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Infection of *Myxobolus turpisrotundus* sp. n. in allogynogenetic gibel carp, *Carassius auratus gibelio* (Bloch), with revision of *Myxobolus rotundus* (s. l.) Nemeczek reported from *C. auratus auratus* (L.)

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Abstract

Infection of a *Myxobolus* species, previously identified as *Myxobolus rotundus,* was detected in 182 of 7892 (2.31%) allogynogenetic gibel carp, *Carassius auratus gibelio,* in a closed pond culture system in China. Morphological and molecular data showed that this myxosporean is a different species from *M. rotundus* parasitizing *Abramis brama* in Europe and is thus designated as a new species, *Myxobolus turpisrotundus*. *M. rotundus* (s.l.) ex *C. auratus auratus* is a synonym of *M. turpisrotundus*. Plasmodia of *M. turpisrotundus* develop in the subepidermal tissues of the body surface resulting in an unaesthetic appearance and causing severe economic losses. Prevalence of infection with the myxosporean plasmodia varied seasonally, increasing in winter and decreasing in spring. Prevalence was positively correlated to host size, but no host sex-specificity was found. No infection was observed in other fish species (grass carp, bighead carp and yellow catfish) reared in the same pond, suggesting that the parasite has a relatively strict host specificity. Plasmodia grew gradually as the parasite developed, and reached up to a maximum 5.6 mm in diameter. Plasmodia ruptured naturally to release the mature spores and host fish completely recovered with no mortality. Release of spores and regeneration of lesions were not correlated with water temperature. Histology showed that plasmodia developed sub-epidermally, and that the wall of the plasmodia was composed of a multiple complex structure, including layers of fibroblasts, a collagenous membrane, melanophores and a layer of cup-like cells of unknown derivation and function. The cup-like cells are in direct contact with presporogonic stages located in the peripheral parts of the large plasmodia. No severe host inflammatory response was seen.

Keywords: allogynogenetic gibel carp, *Carassius auratus gibelio*, *Myxobolus rotundus*, *Myxobolus turpisrotundus*, Myxozoa, prevalence.

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Introduction

Myxozoans are ubiquitous metazoan parasites, some of which are significant pathogens of economically important aquatic animals, especially teleost fish (Kent, Andree, Bartholomew, El-Matbouli, Desser, Devlin, Feist, Hedrick, Hoffmann, Khattra, Hallett, Lester, Longshaw, Palenzeula, Siddall & Xiao 2001; Yokoyama 2003; Canning & Okamura 2004). *Myxobolus* Bütschli, 1882 is the largest genus within the Myxozoa, comprising about 800 species (Eiras, Molnár & Lu 2005; Lom & Dyková 2006; Molnár 2007), of which more than 300 have been reported from freshwater fish in China (Chen & Ma 1998; Zhao, Sun, Kent, Deng & Whipps 2008). However, the validity of some Chinese myxozoan species needs to be re-evaluated because of their incomplete morphological descriptions, insufficient comparison with other known species and recent revision of taxonomic criteria of myxozoans.

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Myxobolus rotundus Nemeczek, 1911 was first described from gills of common bream, *Abramis brama* (L.), in Hungary and subsequently from 27 cyprinid fish species (Eiras *et al.* 2005; Molnár, Székely, Hallett & Atkinson 2009). The species has also been recorded from several fish hosts in Austria, Russia, India and North America (Miroshnichenko 1980; Chen & Ma 1998; Longshaw, Frear & Feist 2003; Molnár 2007). However, more recent reports have demonstrated that *M. rotundus* is a specific parasite of the gill of common bream and that the actinosporean stage develops in *Tubifex tubifex* (Molnár *et al.* 2009; Székely, Hallett, Atkinson & Molnár 2009). Molnár *et al.* (2009) suggested that previous records from other organs of other fish hosts should be revised based on consideration of spore morphology, host range, tissue tropism and molecular analysis. In the monograph of Chen & Ma (1998), incorrectly assigned species possessing an almost identical spore morphology with *M. rotundus* were recorded in gill, kidney, fins, spleen, liver and gonad of *Schizothorax davidi* (Sauvage); gill, body surface, fins, gall bladder and kidney of *Carassius auratus auratus* (L.), kidney of silver carp, *Hypophthalmichthys molitrix* (Valenciennes); and gut of grass carp, *Ctenopharyngodon idella* (Cuvier *et* Valenciennes). Subsequently, a myxosporean with similar spore morphology was found on the body surface of *C. auratus auratus* and identified as *M. rotundus* (Wu & Wang 2000; Lu, Li, Wu & Wang 2002; Zhang, Wang, Wu, Li, Li & Gong 2006). The wide host range and multiple infection sites of the above species must bring into question their validity as *M. rotundus*. In the present study, the validity of '*M. rotundus*' found in *C. auratus auratus* and *C. auratus gibelio* (Bloch) was critically reevaluated by morphological and molecular analyses, as well as tissue tropism.

The development of a sport fishing industry in some Chinese cities has suffered from severe economic losses due to the unaesthetic appearance of fish caused by large numbers of myxosporean cysts on the body surface of allogynogenetic gibel carp, *C. auratus gibelio*, which is one of the main species involved (Zhang 2006). In central China, the culture cycle of allogynogenetic gibel carp is over a 2-year period. To prevent the spread of the myxosporidiosis, farmers usually kill or segregate the infected fish. However, some infected fish without clinical signs are also introduced into sportfishing ponds thus spreading the pathogen.

In the present study, a monthly investigation over three-consecutive years (2004–2006) was conducted in a closed pond culture system in Wuhan City, central China to study the biological characteristics of the parasite and to design possible control strategies. For convenience, in this study all previously recorded *M. rotundus* from China are designated as Chinese *M. rotundus* (s. l.).

