Journal of Applied Biology & Biotechnology Vol. 5 (01), pp. 021-028, Jan-Feb, 2017
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2017.50104

Morphological, histopathological and molecular characterization of *Thelohanellus muscularis* n. sp. (Cnidaria: Myxosporea) infecting head muscles of *Labeo rohita* from Ranjit sagar wetland, Punjab (India)

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ARTICLE INFO

Article history:
Received on: 13/07/2016
Revised on: 06/09/2016
Accepted on: 04/09/2016
Available online: 20/01/2017

Key words:
18S rDNA; muscles; Phylogenetic analysis; *Labeo rohita*; India.

ABSTRACT

In the present study, a new species, *Thelohanellus muscularis* n. sp. infecting head muscles of *Labeo rohita* (Family Cyprinidae) from Ranjit Sagar Wetland, Punjab, India has been described on the basis of its morphology, histopathology and molecular analyses. The plasmodia were visible with the naked eye in the head muscles (opercular region) as round whitish bodies. The size of plasmodia ranged 0.6-0.8 mm each containing about 80-100 myxospores. The myxospores were pyriform in shape, blunt at the anterior end measuring 9.20 ± 0.28 µm × 4.0 ± 0.15 µm in size. Polar capsule was elongately pyriform measuring 5.85 ± 0.08 µm × 3.10 ± 0.11 µm in size. The amplified 18S rDNA product was 900 bp (accession number KT387308). The phylogenetic analysis indicated 99% homogeneity with three other thelohanelloid species recorded from freshwater fishes in India, i.e., *T. filli* (KR340464) infecting gills of *Labeo rohita*, *T.* sp. RA (KR423868) infecting gills of *Catla catla* and *T. jiroveci* (KJ476885) infecting gills of *Labeo rohita*. The prevalence of infection was 25%. The plasmodia and numerous myxospores were recorded in intermuscular fibrillar space in histological sections. Phylogenetic analyses elucidated relationship of the newly described *Thelohanellus muscularis* to other *Thelohanellus* species and supported its position as an independent species.

1. INTRODUCTION

The genus *Thelohanellus* Kudo, 1933 [1] is the sixth most speciose myxozoan after *Myxobolus*, *Myxidium*, *Henneguya*, *Ceratomyxa* and *Chloromyxum* with 108 nominal species [2]. Species belonging to *Thelohanellus* Kudo, 1933 are typically histozoic (rarely coelozoic) infecting almost every organ of the fish. Myxospores are tear shaped or pyriform to broadly ellipsoidal. A single polar capsule is present, either pyriform or tear shaped [3]. Most of the species of *Thelohanellus* have been reported to be non pathogenic to their hosts, however, *T. wuhanensis* [4], *T. hovorkai* [5], *T. nikolskii* [6], *T. kitauei* [7], *T. wangi* [8], *T. bifurcata* [9], *T. filli* [10] have been shown to cause severe morbidity and mortality of infected fish [11-14]. Ranjit Sagar Wetland is located on river Ravi which is about 24 km upstream of Madhopur Headworks in Gurdaspur district, Punjab. It is a manmade, riverine and lacustrine wetland with freshwater ecology. It lies at an altitude of about 540 msl at 32˚ 26´ 30´ ´ N Latitude and 75˚ 43´ 30E Longitude and is spread over an area of 87.60 sq km [15]. The area of different states falling under reservoir is Punjab (3%), Himachal Pradesh (82%) and Jammu & Kashmir (15%). The Ranjit Sagar Wetland is a cold water wetland and occupying largest catchment area (6086 sq. km.) as compared to the other wetlands in the state. For identification of myxosporeans the information generated from molecular phylogeny in addition to morphological traits greatly help in revealing the cryptic and species complexes along with their phylogeographic origin [16, 17]. In North India, many species of myxozoan parasites have been recorded from freshwater fishes in wetlands and aquaculture ponds in Punjab [18-39].

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2.MATERIAL AND METHODS

2.1 Collection and Microscopy

Live specimens of *Labeo rohita* (n= 48) with average length of 15-20 cm were procured from the various catchment sites of Ranjit Sagar Wetland, Punjab, India. Plasmodia present within the head muscle fibres (in opercular) region were removed, teased on a slide and examined under phase contrast microscope (Magnus MLX) to study the myxospore morphology.

