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85 Abstract

Thelohanellus kitauei is a freshwater myxosporean parasite causing intestinal giant cystic disease of common carp. To clarify the life cycle of *T. kitauei*, we investigated the oligochaete populations in China and Hungary. This study confirms two distinct aurantiactinomyxon morphotypes (Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2) from *Branchiura sowerbyi* as developmental stages of the life cycle of *T. kitauei*. The morphological characteristics and DNA sequences of these two types are described here. Based on 18S rDNA sequence analysis, Aurantiactinomyxon type 1 (2048 bp) and Aurantiactinomyxon type 2 (2031 bp) share 99.2–99.4 %, 99.8–100 % similarity to the published sequences of *T. kitauei*, respectively. The 18S rDNA sequences of these two aurantiactinomyxon morphotypes share 99.4 % similarity, suggesting intraspecific variation within the

		taxon, possibly due to geographic origin. Phylogenetic analyses demonstrate the two aurantiactinomyxon types clustered with *T. kitauei*. Regardless, based on 18S rDNA synonymy, it is likely that Aurantiactinomyxon type 1 and 2 are conspecific with *T. kitauei*. This is the fourth elucidated two-host life cycle of *Thelohanellus* species and the first record of *T. kitauei* in Europe.
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ORIGINAL PAPER

The life cycle of *Thelohanellus kitauei* (Myxozoa: Myxosporea) infecting common carp (*Cyprinus carpio*) involves aurantiactinomyxon in *Branchiura sowerbyi*

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Abstract *Thelohanellus kitauei* is a freshwater myxosporean parasite causing intestinal giant cystic disease of common carp. To clarify the life cycle of *T. kitauei*, we investigated the oligochaete populations in China and Hungary. This study confirms two distinct aurantiactinomyxon morphotypes (Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2) from *Branchiura sowerbyi* as developmental stages of the life cycle of *T. kitauei*. The morphological characteristics and DNA sequences of these two types are described here. Based on 18S rDNA sequence analysis, Aurantiactinomyxon type 1 (2048 bp) and Aurantiactinomyxon type 2 (2031 bp) share 99.2–99.4 %, 99.8–100 % similarity to the published sequences of *T. kitauei*, respectively. The 18S rDNA sequences of these two aurantiactinomyxon morphotypes share 99.4 % similarity, suggesting intraspecific variation within the taxon, possibly due to geographic origin. Phylogenetic analyses demonstrate the two aurantiactinomyxon types clustered with *T. kitauei*. Regardless, based on 18S rDNA synonymy, it is likely that Aurantiactinomyxon type 1 and 2 are conspecific with *T. kitauei*. This is the fourth elucidated two-host life cycle of *Thelohanellus* species and the first record of *T. kitauei* in Europe.

Keywords *Thelohanellus kitauei* · Life cycle · Aurantiactinomyxon · 18S rDNA · *Branchiura sowerbyi* · China · Hungary

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Introduction

Myxozoans, a group of obligate parasitic metazoans, play a significant pathogenic role in aquatic vertebrates (mostly fish) worldwide. The myxozoan life cycles are complex and mostly involve both vertebrate and invertebrate hosts, which was first demonstrated in 1984 for *Myxobolus cerebralis* (Wolf & Markiw 1984). Since the pioneering discovery, approximately 50 myxozoan species are confirmed to follow this life cycle pattern that involves the alternation of a myxosporean stage developing in fish with an actinosporean stage in annelid worms (Székely et al. 2014; Eszterbauer et al. 2015).

Initially, life cycle studies of myxozoans were solely performed based on experimental infections. However, most of these studies only replicate partial life cycles (typically fish to worm transmission). It is difficult to conduct holistic transmission experiments for the following reasons: uncertainty of susceptible hosts, deficiency of knowledge of the intrapiscine developmental pathways, and appropriate conditions for infection (Eszterbauer et al. 2015). Along with increasing importance of molecular methods in parasitological studies, the small subunit ribosomal RNA gene (18S rDNA) has been applied as a molecular marker for identifying actinsporean-myxosporean pairs (Bartholomew et al. 1997; Holzer et al. 2004; Eszterbauer et al. 2006; Zhai et al. 2012; Borkhanuddin et al. 2014; Rosser et al. 2015). Compared with the complex, time-consuming and labor intensive nature of

experimental infections, researchers are more inclined to identify actinosporean-myxosporean pairs using molecular analysis.

