Molecular analysis of a novel species, *Gangesia punjabensis* (Family: Proteocephalidae, Subfamily: Gangesiinae) infecting an Indian freshwater cat fish, *Wallago attu* evidencing species complex

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Abstract In the present study, from August 2015 to July 2016, a total of 159 specimens of *Wallago attu* Bloch and Scheider, 1801 vern. mully were collected from the Ropar wetland, Punjab, India and were examined for the presence of intestinal tapeworms. The infection rate was 12.5%, out of which, two fishes were heavily infected with a proteocephalidean cestode belonging to the genus *Gangesia* in the month of February. *Gangesia punjabensis* sp. nov. was identified and described on the basis of morphology and partial amplification of LSU rDNA (28S rDNA). The histopathogenesis caused by these parasites have also been studied in histological sections. About 9–35 number of worms were collected from a single host. The length of worms ranged from 6.0 to 6.2 cm. The strobila consisted of an armed scolex, neck, immature proglottids (approximately 90 in number) and mature proglottids (approximately 7 in number) and gravid proglottids were not detected. Analysis of 28S large subunit (LSU) rDNA sequence of the isolate of *G. punjabensis* n. sp. demonstrated maximum similarity of 99% with *G. bengalensis*, 98% with *G. vachai*, 97% with *G. macrones*. The phylogenetic tree based on the final edited alignment (822 bp) with Neighbour-Joining showed the highest bootstrap value of 100 and placed *G. punjabensis* n. sp. with *G. bengalensis* and *G. macrones* in the same clade showing them to be a species complex. The intestinal mucosal lining exhibited blister formation, haemorrhagia and inflammation due to

the presence of deeply embedded scolices. Histopathology caused was evident due to disruption of villous surface and cellular infiltration. This is the first report of cestodes belonging to the genus *Gangesia* from Ropar wetland, Punjab, India.

Keywords *Gangesia* · Cestode · *Wallago attu* · SEM · LSU rDNA · Histopathology

Introduction

Wetlands are important for fish fauna especially catfishes. Fishes provide good source of proteins, minerals, vitamins etc. In India, 160 species of catfishes belonging to 50 genera and distributed under 13 families viz. Akysidae, Ariidae, Amblycipitidae, Bagridae, Plotosidae, Schilbeidae, Siluridae, Sisoridae, Chacidae, Clariidae, Heteropneustidae, Olyridae, Pangasiidae (Talwar and Jhingran 1991) have been described. *W. attu* (Bl&Sch) syn. *Siluris attu* belonging to the family Siluridae and inhabiting freshwater streams, rivers, reservoirs and commonly found in Harike and Ropar wetlands have been reported as definitive host of many cestode parasites (Hafeezullah 1993; Jadhav et al. 2010). Ropar wetland a unique wetland system located in the lap of Shivalik foothills, is an important habitat of many species and has tremendous ecological value, spread over 1365 Ha. It is located at 31°01'N latitude and 076°30'E longitude, and support as many as 55 species of fishes. In the present study cestode parasites belonging to the genus *Gangesia* were most frequently encountered in the small intestine of *W. attu*. Intestinal fish parasites harm their hosts in a variety of ways. They cause mechanical irritation, injury or atrophy of lining, and may occlude the alimentary canal, blood

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vessels and other ducts, and introduce toxic metabolic byproducts, depriving the fish of nutrition and over and above acting as carriers or vectors of other pathogens. An increasing interest in fish culture as a potential source of extra food is ultimately bound to lead to investigation of fish diseases and pathogens. The scanning electron microscope has been important tool for discerning minute character such as scolex morphology and surface ultrastructure (Scholz et al. 1998; Chervy 2009; Oros et al. 2010). The molecular data based on 28S large subunit (LSU) rDNA sequences of *Gangesia* species reported from Indomalayan region have been used for the confirmation of conspecificity of individual taxa and have assessed their phylogenetic interrelationships (Ash et al. 2012).

