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Morphological and molecular description of *Pallisentis roparensis* n. sp. (Acanthocephala: Quadrigyridae) infecting the freshwater cat fish *Wallago attu* from Ropar Wetland, Punjab, India

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ABSTRACT

The study describes a new species of *Pallisentis* Van Cleave, 1928 infecting the freshwater cat fish *Wallago attu* Bloch and Schneider, 1801 from Ropar wetland, Punjab, India. The morphological characters of *Pallisentis roparensis* include proboscis with 4 circles of 10 hooks each gradually declining in size, first circle of hooks <100 μm in length, 15–16 circles of Y-shaped collar spines and conical trunk spines present up to the posterior end in the females and the anterior region of cement gland in males. Saefftigen's pouch is present and cement gland nuclei are 22–25 in males. The sequences generated for 18S, 28S and ITS1-5.8S-ITS2 molecular markers of the newly described species are nested well among the other comparable sequences from the GenBank. The phylogenetic analyses show the monophyly of the genus *Pallisentis* but point towards the paraphyletic relationship among the three subgenera. The histopathology of fish intestine indicates that the parasite stimulates the inflammatory immune response causing serious injury to the mucosa and dilation of the lymphatic vessels of small intestine.

1. Introduction

The genus *Pallisentis* was created by Van Cleave (1928) with the description of *Pallisentis umbellatus* Van Cleave (1928) as a type species. The diagnostic morphological characteristics of the genus included number of proboscis hooks, number of rows of collar and trunk spines, distribution of trunk spines, position of testes and number of giant nuclei of cement glands. Unfortunately, with the addition of more species in the course of time these traits were observed to exhibit a lot of variability creating difficulty in the taxonomic evaluation. Amin et al. (2000) revised the genus adding some stable morphological parameters like 6–12 proboscis hooks arranged in 4 circles each, two sets of trunk spines separated by a spineless region, single walled proboscis receptacle, syncytial cement gland as the distinctive features of the genus. The studies on Acanthocephala from India prominently show the vast diversity of species from the genus *Pallisentis*. According to the updated key of Gautam et al. (2019) out of the 33 species of the genus *Pallisentis* 28 species have been reported from India and the studies have been mainly confined to the fresh water fishes belonging to families Channidae (15), Nandidae (3), Siluridae (1), Cobitidae (1), Bagridae (1), Cyprinidae (1), Heteropneustidae (1) and Osphronemidae (1). A handful of species have been reported from fish families inhabiting brackish water such as Gobiidae (1), Clupeidae (1), Ailiidae (1) and a marine fish family Caragidae (1). The present host, *Wallago attu* vern. mullee occur widely in the freshwaters of Asian continent and is popular among the edible fishes for its high nutritional value. The population of *W. attu* is rapidly declining in the Indian region due to its over harvesting and lack of proper management (Gupta, 2015). This freshwater catfish has been reported to be infected with various intestinal parasites including Cestodes, Nemerteans, Platyhelminthes, Nematodes and Acanthocephalans which harm the overall health of the fish (Gupta and Narain, 2012; Jasrotia and Kaur, 2017).

The description of most of the Acanthocephalan species is lacking in complete morphological characterization which is based on few specimens and in addition the material cannot be referred due to the unavailability of the type specimens (Amin et al., 2021). The species like *P. channai* Gupta et al. (2015), *P. vinodai* Gupta et al. (2015) and *P. anandai* Gautam et al. (2017) were erected with incomplete morphological and molecular characterization and therefore are more likely to be questioned. Some of the already established Indian species including *P. basiri* Farooqi (1958), *P. guntei* Sahay et al. (1967), *P. clupei* Gupta and Gupta (1980), *P. cavasii*, Gupta and Verma (1980), *P. fasciati*

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Gupta and Verma (1980), *P. guptai* Gupta and Fatma (1986), *P. mehrai* Gupta and Fatma (1986) and *P. jagani* Koul et al. (1991) are sufficiently described morphologically however there is much need to supplement them with molecular data for the cladistic positioning of these species in the phylogenetic tree. So far, molecular data of very few species is available in the database in comparison to the larger number of described species with incomplete and confusing morphological details. Some of the species described by Gautam et al. (2019), Chaudhary et al. (2019), Gautam et al. (2020) and Amin et al. (2021) have been supplemented with the molecular identity based on 18S molecular marker which is helpful to study the genetic divergence among the species in the same geographic location. Earlier to our study, only one species *P. allahabadi* Agarwal (1958) has been isolated from the *W. attu* from India. The present study provides the morphological description with the molecular characterization of a new species of the genus *Pallisentis* from the freshwater cat fish, *W. attu* and histopathological alterations of the intestinal tissue of the infected host fish.

2. Material and methods

A total of 41 freshwater fishes which included fishes from the families Cyprinidae (*Labeo rohita* and *Catla catla*), Channidae (*Channa striata* and *Channa punctatus*) and Siluridae (*Wallago attu*) were procured from the local fish market near the Ropar Wetland, Punjab, India (31.0200° N, 76.5000° E) during the summer season of 2019 and were examined for the presence of acanthocephalan parasites. Out of 14 *Wallago attu* examined, 3 specimens were infected with the presently studied acanthocephalan species indicating prevalence of infection 21.4%. 8–12 worms per fish were counted which included 2–4 males and 6–8 females. The worms were washed in normal saline (0.85%) and kept in distilled water for 1–3 h to evert the proboscis. The specimens were then fixed in 70% alcohol and were later stained in Gower's carmine stain (Gower, 1939) after dehydration in ascending grades of alcohol and mounted in DPX. Line drawings were made with the aid of projection microscope and camera lucida inclined to the microscope. Measurements were taken in the software LAS V4.1 using the microscope Leica DM3000 (Leica Microsystems, CMS GmbH, Wetzlar, Germany). Morphological identification was done considering the classification of the Acanthocephala by Amin (2013) and key to species of *Pallisentis* provided by Amin et al. (2000) and further updated by Gautam et al. (2019). For histopathology, the normal and infected portions of guts were washed properly in distilled water and were fixed in Bouin's fixative. The tissue was washed after 12 h of fixation and dehydrated in ascending series of ethanol and embedded in paraffin wax. The 5 μm thin sections were stained in hematoxylin and eosin and mounted in DPX for observations. Genomic DNA of parasite preserved in 100% alcohol was isolated using Qiagen's DNeasy tissue kit. 18S, 28S and ITS1-5.8S-ITS 2 regions were amplified using PCR and qualitative and quantitative analyses were performed using nanodrop and gel electrophoresis. Primers for the amplification of 18S rRNA, 28S rRNA and ITS1-5.8S-ITS2 gene sequences were referred from García-Varela et al. (2013), García-Varela and Nadler (2005) and Rana and Kaur (2021) respectively. 25 μl of PCR reaction mixture consisted of 2.5 μl 10X PCR buffer, 2 mM $MgCl_2$, 2 μl DNA template, 10 mM each primer and 1U Taq polymerase. Amplified products were purified and sequenced by chain termination method (Sanger et al., 1977). The contig was generated from the multiple sequences for each molecular marker manually and in BioEdit. The sequences for each molecular marker were submitted in NCBI data base and accession numbers were obtained. Almost all the sequences of each molecular marker of the genus *Pallisentis* and other comparable sequences were downloaded from the GenBank for the phylogenetic analyses. Multiple sequence alignment of the data set was done using CLUSTAL W in MEGA X (Kumar et al., 2018). All the positions with less than 95% site coverage in the data set were eliminated. Maximum likelihood phylogenetic trees for each molecular marker were constructed with 1000 bootstrap replicates applying the best fit model mentioned in results respectively, using MEGA X (Kumar et al., 2018). *Mediorhynchus* sp. was chosen as the outgroup taxa while regenerating the phylogenetic tress for all the molecular markers. Genetic distance between the species and substitution patterns were also estimated to analyze the evolutionary changes using the suitable model in MEGA X (Kumar et al., 2018). Tajima's neutrality test (Tajima, 1989) was performed using the dataset to detect the evolutionary selection pressure within the population.

