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Myxobolus oralis sp. n. (Myxosporea: Bivalvulida) infecting the palate in the mouth of gibel carp *Carassius auratus gibelio* (Cypriniformes: Cyprinidae)

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Abstract: During a survey on the myxosporean fauna of gibel carp *Carassius auratus gibelio* (Bloch) in China, a species of *Myxobolus* Bütschli, 1882 that did not conform to any known species was found. The species is characterised by the presence of round to ellipsoidal plasmodia of 2.6–4.0 mm in diameter in the palate of host. Mature spores are obovate in frontal view and lemon-shaped in lateral view, with the following range, mean and standard deviation of dimensions: 10.8–12.8 µm (11.7 ± 0.4 µm) long, 8.2–9.9 µm (8.9 ± 0.4 µm) wide and 6.0–7.5 µm (6.8 ± 0.3 µm) thick. Two polar capsules are pyriform, 4.0–5.5 µm (4.8 ± 0.3 µm) long by 2.9–3.6 µm (3.0 ± 0.2 µm) wide. Polar filaments are coiled, with 5 to 6 turns. A small proportion of spores possesses a short caudal process. Scanning electron microscopy revealed discoid spores with a low sutural ridge and middle bulge. The small subunit ribosomal DNA sequence of this species did not match any available sequences in GenBank. Phylogenetically, this species is sister to *M. nielii* (Nie et Li, 1973) and *M. hearti* Chen, 1998 in a *Henneguya-Myxobolus* clade with robust support. Given the morphological and molecular differences between this species and other *Myxobolus* species, we propose the name *Myxobolus oralis* sp. n. for this parasite from gibel carp.

Keywords: Myxozoa, ultrastructure, SSU rRNA gene, phylogenetics, morphology

Myxobolus Bütschli, 1882 is a genus with the greatest number of described species in the Myxosporea (Lom and Dyková 2006). Some of these species have been reported as significant pathogens of cultured and wild fishes (Chen and Ma 1998, Kent et al. 2001, Lom and Dyková 2006). Specific examples include the well known *Myxobolus cerebralis* Hofer, 1903, which causes whirling disease of salmonids (Gilbert and Granath 2003); *Myxobolus acanthogobii* Hoshina, 1952 that infects the brain of Japanese amberjack *Seriola quinqueradiata* Temminck et Schlegel causing host scoliosis (Yokoyama et al. 2004), and *Myxobolus buckei* Longshaw, Frear et Feist, 2003, which infects the spinal column of cyprinid fishes (Longshaw et al. 2003).

With the increased interest taken in diseases caused by *Myxobolus* species and myxosporeans in general, numerous *Myxobolus* species have been described. In 1991, Landsberg and Lom provided the synopsis of *Myxobolus* species that included 444 nominal species. This expanded greatly in the following 15 years, with Erias et al. (2005) and Lom and Dyková (2006) reporting 744 and 792 nominal species, respectively. Up to the present time, approximately 850 *Myxobolus* species have been described (Molnár 2011). This rapid expansion of nominal species is indicative of the interest in *Myxobolus* species, and also what is likely a great diversity within this genus. Documenting these new species greatly increased the known diversity of myxobolids and prospectively identified species with potential pathological threats to fish.

Gibel carp *Carassius auratus gibelio* (Bloch), a triploid gynogenetic species with fast growth potential, is an important commercial fish species in China. It has been cultured for more than 30 years in China and annual production is estimated at more than 2 million tons (Wang et al. 2011). However, the diseases caused by *Myxobolus* species have been the threat to this important commercial fish and resulted in mass mortality or loss of economic value (Liu et al. 2010a, 2012, Zhang et al. 2010a,b, Xi

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et al. 2011). To generate baseline data on *Myxobolus* diversity in gibel carp, we conducted a survey of these parasites in China. Here, we report a novel *Myxobolus* species infecting the palate in the mouth of gibel carp and describe its morphological, ultrastructural and molecular characteristics.

