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Morphological and molecular characterization of actinosporeans infecting oligochaete *Branchiura sowerbyi* from Chinese carp ponds

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ABSTRACT: We surveyed the actinosporean stages of fish myxosporeans at fish farms in Jiangsu Province, China, from 2011 to 2014. During the surveys, we identified 7 actinosporean types from 4 collective groups: echinactinomyxon (1 type), triactinomyxon (1 type), aurantiactinomyxon (1 type), and neoactinomyxum (4 types), released by the oligochaete *Branchiura sowerbyi*. The morphological characteristics and DNA sequences of these types are described here. Based on 18S rDNA sequence analysis, the actinosporean of echinactinomyxon type CZ with 4 branches at the end of the caudal processes was identified as *Myxobolus wulii*, and the neoactinomyxum type JD was identified as *Thelohanellus wangi* Yuan, Xi, Wang, Xie, Zhang, 2015 (JX458816), a recently nominated species from the gills of allogynogenetic gibel carp *Carassius auratus gibelio*. In addition, actinosporeans of aurantiactinomyxon type JD, neoactinomyxum type CZ-1, neoactinomyxum type CZ-2, and neoactinomyxum type CZ-3 showed high genetic similarity to *T. wuhanensis* (96.3–96.5%), *T. nikolskii* (98.0–99.1%), *T. wuhanensis* (97.8–98.9%), and *T. hovorkai* (98.7–98.9%), respectively. Phylogenetic analyses showed that these actinosporeans were robustly clustered in the *Thelohanellus* spp. clade.

KEY WORDS: Myxozoa · Actinospore · *Myxobolus* · *Thelohanellus*

INTRODUCTION

Actinosporeans from invertebrate hosts (mostly oligochaetes in freshwater and polychaetes in marine environments) are alternative stages of fish-parasitic myxosporeans. Prior to the pioneering research on the life cycle of *Myxobolus cerebralis* by Wolf & Markiw (1984), actinosporeans were considered as independent taxonomic entities, and classified into 20 collective groups with about 150 morphotypes reported in recent years (Özer et al. 2002, Rácz et al. 2005, Lom & Dyková 2006, Yokoyama et al. 2012, Borkhanuddin et al. 2014, Székely et al. 2014). Most actinosporeans have been collected from fish farms (e.g. El-Mansy et al. 1998a, Özer et al. 2002, Eszterbauer et al. 2006, Xi et al. 2013) and natural waters (e.g. El-Mansy et al. 1998b, Székely et al. 2002, Marcucci et al. 2009, Borkhanuddin et al. 2014, Székely et al. 2014), and characterized based on morphological data. However, morphological variability within a given actinosporean type usually confounds exact identification (Hallett et al. 2004, Eszterbauer et al. 2006). Therefore, in recent studies, actinosporeans have been characterized based on genetic data in combination with morphometric data (e.g. Zhai et al. 2012, Xi et al. 2013, Borkhanuddin et al. 2014, Székely et al. 2014).

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Thus far, about 50 actinosporean stages of fish-parasitic myxosporeans have been identified (Yokoyama et al. 2012, Székely et al. 2014). Most of the known actinosporean–myxosporean pairs were identified through experimental infections, and others were determined based on DNA sequence analysis. In general, experimental infections are laborious and time consuming. More importantly, the host oligochaete used must be free from other myxosporean contamination. Analysis of the 18S rDNA sequences in such cases has significantly facilitated accurate and reliable identification of myxozoans (Kallert et al. 2005, Caffara et al. 2009, Marton & Eszterbauer 2011, Urawa et al. 2011, Székely et al. 2014).

Few studies have investigated actinosporeans from oligochaetes in China. To date, only 5 actinosporean types belonging to 4 collective groups (triactinomyxon, raabeia, aurantiactinomyxon, and guyenotia) have been described from the oligochaete *Branchiura sowerbyi* (Wang & Yao 2000, Zhai et al. 2012, Xi et al. 2013). Xi et al. (2013) successfully identified an actinospore of the raabeia type as the intra-oligochaete developing stage of *M. cultus* based on 18S rDNA sequence analysis.

Myxozoans of the genera *Myxobolus* and *Thelohanellus* are important pathogens affecting the culture of allogynogenetic gibel carp *Carassius auratus gibelio*. Some species cause severe fish diseases and mortality, such as *M. honghuensis* forming giant cysts in the pharynx, *M. wulii* infecting and seriously destroying the liver, *M. ampullicapsulatus* forming numerous cysts in the gill, and *T. wuhanensis* developing numerous cysts in the skin (Xi et al. 2013). To understand the transmission and life cycle of these myxosporeans, we carried out several surveys of the actinospore fauna in fish farms. Here, we characterize 7 actinosporean types belonging to 4 collective groups, viz. echinactinomyxon, triactinomyxon, aurantiactinomyxon, and neoactinomyxum, based on morphometric and molecular data. The myxosporean counterparts of these actinosporeans were also identified through 18S rDNA sequence analysis.

MATERIALS AND METHODS

Oligochaete and actinospore collection

The actinosporean fauna surveys were conducted at 4 fish farms in Jiangsu Province, China, from May 2011 to July 2014. The allogynogenetic gibel carp was the main cultured fish species in these farms, and was usually grown with grass carp *Ctenopharyngodon idella*, silver carp *Hypophthalmichthys molitrix*, bighead carp *Aristichthys nobilis*, and blunt snout bream *Megalobrama amblycephala*. Benthic sediment from these farms was dredged and sieved through a 0.3 mm mesh. Oligochaetes were collected and transferred alive to the laboratory in plastic containers with pond water. In the laboratory, oligochaetes were placed in a tray, hand-sorted using a Pasteur pipette or bend-needle, and kept individually in 'cell well' (2×3) plates with dechlorinated tap water. Water from each well was then transferred to an algae-counting chamber (20 × 50 mm) and examined for actinospores under a microscope (Olympus CX41). When floating actinospores were detected, the water was retained and stored at –70°C for DNA extraction. The voucher specimens were stored in 80% ethanol at room temperature. Photomicrographs were taken from fresh actinospores under bright light and phase contrast through a microscope (Nikon ti-u). Morphological characteristics of at least 20 spores were measured based on previous descriptions (Lom et al. 1997, Yokoyama et al. 2012). All measurements were in micrometers (µm). Germ cell counts in the actinospores were determined by gently squeezing the actinospores on slides with a coverslip to disrupt the sporoplasm. Line drawings of actinospores were made based on the images.

Molecular analysis

The water containing the actinospores (10–30) was centrifuged at 2700 × *g* for 10 min and the pellet was dissolved with lysis buffer for microorganisms (TaKaRa). The suspension was incubated at 80°C for 15 min and then stored at –20°C until further use. The partial 18S small-subunit ribosomal DNA (rDNA) sequence was amplified by nested-PCR with the inner-primers ERIB1 and ERIB10 (Barta et al. 1997) and the outer-primers MyxospecF and MyxospecR (Fiala 2006). The actinospore lysate (1/10 of the total PCR reaction) were used as template in the first round of amplification. Amplified products were purified using the QIAquick Gel Extraction Kit and cloned into the pMD-18T vector system (Takara). Positive clones were selected and sequenced using the ABI BigDye Terminator 3.1 Cycle Sequencing Kit on an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). Homologous sequences were determined by performing a BLASTN search at NCBI. DNA sequence similarities (p-distance) were calculated using the MEGA 6.0 software package (Tamura et al. 2013).

