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Protistology

Molecular and morphometric characterization of *Thelohanellus caudatus* (Myxosporea: Myxobolidae) infecting the caudal fin of *Labeo rohita* (Hamilton)

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Summary

Thelohanellus caudatus infecting the caudal fin of carp *Labeo rohita* was characterized morphologically and by 18S rRNA gene sequence analysis. Infection of *Myxobolus* spp. and *Thelohanelus* spp. was more common in *L. rohita* gills with the parasitic frequency index of 66.67% and 50.00%, respectively. The prevalence of fin infecting *Thelohanellus* was 12.50%. The infection rate was low to moderate. The plasmodia on the caudal fin were small, white to pale yellow, elongated and 0.5–0.7 mm in length. The caudal fin of infected fish had up to 3 plasmodia, which contained about 50–60 spores each. Morphologically, *Thelohanellus* species closely resembled *Thelohanellus caudatus*. The comparison of 18S rRNA gene sequences of *T. caudatus* to other *Thelohanellus* spp. available in GenBank revealed only 72–78% homology. The *T. caudatus* (accession number KC865607) was clustered phylogenetically with the clade of freshwater myxosporeans. In the phylogenetic tree, *T. caudatus* formed dichotomy with *T. hovorkai* with low node support. This communication is the first report on molecular characterization of *T. caudatus*.

Key words: aquaculture, *Labeo rohita*, Myxosporea, molecular characterization, phylogenetic relationship, *Thelohanellus caudatus*

Introduction

Aquaculture continues to be the fastest growing animal food producing sector. Diseases are one of the major constraints on aquaculture production. Ulcerative bacterial diseases, myxozoans, monogeans, digeneans, larval cestodes and ectoparasitic crustaceans have been regularly reported. However,

reports of mortalities associated with these pathogens are few. Some of these pathogens are certainly responsible for significant chronic mortalities and poor growth that are reflected in low survival and poor yield. Annual losses of US$ 1.0 million due to disease induced mortality and impaired growth are reported in carp culture in Andhra Pradesh, India (Mohan and Bhatta, 2002).

The fish are vulnerable to various parasitic infections, out of which Myxozoa is emerging as the major group. Myxozoa cause production loss and deaths, and some fish have to be discarded because they are unsightly and not considered fit for human consumption. *Thelohanellus* is a poorly studied myxosporean group, but several species have been precisely described (Lom and Dyková, 2006). These parasites are histozoic and infect freshwater fish. *Thelohanellus* is characterized by pyriform or broadly ellipsoidal spores (valvular view), which look slimmer in sutural view. Spores always have smooth valves and single pyriform polar capsules, with a single coil of the polar filament, or subspherical polar capsules with two coils. Sporoplasms are binucleate, mostly with a spherical polysaccharide inclusion (Lom and Dyková, 2006). *Thelohanellus* spp. can be differentiated by morphological characteristics of their spores and location of plasmodia. Lom and Dyková (1992) enlisted 39 species in this genus. All over the world 75 species of the genus *Thelohanellus* have been reported (Lom and Dyková, 2006). Basu et al. (2006) listed 32 Indian species of the genus *Thelohanellus* Kudo, 1933. In India, Myxozoa have been reported predominantly in three states namely West Bengal (Pagarkar and Das, 1993; Basu and Haldar, 2003; Basu et al., 2006; Acharya and Dutta, 2007), Punjab (Singh and Kaur, 2012a, 2012b) and Andhra Pradesh (Qadri, 1967; Lalitha-Kumari, 1969).

Until recently, only spore morphology served as a means of identification of myxozoan species. Nowadays, molecular methods have become increasingly important in phylogenetic analyses and taxonomic identification of myxosporeans (Kent et al., 2001; Fiala, 2006). Ribosomal DNA sequence analyses enabled correct identification of several myxozoan species (Kent et al., 2001; Liu et al., 2011; Seo et al., 2012; Zhu et al., 2012; Shin et al., 2013). In this study, we present morphological and molecular characterization of *Thelohanellus caudatus* infecting the caudal fin of rohu, *Labeo rohita* (Hamilton) alongside with 18S rRNA based phylogenetic analysis of *Thelohanellus* group and related taxa.

Material and methods

Sampling of fish for myxosporean parasites

Randomly sampled live Indian major carp, rohu *Labeo rohita* (n = 24) from a grow-out composite culture pond of approximately 5000 m^2 in Garia, South 24 Parganas district, West Bengal, India (Lat 22°27′58" N, Long 88°24′17" E) were brought to the laboratory within 30 min of collection in oxygen-filled polythene bags during March 2013. In the laboratory, the length, weight, external symptoms and general health conditions of each fish were recorded immediately. The gills, fins, scales and operculum were removed with least damage and placed in separate Petri-dishes containing filtered water, and examined. The gills on both sides of *L. rohita* and body surface were checked thoroughly for any attached myxosporean parasites. The dorsal, pectoral, pelvic, anal, and caudal fins were cut, placed in separate Petri-dishes and thoroughly examined. Scales on each side were scrapped out along with the mucus, placed on grease free glass slides and examined. The parasitic frequency index (PFI) was calculated as the percentage of hosts ifected with the certain parasite species of the total number of hosts examined. The severity of infection was determined by following the scale proposed by Lightner (1993) with slight modification: 0 = no signs of parasite, 0.5 = a very few scattered signs of parasitic infection; 1 = low parasitic infection; 2 = low to moderate parasitic infection, 3 = moderate parasitic infection, 4 = severe parasitic infection.