MATERIALS AND METHODS

Fish samples

Twenty specimens of gibel carp ranging from 300–450 g in weight were purchased from Baishazhou Fish Market, Wuhan City, Hubei Province, China in November 2011. Fish were transported to the Laboratory of Fish Diseases at the Huazhong Agricultural University in China and held in aquaria, where they were euthanised with 0.2 mg/ml tricaine methanesulfonate (MS-222, Sigma, St Louis, USA) prior to dissection.

Morphological examinations

Gross microscopic examinations of all organs for myxosporean infections were conducted according to Lom and Dyková (1992a) within 24 h after transportation. Plasmodia containing myxospores consistent with those of the genus *Myxobolus* were collected from the palate of gibel carp. Fresh spores from one plasmodium were measured according to Lom and Arthur (1989). Measurements of spores were performed using an Olympus BH2 microscope equipped with an ocular micrometre. Mean and standard deviations of each spore dimension were obtained from fresh mature spores (n = 30). Digitised images were obtained from the fresh wet mounts by a Nikon Eclipse 80i microscope. Line drawings were made based on the digitised images. All measurements are given in micrometres (μm) unless otherwise indicated.

Scanning electron microscopy

Fresh spores were fixed in a solution of 3% glutaraldehyde at 4 °C, dehydrated with CO_2 using the critical point method and sputter coated with gold (Liu et al. 2014). Samples were then examined with a JSM-6390 scanning electron microscope at 20 kV with a working distance of 18 mm.

Transmission electron microscopy

Fresh spores were fixed in 3% glutaraldehyde at 4°C, dehydrated, infiltrated and embedded in Epon812 (Ye et al. 2012). Ultrathin-sections were observed using a HITACHI H-7650 transmission electron microscope at 75 kV.

DNA isolation and sequencing

Genomic DNA was extracted from the spores in a plasmodium fixed in 100% ethanol. The small subunit ribosomal RNA (SSU rRNA) gene was amplified following the procedure of Liu et al. (2014). The PCR products were separated using a 1.0% agarose gel and sequenced directly with an ABI PRISM® 3730XL DNA sequencer (Applied Biosystems Inc., Foster, USA). A contiguous DNA sequence was assembled and deposited in GenBank. A nucleotide-nucleotide BLAST search was conducted to query posted sequences.

Phylogenetic analysis

To evaluate the relationship of the current species to existing myxobolids, 51 sequences were aligned with Clustal X version 1.8 (Thompson et al. 1997). The alignment consisted of the top BLAST search matches and representatives of neighbouring clades based on earlier analyses of the myxobolids (Liu et al. 2010b, Liu et al. 2012). *Ceratomyxa shasta* Noble, 1950 served as an outgroup. Phylogenetic analyses were carried out on this 1 424 character alignment following the procedure of Liu et al. (2010b). A maximum likelihood (ML) analysis was conducted using the general time reversible model (GTR + I + G). Nucleotide frequencies were estimated from the data (A = 0.2347, C = 0.1812, G = 0.2910, T = 0.2932), six rates of nucleotide substitution were [AC] = 1.2947, [AG] = 4.3760, [AT] = 1.8746, [CG] = 0.3513, [CT] = 6.9900, [GT] = 1.0000; proportion of invariable sites = 0.3434; gamma distribution = 0.3748 estimated with six rate categories. Bootstrap confidence values of ML analysis were calculated with 100 replicates. Bayesian analyses were conducted using the evolutionary model as above, with 10^6 generations, tree sampling every 100 generations, with a burn-in of 250 trees.

