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Morpho-molecular Characterization of *Proteocephalus vitellaris* Verma, 1928 (Cestoda: Proteocephalidea) Infecting a Freshwater Fish, *Bagarius yarrelli* Sykes, 1839 Inhabiting Harike Wetland, Punjab, India

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Abstract:
Proteocephalus vitellaris Verma, 1928 infecting *Bagarius yarrelli* Sykes, 1839 vern. goonch inhabiting Harike wetland in Punjab has been redescribed using integrated morpho-molecular approach. Up to five worms were collected from a single host. The total worm length was 27-42mm having globular and unarmed scolex distinctly separated from the neck. Apical sucker was conspicuous, measure 128-160×153-240µm in size, other suckers four in number, bilateral in position and measure 118-144×88-128µm in size. Strobila consisted of 80-85 proglottids with distinct segmentation throughout. Testes small, rounded in shape and 80-90 in number, placed laterally, ovary bilobed, situated at the posterior margin of the proglottid and measure 50-140×166-288µm in size with oviduct arising from the middle, genital pore irregularly alternate, oval in shape, at the centre of the lateral margin of the proglottid, vitellaria follicular oval to rounded, occupying lateral margins of the whole length of the mature proglottid. The present specimens were closely compared with two already known species from India i.e. *P. ritaii* Verma, 1926 and *P. vitellaris* infecting *Rita rita* and *Bagarius yarrelli* respectively and were considered to be closest to the later using various morphological and morphometrical attributes. Phylogenetic analysis based on 18S rRNA and ITS2 were made using Mega 6.0 software. Earlier, this species was recorded from the same host at Allahabad (U.P), India. In the present paper, *P. vitellaris* has been redescribed morphologically as earlier description was deficient and at the molecular level using 18S rRNA and ITS2 markers.

Keywords: *Bagarius yarrelli*, Cestode, Morphology, Phylogeny, *Proteocephalus*, ITS2, 18S rRNA

INTRODUCTION

Proteocephalidean tapeworms are frequently encountered parasites of fishes, amphibians and reptiles, with the highest number of species placed in the genus *Proteocephalus* Weinland, 1858. Numerous species have been reported in the Palaearctic region (Freze, 1965; Priemer, 1982; Schmidt, 1986; Chubb *et al*., 1987; Dubinina, 1987; Scholz, 1989). These tapeworms are cosmopolitan parasites of freshwater fishes exhibiting great intraspecific variations infecting many species of the fish host. These are diagnosed in having distinct external segmentation, one set of gonads per segment, numerous testes, a bilobate ovary at the posterior end of proglottid, follicular and lateral vitellaria, a lateral genital pore and a scolex with four suckers and an apical sucker may be present. Brabec *et al*. (2015) have demonstrated limitation of morphological characters of proteocephalidean and bothriocephalidean cestodes respectively owing to their fluidity. According to de Chambrier *et al*. (2015) 315 valid species have been reported and a large proportion of them being parasites of South American siluriform fishes (Freze, 1965; Rego, 1994). Earlier studies had indicated monophyly of the order Proteocephalidea with one family, Proteocephalidae however studies by Caira *et al*. (2014) established a new order Onchoproteocephalidea having paraphyletic assemblage with hooked tetraphyllidea cestodes. De Chambrier *et al*. (2015) regarded this as a controversial decision because of lack of any morphological synapomorphies. Since there is a limited data of proteocephalidean cestodes from Indian fishes, this work has been undertaken to characterize these highly polymorphic species having enoromous morphological variations. Earlier authors have described a new species *Gangesia punjabensis* infecting *Wallago attu* in India (Jasrotia and Kaur, 2017) using molecular approaches. In the present study, *P. vitellaris* has been described based on morphological and molecular observations. This data which will supplement the earlier data by Verma (1928) on *P. vitellaris* infecting *Bagarius yarrelli* in India. So far only 2 species of the genus *Proteocephalus* have been described in India by Verma, 1926, 1928.

MATERIAL AND METHODS

Fresh specimens of the catfish, *Bagarius yarrelli* vern. goonch were collected from the local fish market near Harike wetland, Punjab and were brought to the laboratory for parasitological investigation. The specimens were collected from anterior part of the small intestine and the prevalence of infection was 10%. The worms were relaxed and stretched in warm 4% formalin over the edge of the beaker, fixed and preserved in fresh 4% formalin for morphology and 95% ethanol for molecular studies. For morphology, worms were stained in Gower's carmine (Gower, 1939), differentiated in 70% acid ethanol (i.e 70% ethanol with 2-3 drops of HCL), dehydrated through alcoholic grades, cleared in xylene and clove oil (eugenal) and were mounted in the DPX. Measurements were taken in micrometer (µm) or otherwise mentioned and line drawings of *P. vitellaris* were made with the help of camera lucida. The identification upto the genus level was done with the help of "Keys to the cestode parasites of vertebrates" by Khalil *et al*. (1994). The frontal section of scolex at 7-9µm thickness were stained with haematoxylin-eosin.

