Research Article

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***Myxobolus taibaiensis* sp. n. (Myxozoa: Myxosporea) infecting the intestinal wall of common carp *Cyprinus carpio* Linnaeus in China**

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Abstract: *Myxobolus taibaiensis* sp. n. was found in the inner intestinal wall of common carp, *Cyprinus carpio* Linnaeus, during the investigation of fish parasite fauna in Lake Taibai, located in the middle reach of the Yangtze River, China. The whitish ellipsoidal plasmodia, up to 2.9 mm long and 1.7 mm wide, developed in the circular muscle layer of the intestinal wall and produced significant compression into adjacent tissues, but no significant inflammatory responses were observed against this infection. Mature spores are oval in frontal view and lemon-like in lateral and apical view, averaging 10.2–11.2 μm ($10.8 \pm 0.2 \mu\text{m}$) in length, 9.1–9.9 μm ($9.6 \pm 0.2 \mu\text{m}$) in width and 6.1–6.6 μm ($6.3 \pm 0.1 \mu\text{m}$) in thickness. Polar capsules are pyriform, equal in size, slightly converging anteriorly, measuring 4.4–5.4 μm ($5.0 \pm 0.2 \mu\text{m}$) in length by 3.2–3.6 μm ($3.4 \pm 0.1 \mu\text{m}$) in width. Polar filaments coiled with four to five turns and arranged perpendicular to the polar capsule length, measuring up to 106 μm . *Myxobolus taibaiensis* sp. n. is morphologically similar to *Myxobolus rotundatus* Achmerov, 1956 which also infects the inner wall of the intestine of common carp. However, the small subunit ribosomal DNA sequence identity was only 94%, generally beyond the intraspecies variation in the genus. Phylogenetically, this new species is sister to *M. rotundatus* and then clusters with *M. shantungensis* Hu, 1965 to form an independent common carp-infecting cluster within the *Henneguya-Myxobolus* clade.

Keywords: common carp, Cyprinidae, intestine, circular muscle layer, Yangtze River

Myxozoa Grassé, 1970 is an important group of metazoans that mainly infect fishes, but with some species parasitising amphibians, reptiles, birds and mammals (Lom and Dyková 2006, Székely et al. 2016). Approximately 2200 myxosporean species have been so far reported over the world, representing high species diversity (Lom and Dyková 2006). However, some of them were described solely based on spore morphology. Given the incredible diversity along with the simplicity of the taxonomic criteria used to differentiation of these species, it is often difficult to discriminate morphologically similar species using spore morphology alone (Chen and Ma 1998, Liu et al. 2013). To avoid the above conundrum, a comprehensive approach including spore morphology, exact location of sporulation, tissue specificity, host specificity and molecular data has been widely accepted for description of novel species (Fiala 2006, Atkinson et al. 2015).

Common carp *Cyprinus carpio* Linnaeus is an important commercial fish species that has been cultured for more than 4000 years in China and its annual production has reached more than 300 million kilograms in China in 2013 (Lin et al. 2014). However, epizootic diseases have

caused severe economic and environmental problems with the rapid development of intensive aquaculture of this species. Virosis, bacteriosis and parasitoses were increasingly reported to be responsible for the mass mortality of reared common carp (Yokoyama et al. 1997, Zhang et al. 2010, Dong et al. 2013, Zhai et al. 2016). Myxosporidiosis represents one of the most important parasitic diseases of common carp in China. About 85 myxosporeans have been so far described in different tissues and organs of common carp in China (Chen and Ma 1998, Dyková et al. 2003, Liu 2014). However, some watersheds have never been investigated, so the species diversity of myxosporeans is probably underestimated in China.

Lake Taibai is located in the middle reach of the Yangtze River where 46 fish species have been reported and the annual fishery product is approximately 4 million kilograms (Jin 2007). To the best of our knowledge, no myxosporean species have been previously recorded in this natural shallow water lake (Chen and Ma 1998). Therefore, we conducted an investigation of fish myxosporean parasites of this lake to enrich our knowledge of the species diversity. In this paper, a new species is described by combining

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spore morphology, ecological features and molecular characteristics.

