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Research Article
Morphological and Molecular Identification of *Paraorygmatobothrium floraformis* in *Rhizoprionodon acutus* off Nellore Coast, Bay of Bengal, India

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Abstract

Background and Objective: Elasmobranchs (Sharks, skates, rays) are frequently available marine fishes which serve as an ideal host for the cestode parasites. *Paraorygmatobothrium floraformis* (Southwell, 1912) Ruhnke, 2011 is a cestode comprising a ubiquitous and specious genus of elasmobranch parasites. This parasite was isolated from the spiral intestine of commercially available elasmobranch fish, *Rhizopriondon acutus* of the Nellore coast, Bay of Bengal. The main objective of this work is to evaluate the morphological criteria, molecular characterization of *Paraorygmatobothrium floraformis* using the 28S ribosomal DNA and to determine the phylogenetic position of this species with bioinformatics tools like BLAST and Mega 6. **Materials and Methods:** The present study is restricted to Nellore Coast, which has a coastline of 169 km. The milk shark, *Rhizopriondon acutus* was collected from the local fisherman and the nearby Coastal areas of Nellore to examine the cestodes from their spiral intestines. They were preserved in FAA (Formalin-10 mL, Alcohol-85 mL and Acetic acid-5 mL) for further morphological analysis. Also, few parasites were fixed in 95% molecular grade ethanol and placed at low temperature of -20°C for further molecular analysis. **Results:** The morphological data was taken from the whole mounts. However, the obtained gene sequences of the cestode parasite were submitted to the Genbank. The gene sequence of the parasite was compared with the available gene sequences of other cestode parasite from other elasmobranchs in the Genbank. The pairwise alignment of gene sequences showed 80% similarity with the order Tetraphyllidea and 5-10% mismatches with the sequences of *Phyllobothriidea* family. Also, the host, *Rhizoprionodon acutus* and Nellore coast serve as new host record and locality records for *Paraorygmatobothrium floraformis* (Southwell, 1912) Ruhnke, 2011. **Conclusion:** Species identification is one of the major difficulties in marine fish parasites. Ribosomal DNA has been widely used for taxonomic studies. The study presents a small molecular phylogenetic analysis of the elasmobranch tapeworms of family *phyllobothridea* and order Tetraphyllidea. These types of analyses can reveal the relationship between different orders, families and species of the cestode parasites from the marine environment.

Key words: *Paraorygmatobothrium floraformis*, molecular analysis, *Rhizoprionodon acutus*, nellore coast, bay of Bengal

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Marine water fish have been model hosts in the study of the community and evolutionary ecology of parasites for the last few decades. Species identification is one of the main problems in marine fish parasites. Parasites are usually small, soft bodied and have few morphological characters. Tapeworms (Platyhelminthes: Cestoda) are obligate internal parasites of vertebrates that display a wide range of body forms, life histories and host associations[1]. Sharks also carry a variety of helminth parasites, of which cestode representation is considered to be rich. Work on cestode parasites of fish was started in 18th Century, but the molecular work came into existence in the recent years i.e., from 20th century. Cestodes are long, slender, white in color measures 10-20 cm long and easily suitable for molecular analysis when compared with other parasites. Cestodes of the family Phyllobothriidae are parasites of elasmobranch fishes and cetaceans. There are approximately 25 valid species under the genus[2]. Phylogenetic relationships of phyllobothriid cestodes have been constructed using morphology, host specificity and biological traits. However, it is still intricate to come up with a consistent conclusion on the phylogenetic relationship between the members of Phyllobothriidae. However, recent studies have shown high level of interspecific variations for molecular characters, which are helpful for characterization of species of *Paraorygmatobothrium* and many new phyllobothrrid species have also been reported using recent molecular biological techniques[3,4]. *Paraorygmatobothrium floraformis* (Southwell[5], Ruhnke[6] comprises a ubiquitous and specious genus of elasmobranchs parasites[5,6]. *Paraorygmatobothrium floraformis* belongs to order Tetraphyllidea. The genus *Paraorygmatobothrium* was erected by Ruhnke[7] with *Paraorygmatobothrium prionacis* (Yamaguti, 1934) Ruhnke[7] as its type taxon. These parasites are thin, small and milky white in color measuring 12-17 cm in length, acraspedote and apolytic. Substantial progress has been made on the phylogenetic studies of tapeworms (Eucestoda) and rDNA clusters are proved to be effective tools for genetic studies[8]. In this study, we used 28S ribosomal DNA of cestode *Paraorygmatobothrium floraformis*. A portion of the nuclear large subunits 28S rDNA gene was selected in resolving interspecific phylogenetic relationships with other cestode groups[1,3,4,9-12]. This ribosomal DNA is used to resolve taxonomic issues of helminth parasites[12-18]. Advantage of using this region is that the repeated copies provide a large number of target sequences for PCR amplification[19] and it is easy to amplify even small quantities of DNA and these markers are also extensively used for taxonomic studies[20]. The present study aimed at identifying this cestode based on morphological studies supplemented with molecular characterization.

