

DNA barcode of Chaetognatha from Indian Waters

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Chaetognatha are the second most abundant zooplankton group in the Indian waters. Precise identification of the species is critical for biogeographical studies. DNA barcodes using mitochondrial cytochrome c oxidase (COI) of seven dominant and frequently occurring species of Chaetognatha from Indian waters are reported here for the first time. Average nucleotide frequencies of COI barcode were A (22.9%), T (34.7%), G (21.3%) and C (21.1%). Kimura-2-Parameter distances within and among species ranged from 0 to 0.050 and 0.050 to 0.400, respectively. Phylogenetic analysis based on maximum likelihood method resolved the species in accordance with morphological traits and close relationship between sister species.

[Keywords: Chaetognatha, COI, DNA barcode, Indian waters]

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Introduction

Chaetognaths (arrow worms) are the most abundant zooplankton in the Indian Ocean¹. They are coelomate animals of relatively small size, with a straight, slender, transparent body displaying perfect bilateral symmetry. Most conspicuous of the external features are the fins, paired lateral and single caudal thin horizontal extensions supported by rays and serving for flotation and equilibration rather than for locomotion. The anterior end forms a well – delimited head bearing a pair of eyes and on either side the characteristic grasping spines (hooks), hard curved spines used in seizing prey and operated by a complex musculature inside the head. The head is also typically armed with arcs of small spines anterior to the mouth. A feature peculiar to the phylum is a fold of body wall that can be drawn over the dorsal and lateral surfaces of the head like a hood. Nervous system consists of ganglia in the head connected by a pair of circumenteric commissures with a large ventral trunk ganglion. Digestive system is a straight tube extending from the mouth located ventrally on the head to the anus found anterior to the tail fin at the tail septum. The chaetognaths are hermaphrodites, the paired

ovaries are situated in the trunk immediately anterior to the tail septum. Testes lie in the tail region posterior to tail septum. Though hermaphrodite cross-fertilization by copulation is the rule. Phylum comprises more than 129 species from all the geographical and vertical ranges of the ocean²⁻⁵. Presently, 31 species of chaetognaths consisting of 4 genera (*Sagitta*, *Pterosagitta*, *Krohnitta* and *Eukrohnita*) have been reported from the Indian Ocean waters, including two new species, viz. *Khronitta balagopali* and *Sagitta meenakshiae* from the Andaman Sea^{6, 7}. Chaetognaths are identified based upon a broad range of characteristics including morphology, ecology and distribution. They display very few diagnostic morphological traits and consequently there is considerable disagreement among the taxonomic schemes suggested by different workers⁸. Subtle differences in the morphology of chaetognath species and their larval stages, including the glass-like transparent nature, few variable traits, and lack of diagnostic features, make the identification extremely difficult even for expert taxonomists using only morphological characters^{9, 10}. Chaetognaths are considered to be rapidly and recently evolving, based on

examination of the 28S gene. Moreover, there has been uncertainty in the placement of certain chaetognath species under the Order Aphragmophora or Phragmophora, including merging of both the orders⁹. More extensive sampling of geographically widely distributed species is needed to determine the accurate levels of intraspecific diversity in chaetognath species. Morphological identification of chaetognaths is difficult, particularly because of the quick decay of the samples after collection. Therefore, additional characteristics other than those currently used for classical taxonomy should also be used for identification of the species.

In fact, in many marine species, mutualism between morphological and molecular approaches to species identification have been demonstrated¹¹. In this context, DNA sequence data, when integrated with existing taxonomic practices, can contribute significantly to the challenges of identifying individuals and increasing the rate of discovering biological diversity¹². Selection of an appropriate gene or gene portion for DNA barcoding is of critical concern. The DNA sequence should have

Materials and Methods

Chaetognaths were collected from diverse locations in the Indian Ocean using a Bongo net (BN, Hydrobios, mesh size 0.2 mm; filtering

sufficiently high rates of divergence to resolve differences among even the most closely related species, with low rates of DNA sequence divergence among individuals of the same species¹³. The COI barcode region is a standardized DNA marker for barcoding animals due to its pattern of variation that is favorable for identifying and differentiating the most closely related species¹⁴. This gene region can also detect genetic divergence of co-specific populations associated with geographic isolation and cryptic speciation¹⁵. The use of DNA sequences as ancillary characters for accurate identification and recognition of chaetognath species is becoming more frequent¹⁶⁻²¹. The COI gene sequences of chaetognath species, *Caecosagittam acrocephala* from the Atlantic Ocean and the Sagami bay have revealed the presence of cryptic species within them¹⁸.