MATERIALS AND METHODS

Morphological analysis

Twenty specimens of common carp ranging from 34.5 cm to 40.5 cm in total length were collected from Lake Taibai off the lakeside of Huangmei country, Hubei province, China. Fish were transported alive to the local laboratory of aquatic animal diseases in oxygenated plastic bags and temporarily kept in aerated aquaria. Fishes were dissected within 1 to 2 days post capture. Samples of gills, skin, kidney, gallbladder, intestine and other organs were routinely examined to find cyst-like plasmodia under naked eyes, stereomicroscope and phase contrast microscope. If dissected fish were infected with myxosporeans, a single plasmodium was isolated from the infected tissues and ruptured with a fine needle on a slide to make wet preparations for morphological observation. The remaining spores from the ruptured plasmodium were preserved in 95% ethanol for molecular analysis. Fresh spores were observed and images captured under an oil immersion objective (1000×) with an Olympus DP10 digital camera. Measurements were taken from 50 randomly selected fresh spores from 5 plasmodia. All measurements are in micrometres (µm) unless otherwise indicated and presented as range (mean ± SD). Some released spores were preserved in glycerine-jelly as reference slide in the laboratory of Fish Diseases, Institute of Hydrobiology, Chinese Academy of Sciences. Additionally, smear slides of mature spores were air-dried and stained with Diff-Quik (NobleRyder, Shanghai, China). Line drawings were made based on the fresh wet mounts and stained slides with the aid of Corel-Draw X6 (Corel, Ottawa, Canada) and Adobe Illustrator software (Adobe Systems, San Jose, CA, USA).

Histological examination

Infected tissue samples containing developing and mature plasmodia were fixed in 10% neutral buffered formalin, dehydrated in ethanol and embedded in paraffin wax. Tissue sections, 4–6 µm thick, were stained with haematoxylin and eosin (H & E) and examined under a light microscope (Olympus DP10).

DNA isolation and sequencing

For DNA extraction, preserved spores in ethanol were centrifuged and washed two times with distilled water to remove the ethanol remnants. Genomic DNA was isolated from tissue homogenates using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Germany), following the manufacturer's recommended protocol for animal tissue. The gDNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Shanghai, China) at 260 nm.

The partial sequence of the SSU rDNA gene was amplified using a primer pair, MyxospecF (Fiala 2006) and 18R (Whipps et al. 2003) in a 25 µl reaction mixture, which comprised 30 ng template DNA, 1×PCR mixture (CWbiotech, Beijing, China), 10 pmol of each primer. Amplification conditions were initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 46 °C for 50 s, 65 °C for 90 s, with a terminal extension at 65 °C for 10 min. The PCR products were electrophoresed in 1.2% agarose gels in Tris-borate-EDTA buffer gel stained with

1% ethidium bromide and then purified with a PCR purification kit (CWbiotech, China). The purified product was cloned into PMD-18T vector system (Takara, Dalian, China) and then sequenced with the ABI BigDye Terminator v 3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyser.

Phylogenetic analysis

Sequences were aligned in BioEdit (Hall 1999) and ambiguous bases clarified using corresponding ABI chromatograms. The consensus sequences were deposited in GenBank with accession number KU928249 and verified as myxozoan by BLAST search. To explore the phylogenetic relationships of the present species with related myxobolids, 43 sequences were retrieved from GenBank and aligned using Clustal X 1.8 program (Thompson et al. 1997) with defaulting setting. The data set for alignment consisted of the top BLAST search matches and representatives of neighbouring clades based on earlier analyses of myxobolids (Molnár et al. 2006, Liu et al. 2016a). The alignment was corrected manually using the alignment editor of MEGA6.0 software (Tamura et al. 2013).

