**Myxobolus lentisuturalis** sp. n. (Myxozoa: Myxobolidae), a new muscle-infecting species from the Prussian carp, *Carassius gibelio* from China

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Abstract. A new highly pathogenic muscle-infecting species of the genus *Myxobolus* Bütschli, 1882 is described from the Prussian carp, *Carassius gibelio* (Bloch, 1782) using spore morphology and SSU rDNA sequence data. Phylogenetic analyses elucidated relationship of the newly described *Myxobolus lentisuturalis* to other *Myxobolus* species and supported its position of an independent species.

After a revision of synonyms, more than four hundred (453) named species were listed within the genus *Myxobolus* Bütschli, 1882 by Landsberg and Lom (1991). Since then the number of recognised species of the genus has grown, most of the species being distinguished from each other solely on morphological criteria. Of all species described to date within the genus *Myxobolus*, thirty only have been characterised also with molecular data, using sequences of SSU rDNA. In the recent review of advances in the understanding of Myxozoa, Kent et al. (2001) stated that molecular data that they used as the basis for conclusions on evolutionary relationships among the Myxozoa were limited and appealed to researchers to extend them. In accordance with the authors of that key review we believe that the only valuable molecular data are those obtained for morphologically well-described species. Unfortunately, spores of many species of the genus *Myxobolus* are very similar to each other, many published descriptions are incomplete or not explicit and many are inconsistent with their documentation. Among hundreds of named species, a small number only is documented by photomicrographs. Inconsistencies in line drawings of the same species produced by different authors make the species identification of new findings sometimes extremely difficult.

In this communication we present a description of a new species of *Myxobolus* coupled with sequence information for SSU rDNA and phylogenetic analyses of molecular data.

**MATERIALS AND METHODS**

In 5 out of 25 specimens of *Carassius gibelio* (Bloch, 1782) (size range 12.0-16.5 cm) collected in September 2001 from Lake Ba’o’an in Hubei Province, China, mutually identical spores of the genus *Myxobolus* Bütschli, 1882 were found. In one fish the infection was manifested by gross deformity, a hump anterior to the dorsal fin. The lesion, limited to skeletal muscles, contained a mass of mature spores. They were cleaned from tissue debris by washing in an ample volume of sterile distilled water and used for morphological and molecular characterisation. Tissue samples were fixed in modified Davidson’s fixative and processed for histology using Histowax (Reichert Jung) as an embedding medium. Histological sections were stained with haematoxylin and eosin (H&E) and Giemsa stains.

Total cell DNA was extracted from spores using the DNeasy™ Tissue Kit (Quiagen, Germany) according to the manufacturer’s protocol. The SSU RNA gene was PCR-amplified with a set of universal eukaryotic primers (5'-AYCTGGTTGATTTGCGAG-3' and 5'-TGATCCATCTGGAGTCACCT-3') (Embley et al. 1992). PCR was carried out in a 25-µl reaction volume using 10 pmol of each primer, 250 µM of each dNTP, and 2.5 µl 10 × PCR Buffer (Takara, Japan) and 1 unit of TaqDNA polymerase (Takara, Japan). The reactions were run on a T3 Thermocycler (Biometra). Conditions were as follows: initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, at 48°C for 2 min, at 72°C for 2 min and a final extension at 72°C for 5 min.
72°C for 10 min. Amplification products were gel-isolated and cloned into pCR® 2.1 TOPO Cloning vector using the TOPO-TA Cloning Kit (Invitrogen). They were sequenced from both strands on an automatic sequencer CEQ™ 8000 (Beckman Coulter) using CEQ DTCs Dye Kit (Beckman Coulter) according to the manufacturer’s protocol.