However, this method has not been tried on the Indian species. This manuscript describes the COI barcode sequence obtained from 7 species of an understudied Phylum Chaetognatha and validation of these sequences for taxonomic assignment.

cone length 250 cm; ring diameter 60 cm). The net was hauled for 10 min at the surface at a ship speed of 2 knots (Fig. 1).

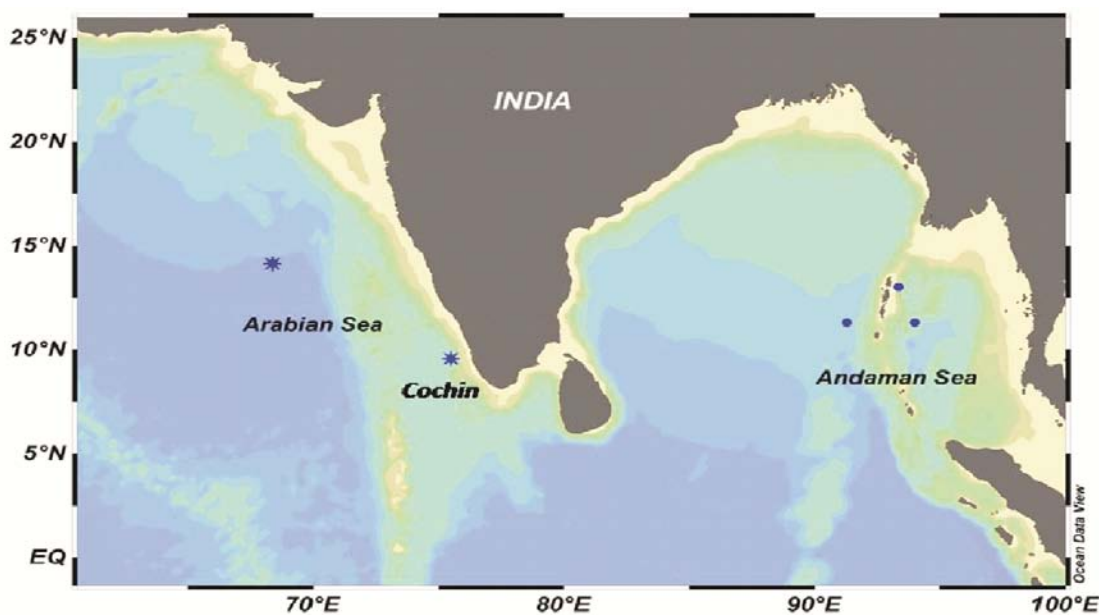


Fig 1: Map showing the collection locations for zooplankton samples

All zooplankton samples were preserved immediately in 95% ethyl alcohol; the alcohol was changed once every 24 hr after collection. Detailed preservation protocols have been

published elsewhere²². Chaetognath specimens were sorted. They were identified based on standard morphological characteristics (Fig. 2).