Molecular analysis

The genomic DNA was isolated by phenol chloroform extraction method (Sambrook and Russell, 2001) from 95% ethanol preserved cestode worms. Immature and mature segments weighing 25mg were crushed and transferred to 1.5 ml microcentifuge tubes. Subsequently 400µl Phosphate buffer saline (PBS), 50µl proteinase K and 50µl of Sodium dodecyl sulfate (SDS) were added, mixed and incubated overnight at 55˚C to digest the tissue completely. Phenol: chloroform (1:1) 300µl was added and centrifuged for 10 min at 10,000 rpm. The upper layer was shifted in a fresh microcentifuge tube and added double amount of chilled isopropanol and well mixed. The DNA was precipitated at -20˚C for 2 hrs and centrifuged at 10,000 rpm for 10 min to obtain the pellet, then washed with 70% ethanol, air dried and eluted in 30µl of 1×TE buffer. Also, the quality of the product was checked by electrophoresis using 0.8% agarose gel. The 18S rRNA was amplified using the primers Ces1 and Ces2 designed from the complete sequence of the 18S rRNA gene of *P. exiguus* and *P. longicollis* (Král'ová *et al*., 1997). The rRNA ITS2 genes was amplified by using the primers Proteo1 and Proteo2 designed the complete sequence of ITS1-ITS2 genes of *Eubothrium crassum* and *E. salvelini* as designed by Král'ová *et al*. (2001). The PCR reaction were performed in 25µl reaction mixture of 2.5µl of 10X buffer,

1µl of each primers, 0.8µl of 2.5mM dNTP, 0.5µl of Taq DNA polymerase, 0.5µl of 50mM Mgcl2, 2.0µl of total DNA and 16.7µl of double purified water. The PCR cycling conditions used for 18S were initial denaturation at 95˚C for 3 min, 30 sec denaturation at 95°C for 33 cycles, 30sec annealing at 60.4°C, 40sec extension at 72°C, 7min final extension at 72°C. The PCR cycling conditions used for ITS2, initial denaturation at 95˚C for 3 min, 30sec denaturation at 95°C for 33 cycles, 30sec annealing at 58.2C, 40sec extension at 72°C, 7min final extension at 72°C. The PCR products was analyzed on a 2% agarose gel containing 0.5µg/ml ethidium bromide in 1×Tris-acetate-EDTA (TAE) buffer run together with DNA ladder (100bp).

DNA sequencing

The PCR amplified products were purified by using Geneipure™ Quick PCR Purification Kit (GeNei™) and sequenced commercially at the Eurofins Genomics Bengaluru Karnataka, India. The accession number of 18S rRNA (KY786339) and ITS2 (KY786338) were obtained after duly submitting sequences to the GenBank.

Phylogenetic analysis

For phylogenetic analysis, the basic local alignment tool was used to analyze the new 18S rRNA 15 additional sequences showing genetic relatedness with homogeneity percentage of 92 to 90 in the GenBank database. In ITS2 (KY786338) 17 additional sequences showing genetic relatedness with homogeneity percentage of 83 to 95 in the GenBank database were analyzed. The outgroup was *Acanthotaenia* sp. (AF267292) from *Varanus exanthematicus*. In 18S rRNA Jukes-cantor model with lowest Bayesian score (BIC) of 7618.442 and Kimura 2-parameter model with lowest Bayesian score (BIC) of 3797.178 (Kimura, 1980) in MEGA6 software (Tamura *et al*., 2013) in ITS2 were used to make genetic distance analyses among closely related species of the genus *Proteocephalus*.

RESULTS

Morphological description (Figures. 1a-c; 2a-d) (Measurements based on 4 specimens)

Parasites 27-42mm in length. Strobila unarmed scolex, 50-60 number of immature proglottids, 18-20 number of mature proglottids. Scolex long, unarmed distinctly separated from neck, apical sucker conspicuous 128-160×153-240µm, having large number of glandular cells filled with dense granular cytoplasm through ducts on its surface (T.S.) (Figure 2d). Suckers four, bilateral in position, 118-144×88-128µm in size. Anterior immature proglottid broader than long 98-272×768-976µm in size. Mature proglottid measure 148-480×726-1440µm in size. Testes small, rounded, 80-900 in number, in one central field, each lobule 12-14×18-20µm in size, occupying whole of space above the ovary. Ovary bilobed, lobes low, pressed along the lower margin of the proglottid, measure 50-140×166-288µm in size, not reaching up to the lateral osmoregulatory ducts (Table 1). Oviduct arise from the middle of the ovary. Vagina posterior to cirrus pouch. Cirrus pouch 125-276µm in length. Vas deferens in the form of fine coiled tube, along with cirrus sac reaching upto 1/4 of the width of mature proglottid or reaching to medial of proglottid. Genital pore alternate irregularly, oval in shape, located at the centre of the lateral margin of proglottids. Vitellaria follicular, oval to rounded shaped, follicles occupying lateral margins of the mature proglottid.

Taxonomic summary *Proteocephalus vitellaris* Verma, 1928

Type host: *Bagarius yarrelli* (Sykes, 1839), Vernacular name: goonch, Family: Siluridae.

Type locality: Harike Wetland, Punjab. It lies at 31.17˚N latitude and 75.20˚E longitudke and is spread over an area of 41 sq km.

Site of infection: Small Intestine.

Holotype: One specimen catalogue no. BY/G-1/ 4.5.2017 submitted in Parasitology Laboratory in the Department of Zoology, Panjab University, Chandigarh, India.

Paratype: Cestode worm stained in Gower's carmine (1 slide catalogue no.: BY/G-2/4.5.2017) and fixed in 4% formalin (catalogue no.: BY/G-3/4.5.2017).