3. Results

Pallisentis roparensis.

3.1. Taxonomic position

Class: Eoacanthocephala Van Cleave, 1948.
Order: Gyracanthocephala Van Cleave, 1936.
Family: Quadrigyridae Van Cleave, 1920.
Subfamily: Pallisentinae Amin, 1985.
Genus: *Pallisentis* Van Cleave, 1928.
Subgenus: *Pallisentis* Van Cleave, 1928.
Species: *roparensis*;

Host and Locality: *Wallago attu* Bloch and Schneider, 1801 from Ropar wetland, Punjab, India (31.0200° N, 76.5000° E);

Site of infection: Small intestine.

Type specimen: Voucher specimens of male and female stained in Gower's carmine deposited in the Museum of the Department of Zoology, Panjab University, Chandigarh, India (A/GC/21.12.2020/2.1 and A/GC/21.12.2020/2.2).

Etymology: specific name "*roparensis*" is derived from the site of sample collection.

Specimens examined: 10 males and 7 females.

3.2. Morphological description (*Fig. 1.*)

Proboscis hooks in 4 circles, 10 hooks per circle, gradually declining in size, hook roots directed posteriorly. Apical organ Y-shaped, with two centrally placed giant nuclei. Both lemnisci tubular, dorsal longer than the ventral. Neck short, unarmed. Trunk divided into two regions, anterior with 15–16 circles of Y-shaped collar spines having comb like base and posterior region with conical trunk spines with dense base.

Male (based on 10 sexually matured specimens): Total length ranges 5.5–9 (7.25) mm, maximum width 304–427 (365.5) μm at proximal region of trunk. Proboscis longer than wider 200–220 (210) × 160–193 (176.5) μm. 4 circles of proboscis hooks, 10 hooks each, H1 79–84 (81.5) μm, H2 63–67 (65) μm, H3 42–45 (43.5) μm, H4 30–37 (33.5) μm. Hook roots shorter than blades HR1 45–53 (49) μm, HR2 40–49 (44.5) μm, HR3 23–29 (26) μm, HR4 20–25 (22.5) μm. Circular muscle band at posterior end of proboscis. Neck unarmed, 200–210 (205) × 160–180 (170) μm. Proboscis receptacle 510–620 (560) × 150–155 (152.5) μm, dorsal lemniscus 1190–1309 (1249.5) × 37–39 (38) μm, ventral lemniscus 930–1285 (1107.5) × 35–38 (36.5) μm. Collar spines 15–16 rows, 22–24 (23) μm in length, spans 413–416 (414.5) μm of body length. Trunk spines conical, 32–35 rows, equally spaced, 16–18 spines in each row, 24–27 (25.5) μm in length, last row ending at anterior region of cement gland, number of spines in posterior circles irregular, 2–4 spines per circle towards the testicular region. Testes two, elongated, tandem, situated in the posterior half of the body. Anterior testis 450-500 (475) × 100–140 (120) μm, slightly longer than posterior testis 430-450 (440) × 130–160 (145) μm. Cement gland elongated 880–890 (885) × 60–70 (65) μm, syncytial with 22–25 (23) nuclei. Cement reservoir, stout, 330–350 (340) × 80–90 (85) μm branching posteriorly into two ducts. Seminal vesicle located posterodorsal to cement reservoir, 400–530 (465) × 0.90–100 (95) μm, tapers to form vas efferens anteriorly and vas deferens posteriorly. Saefftigen's pouch 670–720 (695) × 90–100 (95) μm, ventral in position, parallel to the seminal vesicle, posterior to cement reservoir tapering towards posterior margin

of body. Vas deferens, ducts of saefftigen's pouch and cement reservoir enter bursa. Bursa bell-shaped, 83–87 (85) μm.

Female (based on 7 sexually matured specimens): Total length 7–10.5 (8.75) mm, slightly longer than male, maximum width 380–552 (466) μm at anterior region of trunk. Proboscis squarish, 195–208 (201.5) × 196–209 (202) μm, 4 circles of 10 hooks each, H1 97–98 (97.5) μm, H2 73–76 (74.5) μm, H3 54–58 (56) μm, H4 32–36 (34) μm. HR1 66–75 (70.5) μm, HR2 44–52 (48) μm, HR3 23–30 (26.5) μm, HR4 17–23 (20) μm. Proboscis receptacle 398–521 (459.5) × 171–185 (178) μm. Neck unarmed 200–220 (210) × 160–170 (165) μm. Dorsal lemniscus 1730–1746 (1738) × 37–45 (41) μm, ventral lemniscus 1580–1598 (1589) × 36–44 (40) μm. Collar spines 15–16 rows, 23–30 (26.5) μm in length, spans 451–474 (462.5) μm of body length. Trunk spines conical, 66–73 rows, 16–18 in each row, 15–23 (19) μm in length, spines in posterior rows irregular, 2–4 spines in a row towards the posterior end. Female reproductive system 320–370 (345) μm, uterine bell well developed 80–90 (85) μm with an anterior muscular sphincter, leading into heavily muscular uterus 170–200 (185) μm, vagina 70–80 (75) μm opens into terminal gonopore. Egg 20–30 (25) × 10 μm in size with double membrane, polar elongations of fertilization membrane are absent.

3.2.1. Remarks

Amin et al. (2000) revised the genus *Pallisentis* and provided key to 26 defined species of the genus and later 4 more species were added by Gautam et al. (2019). The new species described in the present study falls under the genus *Pallisentis* due to the presence of two separate regions of trunk spines. Size of the proboscis hooks is observed declining gradually from anterior to the posterior rows and is therefore placed in the subgenus *Pallisentis*.