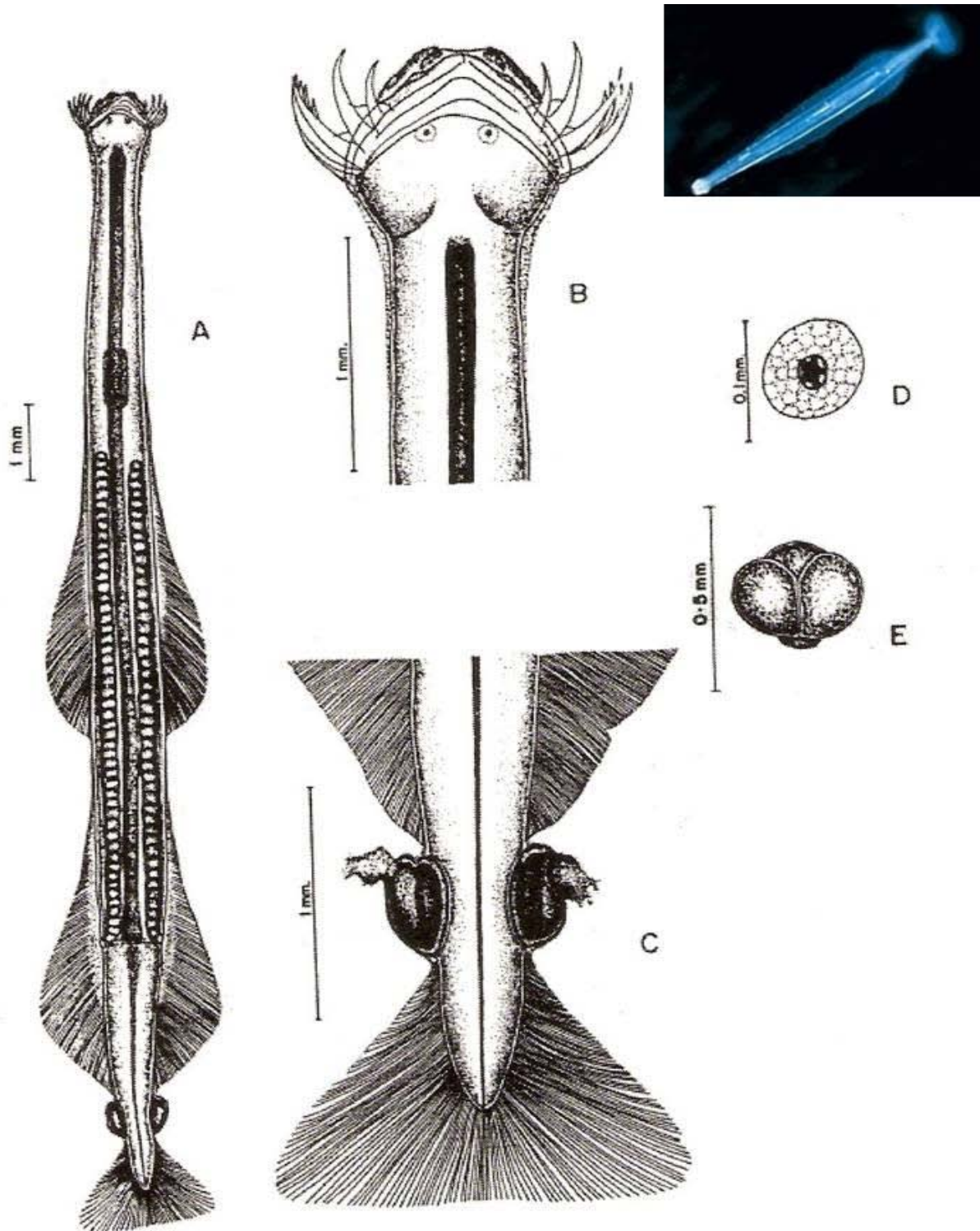


Fig 2: Morphological characteristics of *Sagitta bedoti* Dorsal view; B-Details of head and neck (dorsal view); C-Details of posterior part of tail and seminal vesicles (dorsal view); D-Left eye; E-upper view of arrangement of ova in the ovary. Insert photograph of a chaetognath sp