Light microscopy

The parasite identification was performed in the laboratory according to Lom and Arthur (1989). The myxosporean cysts infecting the caudal fin of *L. rohita* of weight 185 g were isolated carefully. For detailed study, fresh cysts were first taken on clean grease free glass slides and slightly ruptured at one end with a sharp needle. The spores released from the cyst were smeared on clean slides with a few drops of distilled water, covered with cover slips and sealed with Distrene, Plasticizer and Xylene (DPX) for examination under oil immersion (100X) lens. Some of fresh smears were treated with various concentrations of KOH (2-10% w/v) to stimulate polar filament extrusion. The Indian ink method (Lom and Vavrá, 1963) was employed for observing the mucous membrane around the spores. For detection of iodinophilic vacuoles in

the sporoplasm, fresh spores sealed with cover slips, were treated with Lugol's iodine solution and examined at 100× in bright field. Permanent mounting of myxosporean parasites were done by staining with Giemsa solution (HiMedia, Mumbai). Air dried smears were treated with acetone free absolute methyl alcohol for about 8 min to fix the parasites. The stock solution of Giemsa was diluted with phosphate buffer (pH 7.2) in the ratio of 1:2. The slides were then placed on a staining rack and covered with Giemsa working solution for 40 min. The slides were then washed by distilled water and air dried. The slides containing myxosporean spores were observed under oil immersion (100×) lens of Motic BA400 microscope with inbuilt digital camera. The morphometric measurements were done by Motic Image Plus Version2 software. The results are presented in micrometer (μm) as mean± standard deviation (SD).

Scanning electron microscopy

For scanning electron microscopy (SEM), sporogenic plasmodia enclosed within the host tissue (caudal fin) were isolated with the help of sterile forceps and placed on cover slips pre-coated with poly-L-lysine. These were then ruptured, and the envelopes were removed with the tip of a needle. Utmost care was taken to remove entire remnants of the host tissue to free the samples from any foreign particles. The spores were then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at room temperature. Following dehydration in a ethanol gradient, the samples were transferred to a series of absolute alcohol and amyl acetate mixture (3:1, 1:1 and 1:3) for 30 min and pure amyl acetate (Adriano et al., 2002). Amyl acetate was substituted with CO_2 and the samples were dried in a HCP:2 Critical Point Dryer (Hitachi), coated with metallic gold in an IB-2 ion coater, and examined with Hitachi S-530 Scanning Electron Microscope at accelerating voltages of 15 and 20KV.

Molecular analyses

The plasmodia filled with mature spores were ruptured by a sharp needle and the contents were collected carefully in 1.5 mL microfuge tubes. The spores were then centrifuged at 1000×g for 10 min. The DNA was extracted by suspending the spores in 500 μL lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, 0.4 mg/mL Proteinase K) and incubating overnight at 55°C. Then, 500 μL of phenol: chloroform (1:1) was added to the digested spores, mixed gently, and centrifuged at 5200×g for 10 min. The upper phase was transferred to a new tube and mixed with 1/10th volume of sodium acetate (3 M, pH 5.2) and 2 volumes of 96% ethanol (Amresco, USA). If necessary, the extraction step and phenol-chloroform treatment were repeated. The DNA was precipitated at –20°C overnight and pelleted by centrifugation at 10000×g for 30 min. The pellet was washed once with 70% ethanol, air-dried for several minutes and resuspended in 30 μL of molecular biology grade water.

The 18S small subunit ribosomal RNA (18S rRNA) was amplified by Gradient PCR system (Eppendorf Master cycler Pro S) using a set of universal eukaryotic primers - UEP-F, 5′-ACCT GGTTGATCCTGCCAG-3′ and UEP-R, 5′-CT TCCGCAGGTTCACCTACGG-3′ (Barta et al., 1997). The PCR master mix contained 50 ng of genomic DNA, 10 μM of each primer, and 2× PCR TaqMixture (HiMedia, Mumbai). Amplification was done by initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing of primers at 51°C for 30 sec and extension at 72°C for 60 sec. The final extension was at 72°C for 5 min. The PCR products were analysed on a 1.5% agarose (HiMedia, Mumbai) gel containing 0.5 μg/mL ethidium bromide in 1× Tris-acetate- EDTA (TAE) buffer.

Sequence and phylogenetic analyses

The PCR amplified products were sequenced at the Genomics Division, Xcelris Labs Ltd, Ahmedabad, India. The amplified PCR product was first purified using EXO-SAP treatment. The concentration of the purified DNA was determined and subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA) following manufacturers' instructions. Electrophoresis and data analysis were carried out on the ABI 3730xl Genetic Analyzer.

