardt [5]. Ecologically, they are insatiable predators of zooplankton and can have dramatic effects on marine ecosystem structure and functioning.
The ecology and taxonomy of medusozoa and ctenophora have received very less attention in India. Medusozoa is a clade in the phylum Cnidaria, includes the classes Hydrozoa, Scyphozoa, Staurozoa and Cubozoa. Since all groups of cnidarians have not received adequate attention in Indian waters, the number of taxonomical works conducted in the past were found to be limited [6, 7, 8, 9, 10, 11]. A considerable amount of published information is available on

siphonophora from the Atlantic Ocean and some from the Pacific and Indian Oceans [12, 13, 14]. Rengarajan (1974) [15] has identified a total of 47 species from the west coast of India and Daniel (1974; 1966) [16, 17] from the west and east coasts of India, but they were all studies about surface water distribution patterns and morphological features.

Phenotypic plasticity of hydropolyps [18] and scyphistomae [19] as well as morphological divergence associated with geographic distance [20] can render morphological characters of this group more ambiguous. Using morphological characters to accurately assign species based on taxonomy would require comprehensive anatomical studies of every phase in each hydrozoan's life cycle. Species of calycophoran siphonophores including *D. dispar* can have a biphasic life-cycle consisting of polygastric and eudoxid stages that are morphologically distinct or unknown [21]. Other life-history stages such as cysts, plannulae, and actinulae cannot be accurately identified using morphological characters [22]. Morphologically indistinguishable, yet genetically and evolutionarily distinct species i.e., cryptic species can best be discovered through the use of molecular techniques like DNA barcoding [23].

DNA barcodes (short DNA sequences used for species recognition and discrimination) are ancillary and logically independent characters that permit identification of an unknown specimen in terms of a known classification [24]. DNA barcodes are also useful to discover new species, reveal cryptic species and assess taxonomically-significant variation within species with broad or disjunct distributions [25, 26]. The usual DNA barcode region for animals is a 708 base-pair region of mitochondrial Cytochrome Oxidase I (mtCOI), which exhibits favorable levels of divergence within and between species of most hydrozoan groups to allow accurate species identification [22]. In this respect, the objective of the present study was focused on the molecular detection of *D. dispar* by DNA barcoding. In addition, sequence comparisons and phylogenetic relationship of organisms among the family of *Diphyidae* from different geographical regions were studied. This is the first study conducted to characterize *D. dispar* from the Laccadive Sea, off the south-west coast of Arabian Sea, Indian Ocean using the sequence analysis of mtCOI.

2. Materials and Methods

2.1Collection of *Diphyes dispar* for Molecular Analysis.

A biodiversity survey of gelatinous zooplankton from surface waters of the south-west coast of India (off Cochin to Minicoy and Kalpeni islands of Laccadive Sea) was carried out during an oceanographic research on the *FOR/V Sagar Sampada* from 13 July to 02 August 2015.Specimens of zooplankton including *D. dispar* were quantitatively sampled from five stations (Table.1) of the Laccadive Sea, off the south-west coast of Arabian Sea, Indian Ocean using plankton nets. Immediately after net recovery, specimens were examined and gelatinous forms, small fishes, and macro-zooplankton/nekton were removed. Specimens were split into two; one set was preserved in formalin and the other in 95% ethanol for molecular analysis, using protocols described by Bucklin (2000) [27]. Most specimens were analyzed within a few days, but those not analyzed immediately were archived for longer-term storage, and the alcohol was changed 24 hr after collection. Along with *D. dispar* specimens, specimens of other identified gelatinous zooplankton were also shipped to the Kerala University of Fisheries and Ocean Studies, Panangad, Cochin for molecular analysis and DNA barcoding and stored at -80 ºC until use. Specimens designated for barcoding were examined under a stereo zoom microscope soon after collection. For species smaller than ~25 mm, at least one intact individual was retained from at least one collection as a physical voucher and up to three individuals from the remaining collection were removed and the entire organisms extracted. For species larger than ~25 mm, an intact individual from one collection was retained where possible, as long as three other individuals were present from which to remove a small portion for extraction (i.e., at least 4 total individuals). If fewer than four individuals were collected, the smallest portion allowable for DNA extraction was removed from each from a non-taxonomically important region of the specimen [28].

Table 1: Specimen information includes: Species Name, Station No. Voucher Number (V. No.), Collection Location given as Latitude (Lat °N), Longitude (Long °E), and Date Collected (Coll. Date).

