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New record of blunthead pufferfish, *Sphoeroides pachygaster* (Muller & Troschel, 1848) (Tetraodontiformes: Tetraodontidae) from Indian water along with DNA barcode and some biological aspects

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Blunthead pufferfish, *Sphoeroides pachygaster* (Muller and Troschel 1848) was recorded for the first time in Indian seas. A single female specimen of *S. pachygaster* was caught in bottom trawl operation at 263 – 310 m depth of the Eastern Arabian Sea. Morphometric and meristic description are presented in detail and compared with the pertinent records available elsewhere. The specimen collected was fully matured with Gonado Somatic Index (GSI) of 17.9 %. Gonad occupied two-third of the abdominal cavity, ova diameters ranged between 0.45 and 0.6 mm and the absolute fecundity estimated was 0.238 million eggs. Histological studies revealed that, this species performs single spawning strategy and it is the first information on the reproduction of *S. pachygaster* in India. Available reports on the occurrence of this species indicated distribution in the Mediterranean Sea, Atlantic, Indian and Pacific Oceans; however, the present record confirms its distribution in the Arabian Sea. Wide variations in the meristic counts among the specimens of this species collected from various locations are also discussed. Molecular analysis of the present specimen using Mitochondrial 16S rRNA gene sequences, confirmed the identity as *S. pachygaster* with intra specific divergence of 00.0 – 0.04 %. This report is the first well documented, confirmed record and re-description of *S. pachygaster* from the Indian Ocean, which documents a new addition to the family, Tetraodontidae of the Indian ichthyofauna.

[**Keywords**: Arabian Sea, Fecundity, Histology, Mitochondrial 16S rRNA, New record, *Sphoeroides pachygaster*]

Introduction

Pufferfishes, under the family Tetraodontidae Bonaparte 1831, generally occurs in shallow, warm, tropical seas and freshwaters of circum-global areas and also distributed in the tropical and subtropical areas of the Atlantic, Indian and Pacific Ocean[1]. Few species of Pufferfishes like *Arothron firmamentum*, *Canthigaster callisterna*, *C. rivulata*, *Sphoeroides pachygaster* and *Tylerius spinosissimus* occur up to the depth of 450 m[1-3], however the adults are found in 480 m depth, whereas the juveniles are available in less than 100 m also[4]. Pufferfishes are used for human consumption in China, Korea, Japan and Taiwan[5] but, these species had no commercial value as food fish in India. These fishes are unique for their powerful toxin (tetrodotoxin) in their skin and organs, which is a potent neurotoxin as well as strongest marine paralytic toxins[6-7]. The family Tetraodontidae consist of 28 genera with 195 valid species[8-9], of which 8 genera and 32 species are occurring in the Indian waters[10-11]. The genus *Sphoeroides* Anonymous [Lacepède], 1798 is one among the 28 genera of the family Tetraodontidae, sometimes written as *Spheroides*[12], but originally spelled as *Sphoeroides* (Lacepede, 1798) and presently, *Sphoeroides* replaces as *Spheroides*[1]. There are 22 valid species under the genus *Sphoeroides*[1,9,13] across the world waters.

S. pachygaster is found in most of the warmer waters like, Western Atlantic, Gulf of Guinea[14,15], southward to South Africa and also in Pacific Ocean[16,17]. Pertinent literature revealed its distributional range from New Jersey, Western Atlantic to Argentina, and from the African, Atlantic, probably Nigeria to Natal, St. Helena Island in the Atlantic, Philippines and Hawaii in the Pacific[1,4], Gulf of Trieste[17], Mediterranean sea[17,18] and Adriatic Sea[19]. Besides distributional records, few reports on biology of this species are also available *i.e.* on food and feeding[19] and maturity[20,21]. In India, deep

sea puffer fishes like *Amblyrhynchotes spinosissimus*[22], *Torquigener florealis*[23], *Torquigener hypselogeneion*, *Takifugu oblongus*[24], *Sphoeroides honckeni*[25] and *Liosaccus cutaneous*[26] are reported. In this context, the present report is the first well documented and confirmed record with taxonomic, molecular identification and some biological notes of *S. pachygaster* from India, which could be considered as new addition to the family, Tetraodontidae of Indian ichthyofauna.

