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Evolutionary analyses of phylum Chaetognatha based on mitochondrial cytochrome oxidase I gene

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Abstract: Chaetognaths (arrow worms) are an enigmatic group of transparent planktonic invertebrates and play an important role in the marine food web. Their morphological and developmental features have raised extensive debates since the discovery of the phylum in the 18th century. Uncertainty in the phylogenetic placement of certain chaetognath species still exists and is puzzling many scientists who have tried to clarify this task. Studies using a portion of both small subunit ribosomal ribonucleic acid (SSU rRNA) and large subunit ribosomal ribonucleic acid (LSU rRNA) genes when integrated with conventional taxonomy were contributed to resolve taxonomical issues in this group. Here we present the first phylogenetic study of Chaetognatha based on a portion of mitochondrial cytochrome oxidase I (COI) gene and compare our results with the earlier morphological and molecular evolutionary hypotheses. This study includes 16 extant species, representing 8 genera and 6 of which are among the 9 extant families. We recommend the following clade structure for the phylum: Aphragmophora comprising Sagittidae with Pterosagittidae and Krohnittidae included in the Sagittidae and Phragmophora comprising Eukrohniidae, Spadellidae, and Heterokrohniidae. Phylogenetic analyses also supported the division of Phragmophora into two monophyletic groups: the Monophragmophora and Biphragmophora. Moreover, Ctenodontina/Flabellodontina and Syngonata/Chorismogonata suborders were not validated. Precise phylogenetic investigations using various molecular markers and specimens from diverse regions are definitely needed to provide an exact evolutionary concept on this phylum.

Key words: Chaetognatha, evolution, phylogenetic analysis, mitochondrial cytochrome oxidase I gene, Bayesian inference, maximum likelihood

1. Introduction

Chaetognaths are a group of transparent planktonic invertebrates. Their elongated bodies have led to the common name of 'arrow worm' (Jennings et al., 2010). They are found in every marine habitat, from the sea floor to all pelagic zones of coastal waters and the open oceans. Although small in size (2–120 mm), chaetognaths are often abundant, and play an important role in the marine food web as the primary predators of copepods (Bieri, 1991b). Presently, around 130 chaetognath species (100 pelagic and 30 benthic) have been identified in the global oceans (Miyamoto et al., 2014).

Von Ritter-Zahony (1911) and Hyman (1959) divided chaetognaths into four families comprising six genera: Sagitta (Sagittidae), Pterosagitta (Pterosagittidae), Spadella, Eukrohnia and Heterokrohnia (Eukrohniidae), and Krohnitta (Krohnittidae). Tokioka (1965a) reassessed the relationships between families by creating two new orders: the plesiomorphic Phragmophora (presence of a transverse musculature, namely the phragms, and various kinds of glandular structures on the body surface) comprised of Spadellidae and Eukrohniidae, and the consequent Aphragmophora (absence of phragms and few glandular structures). Again, Tokioka (1965a) suggested creating two Aphragmophora suborders — Flabellodontina and Ctenodontina — based on the shape of teeth and hooks and the number of teeth rows. The suborder Flabellodontina only contains the family Krohnittidae, and Ctenodontina contains the families Sagittidae and Pterosagittidae. In his following work, Tokioka (1965b) proposed the paraphyly of Aphragmophora with the Ctenodontina being closer to the Phragmophora than to the Flabellodontina. After the discovery of several deep benthoplanktonic species, Casanova (1985) proposed a slight modification on the hypothesis of Tokioka (1965b). In accordance with his findings, the members of the Phragmophora were split into two new orders: Biphragmophora comprising Heterokrohniidae family and Monophragmophora

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with families Eukrohniidae and Spadellidae. He further divided Biphragmophora, comprising Heterokrohniidae, into subclass Syngonata (having ducts between the genital glands) and Monophragmophora comprising Eukrohniidae and Spadellidae families associated with the Aphragmophora into subclass Chorismogonata (without such ducts). However there has been still uncertainty in the phylogenetic placement of certain chaetognath species under the order Aphragmophora or Phragmophora including merging of both orders.

In this context, molecular data when integrated with conventional taxonomy can contribute to resolve taxonomical issues in this group. The first molecular study of chaetognaths systematics was carried out by Telford and Holland (1997) by focusing on a short portion of the large subunit ribosomal RNA 28S (LSU rRNA) gene. They showed that the Aphragmophora and Phragmophora are natural groups. However, the relationships between several well-supported groups within the Aphragmophora were found to be uncertain. Later, Papillon et al. (2006) carried out an extensive molecular study based on the small subunit ribosomal RNA, 18S (SSU rRNA) isolated from members of six chaetognath families. Besides to their many findings, they added that the Krohnittidae and Pterosagittidae groups should no longer be considered as families as they are included in other groups designated as families. Further, a DNA barcoding analysis carried out by Jennings et al. (2010), who were highly successful at discriminating between the species of chaetognaths, revealed that *Eukrohnia bathypelagica* and *E. hamata* are young sister-species. Recently, Gasmi et al. (2014) conducted an extensive molecular analysis based on SSU and LSU rRNA duplicated genes and combined the molecular results with morphological classification and geometric morphometrics. They suggested the following clade structure for the phylum: (((Sagittidae, Krohnittidae), Spadellidae), (Eukrohniidae, Heterokrohniidae)), with the Pterosagittidae included in the Sagittidae. According to them, the clade formed by Sagittidae and Krohnittidae confirmed the monophyly of Aphragmophora. However, the monophyly of Phragmophora could not be established. The biclassification concepts like Ctenodontina/ Flabellodontina and Syngonata/Chorismogonata hypotheses were also found to be invalid by Gasmi et al. (2014).