Phylogenetic analyses were performed on a selection of thirty four 18S rRNA gene sequences, including the novel sequence, closely related sequences determined by Basic Local Alignment Search Tool (BLAST), and a few other representatives of the Myxobolidae clade (Fiala, 2006). *Buddenbrockia plumatellae* of the class Malacosporea was used as an out-group, because myxosporeans form a sister taxa to the malacosporeans (Canning et al., 2000). Multiple alignments and data analysis were performed by ClustalX (Thompson et al., 1997) and

MEGA5 software (Tamura et al., 2011), respectively. Genetic distance analyses were conducted using the Kimura 2-parameter model (Kimura, 1980). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Maximum Likelihood (ML) analyses were performed using PAUP*4.0b10 (Swofford 2003). Bayesian analyses were conducted by MrBayes v3.2. Parameters were summarized from the posterior probability distribution and generated a tree with posterior probability values (Ronquist and Huelsenbeck, 2003). The nucleotide sequence generated in the present study has been deposited in the GenBank database under accession number KC865607.

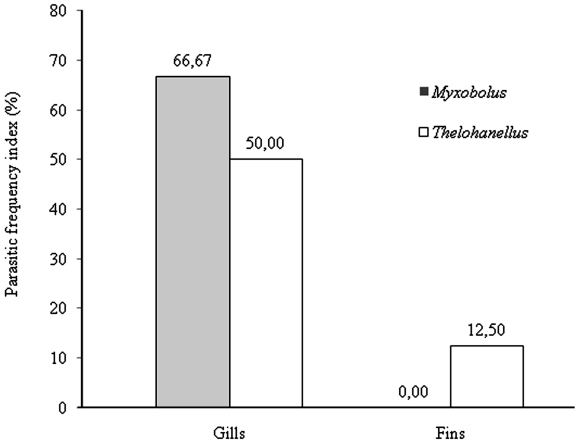


Fig. 1. Prevalence of *Myxobolus* spp. and *Thelohanellus* spp. in cultured *Labeo rohita*.

Results

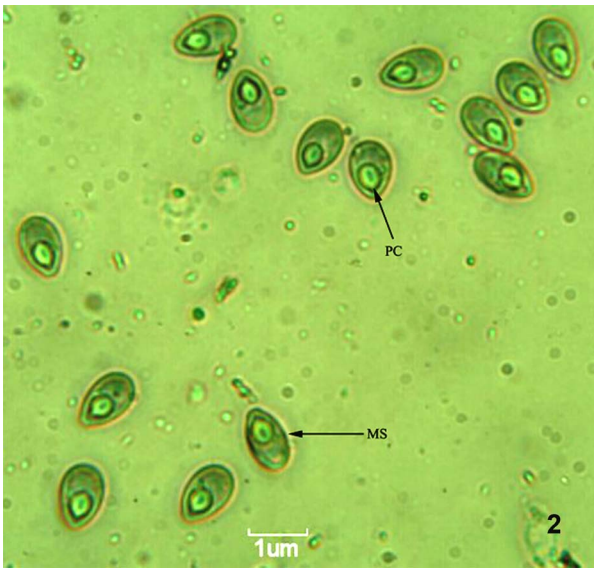
Parasitic infection and morphometry

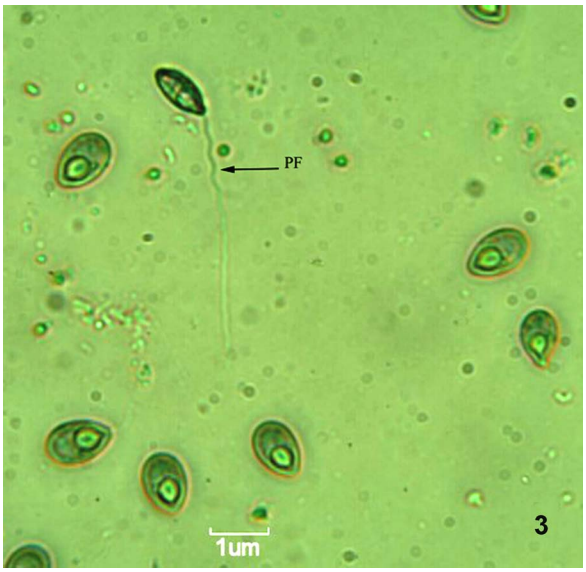
The prevalence of myxosporean parasites such as *Myxobolus* spp. and *Thelohanellus* spp. in the grow-out carp *L. rohita* is presented in Fig. 1. Infection of Myxobolus was more common in *L. rohita* gills with PFI 66.67%. The infection rate was low to moderate. The severity score was in the range of 1-2. *Myxobolus* species was not observed on *L. rohita* fins. Another myxosporean species *Thelohanellus* was also observed on caudal fin (PFI=12.50%) and in gills (PFI=50.00%) of *L. rohita*. *Thelohanellus* sp. infecting the caudal fin of *L. rohita* was further characterized by morphometric and 18S rRNA sequence analysis. Plasmodia were small, white to pale yellow, elongated and 0.5–0.7 mm in length. The caudal fin of infected fish had up to 3 plasmodia. Each plasmodium contained about 50–60 spores. Morphological observations of myxosporean spores isolated from the caudal fin of *L. rohita* are presented in the Table 1. The mature spores measured 15.41±0.48 (14.80-16.60) μm × 10.33±0.79 (9.20-12.90) μm. They were elongated and pyriform in shape with rounded posterior and tapering anterior ends (Fig. 2). The sutural ridge was distinct and straight. There were thin (0.6 μm), smooth and symmetrical shell valves. Parietal folds were absent. The single polar capsule was round to oval with slightly pointed anterior end and broad posterior end. It measured 6.99±0.54 (6.20-8.30) μm × 5.32±0.62 (4.40-6.70) μm and located just beneath the anterior end of the spore. The polar filament formed 5–6 coils within the capsule. When completely extruded, the anterior end of the long thread like polar filament, measuring 70.30±0.91 (69.20-71.40) μm, appeared to be wavy and tapering (Fig. 3). The sporoplasm contained three large nuclei 0.57±0.09 (0.50-0.70) μm in diameter and a large rounded or irregular shaped iodinophilous vacuole 3.02±0.21 (2.70-3.30) μm in diameter (Fig. 4). The spore length (LS)/spore width (WS) ratio of the *Thelohanellus* species in the present study was 1.49 (Table 1).