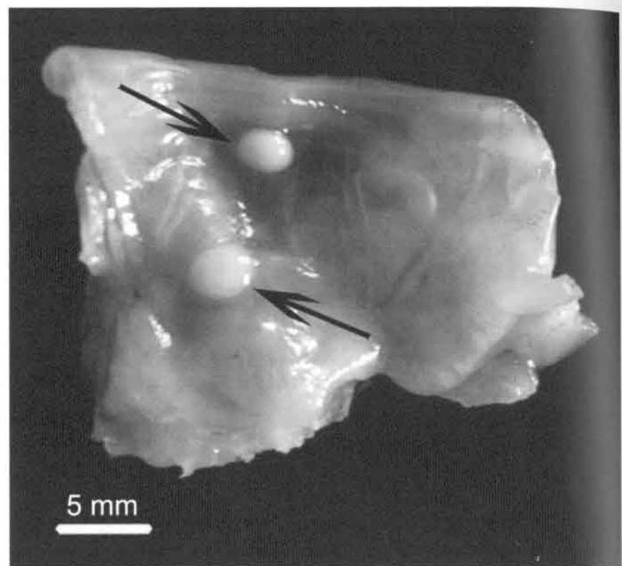

Fig. 1. Maxillary cut from the palate infected with two large plasmodia of *Myxobolus oralis* sp. n. from *Carrasius auratus gibelio* (arrows).

RESULTS

Myxobolus oralis **sp. n.** Figs. 1–4

Plasmodia (Fig. 1) round or ellipsoid, 2.6–4.0 mm, histozoic in palate. Myxospores (Figs. 2A, 3) obovate in frontal view and lemon-shaped in lateral view. Spores (n = 30) 10.8–12.8 (11.7± 0.4) long, 8.2–9.9 (8.9 ± 0.4) wide, 6.0–7.5 (6.8± 0.3) thick. Two polar capsules pyriform, 4.0–5.5 (4.8 ± 0.3) long by 2.9–3.6 (3.0 ± 0.2) wide. Polar filaments coiled with 5–6 turns (Fig. 4A). Two to five V-shaped sutural ridge markings present. Intercapsular appendix small. A small proportion of spores (11%, n = 100) with short tail or much more clearly visible tail (up to 5.2 in length) (Fig. 2B–D). Discoid spores with low sutural ridge and middle bulge, sutural line straight and distinct (Fig. 4B).

Type host: Gibel carp *Carassius auratus gibelio* (Bloch) (Cyprinidae).

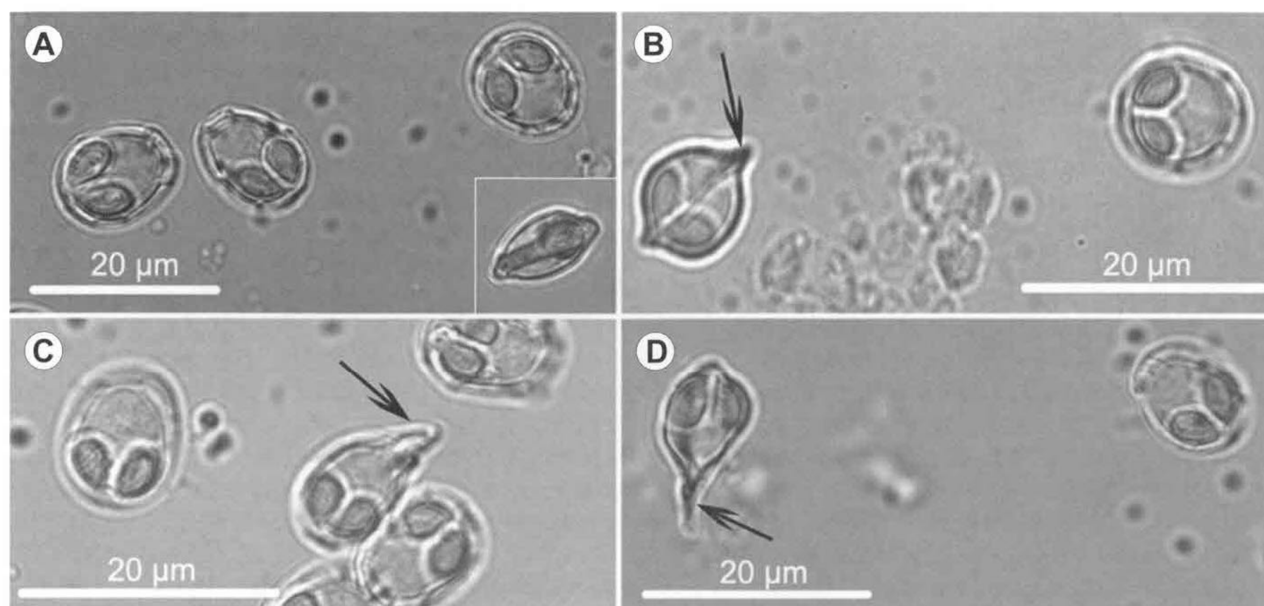

Fig. 2. Photomicrograph of fresh spores of *Myxobolus oralis* sp. n. from *Carassius auratus gibelio*. **A** – normal spores; inset showing spore in sutural view; **B** – a spore with caudal appendage (arrow); **C** – a spore with a short tail (arrow); **D** – a spore with the much elongate tail (arrow).