Sp. Name	St. No.	V. No.	Coll. Location	Lat° N	Long° E	Coll. Date
Diphyes dispar	1	CR.342-Didi-01	Minicoy Off	08°16.040	73° 10.819	22 July 2015
	2	CR.342-Didi-02	Minicoy Coast	08°19.963	73° 08.446	22 July 2015
	3	CR.342-Didi-03	Minicoy Coast	08°14.778	73° 00.686	23 July 2015
	4	CR.342-Didi-04	Minicoy Off	08°33.340	72° 47.070	25 July 2015
	5	CR.342-Didi-05	Kalpeni Off	10°10.328	73° 40.157	29 July 2015

2.2 Molecular Analysis

DNA was purified from individuals of *D. dispar* by salting out procedure of Miller *et al*. (1988) [29]. A portion of the mtCOI gene was amplified with the universal published primers [30] (Table 2). A 660 base-pair region of mtCOI was amplified in a Gene Amp 9600 PCR machine (Applied Biosystems, Inc.). The PCR protocol was 94 ºC for 1min, 45 ºC for 2min, and 72 ºC for 3min, for 40 cycles. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Bands were photographed using a Gel documentation and Analysis System (Bio-Rad). Fragment size was determined with reference to a 100-bp ladder (Fermentas US).

Table 2: Primer Name, Sequence, Size and source for PCR and sequencing primers used in the study

Primer Name	Sequence 5'-3'	Estimated Size	Reference
LCO-1490	GGTCAACAAATCATAAAGATATTGG	660 bp	Folmer *et al*., 1994
HCO-2198	TAAACTTCAGGGTGACCAAAAAATCA		Folmer *et al*., 1994

2.3 DNA Sequencing and Phylogenetic Analysis

Specific amplicons were excised from the agarose gel and extracted using a QIAquick Gel Extraction Kit (Qiagen, Germany), according to the manufacturer's instructions. Sequencing was performed directly from purified PCR amplification products on an applied Biosystems, Inc. (ABI) Model 377 automated DNA sequencer (Foster City, CA) using the forward and reverse primers. The generated consensus sequences were then compared with sequences in Gen Bank using BLAST (Basic Local Alignment Search Tool) on the NCBI (National Centre for Biotechnology Information). Multiple Sequence Alignment (MSA) was performed in ClustalW [31] for partial COI gene sequence with the default parameters. The complete alignment was trimmed to a length of 651 base-pairs for preliminary analysis to confirm the accuracy and validity of the sequences. The verified mtCOI sequence was submitted to the NCBI Gen Bank database using the BARCODE submission portal. The designated Gen Bank accession number can be used to access the Gen Bank record, which includes data and metadata for the specimen: nucleotide sequence in text format, conceptual translations to protein (amino acid) sequence, specimen voucher number, collection date, geospatial coordinates of the collection site, and name of the person collecting the specimens. Gen Bank Accession number is provided in Table 3.

Kimura-2-Parameter (K2P) genetic distances [32] were calculated between barcodes for individuals of the same species and between individuals of different species within the genus of *Diphyes* using MEGA, Ver. 4 [33]. The mtCOI sequences were analyzed using the Neighbor Joining (NJ) algorithm and K2P distances of MEGA Ver. 4 [33] and the resultant tree was bootstrapped using 1,000 sub-replicates.

Table: 3. Taxonomic Group, Number of stations from which DNA sequenced for mtCO1(N), Number of different sequences (N variants), Mean intra-specific Kimura-2-Parameter (K2P), distance and S.D, sequence length in number of base pairs (BP),and Gen Bank Accession Number analyzed for this study

Taxon (Siphonophora)	N	N variants	K2P Mean	Distance S.D.	BP	Accession Number
Diphyes dispar	5	1	0	n/a	651 bp	KU529462

3. Results and Discussion

The PCR amplification of partial mtCOI gene of *D. dispar* is the first instance from the Laccadive Sea, off the south-west coast of Arabian Sea, Indian Ocean and the sequence has been deposited in the NCBI Gen Bank (Accession No. KU529462).The amino acid sequence of the corresponding COI gene was also updated under the accession number AMB61535, which turned out to contain 217 amino acids. Base statistics of the *D. dispar* COI are presented in Table 4.

Table: 4. Base Statistics of *Diphyes dispar* COI.