Materials and method

The fresh specimens of the catfish, *Wallago attu* vern. mullee were collected from the local fish market near Ropar wetland, Punjab and were brought to the laboratory for parasitological investigation. Fishes were longitudinally cut to expose the internal part. The entire alimentary canal was removed and placed in a petridish with physiological saline (0.8% NaCl). Month wise prevalence rate (%) of *Gangesia* worm was maximum (37.5%) in the month of April and highest worm burden (30–37 worms) were collected in the month of January and February. The total prevalence rate during the study period of one year was 25.7%. The tapeworms were gently removed and placed in saline solution, washed several time in saline and counted. The worms were relaxed and stretched in warm 4% formalin over the edge of the beaker and fixed and preserved in fresh 4% formalin for morphology, 4% glutaraldehyde for SEM. For morphology, worms were stained in Gower's carmine, differentiated in 70% acid ethanol (i.e. 70% ethanol with 2–3 drops of HCL), dehydrated through a graded ethanol series, cleared in clove oil (eugenol) and mounted in DPX. Measurements were taken in micrometer (μm) or otherwise mentioned. Line drawings of stained worm were made with the camera lucida. The identification up to the generic level of the tapeworms was done with the help of "Systema Helminthum" by Yamaguti (1959). Mature and gravid proglottids were embedded in paraffin wax, sectioned (T.S.) at 7–9 μm and stained with Delafield's hematoxylin eosin. The worms attached to intestine were kept intact and small pieces of infected intestine and portions of healthy intestine were fixed in Bouin's fixative to study histopathological changes. Fixed tissues were washed, dehydrated through alcoholic grades, cleared in xylene and embedded in paraffin wax (60–62 °C). The blocks were cut at 9 μm and slides were stained with haematoxylin–eosin double staining method and the

section were mounted in the DPX. For scanning electron microscopy, worms were washed 2–3 times in distilled water and fixed in 4% glutaraldehyde at 4 °C, post fixed in 1% osmium tetroxide. The worms were washed in buffer overnight and dehydrated in an ascending series of ethyl alcohol and the specimens were dried in critical point drier, mounted and coated with gold and were photographed with Jeol JSM 6510LV at Sophisticated Instrumentation Centre, Punjabi University, Patiala.

The standard phenol chloroform extraction method (Sambrook and Russell 2001) was used to extract DNA from 100% ethanol preserved cestode worms. Immature and mature segments weighing 25 mg were crushed and transferred to 1.5 ml microcentrifuge tubes. Subsequently 250 μl Phosphate buffer saline (PBS), 50 μl proteinase K and 50 μl of Sodium dodecyl sulfate (SDS) were added, mixed and incubated overnight at 55 °C to digest the tissue completely. Phenol: chloroform (1:1) 300 μl was added and centrifuged for 10 min at 10,000 rpm. The uppermost layer was transferred to a new microcentrifuge tube and mixed with double the amount of chilled isopropanol. The DNA was precipitated at –20 °C for 2 h and centrifuged at 10,000 rpm for 10 min to obtain the pellet, which was washed with 80% ethanol, air dried and suspended in 30 μl of 1 $\times$ TE buffer. Quantification of the product was done on a Nanodrop (MySPEC) spectrophotometer at 175 ng/ μl . Also, the quality of the product was checked on electrophoresis using 0.8% agarose gel. The primer Ces28S Forward (GTCGGTGCACTTCTCTGTG), Ces28S Reverse (CTGGCTTCAACCTACGCAAG) designed according to the sequences of the 28S rRNA gene of *Gangesia bengalensis* (Ash et al. 2012) were used for the amplification of 28S rRNA using Veriti Thermal cycler. The PCR reaction were performed in 25 μl reaction mixture of 2.5 μl of 10 $\times$ buffer, 1.0 of each primers, 0.8 μl of 2.5 mM dNTP, 0.5 μl of Taq DNA polymerase, 0.5 μl of 50 mM MgCl_2 , 2.0 μl of total DNA and 16.7 μl of double purified water. The PCR cycling conditions used included, initial denaturation at 95 °C for 3 min, 30 s denaturation at 95 °C for 33 cycles, 30 s annealing at 60 °C, 40 s extension at 72 °C, 7 min final extension at 72 °C. The PCR products were analyzed on a 2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide in 1 $\times$ Tris–acetate–EDTA (TAE) buffer run together with DNA ladder (100 bp).

The PCR amplified products were purified by using Geneipure™ Quick PCR Purification Kit (GeNei™) and sequenced commercially at the Molecular Diagnostics & Research Laboratories (MDRL) Pvt. Ltd. Chandigarh, India. The accession number of KY018599 was obtained after duly submitting sequences to the GenBank.

For phylogenetic analysis, the basic local alignment tool was used to analyze the new 28S rDNA sequence (KY018599) of cestode worm belonging to the genus

Gangesia, and 24 additional sequences showing genetic relatedness with homogeneity percentage of 91 or above in the GenBank database. The outgroup was *Silurotaenia siluri* (AJ388592) isolated from *Silurus glanis*. Hasegawa-Kishino Yanoparameter model with lowest Bayesian score (BIC) of 2555.662 (Kimura 1980) in MEGA6 software (Tamura et al. 2013) were used to make genetic distance analyses among closely related species of the genus *Gangesia*. Multiple Sequence Comparison by Log-Expectation (MUSCLE) was used for sequence alignment. The tree was generated using Neighbour Joining having 1000 bootstrap values and was proportional to the number of substitutions per site.