The species described in the present study shows closeness with *P. nagpurensis* (Bhalerao, 1931) Baylis (1933) and *P. clupei* Gupta and Gupta (1980) due to the presence of conical trunk spines throughout the length of female while till the anterior of cement gland in male and post equatorial location of testes. The number of hooks on the proboscis arranged in 4 circles, 8–10 per circle in *P. nagpurensis,* with single giant nuclei in the apical organ in contrast to 10 hooks per circle and with two giant nuclei in the apical organ in the present species. In *P. nagpurensis* number of cement gland nuclei is 20–30 with no saefftigen's pouch and sub-terminal gonopore in contrast to the 22–25 cement gland nuclei, presence of saefftigen's pouch and terminal gonopore in the present species. Both the species further differ from each other in the number of rows of trunk spines in females which are 66–73 in the present species in contrast 55–65 in the case of *P. nagpurensis*. Furthermore, the average size of testes of *P. nagpurensis* (anterior testis: 1125 μm, posterior testis: 995 μm) is twice the size of the testes of the present species (anterior testis: 475 μm, posterior testis: 440 μm). In addition to above differences the total body length in both male and female is longer in *P. nagpurensis* in comparison to the present species (male: *P.n* 14.5 vs. *P.r* 7.25; female *P.n* 18 vs. *P.r* 8.75) (Table 1). Further it is added that *P. nagpurensis* has been reported to infect the fishes of the family Channidae while the species under study has been isolated from the host fish belonging to family Siluridae.

The present species differ from *P. clupei* in having 10 hooks per circle instead of 8 hooks per circle. In *P. clupei* rows of collar spines are 12–13 in males and 13–14 in females while in the new species 15–16 rows of Y shaped spines in both sexes are present. The number of cement gland nuclei in *P. clupei* is 9–16 in contrast to 22–25 in the new species although the gonopore is terminal in both the species (Table 1).

3.2.2. Updated key of Gautam et al. (2019) to the species of the genus *Pallisentis*

1. Proboscis hooks in second or third circle declining abruptly in size; cement gland usually small, with few giant

Table 1
Morphometric comparison among the *Pallisentis roparensis* n. sp. and other closely related species of the genus.

Characters	*Pallisentis roparensis* n. sp.(present study)	*Pallisentis nagpurensis* (Bhalerao, 1931) Baylis (1933)	*Pallisentis nagpurensis* (Bhalerao, 1931) Baylis (1933) [a](Rana and Kaur, 2021)	*Pallisentis (P.) clupei* Gupta and Gupta (1980)
Hosts	*Wallago attu*	*Channa striata*	*Channa striata*	*Clupea longiceps*
Locality	Punjab, India	Uttar Pradesh, India	Himachal Pradesh, India	Kerela, India
Male's length (mm)	5.5–9 (7.25)	2.4–19 (10.7)	9-20 (14.5)	8.27–8.64 (8.45)
Proboscis L x W (μm)	200-220 (210) × 160–193 (176.5)	300 × 350 (325)	200-280 (240) × 200–260 (230)	150-210 (180) × 260–310 (285)
Rows of proboscis hooks	4	–	4	4
Number of proboscis hooks in each row	10	8–10	8–10	8
Hooks from ant. To post. (μm)	H1 79–84 (81.5) H2 63–67 (65) H3 42–45 (43.5) H4 30–37 (33.5)	H1-76 H4-30	H1 90–100 (95) H2 75–80 (77.5) H3 60–70 (65) H4 30–40 (35)	H1 110–150 (130) H2 70–90 (80) H3 55–70 (62.5) H4 30–40 (35)
Neck L × W (μm)	200-210 (205) × 160–180 (170)	–	340-440 (390) × 200–240 (220)	–
Rows of collar spines	15–16	12–14	14	12–13
Lemnisci L × W (μm)	Tubular L1 1190–1309 (1249.5) × 37–39 (38) L2 930–1285 (1107.5) × 35–38 (36.5)	–	Coiled	L1 360–950 (655) × 70–80 (75) L2 1250–2070 (1660) × 70–80 (75)
Rows of trunk spines	32–35	30–63	25–35	28–30
Ant. Testis L × W (μm)	450-500 (475) × 100–140 (120)	630-1820 (1225) × 160–370 (265)	1640-1700 (1670) × 380–400 (390)	610-740 (675) × 180–200 (190)
Post. Testis L × W (μm)	430-450 (440) × 130–160 (145)	490-1280 (885) × 170–360 (265)	1400-1500 (1450) × 410–420 (415)	710-750 (730) × 180–190 (185)
Cement gland L × W (μm)	880-890 (885) × 60–70 (65)	1150-1180 (1165)	2520-2600 (2560) × 300–320 (310)	1390-1500 (1445) × 160–200 (180)
Cement gland nuclei	22–25	20–30	20–30	9–16
Cement reservoir L × W (μm)	330-350 (340) × 80–90 (85)	–	660-700 (650) × 200–290 (245)	460-500 (480) × 170–180 (175)
Saefftigen's pouch	Present	–	Absent	Present
Female's length (mm)	7–10.5 (8.75)	– –	7-29 (18)	11.30 –

(continued on next page)

Table 1 (*continued*)

Characters	*Pallisentis roparensis* n. sp.(present study)	*Pallisentis nagpurensis* (Bhalerao, 1931) Baylis (1933)	*Pallisentis nagpurensis* (Bhalerao, 1931) Baylis (1933) [a](Rana and Kaur, 2021)	*Pallisentis (P.) clupei* Gupta and Gupta (1980)
Proboscis L × W (μm)	195-208 (201.5) × 196–209 (202)		200-230 (215) × 240–260 (250)	
Hook from anterior (μm)	H1 97–98 (97.5) H2 73–76 (74.5) H3 54–58 (56) H4 32–36 (34)	–	H1 80–90 (85) H2 60–75 (67.5) H3 50–60 (55) H4 30–40 (35)	–
Neck L × W (μm)	200-220 (210) × 160–170 (165)	–	310-400 (355) × 220–300 (260)	–
Rows of collar spines	15–16	–	14–15	
Lemnisci L × W (μm)	Tubular 1730–1746 (1738) × 37–45 (41) 1580–1598 (1589) × 36–44 (40)	–	Coiled	–
Rows of trunk spines	66–73	–	55–65	61
Vagina L (μm)	70-80 (75)	–	70-80 (75)	–
Uterus L (μm)	170-200 (185)	–	300-320 (310)	–
Uterine bell L (μm)	80-90 (85)	–	90-100 (95)	–
Reproductive system L (μm)	320-370 (345)	–	530- 570 (550)	–
Egg L × W (μm)	20-30 (25) × 10	112 × 70	70-100 (85) × 10–30 (20)	–

[a] Redescribed by Rana and Kaur, 2021.

nuclei————————————————————————————2

Proboscis hooks gradually declining in size posteriorly; cement glands usually long, with many giant nuclei ————————————————————————————-Subgenus *Pallisentis* 12.

2. Proboscis hooks in second circle about half as long as hooks in first circle————Subgenus *Demiduetrospinus* 3

Proboscis hooks in third circle about half as long as hooks in second circle ————Subgenus *Brevitritospinus* 5.