Thelohanellus Kudo, 1933 (Myxosporea, Bivalvulida) is the sixth most speciose genus within the phylum Myxozoa. At present, at least 109 *Thelohanellus* spp. have been described (Yuan et al. 2015). The majority of *Thelohanellus* spp. are seemingly innocuous to their hosts, while others can be severely pathogenic. One of the more well-researched members of the genus is *Thelohanellus kitauei*, which has been implicated in fish kills in common carp (*Cyprinus carpio*) in Asia and can cause considerable economic losses (Liu et al. 2011; Seo et al. 2012; Zhai et al. 2016). While investigations into the host specificity, infection site tropism and genome of *T. kitauei* have taken place, information regarding the extrapiscine developmental stage has remained elusive (Shin et al. 2014; Yang et al. 2014). As such, the source of infection is unknown, although it is presumed the life cycle of *T. kitauei* is similar to other myxozoans, involving an actinosporean stage in an alternate invertebrate host (Seo et al. 2012).

Historically, *T. kitauei* has been reported from common carp in East Asia, mainly referring to Japan, Korea, and China (Egusa & Nakajima 1981; Seo et al. 2012; Zhai et al. 2016). To date, *T. kitauei* has not been reported from Europe or the Americas, even though the common carp is globally distributed.

In the present study, we performed investigations of the actinosporean fauna at Lake Balaton and Kis-Balaton Reservoir of Hungary and freshwater fish ponds in China, and examined the morphology and 18S rDNA sequences of actinosporean stages. The aim of this study was to confirm the identity of two aurantiactinomyxon morphotypes from *Branchiura sowerbyi* found in different biotopes as the counterpart actinosporean stages of *T. kitauei* based on morphological and molecular analyses.

Materials and methods

Sample collection

Oligochaetes were harvested from different sites of Lake Balaton (Keszthely, Tihany, Balatonvilágos, Balatonszemes, Zala channel, Siófok) and Kis-Balaton Reservoir, western part of Hungary (hereinafter referred to as 'natural waters') and cultured fish ponds located at Dongxi Lake and Datong Lake of Hubei Province, China (hereinafter referred to as 'fish ponds'). Sampling from natural waters was carried out from April to October, 2011 and April to September, 2012, while samples were collected in the fish ponds from August to December, 2014 and March to July 2015. Oligochaete samples were taken one to three times each month during the sampling period.

For the natural waters, sediment was collected near water vegetation at about 0.5 to 1 m depth. At each sampling occasion, as much as 40–60 l of mud volume was sieved in situ through 1000 μm mesh size net. Oligochaetes trapped together with debris, vegetation roots and decayed particles were then transferred to the laboratory with minimal lake water. For the fish ponds, sediment was sampled by spade from the accessible shallow water along the ponds. Then, the mud samples were sieved and washed gently with pond water through a 450-μm mesh sieve. The material remaining in the sieve was transferred into plastic boxes containing a small quantity of pond water and transported to the laboratory.

On arrival, the sediments were aerated and supplied with additional fresh dechlorinated tap water. Oligochaetes were hand-sorted from the retained material in trays filled with dechlorinated tap water. Oligochaetes of natural waters were identified according to the key of Timm (1999), while oligochaetes in the fish ponds were identified morphologically following the guidelines of Wang (2002).

Collection and morphological identification of actinospores

Oligochaetes were separated and placed into wells of 24 cell-well plates with 2 ml dechlorinated tap water or distilled water according to the methods of Yokoyama et al. (1991). Plates with worms were held at ambient temperatures and the water in the plates was examined daily for the presence of actinospores by inverted microscopy. Observed actinospores were harvested upon release. Photomicrographs of actinsopores from natural waters were taken from fresh material using both bright-field and phase contrast illumination and a DP-20 digital camera mounted on an Olympus BH-2 microscope. Similarly, photomicrographs of actinospores from fish ponds were taken with a Zeiss Axio Imager A2 fluorescence microscope equipped with Andor Clara CCD camera. Line drawings were made based on these photomicrographs.

Morphological measurements were taken from a variable number of spores, depending on availability from one infected oligochaete. Spore measurements of actinosporean types were performed according to the guidelines of Lom et al. (1997). The number of germ cells was determined by placing actinospores on glass slides and pressing gently on the spores with a coverslip to mechanically release and disrupt the sporoplasm. All measurements were in micrometers (μm).