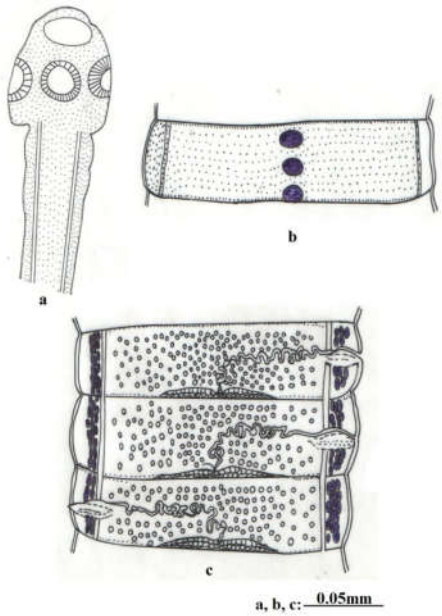

Figure 1: Line drawing of *P. vitellaris* a) Scolex, b) Immature proglottid (ventral view), c) Mature proglottid (ventral view)

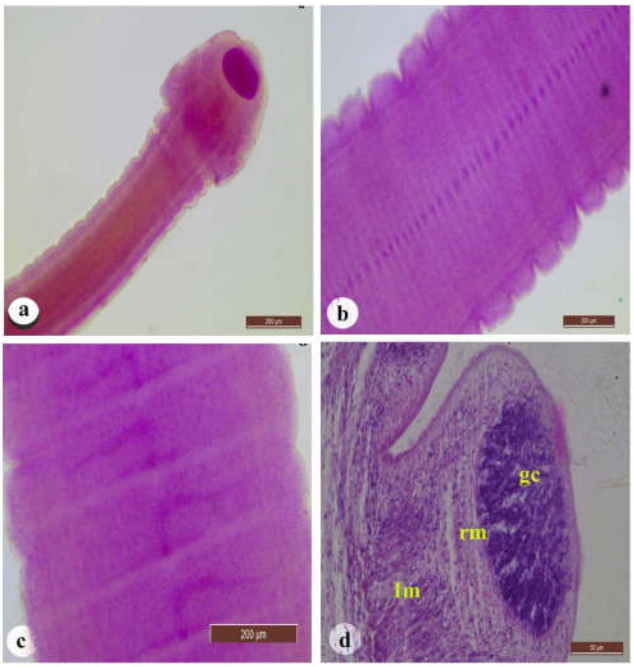

Figure 2: Photomicrograph of *P. vitellaris* a) Scolex, b) Immature proglottid (ventral view), c) Mature proglottid (ventral view) stained with Gower's carmine, d) Frontal section of scolex showing internal longitudinal musculature (Im), retractor muscles (rm), glandular cells (gc) stained with haemotoxylin-eosin

Molecular data (based on one specimen)

The blast analysis of 18S rRNA sequence showed maximum similarity of 92% with *P. sagittus* (KX768935) infecting *Barbatula barbatula*, 92% with *P. midorensis* (AY551130) infecting *Lefuca echigonia*, 91% with *P. percae* (KX786934) infecting *Perca fluviatilis*, *P. tetrastomus* (AF335510) infecting *Hypomesus nipponensis* and 90% with *P. gobiorum* (KX768931) infecting *Apollonia fluviatilis* (Table 2). The blast analysis of ITS2 nucleotide sequence of *P. vitellaris* exhibited maximum similarity of 95% with *P. plecoglossi* (AY551159) infecting *Plecoglossus altiveli* from Japan, 86% with *P. synodontis* (JN005777), 93% with *P. longicollis*, *P. ambiguus* (DQ427097, DQ427096), 94% with *P. ambloplitis* (KT737467) infecting *Micropterus dolomieu* from USA (Table 4). The phylogenetic tree constructed with Maximum likelihood showed highest bootstrap value and formed a separate clade thereby exhibiting monophyly (Figs. 3, 5).

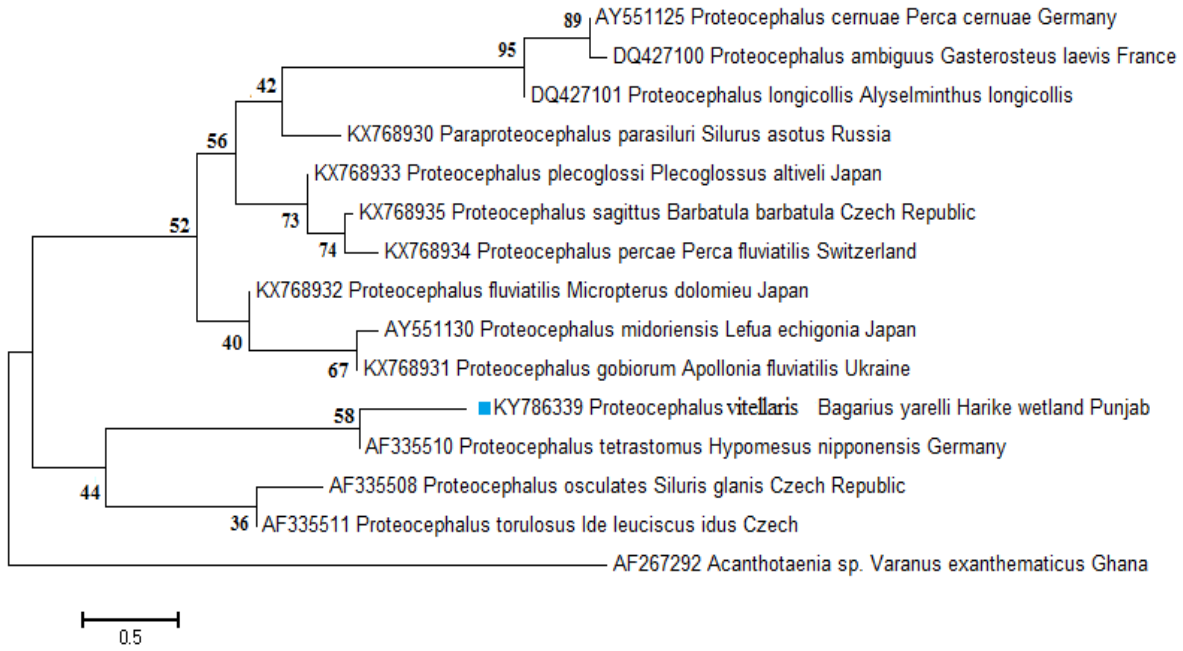

Figure 3: Phylogenetic tree reconstructed by Maximum-Likelihood analysis of 18SrDNA showing the position of *Proteocephalus vitellaris* (KY786339) with other species of family Proteocephalidae