Materials and methods

Epidemiological investigation

A grow-out culture pond (*ca.* 6500 m^2 in area and *ca.* 1.2 m in depth) located in the suburbs of Wuhan City, Hubei Province, China (114°21′ E, 30°32′ N), where the myxosporidiosis is enzootic, was selected to investigate the pattern of infection from December 2004 to May 2006. In this pond, grass carp *C. idellus*, bighead carp, *Aristichthys nobilis* (Richardson), and yellow catfish, *Pelteobagrus fulvidraco* (Richardson), were cultured together with allogynogenetic gibel carp. About 13 allogynogenetic gibel carp and 5–10 of each of the other species of cultured fish were captured monthly by seine net or angling and transported alive to the laboratory. All fish were killed by transection of the spinal cord and necropsied after measurements of body length and weight. All external and visceral organs of sampled fish were examined for the presence of myxosporean plasmodia by naked eye or with a dissection microscope. The overall prevalence was determined based on the total number of allogynogenetic gibel carp counted from the pond in December 2005 at final harvest. Prevalence, mean abundance, and intensity are as defined by Bush, Lafferty, Lotz & Shostak (1997). Water temperature was recorded at every sampling occasion. The correlation of infection with host size, prevalence, intensity and abundance was analyzed. Archival samples of *C. auratus auratus* infected with Chinese *M. rotundus* (s.l.) were examined to determine the possible host sex-specificity, because all cultured allogynogenetic gibel carp are female.

To investigate the dynamics of spore release into the environment, 38 infected fish with a large number of cysts on the body surface were transported to the laboratory and maintained at room temperature (*ca.*18 °C). Remaining infected fish were kept in a field pond. Mean abundance of infection was compared monthly between the fish kept in the laboratory and those in field conditions

subject to temperature changes. All epidemiological data are expressed as means ± SD. Seasonal changes of prevalence and mean abundance were analyzed statistically by one-way ANOVA. The bias of prevalence against host size and sex was tested using a chi-square test. Pearson's correlation was used to determine the effects of water temperature on release of mature spores. All statistical analyses were performed using the software program Statistica 6.0 (StatSoft, Inc.), with the level of significance set at $P < 0.05$.

Parasite identification

To identify the species of myxosporean, wet mounts were prepared using freshly isolated plasmodia. Morphological and morphometrical features of spores were characterized according to Lom & Arthur (1989) by measuring 50 freshly isolated mature spores in wet preparations. Indian ink and 2% Lugol's iodine were used to visualize the mucous envelope and iodinophilic vacuole, respectively. Potassium hydroxide (0.2 M) was applied to fresh spores to induce extrusion of the polar filaments. Spores in fresh mounts were photographed with a Zeiss Axioplan 2 Image and Axiophot 2. Line drawings were made with a Nikon FDX-35. Archival samples of *M. rotundus* (s.l.) ex *C. auratus auratus* which were sampled and stored in formalin by Wu & Wang (2000), Lu *et al.* (2002) and Zhang (2006) were re-examined. All measurements are given as means ± SD (range) in micrometres (μm) unless otherwise stated. Morphological and morphometrical comparisons of this species with previous reported Chinese *M. rotundus* (s.l.) and other related *Myxobolus* spp. were made to confirm its taxonomic position. For histopathology, tissue samples of infected organs were fixed in Bouin's solution and embedded in paraffin wax. Sections cut at 5–6 μm were stained with haematoxylin and eosin (H&E) and observed by light microscopy.

Molecular analysis

Genomic DNA (gDNA) was extracted from freshly isolated plasmodia from different infection sites including the skin, fin, gut wall, pharynx, cranial epidermis and gill arch of infected allogynogenetic gibel carp to confirm the congruence of DNA sequences among the parasites from different sites. The parasite gDNA was isolated by proteinase K digestion overnight at 37 °C, followed by phenol-chloroform protein extraction and ethanol precipitation from the purified spores according to Andree, Székely, Molnár, Gresoviac & Hedrick (1999), and then stored in double distilled water. Partial sequences of SSU rDNA were amplified by the polymerase chain reaction (PCR) with the universal primer-pair, 18e–18g (Hillis & Dixon 1991), followed by the primer-pair specific for the family Myxobolidae, MX5–MX3 (Andree *et al.* 1999). The amplified products were cloned into a T-A cloning vector (pUC57-T, GenScript Corporation), which was used to transform competent Escherichia coli cells (DH5α). Transformants were selected by blue-white screening on plates containing X-Gal and IPTG. The clones containing the target inserts were confirmed by PCR with M13 forward (−41) (GGTTTTCCCAGTCACGAC) and M13 reverse primers (−48) (AGCGGATAACAATTTCACAC). Both strands of the DNA were sequenced from two positive clones using an ABI 377 automated sequencer (Applied Biosystems Inc.). Additional sequence data were obtained by walking primers designed from a previously obtained sequence and contigs were assembled by the SeqMan program included in the DNAStar software package (DNAstar Inc.) and submitted to GenBank under accession number EF690299. The sequence was first compared with the previously registered 726 bp sequence (AY165197) from *C. auratus auratus* and searched by the BLAST program of GenBank to obtain homologous sequences. All selected sequences were globally aligned using ClustalX (Thompson, Gibson, Plewniak, Jeanmougin & Higgins 1997) with default settings and examined manually. The postulated gaps and ambiguously aligned regions were excluded from phylogenetic analysis. Phylogenetic analyses were conducted by the neighbour-joining (NJ) method, the maximum parsimony (MP) method and the Bayesian method, which were implemented in the MEGA3 computer package (Tamura, Dudley, Nei & Kumar 2007), PAUP* 4.0b1 (Swofford 2003) and MrBayes version 3.0b4 (Ronquist & Huelsenbeck 2003), respectively. The NJ tree was constructed using the Kimura two-parameter model (Kimura 1980) and a MP tree was generated using a heuristic search. To estimate the relative branch support of NJ and MP trees, bootstrap analysis with 1000 replicates was conducted. Best nucleotide substitution model, determined by Modeltest 3.7 (Posada & Crandall 1998) was used in ML analysis.