2.2 Histopathology

The muscles containing plasmodia were cut into small pieces and fixed in Bouin's fixative. Tissue samples were dehydrated in ascending grades of ethanol, cleared in xylene, embedded in paraffin wax, sectioned at 6-7µm thickness, stained with Luna's staining method [40] and photographed.

2.3 DNA extraction, PCR amplification and sequencing

The myxospores were collected and fixed in absolute alcohol for molecular and phylogenetic analysis. The parasite DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's instructions. The primers My1F (CTAATCCCGGTAACGAACGA) My10R (CGTCCTCGCAACAAACTGTA) were used for the amplification of 18S rDNA using a Eppendorf Master Cycler Pro S. The PCR was carried out, according to [41] at the final volume of 25 µl using the primers which amplified 900 bp of the 18S rDNA gene.

The amplification reactions were conducted with 45 ng of genomic DNA, 12.5 µl of 1× reaction buffer (Himedia), 1.0 µl of each primers, 1.0 µl of total DNA and 10.5 µl of nuclease free water. Amplification was done by initial denaturation at 95°C for 3 min, followed by 34 cycles of denaturation at 95°C for 30 s, annealing of primers at 57°C for 30 s, extension at 72°C for 1 min 20 s.

The final extension was at 72°C for 10 min. The PCR products were analyzed on a 2% agarose gel and size was estimated by comparison with the 100 bp Plus DNA Ladder. The amplified product was commercially sequenced at Molecular Diagnostic & Research Laboratories, Chandigarh (India).

2.4 Phylogenetic analysis

The phylogenetic analysis was done on a selection of 18S rDNA sequences that comprised the new sequence (KT387308) and 18 additional sequences from closely related sequences showing 88% homogeneity or above in NCBI GenBank database using the basic local alignment tool [42]. *Ceratonova shasta* (AF001579) isolated from *Oncorhynchus mykiss* was taken as an outgroup. Genetic distance analyses were conducted using the Kimura 2-parameter model [43] in MEGA6 software [44]. Included codon positions were 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. The Bayesian phylogenetic analysis was conducted using MrBayes v3.2.2 [45].

Sequence alignment was performed by Multiple Sequence Comparison by Log-Expectation (MUSCLE). The tree was generated using Maximum- Likelihood having 1000 bootstrap values and was proportional to the number of substitutions per site.

3. RESULTS

3.1Vegetative stages

Plasmodia minute, round, creamish-white, measure 0.6-0.8 mm in diameter attached to the muscle fibres of the opercular region, 80-100 myxospores per plasmodium. Clinical signs on the muscles were apparent showing pale appearance (Figure 1).

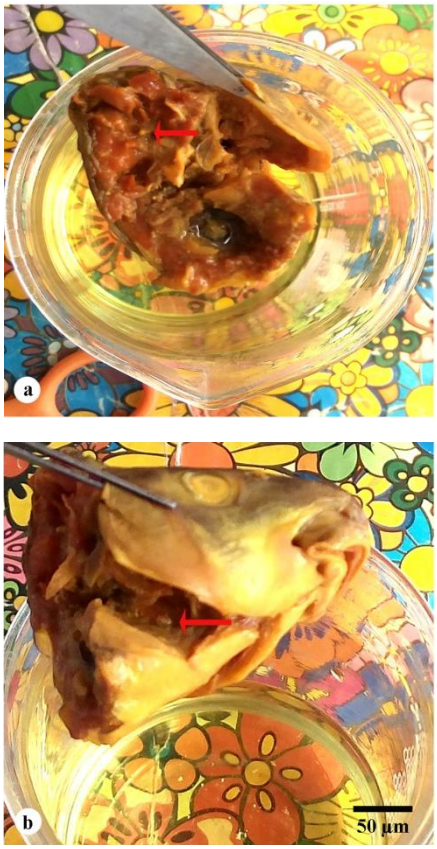

Fig. 1: (a & b) Infected head of *Labeo Rohita* showing plasmodia of T. muscularis n. sp. Located in the muscles.

3.2 Mature myxospores

Myxospores measure 9.20x4.00µm, small-sized pyriform in valvular view having bluntly pointed anterior end and rounded posterior end. Shell valves thin, smooth, symmetrical and measure 0.16 µm in thickness. Sutural line straight. Parietal folds absent. Polar capsule elongately pyriform, eccentrically placed in the myxospore body cavity. Polar capsule occupying more than half of the myxospore body cavity, measure 5.85x3.10µm. Polar filament form 7-9 coils arranged perpendicular to the polar capsule axis. Sporoplasm agranular, homogenous occupying whole of the extracapsular space behind the polar capsule and contain two nuclei and an iodinophilous vacuole (Figure 2, Table 1).