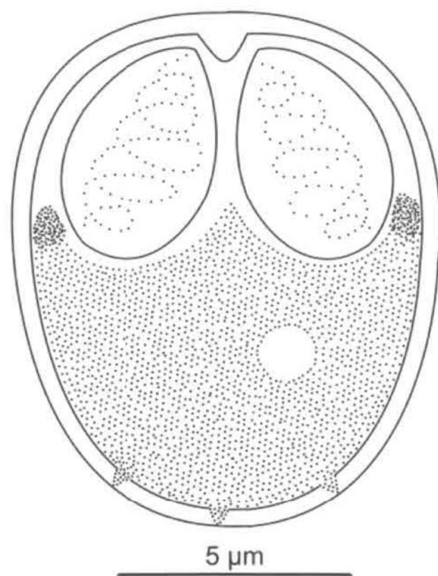

Fig. 3. Line drawing of fresh spore of *Myxobolus oralis* sp. n. from *Carassius auratus gibelio*.

Type locality: Hubei Province, China (30°27'52"N, 114°15'42"E).

Site of infection: Palate of the mouth.

Date of sampling: November 2011.

Prevalence: 10% (n = 20).

Type material: Mature spores fixed by 5% formalin deposited in Laboratory of Fish Diseases, College of Fisheries, Huazhong Agricultural University, Acc. No. MTR20111102.

Etymology: The species is named after its site of infection.

Sequence analysis

An assembled SSU rDNA sequence of 1 615 bases was deposited in GenBank (Acc. No. KC315782). A BLAST search yielded similarities to *Henneguya doneci* Schulman, 1962 (HM146129; 96% over 1 619 bp), *Myxobolus nielii* Landsberg et Lom, 1991 (JQ690358; 95% over 1 619 bp) and *Myxobolus hearti* Chen, 1998 (GU574808; 95% over 1 613 bp). Phylogenetic analysis by both ML and Bayesian analysis yielded trees with similar topology, but differences in nodal support (Fig. 5). Regardless of algorithm, *M. oralis* sp. n. was sister to *M. nielii* and *M. hearti*. There was robust support for the *Henneguya-Myxobolus* clade including *H. doneci*, *M. nielii*, *M. hearti* and the new species.

DISCUSSION

More than 800 nominal *Myxobolus* species have been described thus far throughout the world (Lom and Dyková 2006, Molnár 2011). Given the incredible diversity coupled with the simplicity of the diagnostic stage of these species, it is often difficult to determine the validity of morphologically similar species using spore morphology alone (Molnár 2011). To avoid the above conundrum, recent studies have suggested that host and organ specificity and also tissue tropism should be taken into consideration in species identification (Molnár 1994). In addition, molecular markers are also necessary and important in identification of species of *Myxobolus*, especially for the species developing within identical organs and tissues of the same or closely related fishes (Eszterbauer and Székely 2004, Liu et al. 2012). Therefore, the morphology, organ specificity