Phylogenetic analyses of the actinosporean sequences from this study and myxozoan sequences in GenBank were conducted using MEGA 6.0 based on the partial 18S rDNA sequences. Minimum evolution (ME) analysis was performed using the Tamura-Nei substitution model, with gamma distribution and close-neighbor-interchange algorithm at search level 1. Maximum parsimony (MP) analysis was performed using a tree-bisection-reconnection (TBR) algorithm at search level 1. Maximum likelihood (ML) analysis was performed using a general-time-reversible model with gamma distribution. Phylogeny tests were calculated with 1000 bootstrap replicates.

RESULTS

During the 4 yr survey, 7 types of actinosporeans belonging to 4 collective groups were identified, viz. echinactinomyxon (1 type), triactinomyxon (1 type), aurantiactinomyxon (1 type), and neoactinomyxum (4 types).

Echinactinomyxon type CZ

Description: Spores with 3 elongated, equal sized caudal processes; style absent; caudal processes originate just below the spore body without curving, and with 4 short branches at the distal end. Spore body cylindrical, elongated in side view, 19.2 µm (18.5–20.0) in length, 9.2 µm (8.5–10.2) in width. Polar capsules pear-shaped in side view, 5.8 µm (5.5–6.2) long and 3.0 µm (2.6–3.5) wide. Caudal processes 163.4 µm (158.5–167.2) long and 3.5 µm (3.0–3.8) wide (Fig. 1, Table 1).

Host: *Branchiura sowerbyi*

Locality: Changzhou, Jiangsu Province, China (31.73° N, 119.56° E)

Prevalence: 0.4 % (1/230)

Remarks: The spore morphology of echinactinomyxon type CZ is very similar to echinactinomyxon type 2 of Negredo & Mulcahy (2001), echinactinomyxon type 4 reported by Özer et al. (2002), echinactinomyxon type 1 described by Oumouna et al. (2003), and echinactinomyxon type of Marcucci et al. (2009). However, echinactinomyxon type CZ displayed longer caudal processes and a smaller spore body (Table 1). Unlike most echinactinomyxons reported previously, echinactinomyxon type CZ spores possess 4 small branches at the ends of the caudal processes, which are recorded only in a few actinosporean types. Raabeia type 1 of Özer et al. (2002)

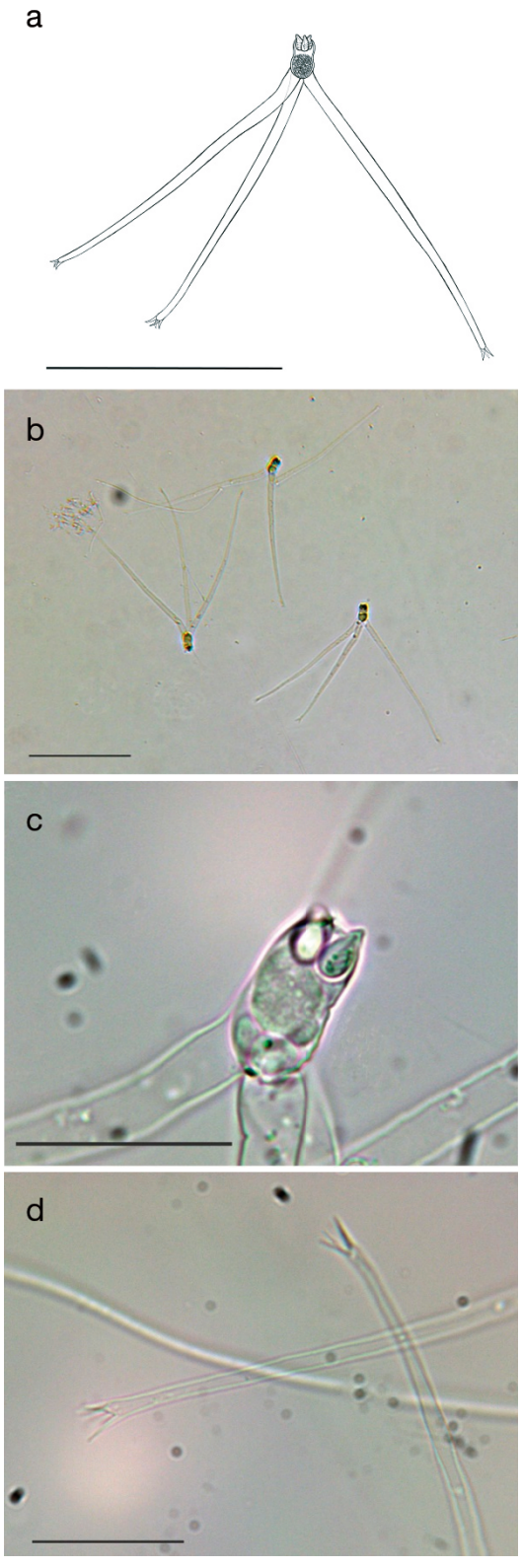

Fig. 1. Echinoactinomyxon type CZ. (a) Line drawing of mature actinospore; (b) waterborne spores; (c) spore body; (d) branched end of the caudal process. Scale bars = (a,b) 100 µm, (c,d) 20 µm

Table 1. Measurements (means, with ranges in parentheses; µm) of freshly released waterborne actinosporeans from the oligochaete host *Branchiura sowerbyi*. L: length; W: width; D: diameter; nd: no data

Actinosporean	Caudal processes		Spore body		Polar capsule		Style	No. of germ cells
	L	W	L	W	L	W	L	
Echinactinomyxon type LY	163.4 (158.5–167.2)	3.5 (3.0–3.8)	19.2 (18.5–20.0)	9.2 (8.5–10.2)	5.8 (5.5–6.2)	3.0 (2.6–3.5)	Absent	nd
Triactinomyxon type CZ	214.0 (203.2–225.8)	13.4 (12.3–13.8)	27.1 (25.3–29.0)	13.2 (11.9–15.4)	6.8 (6.5–7.3)	4.3 (3.9–4.5)	119.7 (112.2–126.5)	16
Aurantiactinomyxon type JD	21.7 (20.0–24.4)	14.0 (11.2–16.4)	15.6	21.2 (17.1–24.0)	D: 2.3 (2.0–2.8)		Absent	>30
Neoactinomyxum type JD	9.3 (7.9–10.3)	25.6 (23.2–29.7)	20.7	22.4 (19.5–25.4)	D: 2.6 (2.0-3.2)		Absent	nd
Neoactinomyxum type CZ-1	8.6 (7.0–10.6)	15.2 (13.0–17.5)	20.5 (18.2–21.3)	28.1 (23.8–31.2)	D: 3.0 (2.8–3.5)		Absent	32
Neoactinomyxum type CZ-2	7.5 (6.6–8.4)	18.4 (16.8–21.5)	18.6 (17.8–19.5)	20.7 (19.8–22.0)	D: 2.7 (2.4–2.8)		Absent	nd
Neoactinomyxum type CZ-3	12.2 (9.0–13.7)	16.0 (15.0–16.7)	18.5	22.1 (21.0–23.2)	D: 2.8 (2.3–3.3)		Absent	nd