Molecular methods

Actinospores harvested from oligochaetes collected from natural waters were initially preserved in 80 % ethanol. Genomic DNA was extracted using the DNeasy™ tissue kit

(animal tissue protocol; QIAGEN, Germany) according to the manufacturer's instructions. A semi-nested PCR system was used for amplification. The first round of PCR reaction was carried out with universal eukaryotic primers ERIB1 and ERIB10 (Barta et al. 1997). In the second round amplification, two semi-nested PCR reactions were performed using two myxozoan primer pairs, MyxospecF (Fiala 2006)-ERIB10 and ERIB1-TKR1 (Seo et al. 2012), respectively. The first round PCR was carried out in a 25-μl reaction mixture comprising 2 μl of extracted genomic DNA, 5 μl of 1 mM deoxyribonucleotide triphosphates (dNTPs; MBI Fermentas), 0.325 μM of each primer, 2.5 μl of 10× DreamTaq buffer (MBI Fermentas), 0.1 μl of DreamTaq polymerase (2 U; MBI Fermentas) and 15 μl of water. The following profile was used to amplify the 18S rDNA region: an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and was completed with terminal extension at 72 °C for 7 min. For the second round of semi-nested PCR reactions, 1 μl of the initial amplified product was used as the template. Compared with the first round PCR, the second round of PCR was performed in a 50-μl reaction mixture and the quantities of each ingredient were doubled. Amplification conditions in the second round followed this profile: 95 °C for 3 min, then 35 cycles at 95 °C for 50 s, 50 °C for 50 s, 72 °C for 1 min 40 s, and terminated with an extension period at 72 °C for 7 min. The PCR products were electrophoresed in 1 % agarose gels in 1× TAE buffer gel stained with ethidium bromide. Amplified DNA was purified with the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc). Purified PCR products were sequenced with myxozoan specific primers listed by Székely et al. (2014) and CR1F (Székely et al. 2015a, 2015b), using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) with an ABI PRISM® 3100 Genetic Analyser (Life Technologies).

Genomic DNA of actinospores harvested from oligochaetes in fish culture ponds was extracted using a TransDirect™ Animal Tissue PCR Kit (TransGen Biotech, Beijing) according to the manufacturer's instructions. The PrimeSTAR® Max DNA Polymerase (TaKaRa) was used to ensure high fidelity sequence. The 18S rDNA was amplified with universal eukaryotic primer pairs 18e (Hillis & Dixon 1991) and 18r (Whipps et al. 2003). PCR was performed in a 50-μl reaction mixture comprising 25 μl 2× PrimeSTAR Max Premix, 0.4 μM of each primer, 16 μl distilled water and approximate 100–150 ng of genomic DNA. Amplification was carried out using a ProFlexTM PCR System (ABI, America) and the amplification profile was as follows: 98 °C for 10 s, 55 °C for 15 s, and terminal elongation at 72 °C for 1 min for 35 cycles. The PCR products were electrophoresed through a 1 % agarose gel in 1× Tris-Acetate-EDTA (TAE) buffer and purified using the Gel Extraction Kit (CWBIO, Beijing). Purified PCR fragments were cloned into pMD-19T vector system (TaKaRa). Positive clones were selected and sequenced with an ABI PRISM® 3730XL DNA sequencer (Applied Biosystems Inc., Foster, USA). The contiguous sequences were assembled according to the corresponding chromatograms with the SeqMan™ utility of the Lasergene software package (DNA Star, Madison, Wisconsin) and submitted to the National Center for Biotechnology Information (NCBI) nucleotide database.

Phylogenetic analyses

Myxozoan nucleotide sequences used in phylogenetic analyses were chosen based on BLAST searches. The highly similar sequences (>80 % match) that were at least 1500 bp in length ($n = 34$) were downloaded from the NCBI nucleotide database. The dataset was aligned with the software MAFFT v. 7.271 (Katoh & Standley 2013), and manually corrected using the BioEdit sequence alignment editor program (Hall 1999). Hypervariable or ambiguous regions were deleted to ensure comparison of homologous positions. Phylogenetic analyses were conducted by Bayesian (BI) and maximum likelihood (ML) methods. The best-fit evolutionary model for BI and ML analyses was determined by MrModeltest v. 2.3 (Nylander 2004), which identified the optimal evolutionary model as the general time reversible model (GTR + I + G) using the Akaike information criteria. Bayesian analysis was conducted in MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003). The length of chains was of 2,000,000 generations with sampling each 100 generations, and the prior 5000 were discarded as burn-in. ML analysis was performed using PhyML v. 3.0 (Guindon et al. 2010). Nucleotide frequencies were estimated from the data (A = 0.2548, C = 0.2029, G = 0.2703, T = 0.2720); six rates of nucleotide substitution were [AC] = 1.1156, [AG] = 3.4511, [AT] = 1.3843, [CG] = 0.7126, [CT] = 5.4490, [GT] = 1.0000; proportion of invariable sites = 0.2339; gamma distribution shape parameter = 0.3271. Bootstrap confidence values were calculated with 100 replicates. The resulting topologies were annotated with MEGA v. 6.06 software package (Tamura et al. 2013) and compared with each other. *Ceratonova shasta* was selected as outgroup.