Table 1: Measurements (µm) and ratio of *T. muscularis* n. sp. isolated from head muscles of *Labeo rohita* (LS length of spore, WS width of spore, LPC length of polar capsule, WPC width of polar capsule, SD standard deviation, CV coefficient of varience).

Characters	Range	Mean Values	SD	CV
LS	9.15-9.25	9.20	0.07	0.00
WS	3.92-4.08	4.00	0.11	0.01
LPC	5.70-5.98	5.98	0.19	0.03
WPC	3.05-3.15	3.10	0.07	0.00
LS/WS		2.30		
Number of filament turns		7- 9		
Parietal folds		Absent		

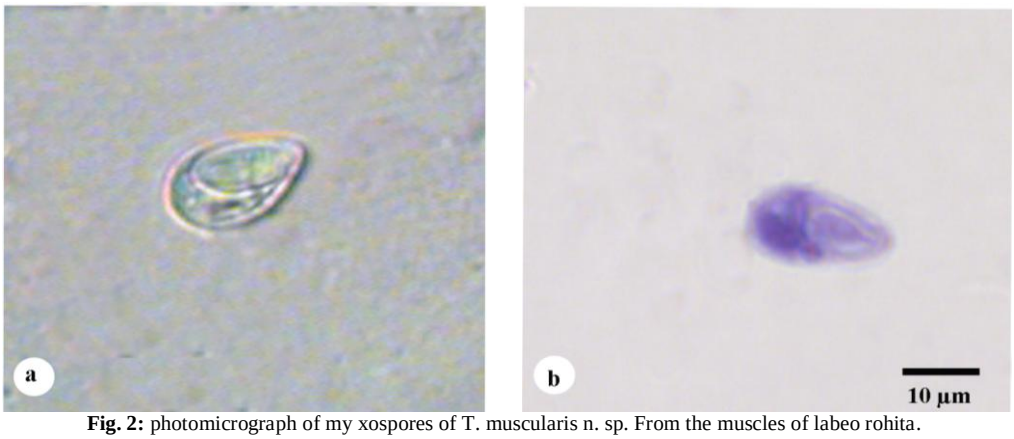

Fig. 2: photomicrograph of my xospores of T. muscularis n. sp. From the muscles of labeo rohita.
a. Fresh my xospore, My xospore stainec in iron- haematoxylin.

3.3 Taxonomic summary

Type-host: *Labeo rohita* vern rohu, (Family: Cyprinidae)

Type-locality: Ranjit Sagar wetland, Punjab, India.

Site of infection: Head muscles.

Type materials: Slide no. M/ZN/16.2.2015 and M/IH/16.2.2015, Parasitology Laboratory, Department of Zoology, Panjab University, Chandigarh (India).

Parasite frequency index (PFI): 25% (12/48)

Clinical symptomatology: Moderately symptomatic, whitish pustules on the muscles and mucous laden body.

Etymology: The specific epithet '*muscularis'* has been given after the name of the tissue location within the host.

3.4 Histopathology

The plasmodia were located in the intermuscular space and were 0.8-1.0 mm in size, forming whitish pustules, cylindrical in shape, containing about 80-100 myxospores. Histologically, masses of myxospores released from disintegrated muscle cells were found between the intact muscle fibres followed by degeneration, necrosis and atrophy. There was accumulation of myxospores in the intermuscular fibrillar space as revealed in transverse sections with myonecrosis along the epaxial end adjacent to vertebral column.

3.5 Phylogenetic analysis

The phylogenetic tree based on the final edited alignment with Maximum- Likelihood showed *T. muscularis* n. sp. in a separate clade with a bootstrap value of 93 comprising *T. seni*, *T. rohitae*, *T. bifurcata*, *T. jiroveci*, *T. filli*, *T.* sp. HK and *T.* sp. RA infecting cyprinids carp from India. The out- group *Ceratonova shasta* phylogenetically clustered distinctly as a separate lineage (Figure 5). Moreover, estimates of evolutionary pair- wise divergence among the sequences of *T. seni*, *T. rohitae*, *T. bifurcata*, *T. jiroveci*, *T. filli*, *T.* sp. HK and *T.* sp. RA were 0.03, 0.05, 0.01, 0.0, 0.0, 0.05 and 0.0 respectively (Figure 6). The nucleotide frequencies were 26.73% (A), 24.21% (T/U), 21.61% (C) and 27.46% (G). The transition/transversion rate ratios are K1= 3.715 (purines) and K2= 5.369 (pyrimidines).