Materials and Methods

A single specimen of *S. pachygaster* (Fig. 1) was caught during the bottom trawl operation made by the vessel MFV *Matsya Varshini* of Fishery Survey of India during the month of March 2019 in-between the area, Lat. 8°41' N, Long. 76°06' E and Lat. 8°44' N, Long. 76°04' E at a depth of 263 – 310 m in Arabian Sea.

Taxonomic study of S. *pachygaster* was carried out using the literature of Shipp[1] and Shipp[15]. Counts and measurements were taken following Fricke[27] and Holden & Raitt[28]. Biometric parameters were measured to the nearest millimetre (mm) using a digital calliper (Mitutoyo ABS lute Digimatte, Japan). The following morphometric measurements and meristic counts were taken, Total length (TL), Standard length (SL), Anal length (AL), and Head length (HL). In addition, Head height (HH), Head width (HW), Snout length (SNL), Opercular length (OL), Body height (BH), Caudal peduncle height (CH), Opercular height (OH), Interorbital space (IS), Head width (HW), Body width (BW), Caudal peduncle width (CW), Eye horizontal diameter (EH), Eye vertical diameter (EV), total weight, liver weight, gonad weight, gutted weight, number of dorsal fin rays, nof anal fin rays, number of pectoral fin rays, number of caudal fin rays (including segmented and unbranched rays) were also documented. Total weight (TW) and eviscerated weight were taken by a digital balance (0.05 g accuracy). To determine the number of vertebrae, X-ray radiograph of the specimen was taken. The specimen was dissected to establish sex and maturity stages and also for histological studies. Small pieces of tissues from different regions of the gonad were taken and fixed in 5 % formalin for the histological studies. Thereafter, the tissue was sectioned transversely with 10 µm thickness and stained with haematoxylin and eosin solutions. Finally, the cell development was observed under the compound microscope (10 - 40X; Labomed CxRIII). The Gonosomatic Index (GSI) was estimated as (GW/BW) × 100. Ovaries were preserved in 10 % formalin. The absolute fecundity was determined using the gravimetric method to count the number of oocytes in weighed sub-samples of formalin-fixed ovaries. Total number of advanced yoked oocytes of the ovary, including hydrated oocytes was also counted, adopting the protocols of Hunter *et al*.[29]. Tissue samples from the dorsal portion of the fish was collected and preserved in 95 % ethanol for the molecular analysis. The specimen was deposited in the Museum of Fishery Survey of India, Kochi, India (FSIKM-TSp-1,223).

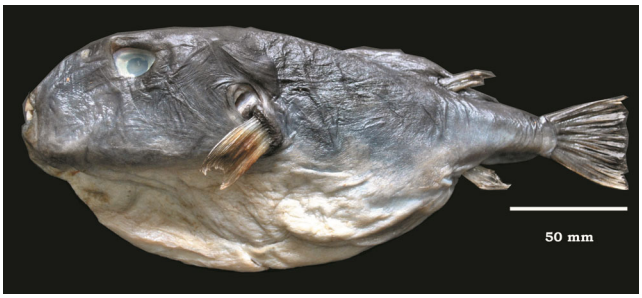

Fig. 1 — *Sphoeroides pachygaster* (Müller & Troschel, 1848) 223 mm TL

The molecular analysis was conducted with 16S rRNA gene sequences. Total genomic DNA was isolated with DNeasy Blood & Tissue Kits (QIAGEN) as per the manufacturer's protocol. The universal primers 16SAR (5'-CG CCTGTTTATCAAA-AACAT-3') and 16SBR (5'-CCGGTCTGA-ACTCAGA-TCACGT-3')[30] were used for PCR amplification. The 16S rRNA gene was amplified in a 25 µl reactions volume with 10x assay buffer (100 mM Tris, 500 mM KCl, pH 9.0) with 20 mM $MgCl_2$, 10 p moles of each primer, 200 µM of each dNTP, 0.25 U TaqDNA polymerase and 50 ng of template DNA. The thermal profile was performed with 35 repetitions of a three-step cycle consisting of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1.5 min including 5 min for initial denaturation at 94 °C and 10 min for the final extension at 72 °C. PCR products were sequenced bi-directionally in resources centre, Cochin. DNA sequences developed in the present study were aligned and edited using BioEdit software with ClustalW algorithm[31,32]. A Maximum Likelihood (ML) analysis and Kimura-2-parameters (K2P) genetic distances were performed using MEGA 10.0.5[(ref. 33)] with 1000 bootstrap replications.