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. KY786339 Proteocephalus vitellaris Bagarius yarelli Harike wetland		0.42	0.41	0.35	0.49	0.63	0.65	0.56	0.36	0.41	0.56	0.56	0.60	0.43	0.72
2. KX768935 Proteocephalus sagittus Barbatula barbatula Czech Repu	0.88		0.07	0.02	0.48	0.37	0.23	0.51	0.03	0.11	0.52	0.45	0.36	0.10	0.46
3. KX768930 Paraproteocephalus parasiluri Silurus asotus Russia	0.86	0.26		0.07	0.57	0.37	0.25	0.55	0.07	0.11	0.55	0.50	0.26	0.04	0.53
4. KX768934 Proteocephalus percae Perca fluviatilis Switzerland	0.79	0.08	0.26		0.46	0.38	0.29	0.53	0.01	0.06	0.53	0.47	0.39	0.10	0.48
5. AF335510 Proteocephalus tetrastomus Hypomesus nipponensis Ge	0.00	1.17	1.43	1.16		0.03	0.67	0.21	0.47	0.37	0.21	0.27	0.07	0.42	0.51
6. AF335508 Proteocephalus osculates Siluris glanis Czech Republic	1.41	1.01	0.99	1.02	0.12		0.63	0.41	0.39	0.46	0.41	0.39	0.03	0.52	0.42
7. AY551130 Proteocephalus midoriensis Lefua echigonia Japan	2.02	0.63	0.65	0.67	1.31	1.37		0.15	0.25	0.40	0.15	0.13	0.63	0.42	0.60
8. AY551125 Proteocephalus cernuae Perca cernuae Germany	2.06	0.00	1.11	1.85	0.62	0.84	0.50		0.54	0.51	0.00	0.01	0.45	0.51	0.68
9. KX768933 Proteocephalus plecoglossi Plecoglossus altiveli Japan	0.81	0.13	0.28	0.05	1.18	1.04	0.66	0.00		0.04	0.54	0.48	0.39	0.10	0.51
10. KX768932 Proteocephalus fluviatilis Micropterus dolomieu Japan	0.89	0.41	0.43	0.22	1.00	1.23	0.00	0.97	0.14		0.51	0.40	0.45	0.07	0.43
11. DQ427100 Proteocephalus ambiguus Gasterosteus laevis France	2.08	1.78	1.09	1.83	0.62	0.84	0.49	0.00	0.00	0.00		0.01	0.44	0.52	0.68
12. DQ427101 Proteocephalus longicollis Alyselminthus longicollis	2.04	0.87	0.00	0.89	0.69	0.83	0.47	0.02	0.91	0.79	0.02		0.40	0.43	0.68
13. AF335511 Proteocephalus torulosus Ide leuciscus idus Czech	1.29	0.95	0.77	1.01	0.28	0.10	1.37	0.86	1.01	1.14	0.86	0.85		0.35	0.39
14. KX768931 Proteocephalus gobiorum Apollonia fluviatilis Ukraine	0.92	0.39	0.15	0.40	1.03	1.22	0.00	2.15	0.40	0.26	2.09	0.83	0.86		0.48
15. AF267292 Acanthotaenia sp. Varanus exanthematicus Ghana	2.12	1.52	1.91	1.60	1.61	1.80	1.96	2.02	1.95	1.08	2.01	2.08	1.01	1.41	

Figure 4: Estimates of evolutionary divergence between the sequences of *Proteocephalus vitellaris* (KY786339) and other species of family Proteocephalidae available in the NCBI GenBank

Table 1: Comparison of present specimens of the genus *Proteocephalus* with the original description of *P. ritaii* Verma, 1926 and *P. vitellaris* Verma, 1928

Species → Characters ↓	***P. vitellaris* (Present study)**	***P. ritaii* Verma, 1926**	***P. vitellaris* Verma, 1928**
Parasite length	27-42mm	125mm	250mm
Number of strobila	80-85	-	-
Width of scolex (WS)	342-344	-	800
Diameter of sucker (DS)	88-128	45	160
DS/WS	0.19-0.22		
Diameter of apical Sucker (DAO)	153-240	-	-
DAO/DS	0.50-0.53	-	-
Length of immature proglottid	98-272	-	-
Width of immature proglottid	768-976	-	-
LHP/WHP	0.25-0.27	-	-
Length of mature proglottid	148-480	-	-
Width of mature proglottid	726-1440	-	-
LHP/WHP	0.30-0.33	-	-
Number of testes	80-90	150-200	250-275
Diameter of testes	18-20	40-60	-
Length of cirrus sac	125-276	192	-
Width of cirrus sac	50-200	128	140
LCS/WCS	1.2-1.4	-	-
LCS/WHP	0.18-0.20	-	-
Width of ovary	166-288	-	-
Length of ovarian lobe	50-140	-	-
Host	*Bagarius yarrelli*	*Rita rita*	*Bagarius yarrelli*
Location	Intestine	Intestine	Intestine
Locality	Harike wetland, Punjab	Allahabad	Allahabad

All measurements are in micrometer unless otherwise mentioned.