The best fit substitution model for constructing the phylogenetic tree was K2+G having the lowest Bayesian score of 3546.077 followed by the Gamma distribution among 5 categories was 0.05, 0.24, 0.58, 1.17 and 2.96 substitutions per site.

All positions containing gaps and missing data were eliminated. Tajima's neutrality test for the nucleotide mutation was also done. The D value was less than 0 and was found to be -1.766589 meaning some of the alleles were present at high frequencies indicating high genetic diversity among myxosporeans.

4. DISCUSSION

4.1 Morphological comparison

The present species was morphologically compared with previously reported *Thelohanellus* species from Indian subcontinent (Table 2).

The present species was closely compared with other *Thelohanellus* species infecting muscles i. e. *T. gangeticus* [56] infecting muscles of *Cheila bacaila* and *T. ophthalmicus* [57] infecting eye muscles of *Catla catla* and was much smaller in size. In addition, the myxospores of *T. seni* (13.71 µm), *T. otebike* (14.85 µm), *T. catlae* (20.4 µm), *T. carassii* (17.0 µm), *T. caudatus* (13.8 µm), *T. boggoti* (11.5 µm), *T. filli* (27.08 µm) and *T. jiroveci* (35.0 µm) were much longer than the present species (9.20 µm). Furthermore, the present species lacked parietal folds hence differed from *T. parastromataei* and *T. misgurni* in which distinct parietal folds were present. The myxospores of the present species also lacked a distinct neck, hence differed from *T. boggoti* and *T. thaili* having distinct neck.

The myxospores of *T. muscularis* n. sp. were characterized in having small-sized pyriform shape in valvular view with bluntly pointed anterior end and rounded posterior end, in this respect, it differed from *T. globulosa* in which myxospores were ovoid to spherical in shape. In addition to this, the present species was compared with *T. batae* and *T. wallogoi*, but different in having eccentrically placed polar capsule as compared to the terminal or central position in the later two.

Table 2: Comparative description of *T. muscularis* n. sp. with morphologically similar species (measurements in micrometer).

Species	Host	Infected organ	Country	LS	WS	LPC	WPC	No. of filament turns
***T. muscularis* n. sp**. (present study)	***Labeo rohita***	**muscles**	India	**9.20**	**4.00**	**5.85**	**3.10**	**7-9**
T. misgurni Kudo, 1933	*Misgurnus anguillicaudatus*	Gall bladder	Japan	14.75	6.65	6.9	3.7	-
T. catlae Chakravarty & Basu, 1948	*Catla catla*	Gills	India	20.4	11.5	10.7	13.9	9-10
T. seni Chakravarty & Basu, 1948	*Catla catla*	branchiae	India	13.71	8.56	6.42	4.52	7-8
T. carassii Akhmerov, 1960	*Carassius auratus gibelio*	Gills	Russia	17.0	10.25	7.75	5.7	-
T. boggoti Qadri, 1962	*Labeo boggot*	Gills	India	11.5	6.8	6.2	3.8	10-11
T. batae Lalitha Kumari, 1969	*Labeo bata*	gill filaments	India	12.3	6.2	7.7	3.0	3-4
T. otebike Allamuratov & Iskov, 1970	*Paracobitis longicauda*	Gills	Uzbekistan	14.85	7.1	7.95	3.55	-
T. jiroveci Kundu & Haldar, 1981	*Labeo rohita, Labeo bata*	Gills	India	35.0	13.0	18.4	7.0	10-12
T. wallagoi Sarkar, 1985	*Wallago attu*	gall bladder	India	9.25	4.8	5.4	2.7	4-5
T. parastromataei Narasimhamurti *et al*., 1990	*Parastromataeus niger*	gall bladder	India	11.18	9.46	8.6	6.88	6-7
T. caudatus Pagarkar & Das, 1993	*Labeo rohita*	caudal and anal fins	India	13.8	9.0	7.0	5.07	6-7
T. globulosa Singh & Kaur, 2012	*Cirrhinus reba*	caudal fin	India	11.67	7.9	5.3	4.8	4-5
T. thaili Singh & Kaur, 2012	*Catla catla*	Gills	India	11.67	7.22	7.30	4.40	4-5
T. filli Kaur *et al.*, 2014	*Labeo rohita*	Gills	India	27.08	10.56	16.63	8.25	10-11

Table 3: Homogeneity of 18S rRNA gene sequences of *Thelohanellus muscularis* n. sp. (Accession number KT387308) and other myxobolids and related taxa available in NCBI GenBank.