Even though ribosomal genes are widely used in molecular phylogenetic studies, it has diminutive limits such as long-branch chain attractions and slow rate of evolutionary change (Towers, 2011). Long-branch chain attractions arise in phylogenetic analyses when rapidly evolving lineages are inferred to be closely related, irrespective of their true evolutionary relationships (Towers, 2011). Hence, other genes, such as mitochondrial cytochrome oxidase I (COI), are also being used to complement and compare the studies carried out by ribosomal genes (Jennings et al., 2010; Ptaszyńska et al., 2012; De Mandal et al., 2014; Peter et al., 2016; Abdelaziz et al., 2019). Application of COI gene for DNA barcoding has become a promising tool for species identification and phylogeny in a wide range of animal taxa (Huang and Ruan, 2018).

At present, 31 species of chaetognaths consisting of 4 genera have been identified in the Indian Ocean (Nair et al., 2015a). The major sampling of our work was conducted in the Arabian Sea, where 25 species of the aforementioned 31 species exist (Nair and Rao., 1973; Nair et al., 2015b). Occurrences of 6 species (*Sagitta bedoti, S. enflata, S. oceania, S. pulchra, S. robusta*, and *K. pacifica*) are so far reported from Cochin backwater system, Southwest coast of India, from where the minor sampling of our study was conducted (Nair, 1972; Nair and Rao, 1973a; Nair and Rao, 1973b; Srinivasan, 1972a, 1972b). To examine the phylogenetic relationship among chaetognath species, 40 nucleotide sequences that represent 8 species (4 genera and 2 families) from off Cochin, South Eastern Arabian Sea, Indian Ocean and Cochin backwater system, Southwest coast of India along with 34 sequences that represent 16 species (8 genera and 6 families) from GenBank were incorporated. We present here a molecular phylogeny concept using COI gene to compare and discuss the previous molecular studies and morphology-based character systems that have traditionally been used to classify this enigmatic phylum.

2. Materials and methods

A biodiversity survey of gelatinous zooplankton from off Cochin, South Eastern Arabian Sea, Indian Ocean was carried out by on board Central Institute of Fisheries Technology (CIFT), Cochin, India fishing vessel Matsya Kumari during the pre-monsoon (March), monsoon (July), and post-monsoon (December) seasons of the year 2017. Survey was also conducted during March 2017 to March 2018 to study the distribution and diversity of chaetognaths in Cochin backwater system, Southwest coast of India. Specimens were quantitatively sampled from epiplanktonic layer of the selected stations. Bongo net of 200 micrometer (μm) mesh size, mouth area 0.28 m^2 was used for the collection. Specimens were preserved in 4% formalin solution for morphological analysis and 95% ethanol solution for molecular analysis using protocols described by Bucklin et al. (2010). For specimens larger than ~25 mm, minimal excised tissue of an individual specimen was removed for DNA extraction and the remaining portion retained as the voucher. For specimens 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 subjected for DNA extraction (Peter et al., 2016). Specimens were examined under a stereo zoom microscope. The identification of chaetognaths was based on taxonomic keys provided by Todd and Laverack (1991). Taxonomical divisions of chaetognath species analyzed in this study are summarized in Table 1.

2.1. Molecular analysis

DNA was purified from individuals of chaetognaths by salting out procedure of Miller et al. (1988). DNeasy (Qiagen, Düsseldorf, Germany) kit, following manufacturer's instruction, was also used to extract DNA from samples, where the salting out procedure failed to yield satisfactory results. A 660 bp region of COI gene was amplified in a Gene Amp 9600 Thermal Cycler machine (Applied Biosystems Inc., California, CA, USA) by using LCO-1490 (5'GTCAACAAATCATAAAGATATTGG3') and HCO-2198 (5'TAAACTTCAGGGTGACCAAAAAATCA3') universal primers (Folmer et al.,1994). The PCR protocol was 94 °C for 1 min, 45 °C for 2 min, and 72 °C for 3 min, for 40 cycles. The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Amplified products were photographed using a gel documentation and analysis system (Bio-Rad)\ and the product size was determined with reference to a 100 bp DNA ladder (Fermentas, US). Specific amplified products were excised from the agarose gel and extracted using a QIAquick gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions. DNA sequencing was performed directly from the purified amplicons on an Applied Biosystems Inc. (ABI, USA) Model 377 automated DNA sequencer (Foster City, CA, USA) using the forward and reverse primers.

BioEdit sequence alignment editor version 7.0.5.2 (Hall, 1999) was used to edit and align the raw DNA sequences. Sequences having noisy peaks were excluded from the analysis. The unsolicited flanking sequences were trimmed and further assessment of insertion or deletions and stop codons were made in MEGA X (Kumar et al., 2018). Multiple sequence alignment and pairwise sequence alignment were performed in all the sequences using ClustalW program implemented in MEGA X (Kumar et al., 2018). Nucleotide variations were carefully monitored and edited manually. Sequences were translated into amino acid sequences using invertebrate mitochondrial codon pattern in the MEGA X (Kumar et al., 2018) for checking the pseudo-gene status. All the sequences were correctly translated into amino acid sequences with their respective starting primes without any internal stop codon.