Seven species *Sagitta bedoti*, *S. hexaptera*, *S. neglecta*, *S. pulchra*, *S. regularis*, *S. robusta* and *Pterosagitta draco* were used for DNA sequence analysis of the barcode region of the COI gene. Sample sizes were depending on the availability of specimens. Total genomic DNA was isolated using the QIAGEN DNeasy kit, using either the tail portion of adult specimens or whole individual juveniles, ensuring no contamination of gut or gut contents. The COI was amplified using primers, L CO-1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO-2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'23. PCR was carried out in a thermal cycler (Master Cycler, Eppendorf, Germany) with the following cycles: 94°C for 3 min as initial denaturation followed by 35 cycles of 94°C for 30 sec, 50°C for 45 sec, 72°C for 1 min and 72°C for 20 min as final extension, resulting in the amplification of approximately 650 base-pair fragment. PCR was performed in a 20 µL reaction volume containing 10 pmol of each primer, 10 mM Tris-HCl (pH 8.3), 0.25 mM of

each dNTP and 1 U of Taq polymerase. Amplified products were resolved in 1.5% agarose gel, stained with ethidium bromide (10 µg.mL⁻¹) and visualized in a gel documentation system (GelLogic1500, Kodak, Japan). The PCR products were cleaned using UltraCleanTMPCR Clean-upTM Kit (Mo Bio Laboratories, Inc.). DNA sequencing was done by direct sequencing of PCR amplification products, according to published protocols and using both forward and reverse primers. Sequencing was carried out in an automated sequencer (ABI PRISM 310 Applied Biosystems, Inc., USA). The sequences were aligned using Sequencher 4.8 (Gene Codes Co., Ann Arbor, MI). All sequences were checked thoroughly for accurate machine reading. Homology search was carried out using BLAST 24. The COI sequences were submitted to the National Center for Biotechnology Information (NCBI) GenBank database and can be accessed using the GenBank Accession Number (Table 1).

Table 1: Species, Voucher Number, GenBank Accession Number and location (Latitude, Longitude)

Species	Voucher No.	GenBank Acc. No.	Latitude	Longitude
<i>Sagitta bedoti</i>	NIOBZC1	JN258001	75.50 E	9.55 N
	NIOBZC2	JN258002	75.50 E	9.55 N
	NIOBZC3	JN258003	75.50 E	9.55 N
	NIOBZC4	JN258004	75.50 E	9.55 N
	NIOBZC5	JN258005	75.50 E	9.55 N
<i>S. hexaptera</i>	NIOBZC17	JN258017	68.39 E	14.15 N
	NIOBZC18	JN258018	68.39 E	14.15 N
	NIOBZC19	JN258019	68.39 E	14.15 N
<i>S. neglecta</i>	NIOBZC20	JN258020	91.30 E	11.30 N
	NIOBZC21	JN258021	93.35 E	13.00 N
	NIOBZC22	JN258022	93.35 E	13.00 N
	NIOBZC23	JN258023	94.00 E	11.30 N
<i>S. pulchra</i>	NIOBZC24	JN258024	68.39 E	14.15 N
	NIOBZC25	JN258025	68.39 E	14.15 N
	NIOBZC26	JN258026	68.39 E	14.15 N
	NIOBZC27	JN258027	68.39 E	14.15 N
<i>S. regularis</i>	NIOBZC28	JN258028	68.39 E	14.15 N
	NIOBZC29	JN258029	68.39 E	14.15 N
	NIOBZC33	JN258033	68.39 E	14.15 N
<i>S. robusta</i>	NIOBZC32	JN258032	93.35 E	13.00 N
	NIOBZC34	JN258034	93.35 E	13.00 N
	NIOBZC35	JN258035	91.30 E	11.30 N
	NIOBZC36	JN258036	94.00 E	11.30 N

	NIOBZC37	JN258037	94.00 E	11.30 N
	NIOBZC8	JN258008	94.00 E	11.30 N
<i>Pterosagitta draco</i>	NIOBZC9	JN258009	93.35 E	13.00 N
	NIOBZC30	JN258030	91.30 E	11.30 N
	NIOBZC31	JN258031	91.30 E	11.30 N