Results

Morphological description (Figs. 1a–c, 2a–d) (measurements based on 10 specimen)

Parasites 62 mm in length. Strobila consisted of an armed scolex, 87–91 number of immature proglottids, 3–5 number of mature proglottids and gravid proglottids were not detected. Scolex knob-like, without neck, $374\text{--}378 \times 388\text{--}391 \mu\text{m}$ in size, consist of four suckers and a retractable rostellum armed with two rows of hooks. Suckers large, circular, $182\text{--}184 \mu\text{m}$ in diameter, overlapping, each having 5–8 rows of fine hooklets along the upper margins.

Fig. 1 Line drawing of *G. punjabensis* n. sp. **a** scolex, **b** rostellar hook, **c** mature proglottid (dorsal view)

Fig. 2 Photomicrograph of *G. punjabensis* **a** scolex, **b** rostellum, **c** mature proglottid stained with Gower's carmine, **d** T.S. of mature proglottid showing single layer of tests (TS) stained with haematoxylin–eosin stain

Rostellum highly retractile, conical, measuring $68.8\text{--}71.1 \times 142\text{--}144\text{ }\mu\text{m}$ in size armed with 2 rows of hooks, 26–28 hooks in each row. Rostellar hooks equal in both the rows, measure $38.2\text{--}40.2\text{ }\mu\text{m}$ in total length of rostellar hooks, $11.3\text{--}13.6\text{ }\mu\text{m}$ length of basal plate and $23.9\text{--}26\text{ }\mu\text{m}$ length of blade of hooks. Immature proglottid broad, measure $132\text{--}134 \times 142\text{--}144\text{ }\mu\text{m}$ in size with developing reproductive organs. Mature proglottid broad, measure $276\text{--}283 \times 332\text{--}334\text{ }\mu\text{m}$ in size. Testes small, rounded $84\text{--}100$ in number, each lobule measure $15.5\text{--}17.7\text{ }\mu\text{m}$ in diameter, occupying whole of the space above the ovary. Testes in single file in the medullary parenchyma (observed in transverse section). Ovary large, butterfly-shaped, measure $188\text{--}191\text{ }\mu\text{m}$ in length and situated at the posterior margin of the proglottid. Oviduct arise from the middle of the ovary. Uterine tube $158\text{--}161\text{ }\mu\text{m}$ in length, uterine pore located ventrally. Vagina opening posterior to cirrus sac, cirrus sac length $35.4\text{--}37.2\text{ }\mu\text{m}$. Vas deferens in the form of fine coiled tube, along with cirrus sac reaching upto $1/4$ of the width of mature proglottid. Genital pore is irregularly alternate, oval in shape, located at the centre of the lateral margin of proglottid. Vitellaria follicular, oval to rounded shaped, follicles occupying lateral margin of the whole length of the mature proglottid.

Prevalence

The total percentage of cestode infection was 41%. With respect to infection of *Gangesia* sp. highest infection of 50% was in the month of January, 2016 and no infection was recorded in September and October, 2015.

Taxonomic summary of *Gangesia punjabensis* sp. nov

Type host: *Wallago attu* (Bloch and Schneider) vern. mullee

Type locality: Ropar Wetland, Punjab

Site of infection: Small Intestine

Etymology: The specific epithet “*punjabensis*” has been given after the locality i.e. state of Punjab, of fish host.

Type specimen: Cestode worm stained in Gower's carmine, deposited in Parasitology Laboratory in the Department of Zoology, Panjab University, Chandigarh, India (catalogue no.: WA/G-1/2015-16)

Paratypes: Cestode worm stained in Gower's carmine (4 Slides: catalogue no.: WA/G-2, G-3, G-4, G-5/2015-16) and fixed in 4% formalin (catalogue no.: WA/G-6/2015-16)

Morphological feature based on scanning electron microscopy (SEM) (observations based on 3 specimens)

Four lobed scolex with suckers on each lobe, suckers directed anterolaterally, no wrinkles on scolex. The suckers are generally round or oval. Rostellum conical in shape, covered with papilliform filitriches and $133\text{ }\mu\text{m}$ in length and $64\text{ }\mu\text{m}$ in width (measurement under SEM). 5–8 number of rows of hooklets present on sucker. Capilliform microtriches on the surface of mature proglottids (Fig. 3a–f).

