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Taxonomy and molecular phylogeny of *Myxobolus bilobus* n. sp. (Myxozoa) parasitizing *Notemigonus crysoleucas* (Cyprinidae) in Algonquin Park, Ontario, Canada

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ABSTRACT: *Myxobolus bilobus* n. sp. (Myxozoa) is described from golden shiner *Notemigonus crysoleucas* in Algonquin Park, Ontario, Canada. Plasmodia develop at the distal end of gill filaments, where they form a novel-shaped plasmodium made up of 2 side-by-side hemispheres joined at a central pore. Surrounding gill tissue is vacuolated, necrotic, and hemorrhagic. Spores of *M. bilobus* n. sp. resemble those of *M. aureatus* Ward, 1919, *M. angustus* Kudo, 1934, *M. spalli* Landsberg & Lom, 1991, and *M. pseudokoi* Li & Desser, 1985 in parasitizing cyprinids and in their pyriform shape. Spores of *M. bilobus* n. sp., however, are much larger (20 to 22.1 µm long, 7.5 to 9.3 µm wide, and 6 µm thick) than those of these other species, and the plasmodium is bi-lobed rather than the typical hollow sphere. A phylogenetic analysis of the 18S rDNA (2014 bp) reveals that *M. bilobus* n. sp. is a member of a clade that includes 11 species of *Myxobolus*, all of which are parasites of cyprinid fishes in North America or Eurasia. The study concludes that *M. bilobus* n. sp. is a member of a clade that has undergone radiation within cyprinid fishes of the 2 continents and that this evolution has involved both host and site switching.

KEY WORDS: Myxozoan · *Myxobolus bilobus* n. sp. · *Notemigonus crysoleucas*

INTRODUCTION

During a study of myxozoan parasites of fishes in Algonquin Park, Ontario, Canada, a species of *Myxobolus* Bütschli, 1882 (Myxozoa) that formed an unusual bi-lobed plasmodium in gill filaments of golden shiner *Notemigonus crysoleucas* was found. The present study describes the parasite as *Myxobolus bilobus* n. sp., reports on the structure of the plasmodium as seen with light microscopy, and determines the phylogenetic relationship of this new species relative to previously studied species in which the 18S rDNA is known.

MATERIALS AND METHODS

Fish and necropsy. A total of 32 adult golden shiner *Notemigonus crysoleucas* from Brewer Lake and Lake Opeongo, Algonquin Park, were collected on 17 to 19 May 2004 by seine net. Fish were killed by cervical incision and necropsied immediately. 'Cysts' (plasmodium and surrounding host tissue) were examined in wet mounts under slight coverslip pressure. About 20 cysts were fixed either in 10% buffered formalin or in absolute ethanol. Formalin-fixed samples were prepared histologically using standard techniques and stained with hematoxylin and eosin, Giemsa, Masson's

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trichrome, or Verhoeff's elastin stain (Presnell & Schreibman 1997). Fresh spores were stained with Lugol's iodine (iodinophilous vacuole) and with 1% methylene blue (mucous coat). Fixed sporoblasts and developed spores were prepared in 1% agar mounts and photographed.

DNA sequencing. Ethanol-fixed specimens were air-dried and incubated in 200 µl TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) with Proteinase K (200 µg ml^{-1}) and SDS (1%) at 55°C for 30 min. After classical extraction using phenol/chloroform, DNA was precipitated from the aqueous phase with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2 vol of 100% ethanol and collected by centrifugation. The pellet was washed with 70% ethanol, air dried, and re-suspended in 60 µl TE buffer. The 18S rDNA was amplified using primers 18r (5′-CTACGGAAACCTTGTTACG-3′) (Whipps et al. 2003) and 18e (5′-CTGGTTGATCCTGCCAGT-3′) (Hillis & Dixon 1991). Approximately 200 ng of the DNA was used as a template in a 50 µl PCR reaction, including 0.4 µM of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 × PCR buffer, and 2 U *Taq* polymerase (Fermentas). Cycling was performed under the following conditions: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C, and 2 min at 72°C, followed by final extension at 72°C for 10 min. A 2014 bp PCR product was excised from an agarose gel and purified using QIAquick Gel Extraction Kit (Qiagen). The purified fragment was cloned into a pDrive cloning-vector (Qiagen). Plasmid DNA from a single clone was sequenced using M13 forward (5′-GT AAAACGACGGCCAG-3′) and M13 reverse (5′-CAGGAAACAGCTATGAC-3′) primers. Primer 18 m (5′-CGCGGTAA TTCCAGCT CCA-3′) was designed to enable sequencing of the middle part of the 18S rDNA. DNA sequencing software (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html) was used to convert the sequence into complimentary strands. The sequences were aligned with BLASTN (http://www.ncbi.nlm.nih.gov/blast/bl2seq/ wblast2.cgi), overlapping sequences were removed, and sequences joined to obtain the full length 18S rDNA.