Table 2: Homogeneity of 18S rRNA gene sequence of *P. vitellaris* (Accession number KY786339) and others *Proteocephalus* available in NCBI Genbank

Cestodes species	Accession number	Organ infected	Host	Country	Homogeneity (%) to *P. vitellaris* (KY786339)
P. sagittus	KX768935	Intestine	*Barbatula barbatula*	Russia	560/560 (92%)
P. parasiluri	KX768930	Intestine	*Silurus asotus*	Russia	560/560 (92%)
P. percae	KX786934	Intestine	*Perca fluviatilis*	Denmark	551/551 (91%)
P. tetrastomus	AF335510	Intestine	*Hypomesus nipponensis*	Germany	551/551 (91%)
P. osculatus	AF335508	Intestine	*Silurus glanis*	Czech Republic	551/551 (91%)
P. midoriensis	AY551130	Intestine	*Lefua echigonia*	Japan	551/551 (92%)
P. cernuae	AY551125	Intestine	*Perca cernuae*	Germany	551/551 (91%)
P. plecoglossi	KX768933	Intestine	*Plecoglossus altivelis altivelis*	Japan	545/545 (91%)
P. fluviatilis	KX768932	Intestine	*Micropterus dolomieu*	USA	545/545 (91%)
P. ambiguus	DQ427100	Intestine	*Pungitius pungitius*	France	545/545 (91%)
P. longicollis	DQ427101	Intestine	*Salmo trutta trutta*	Germany	542/542 (92%)
P. torulous	AF335511	Intestine	*Leuciscus cephalus*	Czech Republic	540/540 (91%)
P. gobiorum	KX768931	Intestine	*Apollonia fluviatilis*	Ukraine	533/533 (90%)
Acanthotaenia sp.	AF267292	Intestine	*Varanus exanthematicus*	Ghana	Outgroup

Table 3: 18S rRNA showing the transitional and transversion substitution obtained by maximum likelihood estimate of substitution matrix Mega6 of *P. vitellaris*

	A	**T/U**	**C**	**G**
A	-	*8.54*	*7.81*	**7.66**
T/U	*8.04*	-	**8.85**	*9.16*
C	*8.04*	**9.68**	-	*9.16*
G	**6.72**	*8.54*	*7.81*	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in *i*talics.

Table 4: Homogeneity of ITS2 gene sequence of *P. vitellaris* (Accession number KY786338) and others *Proteocephalus* available in NCBI Genbank

Cestodes species	Accession number	Organ infected	Host	Country	Homogeneity (%) to *P. vitellaris* (KY786338)
P. syndontis	JN005777	Intestine	*Synodontis schall*	Africa	295/295 (86)
P. pirarara	AY551170	Intestine	*Phractocephalus hemioliopterus*	Brazil	271/271 (83)
P. brooksi	AY551159	Intestine	*Rhamdia guatemalensis*	Mexico	226/303 (94)
P. thymalli	DQ427099	Intestine	*Thymallus arcticus biacalensis*	Russia	224/296 (93)
P. tetrastomus	AB558485	Intestine	*Hypomesus nipponensis*	Japan	224/294(93)
P. percae	DQ427098	Intestine	*Perca fluviatilis*	Denmark	224/300 (93)
P. longicollis	DQ427097	Intestine	*Salmo trutta trutta*	Germany	224/294 (93)
P. ambiguus	DQ427096	Intestine	*Pungitius pungitius*	France	224/294 (93)
P.cernuae	AY551160	Intestine	*Perca cernuae*	Germany	224/294 (93)
P. plecoglossi	AY551171	Intestine	*Plecoglossus altivelis altivelis*	Japan	223/223 (95)
P. sagittus	AY375548	Intestine	*Barbatula barbatula*	Russia	221/265 (93)
P. midoriensis	AY551168	Intestine	*Lefua echigonia*	Japan	221/274 (93)
P. macrocephalus	AY551167	Intestine	*Anguilla Anguilla*	Germany	221/287 (93)
P. fluviatilis	AY551163	Intestine	*Micropterus dolomieu*	USA	221/291 (93)
P. ambloplitis	KT737467	Intestine	*Micropterus salmoides*	USA	219/291 (94)
Acanthotaenia sp.	AF267292	Intestine	*Varanus exanthematicus*	Ghana	Outgroup

Table 5: ITS2 showing the tranitional and transversion substitution obtained by maximum likelihood estimate of substitution matrix Mega6 of *P. vitellaris*

	A	T/U	C	G
A	-	*3.18*	*2.51*	**15.64**
T/U	*2.09*	-	**22.98**	*3.12*
C	*2.09*	**29.07**	-	*3.12*
G	**10.5**	*3.18*	*2.51*	-

Rates of different transitional substitutions are shown in bold and those of transversional substitutions are shown in *i*talics.