Fig. 3 Scanning electron microscope of *G. punjabensis* **a** scolex, **b** armed rostellum, **c** papilliform filitriches on rostellum, **d** hooklets on sucker, **e** magnified view of hooklets, **f** capilliform microtriches on mature proglottid

Histology of mature proglottid (based on 2 specimens)

Testes spherical to oval, forming single file in medullary parenchyma. Vitelline follicles medullary, with some follicles paramuscular (Figure 2d).

Histopathology (Fig. 4a–f) (Based on thin section of 2 infected hosts)

In transverse section (T.S.) of small intestine of *W. attu*, the scolices of *Gangesia punjabensis* n. sp. were detected to be deeply embedded in the mucosal layer. The worms made very intimate contact with the host's intestinal tissue to obtain the nourishment from the host, which resulted in the damage of intestinal mucosa. The attachment resulted in acute mechanical irritation of the epithelium, disruption of the villous surface, desquamated cells, damaged blood

vessels and lamina propria. There was much compression of the epithelium near the embedded worms causing inflammation, vacuolation and damage. Due to the large number of scolices attached there was damage to the lamina propria and formation of inflammatory nodulation in the intestinal wall and increase in number of goblet cell. Large number of inflammatory cells get infiltrated in the lamina propria. The fibroblast, lymphocytes, plasma cells were attached to the intestinal villi surface therefore causing inflammation and nodulation. Numerous mast cells were spotted in sections near the villous surface. However, there was no capsule formation around the parasites within the mucosal lining of the intestinal wall. At the base of the submucosal layer of infected intestine, there was clustering of cell (50–100 s in number) in the form of lymphatic nodules as in higher vertebrates. It has been observed that damage was more evident in heavy infections with *G. punjabensis* therefore regarded as highly pathogenic to the

Fig. 4 Photomicrograph of infected intestine showing **a** clusters of cestodes in the small intestine of fish, *Wallago attu*, **b** inflammatory nodules in the internal lining of small intestine of catfish, *Wallago attu*, **c** T.S. of normal intestine showing villi (V), mucosal (M) and sub-mucosal layers (SM), **d** T.S. of infected intestine showing embedded scolices in the mucosal layer of small intestine, **e** T.S. of infected intestine showing lymphocytes (L), plasma cells (PC) and fibroblasts (FB), **f** T.S. of intestine showing mast cells (MC) on the surface of damaged villi

host of *W. attu*. The worms were not detected in the inner intestinal layers i.e. submucosa and muscularis mucosa. At some of the sites of attachment of scolices, the adjacent villi were noticed to be fused with each other. Also, distortion of villi was evident around the sites of attachment of scolices. At some places the surface epithelium appeared to be removed from the tip of the villi. There was no evidence for the presence of secondary microbial infection. The histopathological changes due to light infection caused by the cestode parasite on the catfish were milder in nature and did not seem to cause any serious damage to the infected intestine. The transverse section of healthy parts of small intestine of the host, *W. attu* showed intact histological architecture and all layers i.e. serosa, muscularis mucosa, submucosa and mucosa were clearly observed. As the place of contact with the cestode, the surface epithelium had been compressed and the cells had become cuboidal, rounded or flattened without definite boundaries, inflammation of lamina propria and thinning of muscle layers had also been observed in the infected areas. Under the mucosa

is the submucosa containing the glands. The infection of *G. punjabensis* caused surface destruction of mucosal epithelium, infiltration of mast cells, plasma cells and damaged villi of intestine. It is also observed that inflammatory nodulation in the intestinal wall was accompanied with haemorrhagic blood vessels and increased number of goblet cells.

Molecular data (based on extraction and amplification of two *Gangesia* worms)

Blast analysis of 28S large subunit (LSU) rDNA sequence of the above sample (accession No. KY018599) demonstrated maximum similarity of 99% with *G. bengalensis* (JX477427, JX477428, JX477429, JX477438) infecting the intestine of *W. attu*, 98% with *G. vachai* (JX477432, JX477437) infecting the intestine of *W. attu* and *Mystus tengara*, 97% with *G. macrones* (JX477433, JX477446, JX477445, JX477444) infecting the intestine of *Sperata seenghala* from India (Table 1). The phylogenetic tree based on the final edited alignment

Table 1 Showing the transitional and transversion substitutions obtained by maximum likelihood estimate of substitution matrix in Mega 6.0 of *G. punjabensis*