The SEM study revealed that the plasmodia were in direct contact with caudal fin rays (Fig. 5). The anterior end of the spore was smooth with suture folds and the sutural protrusion forming a rim around the spores (Fig. 6). The polar filament discharge channel appeared as a dark spot (Fig. 7). On the basis of the morphometric data (Table 1) and their comparison with previous descriptions, the *Thelohanellus* species infecting the caudal fin of *L. rohita* was identified as *Thelohanellus caudatus*.

Molecular comparison

The universal primer sets UEP-F and UEP-R successfully amplified 2048 bp sequence of the 18S rRNA gene from *T. caudatus* (Fig. 8). The novel 18S rRNA sequence of *T. caudatus* had 72-78% identity with *Thelohanellus* spp. such as *T. hovorkai, T. wuhanensis, T. nikolskii* and *T. kitauei* as shown by BLAST. The species most closely related to *T. caudatus* in GenBank was *T. kitauei* with 78% similarity. Phylogenetic analyses based on the alignment of thirty four 1807 bp long sequences placed *T. caudatus* within well supported freshwater clade of Myxosporea. Another marine clade of Myxosporea was poorly (49%) supported in our phylogeny. The topology of the ML tree (not shown) was similar to that of the Bayesian tree (Fig.





Figs 2, 3. Mature spores of *Thelohanellus caudatus* from fresh wet mount preparations. *Abbreviations*: MS — mature spore, PC — polar capsule, PF — polar filament.

9). On both phylogenetic trees, *T. caudatus* formed dichotomy with *T. hovorkai*, though with low node support. Evolutionary pair-wise distances among *T. caudatus* and other analyzed species measured by Kimura-2-Parameter algorithm, ranged from 0.14 (*T. kitauei* and *T. hovorkai*) to 0.32 (*M. pseudodispar*) among the freshwater clade and 0.41 (*M. gadi* and *M. bergense*) to 0.49 *Ceratomyxa* sp. among the marine clade (Table 2).

Discussion

Spore morphology has been used as a basic means to identify myxosporean species. Molnár (1994) characterized myxosporeans as the host, organ and tissue specific organisms, and believed that the host and infection site are important characters for the specific assignment. Due to inadequate drawings of the majority of *Thelohanellus* spp. and

Table 1. Measurements of *Thelohanellus caudatus* spores isolated from the caudal fin of *Labeo rohita*.

Measurement	Range	Mean	SD	SE	CV%
Length of the spore (LS), μm	14.8–16.6	15.41	0.48	0.108	3.13
Width of the spore (WS), μm	9.2–12.9	10.33	0.79	0.17	7.66
Length of the polar capsule (LPC), μm	6.2–8.3	6.99	0.54	0.12	7.85
Width of the polar capsule (WPC), μm	4.4–6.7	5.32	0.62	0.12	11.82
Diameter of iodinophilus vacuole, μm	2.7–3.3	3.02	0.21	0.075	7.01
Number of nuclei	3	–	–	–	–
Diameter of the nucleus, μm	0.5–0.7	0.57	0.09	0.045	15.78
Length of the polar filament, μm	69.2–71.4	70.3	0.91	0.45	1.29
Number of coils	5–6	–	–	–	–
Spore index					
LS/WS	–	1.49	–	–	–
LPC/WPC	–	1.31	–	–	–
LS/LPC	–	2.20	–	–	–
WS/WPC	–	1.94	–	–	–

Notes: "–" – No data. Values are on the basis of 20 observations, SD – standard deviation, SE – standard error, CV – coefficient of variation.