MATERIALS AND METHODS

Study area: The Coastline of Andhra Pradesh is the Second longest coastline with 974 km (605 mi) with an authority over nearly 15,000 km^2 territorial waters, touching 9 districts of Coastal Andhra, including the Nellore District. Nellore is located at 14.43°N 79.97°E. Nellore city is located on the banks of the River Penner and is well known for its aquaculture and agricultural produce. The present study is restricted to Nellore Coast, which has a coastline of 169 km with 12 mandals and Krishnapatnam, Ramayapatnam, Ramatheertham, Katepalli, Kodur, Mypadu, Muttukuru and Tupilipalem beaches are the well-known beaches in the district[21]. The host specimen collections were made from January, 2014 to December, 2016.

Collection of hosts: The milk shark, *Rhizoprionodon acutus* collected from the local fisherman near the Coastal areas of Kavali to Thada and from the local fish markets of Nellore, kavali to Thada are the most commonly available fish species from this coast when compared to other elasmobranch fishes. Fishes of small, medium and alimentary canals of large sized fish were transported to the laboratory and were screened for the presence of the parasites.

Collection of cestodes: The spiral intestine of *R. acutus* was removed and opened with a longitudinal incision. A preliminary examination of the spiral intestine was conducted with stereozoom microscope (LM-52-3621 Elegant) for cestodes. The obtained parasites were divided into three categories for the following analyses.

Morphological analysis: Cestodes were properly pressed and preserved in FAA (Formalin-10 mL, Alcohol-85 mL and Acetic acid-5 mL) for permanent slide preparation, then the specimens were washed with water and stained with alum caramine followed by proper dehydration in several grades of alcohol (50, 75, 90, 95 and 100%) and mounted on glass slide with Canada balsam[22,23]. Line diagrams were drawn with the help drawing tube attachment of Lynx Trinocular Microscope (N-800M). Morphological data were obtained from this whole mounts and ocular micrometer measurements were mentioned in millimeters unless otherwise indicated.

Scanning electron microscopy (SEM) study: Some parasites were stored in 2.5% glutaraldehyde and processed as per protocol for scanning electron microscopy (SEM) studies described elsewhere[24]. The SEM images were taken at different magnifications by Carl Zeiss microscope (ΣIGMA TM) provided by SVU, Tirupati.

Molecular characterization: Few parasites were fixed in 95% molecular grade ethanol for molecular analysis and placed at low temperature -20°C. Total genomic DNA was extracted using the standard Phenol-Chloroform Protocol[25]. The DNA was extracted from the mid-section of worms i.e., from the proglottid region, preserved in 95% ethanol and stored at -20°C and the terminal proglottids and scolex of each was prepared as a whole mount.

Tissue
↓
Centrifuge at 10000 rpm, 5 min
↓
Wash the pellet with 1x PBS (to remove tissue Toxicity)
↓
Centrifuge at 10000 rpm, 5 min
↓
Disturb pellet and resuspend cells in Lysis buffer 800 µL/1 mg
↓
Vortex the whole suspension to achieve uniformity
↓
Add 5 µL of r Nase, allow it to stand for 5 min
↓
Add 25 µL of Proteinase K (if the conc. is 2.4 mg mL^{-1})
↓
Incubate at 55°C for 2 h
↓
Centrifuge at 10000 rpm, 5 min
↓
Discard the pellet. To the supernatant add 10 µL 5M NaCl and 1 µL glycogen (if conc. is 20 mg mL^{-1})
↓
Invert mix and add 500 µL 100% IPA
↓
Incubate at -20°C for 10 min
↓
Centrifuge 12000 rpm, 10 min
↓
Discard soup. Wash the pellet with 500 µL of 75% Ethanol (2 times) at 12000 rpm, 5 min
↓
Discard soup and air dry the pellet
↓
Re suspend the pellet in elution buffer (TE Buffer)