The amplified sequences belonging to DNA barcode region of COI were confirmed by percentage similarity in the NCBI's BLASTn program. Higher percentage similarity (97%–100%) against the reference sequence was used to confirm the identity of the species. The similarity index between the query and the GenBank database sequence has been expressed as significant (97%–100%), moderate (92%–96%) and insignificant (≤91%). All the sequences were submitted to the GenBank.

Table 1. Taxonomical divisions of chaetognath species analyzed in this study.

Phylum	Order	Sub-Order	Family	Genus	Species
Chaetognatha	Aphragmophora	Ctenodontina	Sagittidae	Sagitta	*Sagitta bedoti* *Sagitta robusta* *Sagitta enflata* *Sagitta hexaptera* *Sagitta zetesios*
				Aidanosagitta	*Aidanosagitta neglecta* *Aidanosagitta regularis*
				Zonosagitta	*Zonosagitta pulchra*
			Pterosagittidae	Pterosagitta	*Pterosagitta draco*
		Flabellodontina	Krohnittidae	Krohnitta	*Krohnitta subtilis*
	Phragmophora	Monophragmophora	Eukrohniidae	Eukrohnia	*Eukrohnia hamata* *Eukrohnia bathyantarctica* *Eukrohnia macroneura* *Eukrohnia fowleri*
			Spadellidae	Spadella	*Spadella cephaloptera*
		Biphragmophora	Heterokrohniidae	Heterokrohnia	*Heterokrohnia sp.*

2.2. Phylogenetic analyses

40 nucleotide sequences that represent 8 species from 4 genera and 2 families from the present study (Table 2) along with 34 sequences that represent 16 species from 8 genera and 6 families from GenBank (Table 3) were incorporated to reconstruct phylogenetic relationships among these chaetognath species. Sequences of each species from the present study were from five multiple specimens, which were sampled in different geographic locations of Cochin backwater system, Southwest coast of India and off Cochin, South Eastern Arabian Sea, Indian Ocean, and therefore satisfied the typical criteria of molecular based phylogenetic rules that demands analysis and interpretation with multiple representative specimens under each taxa to be considered for phylogenetic interpretation.

Substitution model of COI sequences in chaetognaths was investigated by MrModeltest v2 program (Nylander, 2008) under Akaike information criterion (AIC). The general time-reversible (GTR) model was selected, with an estimated proportion of Invariant (I) DNA sites, and mutation rates among sites following a Gamma distribution (G). This GTR+I+G model was then used to generate Bayesian and maximum likelihood (ML) phylogenetic trees. The Bayesian tree was obtained with MrBayes 3.2.7 software (Ronquist et al., 2012). Two independent runs of four incrementally heated MCMC chains (one cold chain and three hot chains) were simultaneously run for 1,100,000 generations, with sampling conducted every 500 generations. The convergence of MCMC, which was monitored by determining the average standard deviation of split frequencies, was achieved (<0.01) within 1.1 million generations, and the initial 25% of the tested evolutionary trees were discarded as burn-in. The confidence values of the Bayesian inference tree are presented as the Bayesian posterior probabilities in decimal with the partitioned strategy.

To construct the ML tree, the hill-climbing algorithm of Hillis and Bull (1993) was performed online via the PhyML 3.0 web server (Guindon et al., 2010), using the default options, the chosen GTR+I+G model, and a starting tree made by neighbor joining (NJ). To maintain the consistency with MrBayes, in which the form of the molecular model is specified but parameters are estimated, only the model form was specified in PhyML. Support for nodes in the tree was assessed using the Shimodaira–Hasegawa approximate likelihood ratio test (SH-aLRT), (Shimodaira and Hasegawa, 1999) as implemented in PhyML. The confidence values of the ML tree are presented as the SH-aLRT value (SH-aLRTv) in percentage with the partitioned strategy. Nodes with support values of SH-aLRT ≥ 90 were considered as very robust and values ≥ 80% as robust (Minh et al., 2013; Hoang et al., 2018; Raupach et al., 2019), and specimens that share sister nodes at the tips of the tree are considered to be closely related and possibly as the same species (Hall, 2013).