	A	T	C	G
A	–	<i>3.61</i>	2.98	17.08
T	<i>2.01</i>	–	22.16	<i>4.7</i>
C	<i>2.01</i>	26.83	–	<i>4.7</i>
G	7.32	<i>3.61</i>	2.98	–

Rates of different transitional substitutions are shown in bold and those of transversion substitutions are shown in italics

(822 bp) with Neighbour-Joining showed the highest bootstrap value of 100 and placed *G. punjabensis* n. sp. with *G. bengalensis* (JX477427, JX477429, JX477438) and *G.*

macrones (JX477433) in the same clade showing them to be a species complex. The length of the branches of the above species in the phylogenetic tree supports that they represent a species complex and this clustering also confirmed their paraphyletic origin (Ash et al. 2012).

Discussion

The present species has been compared morphologically with *G. bengalensis* (Verma 1928) infecting *Wallago attu*, *G. agraensis* (Verma 1928) infecting *W. attu*, *G. macrones* (Woodland 1924) infecting *Sperata seenghala*, *G. vachai* (Gupta and Parmar 1988) infecting *Wallago attu* and

Table 2 Comparative description of *G. punjabensis*, with four other species of the genus from Indomalayan region

	Species					
	<i>G. bengalensis</i> Verma, 1928	* <i>G. bengalensis</i> After Ash et al., Verma, 1928 2012	* <i>G. agraensis</i> Verma, 1928	* <i>G. macrones</i> Woodland, 1924	* <i>G. vachai</i> (Gupta and Parmar, 1988)	<i>G. punjabensis</i> (present study)
Characteristic						
Parasite: Length	–	–	–	–	–	60–62 mm
Proglottid number	–	–	–	–	–	96
Scolex length	–	295–355	270–300	160–220	210–235	374–378
Scolex width	430	325–415	305–340	215–275	220–245	388–391
Rostellum width [R]	–	150–190	150–160	110–175	100	142–144
Hook: Number	51	47–54 (24–28 + 23–26)	28–32	80–85	250–450	47–54
Rostellar hooks length	37–43	36–38	24–27	10–13 + 5–7	2–5	38.2–40.2
Rostellar hooks blade length	–	26–33	12–16	8–10 + 2–4	1	23.9–26.1
Rostellar hooks rows	–	Double	Single	Double	4–6	Double
Sucker hooklet rows	–	5–8	2–3	9–12	5–11	5–8
Number of testes	200–more	141–197	142–170	90–140	40–55	84–100
Layers of testes	–	2–3 incomplete	2–3 incomplete	Single	Single	Single
Size of cirrus sac	–	190–245 × 75–105	175–225 × 90–110	255–290 × 75–85	135–155 × 50–65	35.4–37.2 × 78.8–80.1
Opening of vagina in relation to cirrus	–	–	–	–	–	Posterior
Relative size of cirrus sac (LCS/WS)	About 1/3	1/4–1/3	1/5–2/5	1/4	1/4	1/4
Ovarian lobe	–	–	–	–	–	188–191
Host	<i>W. attu</i>	<i>W. attu</i>	<i>W. attu</i>	<i>Sperata seenghala</i>	Catfish	<i>W. attu</i>
Location	Small Intestine	Small Intestine	Small Intestine	Small Intestine	Small Intestine	Small Intestine
Locality	India	West Bengal, India	Cambodia and India	Bangladesh and India	Bangladesh and India	Ropar wetland, Punjab, India

All measurement are in micrometer unless otherwise mentioned

* Data from Ash et al. (2012)

redescriptions by Ash et al. (2012). The present species *G. punjabensis* n. sp. was found to be different in morphology and morphometrics and was characterized in having larger scolex size $374\text{--}378 \times 388\text{--}391 \mu\text{m}$ (*G.b.* $295\text{--}355 \times 325\text{--}415 \mu\text{m}$, *G.a.* $270\text{--}300 \times 305\text{--}340 \mu\text{m}$, *G.m.* $160\text{--}220 \times 215\text{--}275 \mu\text{m}$, *G.v.* $210\text{--}235 \times 220\text{--}245 \mu\text{m}$) and variation in the number of hooks 47–54 (*G.b.* 47–54, *G.a.* 28–32, *G.m.* 80–85, *G.v.* 250–450), number of testes 84–100 (*G.b.* 141–197, *G.a.* 142–170, *G.m.* 90–140, *G.v.* 40–55), size of ovarian lobe $188\text{--}191 \mu\text{m}$ (*G.b.* $490\text{--}665 \mu\text{m}$, *G.a.* $490\text{--}515 \mu\text{m}$, *G.m.* $760\text{--}860 \mu\text{m}$, *G.v.* $405\text{--}510 \mu\text{m}$), 5–8 rows of fine hooklets along the upper margins of sucker (*G.b.* 5–8, *G.a.* 2–3, *G.m.* 9–12, *G.v.* 4–11). The differences in morphometrics have demonstrated that the present specimens were unique and therefore proposed as new to the science and named as *G. punjabensis* n. sp. The present specimens of the genus *Gangesia* were closest morphologically to *G. bengalensis* (Southwell 1913) whose type species was recorded from *Channa striata* and *Labeo rohita* in West Bengal (India). This species had been described and redescribed by many authors since then such as Verma (1928) and Ash et al.