Results

Epidemiological studies

Of 7892 allogynogenetic gibel carp, 182 fish (18.3 cm and 458.6 g average body weight and length, respectively) were infected by the myxosporean (prevalence 2.3%), whereas no infection was observed in grass carp ($n = 26$), bighead carp ($n = 31$) or yellow catfish ($n = 29$) reared in the same pond throughout the investigation period. After small plasmodia first appeared in the jaws, lip, operculum and cranial epidermis of allogynogenetic gibel carp in late July and August 2005, more advanced stages of infection were found in the skin, dorsal, ventral, pectoral, anal and tail fins (Fig. 1a). Infections in the pharynx and gill arches were found after October 2005. The foregut wall is a newly recorded site of infection, found in 6 fish (3.2%) after November 2005 (Fig. 1b). Most infected fish were disfigured due to large numbers of round or ellipsoidal plasmodia ranging from 0.4 to 5.6 mm in diameter on the body surface. These clinical signs occurred after November 2005. The prevalence of infection showed a distinct seasonal pattern with a maximum in winter (15.2%) and a minimum in summer (0%). Mean abundance and intensity of infection were 73.37 ± 95.19 ($n = 408$) and 125.6 ± 63.73 ($n = 218$), respectively. The monthly changes of prevalence, mean abundance and ambient water temperature are shown in Fig. 2. However, it should be noted that the high prevalence and mean abundance after December 2005 resulted from remaining infected fish after most cultured fish were harvested. Mean size of plasmodia in all infection sites increased gradually after the first appearance of the parasite in late August 2005 until November 2005 (data not shown). After plasmodia naturally ruptured to release mature spores, all infected fish recovered by May 2006. No mortalities caused by the parasite occurred during the study period. Comparison of the process of spore release at different temperature, showed that water temperature had no effect ($P > 0.05$) (Fig. 3). Combining the results of the

Figure 1 Allogynogenetic gibel carp heavily infected with *Myxobolus turpisrotundus* sp.n. (a) Disfigured appearance of fish from infection on the body surface (bar = 10 cm). (b) Infected intestine with three large plasmodia in the foregut (arrow) (bar = 10 cm).

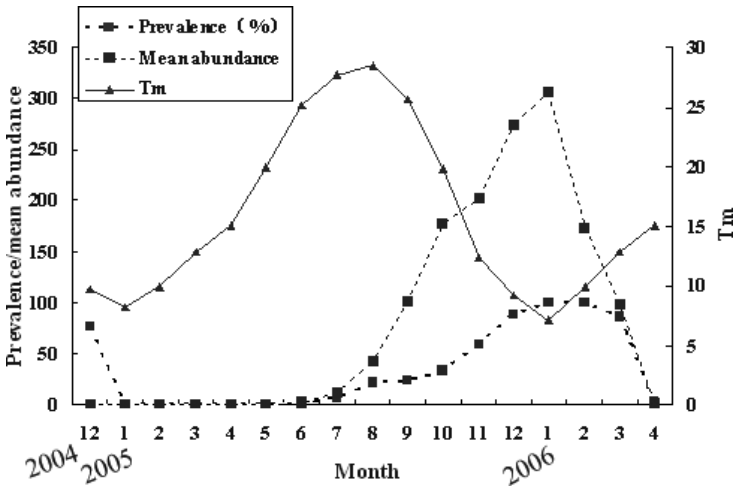

Figure 2 Monthly changes in prevalence and mean abundance of plasmodia of *Myxobolus turpisrotundus* sp.n. infecting cultured allogynogenetic gibel carp from December 2004 to April 2006.

present study with archived samples of infected *C. auratus auratus*, no parasite host sex-specificity was found. Infection occurred mainly in fish larger than 15 cm ($P < 0.01$) in body length; no significant differences in prevalence were found in fish above this size ($P > 0.05$). The mean abundance and intensity of infection were also positively correlated with host size ($P < 0.01$) (Fig. 4).

Morphometric data and descriptions of the species

Examination of archived samples from Ezhou prefecture (Wu & Wang 2000; Lu *et al.* 2002; Zhang 2006) showed that *M. rotundus* (s.l.) ex *C. auratus auratus* shared the same features of spore morphology and infection patterns with those of allogynogenetic gibel carp. Due to the difficulty of reexamination of the archived samples of Chen & Ma (1998), only *M. rotundus* (s.l.) from *C. auratus auratus* and allogynogenetic gibel carp were considered in the present study.

Spore morphology of the present species is as follows: spores in frontal view round or ellipsoidal (Figs 5a & 6a). Spores in sutural view convex in shape, with a straight, thick sutural ridge slightly protruding at the anterior and posterior ends (Figs 5b & 6b). Spore valves thin, symmetrical, smooth and uniform in thickness. No mucous envelope is present. Some spores have 5–6 'V' shaped edge markings on the posterior end of valves (data not shown). Spore length, 9.2 ± 0.3 μm (8.6–10.0); spore width 8.4 ± 0.3 μm (8.2–10.0); spore thickness 5.8 ± 0.2 μm (5.1–6.5). The ratio of spore length and width about 1. Two equal pyriform polar capsules, 4.8 ± 0.1 (4.1–5.1) μm long and 3.0 ± 0.1 (2.5–3.1) μm wide, opening at

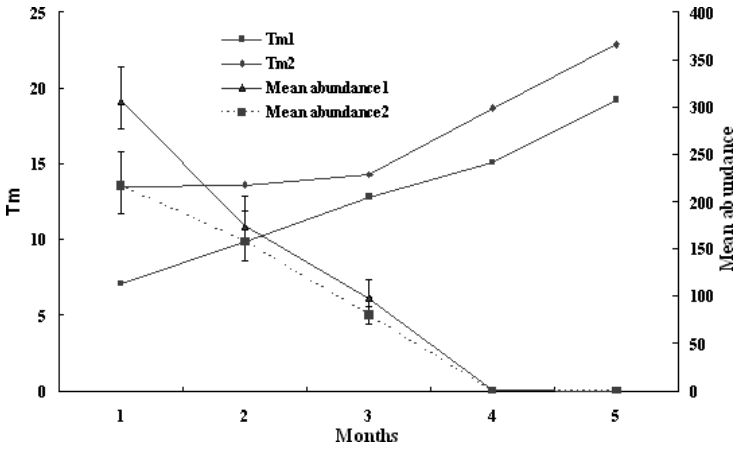

Figure 3 Correlation between ambient water temperature and shedding of plasmodia of *Myxobolus turpisrotundus* sp.n from allogynogenetic gibel carp. Tm1, mean abundance1, and Tm2, mean abundance2, represent field conditions and controlled laboratory conditions, respectively (bar indicates standard deviation).