released from immature oligochaetes also had 4 branches at the tips of caudal processes, but these caudal processes were shorter than the echinactinomyxon type CZ (94.5 vs. 163.4 µm) and the spore body was rounded (length/width: 18.1/15.7 vs. 19.2/9.2 µm). Raabeia type 2 of Özer et al. (2002) released from *Lumbriculus variegatus* showed a rounded spore body (length/width: 18.1/16.1 vs. 19.2/9.2 µm) and 4 branches arranged in 2 pairs at the tips of the caudal processes. The echinactinomyxon type reported by Marcucci et al. (2009) was released from immature oligochaetes (Lumbriculidae) and showed similar morphological characters but had shorter caudal processes compared to the actinospores in our study. The partial 18S rDNA sequence of the echinactinomyxon type CZ, 879 bp long, was obtained from 7 clones (GenBank, accession no. KP642131–2). Sequence variation between clones was 0–0.3% (Table 2). On the basis of the DNA sequences, echinactinomyxon type CZ showed 99.7–99.9% identity to *Myxobolus wulii* (HQ613412) collected from the liver of *Carassius auratus gibelio. M. wulii* is a very common myxosporean in allogynogenetic gibel carp in Jiansu Province.

Triactinomyxon type CZ

Description: Spores with a style and 3 anchor-shaped caudal processes with pointed ends. Spore body barrel-shaped in side view, 27.1 µm (25.3–29.0) long and 13.2 µm (11.9–15.4) wide. Polar capsules pyriform in side view, positioned at the epispore apex, 6.8 µm (6.5–7.3) in length, and 4.3 µm (3.9–4.5) in width. Style 119.7 µm (112.2–126.5) long. Caudal processes slightly curved upwards, tapering toward distal ends, 214.0 µm (203.2–225.8) long and 13.4 µm (12.3–13.8) wide; 16 germ cells present (Fig. 2, Table 1).

Host: *Branchiura sowerbyi*

Locality: Changzhou, Jiangsu Province, China (31.53° N, 119.89° E)

Prevalence: 1% (2/200)

Remarks: The collective group triactinomyxon contains the most diverse actinospore types (>50), representing almost 1/4 of the actinosporeans recorded. Triactinomyxon type CZ differs from other morphotypes reported previously in the group by having a barrel-shaped spore body and anchor-shaped caudal processes that are slightly curved upwards, and the ratio of the caudal processes:style is 1.8. However, it closely resembles triactinosporean type 4 of El-Mansy et al. (1998b), which possessed much longer caudal process (281.7 vs. 214.0 µm) and style (149.0

Table 2. Summary of the genetic similarity between the actinosporean types and the closest myxozoans based on the partial 18S rDNA sequence

Actinosporean	No. of clones sequenced	Sequence identity (%)	GenBank accession no(s).	Closest match in GenBank		
				Similarity (%)	Species	Accession no.
Echinactinomyxon type LY	7	99.7–100	KP642131–32	99.7–99.9	*Myxobolus wulii* ex *Carassius auratus gibelio*	HQ613412
				99.8–99.9	*Myxobolus wulii*	EF690300
				99.7–99.9	*Myxobolus wulii*	KJ725081
Triactinomyxon type CZ	5	99.9–100	JX477771	87.0–90.4	*Chloromyxum legeri*	AY604197
				87.0–89.8	*Sphaerospora oncorhynchi*	AF201373
Aurantiactinomyxon type JD	5	99.9–100	KP642133–34	96.3–96.5	*Thelohanellus wuhanensis*	JQ088179
				96.3–96.5	*Thelohanellus wuhanensis*	HQ613410
Neoactinomyxum type JD	6	99.5–100	KP642135–36	99.6–100	*Thelohanellus* sp.	JX458816
				83.5–84.2	*Thelohanellus wuhanensis*	JQ968687
Neoactinomyxum type CZ-1	15	98.3–99.9	KP642137–38	98.0–99.1	*Thelohanellus nikolskii* ex *Cyprinus carpio*	DQ231156
				98.0–99.1	*Thelohanellus nikolskii*	GU165832
Neoactinomyxum type CZ-2	8	98.3–99.9	KP642139–40	98.3–99.9	Neoactinomyxum type A2	DQ231149
				97.8–98.9	*Thelohanellus wuhanensis* ex *Carassius auratus gibelio*	HQ613410
Neoactinomyxum type CZ-3	12	99.2–99.9	KP642141–42	98.7–98.9	*Thelohanellus hovorkai*	AJ133419
				98.1–98.6	Neoactinomyxum type B2	DQ231151

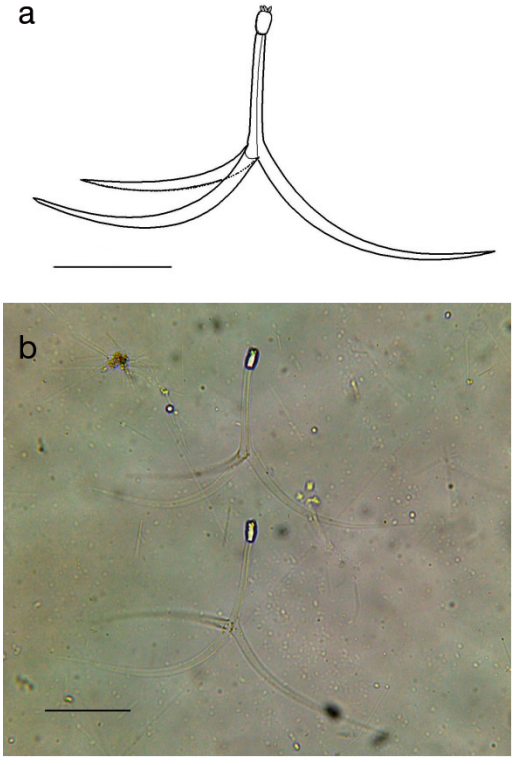

Fig. 2. Triactinomyxon type CZ. (a) Line drawing of mature actinospore; (b) waterborne spores. Scale bars = 100 µm

vs. 119.7 µm). The partial 18S rDNA sequence of triactinomyxon type CZ was obtained from 5 clones, and the consensus sequence (2037 bp) was deposited in GenBank under accession number JX477771. Sequence variation between clones was 0–0.1% (Table 2). Actinosporean type CZ did not match any 18S rDNA sequence in GenBank. The closest sequence similarity was with *Chloromyxum legeri* (AY604197; 87.0–90.4%) and *Sphaerospora oncorhynchi* (AF201373; 87.0–89.8%).