(2012) collected from *W. attu*. As per the morphological and molecular studies Ash et al. (2012) regarded only four species viz *G. bengalensis*, *G. agraensis*, *G. macrones* and *G. vachai* as valid and synonymized many others. The synonyms were as following *Ophryocotyle bengalensis*, *G. wallago*, *G. lucknowia*, *G. sindensis*, *G. spinocirrosa*, *G. kashmirensis*, *G. mehamdabadensis*, *G. indica*, *G. fotedari*, *G. paithanensis*, *G. aurangabadensis*, *G. maharashtrii*, *G. dharurensis*, *G. cirrhinae*, *G. rohita*, *G. seenghali*, *G. rohita*, *G. ramkaei*, *G. bendsurensis*, *G. marathwadensis*, *G. jayakwadensis*. *Gangesia agraensis* Verma 1928 new synonyms and invalid names: *G. haryanae*, *G. sanehensis*, *G. shindei*, *G. wallagoi*, *G. clariusae*, *G. ambikaei*, *G. batrachusi*, *Silurotaenia nybelini*. *Gangesia macrones* Woodland 1924 synonyms *Silurotaenia paithanensis*, *S. barbasi*, *S. macroni*, *S. singhali*, *S. behairvathi*, *S. shastri*, *S. raoii*, *Gangesia mastacembali*. *Gangesia vachai* (Gupta and Parmar 1988) synonym *Silurotaenia vachai* (Table 2).

The successful amplification of 28S large subunit (LSU) rDNA sequence of *G. punjabensis* n. sp. using specific primer sets Ces28S Fd and Ces28S Rv resulted in sequence size of 822 bp. The BLAST analysis indicated maximum

Table 3 Homogeneity of 28S rRNA gene sequence of *G. punjabensis* (Accession number KY018599) and others *Gangesia* available in NCBI GenBank

Cestodes species	Accession number	Organ infected	Host	Country	Homogeneity (%) to <i>G. punjabensis</i> (KY018599)
<i>G. bengalensis</i>	JX477438	Intestine	<i>Wallago attu</i>	India	1467/1467 (99)
<i>G. bengalensis</i>	JX477429	Intestine	<i>Wallago attu</i>	India	1467/1467 (99)
<i>G. bengalensis</i>	JX477428	Intestine	<i>Wallago attu</i>	India	1467/1467 (99)
<i>G. bengalensis</i>	JX477427	Intestine	<i>Wallago attu</i>	India	1467/1467 (99)
<i>G. vachai</i>	JX477432	Intestine	<i>Wallago attu</i>	Bangladesh	1410/1410 (98)
<i>G. vachai</i>	JX477437	Intestine	<i>Mystus cf. tengara</i>	India	1408/1408 (98)
<i>G. macrones</i>	JX477433	Intestine	<i>Sperata seenghala</i>	Bangladesh	1389/1389 (97)
<i>G. macrones</i>	JX477446	Intestine	<i>Sperata seenghala</i>	India	1387/1387 (97)
<i>G. macrones</i>	JX477445	Intestine	<i>Sperata seenghala</i>	India	1387/1387 (97)
<i>G. macrones</i>	JX477444	Intestine	<i>Sperata seenghala</i>	India	1387/1387 (97)
<i>G. agraensis</i>	JX477436	Intestine	<i>Wallago attu</i>	India	1199/1199 (93)
<i>G. agraensis</i>	JX477435	Intestine	<i>Wallago attu</i>	India	1199/1199 (93)
<i>G. agraensis</i>	JX477430	Intestine	<i>Wallago attu</i>	India	1199/1199 (93)
<i>G. agraensis</i>	JX477443	Intestine	<i>Wallago attu</i>	India	1192/1192 (93)
<i>G. agraensis</i>	JX477440	Intestine	<i>Wallago attu</i>	India	1192/1192 (93)
<i>G. agraensis</i>	JX477439	Intestine	<i>Wallago attu</i>	India	1192/1192 (93)
<i>G. agraensis</i>	JX477454	Intestine	<i>Wallago attu</i>	Cambodia	1182/1182 (93)
<i>G. parasiluri</i>	AF286935	Intestine	<i>Silurus asotus</i>	Japan	955/955 (91)
<i>G. oligonchis</i>	JX477452	Intestine	<i>Tachysurus fulvidraco</i>	Russia	941/1078 (91)
<i>G. oligonchis</i>	JX477451	Intestine	<i>Tachysurus fulvidraco</i>	Russia	941/1078 (91)
<i>G. oligonchis</i>	JX477450	Intestine	<i>Tachysurus fulvidraco</i>	Russia	941/1078 (91)
<i>G. oligonchis</i>	JX477449	Intestine	<i>Tachysurus fulvidraco</i>	Russia	941/1078 (91)
<i>G. oligonchis</i>	JX477448	Intestine	<i>Tachysurus fulvidraco</i>	Russia	941/1078 (91)
<i>Silurotaenia siluri</i>	AJ388592	Intestine	<i>Silurus glanis</i>	Czech Republic	Outgroup

