Morphological, histopathological and molecular characterization of *Thelohanellus theinensis* n. sp. (Cnidaria: Myxosporea) infecting an Indian major carp, *Labeo bata* in a cold water wetland in Punjab (India)

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Abstract A myxozoan parasite belonging to the genus *Thelohanellus* Kudo, 1933 (Myxosporea, Bivalvulida) was isolated from the gill lamellae of *Labeo bata* (Hamilton, 1822) inhabiting Ranjit Sagar Wetland in Northern parts of Punjab, India. Histopathology of the infected gills indicated lamellar distortion due to the presence of plasmodia. The intensity of infection was recorded to be moderate as indicated by gill plasmodial index (GPI = 2). The plasmodia were microscopic, ovoid, creamish white, 2.0–4.0 mm in diameter containing 300–400 number of myxospores. The myxospores were broadly pyriform in shape, measuring $8.27 \times 4.41 \mu\text{m}$, having a prominent pore at the anterior end. The polar capsule was pyriform in shape, measuring $4.15 \times 4.10 \mu\text{m}$, containing a polar filament coiled perpendicular to the longitudinal axis of myxospore body making 6–7 turns. At molecular level, the analysis of 18S rDNA sequence of *T. theinensis* n. sp. demonstrated maximum homogeneity of 99 % with *T. filli* (KR340464) as only 2 gaps were found due to the difference of the last 20 base pairs at the 3' end of *T. theinensis* n. sp. The current study emphasizes the need for caution when dealing with members of species complex.

Keywords *Thelohanellus* · Myxosporea · *Labeo bata* · 18S rDNA · Pathogenicity · Species complex

Introduction

Ranjit Sagar Wetland, also known as the Thein Dam is at the boundary of three states of Punjab, Jammu & Kashmir and Himachal Pradesh on river Ravi. 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. The area of different states falling under reservoir is Punjab (3 %), Himachal Pradesh (82 %) and J&K (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. Due to the vast water resources in the state of Punjab and wide spread introduction of *Labeo bata* as food to the water bodies, it has become one of the most widely distributed freshwater fish species in India having high commercial value. Therefore, the knowledge of its pathogens is of utmost importance for fisheries biologists and fish health professionals.

Several *Thelohanellus* species have been described from the world (Yokoyama et al. 2012) as well as from the freshwater fishes from Indian subcontinent (Basu et al. 2015; Kaur and Singh 2008; Sarkar 1985; Singh and Kaur 2012a, b, c, d, 2014, 2015; Kaur and Katoch 2014; Kaur and Gupta 2015). Recent work on the molecular aspects of *Myxobolus* infecting Indian fishes have been done by Szekely et al. (2015), Abraham et al. (2015), Banerjee et al. (2015), Kaur and Gupta (2015), Mondal et al. (2014). Rajesh et al. (2014) studied the 18S rDNA gene sequences of *M. cuttacki* and demonstrated that tissue tropism play an important role in genetic relationship among myxozoan species. Since the morphology of the myxospore offer few characters for identification up to species level, it has become very much important to include molecular sequence data in order to eliminate the inconsistencies occurring in the identification of the species. Myxosporean

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species diversity may often be underestimated when species identifications are limited to morphological features (Bartošová and Fiala 2011). There is no specific measure available to determine what amount of difference in the 18S rDNA exists within and between the species of *Thelohanellus*. Recently, molecular studies suggested host specificity and geographical location of the host as an important factor for discerning the phylogenetic relationships of myxobolids (Carriero et al. 2013). It is also important to assemble and sort out the cryptic species which may lead to better understanding of parasite systematics, evolution of host-parasite interactions, host and tissue specificity of the parasite (Atkinson et al. 2015; Kaur and Attri 2015).