Results

Systematics

Order: Tetraodontiformes L. S. Berg, 1940

Family: Tetraodontidae Bonaparte, 1831

Genus: *Sphoeroides* Anonymous [Lacepède], 1798
Species: *Sphoeroides pachygaster* (Müller & Troschel, 1848)

Synonyms

Liosaccus pachygaster (Müller & Troschel, 1848); *Tetrodon pachygaster* Müller & Troschel, 1848; *Tetrodon cutaneus* Günther, 1870; *Sphaeroides cutaneous* (Günther, 1870); *Spheroides dubius* von Bonde, 1923; *Liosaccus aerobaticus* Whitley, 1928; and *Thecapteryx lioderma* Fowler, 1948.

Material examined: FSIKM-TSp-1,223, 1 Female (SL 189 mm), Arabian Sea, off Kerala, India, Lat. 8°41' N, Long. 76°06' E and Lat. 8°44' N, Long. 76°04' E at 263 – 310 m depth, March 2019.

Holotype: Sphoeroides (Ranzani) ANSP109468, 2 nos, 134 – 137 mm, Florida, ANSP101322 – 1 no, 80.6 mm, Nigeria; ANSP10478, 11 nos, 117 mm, Mozambique. MNHN 8340 (1, 208), Cape of Good Hope; holotype of *Tetraodon laevissimus* of Cuvier and *Stenometopus laevissimus* (Bibron) Troschel and BMNH (2, 183-188), St. Helena.

Syntype: Tetrodon cutaneus Gunther. USNM 168467 (1, 82) Corregidor, Philippine islands. ANSP 75576 (1, 185), Hawaiian Islands, Oahu.

The first diagnostic description of this species was made by Muller and Troschel, however, specimen was missing. Considering the characters available in the record, Shipp[1] reported detailed review by analysing 51 specimens from various museums and synonymised 36 species into single conspecific *Sphoeroides pachygaster*.

Type localities: Tetrodon (Cheilichthys) pachygaster Muller and Trochel 1848a: 677 (original description, Barbados); *Sphoeroides* (also *Sphaeroides, Spheroids*) *Pachygaster.* Jordan 1886b: 605 *Sphaeroides cutaneus*: Smith, 1953, Sea Fishes of Southern Africa: 417 pI. 95, fig. 1190; *Tetrodon cutaneous*: Gunther, 1870, 287 (type locality: St. Helena, Azores); and *Spheroides dubius*: Von Bonde, 1923, 40, pl. 2, fig. 3 (type locality: off Natal, South Africa, 27 fms).

Description

Inflatable body with rounded snout, nostrils was easily visible with the naked eye (Fig. 1); dorsal surface smooth and without a distinct keel from posterior to eyes. Body entirely smooth and prickles totally lacking, which differentiated *S. pachygaster* from other species of the genus *Sphaeroides.* Bands and blotches of spots are present on the body. Interorbital broad and as long as 13 % of SL; pigmentation uniform (Fig. 1), big head and skin without scales. Four large teeth form a beaklike structure with thin lips. Eyes big and oval in shape, vertical and horizontal length almost equal; chin prominent; nasal organ short, papilla erected and nasal pores not equal. Dorsal fin small and situated just above the anal fin with almost same size and shape. Caudal fin slightly concave with white shade on the lower portion. Pelvic fin absent. Dorsal rays 8; anal rays 8; pectoral rays 15 (i,14); caudal fin rays 11(i, 9, i); and vertebrae 19 (9 + 10).

Colouration: Belly white and rough, pectoral fin base black, margin of the pectoral fin white, the ventral side whitish.

DNA sequences: 540 bp of 16S rRNA gene sequences of *S. pachygaster* was generated and submitted to DNA data Bank of NCBI.

