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ORIGINAL PAPER

Occurrence of two myxosporean species, *Myxobolus hakyi* sp. n. and *Hoferellus pulvinatus* sp. n., in *Pangasianodon hypophthalmus* fry imported from Thailand to Europe as ornamental fish

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Abstract Fingerlings of the sutchi catfish *Pangasianodon hypophthalmus*, a favorite food fish in South Asia, is regularly imported by European fish traders and sold in pet fish shops. In shipments from Thailand, a skin and a kidney infection of this fish caused by myxosporean parasites was found both in Hungary and Russia. In the skin of the fish, small millet-sized nodules containing great numbers of a *Myxobolus* species were found, while in the renal glomeruli, spores and sporogonic stages of a *Hoferellus* species developed. The skin-infecting species described as *Myxobolus hakyi* sp. n. had 15.9×6.6-µm-sized spores with elongated polar capsules, while the renal species described as *Hoferellus pulvinatus* sp. n. had roundish spores with a size of 6.5×5.0 µm and had a characteristic pillow-like structure at its posterior end. Besides morphology, histology of infection and 18S rDNA sequences were studied.

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Introduction

Freshwater fish culture in Southeast Asia is one of the fastest-growing branches of agriculture. Endemic species, among them the sutchi catfish, *Pangasianodon hypophthalmus* (Sauvage 1878) are cultured in ponds and cage systems. Fingerlings of the species are often collected and transported to pet fish shops to several European countries. Catfish fry harvested from ponds or natural waters are frequently infected by protozoan and myxosporean parasites. Some of these parasites, primarily those that infect the skin, show conspicuous symptoms easily observable even by non-experts. Some of these symptoms are the small, round, whitish plasmodia of a myxosporean in the skin. Myxosporean infection of the sutchi catfish is relatively well studied. Te et al. (1991; cit. by Arthur and Te 2006) reported on the occurrence of *Myxobolus miyairii* (Kudo 1920) from the gills and a *Ceratomyxa* sp. from the gall bladder of this fish from the Mekong River in Vietnam. Molnár et al. (2006) described two *Myxobolus*, a *Henneguya*, and three *Hennegoides* species from Malaysian cage cultured specimens. Another *Myxobolus* species infecting the muscles of sutchi catfish was described by Székely et al. (2009b). No *Hoferellus* species is known to have occurred in fishes of the Southeast Asian region.

In the present paper, we describe two myxosporean species *Myxobolus hakyi* sp. n. from the skin and *Hoferellus pulvinatus* sp. n. from the kidneys of *Pangasianodon hypophthalmus* fry imported from Thailand to pet shops in Budapest, Hungary and St. Petersburg, Russia.

Materials and methods

Sutchi catfish 7 to 9 cm in length were imported from Thailand to a Hungarian wholesaler of ornamental fish. In some of the fish, small millet-sized nodules were seen in the skin. Similar symptoms were recorded on sutchi catfishes imported from Thailand to a pet shop in St. Petersburg, Russia. Disease symptoms were recorded neither in Hungary nor in Russia. Fishes were carried to laboratories alive. A complete parasitological examination was performed, along with specific investigations on infections involving the skin, muscles, and the kidney in order to find myxosporeans. Before extermination, fish were sedated by clove oil and were given a cervical cut. Skin, fins, gills, and the opened intestine were examined under a stereomicroscope. Pieces of kidney, liver, spleen, and muscle were compressed between two glass plates, examined first under a stereomicroscope, then at 200- to 400-fold magnification in a Zeiss Jenaval compound microscope. *Myxobolus* spores from the isolated and opened cysts were first studied in a wet mount, and then some of the spores were placed in glycerine-jelly onto a slide under a cover slip and preserved as a reference slide.