Aurantiactinomyxon type JD

Description: Spores with 3 broad, triangular caudal processes; style absent; spore body trefoil-shaped in apical view, globular in side view, and entirely within the base of the processes; caudal processes extend slightly downward in side view, rounded at the ends and equal in length; 3 drop-like polar capsules positioned closely at the epispore apex. Spore body 15.6 µm in length (determined from 1 spore), 21.2 µm (17.1–24.0) in width (from the middle of the lobe to the opposite interlobular part). Caudal processes 21.7 µm (20.0–24.4) long and 14.0 µm (11.2–16.4)

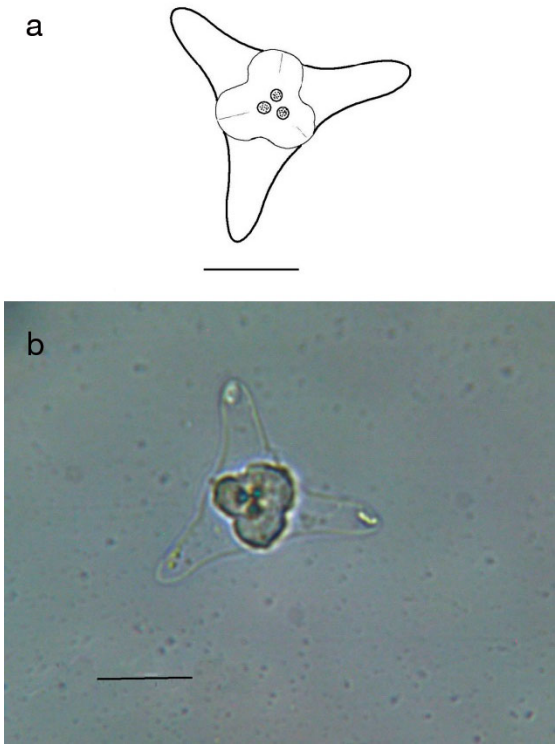

Fig. 3. Aurantiactinomyxon type JD. (a) Line drawing of mature actinospore; (b) waterborne spores. Scale bars = 20 µm

wide. Polar capsules 2.3 µm (2.0–2.8) in diameter; >30 germ cells (Fig. 3, Table 1).

Host: *Branchiura sowerbyi*

Locality: Jiangdu, Jiangsu Province, China (32.67° N, 119.53° E)

Prevalence: 0.7% (1/150)

Remarks: The shape and measurement of this type closely resembled the actinospores of *Thelohanellus hovorkai* described by Yokoyama (1997) and Székely et al. (1998), but with smaller dimensions. The partial 18S rDNA sequence of aurantiactinosporean type JD, 789–905 bp in length, was obtained from 5 clones (GenBank KP642131–2). Sequence variation between clones was 0–0.1% (Table 2). Based on DNA sequence analysis, aurantiactinosporean type JD showed the highest genetic similarity with *T. wuhanensis* (JQ088179 and HQ613410; 96.3–96.5%). *T. wuhanensis* is an important myxosporean pathogen of the allogynogenetic gibel carp, and parasitizes the skin of 1 yr old larval fish.

Neoactinomyxum type JD

Description: Spore elliptical in side view and triangular in apical view; style absent; 3 caudal processes wide, short, crescent-shaped, and equal in length, entirely embracing the spore body at the bases; spore body globular in form; 3 drop-like polar capsules positioned at the epispore apex. Spore body 20.7 µm in length (1 spore), 22.4 µm (19.5–25.4) in width. Caudal processes 9.3 µm (7.9–10.3) long and 25.6 µm (23.2–29.7) wide. Polar capsules 2.6 µm (2.0–3.2) in diameter (Fig. 4, Table 1).

Host: *Branchiura sowerbyi*

Locality: Jiangdu, Jiangsu Province, China (32.65° N, 119.53° E)

Prevalence: 0.4% (1/250)

Remarks: The partial 18S rDNA sequence of neoactinomyxum type JD, 792–887 bp in length, was obtained from 6 clones (GenBank KP642135–6). Sequence variation between clones was 0–0.5% (Table 2). In shape and measurement, neoactinomyxum type JD closely resembled neoactinomyxum type A1 reported by Eszterbauer et al. (2006) from *B. sowerbyi* at a fish farm, and neoactinomyxum type 1 of Borkhanuddin et al. (2014) isolated from *Isochaetides michaelseni* in the Kis Balaton water reservoir in Hungary. However, the DNA sequence analysis did not confirm this morphological similarity. Neoactinomyxum type JD showed less than 83% sequence similarity with neoactinomyxum type A1 (Eszterbauer et al. 2006) and neoactinomyxum type 1 (Borkhanuddin et al. 2014), while it was 99.6–100%

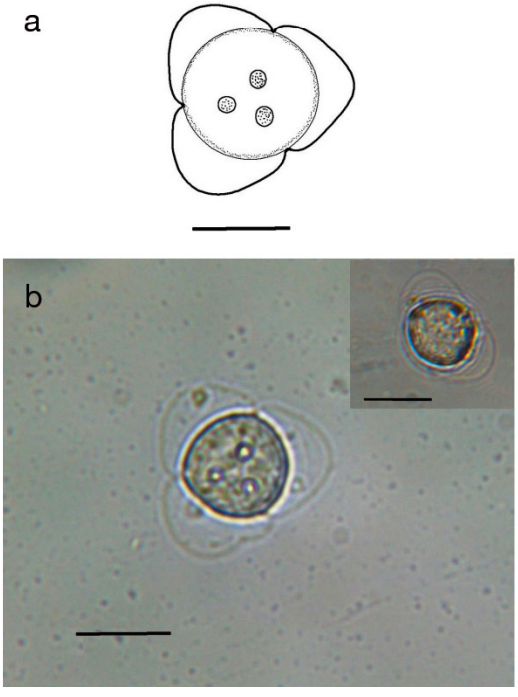

Fig. 4. Neoactinomyxum type JD. (a) Line drawing of mature actinospore; (b) waterborne spores. Inset: side view of the spore. Scale bars = 20 µm

similar to *T. wangi* (Yuan et al. 2015) (JX458816), which was collected from the gills of allogynogenetic gibel carp.

Neoactinomyxum type CZ-1

Description: Spores triangular in apical view, semiglobular in side view; style absent; 3 caudal processes wide, short, triangular-sepal-like, embraced entirely within the spore body at the bases, and extended slightly downward in side view; spore body typically trefoil-shaped, with marked interlobular retraction; 3 drop-like polar capsules positioned at the epispore apex. Spore body 20.5 µm (18.2–21.3) in length, 28.1 µm (23.8–31.2) in width (from the middle of the lobe to the opposite interlobular part). Caudal processes 8.6 µm (7.0–10.6) long and 15.2 µm (13.0–17.5) wide. Polar capsules 3.0 µm (2.8–3.5) in diameter; 32 germ cells (Fig. 5, Table 1).

Host: *Branchiura sowerbyi*

Locality: Changzhou, Jiangsu Province, China (31.78° N, 120.16° E)

Prevalence: 0.4% (3/720)

Remarks: The partial 18S rDNA sequence of neoactinomyxum type CZ-1, 896 bp long, was obtained from 15 clones (GenBank KP642137–8). Sequence variation between the clones was 0.1–1.7% (Table 2). Neoactinomyxum type CZ-1 showed closely similar morphometric characters with neoactinomyxum type B1 of Eszterbauer et al. (2006) and the neoactinomyxum type of Molnár et al. (1999). However, this actinosporean differed significantly from other neoactinomyxum types reported previously by having a different shape of the spore body and caudal processes. Based on DNA sequence analysis, neoactinomyxum type CZ-1 showed the highest genetic similarity to *T. nikolskii* (DQ231156 and GU165832; 98.0–99.1%) collected from common carp.