Materials and methods

Live specimens of *Labeo bata* (Ham.) with an average length of 15–20 cm were collected from the various catchment sites of Ranjit Sagar Wetland and were brought to laboratory in oxygenated polythelene bags. They were killed instantaneously by spiking. 100 fish specimens were dissected and studied during the period. 20 fishes were found to be infected. The infected gills were removed and placed in a petridish containing 0.9 % saline and were fixed in Bouin's and absolute alcohol for histopathological and molecular studies, respectively. The plasmodia were visible under stereozoom binocular and appeared as creamish white pustules on the gills. The plasmodia were teased on a clean slide to liberate myxospores, cover slipped, examined and photographed using Magnus MLX phase contrast microscope. Myxospore measurements were taken with the help of a calibrated ocular micrometer. Myxospore smears were stained with Iron haematoxylin and Ziehl Neelsen to study the morphological features (Kaur and Singh 2008). Tissue from Bouin's fixed gills was dehydrated, embedded in paraffin, and sectioned (5–8 µm thick) and were stained with Luna's method and Iron-haematoxylin (Kaur and Katoch 2014).

Gill plasmodial index (GPI) was determined using the method given by (Kaur and Attri 2015). It was determined based on number of plasmodia counted under a stereozoom trinocular microscope on only one side of the gill of the fish examined (Table 1).

Molecular analysis

The plasmodia preserved in ethanol-fixed gills were teased apart with the help of a sharp needle in a watch glass containing triple distilled water and were transferred into 1.5 ml microcentrifuge tubes. The DNA was extracted from myxospores using the DNeasy Blood & Tissue Kit

Table 1 Parameters for the evaluation of Gill Plasmodial Index (GPI) (Kaur and Attri 2015)

Number of plasmodium per gill	Index (GPI)
0	0 (no infection)
1–5	1 (light infection)
5–10	2 (moderate infection)
10–20	3 (heavy infection)
20–50 or more	4 (severe infection)

(Qiagen) following the manufacture's instructions with little modifications.

The universal primer sets ERIB1-ERIB10 (Barta et al. 1997) were used for the amplification of 18S rDNA. The PCR was carried out at the final volume of 25 µl using the universal primers which amplified the fragments of approx. 1100 bp of the 18S rDNA gene (Andree et al. 1999). The amplification reactions were conducted with 45 ng of genomic DNA, 12.5 µl of reaction buffer, 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 33 cycles of denaturation at 95 °C for 30 s, annealing of primers at 56–58 °C for 30 s, extension at 72 °C for 1 min 50 s. The final extension was at 72 °C for 10 min followed by infinite hold at 4 °C. The PCR products were analyzed on a 2 % agarose gel and size was estimated by comparison with the 100 bp Plus DNA Ladder.

DNA sequencing

The PCR amplified products were commercially sequenced at the Excelris Labs. Ltd. Ahmedabad, Gujarat, India. The edited nucleotide sequence of approx. size 1100 bp obtained from myxospores of *T. theinensis* n. sp. were deposited in GenBank under the accession number of KT387307.

Phylogenetic analysis

The phylogenetic analysis was done on a selection of 18S rDNA sequences that comprised the new sequence (KT387307) and 22 additional sequences from closely related sequences showing 90 % homogeneity or above in NCBI GenBank database using the basic local alignment tool (Fiala 2006). *Ceratonova shasta* (AF001579) isolated from *Onchorhynchus mykiss* was taken as an outgroup. Genetic distance analyses were conducted using the Tamura-Nei parameter model showing the lowest Bayesian score (BIC) of 5189.914 (Kimura 1980) in MEGA6 software (Tamura et al. 2013). The Bayesian phylogenetic analysis was conducted using MrBayes v3.2.2 (Ronquist

Fig. 1 Photomicrograph of infected gills of *Labeo bata* showing Plasmodia of *T. theinensis* n. sp. Scale bar = 50 μ m

and Huelsenbeck 2003). Sequence alignment was performed by Multiple Sequence Comparison by Log-Expectation (MUSCLE). The tree was generated using Neighbour Joining having 1000 bootstrap values and was proportional to the number of substitutions per site. *Ceratonova shasta* (AF001579) was taken as the out-group and was chosen from the group of interest, plus place-holder representatives from the closely related clades identified as potential out-groups. The root was placed between the most distant of these (selected as the out-group) and the rest.