The other part of the spores were collected into Eppendorf tubes and stored at −20°C for subsequent molecular examination. Tissue samples from infected organs containing developing and mature plasmodia were fixed in Bouin's solution, embedded in paraffin wax, cut to 4–5-μm sections, and stained with hematoxylin and eosin. The vitality of spores was checked by adding spores into a 0.4% solution of urea. Spores of a given plasmodium were regarded mature when at least 90% of the spores extruded polar filaments in the solution. Naive spores were studied by Nomarski differential interference contrast of an Olympus BH2 microscope. Fresh spores were photomicrographed with an Olympus DP10 digital camera or recorded on videotapes; spores collected in Russia were studied and photographed with LOMO microscope. All measurements are given in micrometer.

After thawing, samples were homogenized in 1.5-ml microtube with a sterile plastic Eppendorf pestle. Then, microtubes containing the homogenates were filled with dH_2O, mixed by vortexing, and centrifuged at 13,000 rpm for 10 min. Pellets were dissolved in 500 μl lysis buffer (100 mM NaCl, 10 mM Tris, 10 mM EDTA, 0.2% SDS, and 0.4 mg ml^{-1} Proteinase K) and incubated at 55°C for 3 to 4 h. DNA was then purified using the MiniPrep Express Matrix (BIO101, Qbiogene) as described previously by Eszterbauer (2004). DNA was amplified with the primer pair 18e (5′-CTG GTT GAT TCT GCC AGT-3′; Hillis and Dixon 1991) and 18r (5′-CTA CGG AAA CCT TGT TAC-3′; Whipps et al. 2003). The total volume of the PCR reactions was 50 μl, which contained approx. 10 to 50 ng DNA, 1× Taq PCR reaction buffer (MBI Fermentas), 1.5 mM $MgCl_2$, 0.2 mM dNTP mix (Sigma), 25 μM of each primer, and 2 units of Taq DNA Polymerase (MBI Fermentas). Amplification conditions were as follows: 95°C for 50 s, 58°C for 50 s, and 72°C for 80 s for 35 cycles, with a terminal extension at 72°C for 7 min. PCR products were purified with QIAquick Gel Extraction Kit (Qiagen).

The purified PCR product of the kidney sample was cloned with the CloneJET PCR Cloning Kit (Fermentas) following the manufacturer's manual. Eight positive clones were sequenced with sequencing primers supplied with the cloning kit, using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyzer automated DNA sequencer (Applied Biosystems). Furthermore, the purified PCR products of every examined species were sequenced directly in both strands. The following primers were used for direct sequencing: amplification primers 18e and 18r, Myx4r and Act1f by Hallett and Diamant (2001) and MB5 and MB5r described by Eszterbauer (2004). For sequence assembling, the STADEN Sequence Analysis Package version 2001.0 (Staden 1996) was used. DNA sequence similarities were calculated with the Sequence Identity Matrix of the software BioEdit (Hall 1999).

Results

Infection with millet-sized cysts in the skin of the sutchi catfish fry in Hungary was first recorded in 2002. Since then, the infection has repeatedly been diagnosed in sutchi catfish in several shipments each year. In Russia, the infection was first found in 2006 (Fig. 1). Among 100 fingerlings examined by external inspection in Hungary in January 2008, 27 specimens were found to be infected. Ten of the infected fish harbored five to 50 round, whitish plasmodia of 0.5 to 1 mm in the skin slightly emerging over the surface. About the same number of plasmodia was found in the muscles. Most plasmodia contained mature *Myxobolus* spores (Fig. 2). The kidneys of the same fish were infected with a *Hoferellus* species (Fig. 3). Three of the 10 examined kidneys harbored spores in the Hungarian material. Of the 30 fish samples, examined in February, April, and May 2006 in St. Petersburg, *Myxobolus* infection was found in three of the 10 sutchi catfish examined in May, and *Hoferellus* infection was detected in four of the 10 fish examined in April. As to other potential catfish parasites, only *Spironucleus* flagellates and *Thaparocleidus* monogeneans on the gills were found. No external symptoms were recorded in a second sutchi catfish shipment that arrived in Hungary in April 2008. In detailed laboratory examinations, neither *Myxobolus* nor *Hoferellus* infection was found. Spore morphology and measurements