Neoactinomyxum type CZ-2

Description: Spores triangular in apical view, elliptical in side view; style absent; 3 caudal processes wide, short, triangular, with rounded ends, embracing the spore body entirely at the bases; spore body triangular or elliptical in apical view, and elliptical in side view; 3 drop-like polar capsules positioned closely at the epispore apex. Spore body 18.6 µm (17.8–19.5) in length and 20.7 µm (19.8–22.0) wide. Caudal processes 7.5 µm (6.6–8.4) long and 18.4 µm (16.8–21.5) wide. Polar capsules 2.7 µm (2.4–2.8) in diameter (Fig. 6, Table 1).

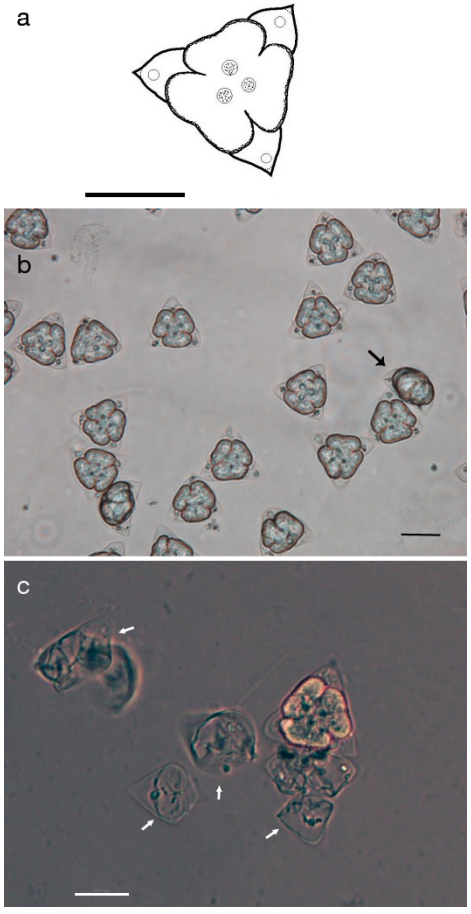

Fig. 5. Neoactinomyxum type CZ-1. (a) Line drawing of mature actinospores; (b) waterborne spores, arrow indicates the spore side-view; (c) waterborne spores, arrows indicate the caudal processes. Scale bars = 20 µm

Host: *Branchiura sowerbyi*

Locality: Changzhou, Jiangsu Province, China (31.78° N, 120.16° E)

Prevalence: 0.6% (4/720)

Remarks: The partial 18S rDNA sequence of neoactinomyxum type CZ-2, 905 bp long, was obtained from 8 clones (GenBank KP642139–40). Sequence variation between clones was 0.1–1.7% (Table 2). The shape and measurements of this actinosporean closely resembled neoactinomyxum type A2 of Eszterbauer et al. (2006). At the DNA level, neoactinomyxum type CZ-2 shared the highest similarity with neoactinomyxum type A2 (Eszterbauer et al. 2006) (DQ231149; 98.3–99.9%) and *T. wuhanensis* (HQ613410; 97.8–98.9%) collected from the skin of allogynogenetic gibel carp.

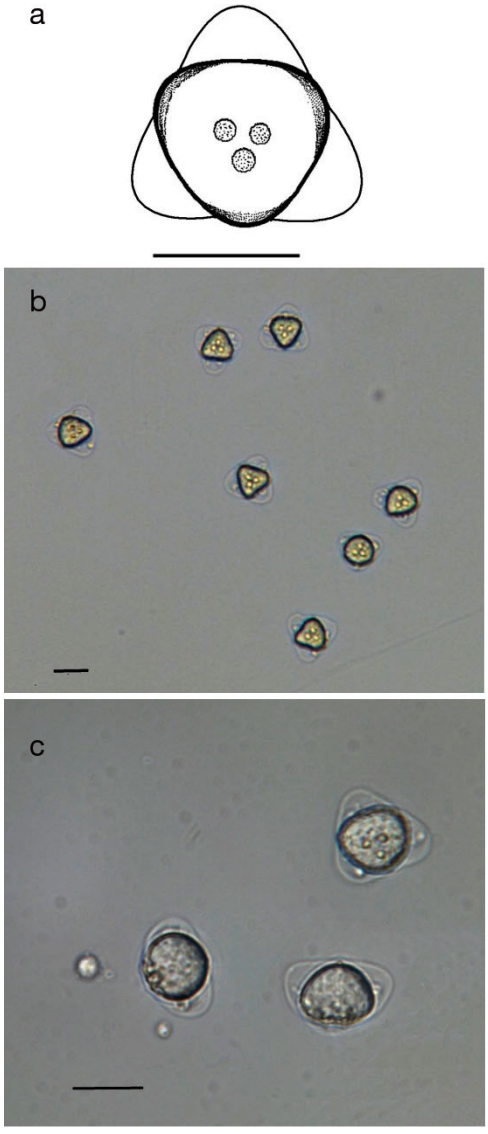

Fig. 6. Neoactinomyxum type CZ-2. (a) Line drawing of mature actinospore; (b,c) waterborne spores. Scale bars = 20 µm

Neoactinomyxum type CZ-3

Description: Spores triangular in apical and side view; style absent; 3 caudal processes short, wide, triangular-shaped, entirely embrace the spore body at the bases; spore body trefoil-shaped in apical view with marked interlobular retraction; 3 drop-like polar capsules positioned closely at the epispore apex. Spore body 18.5 µm in length (determined from 1 spore), 22.1 µm (21.0–23.2) in width. Caudal processes 12.2 µm (9.0–13.7) long and 16.0 µm (15.0–16.7) wide. Polar capsules 2.8 µm (2.3–3.3) in diameter. (Fig. 7, Table 1)

Host: *Branchiura sowerbyi*

Locality: Changzhou, Jiangsu Province, China (31.78° N, 120.16° E)

Prevalence: 0.3% (2/720)

Remarks: The partial 18S rDNA sequence of neoactinomyxum type CZ-3, 903 bp long, was determined from 12 clones (GenBank KP642141–2). Sequence variation between clones was 0.1–0.8% (Table 2). Neoactinomyxum type CZ-3 closely resembled neoactinomyxum type 1 and type 4 reported by El-Mansy et al. (1998a), and differed from neoactinomyxum type B2 of Eszterbauer et al. (2006) in possessing differently shaped ends in the caudal processes (triangular vs. rounded). At the DNA level, neoactinomyxum type CZ-3 showed the highest genetic similarity with *T. hovorkai* from Japan (AJ 133419; 98.7–98.9%), and neoactinomyxum type B2 determined by Eszterbauer et al. (2006) (DQ231151; 98.1–98.6%).