A portion of the nuclear large subunit 28S ribosomal DNA gene was selected; the advantage of this region is easy to amplify even small quantity of DNA[26]. The following primers set are used to amplify the 28S ribosomal DNA the forward primer, LSU (5′-TAGGTCGA CCCGCTGAATTAAGCA-3′) and the reverse primer, 1200R (5′-GCATAGTTCACCATCTTTCGG-3′)[27]. The products were amplified by using AURA Red Taq 2x Master mix and amplified products were purified using Qiagen PCR purification method. Cycling conditions were as follows, Step-1: Initial denaturation at 95°C for 5 min, Denaturation at 95°C for 30 sec, Annealing at 52°C for 30 sec, Extension at 72°C for 30 sec, Step 2: 40 cycles, Final Extension at 72°C for 7 min. The product was purified and sequenced in both directions by an automated sequencer by (Eurofins, Chennai). The sequences obtained were examined by using bioinformatics tool Basic Local Alignment Search Tools (BLAST). The phylogenetic trees of these sequences were drawn using MEGA 6 (Molecular Evolutionary Genetics Analysis) software and in that Test Neighbor-Joining (NJ) analysis were performed and branch support was given by using 500 bootstrap replicates.

RESULTS AND DISCUSSION

Morphological analysis: *Paraorygmatobothrium floraformis* (Southwell[5]) Ruhnke[6] (Fig. 1a-d).

Synonyms: *Anthobothrium floraformis* Southwell, 1912, *Phyllobothrium floraforme* (Southwell[5]) Southwell, 1930.

- Name of the host: *Rhizoprionodon acutus* Ruppell, 1937
- Number of examined and infected hosts: 152
- Number of hosts infected: 39
- Number of parasites collected: 149
- Site of infection: Spiral intestine

Description: The morphological examination of the parasite revealed typical phyllobothriid features: Parasites thin, small and milky white in colour measuring 8-14 cm in length, acraspedote and apolytic. Scolex small 0.24-0.38×0.27-0.3 mm with four sessile, shallow cup shaped bothridia having no suckers. Each bothridia measures 0.14-0.29×0.14-0.23 mm and their margins are thick and slightly crenulated. Neck short and wide 0.23-0.71×0.04-0.06 mm and is followed by a long unsegmented part measuring 4.2-5.8 mm. Strobila consists of 8-9 proglottids. All the proglottids are longer than broad. Immature proglottid less in number measuring 0.50-0.89×0.2-0.3 mm. Mature region starts from 4-6th proglottid of the strobila. Mature proglottids are 3-4 times longer than broad measuring 1.28-1.90×0.21-0.39 mm. Gravid proglottids not obtained. Testes 68-97 in number, spherical to oval and are in two rows on either side of the median longitudinal axis, testes occupy medullary region from anterior