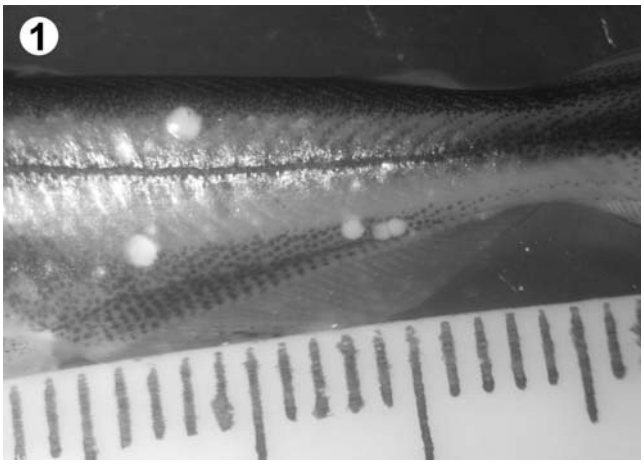

Fig. 1 Infection of the skin of a young sutchi catfish by mature plasmodia of *Myxobolus hakyi* sp. n. from a pet shop in St. Petersburg, Russia

revealed the identity of the myxospore samples originating from Hungary and Russia.

Description of the two species is as follows:

Myxobolus hakyi sp. n.

Type host: Sutchi catfish, *P. hypophthalmus* (Sauvage 1878) syn. *Pangasius sutchi* (Fowler 1937), Pangasiidae.

Type locality: Thailand.

Locality: Imported fish, in a wholesaler of ornamental fish in Budapest, Hungary and fish from pet shops in St. Petersburg, Russia.

Site of tissue development: corium (i.e., dermis) of the skin.

Type material: Spores in glycerin-gelatine, digitized photos of syntype spores, and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-70187. The 18S rDNA sequence of *M. hakyi* sp. n. was deposited in the GenBank under the accession number FJ816269.

Prevalence of infection: 27 of 100 of fish of the size group 7–8 cm in length in Hungary and three of 30 of fish of the size group 5–6 cm in length in Russia.

Intensity of infection: moderate.

Etymology: The species is named after Ha Ky, the well-known Vietnamese fish parasitologist.

Spores: The spores (Figs. 2 and 4a, b) in frontal view are elongatedly ellipsoid with blunted ends. Some spores were blunted at the posterior end and tapered toward the anterior end. In sutural view, they had an elongated shape tapering at both ends with a larger sutural posterior protrusion and a smaller anterior protrusion (Fig. 4c). Length of the spores was 15.9±0.59 (15.0–16.8; N=50), width was 6.6±0.48 (6.0–7.3; N=50), and thickness was 5.6±0.53 (5.0–6.6; N=15). Polar capsules were elongated, different in size, and slightly converging anteriorly. They are 6.3±0.29 (6.0–6.8) long (N=50), 2.3±0.18 (2.9–3.4) wide (N=50), and 2.6±0.12 (2.3–2.8) thick. Six to seven filament coils were arranged perpendicularly to the capsule length in the polar capsule. The length of extruded polar filament was 53 µm in average (N=10). No intercapsular appendix was seen. The width of the suture was 0.9±0.40 m (0.8–1.0). Sutural edge markings were not seen. A single binucleated sporoplasm with round iodinophilous vacuole was present. Mucous envelope was not found. Some aberrant spores had caudal processes.

Molecular findings: The 18S rDNA of two spore samples collected from different fish specimen were sequenced directly. The obtained 1,957-bp-long DNA sequences were identical except for a nucleotide at position 654, where an alteration of nucleotide "A" and "G" was detected. Therefore, this nucleotide was marked as "R" in the consensus DNA sequence submitted to GenBank.