Discussion

The genus *Sphoeroides* is differentiated from other genus of the family tetraodontidae by the presence of lesser dorsal fin count (9 or less); anal fin count (less than 11) and the interorbital width as long as 8 % of the SL[1]. *S. pachygaster* is differentiated from other species of the genus *Sphoeroides* by the absence of scales and prickles on skin. Dorsal surface and skin smooth, body colour of the dorsal surface and the flanks greyish to olive green[1,15]. Morphology, colour, morphometric measurements and meristic counts of the present specimen (*S. pachygaster*) is compared (Tables 1 & 2) with previous descriptions[1,15,17-19,34-36].

Meristic counts and morphometric measurements of this specimen are presented in Tables 1 & 2. The morphometric characters *i.e.* Standard length of *S. phachygaster* were compared with the pertinent literatures (range in parenthesis). Head length 2.5 (2.3 – 3.7) in SL; snout length 5.5 (5.05 – 7.3) in SL; snout to anal 1.26 (1.26 – 1.3) in SL, length of pectoral fin 7.0 (6.4 – 9.8) in SL; body depth 3.5 (3.2 – 4.2) in SL; caudal fin length 5.5 (5.04 – 7 .7) in SL; caudal peduncle length 6.3 (4.7 – 6.8) in SL; caudal fin length 5.5 (5.04 – 7.7). The present specimen exactly corroborated within the range of characters reported elsewhere[17-19,34-39].

Review of pertinent records revealed that fin counts such as pectoral fin rays (varied between 14 – 18), anal and caudal fin rays (Table 2) has wide variation and this may be due to error in counting the branched rays hidden below the skin[27]. In the present study, X Ray photograph was taken for the present specimen to confirm the fin ray counts. Shipp[1] reviewed 51 specimens from several museums,

Table 1 — Comparison of morphometric characters of *S. pachygaster* with pertinent records

Referred literature	Present specimen		Psomadakis *et al.* 2008		Lovrenc *et al.* 2013		Giordano *et al.* 2012		Hamida *et al.* 2009		Carbonara *et al.* 2017	
Morphometric parameters	mm	% in TL	mm	% in TL	mm	% in TL	mm	% in TL	mm	% in TL	mm	% in TL
Total length	223		137		348		280		330		200-355	
Standard length	189	84.8	116	85	300	86.21	245	87.5	295	89.4	175-313	84.4-88.2
Anal fin base length	8	3.6	6	4	15	4.31	10	3.6	11	3.3		
Anal fin length	20	9.0	14	10	37	10.63	24	8.6	30	9.1	28.3-57.2	14.2-16.4
Anal length	150	67.3	92	67	232	66.67	21	7.5	225	68.2	138.-236.	65.0-69.0
Body height	54	24.2	27	20	93	26.72	67	23.9	90	27.3	73.5-140.8	36.6-39.7
Body thickness	39	17.5	28	20	93	26.72	70	25	90	27.3	35.4-67.5	17.7-19.0
Body depth	69	30.9	-	-	-	-	-	-	-	-		
Caudal fin length	34	15.2	23	17	40	11.49	36	12.9	38	11.5	8.2-15.2	3.8-4.3
Caudal peduncle height	28	12.6	10	7	49	14.08	32	11.4	35	10.6		
Dorsal fin base length	8	3.6	6	4	16	4.6	10	3.6	11	3.3		
Dorsal fin length	20	9.0	14	10	35	10	23	8.2	25	7.6		
Eye dia. h	18	8.1	11	8	23	6.61	19	6.8	21	6.4	14.7-28.0	5.6-7.9
Eye dia. vertical	16	7.2	6	4	16	4.6	18	6.4	21	6.4	9.2-16.0	4.1-4.6
Gill opening width	17	7.6	9	7	15	4.31	24	8.6	25	7.6	26.6-49.3	11.5-14.5
Head height	50	22.4	26	19	72	20.69	54	19.3	60	18.2	34.9-58.9	15.8-17.5
Head length	74	33.2	49	36	99	28.45	65	23.2	100	30.3	62.0-123.7	31.0-35.1
Head width	35	15.7	24	18	77	22.13	45	16.1	70	21.2	32.3-61.6	15.2-17.4
Internasal space	19	8.5	12	9	30	8.62	28	10	30	9.1		
Inter orbital width	29	13.0	22	16	38	10.92	28	10	30	9.1	22.1-40.2	11.0-12.5
Nostril greater dia.	6	2.7	4	3	-	-	6	2.1	6	1.8	-	-
Nostril lesser dia.	5	2.2	3	2	-	-	4	1.4	4	1.2	-	-
Pre dorsal length	145	65.0	91	66	220	63.22	20	7.1	215	65.2	-	-
Pectoral fin length	27	12.1	18	13	39	11.21	29	10.4	30	9.1	-	-
Post orbital length	29	13.0	111	81	34	9.77	30	10.7	35	10.6	-	-
Pre pectoral length	84	37.7	54	39	-	-	-	-	-	-	-	-
Snout length	34	15.2	23	17	49	14.08	35	12.5	40	12.1	20.0-55.2	10.0-15.5
Anal fin height	22	9.9	-	-	-	-	-	-	-	-	-	-
Caudal peduncle length	30	13.5	-	-	-	-	-	-	-	-	-	-
Distance DF to AF	40	17.9	-	-	-	-	-	-	-	-	-	-
Dorsal fin height	20	9.0	-	-	-	-	-	-	-	-	-	-
Pectoral fin base	15	6.7	-	-	-	-	-	-	-	-	-	-