3. Trunk spines conical and extending to posterior end of males and females; Saefftigen's pouch absent————————————————————*Pallisentis* (*D.*) *Ophiocephali* (Thapar, 1930) Baylis (1933).

Trunk spines Y-shaped not extending to posterior end of males; Saefftigen's pouch present————————-4.

4. Proboscis hooks in first circle 70–80 long; hook roots recurved, simple; leminci equal; testes equatorial, 580–620 (anterior) and 510–560 (posterior) long cement gland 470–630 long; Saefftigen's pouch 320–390 long; female gonopore terminal————————————————————*Pallisentis* (*D.*) *panadei* Rai (1967)

Proboscis hooks in first circle 100 long; hooks roots stubby knobs; lemnisci unequal; testes pre-equatorial, 950 (anterior) and 700 (posterior) long; cement gland 900 long; Saefftigen's pouch 770 long; female gonopore sub-terminal————————————————————*Pallisentis* (*D.*) *basiri* Farooqi (1958).

5. Trunk spines conical ————————————————————6

Trunk spines Y shaped ————————————————————-9.

6. Trunk spines in many circles, 57–88 in males and 120–149 in females; Saefftigen's pouch absent ————————————————————*Pallisentis* (*B.*) *vietnamensis* Amin et al. (2000)

Trunk spines in fewer circles, up to 27 in males and 36 in females; Saefftigen's pouch present ————————7.

7. Trunk small up to 2.0 mm long in males and 4.5 mm long in females; proboscis hooks in anterior 2 circles similar in size; trunk with 14–18 circles of spines each with 17–24 spines; cement gland less than 200 long-————————————————————————————-*Pallisentis* (*B.*) *guntei* Sahay et al. (1967)

Trunk small up near about 2.0 mm long in males and near about 4.5 mm long in females; proboscis hooks in anterior 2 circles similar in size; trunk with 15–16 circles of spines each with 8–15 spines; cement gland less than 300 long————————————————————*Pallisentis* (*B.*) *jagani* Koul et al. (1991).

Trunk larger, 3.4–6.9 mm long in males and 7.3–15.6 mm long in females; proboscis hooks in second circle slightly smaller that hooks in first circles; trunk with 20–27 circles of spines each with up to 12 spines; cement gland 400–973 long————————————————————8.

8. Female gonopore terminal; length of testes 733–910 (anterior), 785–925 (posterior); cement gland 863–973, and cement reservoir 580–816————————————————-*Pallisentis* (*B.*) *croftoni* Mital and Lal, 1981

Female gonopore terminal; length of testes 1400–1750 (anterior), 1050–1350 (posterior); cement gland not mentioned, and cement reservoir 600–950————————*Pallisentis* (*B.*) *fotedari* Gupta and Sinha (1991).

Female gonopore terminal; length of testes 492–387 (anterior), 352–434 (posterior); cement gland 434–611, and cement reservoir not mentioned————————————————*Pallisentis* (*B.*) *punctatii* Gupta et al. (2015).

Female gonopore sub-terminal; length of testes 475 (anterior), 437 (posterior), cement gland 400, and cement reservoir 361————————————————————*Pallisentis* (*B.*) *allahabadi* Agarwal (1958).

Female gonopore latero-terminal; length of testes 200–590 (anterior), 200–480 (posterior), cement gland 320–590, and cement reservoir 330–640————————————*Pallisentis* (*B.*) *lucknowensis* Gautam et al. (2019).

9. Trunk spines extending to posterior end of males and females; proboscis hooks 10–12 per circles; hooks in anterior circles larger than

100———*Pallisentis* (*B.*) *mehrai* Gupta and Fatma (1986)

Trunk spines not extending to posterior end of males or females; proboscis hooks 6–10 per circles shorter than 100 ———10.

Female less than 4.0 mm long; lemensci ending well above anterior testis, testis small, up to 225(anterior) long; cement gland small, 200–230 long, with 6–8 giant nuclei———*Pallisentis* (*B.*) *cavasii* Gupta and Verma (1980).

10. Female longer than 4.0 mm long; lemnisci may reach anterior testis; testes between 200 and 910 long; cement glands between 172 and 926 long, with 10–18 giant nuclei each———11
11. Proboscis hooks 10 per circles; female proboscis receptacle more than 700 mm long; lemnsci ending well above anterior testis———*Pallisentis* (*B.*) *indica* Mital and Lal (1976)

Proboscis hooks 6–10 per circle; female proboscis receptacle less than 400 long; lemnisci extending to mid-anterior testis———*Pallisentis* (*B.*) *fasciati* Gupta and Verma (1980).

12. Trunk spines conical or Y-shaped, extending to posterior end of at least 1 sex———13

Trunk spines only conical, not extending to posterior end of either sex ———16.

13. Trunk spines conical, extending to posterior end in female only; testes post-equatorial ———14

Trunk spines conical or Y-shaped, extending to posterior end of both males and females; testes equatorial———15.

14. Proboscis hooks in first circles less than 100 long; proboscis receptacle less than 500 long; cement gland with 20–30 giant nuclei; female gonopore sub-terminal ———*Pallisentis* (*P.*) *nagpurensis* (Bhalerao, 1931) Baylis (1933).

Proboscis hooks in first circles 100 or more long; proboscis receptacle more than 800 long; cement gland with 9–16 giant nuclei; female gonopore terminal ———*Pallisentis* (*P.*) *clupei* Gupta and Gupta (1980).

Proboscis hooks in first circles less than 100 long; proboscis receptacle 398–620 long; cement gland 22–25 (mostly 23) nuclei; female gonopore terminal ———*Pallisentis* (*P.*) *roparensis* n. sp.

15. Trunk spines conical, in 28–32 circles in males and 36–76 circles in females; neck separated from proboscis by transverse circular muscle band; cement gland longer than 1.6 mm———*Pallisentis* (*P.*) *garuai* (Sahay et al., 1971) Jain and Gupta (1979).

Trunk spines Y-shaped, in 16–20 circles in males and 25–30 circles in females; no muscles band between neck and proboscis; cement gland less than 0.6 mm long, 10–12 nuclei———*Palllisentis* (*P.*) *guptai* Gupta and Fatma (1986).

Trunk spines Y-shaped, in 28–33 circles in males and 24–42 circles in females; muscles band between neck and proboscis; cement gland less than 0.6 mm long, 7–8 nuclei———*Palllisentis* (*P.*) *unnaoensis* Gautam et al. (2019).

16. Males with Saefftigen's pouch ——— ———17

Males lacking Saefftigen's pouch——— ———22.

17. Trunk spines appearing continuous with collar spines———*Pallisentis* (*P.*) *magnum* Saeed and Bilqees (1971)

Trunk spines well separated from collar spin———18.