Pairwise evolutionary distance based on the genetic variation within and between chaetognath species were determined by the Kimura-2-Parameter method²⁵, using the software program MEGA 4 (Molecular Evolutionary Genetics Analysis) with gap positions ignored on a pairwise basis²⁶. These distances were hierarchically tabulated within each species, and between species –of each genus. A model of DNA sequence evolution was

chosen using Mr Model test v227 under Akaike's Information Criterion (AIC) and the Maximum likelihood (ML) tree was constructed by selecting the GTR+I+G model. In order to further test the confidence of branching orders, bootstrapping using 1,000 replicates values was done. Bootstrap values were assigned to each internal node of the tree and the values >80 are shown in Figure 5

Results and Discussion

The use of COI sequences as DNA barcodes has proven to be useful for marine animal species²⁸. Among zooplankton this technique has been proved successful in copepods²⁹⁻³⁴, euphausiids³⁵, medusae³⁶ and thaliaceans³⁷. The DNA sequence variation of the COI barcode region for chaetognaths from the North Atlantic (including the Mid-Atlantic ridge), South Atlantic, Arctic, and Southern Ocean has been described by Jennings et al.¹⁶. The COII region of *Sagitta setosa* revealed geographic differentiation among oceanographic environments of the eastern North Atlantic and the western Mediterranean, Adriatic, and Black Sea¹⁷⁻¹⁹.

In the present study the DNA barcode variation of seven major species of chaetognaths from the Indian Ocean waters is described. COI gene was sequenced for seven species (*Sagitta bedoti*, *S. hexaptera*, *S. neglecta*, *S. pulchra*, *S. regularis*, *S. robusta* and *Pterosagitta draco*) belonging to two genera (*Sagitta* and *Pterosagitta*). The DNA sequences contained 569 aligned nucleotide positions of which 347, 277, 320 and 9 were conserved, variable, parsimony informative and singleton sites, respectively. No insertions, deletions or stop codons were

observed in any sequence. Analysis revealed overall nucleotide frequencies as follows: A=22.9%, T=34.7%, G=21.3% and C=21.1%. On average, transitional pairs between species (si=75) were more frequent than transversal pairs (sv = 66), with an average si/sv ratio of 1.14. A total of 21 haplotypes were found with an overall haplotype diversity, $h = 0.971$. Two to four haplotypes were observed in each of the seven species. K-2-P genetic distances for pairwise comparisons ranged widely from 0.000 to 0.400, with a mean of 0.275. Thus, COI barcode region showed the pattern of variation that is favorable for identifying and differentiating the most closely related species¹⁴. It also detected the genetic divergence of co-specific populations associated with geographic isolation and cryptic speciation¹⁵. Between individuals of the same species, K-2-P distances ranged from 0.000 to 0.050, with a mean of 0.018 and between individuals of different species, the distances ranged from 0.050 to 0.400, with a mean of 0.300. Average genetic distance value for Chaetognatha is 0.0145¹⁶. Genetic distance within species was found to be the lowest for *S. hexaptera* and the highest for *S. pulchra* (Fig.3).

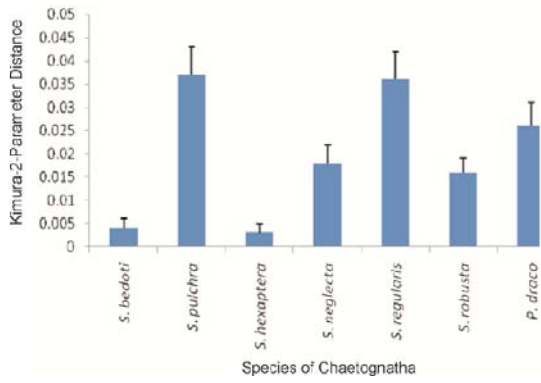


Fig 3: Mean values of Kimura-2-Parameter (K-2-P) genetic distances within the analyzed species of Chaetognatha; bars indicate standard deviations about the mean.