Results

Oligochaetes identified as possible hosts in the present study mainly consisted of *B. sowerbyi*, *Isochaetides michaelseni*, *Limnodrilus hoffmeisteri*, *Nais* sp., *Dero* sp., *Aeolosoma* sp. and *Bothrioneurum vejdovskyanum*. From *B. sowerbyi* (434 and 7321 specimens collected from Hungary and China, respectively), two distinct aurantiactinomyxon morphotypes (Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2) corresponded to known *T. kitauei* deposited in the GenBank

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database. Morphometrics of relevant aurantiactinomyxon types are listed in Table 1. The genetic similarities of aurantiactinoyxon types with *T. kitauei* deposited in the GenBank are summarized in Table 2.

Description of aurantiactinomyxon morphotypes

Aurantiactinomyxon type 1 nov. (Fig. 1a–d)

Description: Spore body is spherical in apical view but with three smooth indentations at the edge of the structure, 19.7 (17.3–23.3) μm in diameter. The caudal processes are of equal length, triangular shaped, tapering to pencil point tips, and curve downwards, measuring 20.4 (18.7–23.3) μm long and 8.9 (7.4–10.0) μm wide at the base. Three polar capsules are located in the middle of the spore body and are pyriform in side view, measuring 3.4 μm long, 2.8 μm wide. Flattened sporoplasmic body reveals at least 28 germ cells (most likely 32).

Host: *Branchiura sowerbyi* Beddard, 1892

Locality: Kis-Balaton Reservoir, Hungary

Date of collection: July, 2011

Prevalence: 0.01 % (1 out of 7755)

GenBank accession no.: KU664643

Remarks: Aurantiactinomyxon type 1 described here closely resembled the Aurantiactinomyxon of *Thelohanellus nikolskii* (Székely et al. 1998) and Aurantiactinomyxon type 'B2' of Eszterbauer et al. (2006), but the caudal processes of the latter two were shorter. Furthermore, our aurantiactinomyxon also showed similar morphological characteristics with Aurantiactinomyxon type 2 of El-Mansy et al. (1998). However, the latter possessed slightly bigger caudal processes (20.4 vs 22.6 μm in mean length and 8.9 vs 11.7 μm in mean width) and was collected from *Limnodrilus* sp. (Table 1). No pansporocysts could be detected from the infected oligochaete. By amplification and sequencing, a total of 2048 bases of 18S rDNA was generated from Aurantiactinomyxon type 1. On the basis of the DNA sequences, Aurantiactinomyxon type 1 showed maximum identity with *T. kitauei*. The contiguous sequence fragment presented a similar percentage of 99.4, 99.4, 99.3, and 99.2 % to the sequences of *T. kitauei* available in GenBank: JQ690367, KR872638, HM624024 and GQ396677, respectively (Table 2).

Aurantiactinomyxon type 2 nov. (Fig. 2a–d)

Description: Spore body is typically trefoil-shaped with obvious interlobular retractions in apical view, 20.9 (19.3–22.1) μm in diameter, and in side view it is ellipsoidal, 18.4 (17.6–23.0) μm in length, 22.3 (21.6–23.0) μm in width (measured from 8 spores). Three equal-sized triangular-sepal-like caudal processes entirely embrace the spore body, and curve downward with pointed ends in side view, measuring 19.7 (17.9–22.3) μm long, 11.6 (9.8–13.0) μm wide at the base. In addition, the caudal processes seem to be composed of two parts, that is, one is round and the other pointed at the end. The nuclei of valve cell locate either distally or proximally in caudal processes. Three polar capsules are spherical in apical view, 2.7 (2.6–2.9) μm in diameter and pyriform in side view, measuring 3.0 (2.8–3.3) μm long, 2.4 (2.2–2.6) μm wide. Number of secondary cells is 32.