collected from various seas around the world and nearly 17 species were brought under *S. pachygaster* as synonyms and were conspecific. He also clarified that these variations in fin ray count are existing due to its geographical variation. According to Shipp[1], *S. pachygaster* is represented by two discrete populations in Atlantic Ocean *i.e.*, Western Atlantic population which has higher pectoral fin count (mean = 16) and spotted, and Eastern Atlantic population has uniform pigmentation and no spots on the body and lesser mean pectoral fin count (14.3). The present specimen had uniform pigmentation on body with 15 pectoral fin rays, which confirmed the range extension of the present collection of *S. pachygaster* from Indian Ocean with more propinquity with Eastern Atlantic Ocean.

Observations on maturity and fecundity

The weight and length of the gonad was 42.5 g and 9.5 cm, respectively. Fully mature gonad, partially spent condition was observed with GSI 17.9 %. Ragonese *et al.*[20] reported the GSI value of *S. pachygaster* ranged between 16 to 30 % during winter to summer seasons in Mediterranean Sea, which is matching with the present findings. The absolute fecundity of the present specimen was 0.238 million eggs, which is similar to the reports from congeneric *S. maculates* from the Atlantic Ocean[21]. However, Ragonese *et al.*[20] reported an absolute fecundity of *S. pachygaster* was 1.5 million eggs in the Mediterranean Sea. In the present specimen, comparatively lower fecundity was observed may be due to offset of breeding season during March, in

Table 2 — Comparison of meristic characters of *S. pachygaster* with published records

	Psoma dakis, 2008	Lover Nac, 2013	Hemida *et al.* 2009	Present specimen	CarboNara *et al.* 2017	Rahman *et al.* 2014	Zachariou & Corsini, 1994	Arculeo *et al.* 1994	Shipp, 1974	Gilhen *et al.* 1985
Dorsal fin rays	9	8	8	8	8	9	8-9	8	8-9 rarely 7	8-9
Anal fin rays	9	8	8	8	7	8	8-9	8	8-9	8-9
Pectoral fin rays	14	14	15	15	14	14	14-15	15	14-17	14-18
Caudal fin rays	11	10	10	11	9	-	9	-	11	-
Vertebrae	19	-	-	19	-	-	-	-	-	19