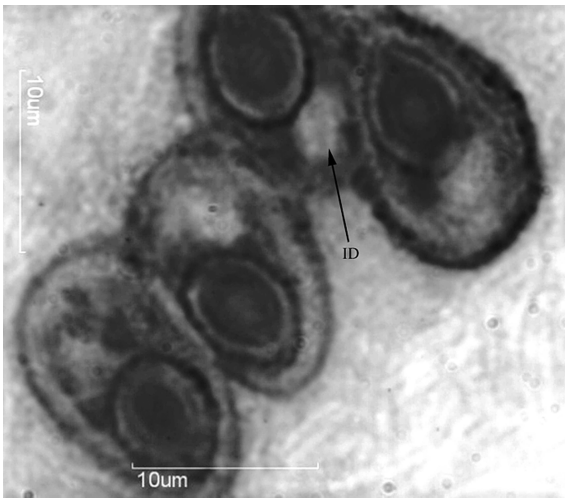


Fig. 4. Giemsa stained spore of *Thelohanellus caudatus*. *Abbreviation*: ID - iodinophilous vacuole.

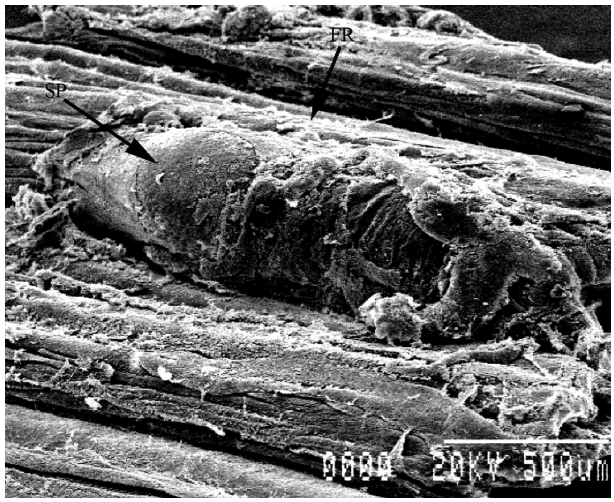
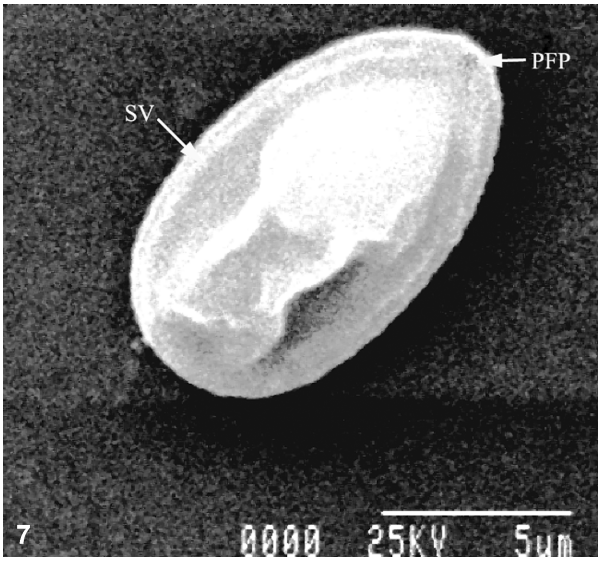


Fig. 5. Scanning electron micrograph of a sporogonic plasmodium attached to the caudal fin ray. *Abbreviations*: SP – sporogonic plasmodium, FR – fin ray.

poor quality of descriptions proper identification of species on the morphological basis is difficult. At the same time, most *Thelohanellus* spp. display strict host specificity and tissue tropism (Akhmerov, 1955; Molnár, 2002). Several *Thelohanellus* spp., other than *T. caudatus*, have been recorded from fins of cyprinid fish. Some of the parasites display extreme variations in their spore size, for example *T. avijiti* and *T. disporomorphus* (Basu and Haldar, 2003; Basu et al., 2006) and *T. habibpuri* from West Bengal (Acharya and Dutta, 2007), *T. deri, T. globulosa, T. haldari, T. kalavatae, T. mrigalae* and *T. rohi* from Punjab (Singh and Kaur, 2012a, 2012b), *T. potaili* (Lalitha-Kumari, 1969) and *T. shortti* from Andhra Pradesh, India (Qadri, 1967), *T. nikolskii* from Amur basin, Russia (Akhmerov, 1955) and *T. wuhanensis* from China (Zhu et al., 2012).