Table 1 Comparison of morphological measurements of the newly identified and the previously reported aurantiactinomyxon types

Actinospore (Reference)	Caudal processes		Spore body	Polar capsules	No. of germ cells	Host
	L	W				
Aurantiactinomyxon type 1 (present study)	20.4 (18.7–23.3)	8.9 (7.4–10.0)	D: 19.7 (17.3–23.3)	L: 3.4 W: 2.8	>26 (*c.* 32)	*Branchiura sowerbyi*
Aurantiactinomyxon type 2 (present study)	19.7 (17.9–22.3)	11.6 (9.8–13.0)	D: 20.9 (19.3–22.1)	L: 3.0 (2.8–3.3) W: 2.4 (2.2–2.6) D: 2.7 (2.6–2.9)	32	*B. sowerbyi*
Aurantiactinomyxon of *Thelohanellus nikolskii* (Székely et al. 1998)	13.4 (11.3–15.5)	9.0 (8.5–9.6)	D: 21.1 (21–21.2)	D: 2.1 (2.0–2.2)	16	*Tubifex tubifex*
Aurantiactinomyxon type 2 (El-Mansy et al. 1998)	22.6	11.7	D: 21.1	L: 2.8 W: 2.0	n.d.	*Limnodrilus* sp.
Aurantiactinomyxon 'B2' (Eszterbauer et al. 2006)	16 (14–20)	8.4 (7–10.6)	D: 19 (18–21)	L: 2.6 (2–4.1)	n.d.	*B. sowerbyi*
Aurantiactinomyxon type JD (Xi et al. 2015)	21.7 (20.0–24.4)	14.0 (11.2–16.4)	L: 15.6 W: 21.2 (17.1–24.0)	D: 2.3 (2.0–2.8)	>30	*B. sowerbyi*

All measurements are in μm and ranges are given in parentheses

L length, *W* width, *D* diameter, *n.d.* no data

Table 2 Genetic similarities of aurantiactinomyxon types from Kis-Balaton Reservoir (Hungary) and fish pond of Honghu (China) with *Thelohanellus kitauei* deposited in the GenBank. Length of 18S rDNA sequences and GenBank accession numbers are shown in parentheses

Myxozoan	1	2	3	4	5	6
1, Aurantiactinomyxon type 1 (2048 bp, KU664643)	100 %					
2, Aurantiactinomyxon type 2 (2031 bp, KU664644)	99.4 %	100 %				
3, *T. kitauei* (2051 bp, JQ690367)	99.4 %	99.9 %	100 %			
4, *T. kitauei* (2048 bp, HM624024)	99.3 %	99.8 %	99.9 %	100 %		
5, *T. kitauei* (2031 bp, KR872638)	99.4 %	99.8 %	99.7 %	99.6 %	100 %	
6, *T. kitauei* (1561 bp, GQ396677)	99.2 %	100 %	99.9 %	99.7 %	99.6 %	100 %

Host: *Branchiura sowerbyi* Beddard, 1892

Locality: Datong Lake, Honghu City, Hubei Province, China

Date of collection: May, 2015

Prevalence: 0.04 % (3 out of 7755)

GenBank accession no.: KU664644

Remarks: The Aurantiactinomyxon type 2 presented here was nearly similar to the Aurantiactinomyxon 'B2' of Eszterbauer et al. (2006), but caudal processes of the latter were relatively smaller, 19.7 (17.9–22.3) vs 16 (14–20) μm in length and 11.6 (9.8–13.0) vs 8.4 (7–10.6) μm in width, and spore body of the latter was incompletely encircled within the base of caudal processes. The shape and measurements of this type also closely resembled the Aurantiactinomyxon type JD of Xi et al. (2015) except the different shape ends of caudal processes (triangular vs rounded). The partial 18S rDNA sequence of Aurantiactinomyxon type 2 was obtained from 3 clones, and the sequences had no variation among 3 clones chosen. The contiguous sequence (2031 bp) was processed to remove vector sequence and subsequently deposited in GenBank database. Based on DNA sequence analysis, Aurantiactinomyxon type 2 showed the highest genetic similarity with *T. kitauei* (JQ690367, HM624024, KR872638 and GQ396677), reaching a similarity percentage of 99.8–100 % (Table 2).

Phylogenetic analyses

Phylogenetic inferences were established based on the two newly obtained 18S rDNA sequences and 34 myxozoan sequences, which consisted of actinospores and their closest related myxospores retrieved from GenBank. Phylogenetic trees constructed by BI and ML analyses had similar topological structure though with different support values at some branch nodes (Fig. 3). Phylogenetic analyses displayed that the two newly identified aurantiactinomyxon types clustered