Phylogenetic analysis was conducted as follows: Maximum likelihood (ML) analysis and Bayesian analyses (BI) was conducted in PhyML 3.0 and Mr. Bayes, respectively (Ronquist and Huelsenbeck 2003, Guindon et al. 2010). The optimal evolutionary model for maximum likelihood and Bayesian analyses were determined using jModelTest 3.07 (Posada 2008) which identified the best evolutionary model as the general time reversible model (GTR+I+G), using Akaike information criteria. Nucleotide frequencies were estimated from the data (A = 0.2413, C = 0.1927, G = 0.2778, T = 0.2881); six rates of nucleotide substitution calculated as AC = 1.3862, AG = 4.2306, AT = 1.9229, CG = 0.6300, CT = 6.0859, GT = 1.000. The proportion of invariable site was 0.3026 and the alpha value of gamma distribution parameter was 0.3868. *Myxidium lieberkuehni* Bütschli, 1882 (X76638) and *Myxidium truttae* Léger, 1931 (AF201374) was used as outgroup. Two independent runs were conducted with 4 chains for 2 million generations for Bayesian analysis. Phylogenetic trees were sampled every 100 generation. The first 25% of the samples were discarded from the cold chain (burninfrac = 0.25). Bootstrap confidence values were calculated with 1000 repetitions for ML. Trees were initially examined in TreeView X (Page 1996).

RESULTS

During the survey of myxosporean fauna in Lake Taibai, five species of *Myxobolus* Bütschli, 1882 were found from the dissected specimens. Among them, *Myxobolus koi* Kudo, 1919, *M. toyamai* Kudo, 1917 and *M. tsangwuensis* Chen, 1954 infected the gills, with infection prevalence of 30% (6/20), 20% (4/20), 15% (3/20), respectively. Coinfection of these three species could be occasionally found. *Myxobolus cyprinicola* Reuss, 1906 and a suspected new species were found to dwell in the outer and inner intestinal wall, respectively, with prevalence of 35% (7/20) and 25 % (5/20). However, no coinfection of these two intestine-infecting species was observed. For the concerned new species, numerous whitish plasmodia were macroscopically visible in the whole intestinal wall after dissecting specimens (Fig. 1A).

Fig. 1. The plasmodia and spores of *Myxobolus taibaiensis* sp. n. from *Cyprinus carpio* Linnaeus. **A** – numerous plasmodia macroscopically visible in the whole intestinal wall; **B** – fresh spores in frontal view.

Fig. 2. Spores of *Myxobolus taibaiensis* sp. n. from *Cyprinus carpio* Linnaeus. **A** – frontal view; **B** – lateral view; **C** – apical view; **D** – folds on the posterior end of spores; **E** – spore with extruded polar filaments stained with Diff-Quik.

Myxobolus taibaiensis sp. n.

Figs. 1–5

ZooBank number for species:

[urn:lsid:zoobank.org:act:39549CFE-6D3D-451E-87DF-EFE0B4C83623](https://zoobank.org/act:39549CFE-6D3D-451E-87DF-EFE0B4C83623)

Morphological description. Plasmodia ellipsoidal, 1.7–2.9 mm in length and 0.9–1.7 mm in width ($n = 20$). Mature spores ellipsoidal in frontal view and lemon-like in lateral and apical view (Figs. 1B, 2A–C). Spores $10.2\text{--}11.2$ (10.8 ± 0.2) in length, $9.1\text{--}9.9$ (9.6 ± 0.2) in width and $6.1\text{--}6.6$ (6.3 ± 0.1) in thickness ($n = 50$). Spore valves smooth and symmetrical (Fig. 3). Polar capsules pyriform, equal in size, situated on anterior end of spores, slightly converging anteriorly, $4.4\text{--}5.4$ (5.0 ± 0.2) in length and $3.2\text{--}3.6$ (3.4 ± 0.1) in width, occupying half body of spores.

Polar filaments four to five turns, perpendicular to capsule length. Polar filament up to 106 long after completely extruded, about tenfold of spores length (Fig. 2E). No folds on posterior end of mature spores, but three to four folds clearly visible in some spores (Fig. 2D).

Type host: Common carp *Cyprinus carpio* Linnaeus (Cyprinidae).

Site of infection: The circular muscle layer of inner intestinal wall.