3. Results

3.1. Molecular phylogenetic analyses

This study includes 16 extant species, which represent 8 genera and 6 of the 9 extant families. The alignment of 74 sequences of COI gene was 433 bp long after trimming. The optimal gene trees produced by Bayesian and ML trees were almost identical, in which the tip branches within species were short, and species were separated by much longer branches (Figures 1 and 2). Sequences were clustered strongly by species in all cases by both trees. The best model of evolution estimated with MrModeltest v2 was the GTR+I+G model (log likelihood = –7470.08779). Phylogenetic trees were rooted on the monophyletic assemblage consisting of Eukrohniidae, Spadellidae, and Heterokrohniidae families. The species of chaetognaths yielded two major monophyletic groups viz.: division I and division II. Division I group comprised Aphragmophora and division II comprised Phragmophora (Figures 1 and 2). The Aphragmophora division encompassed the family Sagittidae comprising Pterosagittidae and Krohnittidae families and Phragmophora division encompassed Eukrohniidae, Spadellidae, and Heterokrohniidae families. The node separating division I from division II was very well supported by both Bayesian and ML analyses (1/96.3, pp/SH-aLRTv). Similarly, the division II was also very well supported monophyletically by support values (1/96.3, pp/ SH-aLRTv). Further exploration of the data revealed that most other internal nodes are also strongly supported by both analyses. Statistical values obtained from the Bayesian and ML methods are represented on the corresponding Bayesian and ML topologies (Figures 1 and 2). All the families (Eukrohniidae 1/81.9, Spadellidae 1/100, Heterokrohniidae 1/100 and Sagittidae comprising Pterosagittidae and Krohnittidae 1/96.3) were highly supported by both trees (Figures 1 and 2).

Regarding the species studied, the division I group (Aphragmophora) encompassed *Aidanosagitta neglecta, A. regularis, Krohnitta subtilis, Pterosagitta draco, Sagitta bedoti, S. enflata, S. hexaptera, S. robusta, S. zetesios,* and *Zonosagitta pulchra*. Krohnittidae, the monogeneric family comprising *K.subtilis* ascended as the sister-species to *S.enflata* with Bayesian and ML support values of 1 and 94.5, respectively (Figures 1 and 2). Though, there are occurrence of two more species from the world oceans, COI sequences from *K. subtilis* was the only one representative of Krohnittidae at the GenBank. *P. draco*, the only living representative of the Pterosagittidae, placed within the clade Sagittidae in both phylogenetic trees. Hence, monophyly of Sagittidae were not recovered not

Table 2. Details of the sequences and GenBank accession numbers obtained from this study.

Sl No	Species	Voucher No.	Geographical Location	Latitude (N) Longitude (E)	GenBank Accession No.
1	*Sagitta enflata*	CR.MK-SE-01	Off Cochin, Arabian Sea	09°57'–76°11'	MH500023
		CR.MK-SE-02	Off Cochin, Arabian Sea	09°55'–76°07'	MH500024
		CR.MK-SE-03	Off Cochin, Arabian Sea	09°54'–76°06'	MH500025
		CR.LB-SE-01	Cochin Backwaters	10°01' – 76°26'	MH500026
		CR.LB-SE-02	Cochin Backwaters	09°96' –76°25'	MH500027
2	*Sagitta robusta*	CR.MK-SR-01	Off Cochin, Arabian Sea	09°57'–76°11'	MH444759
		CR.MK-SR-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH444760
		CR.MK-SR-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH444761
		CR.LB-SR-01	Cochin Backwaters	10°01' –76°26'	MH444762
		CR.LB-SR-02	Cochin Backwaters	09°96' –76°25'	MH444763
3	*Zonosagitta pulchra*	CR.MK-ZP-01	Off Cochin, Arabian Sea	09°57' –76°11'	MH444742
		CR.MK-ZP-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH444743
		CR.MK-ZP-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH444744
		CR.LB-ZP-01	Cochin Backwaters	09°96' –76°25'	MH444745
		CR.LB-ZP-02	Cochin Backwaters	09°96'–76°25'	MH444746
4	*Sagitta bedoti*	CR.MK-SB-01	Off Cochin, Arabian Sea	09°57'–76°00'	MH752193
		CR.MK-SB-02	Off Cochin, Arabian Sea	09°55'–76°07'	MH752194
		CR.MK-SB-03	Off Cochin, Arabian Sea	09°54'–76°06'	MH752195
		CR.LB-SB-04	Cochin Backwaters	10°01'– 76°26'	MH752196
		CR.LB-SB-05	Cochin Backwaters	09°96' –76°25'	MH752197
5	*Aidanosagitta neglecta*	CR.MK-AN-01	Off Cochin, Arabian Sea	09°57' –76°11'	MH388294
		CR.MK-AN-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH388295
		CR.MK-AN-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH388296
		CR.MK-AN-04	Off Cochin, Arabian Sea	09°53' –76°05'	MH388297
		CR.MK-AN-05	Off Cochin, Arabian Sea	09°52' –76°03'	MH388298
6	*Sagitta hexaptera*	CR.MK-SH-01	Off Cochin, Arabian Sea	09°57' –76°11'	MH649351
		CR.MK-SH-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH649352
		CR.MK-SH-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH649353
		CR.MK-SH-04	Off Cochin, Arabian Sea	09°53' –76°05'	MH649354
		CR.MK-SH-05	Off Cochin, Arabian Sea	09°52' –76°03'	MH649355
7	*Pterosagitta draco*	CR.MK-PD-01	Off Cochin, Arabian Sea	09°57' –76°11'	MH649361
		CR.MK-PD-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH649362
		CR.MK-PD-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH649363
		CR.MK-PD -04	Off Cochin, Arabian Sea	09°53' –76°05'	MH649364
		CR.MK-PD-05	Off Cochin, Arabian Sea	09°52' –76°03'	MH649365
8	*Aidanosagitta regularis*	CR.MK-AR-01	Off Cochin, Arabian Sea	09°57' –76°11'	MH649356
		CR.MK-AR-02	Off Cochin, Arabian Sea	09°55' –76°07'	MH649357
		CR.MK-AR-03	Off Cochin, Arabian Sea	09°54' –76°06'	MH649358
		CR.MK-AR-04	Off Cochin, Arabian Sea	09°53' –76°05'	MH649359
		CR.MK-AR-05	Off Cochin, Arabian Sea	09°52' –76°03'	MH649360

Table 3. Details of the sequences and GenBank accession numbers obtained from previous studies and used in the present analyses.