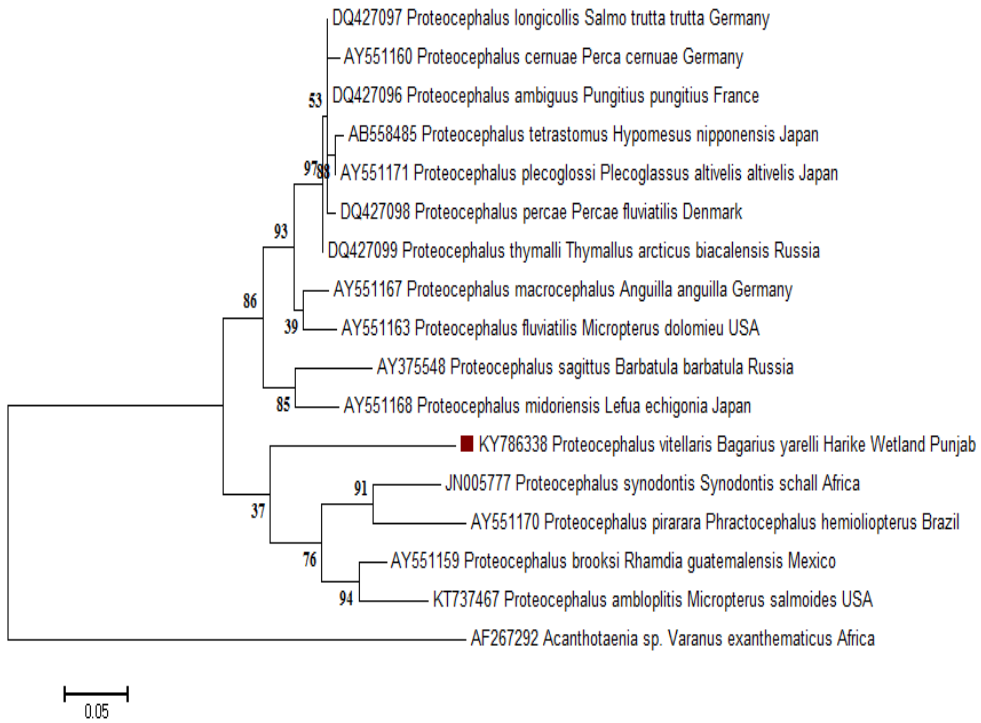

Figure 5: Phylogenetic tree reconstructed by Maximum-Likelihood analysis of ITS2 showing the position of ***Proteocephalus vitellaris*** **(KY786338) with other species of family Proteocephalidae**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. KY786338 Proteocephalus vitellaris Bagarius yarelli Harike Wetland		0.05	0.06	0.05	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.08	0.07	0.06	0.06	0.05	0.19
2. JN005777 Proteocephalus synodontis Synodontis schall Africa	0.22		0.04	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.07	0.07	0.07	0.07	0.06	0.24
3. AY551170 Proteocephalus pirarara Phractocephalus hemioliopterus	0.28	0.20		0.04	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.06	0.05	0.05	0.06	0.24
4. AY551159 Proteocephalus brooksi Rhamdia guatemalensis Mexico	0.24	0.27	0.22		0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.07	0.07	0.06	0.07	0.03	0.24
5. AB558485 Proteocephalus tetrastomus Hypomesus nipponensis Ja	0.30	0.30	0.25	0.28		0.01	0.01	0.01	0.01	0.01	0.01	0.04	0.04	0.03	0.02	0.06	0.24
6. DQ427099 Proteocephalus thymalli Thymallus arcticus biacalensis I	0.29	0.29	0.23	0.27	0.02		0.01	0.00	0.00	0.01	0.01	0.04	0.04	0.03	0.02	0.06	0.19
7. DQ427098 Proteocephalus percae Percae fluviatilis Denmark	0.29	0.29	0.23	0.27	0.03	0.02		0.00	0.01	0.01	0.01	0.04	0.04	0.03	0.02	0.06	0.19
8. DQ427097 Proteocephalus longicollis Salmo trutta trutta Germany	0.29	0.28	0.24	0.27	0.02	0.01	0.02		0.00	0.01	0.01	0.04	0.04	0.03	0.02	0.06	0.19
9. DQ427096 Proteocephalus ambiguus Pungitius pungitius France	0.29	0.29	0.23	0.27	0.02	0.00	0.02	0.01		0.01	0.01	0.04	0.04	0.03	0.02	0.06	0.19
10. AY551160 Proteocephalus cernuae Perca cernuae Germany	0.30	0.31	0.25	0.28	0.03	0.03	0.04	0.02	0.03		0.01	0.05	0.04	0.03	0.03	0.06	0.21
11. AY551171 Proteocephalus plecoglossi Plecoglassus altivelis altive	0.31	0.30	0.24	0.27	0.02	0.03	0.04	0.02	0.03	0.04		0.04	0.04	0.03	0.02	0.06	0.21
12. AY375548 Proteocephalus sagittus Barbatula barbatula Russia	0.37	0.32	0.27	0.33	0.22	0.20	0.21	0.20	0.20	0.23	0.20		0.03	0.05	0.05	0.07	0.29
13. AY551168 Proteocephalus midoriensis Lefua echigonia Japan	0.31	0.30	0.26	0.33	0.21	0.20	0.21	0.20	0.20	0.22	0.20	0.16		0.05	0.05	0.07	0.28
14. AY551167 Proteocephalus macrocephalus Anguilla anguilla Germa	0.30	0.32	0.26	0.29	0.15	0.15	0.15	0.14	0.15	0.16	0.15	0.24	0.24		0.03	0.07	0.27
15. AY551163 Proteocephalus fluviatilis Micropterus dolomieu USA	0.28	0.31	0.25	0.31	0.12	0.12	0.12	0.12	0.12	0.14	0.13	0.23	0.24	0.15		0.07	0.24
16. KT737467 Proteocephalus ambloplitis Micropterus salmoides USA	0.24	0.28	0.27	0.15	0.30	0.30	0.29	0.29	0.29	0.29	0.30	0.34	0.32	0.32	0.34		0.23
17. AF267292 Acanthotaenia sp. Varanus exanthematicus Africa	0.74	0.90	0.89	0.89	0.92	0.75	0.74	0.75	0.74	0.81	0.81	1.09	1.04	0.99	0.91	0.88	