Results and discussion

Morphological characteristics of *T. theinensis* n. sp.
Vegetative stages (Plasmodia) (Fig. 1)

Round to irregular, large white, 3–5 plasmodia per gill, present within a cavity and measure 0.5–4.0 mm in diameter. 300–400 of myxospores present per plasmodia in different stages of development.

Myxospore description (Figs. 2a–c, 3a–d)
(Measurements based on 15 myxospores in frontal view) (Table 2).

Myxospores measure $8.12\text{--}8.43 \times 4.20\text{--}4.63 \mu\text{m}$, broadly pyriform in frontal view having a prominent pore at the anterior end. Shell valves thick, symmetrical and $0.6 \mu\text{m}$ in thickness. Polar capsule measure $4.15\text{--}4.68 \times 4.10\text{--}4.63 \mu\text{m}$, pyriform with distinct neck at the anterior end. It covers more than half of the myxospore body cavity. Polar filaments form 6–7 coils arranged obliquely to the polar axis, $7.2 \mu\text{m}$ in length when extruded. Sporoplasm agranular, homogenous occupying whole of the extracapsular space beneath the polar capsule containing three nuclei and a large iodophilous vacuole.

Fig. 2 Line drawing of myxospores of *T. theinensis* n. sp. **a** Ziehl-Neelsen stain (frontal view), **b** Extruded polar filament (sutural view), **c** Iron-Haematoxylin stain (frontal view)

Taxonomic summary of *T. theinensis* n. sp.

Host	<i>Labeo bata</i> (Ham.) vern. bata, Family-Cyprinidae
Locality	Ranjit Sagar Wetland, Punjab, India
Site of infection	Gills
Type material	Paratypes are myxospores stained in Ziehl-Neelsen and Iron-haematoxylin, Deposited in the Parasitology Laboratory, Department of Zoology, Panjab University, Chandigarh (India). Slide no. M/ZN/23.07.2013 and M/IH/23.07.2013
Parasite Frequency Index	20 % (20/100)
Gill Plasmodial Index	2
Plasmodial type	Interlamellar Epithelial type (LE2)

Fig. 3 Photomicrograph of myxospores of *T. theinensis* n. sp. **a** Fresh myxospore under phase contrast microscope, **b** Fresh myxospore with extruded polar filament, **c** Myxospore stained with Iron-Haematoxylin stain, **d** Myxospore stained with Ziehl-Neelsen. Scale bar 10

Table 2 Measurements (μm) and ratio of *T. theinensis* n. sp. isolated from gills of *Labeo bata* (SD standard deviation CV coefficient of variance)

Characters	Range	Mean values	SD	CV
Length of myxospore (LS)	8.12–8.43	8.277	0.21	2.53
Width of myxospore (WS)	4.20–4.63	4.416	0.30	6.79
Length of polar capsule (LPC)	4.15–4.68	4.416	0.37	8.37
Width of polar capsule (WPC)	4.10–4.63	4.366	0.37	8.47
Ratio: LS/WS		1.872		
Number of coils		6–7		
Parietal folds		Absent		

Clinical symptomatology Moderately symptomatic, creamish white pustules on the gills and mucous laden body surface

Etymology The specific epithet '*theinensis*' has been given after the common name of Ranjit Sagar wetland

Molecular data

The edited nucleotide sequence obtained from myxospores of *T. theinensis* n. sp. were deposited in the GenBank under the accession number of KT387307. Analysis of 18S small subunit (SSU) rDNA sequence of the isolate demonstrated maximum homogeneity of 99 % with *T. filli* (KR340464) infecting the gills of *Labeo rohita* from India. The

phylogenetic tree based on the final edited alignment (1100 bp) with Neighbour-Joining showed the highest bootstrap value of 99 and placed *T. theinensis* n. sp. with *T. filli*, *T. bifurcata*, *T. seni*, *T. rohita*, *T. jiroveci* and *T. catlae* in the same group showing them to be a species complex. The length of the branches of the above species in the phylogenetic tree supports that they represent a species complex and this clustering also confirmed their paraphyletic origin (Atkinson et al. 2015; Bartošová and Fiala 2011; Kaur and Gupta 2015).