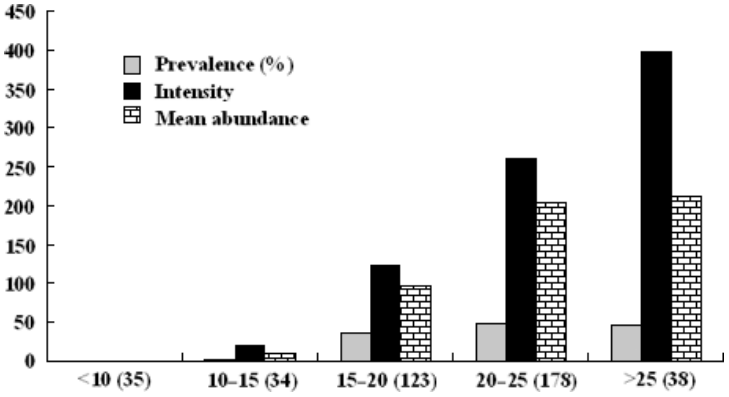

Figure 4 Prevalence, intensity and mean abundance of *Myxobolus turpisrotundus* sp.n plasmodia in relation to fish length. Number in parentheses indicates the number of fish sampled.

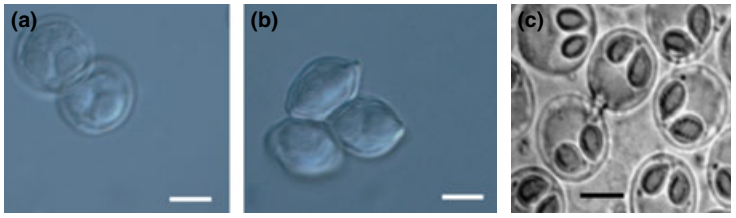

Figure 5 Fresh spores of *Myxobolus turpisrotundus* sp.n from the lips of allogynogenetic gibel carp and fresh spores of *Myxobolus rotundus* from gill of common bream (bars = 10 μm). (a) *M. turpisrotundus* sp.n spores in frontal view; (b) *M. turpisrotundus* sp.n spores in sutural view and side view; (c) *M. rotundus* spores in frontal view (provided by Dr C. Székely).

the anterior part of spores and tapering towards the discharging canals of the polar filaments. The ratio of polar capsule length to spore length is usually above 0.5 (Fig. 6c). Polar filaments coiled with 5–6 turns in polar capsules, situated perpendicularly to the longitudinal axis of the capsule. A remarkable intercapsular process is observed at the anterior end of the spore. Sporoplasm with two nuclei is uniform in density and occupies less than half the volume of the spore. A distinct iodinophilous vacuole is present. No extruded polar filament was observed even after stimulation with potassium hydroxide. Sporogenesis is asynchronous, with trophozoites and pre-sporogonic stages located in the periphery and mature spores at the centre of plasmodia. Histology showed that plasmodia developed sub-epidermally in several host organs and comprised a multiple, complex structure, which included a layer of fibroblasts, a collagenous membrane, melanophore and cup-like cells of unknown origin and function. The cup-like cells contacted directly with pre-sporogonic stages located in the peripheral parts of the large plasmodia. No severe inflammatory responses were found (Fig. 7).

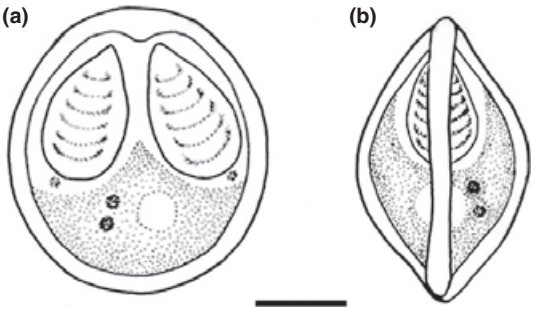

Figure 6 Schematic drawings of myxospore of *Myxobolus turpisrotundus* sp.n.(bar = 5 μm). (a) Frontal view. (b) Sutural view.

Taxonomic summary of *Myxobolus turpisrotundus* sp. n. (Figs 5, 6, 7)

Type hosts: *Carassius auratus auratus* (L.) (Cyprinidae: Cypriniformes), *Carassius auratus gibelio* (Bloch)

Type locality: Ezhou Prefecture, Hubei Province, China

Sites of infection: The subepidermal tissues of skin, lips, jaws, gill operculum, dorsal fins, pectoral fins, ventral fins, anal fins, tail fins, gill arches, pharynx and intestine wall.