homogeneity of 99% with *G. bengalensis* (JX477427, JX477429, JX477438) infecting the small intestine of *Wallago attu* from India with 5 gaps, 98% with *G. vachai* with 12 gaps, 97% with *G. macrones* with 20 gaps and 93% with *G. agraensis* with 21 gaps (Table 3). The phylogenetic tree based on Neighbour-Joining showed the highest bootstrap value of 100 and placed *G. punjabensis* n. sp. with *G. bengalensis* in the same clade (Fig. 5). However, estimates of evolutionary pair-wise divergence was nil (0.00) (Fig. 6). The estimation of pattern of nucleotide substitution considering 25 sequences using HKY + G as the best substitution model with lowest Bayesian Information Criterion (BIC) 2555.662. A = 15.14%, T/U = 27.13%, C = 22.41%, G = 35.33% were the nucleotide frequencies estimated, [AC] = 2.98, [AG] = 17.08, [AT] = 3.61, [CG] = 4.7, [CT] = 26.83, [GT] = 3.61 were rate of nucleotide substitution; 0.00, 0.03, 0.21, 0.83, 3.94 were 5 substitutions. There were a total of 445 positions in the final dataset. The phylogenetic tree generated has demonstrated two main clades (Bootstrap value—66), one larger clade with *Gangesia* species having sequences from Indomalayan region i.e. India, Bangladesh and Cambodia. The second smaller clade

included *Gangesia* species in two well defined subclades (bootstrap value—99), one from Japan (*G. oligonchis*) and second from Russia (*G. parasiluris*). The larger main clade had two subclades the first having *G. bengalensis*, *G. macrones*, *G. vachai* and the present species, *G. punjabensis*, all sequences from India and Bangladesh (bootstrap—100). The second clade had *G. agraensis* sequences reported from Cambodia and India (bootstrap—99). This clearly indicate that *G. bengalensis*, *G. macrones*, *G. vachai*, *G. agraensis* and *G. punjabensis* n. sp. are within the same clade in the phylogenetic tree with high bootstrap support and confirmed their paraphyletic origin with significant genetic relatedness thereby forming a species complex.

It has been shown that the number of nominal species of proteocephalidean cestodes described as members of *Gangesia* and *Silurotaenia* from freshwater fishes in the Indomalayan region exceeds that of actually valid taxa, similarly, as in cestodes of the order Caryophyllidae instead of 48 species in two genera, only four species of *Gangesia* were valid on the basis of molecular study made by Ash et al. (2012). Therefore, the genetic relatedness has been clearly demonstrated in the present study among

Fig. 5 Phylogenetic tree generated by Neighbour Joining analysis showing the phylogenetic of *G. punjabensis* (KY018599) with others valid *Gangesia* taxa of the Indomalayan region along with Palearctic taxa available. GenBank accession number given and number above nodes indicates boot-strap confidence value. Scale bar amount of inferred evolutionary change along branch lengths