Prevalence: 25% (5/20).

Type locality: Lake Taibai, Huangmei Country, Hubei Province, China ($29^{\circ}56'05''\text{N}$; $115^{\circ}47'28''\text{E}$).

Type material: Syntype specimens of spores in glycerin gelatin, Diff-Quik stained slide and H & E stained section deposited in the Laboratory of Fish Diseases, Institute

of Hydrobiology, Chinese Academy of Science, Acc. No. MTR20151208. The partial 18S rDNA sequence was deposited in the GenBank under the accession number KU928249.

E t y m o l o g y : The species is named after type locality, Lake Taibai.

Remarks. Until now, 18 *Myxobolus* species were found in the intestine of common carp in China. *Myxobolus taibaiensis* sp. n. can be differentiated from morphologically most similar species (Table 1), as follows. The new species can be distinguished from *M. cyprinicola* Reuss, 1906 and *M. rotundatus* Achmerov, 1956 by the different shape of the anterior end of spores (tapered vs round). In addition, *M. cyprinicola* has a bigger iodophilous vacuole and higher number of turns of polar filaments (7–8 vs 4–5) than that of *M. taibaiensis*. Both, *M. rotundatus* and *M. taibaiensis*, infect the inner intestinal wall of common carp, but the former species possesses a smaller polar capsule and the ratio of the width of polar capsules to the length. Sequence similarity of 94% between *M. rotundatus* and *M. taibaiensis* further supports that they are two different species. *Myxobolus ornatus* Achmerov, 1960, *M. oviformis* Thélohan, 1892 and *M. linghuensis* Chen, 1954 possess a tapered anterior end in which they resemble the new species. However, *M. taibaiensis* can be easily distinguished

Fig. 3. Schematic drawings of myxospore of *Myxobolus taibaiensis* sp. n. from *Cyprinus carpio* Linnaeus. **A** – frontal view; **B** – sutural view.

from *M. ornatus* by its lack of a distinct intercapsular appendix. In addition, *M. taibaiensis* can be discriminated from *M. oviformis* and *M. linghuensis* by its shorter spore, bigger ratio of the length to the width of spores and fewer turns of polar filaments (4–5 vs 6–7 and 7–8). Furthermore, *M. oviformis* possesses intercapsular appendix and smaller plasmodium (0.19 mm × 0.18 mm in diameter vs

Fig. 4. Histological sections of the intestine of common carp *Cyprinus carpio* Linnaeus infected by *Myxobolus taibaiensis* sp. n. **A** – two plasmodia (p) overgrown by the circular muscle layer (cm) of intestinal wall and bulged into longitudinal muscle layer (lm) and submucosa layer (sb); **B** – smaller plasmodium (p) from Fig. 4A, showing the boundary between longitudinal muscle layer (lm) and circular muscle layer (cm) of intestinal wall of host, ec – ectoplasm; **C** – the connective tissue (ct) surrounding plasmodium (p); **D** – shrinking of adjacent smooth muscle tissues (arrow) caused by mechanical compression of the huge plasmodium (p).

Fig. 5. Phylogenetic tree generated by Bayesian analysis (BI) of the aligned partial SSU rRNA gene sequences of *Myxobolus taibaiensis* sp. n. and related myxosporeans, rooted at *Myxidium lieberkuehni* Bütschli, 1882 and *Myxidium truttae* Léger, 1931. Genbank accession numbers are given adjacent to species names. Numbers at nodes indicate bootstrap support values by BI and maximum likelihood, respectively. Asterisks are shown where values exceeded 95% and dashes shown for value under 50%. The new species in the present paper is in bold and the intestine-infecting species are within the boxes. The *Myxobolus*-*Henneguya* clade, including several common carp-infecting and gibel carp-infecting species, is marked in a dashed box. The order of hosts of all selected species for phylogenetic analysis was pointed out.

1.7–2.9 mm × 0.9–1.7 mm). Folds could be observed easily on the posterior end of *M. taibaiensis* and *M. oviformis*, but, their shape is different (V shape vs round shape).