Myxozoan	Accession number	Organ infected	Host	Country	Query cover	Homogeneity (%) to *T. muscularis* n. sp. (KT387308)
T. filli	KR340464	Gills	*Labeo rohita*	India	99	1668/1668 (99)
T. sp. RA	KR423868	Gills	*Cirrhinus mrigala*	India	99	1629/1629 (99)
T. jiroveci	KJ476885	Gills	*L. rohita*	India	98	1611/1611 (99)
T. bifurcata	KJ476886	Gills	*L. rohita*	India	99	1594/1594 (98)
T. T. seni	KJ476884	Gills	*L. rohita*	India	98	1580/1580 (98)
Thel T. rohitae	KF170927	Gills	*L. rohita, L. bata*	India	98	1480/1480 (96)
T. sp. HK	KP792568	Gills	*C. catla*	India	93	1423/1423 (97)
Thelo T. catlae	KJ476881	Gills	*C. catla*	India	98	1319/1319 (93)
T. sp. KLT	KM401440	skin, gill arch	*L. rohita*	Myanmar	98	1219/1219 (91)
T. kitauei	HM624024	intestine	*Cyprinus carpio nudus*	South Korea	98	1214/1214 (91)
Thelo T. wuhanensis	HQ613410	Skin	*Carassius auratus gibelio*	China	98	1212/1212 (91)
Thelo T. nikolskii	GU165832	Fins	*C. carpio*	China	97	1098/1098 (89)
T. macrovacuolaris	KU160631	Palate	*C. carpio*	China	73	795/795 (88)
Myxo T. hovorkai	DQ231155	Abdomen	*C. carpio*	Hungary	70	778/778 (89)
T. sp. YL	KC843624	Skin	*C. auratus gibelio*	China	61	737/737 (96)
T. sp. JZ	JX458816	Gills	*C. auratus gibelio*	China	80	723/1215 (96)
Myxobolus margitae	EU598803	Gills	*Alburnus alburnus*	Hungary	48	723/723 (96)
Ceratonova shasta	AF001579	intestinal tissues	*Oncorhynchus mykiss*	USA		Outgroup

4.2 Molecular comparison

The primer sets My1F and MY10R successfully amplified the 18S rRNA gene of size 900 bp (Figure 3). The edited nucleotide sequence obtained from myxospores of *T. muscularis* n. sp. were deposited in the GenBank under the accession number of KT387308. The BLASTn analysis of *T. muscularis* n. sp. showed maximum homogeneity with *T. filli* (KR340464; 99% similarity over 1668 bp) infecting the gills of *L. rohita* from India, *T.* sp. RA (KR423868; 99% similarity over 1629 bp) infecting the gills of *C. mrigala* from India, *T. jiroveci* (KJ476885; 99% similarity over 1611 bp) infecting the gills of *L. rohita* from India, *T. bifurcata* (KJ476886; 90% similarity over 1594 bp) infecting the gills of *L. rohita* from India and *T. seni* (KJ476884; 98% similarity over1580 bp) infecting gills of *Labeo rohita* from India (Table 3). The high homogeneity values between *T. muscularis* n. sp. and above mentioned species could be due to the same order/family of host and same geographical location. The Tajima's neutrality test suggests that some of the alleles were present at high frequencies indicating significant genetic diversity among myxosporeans.

In view of the above differences, the present species under study has been proposed as new to the science and named as *T. muscularis* n. sp. through this communication.