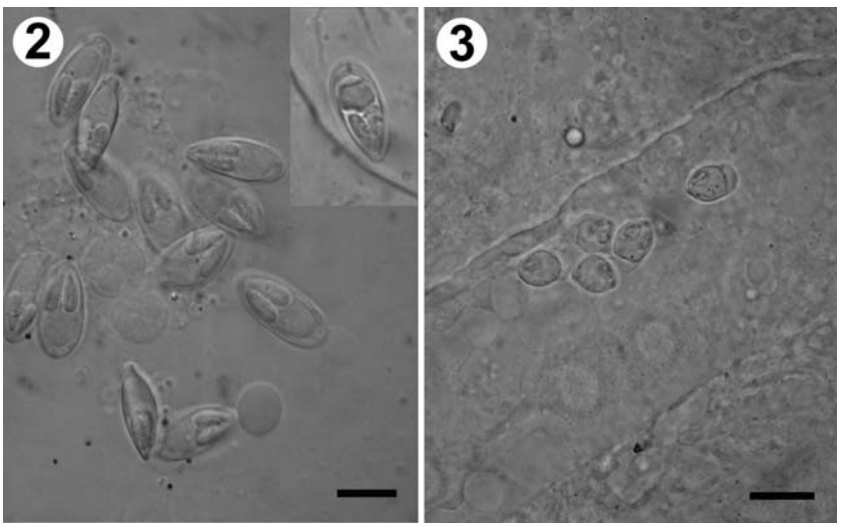

Fig. 2 Spores of *Myxobolus hakyi* sp. n. Spores from a skin cyst received from a Hungarian petfish shop. *Inset* A spore from a muscle cyst developed in the connective tissue of an intermuscular septum. *Bar*=10 µm

Fig. 3 *H. pulvinatus* sp. n. spores (*arrows*) in a renal tubule. H&E. *Bar*=10 µm

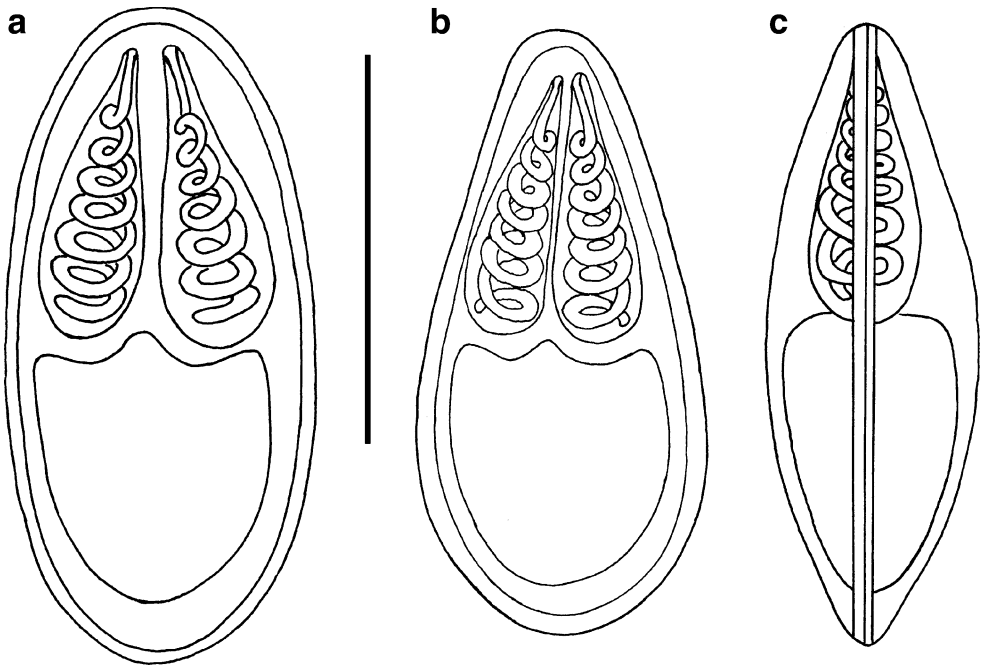

Fig. 4 Schematic drawings on the spores of *M. hakyi* sp. n. **a** Elliptic spores in frontal view; **b** spores somewhat tapering toward the anterior end in frontal view; **c** spores in sutural view. *Bar*=10 μm