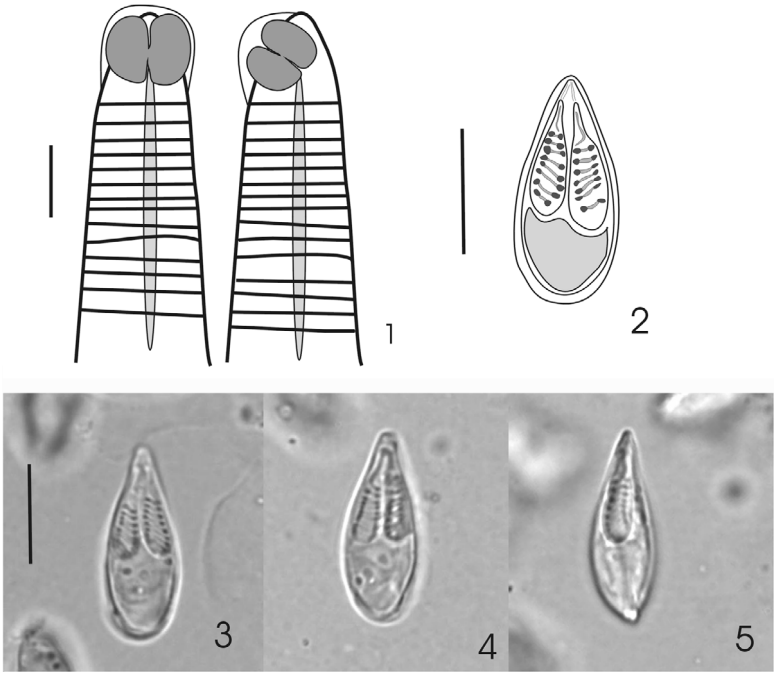
Molecular phylogeny. Sequences were compared using Clustal X software (Thompson et al. 1997). Phylogenetic analyses were conducted using PAUP Version 4.0b10 (Swofford 2001) and included parsimony, maximum-likelihood, and neighbor-joining distance methods. The phylogeny was calculated using neighbor-joining methods and bootstrap analysis with 1000 replicates with *Ceratomyxa shasta* Noble, 1950 (AF001579) as the outgroup. All morphological measurements are presented in micrometers unless stated otherwise.

RESULTS

Myxobolus bilobus n. sp.

Description: Plasmodium typically bi-lobed, up to 1 mm in diameter (Fig. 1). Disporoblastic, with asynchronous spore development (see Fig. 3). Spores (Figs. 2 to 5) pyriform in frontal view, relatively large, 21 ± 0.6 (20 to 22.1) (N = 13) long, 8.4 ± 0.5 (7.5 to 9.3) wide, 6 thick; length-to-width ratio 1:2.49 (±0.16) (1:2.31 to 1:2.81). Polar capsules pyriform, with distinct neck region, dissimilar in size (paired *t*-test, $t = -4.26$, $p = 0.001$, N = 13); large capsule 10.8 ± 0.7 (9.2 to 12) (N = 13) long, 2.7 ± 0.2 (2.2 to 3.6) wide; small capsule 10.1 ± 0.7 (9.1 to 11) (N = 13) long, 2.8 ± 0.2 (2.4 to 3.2) wide. Polar capsules converging anteriorly, but not crossing and not reaching spore apex; 7 to 9 tight polar filament coils, arranged perpendicular to capsule length. Intercapsular appendix, iodinophilous vacuole and mucous coat absent.

Type host: Golden shiner *Notemigonus crysoleucas* (Cyprinidae).