There was overlap in gene divergence between intraspecific and interspecific comparisons (Fig. 4). Interspecific genetic distance was found to be the highest (0.399) between *P. draco* and *S. hexaptera* and the lowest (0.059) between *S. regularis* and *S. robusta* (Table 2). COI detected a close relationship between *S. bedoti* and *S. pulchras* and between *S. robusta* and *S. regularis*. Our results clearly indicate that the barcode region of COI is an accurate and reliable identification tool for the species of chaetognaths. Absence of pseudogenes due to lack of stop codons in the sequences was in congruence with an earlier study³⁸. Intraspecific diversity levels might have been underestimated in this study due to several factors, including the restrictive sampling and variation in sample sizes.

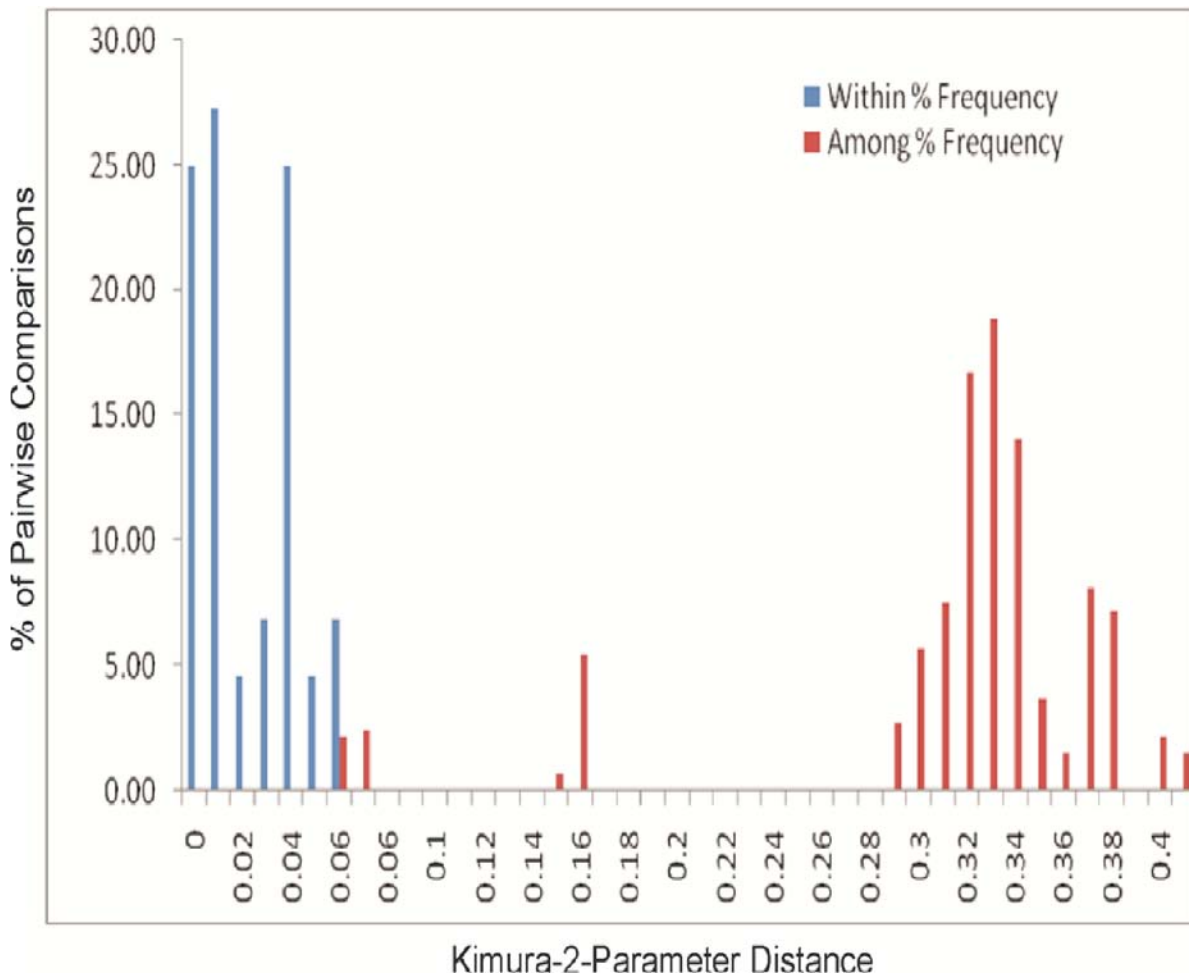


Figure 4: Percentage of pair-wise comparisons versus Kimura-2-Parameter genetic distances for within (blue) and among (red) species comparisons.