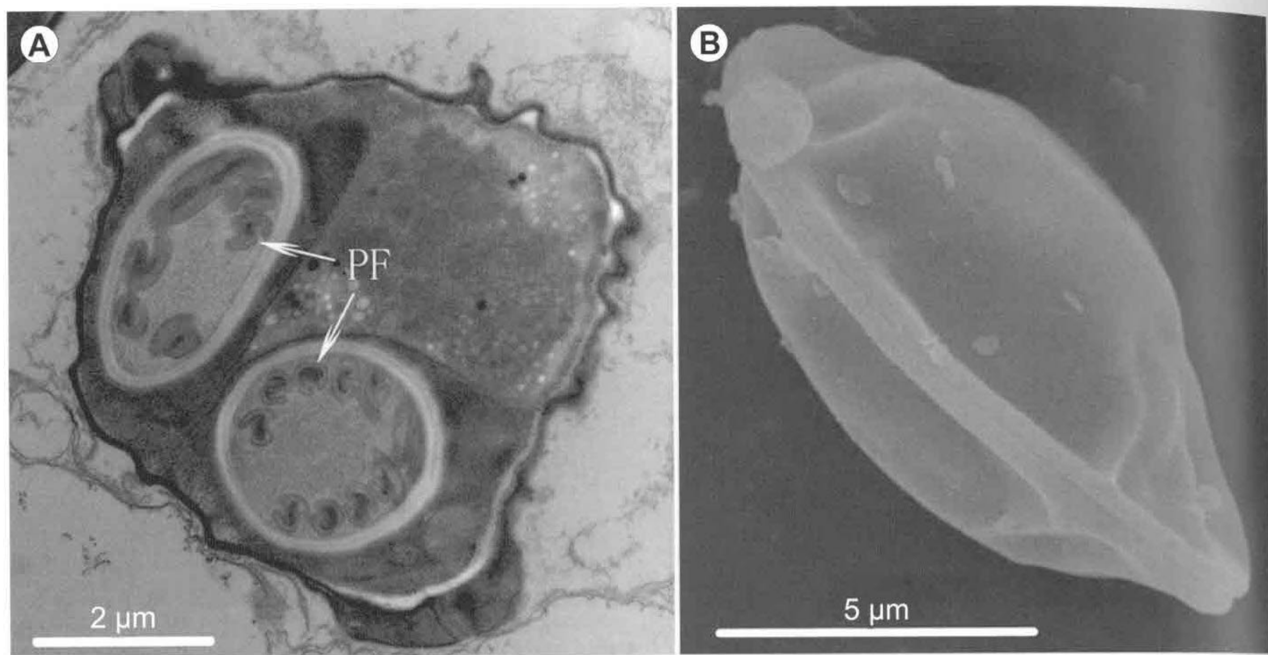

Fig. 4. Spores of *Myxobolus oralis* sp. n. from *Carassius auratus gibelio*. **A** – transmission electron micrograph of transverse section of a spore showing polar capsules and polar filaments (PF); **B** – scanning electron micrograph of the spore in sutural view with straight sutural line.

Table 1. Comparison of measurements of spores of *Myxobolus oralis* sp. n. with morphologically similar species. All measurements are in micrometres (µm), with mean ± standard deviation (if available), and range in parentheses.

Parasite	*M. oralis* sp. n.	*M. changkiangensis* Chen, 1998	*M. gibelio* Yukhimenko, 1986	*M. platyrostris* Akhmerov, 1960	*M. pyramidis* Chen, 1998	*M. sphaericus* Landsberg et Lom, 1991
Source	Present study	Chen and Ma (1998)	Yukhimenko (1984)	Akhmerov (1960)	Chen and Ma (1998)	Eiras et al. (2005)*
Host	*C. auratus gibelio*	*C. auratus auratus*	*C. auratus gibelio*	*C. auratus gibelio*	*C. auratus auratus*	*C. auratus gibelio*
Infected organ	oral	gall-bladder	gills, fins, kidneys	-	gills	kidneys
SB shape	obovate	ovate or long-ovate	obovate	angular ovate	pyriform	round
SB length	11.7 ± 0.4 (10.8–12.8)	12.2 (10.8–13.4)	10.5–12.6	12	10.2 (9.6–11.0)	8.5–12
SB width	8.9 ± 0.4 (8.2–9.9)	8.8 (8.4–9.6)	7.4–10.0	8	10.0 (8.4–10.8)	9–11
SB thickness	6.8± 0.3 (6.0–7.5)	7.2 (6.8–7.6)	6.0	-	6.0	6
Polar capsule length	4.8 ± 0.3 (4.0–5.5)	6.7 (6.0–7.2)	3.6–5.3	4	5.2 (4.5–6.0)	4.5–5.5
Polar capsule width	3.0 ± 0.2 (2.9–3.6)	3.4 (3.1–3.6)	2.6–3.5	2.8	3.3 (3.0–3.6)	2.6–3.5
Intercapsular process	Small	Small	Small	Non-existent	Distinct	-
No. filament turns	6–7	5–6	-	-	5–6	-

* data from Eiras et al. (2005) synopsis, not original description; SB – spore (body).

and molecular characteristics of the present species were studied.

