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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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. wuhanismis* [4], *T. hovorkai* [5], *T. nikolskii* [6], *T. kitauei* [7], *T. wangii* [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’ 30” E 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 myxosporean parasites have been 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* species to other *Thelohanellus* species and supported its position as an independent species.

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 (CGTCTCGCAAACACTGTA) 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

3.1 Vegetative 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>
<thead>
<tr>
<th>Characters</th>
<th>Range</th>
<th>Mean Values</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
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<td>LS</td>
<td>9.15-9.25</td>
<td>9.20</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>WS</td>
<td>3.92-4.08</td>
<td>4.00</td>
<td>0.11</td>
<td>0.01</td>
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<tr>
<td>LPC</td>
<td>5.70-5.98</td>
<td>5.98</td>
<td>0.19</td>
<td>0.03</td>
</tr>
<tr>
<td>WPC</td>
<td>3.05-3.15</td>
<td>3.10</td>
<td>0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>LS/WS</td>
<td></td>
<td>2.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of filament turns</td>
<td></td>
<td>7-9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parietal folds</td>
<td></td>
<td>Absent</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 variance).
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. The present species was closely compared with other *Thelohanellus* species infecting muscles i.e. *T. otebike* (14.85 μm), *T. caudatus* (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 whereas the present species infected 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. caudatus* (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 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 baccala* 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. caudatus* (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. wallagoi*, 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).

<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Infected organ</th>
<th>Country</th>
<th>LS</th>
<th>WS</th>
<th>LPC</th>
<th>WPC</th>
<th>No. of filament turns</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. muscularis</em> n. sp. (present study)</td>
<td><em>Labeo rohita</em></td>
<td>muscles</td>
<td>India</td>
<td>9.20</td>
<td>4.00</td>
<td>5.85</td>
<td>3.10</td>
<td>7-9</td>
</tr>
<tr>
<td><em>T. misgurni</em> Kudo, 1933</td>
<td><em>Misgurnus anguillicaudatus</em></td>
<td>Gall bladder</td>
<td>Japan</td>
<td>14.75</td>
<td>6.65</td>
<td>6.9</td>
<td>3.7</td>
<td>-</td>
</tr>
<tr>
<td><em>T. catlae</em> Chakravarty &amp; Basu, 1948</td>
<td><em>Catla catla</em></td>
<td>Gill</td>
<td>India</td>
<td>20.4</td>
<td>11.5</td>
<td>10.7</td>
<td>13.9</td>
<td>9-10</td>
</tr>
<tr>
<td><em>T. seni</em> Chakravarty &amp; Basu, 1948</td>
<td><em>branchedia</em></td>
<td>Gill</td>
<td>India</td>
<td>13.71</td>
<td>8.56</td>
<td>6.42</td>
<td>4.52</td>
<td>7-8</td>
</tr>
<tr>
<td><em>T. carassii</em> Akhmerov, 1960</td>
<td><em>C. auratus gibelio</em></td>
<td>Gill</td>
<td>Russia</td>
<td>17.0</td>
<td>10.25</td>
<td>7.75</td>
<td>5.7</td>
<td>-</td>
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<tr>
<td><em>T. bogotti</em> Qadri, 1962</td>
<td><em>Labeo bogott</em></td>
<td>Gill</td>
<td>India</td>
<td>11.5</td>
<td>6.8</td>
<td>6.2</td>
<td>3.8</td>
<td>10-11</td>
</tr>
<tr>
<td><em>T. batae</em> Lalitha Kumari, 1969</td>
<td><em>Labeo bata</em></td>
<td>Gill filaments</td>
<td>India</td>
<td>12.3</td>
<td>6.2</td>
<td>7.7</td>
<td>3.0</td>
<td>3-4</td>
</tr>
<tr>
<td><em>T. otebiike</em> Allamuratov &amp; Iskov, 1970</td>
<td><em>Paracottus longicauda</em></td>
<td>Gill</td>
<td>Uzbekistan</td>
<td>14.85</td>
<td>7.1</td>
<td>7.95</td>
<td>3.55</td>
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<tr>
<td><em>T. jiroveci</em> Kundu &amp; Haldar, 1981</td>
<td><em>Labeo rohita, Labeo bata</em></td>
<td>Gill</td>
<td>India</td>
<td>35.0</td>
<td>13.0</td>
<td>18.4</td>
<td>7.0</td>
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<td><em>T. wallagot</em> Sarkar, 1985</td>
<td><em>Wallago atta</em></td>
<td>Gall bladder</td>
<td>India</td>
<td>9.25</td>
<td>4.8</td>
<td>5.4</td>
<td>2.7</td>
<td>4-5</td>
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<td><em>T. parasstromataei</em> Narasimhamurthi et al., 1990</td>
<td><em>Parastromataeus nigro</em></td>
<td>Gall bladder</td>
<td>India</td>
<td>11.18</td>
<td>9.46</td>
<td>8.6</td>
<td>6.88</td>
<td>6-7</td>
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<td><em>T. caudatus</em> Pagarkar &amp; Das, 1993</td>
<td><em>Labeo rohita</em></td>
<td>caudal and anal fins</td>
<td>India</td>
<td>13.8</td>
<td>9.0</td>
<td>7.0</td>
<td>5.07</td>
<td>6-7</td>
</tr>
<tr>
<td><em>T. globolosa</em> Singh &amp; Kaur, 2012</td>
<td><em>Cirrhinus reba</em></td>
<td>caudal fin</td>
<td>India</td>
<td>11.67</td>
<td>7.9</td>
<td>5.3</td>
<td>4.8</td>
<td>4-5</td>
</tr>
<tr>
<td><em>T. thalii</em> Singh &amp; Kaur, 2012</td>
<td><em>Catla catla</em></td>
<td>Gill</td>
<td>India</td>
<td>11.67</td>
<td>7.22</td>
<td>7.30</td>
<td>4.40</td>
<td>4-5</td>
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<tr>
<td><em>T. filli</em> Kaur et al., 2014</td>
<td><em>Labeo rohita</em></td>
<td>Gill</td>
<td>India</td>
<td>27.08</td>
<td>10.56</td>
<td>16.63</td>
<td>8.25</td>
<td>10-11</td>
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</table>