18. Proboscis hooks 10 per circles, each embedded in thickened cuticular rim; trunk spines with cuticular comb like thickening; males with additional circles of post-testicular trunk spines; testes pre-equatorial———*Pallisentis* (*P.*) *kalariai* Khan and Bilqees (1985)

Proboscis hooks eight per circles, each embedded in thickened cuticular rim; trunk spines with no cuticular comb like thickening; males with no post-testicular trunk spines; testes pre-equatorial———*Pallisentis* (*P.*) *meyeri* Gautam et al. (2019).

Proboscis hooks 8–10 per circle; no cuticular thickening at base of proboscis hooks or trunk spines; no post-testicular trunk spines; testes not pre-equatorial——— ———19.

19. Female trunk spines in 36–73 circles each with 8–14, extending to just anterior to posterior end; lemnisci unequal; testes small, less than 0.5 mm long ———*Pallisentis* (*P.*) *gomtii* Gupta and Verma (1980)

Female trunk spines in 35–75 each with 16–22 circles, not extending to posterior end; lemnisci equal; testes small, less than 0.5 mm (270–480 anterior, 260–440 posterior)———*Pallisentis* (*P.*) *amini* Gautam et al. (2019).

Female trunk spines in 10–20 circles, extending only to anterior third of trunk; lemnisci unequal; testes small, less than 1.0 mm long ———20.

Female trunk spines in 10–20 circles, extending only to anterior third of trunk; lemnisci equal; testes large, more than 1.0 mm long ———21.

20. Proboscis hooks 8 per circle; female trunk spines in 11 circles, 24–30 in each circles testes post-equatorial; cement gland 208 long with 18 nuclei———*Pallisentis* (*P.*) *chongqingensis* Daoyuan and Zikun (1993)
21. Proboscis hooks 8 per circle; testes equatorial; cement gland 2.2–3.0 mm long———*Pallisentis* (*P.*) *sindensis* Khan and Bilqees (1987)

Proboscis hooks 10 per circle; testes post equatorial; cement gland short, 0.7–1.6 long———*Pallisentis* (*P.*) *gaboes* 24, Van Cleave (1928).

22. Proboscis hooks 6–7 per circle ——— ———23

Proboscis hooks 8–12 per circle ——— ———24.

23. Proboscis hook 6 per circle; anterior hooks 89–119 long; cement gland with 16 giant nuclei———

———————————————————————————*Pallisentis* (*P.*) *umbellatus* Van Cleave (1928)

Proboscis hook seven per circle; anterior hooks 60–70 long; cement gland with 10–12 nuclei———————————————————————————*Pallisentis* (*P.*) *pesteri* (Tadros, 1966) Chowhan et al. (1987).

24 Cement glands with 12–14 giant nuclei; lemnisci equal———————————————————25

Cement glands with 23–25 giant nuclei; lemnisci unequal———————————————————26.

25. Proboscis hooks 8 per circle; collar spines in 6–7 circles in 8–13 circles each with 29–40 spines; trunk spines in 8–13 circles each with 30–41 spines with sclerotized, large, variably shaped beds; testes longer than 0.7 mm————————*Pallisentis* (*P.*) *celatus* Van Cleave (1928)

Proboscis hooks 10–12 per circle; collar spines in 15–17 circles each with 18–20 spines; trunk spines in 21–22 (males), 67 (females) circles each with 16–20 simple triangular spines; testes 0.28 mm–0.42 long-———————————————————————————————————————*Pallisentis* (*P.*) *colisai* Sarkar (1954).

26. Proboscis hooks 93, 80, 60, 33 long (from anterior); trunk spines in 44–55 circles, each with 16–20 spines; female gonopore posterior-ventral———————————————————*Pallisentis* (*P.*) *nandai* Sarkar (1953)

Proboscis hooks 62–64, 49–54,36–46, 24–28 long (from anterior); trunk spines in 25–26 circles, each with 10 spines; female gonopore terminal———————————————*Pallisentis* (*P.*) *singaporensis* Khan and Ip (1988).

3.3. Molecular characterization

The sequences generated for 18S rRNA, 28S rRNA, ITS1-5.8S-ITS2 gene markers were submitted to the NCBI database. The amplicon size is 1733 base pairs for small subunit ribosomal RNA gene (18S), 1528 base pairs for large subunit ribosomal RNA gene (28S) and 1171 base pairs for internal transcribed spacer 1-5.8S ribosomal RNA gene-internal transcribed spacer 2 and have been assigned MW421631, MW421634 and MW421633 accession numbers respectively. The other comparable sequences for the reconstruction of phylogenetic tree have been obtained from the GenBank.

The maximum likelihood tree obtained for 18S rRNA gene marker included almost all the *Pallisentis* sequences from the database at least with the generic identity (Fig. 2.). The analysis involved 28 nucleotide sequences with total 499 positions in the final data set and all the positions containing gaps and missing data were eliminated. The phylogenetic tree was reconstructed using maximum likelihood method with highest log likelihood value −1939.4977 based on Kimura 2-parameter model (Kimura, 1980). The rate of various transitional substitutions is observed to be 12.50 while the rate of various transversionsal substitutions is 6.25; the value of estimate of the transition/transversion bias (R) is 1. D value for the Tajima's neutrality test (Tajima, 1989) is below the state of equilibrium speculating a selective sweep or population expansion. The phylogenetic tree initially bifurcated into two clades separating the isolates of the genus *Pallisentis* from the *Acanthosentis*. The clade including all the sequences of *Pallisentis* bifurcated in