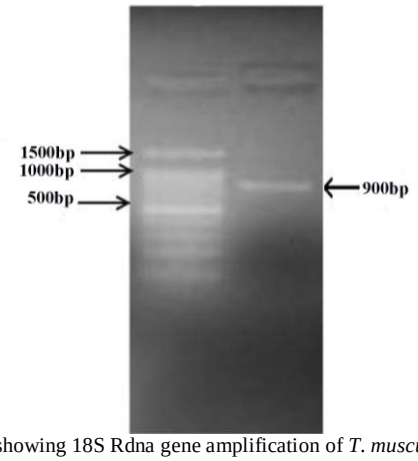

Fig. 3: Agarose gel (2%) showing 18S Rdna gene amplification of *T. muscularis* n. sp. From *Labeo rohita.*

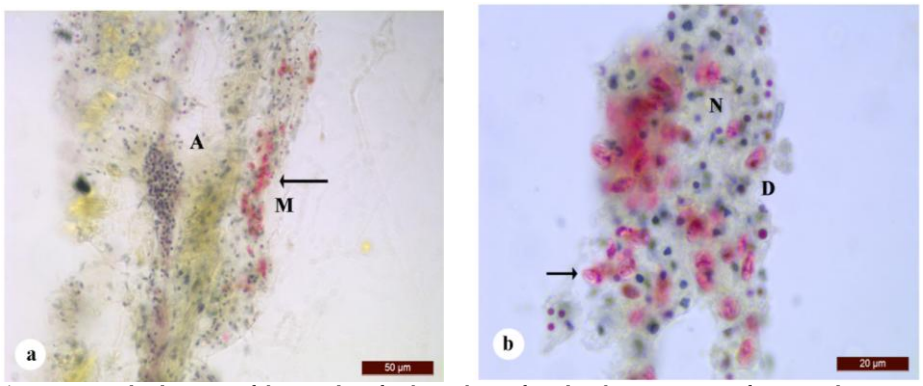

Fig. 4: Longitudinal section of the muscles of Labeo rohita infected with my xospores of T. muscularis n. sp.
(A- atrophy of cells, M-myxospores, D-degeneration of cells, N-necrosis)
a-400x, b- 1000x.