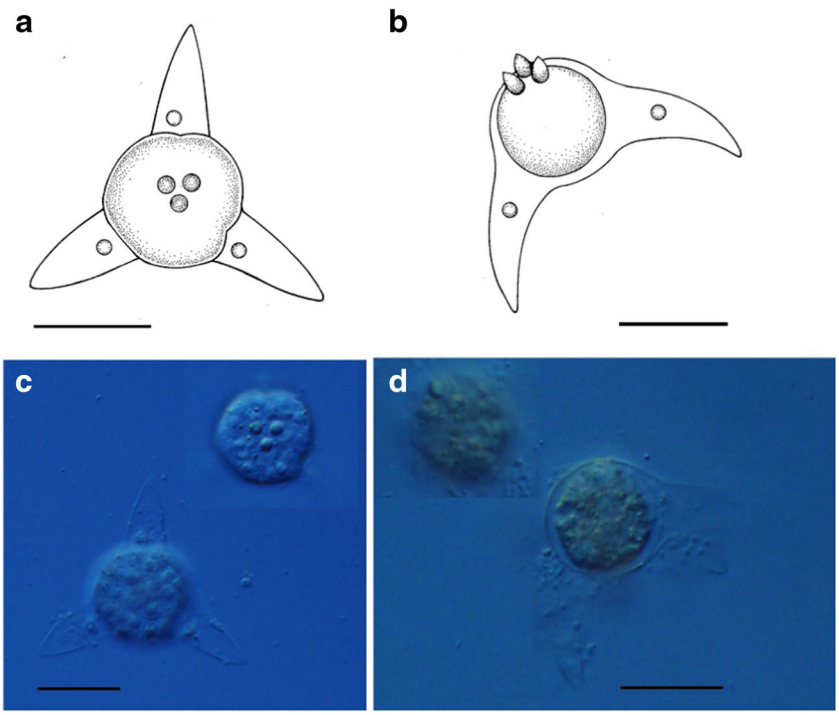

Fig. 1 Aurantiactinomyxon type 1 nov. **a** Line drawing of apical view of a mature spore. **b** Line drawing of side view of a mature spore. **c** Apical view of a waterborne spore. **d** Side view of a waterborne spore. *Scale bars* = 20 μm

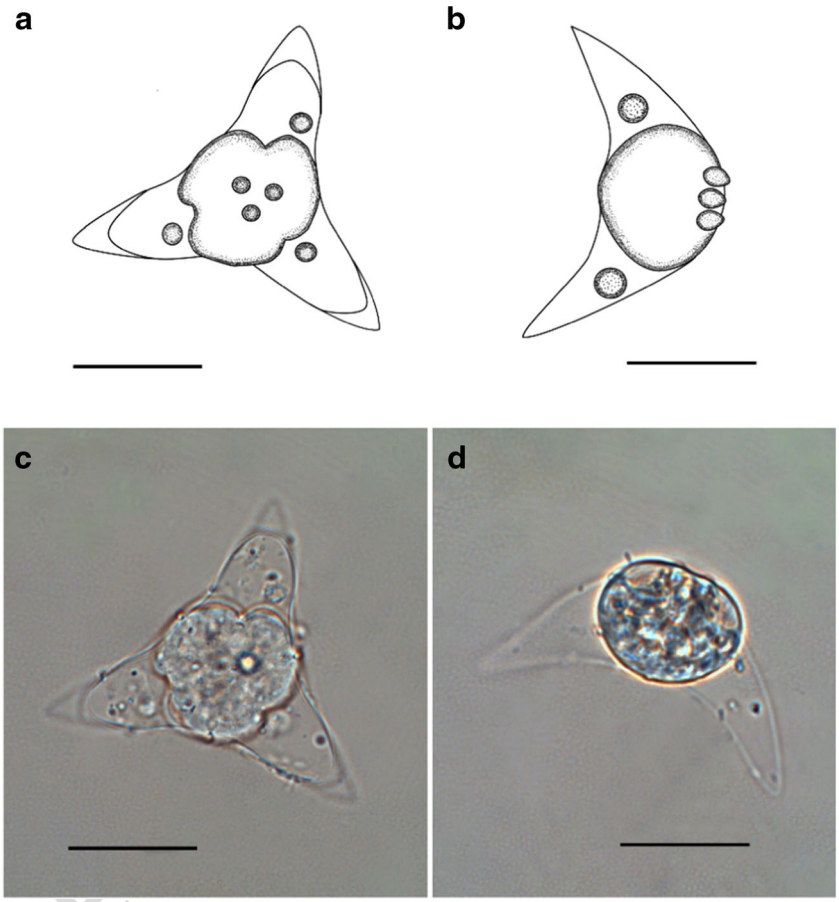

Fig. 2 Aurantiactinomyxon type 2 nov. **a** Line drawing of apical view of a mature spore. **b** Line drawing of side view of a mature spore. **c** Apical view of a waterborne spore. **d** Side view of a waterborne spore. *Scale bars* = 20 μm

with *T. kitauei* and formed a sister relationship with *Thelohanellus hovorkai*, which integrated *Thelohanellus wuhanensis* and *T. nikolskii* to jointly constitute an independent *Thelohanellus* clade. The phylogenetic trees also showed *Thelohanellus* species had a close relationship with some *Myxobolus* species, which were consistent with several previous reports (Shin et al. 2014; Yuan et al. 2015).