Sl No.	Species	Voucher No.	Geographical Location	GenBank Accession No.
1	*Sagitta robusta*	NIOBZC34	Indian Ocean; India	JN258034
2	*Sagitta robusta*	NIOBZC32	Indian Ocean; India	JN258032
3	*Aidanosagitta regularis*	NIOBZC29	Indian Ocean; India	JN258029
4	*Aidanosagitta regularis*	NIOBZC28	Indian Ocean; India	JN258028
5	*Pterosagitta draco*	NIOBZC9	Indian Ocean; India	JN258009
6	*Pterosagitta draco*	NIOBZC8	Indian Ocean; India	JN258008
7	*Sagitta bedoti*	NIOBZ 2	Cochin Backwaters; India	FJ648784
8	*Sagitta bedoti*	NIOBZC4	Indian Ocean; India	JN258004
9	*Zonosagitta pulchra*	NIOBZC26	Indian Ocean; India	JN258026
10	*Aidanosagitta neglecta*	NIOBZC20	Indian Ocean; India	JN258020
11	*Aidanosagitta neglecta*	NIOBZC23	Indian Ocean; India	JN258023
12	*Aidanosagitta neglecta*	Y16S9	South China Sea	KY882130
13	*Aidanosagitta neglecta*	Y16S16	South China Sea	KY882131
14	*Sagitta hexaptera*	NIOBZC17	Indian Ocean	JN258017
15	*Sagitta hexaptera*	NIOBZC18	Indian Ocean	JN258018
16	*Sagitta enflata*	St.9-2	South China Sea	KX009863
17	*Sagitta enflata*	St.9-19	South China Sea	KX009873
18	*Krohnita subtilis*	SP9CH	Arabian Sea	FJ538305
19	*Sagitta zetesios*	UCONN:Ch11.2.1	Atlantic Ocean: northern Mid- Atlantic Ridge	GQ368425
20	*Sagitta zetesios*	UCONN:Ch11.1.2	Atlantic Ocean: northern Mid- Atlantic Ridge	GQ368423
21	*Eukrohnia hamata*	UCONN:Ch19.4.1	Arctic Ocean	FJ602473
22	*Eukrohnia hamata*	UCONN:Ch19.9.3	Atlantic Ocean: southeast region	GQ368390
23	*Eukrohnia hamata*	G25	Atlantic Ocean	KC633127
24	*Eukrohnia bathyantarctica*	UCONN:Ch03.1.10	Atlantic Ocean: near northern Mid-Atlantic Ridge	GQ368380
25	*Eukrohnia bathyantarctica*	UCONN:Ch03.1.7	Atlantic Ocean: near northern Mid-Atlantic Ridge	GQ368377
26	*Eukrohnia macroneura*	UCONN:Ch19.6.2	Atlantic Ocean: northeast region	GQ368392
27	*Eukrohnia macroneura*	UCONN:Ch19.6.3	Atlantic Ocean: northeast region	GQ368393
28	*Eukrohnia fowleri*	UCONN:Ch02.3.1	Atlantic Ocean: northeast region	GQ368387
29	*Spadella cephaloptera*	SOR-23	France: Calanque de Sormiou	KP843795
30	*Spadella cephaloptera*	SOR-26	France: Calanque de Sormiou	KP843798
31	*Spadella cephaloptera*	SOR-24	France: Calanque de Sormio	KP843796
32	*Spadella cephaloptera*	SOR-25	France: Calanque de Sormiou	KP843797
33	*Heterokrohnia sp.*	UCONN:Ch26.1.1	Arctic Ocean	FJ602474
34	*Heterokrohnia sp.*	UCONN:Ch26.1.2	Arctic Ocean	FJ602475

only because of the inclusion of *P. draco* but also that of *K. subtilis* is sister to *S. enflata* species (Figures 1 and 2).

According to the rooted topology obtained on the analyses of division II (Phragmophora) group, Eukrohniidae, Spadellidae, and Heterokrohniidae were rooted by a monophyletic assemblage with well supported values (1/96.3). As stated by Gasmi et al. (2014) using the molecular phylogeny by SSU and LSU rRNA genes, within the Eukrohniidae, *Eukrohnia fowleri* appeared basal with the other species under the genus by both analyses. It remarkably revealed that *E. bathyantarctica* and *E. hamata* are probably young sister-species (Figures 1 and 2). Our results unambiguously confirmed the monophyly of Eukrohniidae, since *Eukrohnia bathyantarctica, E. fowleri, E. hamata,* and *E. macroneura,* produced a unique assemblage with a support of 1/81.9. Both Spadellidae (1/100) and Heterokrohniidae (1/100) families were analyzed with a single set of available species at the