Gen Bank ID: KU529462	G+C Content = 33.8%	A+T Content = 66.2%
Nucleotide	**Count**	**Percentage**
A	187	28.7
T	244	37.5
G	120	18.4
C	100	15.4

It can be seen from the table that the fragment is rich in AT content as expected with Thymine (T), 37.5% occurring most frequently followed by the others in the order Adenine (A), 28.7%, Guanine (G), 18.4% & Cytosine (C), 15.4%. Analysis of the result revealed that the AT % stood at 66.2 in comparison to GC % at 33.8. DNA from different sources has different ratios of the A-to-T and G-to-C base pairs. DNAs isolated from organisms that live in hot springs or higher temperature profile climate have a higher GC content, which takes advantage of the increased thermal stability of the GC base pair [34]. Due to the robustness endowed to the genetic materials in high GC organisms, it was commonly believed that the GC content played a vital part in adaptation temperatures [35]. High GC content genomes are thermodynamically more stable and can survive the extra molecular collisions of higher energy of those environments more readily. Selection for higher thermal stability has also been suggested to explain the evolution of GC-rich regions in the genomes of homeothermic vertebrates in contrast to their GC-poor homologs found in poikilothermic (i.e., cold-blooded) groups [36]. Zooplankton are poikilothermic, so their physiological processes are highly sensitive to temperature [37]. Concurred to these statements, the composition of nucleotides of the family *Diphyidae* showed clear bias to nucleotide 'AT'. The average percentage of nucleotides A, T, G, C present in the COI sequence of the *Diphyidae* species were in the concentrations A - 25.9, T - 38.5, G - 19.4, C - 16.2 respectively (Table 5).

The protein entry was subjected to family confirmation by searching the Smart BLAST database and the result indicated a very high and significant match confirming our sequence to be a part of Cytochrome C Oxidase subunit1 family with good query coverage (Fig. 1). The Smart BLAST search indicated the sequenced segment to be closely related to *dispar* species of the family *Diphyidae* (Fig. 1).

The Kimura-2-Parameter/Neighbor Joining (K2P/NJ) tree grouped haplotypes assigned to the same species within the same cluster (Fig. 2). In no case was a species haplotype assigned to an incorrect or different species. Sequence divergence (measured as K2P distance) between individuals of the same species *viz., dispar* ranged from 0.010 to 0.058 (mean 0.033, s.d.0.007). K2P genetic distances were significantly lower between individuals of the same species than between individuals of different species within the *Diphyes* genus. Inter-specific sequence divergence between species of the same genus ranged from 0.133 for *Diphyes spp*., 0.172 for *Eudoxoides spp*., 0.003 for *Sulcularea spp* and 0.466 for *Lensia spp*. There was no overlap between the intra-specific and inter-specific variation within each genus, thus maintaining the barcoding gap for these genera. However, the number of exemplar sequences is insufficient to conduct a rigorous analysis of genetic variance; the statement of

"barcoding gap" is dependent on the present sampling pattern. Therefore, an expanded database study of siphonophores from different geographical regions are needed to reveal the actual "barcode gap" of this group of organisms.

The phylogenetic tree (Fig. 2) revealed that the clade belonging to the genus *Diphyes* was grouped together with a good bootstrap score of 95. Similarly the species belonging to the *Eudoxoides* genus formed a single clade with a bootstrap score of 91. Species of all genera for which multiple individuals were analyzed yielded consistent results (100%), with an exception in *D. dispar sp*. where, multiple barcodes were resolved by boot-strap values of 96–100%. Closer examination of intra-specific variation is useful to reveal cryptic species and analyze geographic distribution of lineages, or phylogeography [38]. Among zooplankton, mtCOI is a useful marker for large-scale population genetic differentiation and phylogeography [39].

Evolutionary pattern of partial mtCOI gene sequence of first Indian isolate was checked with available polar water *D. dispar* isolates that were collected from NCBI-Nucleotide database. Interestingly, specimens obtained from the polar waters *viz.*, Artic, Atlantic and Northeast Pacific Oceans [22] were clubbed together as a separate clade with a bootstrap score of 99.There was a clear segregation between the tropical water isolate of the present study and the polar water isolates downloaded from the NCBI Gen Bank. The present study isolate (KU529462) emerged as a sister taxa to the clade formed by polar water isolates. Intensive analysis of the tree revealed that *D. chamissonis* and *D. dispar* have had a More Recent Common Ancestor (MRCA) than *D. bojani.* The phylogenetic tree constructed from the sequence of the present study isolate was genetically more similar to the taxa GQ119972 as shown in Figure 2. Hence, an expanded database of DNA barcodes is required to improve the accuracy of species identification for this ecologically important and taxonomically challenging group of organisms.