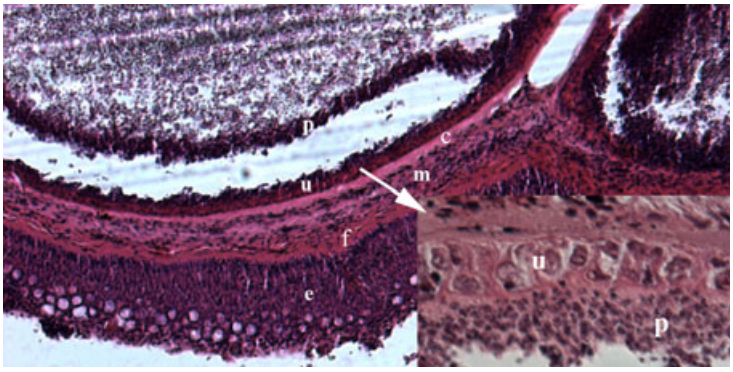

Figure 7 Histological sections of the skin of allogynogenetic gibel carp infected by *Myxobolus turpisrotundus* sp.n. showing the complex structure of plasmodia: p, pre-spore stages of *M. turpisrotundus* distributed at the periphery of plasmodia; u, cup-like cell with unknown derivation and function; c, collagenous fibroblast membrane; f, fibroblast layer; m, melanophore layer; e, epithelial cell of host (×400). Inset showing higher magnification of u and p (×800).

Prevalence of infection: 2.31% (182 out of 7892).

Type material: Syntype specimens of spores in glycerin gelatin and H&E-stained sections deposited in the Laboratory of Fish Diseases, Institute of Hydrobiology, Chinese Academy of Sciences, accession no. MTR20051226.

Etymology: Turpisrotundus is a composite of the Latin *turpis* (= ugly) and *rotundus* (= round), denoting the disfigured appearance of the host fish due to the parasite infection and the roundish spores.

Taxonomic remarks: Because a round spore shape is a common feature of many myxosporeans species, it is not easy to differentiate them when spore sizes overlap. Out of about 80 *Myxobolus* species infecting goldfish, crucian carp and allogynogenetic gibel carp, *M. turpisrotundus* displays similar morphological characteristics with the erroneously designated *M. rotundus*, *Myxobolus nielii* (syn. *Myxosoma sinensis* Nie et Li, 1973), *Myxobolus sphaerica* (Fujita, 1924), *Myxobolus lieni* (syn. *Myxosoma lieni* Nie et Li, 1973), *Myxobolus pyramidis*, *Myxobolus rotundatus*, *Myxobolus asianensis*, *Myxobolus auratus* (syn. *Myxobolus orbiculatus*), *Myxobolus obiquus* Kudo, 1934, *Myxobolus pseudoparvus* Li et Nie, 1973, *Myxobolus dispar*, *Myxobolus valatus* Li et Nie, 1973, *Myxobolus gibelioi* Wu et Wang, 1982, *Myxobolus tuberculus* Nie et Li, 1992. However, the spore of *M. nielii* is oval in shape, with slightly tapering anterior end and usually 5-15 'V' shaped edge markings located in the posterior end of the spore, compared with the round shape and 5–6 'V' shaped edge markings seen in spores of *M. turpisrotundus*. Spore lengths for *M. sphaerica* and *M. gibelioi* Wu et Wang, 1982 are shorter than spore width, whereas the ratio of spore length to spore width for the present species is more than 0.5. In addition, the number of polar filament coils of *M. sphaerica* (4–5) is less than that of *M. turpisrotundus* (5–6). Also, the plasmodia of the former species are small (about 20 μm in diameter), compared with the latter (above 200 μm in diameter). The spores of *M. lieni* and *M. pseudoparvus* Li and Nie, 1973 are smaller than those of *M. turpisrotundus*. The spores of *M. pyramidis* are pyriform with slightly narrow anterior end, compared with the round spores of *M. turpisrotundus*. *M. rotundatus* can be differentiated from *M. turpisrotundus* by its' thicker spore and small intercapsular process. The spores of *M. auratus* and *M. tuberculus* are bigger than those of *M. turpisrotundus*. The anterior end of spores of *M. asianensis* is narrower than that of *M. turpisrotundus*. *M. obliquus* differs from *M. turpisrotundus* in the width of the posterior end of spores. *M. dispar* is distinct from *M. turpisrotundus* in possessing two unequal polar capsules. The spores of *M. valatus* Li and Nie, 1973 have a remarkable lace-like posterior membrane and 8–9 coils of the polar filament. Some other *Myxobolus* species also share similar morphology with *M. turpisrotundus*, including *Myxobolus chungnanensis* infecting the kidney and gills of *C. idella* and *Myxobolus hoshinai* infecting the integument of the skins, fins, mouth and gills of *Cyprinus carpio* L. However, the intercapsular process of spores of *M. chungnanensis* is smaller than that of *M. turpisrotundus* and the anterior end of *M. hoshinai* spores is wider than the posterior end, in contrast to *M. turpisrotundus*. The previous erroneously identified *M. rotundus* (s.l.) in the monograph of Chen & Ma (1998) cannot be differentiated from *M. turpisrotundus* solely on spore morphology. However, the infection sites and size of plasmodia are different from *M. turpisrotundus*, according to the descriptions of Chen & Ma (1998). *Myxobolus turpisrotundus* is difficult to discriminate morphologically from *M. rotundus* although there are some slight differences, e.g. the relative size of spore and

intercapsular process and the ratio of the length of polar capsule to the length of spore. However, the spore morphology of *M. rotundus* (s.l.) ex *C. auratus auratus* is almost identical with that of *M. turpisrotundus*. Detailed morphological comparisons between the original *M. rotundus, M. turpisrotundus* and similar *Myxobolus* species can be found in Tables 1 & 2.