Phylogenetic analyses

Phylogenetic analyses were performed on 13 newly obtained 18S rDNA sequences and 83 myxozoan sequences, which consisted of actinosporeans and their closest related myxosporeans retrieved from GenBank. The phylogenetic trees constructed using ME, MP, and ML methods showed similar topological structures, but several branches were clustered in different positions (Fig. 8). In the phylogenetic trees, the echinactinomyxon type CZ was firmly clustered with

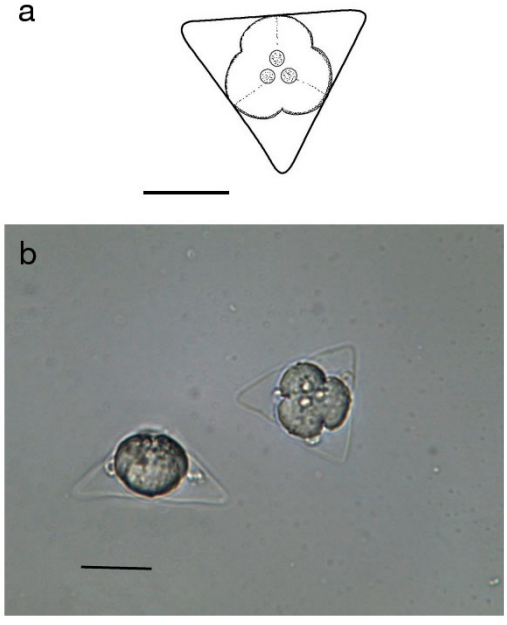

Fig. 7. Neoactinomyxum type CZ-3. (a) Line drawing of mature actinospores; (b) waterborne spores. Scale bars = 20 µm

Myxobolus wulii. Triactinomyxon type CZ was clustered with *Sphaerospora oncorhynchi* (AF201373) and echinactinomyxon type 1 (AJ582000), but showed long phylogenetic branches. The actinosporeans of the collective groups aurantiactinomyxon and neoactinomyxum examined here were all clustered in the clade consisting of myxosporeans *Thelohanellus* spp. Aurantiactinomyxon type JD formed a separate branch and closely clustered with a sub-clade formed by neoactinomyxum type CZ-2, neoactinomyxum type A2 reported by Eszterbauer et al. (2006) (DQ 231149), and the myxosporean *T. wuhanensis* (JQ 088179, HQ613410, JQ968687). Neoactinomyxum type JD was firmly clustered with *T. wangi* (JX458816) and formed a separate branch. Neoactinomyxum type CZ-1 was clustered with *T. nikolskii* (DQ231156, GU165832) with a robust branch support. Neoactinomyxum type CZ-1 was firmly located in the sub-clade comprising *T. hovorkai* (AJ133419), neoactinomyxum type 1 of Borkhanuddin et al. (2014) (KJ152183), and neoactinomyxum types B1 (DQ231150) and B2 (DQ231151, DQ231152) (Eszterbauer et al. 2006).

DISCUSSION

In China, about 570 myxosporeans have been recorded, with some species causing severe pathogenesis in cultured and wild fish (Chen & Ma 1998, Zhang et al. 2010, Xi et al. 2011). However, studies on the actinosporean stage of fish parasitic myxosporeans are scarce. In this report, we identified 7 actinosporean types from the oligochaete *Branchiura sowerbyi* in carp pond sediments. These belonged to the 4 collective groups echinactinomyxon, triactinomyxon, aurantiactinomyxon, and neoactinomyxum. The number of actinosporeans reported in China has now increased to 12, including actinosporean raabeia, guyenotia, aurantiactinomyxon types described by Xi et al. (2013), 1 triactinosporean type of Zhai et al. (2012), and 1 triactinosporean type of Wang & Yao (2000). However, all of these actinospores examined were limited to the oligochaete host *B. sowerbyi.* In geographic locations other than China, actinosporeans have been detected in many kinds of freshwater oligochaetes, such as *Limodrilus hoffmeisteri*, *Tubifex tubifex*, *Lumbriculus variegatus*, and *Isochaeides michaelseni* (e.g. Özer et al. 2002, Székely et al. 2014). In the fish ponds that we examined, *B. sowerbyi* was the dominant species in the oligochaete community, although a few other oligochaetes were collected. In contrast, benthic oligochaetes in natural waters, such as Lake Taihu, have higher species diversity, and *L. hoffmeisteri* is usually the dominant species (Cai et al. 2010, Chen et al. 2013). Therefore, a more extensive investigation will be needed to reveal the actinospore diversity in China.

By comparing their morphological characters, the actinosporeans examined in this study were characterized as echinactinomyxon type CZ, triactinomyxon type CZ, aurantiactinomyxon type JD, neoactinomyxum type CZ-1, and neoactinomyxum type CZ-3, respectively, and differed markedly from the previously reported actinosporean types in their spore shape and the dimensions of the caudal processes. The limited number of distinguishable morphological features in actinospores usually makes correct identification difficult, thus DNA sequence analyses are essential to accurately describe actinosporeans (Hallett et al. 2004, Eszterbauer et al. 2006, Caffara et al. 2009). At the DNA level, the high genetic divergence between the actinosporeans identified in this study and the actinosporeans retrieved from GenBank also confirmed that they were new actinosporean types. Neoactinomyxum type JD closely resembled neoactinomyxum type A1 reported by Eszterbauer et al. (2006) and neoactinomyxum type 1 of Borkhanuddin et al. (2014), although the genetic similarity between them was less than 84.0%. Neoactinomyxum type CZ-2 also closely resembled neoactinomyxum type A2 of Eszterbauer et al. (2006) (DQ231149), and 4 of 8 sequenced clones showed 99.7–99.9% sequence identity over a 692 bp 18S rDNA fragment, but the other 4 clones showed only 98.4–98.3%. Whether the neoactinomyxum type A2 examined comprises 2 genotypes or represents distinct species requires further analysis.

Analyses of DNA sequence data of the actinosporeans revealed that the 18S rDNA sequence of echinoactinomyxon type CZ was 99.7–99.9% similar to the fish myxosporean *Myxobolus wulii* (Landsberg & Lom, 1991) (syn., *Myxosoma magna* Wu & Li, 1986, *Myxobolus guanqiaoensis* Wu & Wang, 1997). Sequence identity among 7 clones from this actinosporean was very high (99.7–100%), indicating that echinoactinomyxon type CZ correspond to the actinosporean stage of *M. wulii*, an important pathogenic myxozoan of the goldfish *Carassius auratus auratus* and allogynogenetic gibel carp in Japan and China. Infection by *M. wulii* results in a swollen abdomen and destruction of liver tissues in the fish (Zhang et al. 2010). This is the first study to report 2 distinct spore stages in the life cycle of *M. wulii* based on DNA sequences.