The SSU rRNA gene sequences were aligned using Clustal X program (Thompson et al. 1997) with various alignment parameters. In order to identify the most accurate position of M. lentisuturalis in the myxosporean tree, the dataset of sequences covering maximum of myxosporean species available in GenBank was used for the first alignment. Polypodium hydriforme, a parasitic narcomedusan, was chosen as outgroup. The alignment was corrected by eye using the BioEdit sequence alignment editor (Hall 1999). Ambiguously aligned regions were excluded. The second alignment included sequences of species related to M. lentisuturalis. Ceratomyxa shasta, Myxidium truttae and Myxidium sp. (Accession No. U13829) were selected as outgroups based on results from analysis of the first alignment. Phylogenetic analyses were performed using maximum parsimony (MP) and distance methods (minimum evolution). Methods were carried out with the program package PAUP*, Version 4.0b8a (Swofford 2001). The MP analysis was done using heuristic search with random addition of taxa (10 replications) and the ACCTRAN option. Gaps were treated as missing data and transition/transversion (Tv/Ts) ratios of 1:1, 1:2, 1:3 and 1:4. The distance method was executed using heuristic search with the minimum evolution as the objective setting. We used K2P and GTR substitution models. Genetic distances were calculated with K2P algorithm. Clade support was assessed with bootstrapping (500 replicates for MP, 1000 replicates for distance method).

RESULTS

Myxobolus lentisuturalis sp. n. Figs. 1-3

Description of fresh spores (light microscopy). Spores (Figs. 1-3) ellipsoidal, 11.8 (11.2-12.4) µm long, 7.6 (7.2-8.4) µm wide and 5.2 µm thick (n = 20). Results of image analysis: L = 11.82 ± 0.36, median 12; W = 7.64 ± 0.30, median 7.6. Mucus envelope lacking. Shape of spores uniform (Figs. 2, 3). Two equal-sized pyriform polar capsules 4.2 (4.0-4.4) µm long × 2.5 (2.0-2.8) µm wide, with long axes parallel to long axis of spore. Posterior end of polar capsules slightly exceeding mid-spore length, (ratio 1.16). Thickening of apical part of suture bulges inwards and separates points of polar capsules at a distance of 2.4-2.8 µm. Polar filaments coiled four times. Sutural folds very difficult to discern. Of the features discriminating Myxobolus lentisuturalis from other species with similar shape of spores, the most important was the position of polar capsules as seen in frontal view (Figs. 1-3).

Type host: Carassius gibelio (Bloch, 1782) (Cypriniformes, Cyprinidae).

Site of infection: Muscle tissue (m. laterodorsalis). Plasmodia developed in muscle fibres (Fig. 4).

Fig. 1. Myxobolus lentisuturalis sp. n., frontal view of a mature spore.

Type locality: Lake Bao’an, Hubei Province, China.

Prevalence: Five of 25 fish examined (20%) were infected.

Type material: Slides with stained spores are deposited in the Institute of Parasitology, Academy of Sciences of the Czech Republic, České Budějovice (Cat. Nos. H-PM-060 to 063) and in the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, P. R. China.

Etymology: The name is derived from a conspicuous morphological feature, lens-like thickening of the valve suture bulging inwards and separating points of polar capsules. (Lens, genit. lentis, Latin, a lens.)

SSU rDNA sequence data and phylogenetic analyses

The length of complete sequence of M. lentisuturalis was 2107 bp including regions corresponding to forward and reverse primers. G+C content was 47.7%. The sequence was deposited in the GenBank under accession number AY119688. The phylogenetic analyses were based on a final alignment (2217 nucleotide sites) consisting of 49 taxa, from which ambiguously aligned areas (240 nucleotide sites) were removed. The dataset for this alignment was chosen upon the preliminary results based on the first alignment and consisted of Myxobolus-related species. The most closely related species shown by the SSU rRNA gene analyses were Myxobolus xiao and Myxobolus sp. from Catostomus commersoni (Acc. No. AF378343). The distances computed from the alignment revealed 91.4% and 91.8% similarity between M. lentisuturalis and two sequences of M. xiao. Myxobolus sp. (Acc. No. AF378343) from Catostomus commersoni was 88.2% similar to M. lentisuturalis. The similarity to other myxosporean species was about 70%.