Molecular data

All sequences amplified from plasmodia of *M. turpisrotundus* isolated from different sites of infection were identical. The similarity search by BLAST showed that the sequence of *M. turpisrotundus* was 98% identical with a previously cloned fragment (AY165179) which is 726bp in length and included several ambiguous sites, and was then most similar to *Sphaerospora molnari* (AF378345), *Sphaerospora renicola* (AY735410) and *Henneguya doneci* (EU344898), with sequence identities of 92%, 92% and 94%, respectively. However, it was significantly different (87%) from *M. rotundus* (EU710583) from common bream and its triactinospore stage, which share 100% sequence identity with *Triactinomyxon* sp. 'type 4' and 99% identity with *Myxobolus parviformis* (AY836151). Congruent phylogenetic tree topologies were obtained regardless of the tree-constructing method used (ML, MP, NJ and Bayesian inference), albeit minor differences existed between species not discussed here and between slightly different bootstrap values. The phylogenetic relationships of selected species are consistent with previous reports (Fiala 2006; Jirků, Fiala & Modrý 2007) and representatives of *Myxobolus* species were intermixed with the morphologically distinct *Sphaerospora* spp., *Thelohanellus* spp. and *Henneguya* spp. *M. turpisrotundus* falls within a well-supported mixed clade which includes *S. molnari, S. renicola* and *H. doneci*. Interestingly, the spores of all these species are round (Fig. 8). However, *M. rotundus* clustered well with *M. parviformis* forming a sister branch with *Myxobolus impressus,* although there are many differences in spore morphology between them.

Discussion

Due to the simplicity of myxospore structure and the few morphological keys for taxonomy, species identification and differentiation between myxosporean species possessing similar morphological characteristics are often problematic. Synonyms or anonyms possibly still exist in described species, especially for *Myxobolus* species which are the richest in diversity in the Myxozoa. Molecular data combined with spore morphology, host specificity and tissue affinity are now accepted for identification of a new species and redescription of inadequately described species (Eszterbauer 2004; Molnár 2007). In China, a monograph of the Myxozoa was published in 1998 which recorded 575 species assigned to 23 genera from cultured and wild fish throughout the country. It has greatly increased the species richness of the Myxozoa (Chen & Ma 1998; Kent *et al.* 2001), however, some species were inadequately described or identified without detailed tissue tropism and molecular data. This is apparently due not only to poor reference to published descriptions but also technical limits at that time, resulting in several taxonomic confusions. *M. rotundus* was originally described in *A. brama* in Hungary and subsequently found in *Gobio gobio* (L.) by Landsberg & Lom (1961). Subsequently, the parasite was recorded in a wide range of hosts, especially cyprinid fish. However, most species descriptions were based solely on spore morphology. No complete data sets for *M. rotundus,* including the life cycle were available until the recent papers by Molnár *et al.* (2009) and Székely *et al.* (2009), where the species was considered as specific for *A. brama* and *T. tubifex*. Thus, it was necessary to re-evaluate the validity of '*M. rotundus*' reported from other fish hosts. In the present study, based on morphological data, tissue tropism and molecular analysis, we conclude that the species from *C. auratus auratus* and allogynogenetic gibel carp in China are conspecific (Wu & Wang 2000; Lu *et al.* 2002; Zhang 2006), and the parasite is designated as *M. turpisrotundus* sp. n., distinct from *M. rotundus*. However, the validity of *M. rotundus* (s.l.) from different organs in genetically distant fish hosts recorded by Chen & Ma (1998) remains unresolved. It is likely that they are a collective species, given that most *Myxobolus* spp. have strict host and tissue specificities (Molnár 1994; Kent *et al.* 2001). Morphologically, *M. turpisrotundus* resembles many *Myxobolus* spp. with round spores of similar size (about 10 μm spore length). However, there are considerable differences in their tissue tropism, host specificity and geographical distribution. In addition, molecular evidence based on SSU rRNA can differentiate *M. turpisrotundus* from those morphologically similar species with sequences

Table 1 Biometrical comparisons between *Myxobolus turpisrotundus* sp.n. and *Myxobolus rotundus* s.l. Nemeczek, 1911

	Myxobolus rotundus Nemeczek 1911 (Eiras *et al.* 2005)	*M. rotundus* (Miroshnichenko 1980)	*M. rotundus* (Molnár *et al.* 2009)	*M. rotundus* (s.l) (Chen & Ma 1998)	*M. rotundus* (s.l) (Wu & Wang 2003)	*Myxobolus turpisrotundus* (s.l) (Present study)
Host	*Abramis brama; Gobio gobio*	*A. brama* and other fish in the Ukrainian SSR	*A. brama*	*Hypophthalmichthys molitrix; Ctenopharyngodon idella; Schizothorax davidi; C. auratus auratus*	*C. auratus auratus*	*C. auratus gibelio*
Infection sites	Gills	Gills	Capillary network of gill lamellae	Gills, fins, kidney, intestine, liver, spleen, gonad, urinary bladder, gall bladder	Body surface, cranium, gills, lips, fins	Subepidermal tissues of skin, lip, jaw, fins, gill arches, operculum, pharynx, intestine wall
Cyst size	1300 × 1500	Unavailable	60–180	65.4 (32–120) × 50 (30–90)	820 (320–1250) × 730 (370–950)	200–5600
SS	Roundish	Roundish	Roundish	Roundish or ellipsoidal	Roundish	Roundish
LS	10	9–10.8	10.6 (9–12)	9.2 (8.4–9.8)	9.32 (8.06–9.89)	9.2 ± 0.3 (8.6–10.0)
WS	9.8	8.1–9.8	9.5 (8–11)	8.2 (7.8–8.4)	8.31 (7.56–8.64)	8.4 ± 0.3 (8.2–10.0)
TS	3	5–6	6.2 (5.5–6.5)	5.5 (5.4–5.8)	5.67 (5.17–6.44)	5.8 ± 0.2 (5.1–6.5)
PCS	Pyriform	Pyriform	Pyriform	Pyriform	Pyriform	Pyriform
LPC	3.8–5	4.0–5.3	4.9 (4.5–5.0)	4.5 (3.6–4.8)	4.65 (3.81–5.53)	4.8 ± 0.1 (4.1–5.1)
WPC	–	2–3	3.2 (3–5)	2.8 (2.4–3)	3.10 (2.72–3.34)	3.0 ± 0.1 (2.5–3.1)
CPC	–	–	6	5–6	7	5–6
RP	–	–	< 1/2	< 1/2	> 1/2	> 1/2
IP	–	Small	Small	Large	Large	Large

SS, shape of spore; LS, length of spore; WS, width of spore; TS, thickness of spore; SPC, shape of polar capsule; LPC, length of polar capsule; WPC, width of polar capsule; CPC, coils of polar filaments, ratio of the length of polar capsule to the length of spore (RP) and intercapsular process (IP).