Discussion

The current study revealed the oligochaete *B. sowerbyi* is an extrapiscine host in the life cycle of *T. kitauei* and demonstrated two phenotypic aurantiactinomyxon, namely Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2, as the developmental stages of the life cycle of *T. kiatuei* by morphological and molecular analyses (Fig. 4).

In terms of morphology, Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2 had obvious distinctions. The spore body of Aurantiactinomyxon type 2 was typically trefoil-shaped with conspicuous interlobular retractions in apical view while that of Aurantiactinomyxon type 1 was spherical despite three inconspicuous indentations at the edge of the structure. In addition, the caudal processes of Aurantiactinomyxon type 2 were unique and seemed to be composed of two parts: one is round and the other pointed at the end. It is noticeable that the special structure of caudal processes is only reported in the Aurantiactinomyoxn type 2 and type 4 of Özer et al. (2002). At DNA level, Aurantiactinomyxon type 2 presented here showed maximum identity with *T. kitauei* and shared a similar percentage of 99.8–100 % to the sequences of *T. kitauei* available in GenBank (Table 2), which strongly suggests that Aurantiactinomyxon type 2 and *T. kitauei* are conspecific. The 18S rDNA sequence of Aurantiactinomyxon type 1 was 99.2–99.4 % similar to *T. kitauei* deposited in the GenBank and that of two aurantiactinomyxon types described here exhibited 99.4 % similarities to each other (Table 2), suggesting that this taxon have intraspecific variability, possibly due to different sampling locations. Hallett et al. (2004) reported that different morphometrical triactinomyxon types exhibited by morphological similarity were actually the same genotype. This was later supported by Eszterbauer et al. (2006), who similarly reported the same genotype can represent multiple morphotypes, suggesting that actinospore classification based solely on traditional morphological features can lead to erroneous associations. Therefore, DNA sequence analysis is recommended in species descriptions.