The studied *Thelohanellus* sp. morphologically closely resembled *T. caudatus*, a species described by Pagarkar and Das (1993) from the cultured carp *L. rohita* in West Bengal, India, but slightly differed from it by larger spores. Pagarkar and Das (1993) observed



Figs 6, 7. Scanning electron micrographs of mature spores of *Thelohanellus caudatus*. *Abbreviations*: PC – polar capsule, SL – suture line, PFP – polar filament pore, SV – smooth valve.

this parasite on rays of caudal and anal fins of *L. rohita*. Singh and Kaur (2012b) also recorded this parasite on the caudal fin of *L. calbasu* from the Kanjali wetland, Punjab, India. Although the polar filaments formed 5-6 coils within the capsule in all isolates, the spore measurements of the present isolate (Table 1) were slightly higher than the ones recorded in the previous descriptions (Pagarkar and Das, 1993; Singh and Kaur, 2012b). This discrepancy might be connected with the usage of software for spore measurements in the present study; while the previous descriptions (Pagarkar and Das, 1993; Singh and Kaur, 2012b) relied on ocular micrometer. Locality, ecology and fish age might be also responsible for minor variations in morphometric data and prevalence of the parasite. Moshu and Molnár (1997) recorded spores of larger size in older fish more frequently than in fingerlings. They also recorded the locality-dependent and poor ecological condition-related variations of the morphometric data on *T. nikolskii*. Still, the length/width ratios of spores (1.49), and spore capsules (1.31) of *T. caudatus* (Table 1) were similar (LS/WS=1.53; LPC/WPC=1.38) to West Bengal isolate (Pagarkar and Das, 1993), but differed (LS/WS=2.14; LPC/WPC=1.36) from those of Punjab isolate (Singh and Kaur, 2012b). All isolates exhibited the host (*Labeo* spp.) and tissue (caudal fin) specificity. Perhaps slight differences in size and shapes reflect natural variations among populations.

Our ssu rDNA based phylogenetic analyses are in accord with previously published (Fiala, 2006) and support existence of marine and freshwater clades. *Thelohanellus caudatus* was clustered with freshwater clade of myxosporeans, but distinctly separated from other *Thelohanellus* spp. It formed dichotomy with *T. hovorkai* with low node support (Fig. 9). Further, the novel sequence of *T. caudatus* has relatively little homology with *Thelohanellus* spp. from Genbank, may be because the latter are from other geographical regions. This is the first record of molecular characterization of *T. caudatus* from India.

Several earlier studies confirmed the importance of the features such as the host, tissue tropism and geographical location, and found them to correlate with the results of molecular data based on the comparison of 18S rRNA gene sequences (Kent et al., 2001; Liu et al., 2011; Seo et al., 2012; Zhu et al., 2012; Shin et al., 2013). In our study, sequence homogeneity of fin-infecting *T. caudatus* with other *Thelohanellus* spp. exhibiting different tissue tropism was variable ranging from 70 to 78%, which contradicts the results of Shin et al. (2013).

The prevalence of *Thelohanellus* infection on the caudal fin of *L. rohita* reported here was 12.5% versus 25.0% in *L. calbasu* (Singh and Kaur, 2012b). High incidence of myxosporean infection on gills was usually accompanied by poor growth, uneven size, big head, and poor body muscle ratio of fish hosts, like *L. rohita* and other Indian major and minor carps inhabiting fish ponds, though major disease symptoms were not recorded. At the same time, high frequency of myxosporean infections could become an economic problem due to decrease of fish commercial value. The pathogenesis of the diseases caused by most gill- and fin-infecting myxosporeans is largely unclear. Yet, observations of high prevalence of myxosporean infection in association with poor fish growth may indicate that the carps cultured in ponds experience respiratory and physiological stress due to the parasitic infection. At the same time, our observations suggest that infection of the caudal fin with *T. caudatus* is insignificant for the fitness of the host *L. rohita*.

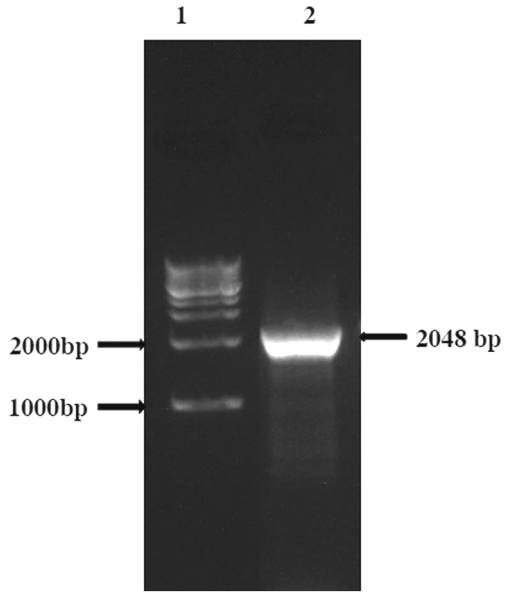


Fig. 8. Agarose gel (1.5%) showing 18S rRNA gene amplification of *Thelohanellus caudatus* from the caudal fin of *Labeo rohita*. Lane 1: 1kb DNA Ladder (Takara Bio Inc., Japan); Lane 2: *Thelohanellus caudatus*.