Figure 6: Estimates of evolutionary divergence between the sequences of *Proteocephalus vitellaris* (KY786338) and other species of family Proteocephalidae available in the NCBI GenBank

DISCUSSION

The present species *P. vitellaris* Verma, 1928 infecting small intestine of *B. yarrelli* was identified in having a well marked prominent scolex 342-624µm wide, with distinct neck, more bilaterally situated suckers, 118-144×88-128µm in size and prominent large sized apical sucker 153-240µm in width. The measurements were closely compared with the original description. Since the original description was not complete, an attempt has been made to supplement the morphological and morphometric data, however some variation were noted in the total length of worm (27-42 vs 250mm), width of

scolex (800 vs 342-344µm), diameter of sucker (160 vs 88-128µm), no. of testes (250-275 vs 80-90) and width of cirrus sac (140 vs 50-200µm) (Table 1). The present specimens of the genus *Proteocephalus* were compared with other species from India and other parts of the world in general morphology and morphometric of scolex, apical sucker, male and female reproductive systems. This includes the species which have been recorded in the blast analysis using 18S rRNA and ITS2 gene markers.

Scolex

In contrast to the present species the scolex in *P. ambiguus* was smaller and rounded in shape, 157-220µm with indistinct neck, bilateral suckers, smaller to vestigial apical sucker, 20-32µm in diameter which appeared flattened on the apical surface. In *P. macrocephalus* and *P. sagittus*, the scolices were rounded with lateral or sublateral suckers. However, the apical sucker was vestigial and much smaller in the former and absent in the latter. In *P. torulosus*, *P. brooksi* and *P. gobiorum* the apical sucker is absent in contrast to a prominent and large one in the present species.

The scolices of *P. midorensis* and *P. pirarara* were different in shape, scolex <300µm in width in the former and scolex wider than neck 485-720µm, suckers uniloculate, oval, directed anteriorly, 200-290µm in diameter in the latter. Furthermore, the apical sucker absent in both of them. *P. plecoglossi* scolex was smaller and rounded in shape. Also, differed from the present species in having suckers which were placed posteriorly close to the neck and in having much smaller apical sucker 4-7µm in diameter. *P. longicollis* and *P. percae* differed from the present species in having smaller, rounded and tapering anteriorly with distinct and narrower neck than the scolex in the former and wider than the scolex in the latter. The suckers were posteriorly placed in the former and lateral to sublateral in the latter. Furthermore, the apical sucker was much smaller in *P. longicollis* 22-86µm in diameter in comparison to 152-154µm in diameter in the present species. In *P. percae* the apical sucker was small, vestigial and flattened in shape.

P. cernuae differed from the present species in having scolex blunt at the anterior end with a neck slightly narrower than scolex. Furthermore, it also differed in having smaller anterolaterally placed suckers and a vestigial apical sucker which was also flattened to slightly elliptical in shape representing only about 10% of scolex width. The scolex in *P. tetrastomus* was semi-spherical, 230-307µm in width with sublaterally situated larger suckers 99-134µm in diameter. However differed from the present species in having strongly reduced apical sucker. Also in *P. fluviatilis* the apical sucker was widely oval and vestigial in comparison to the present species.

The scolex in *P. thymalli* was longer and 376-780µm in wider with distinct neck, large suckers 147-227µm in diameter directed anteriorly and the apical sucker was quite small, vestigial, flattened and 58-96µm in diameter. In *P. synodontis* the scolex was pyramidal in shape with deeply embedded suckers, apical sucker (absent in some specimens) with numerous gland cells occupying an area of 152-160µm in diameter. *P. ambloplitis* differed from the present species in having external apical sucker and a subcuticular organ. *P. parasiluri* differed in having globose scolex, 1600-1920×2000-4560µm in size, without distinct neck, four suckers spherical, directed anteriorly in the central field of scolex, 380-690µm in diameter, apical sucker anterior to them, 110-220µm in diameter. The present species was also compared with *P. osculates* in having large scolex, 352-440µm wide with prominent large lateral or sublateral suckers 108-139µm in diameter, well developed apical sucker 64-86µm in diameter, strongly muscular with a deep and numerous bottle shaped gland cells.

Testes

In the present species the testes were small, rounded, medullary, spherical to oval, 12-14×18-20µm in size, 80-90 in number per proglottid, 1-2 layers in cross section. *P. vitellaris* in having testes 250-275 in number and *P. ritaii* having larger sized testes 40-60µm, 150-200 in number. In this respect the present species differed from *P. tetrastomus* in having testes in 1 layer, 33-58 in number. In *P. sagittus*, *P. midorensis* and *P. gobiorum* having >40 testes a per proglottid, arranged in 1-2 layers. In *P. synodontis* testes medullary, 95-133 in number and *P. pirarara* in having testes medullary, 69-93 in number. *P. brooksi* differed in having 108-175 number of testes, averaging 28 preporal, 38 postporal and 72 aporal. In *P. parasiluri* differed in having 142-218 in number per proglottid.

Ovary

In the present species the ovary was bilobed, measures 22-24×165-167µm in size and low, pressed along the lower margin of the proglottid. Ovary reaching not up to the lateral osmoregulatory ducts. In this respect the present species differed from *P. sagittus* in having ovary 364-591µm in width. *P. midorensis* was differed in having large sized ovary with much wider lobes and several transverse branches. *P. synodontis* also differed in having bilobed compact medullary ovary with a small outgrowth on the surface. In *P. pirarara*, the ovarian lobes were dorsomedullary, bilobed, folliculate with numerous dorsal follicles. *P. parasiluri* differed in having ovary transversely elongated triangular, bilobed, increasing in length but decreasing in width with proglottid growth, 190-400×400-1280µm in size.