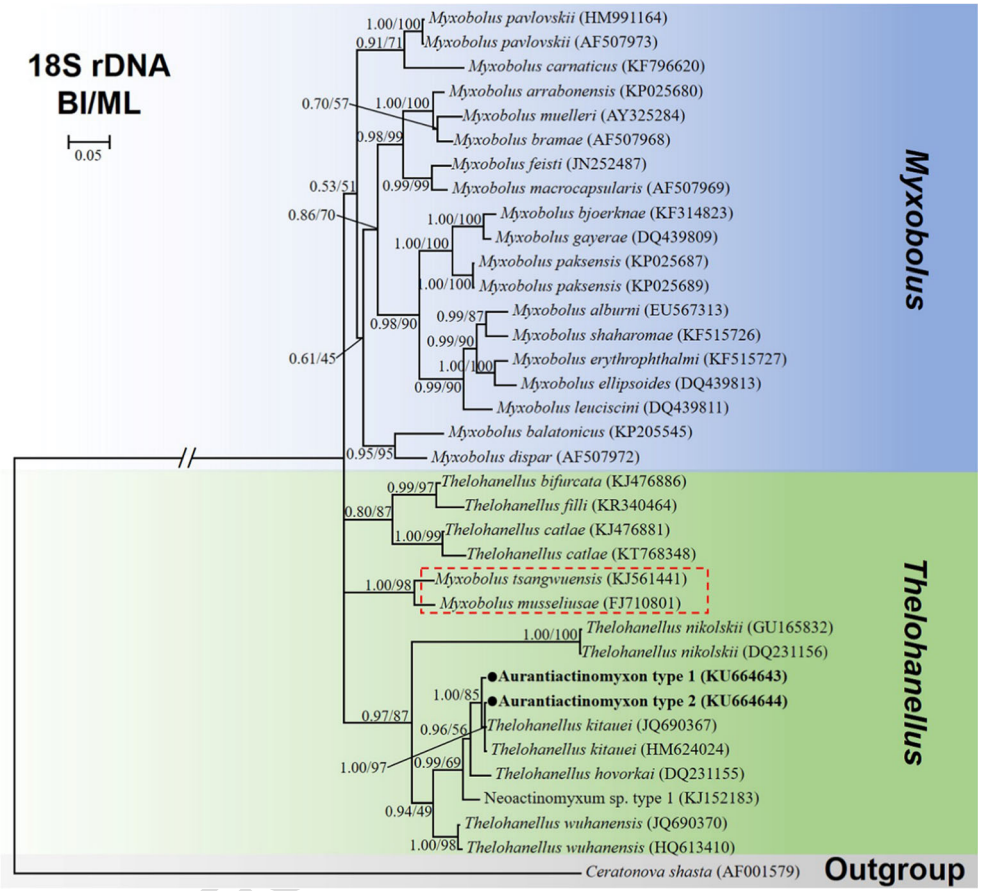

Fig. 3 Phylogenetic positions of Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2 based on 18S rDNA data analyzed using Bayesian analysis (BI). Numbers at nodes represent bootstrap support values of Bayesian posterior probabilities and maximum likelihood (ML). GenBank accession numbers are in parentheses. *Dotted box* represents two *Myxobolus* species are separated from the major *Myxobolus* clade. *Ceratonova shasta* is taken as outgroup. Aurantiactinomyxon type 1 and Aurantiactinomyxon type 2 examined in this study are in *bold*

In North China, common carp are the most prevalent cultured fish species with the highest production. *T. kitauei*, the pathogenic agent of intestinal giant-cystic disease of common carp, has the potential to cause catastrophic fish kills and severe economic losses in the aquaculture industry of North China (Chen & Ma 1998). Interestingly, the myxospore stage of *T. kitauei* has not been reported from Europe. Molnár (2009) hypothesized that European common carp originated from the Far-Eastern Amur-Chinese geographical region. During the long migration from China to Europe, the common carp lost its original parasite fauna. Then, after introduction of the Amur wild common carp and the color carp from Asia to Europe, several 'old' parasites of the common carp have been introduced and are gradually expanding. This would suggest that more recently described parasites of carp, at present known only in China, Japan and the Amur Basin might also make their way to Europe (Székely et al. 2015a, 2015b). This is the first report on the actinosporean stage of *T. kitauei* from Europe based on morphological, molecular and phylogenetic analyses. The Aurantiactinomyxon type 1 identified in the current study indicates that further investigations are urgently needed to find the myxosporean stage of *T. kitauei* in fish host, most probably, common carp in Europe.

So far, only three life cycles of *Thelohanellus* species have been elucidated, all with *B. sowerbyi* as invertebrate hosts. *T. hovorkai* and *T. nikolskii* involve aurantiactinomyxon as intraoligochaete developmental stages (Yokoyama 1997; Székely et al. 1998). *Thelohanellus wangi* involves

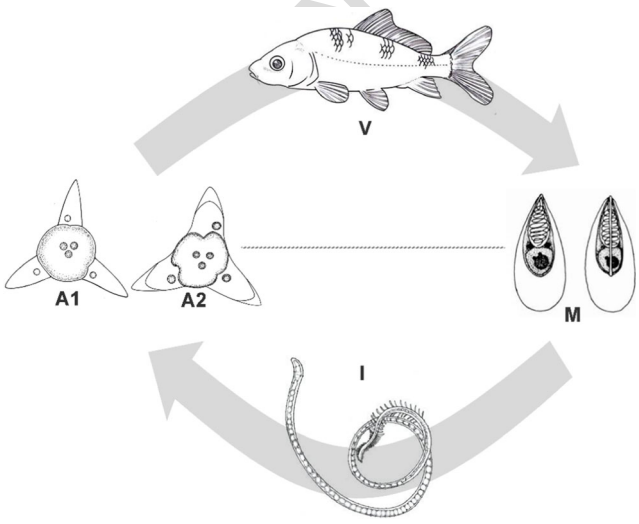

Fig. 4 Schematic illustration of *T. kitauei* life cycle: Aurantiactinomyxon type 1 (A1) and Aurantiactinomyxon type 2 (A2) actinospores infect the vertebrate host common carp (V) in which then myxospores (M) infecting the invertebrate host *B. sowerbyi* (I) develop

neoactinomyxum as extrapiscine alternating stage (Xi et al. 2015). This study confirms two distinct aurantiactinomyxon morphotypes as developmental stages of the life cycle of *T. kitauei* by morphological and molecular analyses and constitutes the fourth description of life cycle of *Thelohanellus* species.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent Informed consent was obtained from all individual participants included in the study. The publication only reflects the views of authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

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