Histopathology (Fig. 4a–d)

The gills were mucous laden with minute creamish plasmodia. The plasmodia were located in the gill lamellae resulting in their dilation. These plasmodia were packed with developing stages as well as with mature myxospores. This type of tissue location within the gills corresponded with the ‘interlamellar epithelial type (LE2)’ exceeding the length of the gill lamella between the neighbouring

lamellae as described by Molnár (2002). The sections of the infected gills showed that the functional respiratory areas i.e. gill lamellae were highly affected by the infection thereby reducing the surface of air exchange leading to anoxic condition of the fish host.

Morphological comparison (Table 3)

The present species has been compared with *T. catlae* Chakravarty and Basu 1948 from gills of *Catla catla*; *T. boggoti* Qadri 1962 from gills of *Labeo boggoti*; *T. batae* Lalitha Kumari 1969 from gill filaments of *Labeo bata*; *T. wallagoi* Sarkar 1985 from gall bladder of *Wallago attu*; *T. caudatus* Pagarkar and Das 1993 from caudal and anal fins of *Labeo rohita*; *T. bifurcata* Basu and Haldar 1999 from gill lamellae of *Labeo rohita* and *Catla catla*; *T. avijiti* Basu and Haldar 2003 from dorsal fin of *Labeo rohita*; *T. chandannagarensis* Basu and Haldar 2003 from gill filaments of *Catla catla*; *T. filli* Kaur et al. 2014a from gills of *Labeo rohita*; *T. kalbensi* Singh and Kaur 2014 from gills

Fig. 4 Histopathology of gills of *Labeo bata* infected with *T. theinensis* n. sp. **a** Saggital section showing developing Plasmodia (DP), necrosis of cells (N) and hyperplasia, **b** Gills showing Interlamellar (LE2) Plasmodia (P) (400×), **c** Magnified view of infected gill showing deformed gill lamellae (DGL) forming Plasmodium enclosing numerous myxospores (S) (1000×). Scale bar 50 pm

Table 3 Comparative description of *T. theinensis* n. sp. with closely related species (measurements in micrometer)

Species	Host	Infected organ	LS	WS	LPC	WPC	No. of filament turns
<i>T. theinensis</i> n. sp. (present study)	<i>Labeo bata</i>	Gill lamellae	8.27	4.41	4.41	4.36	5–6
<i>T. catlae</i> Chakravarty & Basu, 1948	<i>Catla catla</i>	Gill	20.4	11.5	10.7	13.90	9–10
<i>T. boggoti</i> Qadri, 1962	<i>Labeo boggut</i>	Gills	11.5	6.8	6.2	3.8	10–11
<i>T. batae</i> Lalitha kumari, 1969	<i>Labeo bata</i>	Gill filaments	12.3	6.2	7.7	3.0	3–4
<i>T. wallagoi</i> Sarkar, 1985	<i>Wallago attu</i>	Gall bladder	9.2	4.8	5.4	2.7	4–5
<i>T. caudatus</i> Pagarkar & Das, 1993	<i>Labeo rohita</i>	Caudal and anal fins	13.8	9.0	7.0	5.07	6–7
<i>T. bifurcata</i> Basu and Haldar, 1999	<i>Labeo rohita</i> , <i>Catla catla</i>	Gill lamellae	34.89	9.21	23.3	6.6	15–20
<i>T. avijiti</i> Basu & Haldar, 2003	<i>Labeo rohita</i>	Dorsal fin	14.0	9.7	6.0	4.0	6–8
<i>T. chandannagarensis</i> Basu & Haldar, 2003	<i>Catla catla</i>	Gill filaments	12.5	6.7	5.1	3.1	8–10
<i>T. filli</i> Kaur et al., 2014	<i>Labeo rohita</i>	Gills	27.08	10.56	16.63	8.25	10–11
<i>T. kalbensi</i> Singh & Kaur, 2014	<i>Labeo calbasu</i>	Gills	9.5	4.9	4.8	3.2	5–6
<i>T. lamelliformis</i> Singh & Kaur, 2014	<i>Catla catla</i>	gill lamellae	10.27	4.9	3.8	2.6	6–7
<i>T. neocyprini</i> n. sp. Attri, 2015 (Unpb. data)	<i>Catla catla</i>	gill filaments	34.0	9.05	18.3	5.89	11–12