Myxobolus lentisuturalis clusters with the freshwater clade of myxosporeans represented mostly with Myxobolus and Henneguya species. In all performed analyses (MP – Tv/Ts = 1:1, 1:2, 1:3, 1:4 and distance methods) this clade consisted of three well-supported subclasses corresponding to previous studies (Kent et al. 2001). The subclass containing M. lentisuturalis included other Myxobolus species (e.g., M. cerebralis) and also Sphaerospora truttae and two species of Henneguya.
Myxobolus lentisuturalis constitutes a strongly supported clade with M. xiaoi and Myxobolus sp. from C. commersoni (Fig. 8). The ancestral position of M. lentisuturalis that resulted from MP with TvdTs = 1:2 (three most parsimonious trees, 9669 steps) was weakly supported (57%), but this topology remained unchanged in MP with TvdTs = 1:3 (18 most parsimonious trees, 12536 steps) and 1:4 (six most parsimonious trees, 15395 steps). Distance method with K2P and GTR model of evolution and MP with TvdTs = 1:1 (six most
**Fig. 8.** Maximum parsimony tree of the SSU rRNA gene sequences of myxosporeans rooted at *Ceratomyxa shasta*, *Myxidium truttae* and *Myxidium* sp. (Tv/Ts = 1:2, 9669 steps). Bootstrap values (MP Tv/Ts = 1:1, MP Tv/Ts = 1:2, distance method K2P) are indicated for nodes gaining more than 50% support. GenBank accession numbers are in parentheses. The scale is given under the tree.
Although similar in shape according to descriptions and
some of the drawings available, their spores differ in
diagnostic features. Spores of *M. notropis* are 13.7
(12.0-15.6) μm long, 8.6 (7.8-9.0) μm wide and have
many sutural ridges. Spores of *M. carassii*, with
the average length (13.6 μm) exceeding the range of our
material, have polar capsules with convergent anterior
points and six to eight coils of polar filament. The
measurements of spores in our material correspond with
those given in the description of *Myxobolus kubanicus*
Bykhovskaya-Pavlovskaya et Bykhovski, 1940, the
species common for all *Carassius* hosts in China (*C.
auratus auratus*, *C. carassius* and *C. gibelio*). *Myx-
obolus kubanicus* has been documented in a series of line
drawings showing surprising variability of spores (Chen
1973, Shulman 1984, Zhang and Li 1990, Chen and Ma
1998). Since gross lesions induced by *M. lentisuturalis*
were similar to those depicted in Chen and Ma (1998)
for infection with *M. carassii* and *M. kubanicus*, we
analysed carefully the descriptions and drawings of the
latter species available in the literature (Shulman 1966,
1984, Chen 1973, Zhang and Li 1990, Chen and Ma
1998). Only one among twelve simplified line drawings
of *M. kubanicus* resembles our spores, while triangular
intercapsular processes, sutural ridges and convergent
polar capsules of different size testify that *M. kubanicus*
is a distinct species.

Among 65 *Myxosoma* and *Myxobolus* species listed
for *C. auratus auratus* in Chen and Ma (1998), another
four species (*Myxosoma pfrille* Fantham, Porter et
Richardson, 1939; *Myxobolus hypseleotris* Chen, 1998;
*M. ochengensis* Chen, 1998; and *M. inflatus* Chen, 1998)
have similar shape of spores, but other features distinguish them from our material. The
thickening of anterior part of spore suture is absent in all
of them. In addition, the polar capsules of *M. pfrille*, *M.
hypseleotris* and *M. ochengensis* are clearly convergent.
Spores of *M. inflatus* have a relatively large inter-
capsular process with a narrow base.

Contrary to variability depicted for many species of the
genus *Myxobolus* parasitising cyprinids, the
uniformity of shape of spores of *M. lentisuturalis* was
conspicuous, probably due to the fact that basically one
infrapopulation was evaluated while from the other four
host species only a small number of spores was
available and used for study.

Phylogenetic relationships

In accordance with the recent review of *Myxozoa*
(Kent et al. 2001) the freshwater clade of myxosporo-
ens branched in our phylogenetic analyses into three well-
supported subclades. Kent et al. (2001) did not include
in their phylogenetic analysis two partial sequences of
*M. xiaoi* and the sequence of *Sphaerospora truttae*
recently submitted to GenBank. Based on our results
both species are closely related to *M. lentisuturalis*. The
addition of these sequences changed the internal branch-
ing pattern of the subclade. *Myxobolus lentisuturalis*
clustered with *Myxobolus* sp. from white sucker (Acc.
No. AF378343) and with *M. xioai*, *Henneguya zschokkei* and *H. salminicola* clustered with *M. squamalis* and *Myxobolus* sp. from rainbow trout (Acc. No. AF378342).

In conclusion, phylogenetic analyses show a clearly independent position of *M. lentisuturalis* among the *Myxobolus* species sequenced to date. Although to understand species-level boundaries is often difficult, the comparison with very weakly resolved *M. cerebralis* and closely related species allow us to consider *M. lentisuturalis* as an independent species.

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