Table 2: Pairwise Kimura-2-Parameter (K-2-P) genetic distances for the seven species of Chaetognaths. Above diagonal: mean K-2-P genetic distance; below diagonal: standard deviation.

	<i>P. draco</i>	<i>S. bedoti</i>	<i>S. hexaptera</i>	<i>S. neglecta</i>	<i>S. pulchra</i>	<i>S. regularis</i>	<i>S. robusta</i>
<i>P. draco</i>		0.029	0.031	0.025	0.027	0.023	0.024
<i>S. bedoti</i>	0.366		0.026	0.027	0.015	0.025	0.025
<i>S. hexaptera</i>	0.399	0.316		0.029	0.026	0.028	0.029
<i>S. neglecta</i>	0.332	0.333	0.374		0.023	0.025	0.026
<i>S. pulchra</i>	0.339	0.155	0.323	0.295		0.024	0.025
<i>S. regularis</i>	0.311	0.332	0.365	0.317	0.294		0.008
<i>S. robusta</i>	0.310	0.328	0.368	0.325	0.306	0.059	

ML tree showed clear resolution of the seven species of chaetognaths based on COI sequence variation (Fig. 5). For each species, the bootstrap value for clusters of barcodes was 100%. The ML of COI barcode region variation resolved two clusters of species supported with 90 - 100% bootstrap values. The first cluster was formed by congeneric species *S. bedoti* and *S. pulchra*, which was supported with 100% bootstrap value. The second cluster was formed by the co-generic species *S. robusta* and *S. regularis* (100% bootstrap value). In the ML tree of the Indian species, sequences of previously published data³⁸⁻⁴⁰ have been

included. We found a close relationship of species from Indian waters to other chaetognath genera viz. *Eukrohnia*, *Solidosagitta* and *Decopisagitta* from other waters³⁸. The *Decopisagitta* and *Solidosagitta* are the genera named under non-classical taxonomy, but under classical taxonomy, they are now placed under genus *Sagitta*. Chaetognaths from Indian waters also showed association with *Eukrohnia hamata*, *E. bathyantartica*, *E. microneura*, and *E. fowleri* with 99% bootstrap value (Fig. 5).