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Table 2. Estimates of evolutionary divergence between

		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*T. kitauei*/HQ115585														
2	*T. hovorkai*/AJ133419	0.01													
3	*T. kitauei*/GQ396677	0.00	0.01												
4	*T. kitauei*/JQ690367	0.00	0.01	0.00											
5	*T. kitauei*/HM624024	0.00	0.01	0.00	0.00										
6	*T. hovorkai*/DQ231155	0.00	0.01	0.00	0.00	0.00									
7	*T. wuhanensis*/JQ088179	0.01	0.02	0.01	0.01	0.01	0.01								
8	*T. wuhanensis*/HQ613410	0.01	0.02	0.01	0.01	0.01	0.01	0.00							
9	*T. wuhanensis*/JQ968687	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.00						
10	*T. wuhanensis*/JQ690370	0.01	0.02	0.01	0.01	0.01	0.01	0.00	0.00	0.00					
11	*T. wuhanensis*/AY165181	0.03	0.04	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.03				
12	*T. hovorkai*/DQ445295	0.03	0.04	0.03	0.03	0.03	0.03	0.02	0.02	0.02	0.02	0.05			
13	*T. sinensis*/DQ452013	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.05	0.08	0.06		
14	*M. pavlovskii*/AF507973	0.03	0.04	0.03	0.03	0.03	0.03	0.04	0.04	0.04	0.04	0.07	0.04	0.03	
15	*M. bramae*/AF507968	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.07	0.06	0.06	0.09	0.07	0.05	0.04
16	*M. muelleri*/AY325284	0.06	0.07	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.09	0.07	0.05	0.04
17	*M. macrocapsularis*/AF507969	0.06	0.07	0.06	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.09	0.07	0.05	0.04
18	*M. dispar*/AF507972	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.05	0.05	0.05	0.08	0.05	0.05	0.03
19	*T. nikolskii*/GU165832	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.05	0.08	0.06	0.08	0.07
20	*T. nikolskii*/DQ231156	0.06	0.07	0.06	0.06	0.06	0.06	0.05	0.06	0.05	0.05	0.08	0.06	0.08	0.07
21	*M. musculi*/AF380141	0.16	0.17	0.16	0.16	0.16	0.16	0.15	0.16	0.15	0.15	0.19	0.16	0.17	0.15
22	*M. pseudodispar*/AF380145	0.16	0.17	0.16	0.16	0.16	0.16	0.15	0.15	0.15	0.15	0.18	0.15	0.17	0.14
23	*M. cyprini*/AF380140	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.16	0.18	0.15
24	*T. zahrahae*/EU643622	0.11	0.12	0.11	0.11	0.11	0.12	0.12	0.12	0.12	0.12	0.15	0.12	0.12	0.11
25	*M. cerebralis*/AF115254	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.15	0.17	0.15
26	***T. caudatus*/KC865607**	**0.14**	**0.14**	**0.14**	**0.14**	**0.14**	**0.14**	**0.15**	**0.15**	**0.15**	**0.15**	**0.18**	**0.17**	**0.19**	**0.17**
27	*T. toyamai*/HQ338729	0.12	0.13	0.12	0.12	0.12	0.11	0.12	0.12	0.12	0.12	0.15	0.12	0.13	0.12
28	*H. ictaluri*/AF195510	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.16	0.16	0.15
29	*H. exilis*/AF021881	0.16	0.17	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.19	0.17	0.16	0.16
30	*M. bergense*/JN033231	0.30	0.31	0.30	0.30	0.30	0.30	0.30	0.31	0.30	0.30	0.34	0.30	0.31	0.31
31	*M. gadi*/GQ890673	0.30	0.31	0.30	0.30	0.30	0.30	0.30	0.31	0.30	0.30	0.34	0.30	0.31	0.31
32	*C. shasta*/AF001579	0.35	0.36	0.35	0.35	0.35	0.35	0.34	0.34	0.34	0.34	0.38	0.35	0.36	0.36
33	*Ceratomyxa sp.*/DQ333432	0.38	0.39	0.38	0.38	0.38	0.38	0.39	0.40	0.39	0.39	0.44	0.40	0.42	0.40
34	*B. plumatellae*/AY074915	0.43	0.45	0.43	0.43	0.43	0.43	0.43	0.44	0.43	0.43	0.45	0.46	0.46	0.45

Notes: B – *Buddenbrockia,* C – *Ceratomyxa,* H – *Henneguya,* M – *Myxobolus,* T – *Thelahanellus.*

sequences of Myxosporea and Malacosporea.

15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
0.01																			
0.03	0.02																		
0.05	0.05	0.05																	
0.08	0.07	0.08	0.07																
0.08	0.07	0.08	0.07	0.00															
0.15	0.16	0.15	0.17	0.17	0.17														
0.15	0.16	0.15	0.16	0.17	0.17	0.01													
0.14	0.15	0.14	0.17	0.17	0.17	0.01	0.02												
0.12	0.11	0.11	0.12	0.13	0.13	0.15	0.15	0.14											
0.16	0.16	0.15	0.17	0.18	0.18	0.15	0.17	0.16	0.15										
0.19	**0.20**	**0.20**	**0.18**	**0.21**	**0.21**	**0.31**	**0.32**	**0.31**	**0.26**	**0.30**									
0.11	0.12	0.12	0.13	0.16	0.16	0.12	0.13	0.12	0.11	0.13	0.24								
0.16	0.15	0.15	0.16	0.20	0.20	0.18	0.18	0.19	0.17	0.14	0.30	0.15							
0.16	0.16	0.16	0.17	0.20	0.20	0.18	0.18	0.19	0.16	0.16	0.31	0.15	0.02						
0.33	0.33	0.34	0.31	0.34	0.34	0.36	0.36	0.36	0.34	0.35	0.41	0.32	0.32	0.00					
0.33	0.33	0.34	0.31	0.34	0.34	0.36	0.36	0.36	0.34	0.35	0.41	0.32	0.32	0.00	0.09				
0.38	0.38	0.38	0.37	0.37	0.37	0.36	0.34	0.36	0.36	0.39	0.48	0.35	0.40	0.19	0.22	0.19			
0.40	0.42	0.42	0.42	0.42	0.42	0.43	0.44	0.43	0.43	0.42	0.49	0.38	0.41	0.31	0.31	0.31	0.18		
0.46	0.46	0.45	0.45	0.48	0.48	0.42	0.41	0.42	0.48	0.45	0.55	0.41	0.45	0.34	0.40	0.34	0.54	0.49	