Figs. 1 to 5. Plasmodia and spores of *Myxoblus bilobus* n. sp. from *Notemigonus crysoleucas* in Algonquin Park, Ontario, Canada. Fig. 1. Line drawing of bi-lobed plasmodia at the distal tip of gill filaments. Scale bar = 1 mm. Fig. 2. Line drawing of a typical spore. Scale bar = 10 µm. Figs. 3 & 4. Photomicrographs of developed spores in frontal view. Scale bar = 10 µm. Fig. 5. Developed spore in lateral view. Scale for Figs. 4 & 5 as for Fig. 3

Type locality: Brewer Lake, Algonquin Park, Ontario (45° 35′ N, 78° 19′ W).
Other localities: Lake Opeongo, Algonquin Park, Ontario.
Site of tissue development: Distal gill filaments.
Type material: A Giemsa-stained histological section of a plasmodium containing developed spores has been deposited as a syntype slide to the United States National Parasite Collection (USNPC) (Accession Number 96438), Beltsville, Maryland, USA.
Etymology: The species is named after its bi-lobed plasmodium.
Prevalence of infection: 63.3 % (19 of 30).
Intensity of infection: 1 to 5 'cysts'.
Histology of infection: Plasmodia were bi-lobed in cross-section, consisting of 2 hemispheres joined by a central pore. They were fluid filled and contained loosely arranged, peripheral sporoblasts and debris. The outer margin was a fibrous, eosinophilic layer, measuring 3 to 10 µm thick (Fig. 6), that stained deep red with Masson's trichrome and deep blue with Verhoeff's elastin stain (the latter 2 colors being negative for elastin). Its outer surface was smooth, the inner surface irregular. Hyperplastic epithelium and connective tissue that surrounded the plasmodia were typically vacuolated, necrotic, and hemorrhagic (Fig. 6). In freshly killed hosts the 'cysts' were fragile and, if touched with fine tweezers, readily burst and released a cloud of spores.

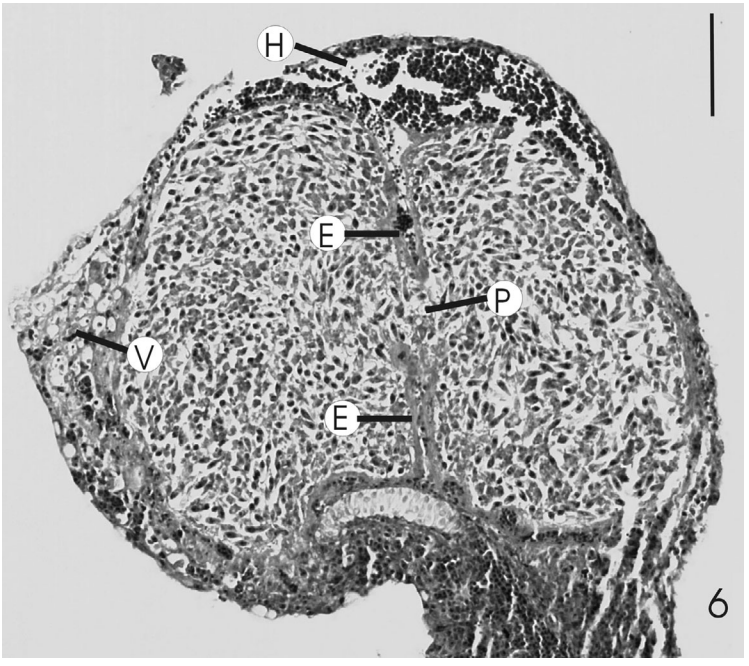
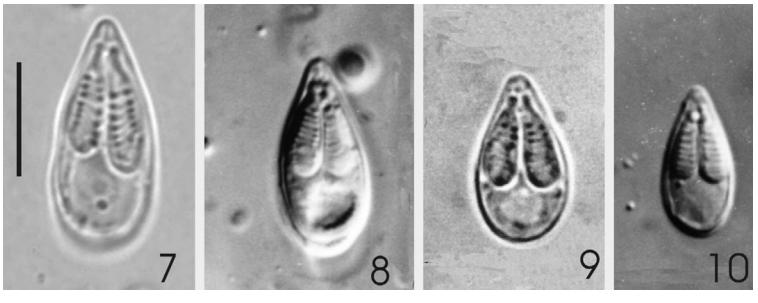


Fig. 6. *Myxobolus bilobus* n. sp. Histological section through a 'cyst' showing the central pore (P), the presumed ectoplasm (E), vacuolated and necrotic epidermis (V), and a hemorrhagic (H) area. Scale bar = 100 µm. Hematoxylin and eosin stain