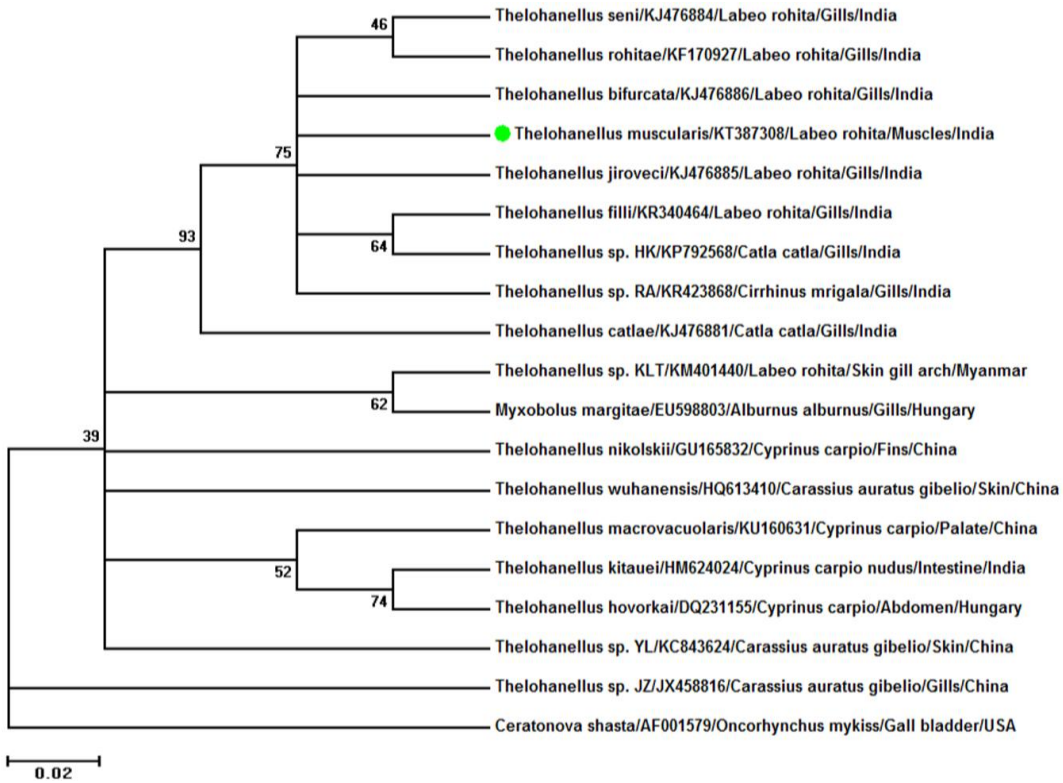

Fig. 5: Phylogenetic tree generated by maximum- likelihood showing the phylogenetic position of T. muscularis n. sp. (KT387308) with other myxosporeans. Genbank accession numbers, organ, host and country names are given and number above nodes indicates boot-strap confidence values. Ceratonova shashta was taken as the out-group. Scale bar: amount of inferred evolutionary change along the branch lengths.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Thelohanellus muscularis																			
2. Thelohanellus filli	0.00																		
3. Thelohanellus sp. RA	0.01	0.00																	
4. Thelohanellus jiroveci	0.00	0.00	0.00																
5. Thelohanellus bifurcata	0.01	0.01	0.01	0.01															
6. Thelohanellus seni	0.02	0.02	0.02	0.02	0.03														
7. Thelohanellus rohitae	0.03	0.03	0.04	0.04	0.04	0.05													
8. Thelohanellus sp. HK	0.02	0.01	0.02	0.02	0.02	0.03	0.05												
9. Thelohanellus catlae	0.02	0.01	0.01	0.01	0.02	0.03	0.05	0.03											
10. Thelohanellus sp. KLT	0.04	0.04	0.04	0.04	0.04	0.06	0.07	0.05	0.05										
11. Thelohanellus kitauei	0.02	0.02	0.03	0.03	0.03	0.04	0.05	0.03	0.03	0.03									
12. Thelohanellus wuhanensis	0.03	0.03	0.03	0.03	0.04	0.05	0.06	0.04	0.04	0.03	0.01								
13. Thelohanellus nikolskii	0.06	0.06	0.06	0.06	0.07	0.08	0.09	0.07	0.06	0.07	0.05	0.05							
14. Thelohanellus macrovacuolaris	0.03	0.03	0.03	0.03	0.04	0.05	0.06	0.04	0.04	0.03	0.01	0.02	0.06						
15. Thelohanellus hovorkai	0.02	0.02	0.03	0.03	0.03	0.04	0.05	0.03	0.03	0.03	0.00	0.01	0.05	0.01					
16. Thelohanellus sp. YL	0.04	0.04	0.04	0.04	0.04	0.06	0.07	0.05	0.04	0.05	0.03	0.03	0.07	0.03	0.03				
17. Thelohanellus sp. JZ	0.05	0.05	0.05	0.05	0.05	0.07	0.08	0.06	0.05	0.04	0.04	0.03	0.07	0.03	0.04	0.03			
18. Myxobolus margitae	0.05	0.05	0.05	0.05	0.05	0.07	0.08	0.06	0.06	0.05	0.04	0.04	0.08	0.05	0.04	0.05	0.06		
19. Ceratonova shasta	0.22	0.21	0.21	0.21	0.22	0.24	0.25	0.22	0.21	0.22	0.21	0.20	0.23	0.20	0.21	0.21	0.21	0.23	

Fig. 6: Estimates of evolutionary divergence between the sequences of T. muscularis n. sp. (KT387308) and other myxosporeans available in NCBI GenBank.

5. CONCLUSIONS

The present study deals with the identification of a new myxosporean parasite, *T. muscularis* n. sp. infecting the head muscles of *Labeo rohita* from Ranjit sagar wetland, Punjab (India). The 18S rDNA molecular marker was used to study the phylogeny of the parasite. Histopathogenesis indicated intermuscular space as the tissue location causing deformation and damage to muscle cells. The present study further supported the formation of species complex among the members of the genus *Thelohanellus* recorded from the same geographical location and cyprinid host.

6. ACKNOWLEDGEMENTS

Financial support and sponsorship: The authors acknowledge financial support by University Grants Commission (UGC), Govt. of India under CAS grant.

Conflict of Interests: There are no conflicts of interest.

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How to cite this article:

Kaur H, Gupta A. Morphological, histopathological and molecular characterization of *Thelohanellus muscularis* n. sp. (Cnidaria: Myxosporea) infecting head muscles of *Labeo rohita* from Ranjit sagar wetland, Punjab (India). J App Biol Biotech. 2017; 5 (01): 021-028. DOI: 10.7324/JABB.2017.50104