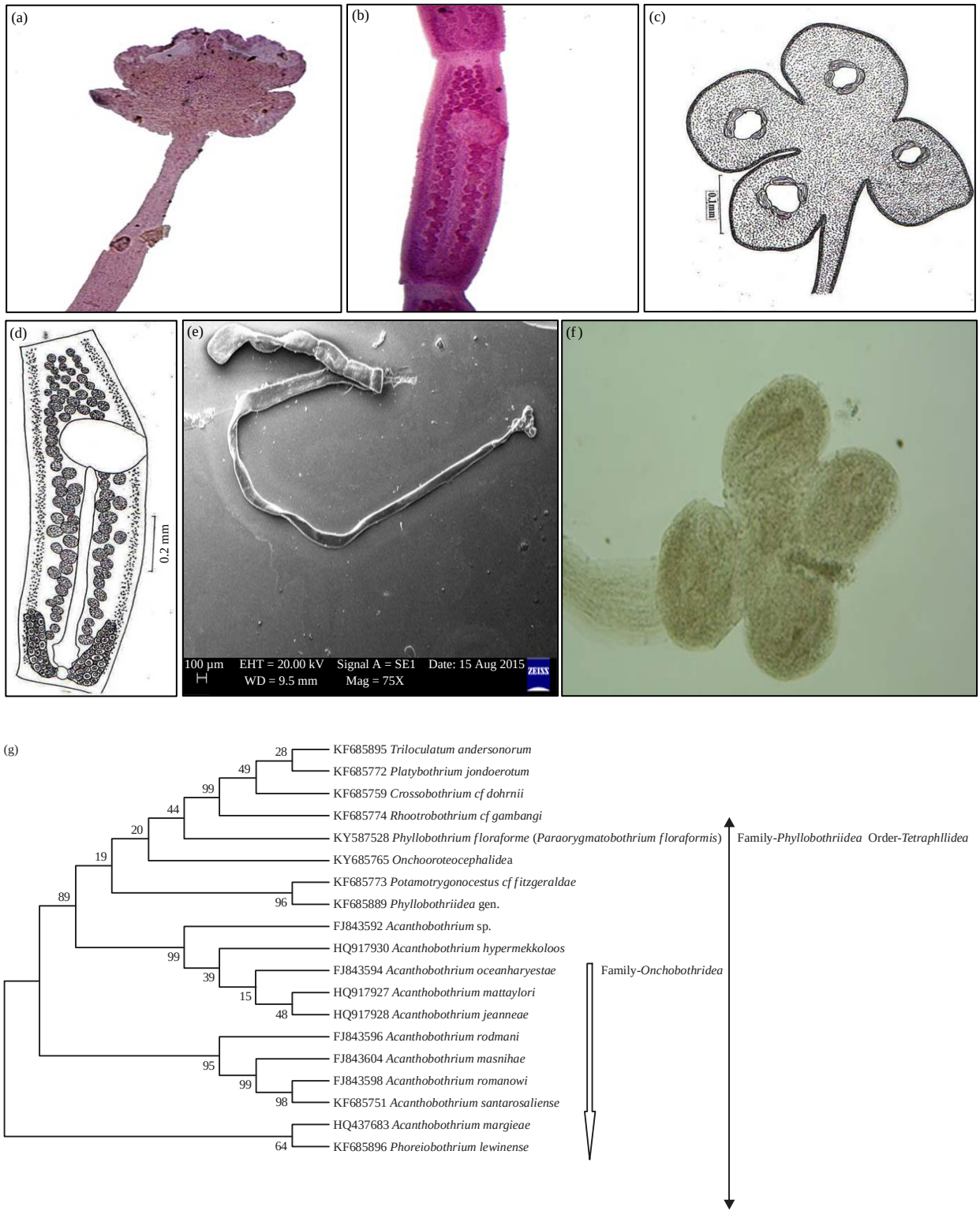

Fig. 1(a-g) (a) Microphotograph of Scolex of *Paraorygmatobothrium floraformis* 100X, (b) Microphotograph of mature proglottid of *P. floraformis*, (c) Line diagram of scolex of *P. floraformis*, (d) Line diagram of mature proglottid of *P. floraformis*, (e) Entire Worm-*P. floraformis* (SEM) 75X, (f) Original *Paraorygmatobothrium floraformis*-Scolex (light microscope) 100X and (g) Phylogenetic relationship of cestode parasties

Table 1: Pairwise alignment of sequence of *Phyllobothriidea* genus 10 n.sp. from elasmobranch fish, this sequence taken from gene bank (KF685889) for comparison with *P. floraformis*