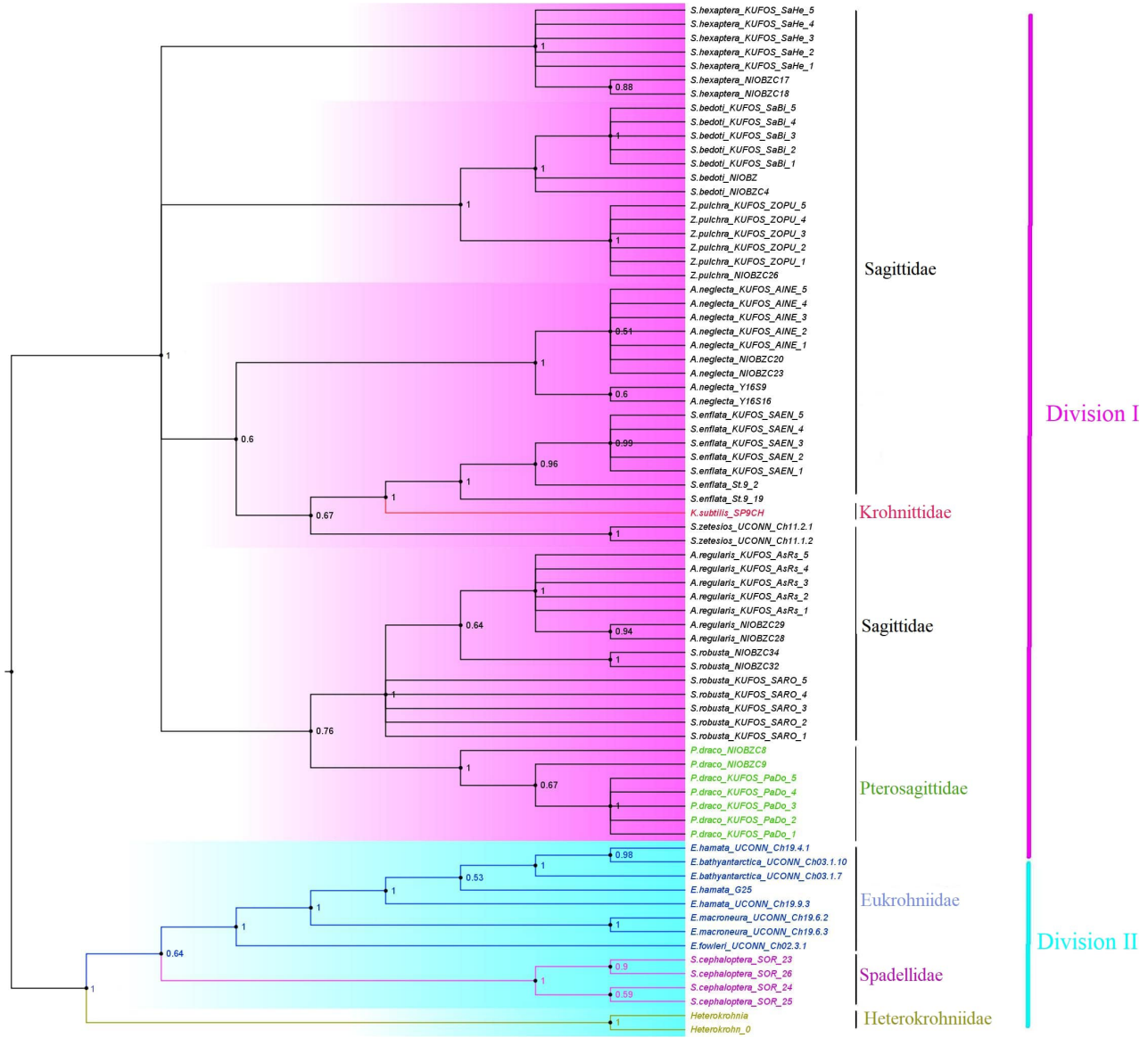

Figure 1. The Bayesian tree based on the analysis of COI gene sequences. The confidence values are presented on the nodes.

GenBank. Based on the available set of sequences deposited at the GenBank, the study was able to place the families Eukrohniidae and Spadellidae (Monophragmophora) in a single clade but with low robust values (0.64/54) and family Heterokrohniidae (Biphragmophora) as another unique clade with very high robust values (1/100) (Figures 1 and 2).

4. Discussion

4.1. Division I- Aphragmophora and Ctenodontina/ Flabellodontina hypothesis

Studies on the internal systematics in chaetognaths (Nielsen, 2001; Papillon et al., 2006; Perez et al., 2014) revealed two major groups, Phragmophora and Aphragmophora, on the basis of the occurrence of the phragms. Throughout the debate on chaetognath evolutionary trends, authors like Tokioka (1965a) and Casanova (1985) agreed to consider the presence of phragms as a plesiomorphic state but with slightly different hypotheses. Salvini-Plawen (1986) suggested a radically different concept which contradicted the primitiveness of phragms and identified Pterosagittidae as the sister group to all remaining families.