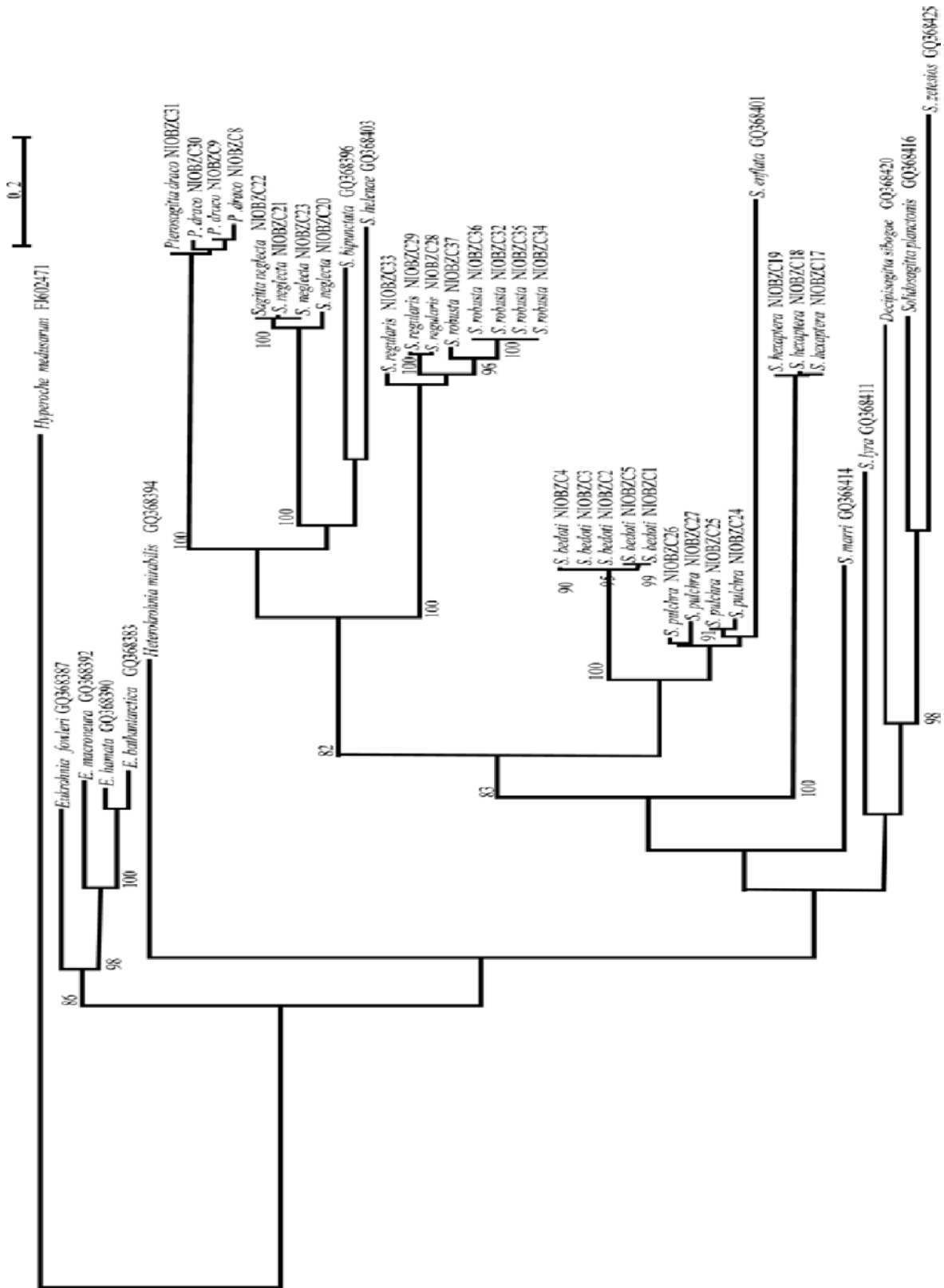


Fig 5: Maximum Likelihood tree for COI barcodes of *Chaetognath* species, showing topology and bootstrap values. Also included in the analysis previously published sequences¹⁶ indicated by GENBANK Accession Numbers following the species name. Numbers after species names are voucher numbers for sequences determined by this study (see Table 1).

The species under genus *Eukrohnia* are either meso or bathypelagic in nature. For various practical applications, including ocean observing, monitoring of ecosystem services and health, prediction of responses to climate change, among others, the accurate identification and clear demarcation of species limits are critically needed³⁴. The DNA barcode analyses will also reveal cryptic and unrecognized species, and solve mysteries about the functioning of ocean ecosystems. The increased use of DNA markers, including the COI barcode region, for identification of species in the Indian Ocean waters will accelerate the rate of discovery of new species and thus the accurate assessment of biodiversity in this region. A DNA barcode library is also the first

step towards future biodiversity analysis by environmental barcoding' or high-throughput next-gene sequencing of unsorted samples⁴¹. This study reports new DNA sequence data for chaetognath species from the Indian Ocean waters, which can be used to accurately identify and discriminate these abundant and ecologically important – but systematically challenging – species. A comprehensive database or library of DNA barcode sequences for zooplankton will enable in characterizing the biodiversity hotspot regions of the Indian Ocean and also facilitate progress towards understanding the ecology, biogeography, and evolution of Chaetognatha, an important zooplankton group.

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