The morphology of the myxosporean spores described in this paper is consistent with that of *Myxobolus*. When compared with species of *Myxobolus* previously described (Chen and Ma 1998, Erias et al. 2005, Zhao et al. 2008, Zhang et al. 2010a, Liu et al. 2012), the present species is distinct. *Myxobolus oralis* sp. n. resembles the following species: *Myxobolus pyramidis* Chen, 1998; *Myxobolus changkiangensis* Chen, 1998; *Myxobolus sphaericus* (Fujita, 1924); *Myxobolus platyrostris* Akhmerov, 1960; and *Myxobolus gibelio* Yukhimenko, 1986 (Table 1).

However, *M. oralis* sp. n. can be distinguished as follows. *Myxobolus pyramidis* infects gills of gold fish rather than the palate and bears pyriform spores(*vs* ovoid spores in *M. oralis*). *Myxobolus changkiangensis* has a distinctly longer polar capsule than *M. oralis*. The morphometric data of *M. sphaericus* and *M. platyrostris* are incomplete, making accurate comparison challenging. However, they could be distinguished from *M. oralis* by the reported round and square spore shape, respectively. Despite superficial similarity of *M. gibelio* to *M. oralis*, there is a distinct pit at the anterior end of the *M. gibelio* spore, which is absent in *M. oralis*. In addition, these two *Myxobolus* species show different site of infection with *M. gibelio* infecting the gills, fins and kidneys of host.

Molecular biological methods have become essential in identification of myxosporeans (Kent et al. 2001,