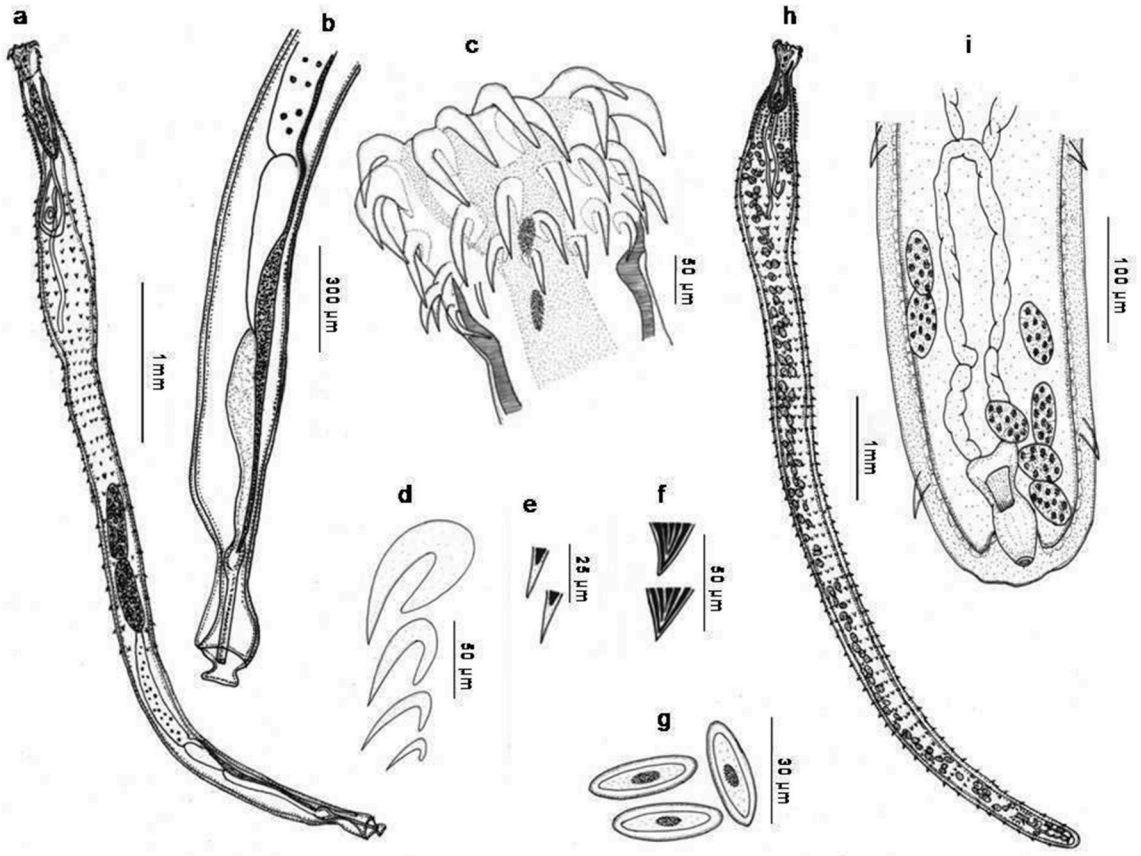

Fig. 1. Line drawings of specimens of *Pallisentis roparensis* from *Wallago attu*. a-male; b-posterior end of the male; c-proboscis (female); d-hooks of the proboscis declining gradually in the size; e−conical trunk spines; f- Y-shaped collar spines; g-mature egg; h-female; i-posterior end of the female.

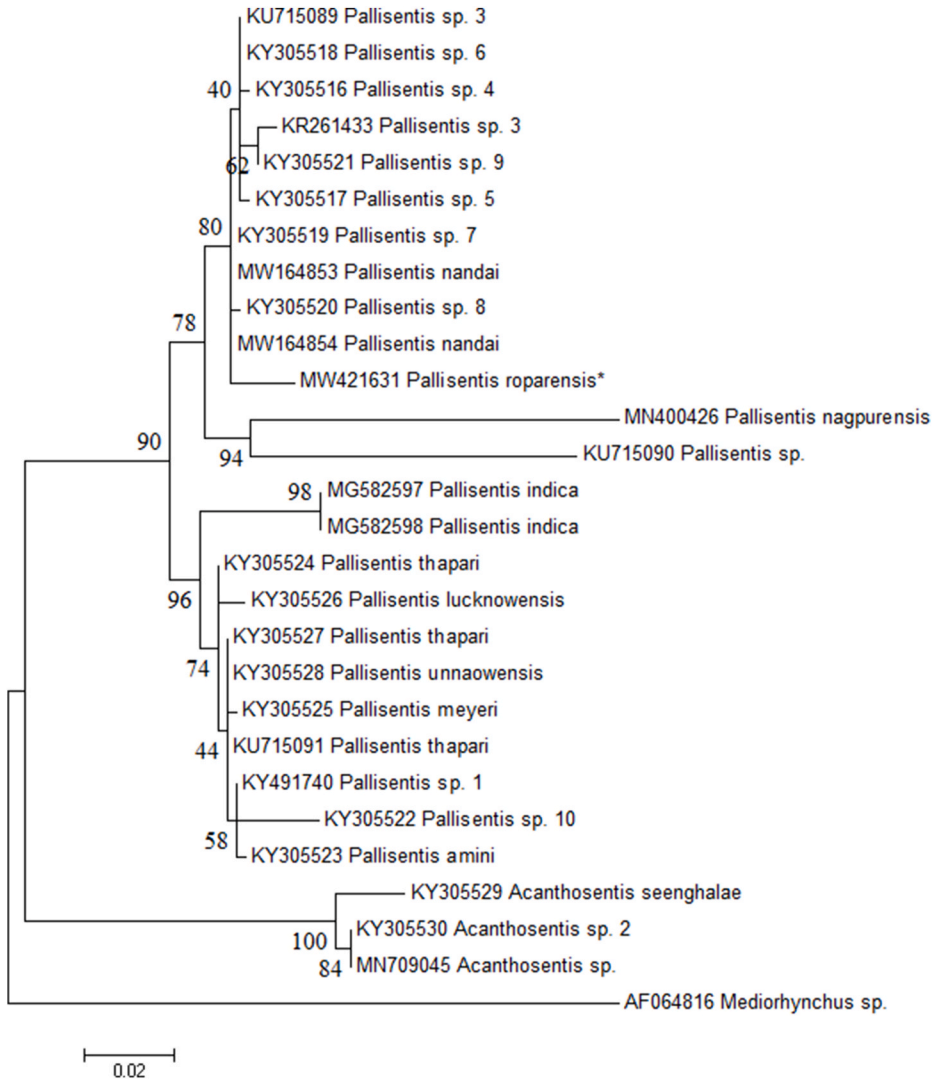

Fig. 2. Maximum likelihood tree generated using 18S rRNA gene sequence of *Pallisentis roparensis* and the sequences of related taxa downloaded from GenBank. Numbers near internal nodes show ML bootstrap clade frequencies.

two subclades with the maximum bootstrap score. The subclade 1 included the sequences of the new species generated in present study (MW421631) along with the isolates of *P. nandai* (MW164853 and MW164854), *P. nagpurensis* (MN400426) and other unidentified *Pallisentis* sequences reported from India. The genetic distance between *P. roparensis* (MW421631) and *P. nandai* (MW164853 and MW164854) is 0.014 and between *P. roparensis* (MW421631) and *P. nagpurensis* (MN400426) 0.106. The subclade 2 included few of the recently characterized species of the genus.

The maximum likelihood phylogenetic tree regenerated for 28S using Kimura 2-parameter model (Kimura, 1980) which included 8 nucleotide sequences with 1204 positions in the final dataset (Fig. 3.) having the highest log likelihood value −3708.992. The substitution pattern showed rate of transitions and transversions as 13.92 and 5.54

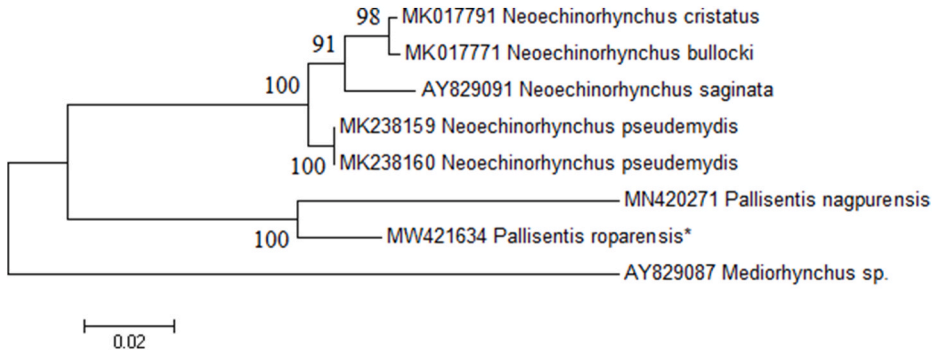

Fig. 3. Maximum likelihood tree generated using 28S rRNA gene sequence of *Pallisentis roparensis* and the sequences of related taxa downloaded from GenBank. Numbers near internal nodes show ML bootstrap clade frequencies.