P. floraformis	CCAATGACCACATCATGGGACGGGCCGGTGATGCGCCCACTGCTCTTTARCCTGATGCTG
Phyllobothriidea	CCAATGACCACATCATGGGACGGGCCGGTGATGCGCCCACCGCTCTT-AGCCTGATGCTG
	* * *
P. floraformis	ACA---T TAGGCTGATGCGAGTAACAGCAGGATCTCACCTTACTCCGAGA-CAAGTCGAA
Phyllobothriidea	ACAAAGC TAGGCTGATGCGAGTAACAGCAGGATCTCACC TCACTCCGAGA ACAAGTCGAA
	* * * * * *
P. floraformis	GCTTTACTTTCACTGCGCCTTTGGGTTTCGTA-ACGCCCATTGACTCGCATACATGTTA
Phyllobothriidea	GCTTTACTTTCACTGCGCCTTTGGGTTTCGTAGGAC-CCC-TTGACTTGCATACATGTTA
	** * * *
P. floraformis	AACTCCTTGGTCCGTGTTTCAARACGGGTCGGGTAGCTCGTCTACCACTACACCACTGAC
Phyllobothriidea	AACTCCTTGGTCCGTGTTTCAAG ACGGGTCGGGTAGCTCGTCTACCACTACACCACTGAC
	*
P. floraformis	TATTAGGCCCACTGCATCCCGTGCT GACAACATCATCCGC TGCCTATCCCG AGTCAAGCC
Phyllobothriidea	TATTTGGCCCACCACACCCCACACAGGGAGCACCATCCACAACCCATCCTG CATC AGGCC
	* ** * *** * ** * * ** * * ** *
P. floraformis	ACTGCCGGGCCTACTTTGCGCAGGCGACACGACAATCAGACCCAACACCGGGCARACAAT
Phyllobithriidea	ACTGCCGAGCCCACTTTGCGCAGGCGACACGACAATCAGACCCAACAACAGGCAGACTGC
	* * * * ***
P. floraformis	AACGACACT-GCCACTGGTGCCACTTGTAACATGGGCACARGACACATTTGAGGCCGAT
Phyllobothriidea	AATGGCACCCCGCCGCT TAA-CCACTTGTAACATGGGCGCGGGATGCATTTGAGGCCGAT
	* * *** * *** * * ** **
P. floraformis	AACTCCGGTGCCTTGCACAGTTATTCTTACCACCWTGTCCCACCAAACC
Phyllobothriidea	AAACCCGGTGCCTTGCACAGTTATTCTTACCACC ATGGCCCACCAGACC
	** * * *

*Indicates the difference in the nucleotide sequence of *P. floraformis* and *Phyllobothriidea genus*

to ovarian region leaving space to cirrus and central uterine sac. Testes measure 0.03-0.07 mm in diameter. Cirrus sac large, oval present in the anterior third of proglottid measuring 0.12-0.25×0.19-0.22 mm. Genital pores irregularly alternate. Ovary bilobed, U-Shaped and posterior. Ovarian lobes are connected by an isthmus in the posterior region. Each lobe measures 0.2-0.3×0.05-0.10 mm. Vitellaria granular, forms strands on either side, extending the entire cortical length of the proglottid. Uterus is narrow tube, originates from ovarian isthmus and extends up to the level of cirrus sac. All these morphological characters are in consistence with those of *Paraorygmatobothrium floraformis* species.

SEM description: The SEM (Fig. 1e) and LM (Fig. 1f) studies of scolex shows four shallow cup like bothridia with no suckers. Neck is long and strobila consists of proglottids. Immature proglottid is less in number and mature proglottids are broad longer than immature proglottids.

Molecular analysis: Sequence length ranged from 364-478 base pairs. The nucleotide sequence data reported in this paper were submitted to the gene bank by the name *Phyllobthrium floraforme*, its accession number (KY587528) which is a synonym of *Paraorgymatobothrium floraformis*. The nucleotide sequences obtained from PCR were subjected to BLAST. The BLAST hits results showed that the sequences of the Order Tetraphyllidea and families belonging to *Phyllobothridea* and *Onchobothridea* species are closer to this sequence with maximum similarity.

Phylogenetic analysis: For the purposes of phylogenetic analyses, molecular data were generated for 1 specimen. Phylogenetic tree were obtained by comparing the cestode parasite sequence of other elasmobranch fishes from different geographical isolates. All 28S ribosomal DNA sequences were aligned by using MEGA 6 Software. The present sequences showed close similarities to the sequences of Order Tetraphyllidea confirming the species to this order. Within the order Tetraphyllidea, the *Paraorygmatobothrium floraformis* sequences showed maximum (90%) similarity with the sequences of other isolates in *Phyllobothriidea* family[26] (e.g.,-Genbank KF685889). The various species of genus *Acanthobothrium* of the family Onchobothriidae also showed 80-86% similarity with the present sequence (Fig. 1g). The Phylogenetic tree was drawn by comparing the present gene sequence with the 18 deposited gene sequences of cestodes collected from various elasmobranchs. The Pairwise alignment of *Paraorygmatobothrium floraformis* and *Phyllobothriidea* genus 10 n. sp taken from Gen bank (KF685889) showed only 10% mismatches as both the parasites belonged to same order (Table 1).