The DNA sequence of neoactinomyxum type JD showed 99.6–100% sequence identity with *Thelo-*

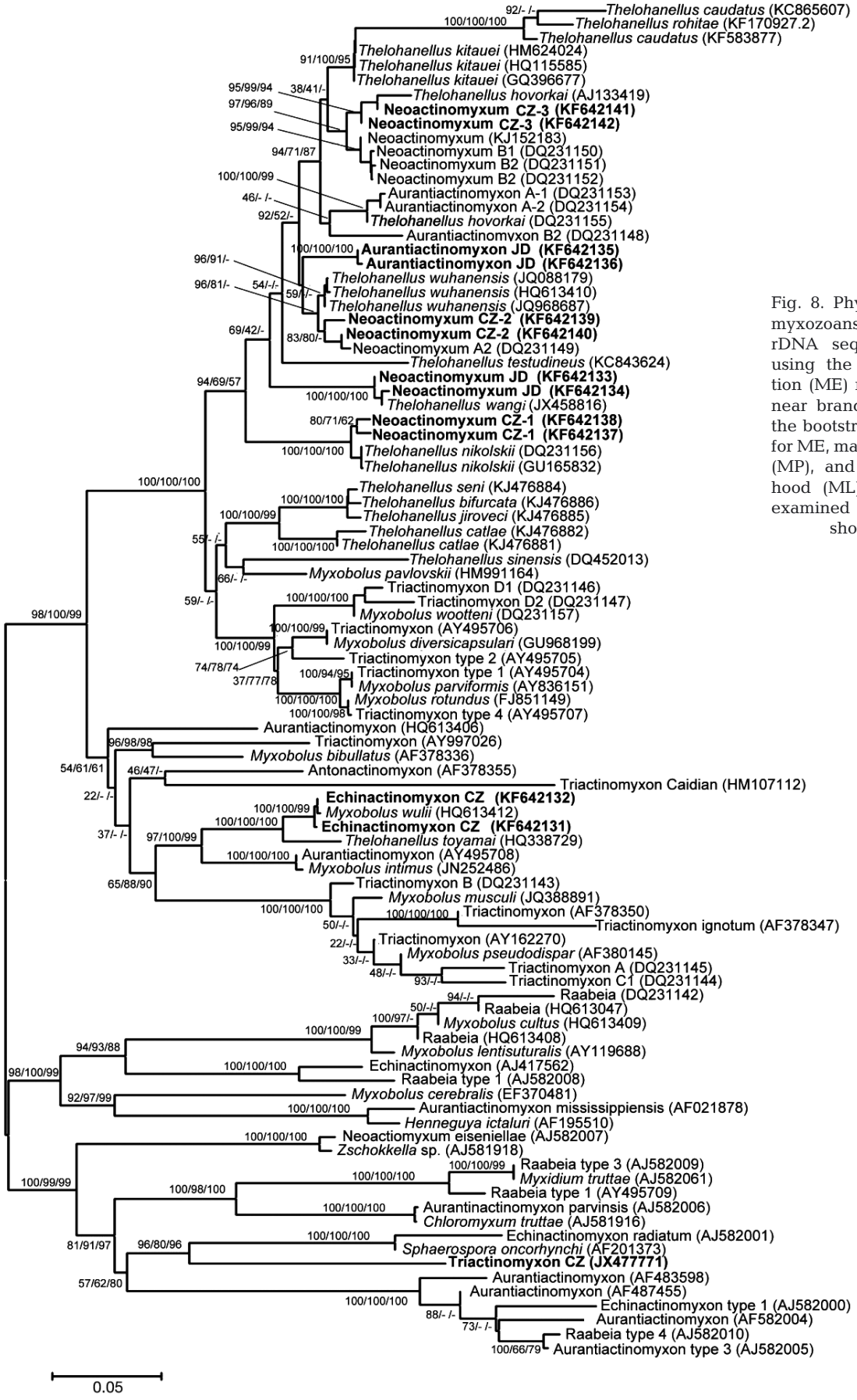

Fig. 8. Phylogenetic tree of myxozoans based on the 18S rDNA sequences analyzed using the minimum evolution (ME) method. Numbers near branch nodes indicate the bootstrap support values for ME, maximum parsimony (MP), and maximum likelihood (ML). Actinosporeans examined in this study are shown in **bold**

hanellus wangi, a newly discovered and nominated species detected from the gills of allogynogenetic gibel carp at a fry nursery farm (Yuan et al. 2015). We have also found and identified the actinosporean *T. wangi* released by *B. sowerbyi* from a different fish farm.

In the phylogenetic trees, neoactinomyxum type CZ-1 clustered with *T. nikolskii*, a fish myxosporean that develops large plasmodia on the fins of common carp. Their sequence similarity was 98.0–99.1%, which is consistent with the genetic variation between the 15 clones of neoactinomyxum type CZ-1 (98.3–99.9%). The intraoligochaete development of *T. nikolskii* was previously demonstrated by Székely et al. (1998), and its actinospore stage, a neoactinomyxum type, was detected in experimentally infected *T. tubifex*. However, neoactinomyxum type CZ-1 reported here differed markedly from the neoactinomyxum type reported by Székely et al. (1998) in the shape of the spore body (trefoil-shaped vs. globate) and caudal processes (triangular-sepal-like vs. triangle-shaped). Ruidisch et al. (1991) described the actinospore stage of *Myxobolus pavlovskii* as a hexactinomyxon type after a laboratory infection. This report was later found to be inconsistent with the results of Marton & Eszterbauer (2011), who found an echinactinomyxon-type actinospore with 100% 18S rDNA sequence identity with *M. pavlovskii*. Therefore, DNA sequence analysis is essential for artificial infection studies to avoid misidentification caused by contamination from other myxozoans.

In China, about 50 myxosporeans in the genus *Thelohanellus* have been described from different fishes (Chen & Ma 1998). However, 18S rDNA sequences have been determined for only a few species, including *T. kitauei* and *T. wuhanensis*. The limited comparative DNA data from myxosporeans constrained the specific identification of actinosporeans. Although the actinospores aurantiactinomyxon type JD, neoactinomyxum type CZ-2, and neoactinomyxum type CZ-3 did not match any myxospore entity found in GenBank, their high genetic similarity to *T. wuhanensis* and *T. hovorkai* (96.3–96.5%, 97.8–98.9%, and 98.7–98.9%, respectively; Table 2) and being clustered robustly in the clade of *Thelohanellus* spp., suggested that these actinosporeans have close affinities to myxosporeans of the genus *Thelohanellus*.

In conclusion, by morphological and molecular characterization, we have described and identified 7 actinosporean types released by the oligochaete *B. sowerbyi* from carp ponds. However, further studies are required to evaluate the actual actinosporean diversity at the fish farms, and to identify additional myxosporeans corresponding to the actinospores reported here.