Remarks: *Myxobolus* spp. known from sutchi catfish differ from this new species both in shape and in site selection. In its spore morphology, *Myxobolus pangasii* (Molnár et al. 2006) infecting the spleen serosa of sutchi catfish resembles *M. hakyi* sp. n., as it also has elongated spores and elongated polar capsules. However, its similar-sized spores (13.5–15×6–7.8) taper toward the anterior end more sharply. *M. pangasii* (GenBank acc. no. FJ816270) and *M. hakyi* sp. n. also differ from each other at the genetic level as the two species showed only 97.9% similarity in their 18S rDNA sequence. The other species, *Myxobolus baskai* (Molnár et al. 2006), known from the gills of sutchi catfish has similar-sized spores (13.5–15×10.5–11), but it differs from *M. hakyi* sp. n. by its less elongated, ovoid spores with drop-like polar capsules. *Myxobolus terengganuensis* (Székely et al. 2009b), a species found in the muscle of sutchi catfish, is somewhat different in shape. The spore width of the latter species is larger than the spore length. Of the *Myxobolus* spp. described from other silurid fishes, two species, *M. miyairii* and *Myxobolus gigi*, resemble *M. hakyi* sp. n. the best by their elongated ellipsoid spore shapes. *M. miyairii* (Kudo 1919) from the intestine of *Parasilurus asotus* has about the same sized spores, but their polar capsules are shorter than the capsules of the present species. As for the spores of *M. gigi* (Fujita 1927), the parasite of the kidney of *Pseudobagrus fulvidraco*, one of the polar capsules, is slightly smaller than those of *M. hakyi* sp. n. The shape of the spores *M. hakyi* sp. n. is also similar to *Myxobolus brasiliensis* (Casal et al. 1996), a parasite of an Amazonian silurid fish *Bunocephalus coracoideus*, but the spores of the latter species are smaller than those of *M. hakyi* sp. n. Of the non-silurid Far-East fishes, *Myxobolus macropodusi* (Chen and Ma 1998), a parasite of the skin of the paradise fish *Macropodus sinensis*, resembles the best this new species by its large elongated ellipsoidal spores. However, the polar capsules of the latter species are shorter than those of *M. hakyi* sp. n.; furthermore, the genetic difference between the fish hosts is also wide. Elongated-shaped spores of *Myxobolus mariulensis* (Sarkar et al. 1985), a parasite of the Indian ophiocephalid fish *Channa marulius*, and spores of *Myxobolus cuneus* (Adriano et al. 2006), a parasite of the Brasilian characid fish *Piaractus mesopotamicus*, also resemble *M. hakyi* sp. n., but both species have extremely long polar capsules. Of the seven *Myxobolus* species described from Malaysian fishes by Székely et al. (2009a, b), the spores of *Myxobolus leptobarbi* (GenBank acc. no. EU643623), a species of the cyprinid fish *Leptobarbus hoevenii*, are oval in shape, but the spores of this species have longer polar capsules than *M. hakyi* sp. n., and the two species also differ in their 18S rDNA sequence as they share only 76.4% of identical nucleotides over a 1,333-bp-long aligned DNA fragment. BLASTn search revealed *Myxobolus pangasii* (FJ816270) as the genetically most similar species to *M. hakyi* sp. n. with 97.9% similarity.