DISCUSSION

The genus *Paraorygmatobothrium* was erected by Ruhnke[7] with *Paraorygmatobothrium prionacis* (Yamaguti, 1934) Ruhnke[7], as its type taxon. The species *Paraorymatobothrium floraformis* (Southwell[5]) Ruhnke[6] was reported from the spiral intestines of *Carcharias bleekeri* (=*Carcharhinus* sorrah) from Ceylon, Srilanka. This species was earlier described as *Anthobothrium floraformis* by Southwell[5] but subsequently transferred the species to the genus *Phyllobothrium* which was erected by Van Beneden[28] with *Phyllobothrium lactuca* as its type taxon. Nearly 90 valid species have been described under the genus *Phyllobothrium* which also includes *Phyllobothrium floraforme* as valid species[29]. However, Ruhnke[6] further synonymized the *Phyllobothrium floraforme* as *Paraorygmatobothrium floraformis*. The Light microscopic and SEM observations of the present specimens clearly shown the presence of four sessile, shallow cup shaped bothridia having no suckers which is the distinguishing character of *Phyllobothriid* species. The present specimen comes closer to *Paraorygmatobothrium floraformis* Southwell[5] Ruhnke[6] in all morphological characters, but differs in the host, locality and a few differences in measurements. Hence, the host, *Rhizoprionodon acutus* and Nellore coast can serve as new host record and locality records for *Paraorygmatobothrium floraformis* (Southwell[5]) Ruhnke[6]. The use of ribosomal DNA or the rDNA clusters for taxonomic studies have gained a recent attention of parasitologists. In analysis of sequences of the rDNA ITS2 and ITS1 and comparing with the so far available gene sequences in genbank, the sequence of this parasite showed close similarities to the sequences of Order Tetraphyllidea[26,30], thus, confirming the species to this order. Within the order Tetraphyllidea, the *Paraorygmatobothrium floraformis* sequences showed close similarity (high boot strap value of 90% and above) with the sequences of other isolates in *Phyllobothriidea* family[26] and showed 80-90% similarity with various species of the genus *Acanthobothrium* (e.g., Genbank No. GQ470108, GQ470110, GQ470112, GQ470113) of the family Onchobothriidae[30]. If the bootstrap value is 70% or higher than the topology at that branch is considered reliable or correct[31]. This result is reliable with all previous molecular analysis that includes different parasitic order and parasitic species[32-36]. In the present study, the pairwise alignment of gene sequences of *Paraorygmatobothrium floraformis* with several other available isolates in the genbank showed almost 10-12% mismatches (e.g., Genbank No. AF286953)[1,26]. Hence, on the basis of morphological similarities with earlier studies supplemented by close matching of the ITS sequences of the parasites found in the spiral intestine of sharks in the study area is indeed *Paraorygmatobothrium floraformis* Southwell[5], Ruhnke[6].

CONCLUSION

Elasmobranchs are very important in commercial fisheries in various parts of the world including India. Most are excellent as human food but the cestode representation in their spiral intestine is very heavy and species identification will be difficult through morphological analyses. Hence, Ribosomal DNA has been widely used to resolve the taxonomic issues in helminth identification. This study presents a small molecular phylogenetic analysis of the elasmobranch tapeworms of family *phyllobothridea* and order Tetraphyllidea. This is the first examination of molecular analysis of cestode parasites from elasmobranchs of the Nellore coast andhra Pradesh. These types of analyses can reveal the interrelationship between different orders, families and species of the cestode parasites from the marine environment.

SIGNIFICANCE STATEMENT

There are only few results regarding molecular analysis on cestode parasite from India this study discloses the truth that the species identification of the parasite through morphological analyses can be well authenticated only through molecular analyses. These types of studies can help the young researchers to commence more advanced studies on the parasites based on such type of studies. Also, it can diminish the ambiguity regarding the taxonomic identification of the parasites and can provide an accurate host-parasite database to help imminent researchers in this field.

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