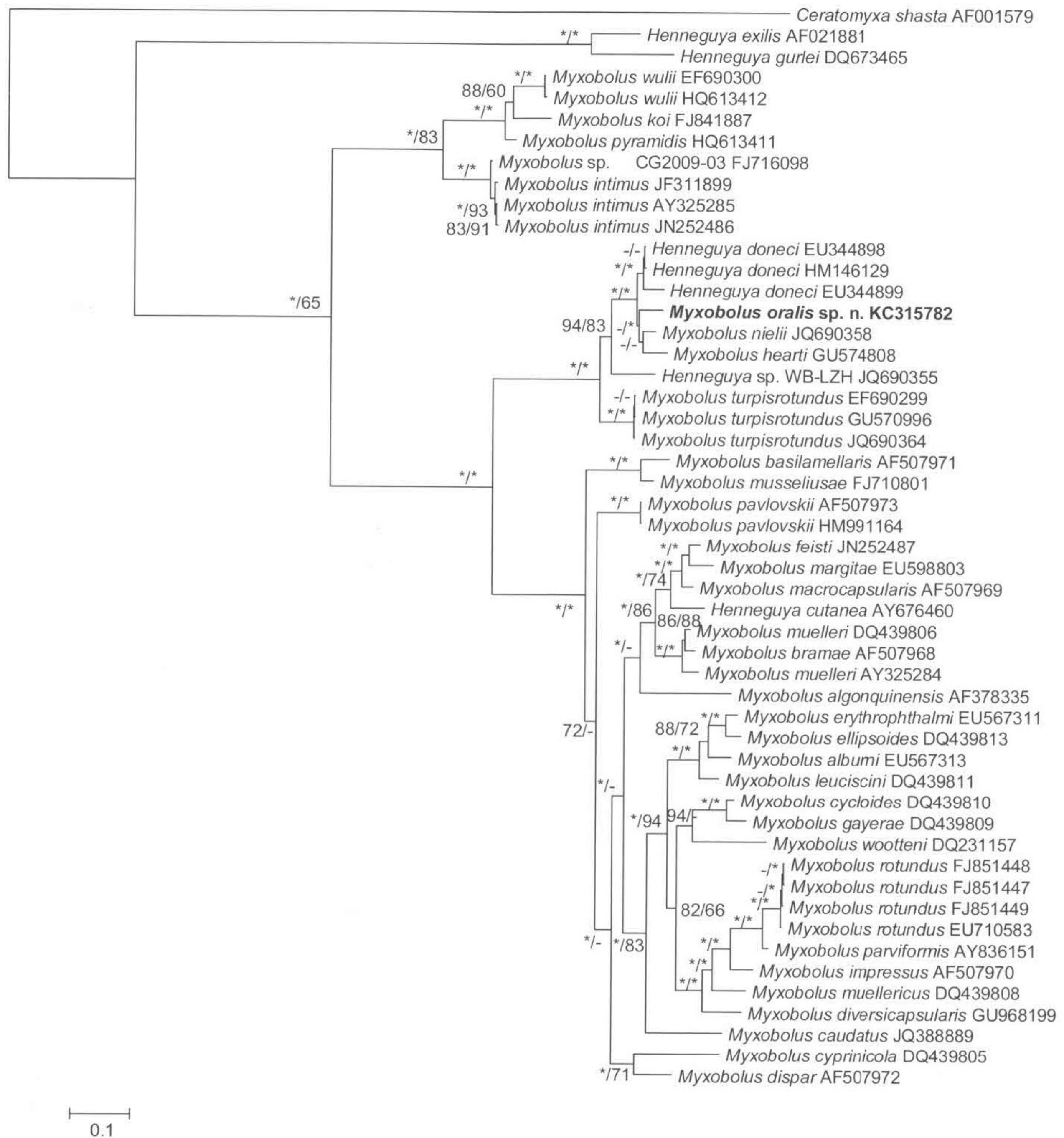

Fig. 5. Phylogenetic tree generated from Bayesian analysis of SSU rRNA gene sequences of *Myxobolus oralis* sp. n. from *Carassius auratus gibelio* and related myxobolids. GenBank accession numbers are listed adjacent to species names. Support values in percent units at branching points are listed as: Bayesian posterior probabilities/bootstrap values from ML analysis. Asterisks are shown where values exceeded 95%. Dashes are shown for values under 60%.

Lom and Dyková 2006, Molnár 2011), but many nominal species have not been sequenced. This is the case for *M. changkiangensis*, *M. sphaericus*, *M. platyrostris* and *M. gibelio*. DNA sequence is available for *M. pyramidis* (HQ613411), but this species was just 91% similar to *M. oralis*. In addition, a BLAST search indicated that the DNA sequence of *M. oralis* did not match any other myxosporean sequences in GenBank, sharing 96%, 95%, 95% similarity with *H. doneci*, *M. nielii* and *M. hearti*, which are generally outside the intra-specific sequence variation of what has been reported for species of myxosporeans (Whipps et al. 2004, Molnár et al. 2006, Whipps and Diggles 2006, Whipps and Kent 2006, Ferguson et al. 2008).

The genera *Henneguya* Thélohan, 1892 and *Myxobolus* are distinguished by the presence and absence of caudal appendages (Lom and Dyková 1992b). However, phylogenetic studies based on the ribosomal DNA sequence data do not support a separation of these two genera (Kent et al. 2001, Fiala 2006, Fiala and Bartošová 2010, Liu et al. 2010b, Carriero et al. 2013). Kent et al. (2001) speculated that the caudal appendage of *Henneguya* spp. was not a valid feature for characterisation of the genus. Recently, some *Myxobolus* species have been reported with appendages similar to those of *Henneguya* species (El-Mansy 2005, Bahri 2008, Liu et al. 2010b, 2013a), which supports the opinion of Kent et al. (2001). In the present study, we also observed 11% of the spores with caudal appendage. In addition, phylogenetic analysis showed *M. oralis* was placed sister to *M. nielii* and *M. hearti* in a *Henneguya-Myxobolus* clade with robust support. The observation of some proportion of *Myxobolus* spores with appendages further complicates the reliance of this single character to differentiate *Henneguya* from *Myxobolus*.

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