Table 2 Comparisons of *Myxobolus turpisrotundus* sp.n with related *Myxobolus* species with roundish or oval spores and overlapping spore size

Species	LS	WS	TS	LPC	WPC	IS	Ref.
Myxobolus turpisrotundus sp.n.	9.2 ± 0.3 (8.6–10.0)	8.4 ± 0.3 (8.2–10.0)	5.8 ± 0.2 (5.1–6.5)	4.8 ± 0.1 (4.1–5.1)	3.0 ± 0.1 (2.5–3.1)	Under epithelial tissues	Present study
M. nielii	10.0 (8.0–12)	8.6 (8.4–9.6)	6.0	4.7 (4.2–5.0)	2.9 (2.4–3.0)	Gills, kidney, fins, intestine, bile bladder, spleen, urinary bladder, heart, throat	Chen & Ma (1998)
M. sphaericus	8.5–12	9.0–11.0	6.0	4.5–5.5	2.6–3.5	Gills, kidney, intestine	Chen & Ma (1998)
Myxosoma lieni	7.2–7.4	7.2–7.4	4.8–5.0	3.8 (3.6–4.2)	2.8 (2.6–3.0)	Gills, kidney, urinary bladder, intestine, liver, gall bladder, brain, spleen, muscle	Chen & Ma (1998)
M. rotundatus	10.1 (9.2–10.8)	9.2 (8.4–9.6)	7.2 (7.0–7.4)	4.6 (4.2–4.8)	2.7 (2.1–2.9)	Stomach, hindgut, gills, gall bladder, urinary bladder, kidney, gut wall, fins	Chen & Ma (1998)
M. asianensis	11.0 (10.8–12.0)	9.6 (9.4–10.0)	6.2 (6.0–6.6)	5.0 (4.6–5.4)	3.4 (3.2–3.6)	Gills, body surface, gut	Chen & Ma (1998)
M. pyramidis	10.5 ± 1.1 (9.6–12.0)	10.2 ± 0.9 (9.2–11.5)	6.1 ± 0.2 (5.8–6.3)	5.5 ± 0.7 (4.5–6.3)	3.5 ± 0.2 (3.0–4.1)	Gill filaments	Zhang *et al.* (2006)
M. obliquus	8–9	7–8	5–6	4.5	2	Muscle	Eiras *et al.* (2005)
M. pseudoparvus	8.6 (8.2–9.6)	8.1 (7.2–9.0)	6.1 (6.0–6.2)	3.7 (3.4–3.0)	2.5 (2.2–2.6)	Body surface, gills, fins, spleen	Chen & Ma (1998)
M. valatus	9.4 (8.4–9.6)	8.2 (7.4–8.4)	6.0–7.0	5.0 (4.8–5.4)	3.1 (3.0–3.4)	Gill lamellae, body surface	Chen & Ma (1998)
M. gibelioi	9.0–11.7	9.5–13.5	5.4–8.1	5.4–6.3	4.8–5.4	Gills	Chen & Ma (1998)
M. tuberculus	13.6–14.2	13.0–14.0	7.0–7.5	6.4–7.2	3.0–4.0	Heart, urinary bladder	Chen & Ma (1998)
Myxobolus bramae	10–12	8–10	4.5–6.5	4–4.5	2.3–3.5	Gill lamellae	Molnár *et al.* (2009)
M. anguilli	10.8–12.4	10.8–12.0	5.6–6.4	4.8–5.6	3.2–4.0	Gills, mouth	Chen & Ma (1998)

LS, length of spore; WS, width of spore; TS, thickness of spore; LPC, length of polar capsule; WPC, width of polar; IS, infection sites.

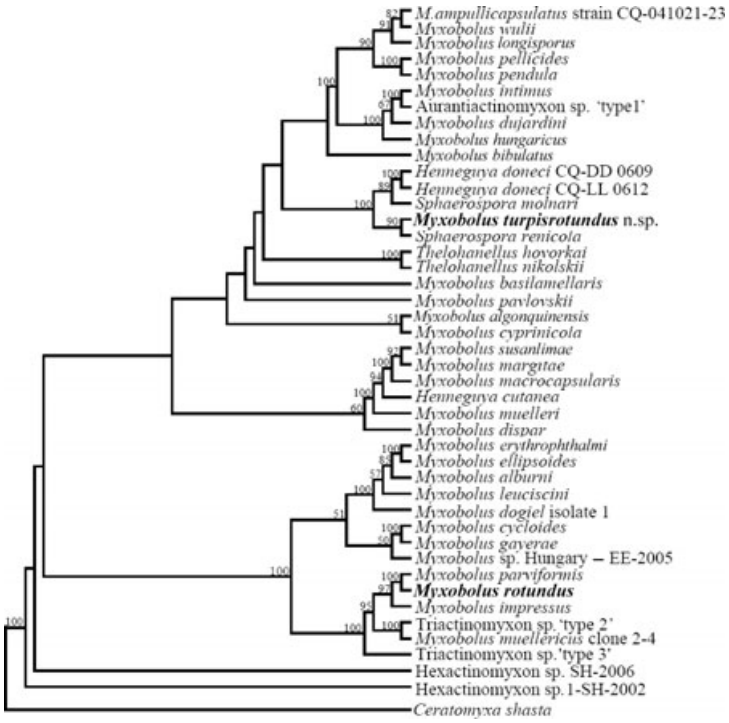

Figure 8 Phyogenetic tree generated by maximum likelihood analysis of the aligned SSU rRNA gene sequences of selected species, with evolutionary model HKY and transition/tranversion set at 4. Numbers at nodes indicate bootstrap confidence values (100 replications). *Myxobolus turpisrotundus* sp.n. and *Myxobolus rotundus* highlighted.