Table 4 Homogeneity of 18S rRNA gene sequences of *T. theinensis* n. sp. (Accession number KT387307) and other myxosporeans available in NCBI GenBank

Myxozoan	Accession number	Organ infected	Host	Country	Homogeneity (%) to <i>T. theinensis</i> n. sp. (KT387307)
<i>T. filli</i>	KR340464	Gills	<i>Labeo rohita</i>	India	2180/2180 (99)
<i>T. bifurcata</i>	KJ476886	Gills	<i>Labeo rohita</i>	India	1814/1814 (95)
<i>T. jiroveci</i>	KJ476885	Gills	<i>Labeo rohita</i>	India	1801/1801 (95)
<i>T. seni</i>	KJ476884	Gills	<i>Labeo rohita</i>	India	1781/1781 (95)
<i>T. wuhanensis</i>	KJ813820	Skin	<i>Carassius carassius</i>	China	821/821 (94)
<i>T. rohita</i>	KM252682	Gills	<i>Labeo rohita</i>	India	1535/1535 (93)
<i>M. tauricus</i>	JQ388896	Muscle, bone and fins	<i>Barbus barbus</i>	Ukraine	1347/1347 (92)
<i>T. hovarkai</i>	AJ133419	Gills	<i>Carassius carassius</i>	Japan	769/769 (92)
<i>T. catlae</i>	KJ476881	Gills	<i>Catla catla</i>	India	1563/1563 (91)
<i>M. pavloskii</i>	AF507973	Gills	<i>Aristichthys nobilis</i>	Hungary	1450/1450 (91)
<i>M. susanlimae</i>	EU598805	Gills	<i>Alburnus alburnus</i>	Hungary	1260/1260 (91)
<i>M. macrocapsularis</i>	FJ716095	Gill filaments	<i>Blicca bjoerkna</i>	Hungary	1234/1234 (91)
<i>M. allotypica</i>	KJ725075	Gills	<i>Hypophthalmichthys molitrix</i>	China	1417/1417 (90)
<i>Henneguya cutanea</i>	AY676460	Gills	<i>Abramis brama</i>	Hungary	1397/1397 (90)
<i>M. algonquinensis</i>	AF378335	Ovary	<i>Notropis cornutus</i>	Canada	1395/1395 (90)
<i>T. sp.</i>	KM401440	Skin and gill arch	<i>Labeo rohita</i>	Myanmar	1393/1393 (90)
<i>T. kituae</i>	HQ115585	Intestinal wall	<i>Cyprinus carpio</i>	China	1332/1332 (90)
<i>M. feisti</i>	EU598804	Gill filaments	<i>Rutilus rutilus</i>	Hungary	1251/1251 (90)
<i>M. dogieli</i>	EU003977	Heart	<i>Abramis brama</i>	Hungary	1234/1234 (90)
<i>T. sinensis</i>	DQ452013	Gills and skin	<i>Channa maculata</i>	China	887/887 (90)
<i>Ceratonova shasta</i>	AF001579	Intestinal tissues	<i>Oncorhynchus mykiss</i>	USA	Outgroup

of *Labeo calbasu*; *T. lamelliformis* Singh and Kaur 2014 from gill lamellae of *Catla catla*; *T. neocyprini* Attri 2015 from gill filaments of *Catla catla*. The myxospores of the present species, *T. theinensis* n. sp. was compared with all the above mentioned species and was found different in morphology and morphometrics. The myxospores of the

present species are characterized in having broadly pyriform shape without intercapsular process and a prominent pore at the anterior end. The polar capsule is single, pyriform with distinct neck. The present species differed from *T. filli*, *T. bifurcata* and *T. neocyprini* and *T. catlae* in having smaller myxospores. Furthermore, it also differed