Comments: *Myxobolus bilobus* n. sp. resembles *M. aureatus* Ward, 1919, *M. angustus* Kudo, 1934, *M. spalli* Landsberg & Lom, 1991, and *M. pseudokoi* Li & Desser, 1985 in that they develop within cyprinid hosts in North America and they have similar pyriform-shaped spores (Table 1). Spores described herein are longer than those of the other species (20 to 22.1 µm vs. ≤16.6 µm), giving the spore a narrow pyriform appearance. In addition, the polar capsules are dissimilar in length, with the larger one being generally longer (9.1 to 12 µm vs. ≤9.5 µm) than those of the other species (Table 1). The bi-lobed plasmodium of *M. bilobus* n. sp. is also novel among these and other species within the genus. A typical spore of *M. bilobus* n. sp. (see Fig. 11) is compared to those of *M. angustus*, *M. aureatus*, and *M. pseudokoi* at identical magnifications to illustrate the morphological differences (Figs. 7 to 10).

Molecular phylogeny: Sequence data for a 2014 bp region of the 18S rDNA (GenBank DQ008579) was successfully determined. The topology of the phylogenetic trees produced using parsimony, maximum-likelihood and neighbor-joining methods were the same. *Myxobolus bilobus* n. sp. occurred, with high bootstrap support, within a clade (Fig. 11) that included 10 other species of *Myxobolus* from gill or muscle tissue of various cyprinid fishes from North America or Eurasia.



Figs. 7 to 10. *Myxobolus* spp. A comparison of formalin-fixed spores. Fig. 7. *M. bilobus* n. sp. collected in the present study (scale bar = 10 µm) Fig. 8. *M. angustus* Kudo, 1934 collected from the gills of *Pimephales promelas* in August 1993, Duluth, Minnesota. Fig. 9. *M. aureatus* from the fins of *Pimephales promelas*, September 1986, as reproduced from Lom et al. (1992, their Fig. 2). Fig. 10. *M. pseudokoi* Li & Desser, 1985 collected from the gills of *Luxilus cornutus* in June 1994, Lake Opeongo, Algonquin Park. All spores at similar magnifications

Table 1. *Myxobolus* spp. A comparison of host species, known localities, cyst features, tissue sites, and spore morphometrics of *Myxobolus bilobus* n. sp. with 5 similar species from cyprinid fishes in North America. PCs: polar capsules

Species	Host	Locality	Cyst features/tissue site	Morphometrics	Source
Myxobolus aureatus Ward, 1919	Type host: *Notropis anogenus* Other hosts: *Pimephales notatus*, *P. promelas*	Type locality: Lake Erie Other localities: Lake Decatur, Rock River and Fox River, Illinois	Large cysts in the fins, often pigmented black	Fixed spores pyriform, 14–16.6 by 7.3–8.6. PCs 6.2–9.4 by 2.4–3.6; 6 to 10 filament coils	Lom et al. (1992)
Myxobolus angustus Kudo, 1934	Type host: *Pimephales vigilax*	Type locality: Illinois River at Merodosia Bay, Illinois	Cysts in gill filaments up to 0.1 by 0.26 mm	Fixed spores pyriform, 14–15 by 7–8 by 6–7. PCs 8–9.5 by 2.5–3. Large iodinophilous vacuole	Kudo (1934)
Myxobolus bilobus n. sp.	Type host: *Notemigonus chrysoleucas*	Type locality: Brewer Lake, Algonquin Park, Ontario Other localities: Lake Opeongo, Algonquin Park	Bi-lobed cysts in distal region of gill filaments, up to 1 mm in diameter	Fixed spores pyriform, 20–22.1 by 7.5–9.3 by 6. PCs 9.1–12 (large)/ 9.1–11 (small) by 7–11. Tight filament coils	Present study
Myxobolus spalli Landsberg & Lom, 1991 Syn. *Myxosoma cyprini*	Type host: *Notropis lutrensis* Other hosts: *Notemigonus crysoleucas*	Type locality: Little Stillwater Creek, Payne County, Oklahoma	Small cysts 0.3 mm in diameter, located basally in secondary lamellae	Fresh spores pyriform, 14.4–15.0 by 7.5–8.3 by 7.1–8.0. PCs 6.7–7.5 by 2.8–3.0; 9 coils. Posterior mucous envelope Fixed spores, 13.5–15.5 by 8.5–9.2 by 6.0–6.8. PCs 5.6–8.4 by 2.5–3.2	Spall (1974)
Myxobolus notemigoni Lewis & Summerfelt, 1964	Type host: *Notemigonus crysoleucas*	Type locality: Paragould, Arkansas Other localities: Southern Illinois	White cysts up to 0.9 mm embedded in tissues beneath scale and above dermis of body trunk	Fresh spores ovoid, 11.8 by 8.95 by 7.53. PCs 4.1 by 3.37; 6 to 8 coils. Iodinophilous vacuole present	Lewis & Summerfelt (1964)
Myxobolus pseudokoi Li & Desser, 1985	Type host: *Luxilus cornutus*	Type locality: Lake Sasajewun, Algonquin Park, Ontario Other localities: Lake Opeongo, Algonquin Park	Cysts in gills and skin, up to 0.12 by 0.5 mm	Fixed spores pyriform, 11.5–14 by 6–7 by 5. PCs 6.0–7.5 by 2–3. Prominent posterior mucous envelope. Iodinophilous vacuole present	Li & Desser (1985)