Table: 5. Nucleotide frequencies of the COI sequence of the *Diphyidae species*: All frequencies are given in percentage

Accession No| Species Name	**Nucleotide Frequencies in percentage**				
	T(U)	C	A	G	Total
KU529462.1| Diphyes dispar isolate KUFOS	37.5	15.4	28.7	18.4	651.0
AY937367.1| Diphyes dispar	39.6	13.9	28.1	18.3	661.0
GQ119973.1| Diphyes dispar	38.7	14.2	27.5	19.6	775.0
GQ119972.1| Diphyes dispar	39.5	13.1	27.4	20.0	1044.0
JQ353744.1| Muggiaea atlantica	38.6	16.2	26.1	19.2	687.0
KF977297.1| Muggiaea atlantica	39.2	15.9	25.9	19.0	630.0
GQ119969.1| Diphyes bojani	38.4	15.3	27.2	19.2	766.0
GQ119967.1| Diphyes bojani	38.5	14.6	26.8	20.1	850.0
KF977289.1| Lensia subtiloides	34.8	15.9	27.0	22.3	422.0
KF977269.1| Diphyes chamissonis	39.9	13.8	27.3	19.1	682.0
KF977266.1| Diphyes chamissonis	39.9	13.7	27.5	18.9	681.0
GQ120066.1| Lensia campanella	39.1	18.4	24.0	18.4	792.0
GQ120050.1| Sulculeolaria quadrivalvis	34.6	19.3	23.1	23.1	642.0
AY937378.1| Sulculeolaria quadrivalvis	34.8	19.4	23.4	22.4	661.0
GQ120013.1| Lensia cf. multicristata	33.7	27.1	21.1	18.1	630.0
GQ120011.1| Lensia cf. multicristata	33.4	27.0	21.0	18.6	634.0
GQ120010.1| Lensia meteori	37.9	14.7	28.5	19.0	860.0
GQ120009.1| Lensia hotspur	41.2	12.7	27.2	18.8	628.0
GQ120008.1| Lensia grimaldi	40.8	13.4	27.1	18.7	635.0
GQ120006.1| Lensia fowleri	37.1	15.2	29.1	18.6	633.0
GQ120003.1| Lensia Exeter	37.4	18.2	26.4	18.0	617.0
GQ120002.1| Lensia conoidea	41.1	12.7	27.7	18.5	822.0
GQ120066.1| Lensia campanella	39.1	18.4	24.0	18.4	792.0
JQ353739.1| Lensia campanella	38.2	19.0	24.5	18.4	642.0
GQ120000.1| Lensia Achilles	41.0	14.2	26.0	18.8	831.0
GQ119983.1| Eudoxoides spiralis	38.3	16.6	24.1	20.9	812.0
GQ119982.1| Eudoxoides spiralis	37.6	17.9	24.3	20.2	593.0
GQ119978.1| Eudoxoides mitra	40.3	14.4	25.0	20.3	792.0
GQ119977.1| Eudoxoides mitra	40.8	13.7	24.8	20.6	838.0
Avg.	**38.5**	**16.2**	**25.9**	**19.4**	**713.9**

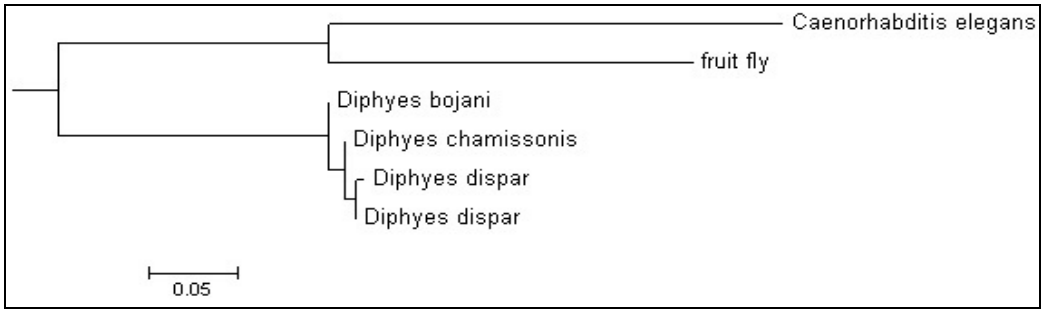

Fig 1: Smart BLAST database Evolutionary Relationships of Taxa: A concise summary of three species of the genus, *Diphyes* best matches in the sequence database together with the two best matches from well-studied reference species (*Caenorhabditis elegans* and *Drosophila melanogaster*-fruit fly) showing phylogenetic relationships based on multiple sequence alignment and conserved protein domains.