Fig. 5 Phylogenetic tree generated by Neighbour Joining analysis showing the phylogenetic position of *T. theinensis* n. sp. (KT387307) with other myxosporeans. GenBank accession numbers are given and number above nodes indicates boot-strap confidence values. Scale bar amount of inferred evolutionary change along branch lengths

from *T. caudatus*, *T. chandannagarensis*, *T. lamelliformis* in the position of the polar capsule as the polar capsule and it also occupied more than half of the myxospore body in *T. theinensis* n. sp. as compared to half in the case of above three species. The present species also differed from *T. boggoti*, *T. batae*, *T. wallagoi*, *T. avijiti* and *T. kalbensi* in having a prominent pore at the anterior end. In view of the above differences in morphology and morphometrics, the present species under study has been proposed as new to science and named as *T. theinensis* n. sp.

Molecular comparison (Table 4)

The universal primer sets ERIB1-ERIB10 successfully amplified the 18S rRNA gene of approx. size 1100 bp. The edited nucleotide sequence obtained from myxospores of *T. theinensis* n. sp. were deposited in the GenBank under the accession number of KT387307. The BLASTn analysis of *T. theinensis* showed maximum homogeneity of 99 % with *T. filli* (KR340464) infecting the gills of *Labeo rohita* from India. The phylogenetic tree based on the final edited alignment (1100 bp) with Neighbour-Joining showed the highest bootstrap value of 99 and placed *T. theinensis* n. sp. with *T. filli*, *T. bifurcata*, *T. seni*, *T. rohatae*, *T. jiroveci* and

T. catlae in the same group (Fig. 5). Moreover, estimates of evolutionary pair-wise divergence among the sequences of *T. filli* and *T. jiroveci* were measured as nil (0.00), while it was 0.02 for *T. seni*, 0.05 for *T. catlae* and 0.10 for *T. rohatae* (Fig. 6). This clustering confirmed their paraphyletic origin and formed a species complex. In order to estimate the pattern of nucleotide substitution involving 23 sequences, K2 + G was used as the best substitution model having lowest Bayesian Information Criterion (BIC) of 4229.670. Nucleotide frequencies were estimated from the data (A = 25.9, C = 20.1, G = 27.9, T/U = 26.1), five rates of nucleotide substitution were [AC] = 3.09, [AG] = 18.82, [AT] = 3.09, [CG] = 3.09, [CT] = 18.82, [GT] = 3.09.; proportion of invariable sites = 10.2852 %; gamma distribution = 0.4980 estimated with five rate categories. All positions containing gaps and missing data were eliminated. There were 408 positions in the final dataset.

In India, despite several reports of the occurrence of diseases caused by myxosporidians (brain myxoboliosis in *Labeo rohita* (Das et al. 1989), gill and renal myxoboliosis in 15 day old fry of *Catla catla* (Mohan and Shankar 1995), recent studies dealing with pathology remained few and fragmentary. Many taxonomic descriptions do not