Histopathology. Large plasmodia of ellipsoidal shape started their development in the circular muscle layer of the intestinal wall of common carp (Fig. 4A,B). Ectoplasm of the plasmodia constituted an intensive eosinophilic wall material (Fig. 4B). Plasmodia were surrounded by a layer of connective tissues (Fig. 4A–D) where collagen fibres and large amounts of nuclei of fibroblasts were observed (Fig. 4A–C). The development of plasmodia was asynchronous and produced significant mechanical compression of adjacent tissues, even bulging into the intestinal mucous layer. The involved circular muscle was remarkably thicker than that of unaffected tissues (Fig. 4A,B,D).

No distinct inflammatory response was observed in the infection focus.

Molecular characterisation. A partial SSU rDNA sequence obtained for *M. taibaiensis* was 1 737 bp long including regions corresponding to forward and reverse primers. The sequence containing 46.69% GC bases, was deposited in the GenBank under accession number KU928249. A BLAST search indicated that this sequence was not identical to any myxosporean sequences available in GenBank, but showed high similarity to the following species: *Myxobolus shantungensis* Hu, 1965 (KJ725079, 96% over 947 bp), *Henneguya doneci* Schulman, 1962 (LC011456, 95% over 1 679 bp), *M. nielii* (Nie et al., 1973) (JQ690358, 95% over 1 649 bp), *M. turpisrotundus* Zhang, Wang, Li et al., 2010 (JQ690364, 94% over 1 633 bp), *M. hearti*

Table 1. Morphological comparison of spore of *Myxobolus taibaiensis* sp. n. with similar species; all measurements in micrometres, minimum–maximum with mean ($\pm$ SD) in parentheses.

Species	Site of infection	Shape	Length of spores	Width of the spores	Thickness of spores	Length of polar capsule	Width of polar capsule	CPF	Reference
<i>M. cyprinicola</i> Reuss, 1906	intestine	EL	9.8–12.0 (10.9)	7.2–8.4 (8.0)	6.0–6.2 (6.1)	4.3–5.2 (4.8)	2.2–3.1 (2.5)	7–8	Chen and Ma 1998
<i>M. linghuensis</i> Chen, 1954	intestine	oval	10.2–13.2 (11.0)	8.4–9.6 (9.3)	6.0–7.2 (7.0)	4.8–5.4 (4.9)	2.6–3.4 (2.8)	7–8	Chen and Ma 1998
<i>M. ornatus</i> Achmerov, 1960	intestine	EL	9.8–12.0 (10.9)	9.0–10.8 (9.8)	7.0–7.2 (7.1)	4.8–5.4 (5.0)	3.6–3.8 (3.7)	6–7	Chen and Ma 1998
<i>M. oviformis</i> Thélohan, 1892	intestine	oval	10.8–12.0 (11.2)	8.4–9.6 (9.0)	6.0–6.6 (6.3)	4.8–6.0 (5.6)	2.6–2.8 (2.7)	6–7	Chen and Ma 1998
<i>M. rotundatus</i> Achmerov, 1956	IW	EL	9.2–10.8 (10.1)	8.4–9.6 (9.2)	7.0–7.4 (7.2)	4.2–4.8 (4.6)	2.1–2.9 (2.7)	4–5	Chen and Ma 1998
<i>M. taibaiensis</i> sp. n.	IW	oval	10.2–11.2 (10.8 $\pm$ 0.2)	9.1–9.9 (9.6 $\pm$ 0.2)	6.1–6.6 (6.3 $\pm$ 0.1)	4.4–5.4 (5.0 $\pm$ 0.2)	3.2–3.6 (3.4 $\pm$ 0.1)	4–5	Present study

IW – intestinal wall; EL – ellipsoidal; CPF – coils of the polar filaments.

Chen, 1998 (GU574808, 94% over 1 619 bp), *H. globulata* Ye et Wang, 2012 (JQ690355, 93% over 1 621 bp) and *M. rotundatus* (AY165180, 94% over 660 bp). BI and ML analyses generated trees of highly similar topologies with different support values at some branch nodes. *Myxobolus taibaiensis* is sister to *M. rotundatus* and then clusters with *M. shantungensis* to form an independent common carp-infecting group within the *Myxobolus-Henneguya* clade.