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.

<table>
<thead>
<tr>
<th>Myxozoa</th>
<th>Accession number</th>
<th>Organ infected</th>
<th>Host</th>
<th>Country</th>
<th>Query cover</th>
<th>Homogeneity (%) to <em>T. muscularis</em> n. sp. (KT387308)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. filli</em></td>
<td>KR340464</td>
<td>Gills</td>
<td><em>Labeo rohita</em></td>
<td>India</td>
<td>99</td>
<td>1668/1668 (99)</td>
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<tr>
<td><em>T. sp. RA</em></td>
<td>KR423868</td>
<td>Gills</td>
<td><em>Cirrhinus miragala</em></td>
<td>India</td>
<td>99</td>
<td>1629/1629 (99)</td>
</tr>
<tr>
<td><em>T. jiroveci</em></td>
<td>KJ476885</td>
<td>Gills</td>
<td><em>L. rohita</em></td>
<td>India</td>
<td>98</td>
<td>1611/1611 (99)</td>
</tr>
<tr>
<td><em>T. bifurcata</em></td>
<td>KJ476886</td>
<td>Gills</td>
<td><em>L. rohita</em></td>
<td>India</td>
<td>99</td>
<td>1594/1594 (98)</td>
</tr>
<tr>
<td><em>T. wallagot</em></td>
<td>KJ476884</td>
<td>Gills</td>
<td><em>L. rohita</em></td>
<td>India</td>
<td>98</td>
<td>1580/1580 (98)</td>
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<td><em>Thel.</em></td>
<td>KJ476887</td>
<td>Gills</td>
<td><em>L. rohita, L. bata</em></td>
<td>India</td>
<td>98</td>
<td>1480/1480 (96)</td>
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<td><em>T. sp. HK</em></td>
<td>KJ922568</td>
<td>Gills</td>
<td><em>C. catla</em></td>
<td>India</td>
<td>93</td>
<td>1423/1423 (97)</td>
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<td><em>Thel. T. caudata</em></td>
<td>KJ476881</td>
<td>Gills</td>
<td><em>C. catla</em></td>
<td>India</td>
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<td>1319/1319 (93)</td>
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<td><em>T. sp. KLT</em></td>
<td>KM401440</td>
<td>skin, gill arch</td>
<td><em>L. rohita</em></td>
<td>Myanmar</td>
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<td>1219/1219 (91)</td>
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<td><em>T. kitaei</em></td>
<td>HM624024</td>
<td>intestine</td>
<td><em>Cyprinus carpio nudus</em></td>
<td>South Korea</td>
<td>98</td>
<td>1214/1214 (91)</td>
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<td><em>Thel. T. wahanensis</em></td>
<td>HQ613410</td>
<td>Skin</td>
<td><em>Carias auratus gibelio</em></td>
<td>China</td>
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<td>1212/1212 (91)</td>
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<td><em>Thel. T. nikolskii</em></td>
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<td>Fins</td>
<td><em>C. carpio</em></td>
<td>China</td>
<td>97</td>
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<td><em>T. macrovacularis</em></td>
<td>KU160633</td>
<td>Palate</td>
<td><em>C. carpio</em></td>
<td>China</td>
<td>73</td>
<td>795/795 (88)</td>
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<td><em>Myxobolus margaritae</em></td>
<td>DQ23155</td>
<td>Abdomen</td>
<td><em>C. carpio</em></td>
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<td>70</td>
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<td>KC843624</td>
<td>Skin</td>
<td><em>C. auratus gibelio</em></td>
<td>China</td>
<td>61</td>
<td>737/737 (96)</td>
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<td><em>T. sp. IZ</em></td>
<td>JX458816</td>
<td>Gills</td>
<td><em>C. auratus gibelio</em></td>
<td>China</td>
<td>80</td>
<td>723/1215 (96)</td>
</tr>
<tr>
<td><em>Ceratobucca</em></td>
<td>EU598803</td>
<td>Gills</td>
<td><em>Alburnus alburnus</em></td>
<td>Hungary</td>
<td>48</td>
<td>723/722 (96)</td>
</tr>
<tr>
<td><em>Ceratobucca</em></td>
<td>AF001579</td>
<td>intestinal tissues</td>
<td><em>Onchorynchus mykiss</em></td>
<td>USA</td>
<td></td>
<td>Outgroup</td>
</tr>
</tbody>
</table>

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 myxosporae of *T. muscularis* n. sp. were deposited in the GenBank under the accession number 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. auratus* 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 over 1580 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 myxosporae.

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 myxospores 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.
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

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