Vagina

In the present species, *P. vitellaris* the vagina opens posterior to the cirrus sac without vaginal sphincter occupying ratio 1/4 of proglottid width. In the case of *P. plecoglossi* and *P. percae* vaginal sphincter was well developed with elongated cirrus sac representing 1/3-2/5 of proglottid width. However, in *P. tetrastomus*, the vagina was without well developed sphincter. In *P. macrocephalus*, the vaginal canal was widened, thick walled distally, formed seminal receptacle anterior to the ovarian isthmus. However, short cirrus sac represented 1/8-1/4 of proglottid width. In contrast *P. fluviatilis* the vaginal canal was without prominent thickening in the distal part, seminal receptacle present anteriodorsal to the ovarian isthmus. In *P. sagittus* the vagina opens anterior to cirrus sac, without vaginal sphincter however in the case of *P. synodontis*, *P. pirarara* and *P. brooksi* the vagina anterior (68%) or posterior (32%) to cirrus, never crossing cirrus sac. Vagina sphincter was also absent in *P. brooksi* but in the case of *P. pirarara* terminal thick part and small muscular sphincter was present. *P. parasiluri* also the vagina open anterior or posterior to cirrus pore, without sphincter.

Vitelline follicles

In the present species the vitelline follicular were medullary. In this respect it resembled *P. synodontis* and *P. pirarara* in having follicular medullary vitellaria, formed by small follicles in two lateral bands, situated ventrally and differed from *P. brooksi* in having vitellines follicles paramuscular. *P. parasiluri* also differed in having vitelline follicles distributed in a band curved at right angles on each side of medullary region.

The Ces1 and Ces2 primers successfully amplified the 18S rRNA gene of approx. size 400bp. The blast analysis indicated maximum homogeneity of 92% with *P. sagittus* infecting *Barbatula barbatula* from Russia with 7 gaps, 92% with *P. midorensis* infecting *Lefua echigonia* from Japan with 9 gaps, 92% with *P. longicollis* infecting *Salmo trutta trutta* from Germany with 5 gaps, 91% with *P. percae*, *P. tetrastomus*, *P. cernuae*, *P. plecoglossi*, *P. fluviatilis*, *P. ambiguus* with 7 gaps and 90% with *P. gobiorum* infecting *Apollonia fluviatilis* from Lake kita, Ukraine with 9 gaps (Table 2). The best substitution model with lowest BIC score 7618.442 using Jukes-Cantor (JC) and showed 0.02, 0.26, 0.14, 0.12, 0.50 divergence with 14 *Proteocephalus* species (Fig. 4). The data estimated from nucleotide frequencies were A=23.96%, T/U=24.46%, C=23.27%, G=27.30% and nucleotide substitution were [AC]=7.81, [AG]=7.66, [AT]=8.54, [CG]=9.16, [CT]=9.68, [GT]=8.54. The final edited alignment (400bp) with Maximum likelihood showed two clades. Therefore, the first clade having two subclades- upper subclade and lower subclade. However, in the lower clade there cluster the present species *P. vitellaris* (KY786339), *P. tetrastomus* (AF335510), *P. osculates* (AF335508), *P. torulosus* (AF335511) from Germany and Czech Republic (Fig. 3).

The Proteo 1 and Proteo 2 primers sucessfully amplified the ITS2 rRNA gene of approx. size 410 bp. The BLAST analysis indicated maximum homogeneity of 95% with *P. plecoglossi* infecting *Plecoglossus altiveli* from Japan with 0 gaps, 94% with *P. brooksi* infecting *Rhamdia guatemalensis* from Mexico with 2 gaps, 94% with *P. ambloplitis* infecting *Micropterus salmoides* from USA with 4 gaps, 86% with *P. syndontis* infecting *Synodontis schall* from Africa with 6 gaps, 83% with *P. pirarara* infecting *Phractocephalus hemioliopterus* from Brazil with 19 gaps (Table 4). The best substitution model with lowest BIC score 3797.178 using K2+G and showed 0.01, 0.02, 0.04, 0.15, 0.16 divergence with 16 *Proteocephalus* species (Fig. 6). The data estimated from nucleotide frequencies were A=19.20%,

T/U=29.16%, C=23.05%, G=28.60% and nucleotide substitution were [AC]=2.59, [AG]=13.19, [AT]=3.6, [CG]=3.58, [CT]=31.22, [GT]=3.6. The sequencing of the ITS2 rRNA gene of *P. vitellaris* resulted in a total of 410 bp and the phylogenetic tree based on the final alignment with Maximum likelihood showed two clades. In lower clade, the present species *P. vitellaris* (KY786338) in a separate branch and stood alone in one clade (Fig. 5). Therefore, the morphological and molecular studies (18S and ITS2 markers) supports the 14 valid species of the genus *Proteocephalus* (Scholz *et al*., 2007).

CONCLUSION

This paper deals with morphological, morphometric and molecular characterization *of P. vitellaris* using amplification of 18S rRNA and ITS2 and phylogenetically analysis are important for formation of cryptic species. The parasites were collected from the small intestine of a freshwater catfish, *Bagarius yarrelli* inhabiting Harike wetland Punjab, India. It was earlier reported from the same host at Allahabad (India) by Verma (1928).

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