DISCUSSION

Molnár (2002) summarized the microhabitats within the gill filaments, lamellae, and arches of fishes that are utilized by myxozoans, emphasizing the need to include the exact site of development in species descriptions so as to go beyond 'gills'. In the present study we report that *Myxobolus bilobus* n. sp. develops consistently in tissues near the distal tip of the filament. The exact site of development was not determined because initial developmental stages were absent from the fish examined. The lack of positive staining for elastin, a main structural component of fish arteries (Roberts 1989), at the margin of the cyst suggests that development does not take place within the afferent artery, a common site of development of myxobolids within the distal tissues of the gill filament (Molnár 2002).

Host tissue surrounding the developed plasmodium of *Myxobolus bilobus* n. sp. undergoes degeneration and necrosis, leading to the hemorrhagic cyst that is sensitive to rupturing as observed during necropsy. Spontaneous rupturing of gill-dwelling plasmodia is known to occur (Dyková & Lom 1978, Cone 1994, Molnár 1998). The process has been linked to an intense inflammatory reaction (Dyková & Lom 1978) that ultimately leads to dispersal of myxospores to the outside environment (Cone 1994). In the case of *M. bilobus* n. sp. there is an extensive build-up of hyperplastic tissues, and we suspect that significant necrosis of this tissue is necessary before spores can be effectively released.

Gill-dwelling myxobolids typically produce a spherical to oval plasmodium comprised of a relatively thin, peripheral ectoplasm containing a germinal layer of sporoblasts and a fluid-filled inner endoplasm containing a mass of irregularly arranged spores and debris. The plasmodium can be taxonomically important in situations in which they occur at a specific site within gill tissue (Molnár 2002), when the fully developed plasmodium is noticeably small or