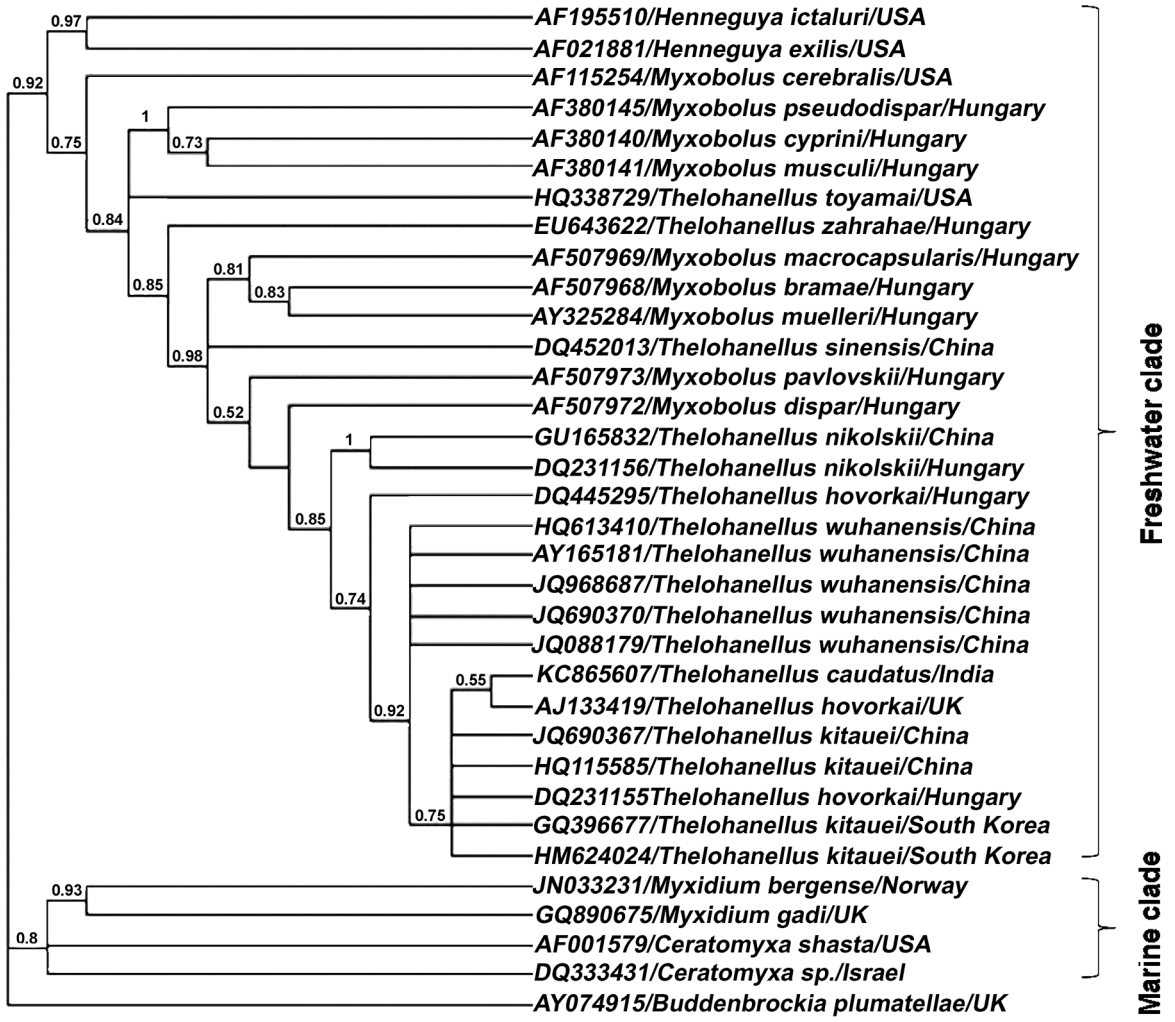

Fig. 9. Molecular phylogenetic tree produced by Bayesian analysis. The GenBank accession number and Geographical location are provided for each species. Scale bar: amount of inferred evolutionary change along the branch lengths.

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