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1. <i>Thelohanellus filli</i> /KR340464		0.00	0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.07
2. <i>Thelohanellus theinensis</i> /KT387307	0.00		0.00	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.07
3. <i>Thelohanellus jiroveci</i> /KJ476885	0.00	0.00		0.01	0.01	0.01	0.02	0.01	0.02	0.01	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.00	0.07
4. <i>Thelohanellus seri</i> /KJ476884	0.01	0.01	0.02		0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.07
5. <i>Thelohanellus catiae</i> /KJ476881	0.03	0.03	0.04	0.05		0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.07
6. <i>Thelohanellus rohithae</i> /KM252682	0.06	0.06	0.07	0.08	0.10		0.02	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.03	0.03	0.03	0.03	0.02	0.03	0.03	0.01	0.09
7. <i>Myxobolus pavlovskii</i> /AF507973	0.07	0.07	0.07	0.08	0.08	0.15		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.06
8. <i>Myxobolus allotypica</i> /KJ725075	0.07	0.07	0.07	0.08	0.08	0.15	0.01		0.01	0.01	0.01	0.01	0.02	0.01	0.02	0.02	0.01	0.01	0.01	0.02	0.02	0.02	0.06
9. <i>Henneguya cutanea</i> /AY676460	0.08	0.08	0.08	0.10	0.10	0.16	0.07	0.07		0.01	0.02	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.07
10. <i>Myxobolus algonquensis</i> /AF378335	0.06	0.06	0.06	0.07	0.07	0.14	0.05	0.06	0.05		0.01	0.01	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.06
11. <i>Thelohanellus</i> sp./KM401440	0.08	0.08	0.08	0.10	0.09	0.16	0.04	0.06	0.08	0.06		0.02	0.01	0.01	0.02	0.02	0.01	0.02	0.01	0.01	0.02	0.02	0.06
12. <i>Myxobolus tauricus</i> /JQ388896	0.05	0.05	0.05	0.06	0.06	0.13	0.05	0.05	0.07	0.06	0.07		0.01	0.01	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.01	0.06
13. <i>Thelohanellus kitauei</i> /HQ115585	0.07	0.07	0.08	0.09	0.08	0.15	0.07	0.08	0.08	0.08	0.06	0.07		0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.01	0.02	0.06
14. <i>Myxobolus susaninae</i> /EU598805	0.07	0.07	0.07	0.09	0.09	0.15	0.06	0.07	0.02	0.04	0.07	0.06	0.07		0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.06
15. <i>Myxobolus feisti</i> /EU598804	0.08	0.08	0.08	0.10	0.09	0.16	0.07	0.07	0.03	0.05	0.08	0.07	0.09	0.01		0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.07
16. <i>Myxobolus macrocapsularis</i> /FJ716095	0.08	0.08	0.08	0.10	0.08	0.16	0.07	0.07	0.03	0.04	0.09	0.07	0.10	0.03	0.03		0.01	0.01	0.01	0.02	0.02	0.02	0.07
17. <i>Myxobolus dogieli</i> /EU003977	0.08	0.08	0.08	0.09	0.09	0.16	0.05	0.06	0.06	0.05	0.07	0.06	0.07	0.04	0.06	0.06		0.01	0.01	0.01	0.02	0.02	0.06
18. <i>Myxobolus rutili</i> /GU968201	0.08	0.08	0.08	0.09	0.10	0.16	0.05	0.06	0.06	0.06	0.07	0.06	0.07	0.05	0.06	0.06	0.01		0.01	0.01	0.02	0.02	0.06
19. <i>Thelohanellus sinensis</i> /DQ452013	0.06	0.06	0.07	0.08	0.08	0.14	0.04	0.04	0.06	0.05	0.06	0.05	0.08	0.05	0.06	0.05	0.06	0.06		0.02	0.02	0.01	0.06
20. <i>Thelohanellus wuhanensis</i> /KJ813820	0.07	0.07	0.08	0.09	0.09	0.15	0.06	0.07	0.08	0.08	0.06	0.07	0.02	0.07	0.08	0.09	0.06	0.06	0.07		0.01	0.02	0.06
21. <i>Thelohanellus hovorkai</i> /AJ133419	0.09	0.09	0.09	0.10	0.10	0.17	0.08	0.10	0.09	0.10	0.08	0.08	0.02	0.08	0.10	0.11	0.09	0.08	0.09	0.03		0.02	0.06
22. <i>Thelohanellus bifurcata</i> /KJ476886	0.00	0.00	0.01	0.02	0.04	0.07	0.07	0.07	0.09	0.07	0.09	0.06	0.08	0.08	0.09	0.09	0.08	0.08	0.07	0.08	0.10		0.07
23. <i>Ceratomyxa shasta</i> /AF001579	0.50	0.50	0.50	0.53	0.52	0.65	0.45	0.45	0.53	0.46	0.46	0.45	0.44	0.47	0.49	0.51	0.42	0.42	0.46	0.41	0.47	0.52	