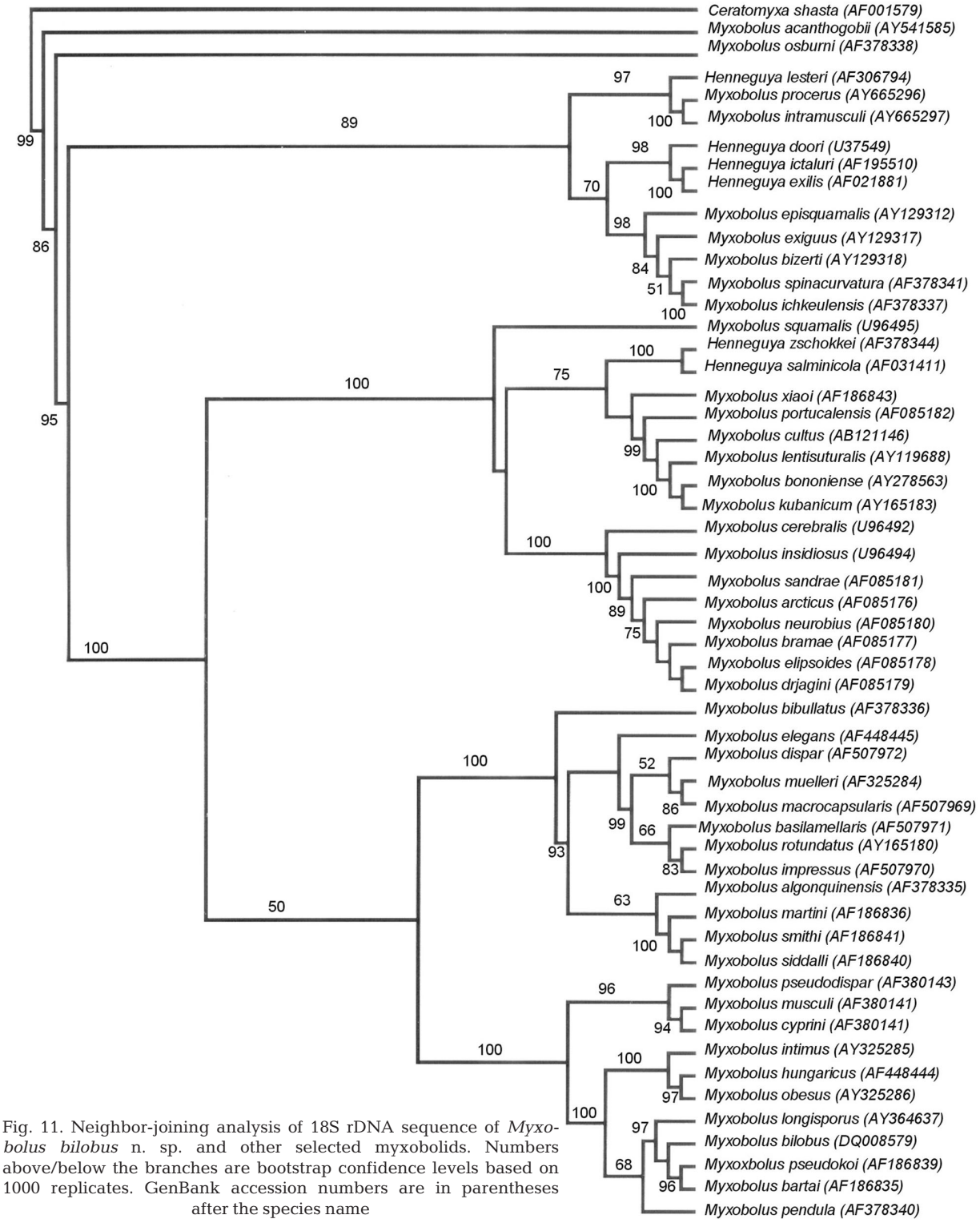


Fig. 11. Neighbor-joining analysis of 18S rDNA sequence of *Myxobolus bilobus* n. sp. and other selected myxobolids. Numbers above/below the branches are bootstrap confidence levels based on 1000 replicates. GenBank accession numbers are in parentheses after the species name

large (Price & Mellen 1980), when the relative thickness and cellular makeup of the ectoplasm is novel (Mavor 1916, Martyn et al. 2002), and/or when the plasmodium is freeform, engulfing, but not destroying, the capillary bed (Cone & Wiles 1985). The bi-lobed plasmodium of *Myxobolus bilobus* sp. appears to be another example of such diagnostic use of the plasmodium. The preliminary histochemistry suggests that the prominent eosinophilic layer at the outer edge of the plasmodium is entirely ectoplasm, devoid of any

incorporation of host cells. The facts that the apparent ectoplasm is continuous throughout the periphery and that the endoplasm of the 2 spheres are connected via the central pore suggest that the bi-lobed form arises from a single plasmodium and not from fusion of 2 smaller ones.

Easy et al. (2005) reviewed the application of 18S rDNA to the taxonomy of species of *Myxobolus* and other myxozoans and concluded that intraspecific variation in sequence alignment was typically <1%. The present study supports this conclusion because *M. bilobus* has only ≤96% similarity in alignment of the 18S rDNA with other studied species in the genus.

The phylogenetic analysis reveals that *Myxobolus bilobus* n. sp. is a member of a clade that has undergone a major radiation within cyprinid fishes in North America and Eurasia. Members include *M. pseudodispar*, *M. musculi*, *M. cyprini*, *M. intimus*, *M. hungaricus*, *M. obesus*, *M. longisporus*, *M. pseudokoi*, *M. bartai*, *M. pendula*, and *M. bilobus* n. sp., with those species from the separate continents grouping together (the exception being *M. longisporus* which aligns with the North American species). It appears that the ancestor to this clade was a parasite of a cyprinid host(s) prior to the separation of North America and Europe/Eurasia. Following geographical isolation and subsequent evolution of cyprinids on the 2 continents, radiation of this myxozoan lineage was complex, involving both host and site switching. We conclude this because these parasites are host specific and occupy as a group a variety of sites within gill and muscle tissues (Molnár & Baska 1999, Salim & Desser 2000, Molnár et al. 2002, Dyková et al. 2003, Rácz et al. 2004).

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