DISCUSSION

Traditional taxonomy of myxosporean parasites depended upon the spore morphology, but the simplicity and plasticity of mature myxospores caused taxonomic problems (Fiala 2006, Liu 2014). A comprehensive approach of combining morphological features, host, tissue tropism and molecular data has been widely accepted to resolve the above problem, especially discriminating morphologically similar species (Liu et al. 2016b). In the present work, *M. taibaiensis* sp. n. is morphologically difficult to discriminate from *M. rotundatus* (Table 1). Based on morphological differences, including slightly pointed anterior end, bigger polar filament of *M. taibaiensis*, we thought that these two species are different, even though they both infect the inner intestinal wall of common carp. However, their sequence similarity of only 94% can be thought to be beyond the intraspecific variation (typically >2% intraspecific SSU sequence difference). Therefore, *M. taibaiensis* is molecularly different from *M. rotundatus*.

Tissue specificity has been considered a very important criterion for myxosporean identification, especially species of *Myxobolus*. (Molnár 2002, Fiala 2006). About 60 among more than 800 species of *Myxobolus* were found to infect fish intestine (Eiras et al. 2005, 2014), but only five species were described by the current approach (Molnár et al. 2006, 2009, Liu 2014, Liu et al. 2016a). These are *M. shaharomae* Molnár, Eszterbauer, Marton, Cech et Székely, 2009, *M. erythrophthalmi* Molnár, Eszterbauer, Marton, Cech et Székely, 2009, *M. gayerae* Molnár, Marton, Eszterbauer et Székely, 2007, *M. cyprinicola* and *M. miyairii* Kudoa, 1919. *Myxobolus miyairii* developed numerous visible plasmodia in the whole intestinal wall of its host, with same tissue tropism as *M. taibaiensis*, but they can be

easily differentiated by the morphology of spore, size of plasmodia (0.32–0.78 mm vs 1.7–2.9 mm $\times$ 0.9–1.7 mm) and the distantly related fish hosts (Siluriformes vs Cypriniformes). Both *M. cyprinicola* and *M. taibaiensis* infect common carp, but *M. cyprinicola* dwells in the subepithelium of lamina propria rather than circular muscle layer of the intestine and its plasmodia are smaller than those of the latter (0.7 mm vs 1.7–2.9 mm $\times$ 0.9–1.7 mm). *Myxobolus shaharomae*, *M. erythrophthalmi* and *M. gayerae* have not been reported from China. Among them, the plasmodia of *M. shaharomae* and *M. erythrophthalmi* develop in the blood vessel whereas *M. gayerae* in the propia and submucosa layers of the intestinal wall of chub *Squalius cephalus* (Linnaeus), surrounded by the loose connective tissues.

The present phylogenetic analysis further demonstrates that all of these six intestine-infecting species of *Myxobolus* are evolutionally distant. Our result also support the previous conclusion that tissue specificity of sporulation can truly indicate evolutionary relationship (Molnár et al. 2006, 2009), because *M. taibaiensis* and *M. cyprinicola*, with different tissue tropism, appear in two different clades. Interestingly, *M. shantungensis* showed close genetic affinity with *M. taibaiensis*, although *M. shantungensis* was reported from gill arches of common carp and is morphological different from the new species. However no detailed data on tissue tropism of *M. shantungensis* are available.

The increasing number of reports of *Henneguya*-like spores within the plasmodia of *Myxobolus* species provides strong evidence that these two genera have close evolutionary relationships (Liu et al. 2010, 2016a). Our results also show that *M. taibaiensis* clusters within the *Myxobolus-Henneguya* clade among which all species infect common carp and gibel carp, *Carassius auratus gibelio* Bloch.

In summary, the present work further evidences that molecular data are important for the identification of myxosporean species and *M. taibaiensis* was proved to be a new species distinguished from congeners by combining morphological, ecological and molecular data.

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