respectively with the R value of 1.26. The value obtained from the Tajima's neutrality test (Tajima, 1989) using 28S dataset is also observed to be below the equilibrium. The phylogenetic tree showed clustering of available sequences of *P. nagpurensis* (MN420271) and sequence generated in this study (MW421634) placed distinctly from the other sequences of order Neoechinorhynchida. The genetic distance between *P. roparensis* (MW421634) and *P. nagpurensis* (MN420271) is 0.090. The 28S sequence of *P. ophiocephali* (KF700099) showed the least nucleotide match (sequence divergence value 1.071 and 1.101 from *P. nagpurensis* and *P. roparensis* respectively) with the genetic distance much more than the average value (0.29) and is therefore not included in the analysis.

The phylogenetic tree regenerated using ITS1-5.8S-ITS2 gene markers included 11 nucleotide sequences and 610 positions in final dataset. The Tamura (+G + I) model (Tamura 1992) was used to compute the phylogenetic tree with highest log likelihood value −3435.4573. Rates of different transitional and transversionsal substitutions ranged from 13.19–17.18 and 4.26–5.55 respectively with R value 1.52. The D value of Tajima's neutrality test was observed to be above the equilibrium. The tree showed initial clustering of members of the *Acanthosentis* into one clade and sequences of the *Pallisentis* into another clade (Fig. 4.). The second clade further divided into two sub clades one including *P. indica* (MG737588 and MG737587) and other including the new species (MW421633), *P. nagpurensis* (MN720108) and *P. nandai* (MW182514 and MW182515). The genetic distance between closely related species *P. roparensis* (MW421633) and *P. nagpurensis* (MN720108) is 0.010 while the genetic distance between *P. roparensis* (MW421633) and *P. nandai* (MW182514 and MW182515) is 0.015. The genetic distance between *P. roparensis* (MW421633) and *P. indica* (MG737588 and MG737587) is 0.196. The different phylogenetic analyses conducted for the regeneration of maximum likelihood and maximum parsimony methods based on 18S, 28S and ITS1-5.8S-ITS2 gene markers notably show the distinct identity of the species described in the present study.

3.4. Histopathology

Transverse section of hematoxylin and eosin stained tissue of uninfected fish shows normal intestine architect (Fig. 5 a). Intestinal wall of the fish consists of epithelium, lamina propria, stratum compactum, stratum granulosum, circular and longitudinal muscle layers and an outer serosa. In the present study regular morphology of the intestinal villi with a continuous mucosal epithelium is observed. Resident macrophages of the intestine are visible throughout the section. In comparison to the normal morphology, few changes were observed in case of the transverse section of the host intestine infected with the parasite. Desquamation of the intestinal villi with the proliferation of granulocytes and macrophages due to the inflammation are observed throughout the section (Fig. 5d). Severely damaged mucosa, ruptured intestinal villi with a much shorter length were also observed while irregular branching was visible at few sites (Fig. 5b and c). The dilation of lymphatic vessels is significantly visible in the infected fish intestine.

4. Discussion

The present study reports the morphological as well as the molecular description of *P. roparensis* n. sp. from Punjab, India. The sequences generated in the present study for each molecular marker are well nested within the cluster of the other isolates of *Pallisentis* retrieved from the GenBank with the significant bootstrap values in the phylogenetic trees reconstructed in the study. Due to the recent surge in descriptions of many new species of the genus *Pallisentis* (Gupta et al., 2015; Gautam et al., 2019, 2020) molecular characterization of species is needed. Initially the sub-generic classification devised by Golvan (1959) based on the number of proboscis hooks per circle created more confusion since many species show range in the number of proboscis hooks per circle. The description of some species with either the overlapping characters of more than one subgenus or not falling in any of the subgenus necessitated the revision of the genus. Amin et al. (2000) provided a key to species formulating three sub-genera viz. *Demidueterospinus*, *Brevitritospinus* and *Pallisentis* based on the more consistent morphological characteristics like the difference in the size of the proboscis hooks from anterior to posterior circles, size of the cement gland and number of the giant nuclei of the cement gland. This sub-generic classification resolved the uncertainty related to the placement of different species within the genus contemporarily and was followed by Amin (2013), Chaudhary et al. (2019), Gautam et al. (2019), Gautam et al. (2020), and Amin et al. (2021). Lately, the phylogenetic analyses based on the 18S gene marker by Chaudhary et al. (2019), Gautam et al. (2020) and Rana and Kaur (2021) have shown monophyletic origin of the genus *Pallisentis* but create ambiguity within the genus. The clustering of the sequences of the genus *Pallisentis* into two subclades cannot be explained at this stage due to the unidentified *Pallisentis* sequences submitted to the GenBank mainly after 2015. Also, the phylogenetic placement of the species within the genus *Pallisentis* do not show any trend related to the sub-generic parameters observed morphologically by various workers and shows paraphyly among the three sub-genera. Moreover, if we carefully look at the morphology of the species established by Gautam et al. (2019) and Gautam et al. (2020), some of the taxonomically important characters like length of the proboscis hooks,

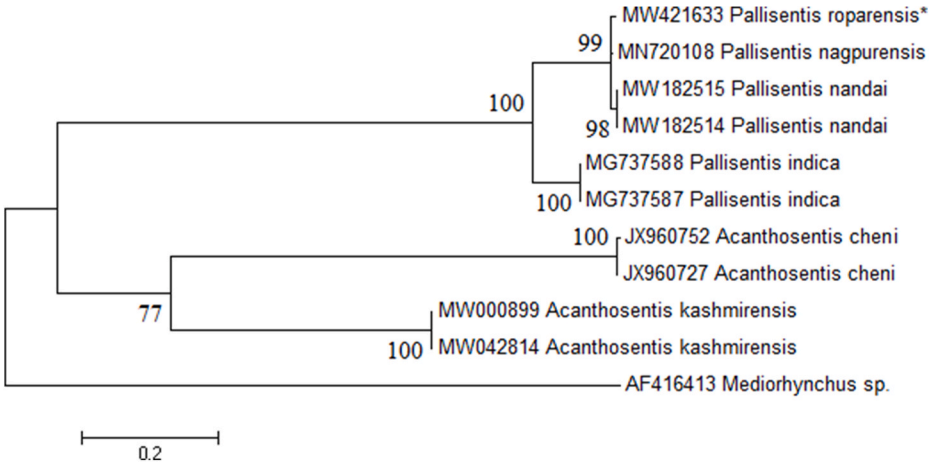

Fig. 4. Maximum likelihood tree generated using ITS1-5.8S-ITS2 gene sequence of *Pallisentis roparensis* and the sequences of related taxa downloaded from GenBank. Numbers near internal nodes show ML bootstrap clade frequencies.