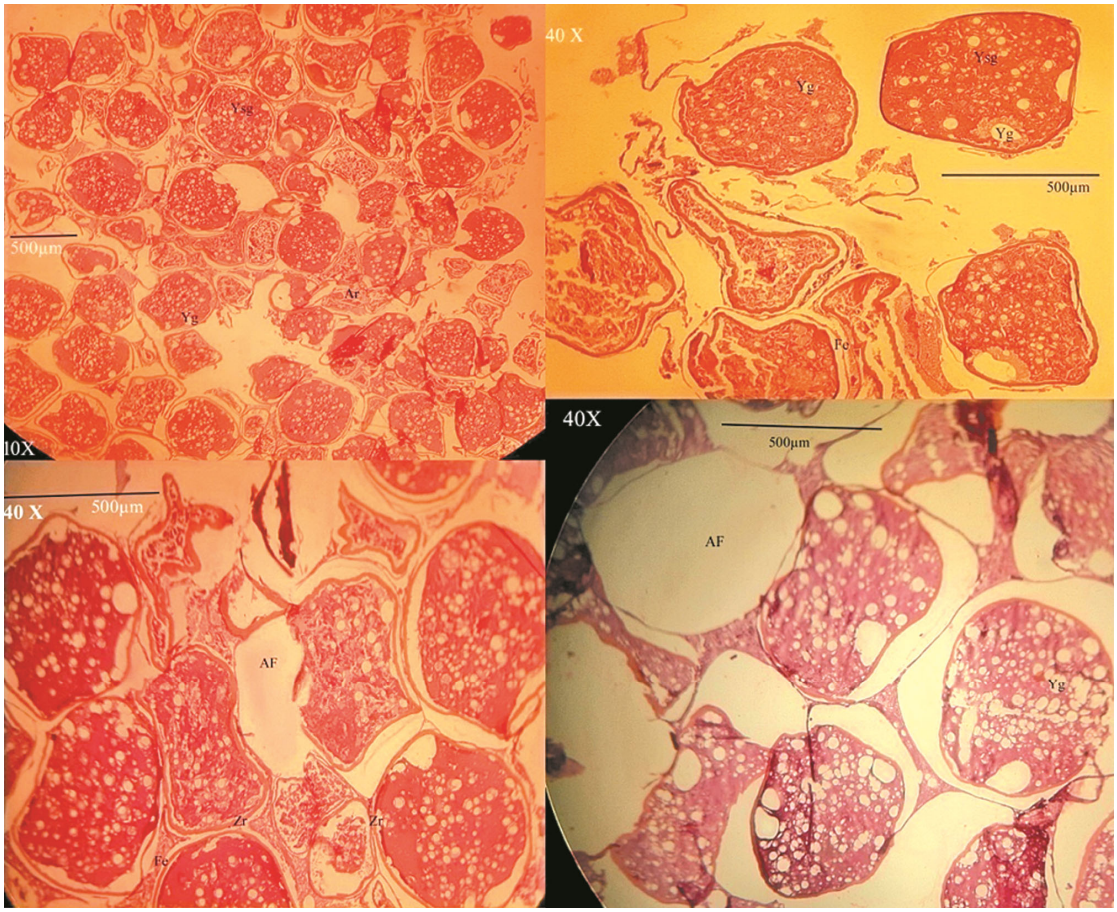

Fig. 2 — Histological section of gonad of *S. pachygaster* from the Indian water (10 µm thickness observed at 10X and 40X magnification). Fe: Follicular epithelium cells, Zr: Zona radiata, Yg: Yolk globules, Ysg: Egg yolk granules, and Af: Atretic follicle

general, annual potential fecundity should be fixed prior to onset of spawning[29]. Ova diameter of the present specimen ranged between 0.45 and 0.6 mm, whereas, Ragonese *et al.*[20], reported 0.35 to 0.7 mm. Histological analysis of gonad revealed that it was partly spent and all the eggs were almost in uniform stage (Fig. 2a – d) which indicated *S. pachygaster* is a total spawner. Moreover, atretic follicles among the oocyte appeared (Fig. 2), which indicated the post-ovulation stage[40] of *S. pachygaster* during the period under report, *i.e.*, mid of March, so breeding season may possibly fall between February and March. The present finding was corroborated with the findings of Ragonese *et al.*[20] who reported that the breeding season of this species starts from late winter and continue till summer in the Mediterranean Sea where the peak spawning was observed during February. The present observation could only be confirmed on analysis of more numbers of specimens during various season in future.