Later, Bieri (1991a) pointed out a possible relationship between *P. draco* and species belonging to the family Sagittidae. The inclusion of *P. draco* within Sagittidae has been corroborated by many reports (Harzsch et al., 2009; Gasmi et al., 2014). In agreement with these reports, our study also showed an assemblage of *P. draco*

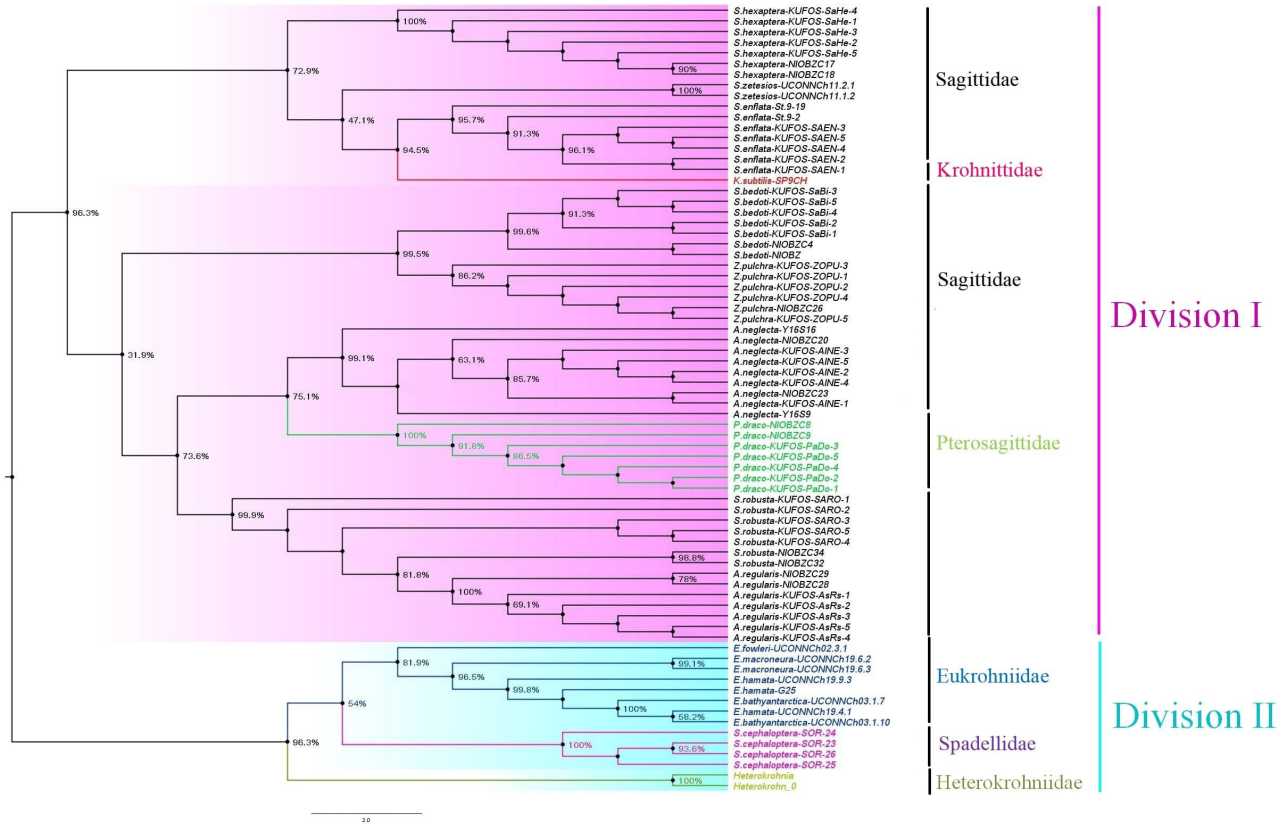

Figure 2. The maximum likelihood tree based on the analysis of COI gene sequences. The confidence values are presented on the nodes.

(Pterosagittidae) to that of Sagittidae species. Although there is only one species that was taken into account from Krohnittidae, the *K. subtilis* ascended as sister-species to *S. enflata* by both analyses and showed a close assemblage to that of Sagittidae species. As stated by Gasmi et al. (2014) using both morphological and molecular data, monophyly of Sagittidae were not retrieved in our analyses and revealed that Sagittidae is strictly paraphyletic. Hence, we propose that the Aphragmophora division encompassed Sagittidae comprising Pterosagittidae and Krohnittidae families and our analyses revives the concept of Aphragmophora, a clade invalidated by Papillon et al. (2006). In parallel to our findings, the first molecular study conducted by Telford and Holland (1997) using LSU rRNA gene upheld the concept Aphragmophora by including Sagittidae, Ptreosagittidae, and Krohnittidae under a unique clade. Again, a recent phylogenetic study conducted by Gasmi et al. (2014) using both SSU and LSU rRNA genes were also supported the monophyly of Aphragmophora with the Pterosagittidae included in the Sagittidae. However, our findings undermined an earlier hypothesis proposed by Papillon et al. (2006) using 26 sequences of the SSU rRNA isolated from members of six extant families. According to them, the order Aphragmophora is monophyletic without *Pterosagitta draco*, the only living representative of pterosgittidae family.

Finally, moving on to Tokioka's biclassification concept of Aphragmophora into two sub-orders (Flabellodontina containing the family Krohnittidae and Ctenodontina containing families Sagittidae and Pterosagittidae), our study established that Sagittidae sensu stricto is a paraphyletic assemblage from which *P. draco* and *K. subtilis* derives. Morphological studies conducted by many scientists were already disproved this concept and added that further division of Aphragmophora into Ctenodontina/Flabellodontina is not relevant (Salvini-Plawen, 1986; Casanova, 1996 and Gasmi et al., 2014). Later, Papillon et al. (2006) and Gasmi et al. (2014) using the molecular phylogeny of a portion of ribosomal (rRNA) genes also disproved this biclassification concept. Hence, the Ctenodontina and Flabellodontina concept and the hypothesis based on the structure of the cephalic armature were not supported.