Fig. 6 Estimates of evolutionary divergence between the sequences of *T. theinensis* n. sp. (KT387307) and other myxobolids available in NCBI GenBank

record information on pathology. To date, no commercially chemotherapeutants and vaccines are available to cope with myxosporeans infections, and the future myxosporidiosis control can only be depended on the knowledge of the biology of myxozoans (Yokoyama et al. 2012; Xi et al. 2013). It was found that myxosporeans with different morphology and size can be closely related and concluded that morphological features alone may not be good indicators of genetic relationships. We also found that partial 18S rDNA analysis greatly facilitated the process of clarity in species identification.

Discussion

On close comparison between the spores of *T. filli* and *T. theinensis* n. sp. it is found that sufficient differences in the shape of the myxospores was found (elongate pyriform in valvular view, tapering anteriorly with bluntly pointed anterior end and broad rounded at the posterior end for *T. filli* and broadly pyriform in frontal view

having a prominent pore at the anterior end for *T. theinensis* n. sp.) respectively. Furthermore, the size was much larger than *T. theinensis* n. sp. (27.08×10.56 for *T. filli* and 8.27×4.41 for *T. theinensis* n. sp.) In addition to this, the tissue location of these two closely related species is variable i.e. Intrafilamental in the case of *T. filli* and Interlamellar epithelial type in the case of *T. theinensis* n. sp. Histologically, *T. filli* was located within the gill filament. Due to the presence of spores within the entire length of the gill filament, the blood vessels were dilated and bounding endothelial cells were highly compressed (Kaur et al. 2014b). The gills infected with *T. theinensis* n. sp. were mucous laden with minute creamish plasmodia. The plasmodia were located in the gill lamellae resulting in the dilation of the lamellae. These plasmodia were packed with developing stages as well as with mature myxospores. This type of tissue location within the gills corresponded with the 'interlamellar epithelial type (LE2)' exceeding the length of the gill lamella between the neighbouring lamellae as described by Molnár (2002).

At molecular level, the analysis of 18S rDNA sequence of *T. theinensis* n. sp. demonstrated maximum homogeneity of 99 % with *T. filli* (KR340464) as only 2 gaps were found due to the difference of the last 20 base pairs at the 3' end of *T. theinensis* n. sp. The current study emphasizes the need for caution when dealing with members of species complex. This is suggested by the placement of both species within the same clade in the phylogenetic tree with high bootstrap support and confirming them to be of paraphyletic origin.

There are evidences from the work done on myxozoans by earlier workers (Fiala 2006; Bartošová and Fiala 2011; Atkinson et al. 2015) showing the instances when spore morphology was difference but the molecular data was identical or vice versa. Banerjee et al. 2015 studied the molecular characterization of *M. catmrigalae* and *T. quadrii* infecting gills and epithelium of Indian major carps of *Cirrhinus mrigala* and *Catla catla* (Hamilton, 1822). Even the previous studies conducted by the authors have indicated the presence of species complex infecting gills and related organs of the members of the Cyprinids carp (Kaur and Gupta 2015; Kaur and Attri 2015). In the case of *T. seni*, *T. jiroveci*, *T. bifurcata*, *T. catlae* and *T. rohita* there was a considerable morphological difference but it showed homogeneity of 93–99 % and the phylogenetic tree based on the final edited alignment with Neighbour-Joining placed all of them in the same clade along with *T. theinensis* n. sp. and *T. filli* showing them to be a species complex (highlighted Fig. 5).

Conclusion

Myxosporean species diversity may often be underestimated when species identifications are limited to morphological features. There is no specific measure available to determine what amount of difference in the 18S rDNA exists within and between the species of *Thelohanellus*.

The consistent sequence difference coupled with the statistically significant difference in spore length leads us to confirm that both *T. filli* and *T. theinensis* n. sp. actually represent separate species. Hence, *T. theinensis* n. sp. is another addition to the species complex.

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Compliance with ethical standards

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

Ethical approval Not required as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

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