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LITERATURE CITED

Barta JR, Martin DS, Libetator PA, Dashkevicz M and others (1997) Phylogenetic relationships among eight *Eimeria* species infecting domestic fowl inferred using complete small subunit ribosomal DNA sequences. J Parasitol 83: 262–271

Borkhanuddin MH, Cech G, Molnár K, Nemeth S, Székely C (2014) Description of raabeia, synactinomyxon and neoactinomyxum developing stages of myxosporeans (Myxozoa) infecting *Isochaetides michaelsei* Lasto kin (Tubificidae) in Lake Balaton and Kis-Balaton Water Reservoir, Hungary. Syst Parasitol 88:245–259

Caffara M, Raimondi E, Florio D, Marcer F, Quaglio F, Fioravanti ML (2009) The life cycle of *Myxobolus lentisuturalis* (Myxozoa: Myxobolidae), from goldfish (*Carassius auratus auratus*), involves a Raabeia-type actinospore. Folia Parasitol 56:6–12

Cai YJ, Gong ZJ, Qin BQ (2010) Community structure and diversity of macrozoobenthos in Lake Taihu, a large shallow eutrophic lake in China. Biodivers Sci 18:50–59

Chen QL, Ma CL (1998) Fauna Sinica invertebrata, Vol 15. Myxozoa, Myxosporea. Science Press, Beijing

Chen LP, Gao X, Niu CJ, He PM, Liu QG, Sun YJ, Hu ZG (2013) Community structure of zoobenthos in spring and their application to bioassessment of water quality in Taihu Lake Watershed. J Fish China 37:1679–1688

El-Mansy A, Székely C, Molnár K (1998a) Studies on the occurrence of actinosporean stages of fish myxosporeans in Lake Balaton, Hungary, with the description of triactinomyxon, raabeia and aurantiactinomyxon types. Acta Vet Hung 46:437–450

El-Mansy A, Székely C, Molnár K (1998b) Studies on the occurrence of actinosporean stages of fish myxosporeans in a fish farm of Hungary, with the description of triactinomyxon, raabeia, aurantiactinomyxon and neoactinomyxon types. Acta Vet Hung 46:259–284

Eszterbauer E, Marton S, Racz ZO, Letenyei M, Molnár K (2006) Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification. Syst Parasitol 65: 97–114

Fiala I (2006) The phylogeny of Myxosporea (Myxozoa) based on small subunit ribosomal RNA gene analysis. Int J Parasitol 36:1521–1534

Hallett SL, Atkinson SD, Erséus C, El-Matbouli M (2004) Molecular methods clarify morphometric variation in triactinomyxon spores (Myxozoa) released from different oligochaete hosts. Syst Parasitol 57:1–14

Kallert DM, Eszterbauer E, Erséus C, El-Matbouli M, Haas W (2005) Life cycle studies of *Myxobolus parviformis* sp.

n. (Myxozoa: Myxobolidae) from bream. Dis Aquat Org 66:233–243

Lom J, Dyková I (2006) Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitol 53:1–36

Lom J, McGeorge J, Feist SW, Morris D, Adams A (1997) Guidelines for the uniform characterisation of the actinosporean stages of parasites of the phylum Myxozoa. Dis Aquat Org 30:1–9

Marcucci C, Caffara M, Goretti E (2009) Occurrence of actinosporean stages (Myxozoa) in the Nera River system (Umbria, central Italy). Parasitol Res 105:1517–1530

Marton S, Eszterbauer E (2011) The development of *Myxobolus pavlovskii* (Myxozoa: Myxobolidae) includes an echinactinomyxon-type actinospore. Folia Parasitol 58:157–163

Molnár K, El-Mansy A, Székely C, Baska F (1999) Experimental identification of the actinosporean stage of *Sphaerospora renicola* Dyková & Lom 1982 (Myxosporea: Sphaerosporidae) in oligochaete alternate hosts. J Fish Dis 22: 143–145

Negredo C, Mulcahy MF (2001) Actinosporean infection in oligochaetes in a river system in southwest Ireland with descriptions of three new forms. Dis Aquat Org 46:67–77

Oumouna M, Hallett SL, Hoffmann RW, El-Matbouli M (2003) Seasonal occurrence of actinosporeans (Myxozoa) and oligochaetes (Annelida) at a trout hatchery in Bavaria, Germany. Parasitol Res 89:170–184

Özer A, Wootten R, Shinn AP (2002) Survey of actinosporean types (Myxozoa) belonging to seven collective groups found in a freshwater salmon farm in Northern Scotland. Folia Parasitol 49:189–210

Rácz O, Eszterbauer E, Molnár K (2005) Hungactinomyxon, a new actinosporean type and collective group (Myxozoa) from *Branchiura sowerbyi* Beddard. Syst Parasitol 61:107–113

Ruidisch S, El-Matbouli M, Hoffmann RW (1991) The role of tubificid worms as an intermediate host in the life cycle of *Myxobolus pavlovskii* (Ahmerov, 1954). Parasitol Res 77:663–667

Székely C, El-Mansy A, Molnár K, Baska F (1998) Development of *Thelohanellus hovorkai* and *Thelohanellus nikolskii* (Myxosporea: Myxozoa) in oligochaete alternate hosts. Fish Pathol 33:107–114

Székely C, Urawa S, Yokoyama H (2002) Occurrence of actinosporean stages of myxosporeans in an inflow brook of a salmon hatchery in the Mena River System, Hokkaido, Japan. Dis Aquat Org 49:153–160

Székely C, Borkhanuddin MH, Cech G, Kelemen O, Molnár K (2014) Life cycles of three *Myxobolus* spp. from cyprinid fishes of Lake Balaton, Hungary involve triactinomyxon-type actinospores. Parasitol Res 113:2817–2825

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729

Urawa S, Freeman MA, Johnson SC, Jones SRM, Yokoyama H (2011) Geographical variation in spore morphology, gene sequences, and host specificity of *Myxobolus arcticus* (Myxozoa) infecting salmonid nerve tissues. Dis Aquat Org 96:229–237

Wang GT, Yao WJ (2000) First report on the actinosporean in oligochaetes in China. Acta Hydrobiol Sin 24:198–200

Wolf K, Markiw ME (1984) Biology contravenes taxonomy in the Myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. Science 225:1449–1452

Xi BW, Xie J, Zhou QL, Pan LK, Ge XP (2011) Mass mortality of pond-reared *Carassius gibelio* caused by *Myxobolus ampullicapsulatus* in China. Dis Aquat Org 93:257–260

Xi BW, Zhang JY, Xie J, Pan LK, Xu P, Ge XP (2013) Three actinosporean types (Myxozoa) from the oligochaete *Branchiura sowerbyi* in China. Parasitol Res 112: 1575–1582

Yokoyama H (1997) Transmission of *Thelohanellus hovorkai* Achmerov, 1960 (Myxosporea: Myxozoa) to common carp *Cyprinus carpio* through the alternate oligochaete host. Syst Parasitol 36:79–84

Yokoyama H, Grabner D, Shirakashi S (2012) Transmission biology of the Myxozoa. In: Carvalho ED, David GS, Silva RJ (eds) Health and environment in aquaculture. In Tech, Rijeka and Shanghai, p 3–42

Yuan S, Xi BW, Wang JG, Xie J, Zhang JY (2015) *Thelohanellus wangi* n. sp. (Myxozoa, Myxosporea), a new gill parasite of allogynogenetic gibel carp (*Carassius auratus gibelio* Bloch) in China, causing severe gill myxosporidiosis. Parasitol Res 114:37–45

Zhai YH, Zhou L, Gui JF (2012) Identification and characterization of one novel type of triactinospomyxon with short spore axis. Parasitol Res 110:2385–2393

Zhang JY, Yokoyama H, Wang JG, Li AH and others (2010) Utilization of tissue habitats by *Myxobolus wulii* Landsberg et Lom, 1991 in different carp hosts and disease resistance in allogynogenetic gibel carp: redescription of *M. wulii* from China and Japan. J Fish Dis 33:57–68

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