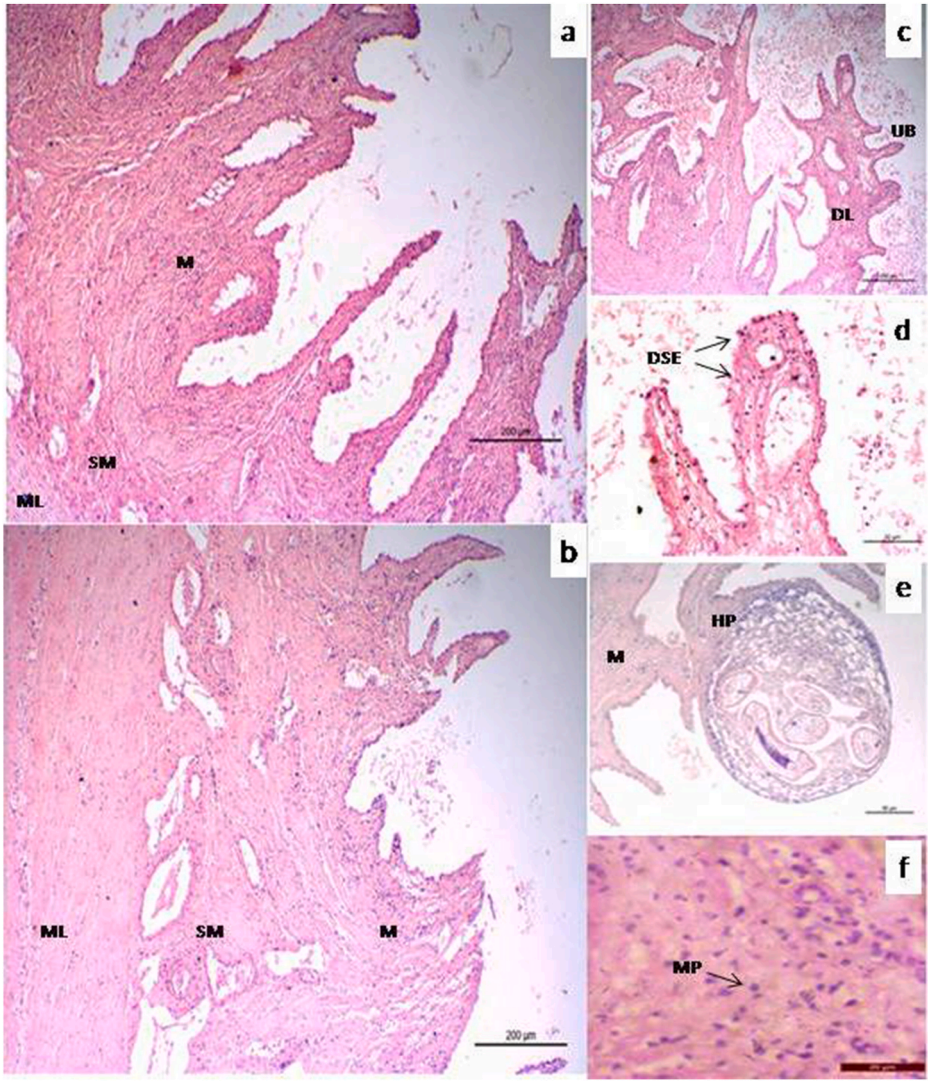

Fig. 5. a-histological section of small intestine of uninfected fish (*Wallago attu*) showing intestinal villi with a continuous epithelium; b-ruptured intestinal villi of the infected fish host; c-unusual branching of villi and dilated lymphatic vessels in mucosa of infected small intestine; d-abrasion and desquamation of the mucosal epithelium in infected fish; e–hyperplasia of the intestinal villi at the site of parasite attachment; f-magnified view of the infiltrated immune cells in the sub mucosal layer of the infected intestine (M-mucosa, SM-submucosa, ML-muscularis, DL-dilated lymphatic vessels, UB-unusual branching, DSE-desquamated intestinal epithelium, HP- hyperplasia, MP- macrophage).

number of collar and trunk spines, size of the testes, size of the cement gland and the number of nuclei of cement gland are often overlapping but the molecular data based on 18S molecular marker shows notable genetic difference (Gautam et al., 2020) within these species. The present analysis included 24 sequences of the genus *Pallisentis* based on 18S gene marker in which the isolate of *P. roparensis* clustered in a sub clade which includes the sequence of the morphologically close species *P. nagpurensis* with the genetic divergence of 0.106 while the molecular data related to the *P. clupei* is not available. It can be hypothesized that this similarity in the morphology maybe due to the continuous evolution of such species from a common ancestor and indicates the species complex pattern of closely related species from same geographical area.

The lacuna to elucidate the interspecies relationships within the genus lies due to the unavailability of molecular data on the previously reported species except a few. Classification of the genus into subgenera based on morphological characters by Amin et al. (2000) shall only be completely supported with molecular confirmation. Moreover, the combination of genetic markers and possibly large amplicon size is a desirable approach for molecular investigations before coming to any conclusion (García-Varela and Nadler, 2006). The phylogenetic trees regenerated in this study show the clustering of all the sequences of genus *Pallisentis* within the single clade which further bifurcated into two subclades on the basis of 18S although nothing much can be interpreted regarding the relationships within the genus because of the limited molecular data in comparison to the number of morphologically described species.

Host immune response against the acanthocephalan worms mainly depends on density of the worms and depth of the parasite penetration into the host intestine (Taraschewski, 2000). Acanthocephalan parasites have been reported to damage the intestinal folds and muscular layers of the intestine and induce a complex host response (Bullock, 1963). The extent of injury to the host intestine also depends on the parasite according to the presence or absence of a proboscis bulb, proboscis length and the nature of spination which is highly variable within the taxa. Not much has been documented so far about the histopathological alterations caused by the infection of the *Pallisentis* species in the host intestine. The present study shows the infiltration of granulocytes and increase in the number of macrophages at the site of infection also reported by Sanil et al. (2011) caused by the infection of *Tenuiproboscis* sp. in *Lutjanus argentimaculatus*. A significant mechanical damage to the mucosa and intestinal folds was observed in case of the infected intestine which is due to the continuous irritation of the outer layers of the intestine because of the proboscis hooks and spination of the parasite. The unusual branching of intestinal villi observed in case of the infected tissue section in the present study is not being reported in the previous studies of Sanil et al. (2011), Amin et al. (2018) and Verma and Saxena (2018).

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Comment on ethics

Ethical clearance has been obtained from the Institutional Animal Ethics Committee (IAEC) of Panjab University (Approval no.: PU/45/99/CPCSEA/IAEC/482).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.10.011.

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