in GenBank. Phylogenetic analysis in the present and previous studies (Lu & Nie 2004; Fiala 2006) clearly showed that *M. turpisrotundus*, *M. rotundatus*, *H. doneci*, *S. renicola* and *S. molnari* formed the same clade with a high bootstrap value. Interestingly, the five species possess round spores, suggesting that spore morphology has congruence with molecular phylogeny to some extent. Similar relationships have been reported in other groups of *Myxobolus* species (Salim & Desser 2000; Eszterbauer 2004; Zhao *et al.* 2008). In addition, all the five species mentioned above develop in subepidermal tissues although from different organs. It is obviously important to combine the morphological features proposed by Lom & Arthur (1989) with host specificity, tissue affinity and molecular evidence in interpretation of myxozoan phylogeny. Additional molecular markers should be applied more broadly to Myxozoa to validate phylogeny based on SSU rRNA because a single gene may produce biased results, e.g. a long branch attraction problem with the use of ribosomal genes (Siddall & Whitting 1999; Fiala & Bartošová 2007).

The seasonal occurrence of myxosporean plasmodia has been widely reported for many histozoic plasmodia-forming species, including *Kudoa ciliatae* parasitizing *Sillago maculate* Quoy & Gaimard (Hallett, O'Donoghue & Lester 1997), *Thelohanellus wuhanensis* (Wang, Yao, Wang & Lu 2001) and *M. gibelioi* (Wang, Yao, Gong, Wang & Nie 2003) infecting *C. auratus gibelio*, *Myxobolus magellanicus* infecting *Galaxias maculatus* (Flores & Viozzi 2001) and *Myxobolus intimus* parasitizing *Rutilus rutilus* (L.) (Rácz, Székely & Molnár 2004). The highest prevalence and abundance of *M. turpisrotundus* plasmodia occurred in winter and the lowest in spring and summer. The unaesthetic appearance caused by *Myxobolus turpisrotundus* has resulted in large economic losses of allogynogenetic gibel carp. Fish farmers have generally killed or isolated infected fish to prevent further spread of the disease, but the results of the present study indicate that *M. turpisrotundus* does not cause directly mortality, and that infected fish can recover completely. Thus, it is recommended that infected fish are temporarily kept in enclosed conditions until late spring or early summer to let them recover naturally, and to prevent the release of spores into culture ponds to cause re-infection.

Biological and environmental factors, including the host susceptibility, appearance and concentration of infectious stages, substrate type, water temperature and water flow velocity have been reported to influence the severity of infection with

myxozoan species (Kent *et al.* 2001; Steinbach Elwell, Kerans, Rasmussen & Winton 2006; Hallett & Bartholomew 2008; Bjork & Bartholomew 2009). Among environmental factors, water temperature has been suggested to have a considerable effect on the development of myxozoans in fish and invertebrate hosts, as well as spore release from the host (Kent *et al.* 2001; Yokoyama 2003; Canning & Okamura 2004). However, in the present study, the release of mature spores of *M. turpisrotundus* into the water has been found to be unrelated to water temperature. Spore release while the host fish is still alive, rather than retention within the host until its predation or death, possibly represents a highly evolved host-parasite relationship (Atkinson, Hallett & Bartholomew 2007). This is a common feature of most myxozoan parasites which do not causes severe reduction of host fitness. Based on the seasonal pattern of occurrence of *M. turpisrotundus* and the life cycles of histozoic myxozoan species, it is suggested that the corresponding actinosporean infects allogynogenetic gibel carp from April to June when the water temperature increases to above 18 °C.

The absence of infection of *M. turpisrotundus* in grass carp, bighead carp and yellow catfish suggests that the myxosporean has a relatively strict host specificity, similar to other myxozoans including *Myxobolus cerebralis* (El-Matbouli, Hoffmann, Schoel, McDowell & Hedrick 1999; Wagner, Arndt & Brough 2002; Hedrick, McDowell, Marty, Fosgate, Mukkatira, Mykebust & El-Matbouli 2003; Kallert, Eszterbauer, Grabner & El-Matbouli 2009), *Kudoa thysites* (Moran, Whitaker & Kent 1999) and *Ceratomyxa shasta* (Ching & Munday 1984). The resistance mechanisms of different fish hosts to myxosporean species remain unresolved, but differences in innate immunity have been suggested as a cause (Baerwald, Welsh, Hedrick & May 2008). Additionally, the feeding habit and habitat differences of fish species may partially explain the different susceptibility of host fish to myxosporean species (Xiao 1988). The grass carp is herbivorous and pelagic and perhaps less likely to come into contact with infected oligochaete hosts or actinosporean stages, which may explain why this species is less infected by myxosporeans than other widely cultured fish in China (Chen & Ma 1998).

Although all allogynogenetic gibel carp are female, some male *C. auratus auratus* were found to be infected with *M. turpisrotundus* from archived samples. This suggests that infection of *M. turpisrotundus* is not host sex-specific. However, a strong relationship of the parasite with host size was evident. No infection was found in fish smaller than 10 cm in body length, while prevalence and abundance significantly increased with increasing size of host fish. Conflicting results of the relationship between myxosporean infection and host size have been reported for other myxosporean species (e.g. Sitja-Bobadilla & Alvarez-Pellitero 1993; Molnár 1998; Flores & Viozzi 2001; Rácz *et al.* 2004). A cumulative effect of parasite load is a possible reason for the high prevalence of infection with *M. turpisrotundus* in larger fish. Alternatively, it is possible that the significant differences in prevalence and abundance between different sizes of host fish were due to seasonality of the life cycle of the parasite in relation to the life history of the host.

In conclusion, the present study confirmed that *M. turpisrotundus* sp. n. is clearly different from *M. rotundus* based on morphological features, tissue tropism, seasonal patterns of infection and molecular data. Future studies on the life cycles of *M. turpisrotundus* are required to clarify several biological characteristics of the parasite which remain uncertain.

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