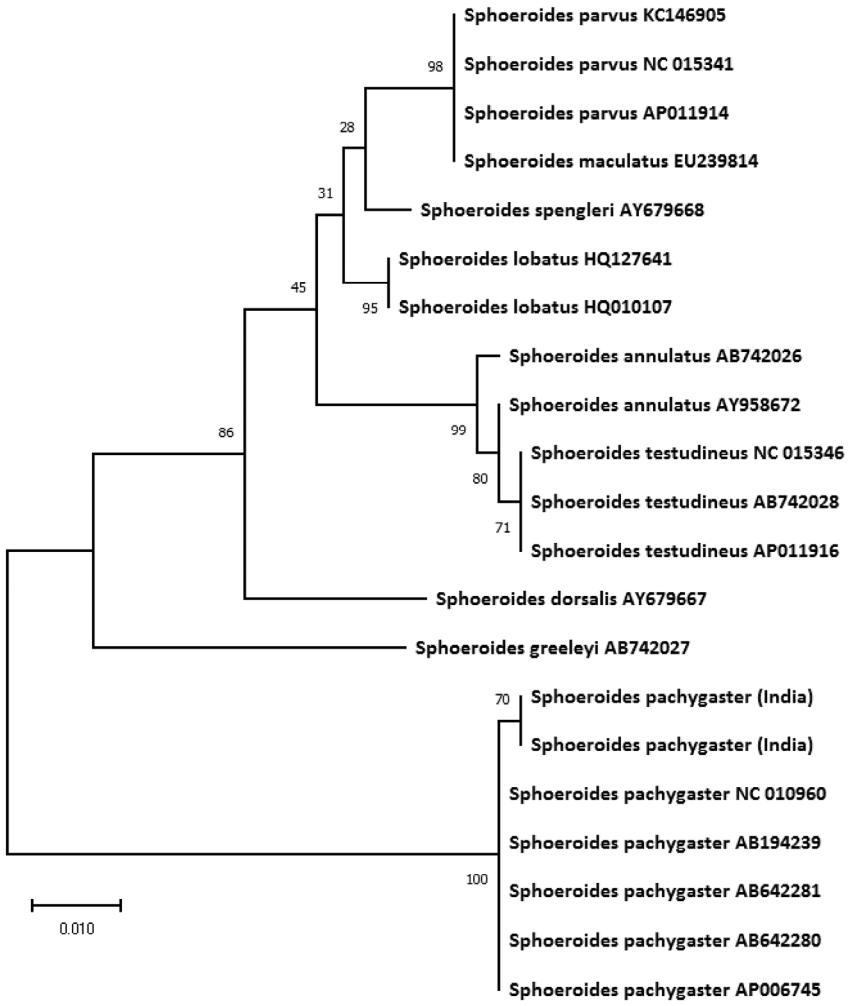

Fig. 3 — Phylogenetic tree topology for *Sphoeroides pachygaster* (Müller & Troschel, 1848) with sister species based on 16S gene sequences using maximum likelihood method

In molecular analysis, a fragment of 540 base pairs was sequenced for 16S gene. The phylogenetic tree was constructed for present material with other available sequences of genus *Sphoeroides* retrieved from the NCBI. The ML analysis provide that the specimens from the Indian material was well formed with NC010960 form a robust monophyletic clad with other sister species (Fig. 3). Pair-wise genetic distances were estimated, where interspecific divergence showed ranges from 2.4 to 12.7 %. Intraspecific divergence ranged from 0 to 0.04 %. Overall, molecular analysis is significantly supporting to the morphological identification of the species from the Indian water.

Conclusion

This report is the first well documented, confirmed record with re-description of Blunthead pufferfish, *Sphoeroides pachygaster* (Muller and Troschel 1848) from the Indian Ocean, which documented a new addition to the family, Tetraodontidae of the Indian ichthyofaunal diversity. Biological information on this species is an initiative to understand the population and stock status of *S. pachygaster* in the Indian Ocean. Molecular analysis of the present specimen using Mitochondrial 16S rRNA gene sequences, confirmed the identity as *S. pachygaster* with intra specific divergence of 00.0 – 0.04 %.

Acknowledgments

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Conflict of Interest

The Authors hereby declares that they do not have any conflict of interest.

Ethical Statement

This manuscript is the authors' own original work, which has not been previously published elsewhere. This manuscript is not currently being considered for publication elsewhere. Further it is to state that the work reflects the authors' own research and analysis in a truthful manner.

Author Contributions

SR collected the specimen, identified and prepared initial draft; TTA, PP & KKL contributed in genetic studies and preparation of manuscript; and others (SPV, NU & AEA) contributed in identification and preparation of manuscript.

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