4.2. Division II- Phragmophora and validity of Biphragmophora/ Monophragmophora and Syngonata/ Chorismogonata hypotheses

According to our results, earlier classification which included Eukrohnia, Heterokrohnia, and Spadella in a

single family viz., Eukrohniidae as proposed by Von Ritter-Zahony (1911) and Hyman (1959) is invalid. In parallel to the statement proposed by Gasmi et al. (2014) who used SSU and LSU rRNA genes, both the Bayesian and ML trees formed by COI gene were able to separate the species of Eukrohniidae, Spadellidae, and Heterokrohniidae in three separate clades. As stated by Telford and Holland (1997) who used the LSU rRNA gene, the grouping of Eukrohniidae, Spadellidae, and Heterokrohniidae under the monophyletic division of Phragmophora is found well supported for the available molecular datasets studied and thereby invalidated Gasmi's concept of paraphyly of Phragmophora (Gasmi et al., 2014). Again, our results underscored an earlier morphological hypothesis proposed by Tokioka (1965a, 1965b) and Salvini-Plawen (1986) regarding the monophyly of Phragmophora and undermined their concept of inclusion of Heterokrohniidae under Eukrohniidae.

Our study unambiguously confirmed the monophyly of Eukrohniidae, since *Eukrohnia bathyantarctica, E. fowleri, E. hamata,* and *E. macroneura* produced a unique assemblage with support values 1/81.9. This result was in accordance with recent phylogenetic analyses where a close relationship was observed in species under the family Eukrohniidae (Jennings et al., 2010, Gasmi et al., 2014). The molecular analyses supported the division of Phragmophora into two monophyletic groups, the Monophragmophora and Biphragmophora. Phylogenetic trees showed Casanova's concept of Monophragmophora (Eukrohniidae and Spadellidae) as a natural group, yet with low robust values (0.64/54). In agreement with the Casanova's hypothesis, when placed Heterokrohniidae under the sub-division Biphragmophora, the available set of sequences of Heterokrohnia species produced a distinctive clade. Hence, the subdivisional concept of Biphragmophora was found true and rejected the statement proposed by Papillon et al. (2006). However, to definitely conclude such a sister-group relationship between these three families (Eukrohniidae, Spadellidae and Heterokrohniidae), broader COI gene sequences from various species of Heterokrohniidae, meso-bathyplanktonic Eukrohniidae, and representative of Hemispadella genus, a link between the families Heterokrohniidae and Spadellidae, (Casanova, 1996) need to be studied. Moving on to the biclassification concept of Casanova in to Syngonata and Chorismogonata, a clear separation was detected between the species under Phragmophora and Aphragmophora, and thereby the Syngonata and Chorismogonata hypothesis found undermined. Earlier studies conducted by Papillon et al. (2006) and Gasmi et al. (2014) already rejected the Syngonata and Chorismogonata hypothesis.

Although this study provides some coverage of species of phylum Chaetognatha, it is not a complete analysis of ca. 130 chaetognath species from the global oceans (Miyamoto et al., 2014). Taxonomic coverage was uneven for Heterokrohniidae, Krohnittidae, and Spadellidae families. Hence, an expanded database of chaetognaths COI barcodes is needed to improve the accuracy of species identification and phylogeny of this complex group of organisms. Further, it is well known that an evolutionary tree (gene tree) constructed from DNA sequences for a genetic locus does not necessarily approve with the tree that represents the real evolutionary pathway of the species involved (species tree). Therefore, one has to use DNA sequences from various loci that have evolved independently of each other to predict the actual evolutionary relationship of organisms (Pamilo et al., 1988). Although we used only a single set of gene locus (COI) in our analyses, we were able to compare our results with previously proposed major hypotheses using various molecular loci and thereby provided new insights into the evolutionary relationships of chaetognaths.

5. Conclusion

The first molecular phylogenetic analyses of the chaetognath COI barcodes served as an accurate tool for species identification and evolution. Based on the sequences obtained from our study and a set of sequences retrieved from the GenBank, we hereby propose that the traditional concept of division into Aphragmophora and Phragmophora is supported. In light of our analyses, we recommend the following clade structure for the phylum: Aphragmophora comprising Sagittidae with Pterosagittidae and Krohnittidae included in the Sagittidae and Phragmophora comprising Eukrohniidae, Spadellidae, and Heterokrohniidae.

Moreover, the suborders concepts of Ctenodontina/Flabellodontina and Syngonata/Chorismogonata are found to be invalid. Phylogenetic analyses also support the division of Phragmophora into two monophyletic groups, the Monophragmophora and Biphragmophora. Hence, we suggest that molecular taxonomy combined with proper morphological identification is crucial for improving the comprehensive understanding of this mysterious group of organisms. Precise phylogenetic investigations using various molecular markers and specimens from diverse regions are definitely needed to provide an exact evolutionary concept on this enigmatic phylum.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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