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. KY018599 <i>Gangesia punjabensis</i> Wallago attu India																									
2. JX477438 <i>Gangesia bengalensis</i> Wallago attu India	0.00																								
3. JX477429 <i>Gangesia bengalensis</i> Wallago attu India	0.00	0.00																							
4. JX477428 <i>Gangesia bengalensis</i> Wallago attu India	0.00	0.00	0.00																						
5. JX477427 <i>Gangesia bengalensis</i> Wallago attu India	0.00	0.00	0.00	0.00																					
6. JX477432 <i>Gangesia vachai</i> Wallago attu Bangladesh	0.01	0.00	0.00	0.00	0.00																				
7. JX477437 <i>Gangesia vachai</i> Mystus cf. tengara India	0.01	0.01	0.01	0.01	0.01	0.00																			
8. JX477433 <i>Gangesia macrones</i> Sperata seenghala Bangladesh	0.00	0.00	0.00	0.00	0.00	0.00	0.01																		
9. JX477446 <i>Gangesia macrones</i> Sperata seenghala India	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00																	
10. JX477445 <i>Gangesia macrones</i> Sperata seenghala India	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00																
11. JX477444 <i>Gangesia macrones</i> Sperata seenghala India	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00															
12. JX477436 <i>Gangesia agraensis</i> Wallago attu India	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04														
13. JX477435 <i>Gangesia agraensis</i> Wallago attu India	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.00													
14. JX477430 <i>Gangesia agraensis</i> Wallago attu India	0.04	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.04	0.04	0.04	0.00	0.00												
15. JX477443 <i>Gangesia agraensis</i> Wallago attu India	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.00	0.00	0.00											
16. JX477440 <i>Gangesia agraensis</i> Wallago attu India	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.00	0.00	0.00	0.00										
17. JX477439 <i>Gangesia agraensis</i> Wallago attu India	0.05	0.04	0.04	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.00	0.00	0.00	0.00	0.00									
18. JX477454 <i>Gangesia agraensis</i> Wallago attu Cambodia	0.04	0.04	0.04	0.04	0.04	0.05	0.05	0.04	0.04	0.04	0.04	0.01	0.01	0.01	0.01	0.01	0.01								
19. JX477452 <i>Gangesia oligonchis</i> Tachysurus fulvidraco Russia	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.04	0.05	0.04	0.00						
20. JX477451 <i>Gangesia oligonchis</i> Tachysurus fulvidraco Russia	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.05	0.04	0.00	0.00					
21. JX477450 <i>Gangesia oligonchis</i> Tachysurus fulvidraco Russia	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.05	0.04	0.00	0.00					
22. JX477449 <i>Gangesia oligonchis</i> Tachysurus fulvidraco Russia	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.05	0.04	0.00	0.00	0.00				
23. JX477448 <i>Gangesia oligonchis</i> Tachysurus fulvidraco Russia	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.04	0.04	0.04	0.04	0.05	0.04	0.00	0.00	0.00	0.00			
24. AF286935 <i>Gangesia parasituri</i> Silurus asotus Japan	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.05	0.05	0.05	0.05	0.04	0.04	0.04	0.04	0.04	0.04	0.04	0.01	0.01	0.02	0.01	0.01		
25. AJ388592 <i>Silurotaenia siluri</i> Silurus glanis Czech Republic	0.11	0.11	0.11	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.11	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.11	

Fig. 6 Estimate of evolutionary divergence between the sequences of *G. punjabensis* (KY018599) and others *Gangesia* available in NCBI GenBank

various other species of the genus *Gangesia* which means that there is formation of a species complex.

In histological sections the scolices of *G. punjabensis* sp. nov. worms were deeply embedded in the intestinal mucosa and villi encircled the scolices thereby disturbed the intestinal architecture. There was complete destruction of villi and mucosal epithelium of intestine at the point of attachment of the scolex. There was much compression of the epithelium near the embedded worms causing inflammation, vacuolation and damage. The infection of *Fasciola buski* causing surface desquamation of mucosal epithelium have been reported by Haque and Siddiqui (1978) and destruction of mucosal epithelium and villi of intestine was also reported by them. Our finding were similar to those of Haque and Siddiqui who observed surface desquamation and damaged lamina propria. In addition, there was increase in number of goblet cells especially in the areas with inflammatory nodules. Hiware (2008) reported that the intestinal layer (internal epithelium, submucosa, muscularis layer) can be crossed by majority of helminthes and reach up to serosa and therefore were dangerous and destructive parasites to the definitive host.

Conclusions

The morphological and molecular data supports clustering of a species complex with already established four species. The phylogenetic studies indicate clusters of *G. punjabensis* with other valid species of the same genus. Furthermore, the intraspecific variations at the morphological

and molecular level demonstrated the formation of species complexes and cryptic species which needs to explored using more molecular markers in future studies for support and validity.

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Compliance with ethical standards

Conflict of interest There is no conflict of interest to disclose.

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