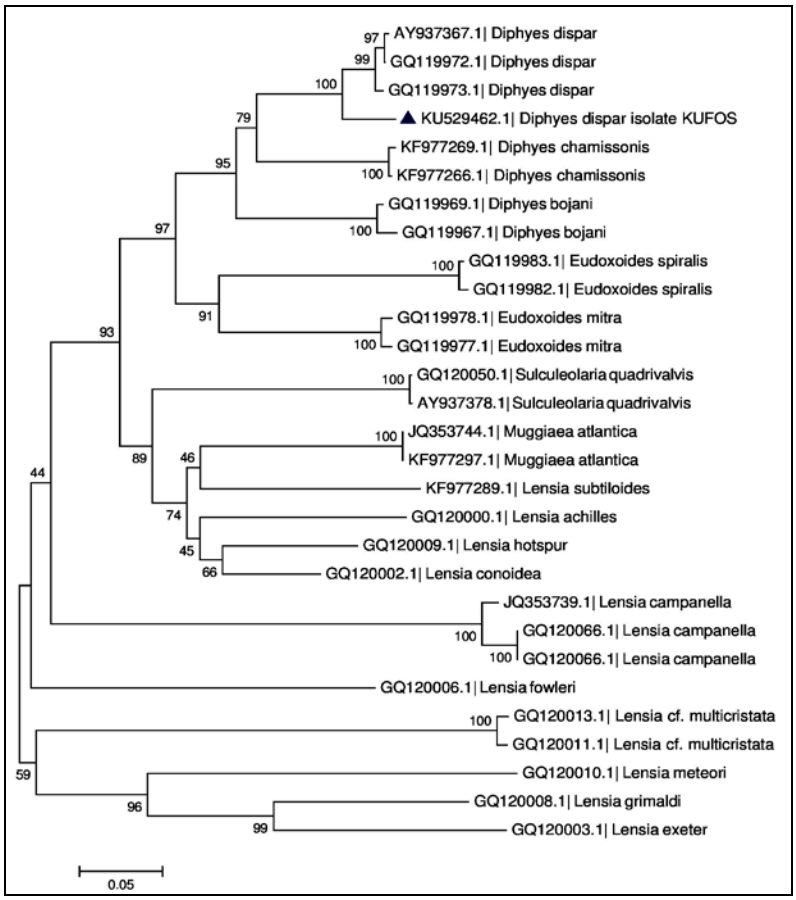

Fig 2: Evolutionary Relationships of Taxa: The evolutionary history was inferred using the Neighbor-Joining method [40].The optimal tree with the sum of branch length = 2.89747305 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [41]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura-2-Parameter method [32] and are in the units of the number of base substitutions per site. The analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 400 positions in the final dataset. Evolutionary analyses were conducted in MEGA 4 [33].

4. Conclusions

This study is the first report on the molecular characterization of *D. dispar* from the Laccadive Sea, off the south-west coast of Arabian Sea, Indian Ocean, contributing to the knowledge of their global distribution. The sequenced segment was found to be 651 bp long and rich in AT content. It was confirmed from the Smart BLAST database search that the sequenced segment belongs to the Cytochrome Oxidase subunit 1 family and also shows close proximity towards the family of *Diphyidae* to which it belongs. From the phylogenetic studies we also infer that the sequence was clustered with an entry corresponding to *Diphyes spp*., with a high bootstrap score. The sequence divergence between the new COI sequence for *D. dispar* and those in the Gen Bank was about 5%. This divergence could either mean that the species has relatively high intraspecific variation (given the crude 3-5% divergence cutoff that is often applied to putative species in barcoding analyses) or that the Indian sequences represent a distinct or cryptic species. Hence, an expanded database of hydrozoan barcodes will improve the usefulness of DNA barcoding and the accuracy of species identification.

In light of the foremost position of zooplankton in ocean food webs, their usefulness as rapid responders to environmental change, and the increasing scarcity of taxonomists, the use of DNA barcodes is an important and useful approach for rapid analysis of species diversity and distribution. Considering the difficulty in identifying the most diverse world of gelatinous zooplankton groups, this exercise will be of great help and the sequence will serve as a key to unlock the mysteries of species diversity in the open ocean pelagic realm.

5. Abbreviations

BLAST: Basic Local Alignment Search Tool; K2P: Kimura-2-Parameter; mtCOI: mitochondrial Cytochrome Oxidase I; MEGA: Molecular Evolutionary genetic analysis; MRCA: More Recent Common Ancestor; MSA: Multiple Sequence Alignment; NCBI: National Center for Biotechnology Information; NJ: Neighbor Joining.

6. Competing interests

The authors declare that they have no competing interests.

7. Acknowledgements

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