Histology: The normal structure of the sutchi catfish's skin is composed of a relatively thin layer of non-hornifying multilayered epithelium and a thin corium underlined with a thick dense layer of collagenic connective tissue (i.e., subcutis). In some parts of the skin, a layer of large mucin-producing cells (goblet cells) are located inside the multilayered epithelium (Fig. 5). In other parts of the skin, the loose connective tissue layer (*corium*), connecting the epithelium from one side and the muscles from the

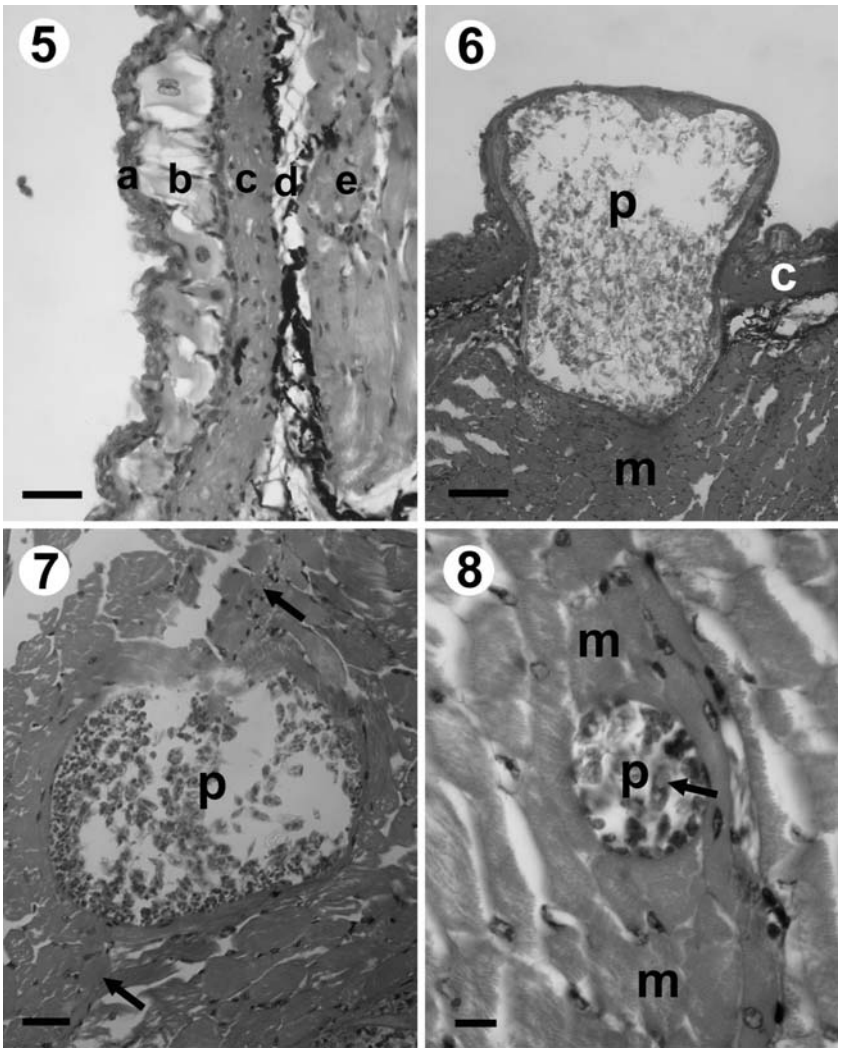

Fig. 5 Histological structure of the skin of a sutchi catfish. *a* Epithelium, *b* layer of the mucus cells, *c* layer of dense connective tissue, *d* melanin containing layer of the dermis, *e* muscles. H&E. *Bar*=75 μm

Fig. 6 Mature plasmodium (*p*) of *M. hakyi* sp. n. in the skin developing in the connective tissue (*c*), emerging over the skin surface and submerging deep into the muscles (*m*). H&E. *Bar*=50 μm

Fig. 7 A large, mature plasmodium (*p*) filled by the spores of *M. hakyi* sp. n. developing in an intermuscular septum (*arrows*). H&E. *Bar*=20 μm

Fig. 8 Plasmodium (*p*) of an unidentified *Myxobolus* sp. developing intracellularly in a muscle cell (*m*). Among sporoblastic stages, a single spore (*arrow*) is seen. H&E. *Bar*=10 μm

other side to the dense collagenic connective tissue, harbors large numbers of melanin containing cells. *Myxobolus* plasmodia in the skin are located in the loose connective tissue under the dense collagenic tissue. A part of the plasmodium protrudes over the skin surface, while another part is deeply introduced to the muscles (Fig. 6). Both the epithelium and the connective tissue layers above the plasmodium became thin. The plasmodium was filled with matured spores, but close to the ectoplasm, some early sporogonic stages were also found. In the musculature, two types of plasmodia were found. Relatively large plasmodia about the size of the ones found in the skin were located inside the intramuscular septal connective tissue (Fig. 7). These plasmodia contained spores morphologically corresponding to the ones found in skin plasmodia. The other types of plasmodia were located intracellularly in the sarcoplasm of muscle cells (Fig. 8). These plasmodia were in the stage of early plasmodial development, and young spores were only occasionally found in the central region of plasmodia. Spores from these plasmodia, which most likely belong to another species, have not been studied in details yet.

Hoferellus pulvinatus sp. n.

Type host: Sutchi catfish *P. hypophthalmus* (Sauvage 1878) syn. *P. sutchi* (Fowler 1937), Pangasiidae.

Type locality: Thailand.

Locality: Imported fishes, in a wholesaler of ornamental fish in Budapest, Hungary and St. Petersburg, Russia.

Site of tissue development: Glomeruli in the kidney.

Type material: Spores in glycerin-gelatine, digitized photos of syntype spores, and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, coll. no. HNHM-70188.

Prevalence of infection: three of 10 of fish of 7–8 cm in length (in Hungary) and four of 10 of fish of 5–6 cm in length (in Russia).

Intensity of infection: intensive.

Etymology: The Latin name of the species derives from the pillow-like structure over the caudal end of the spores.

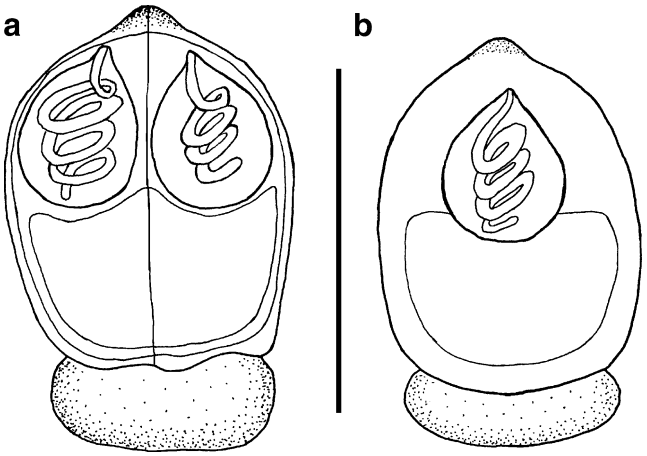

Fig. 9 Schematic drawings on the spores of *H. pulvinatus* sp. n. **a** Apical (sutural) view, **b** side view. *Bar*=10 μm

Spores: The spores (Figs. 3 and 9a) in apical (sutural) view are subspherical flattened posteriorly. They have a small protrusion at the anterior end and a pillow-like structure above the posterior end. In some spores, the flattened end shows two small protrusions laterally and one protrusion centrally. Suture is slightly visible. In side view (Fig. 9b), the spores have about the same shape but they are somewhat slender. Length of the spores was 6.5±0.28 (6.0–7.2; *N*=30), width was 5±0.20 (4.7–5.3; *N*=20), and thickness was 5.6±0.20 (5.3–5.9; *N*=30). Two drop-like polar capsules are located at the two sides of the suture. They are 3±0.42 (2.4–3.2) long (*N*=30) and 2.5±0.19 (2.1–2.7) wide (*N*=30). Three filament coils were arranged loosely and perpendicularly to the capsule length in the polar capsule. The pillow-like attachment is 1.7±0.11 (1.5–1.8; *N*=30) long, 4.6±0.04 (4.0–5.0; *N*=30) wide, and 4±0.01 (3.8–4.0; *N*=8) thick. The anterior protrusion measures 0.4–0.6. No intercapsular appendix was seen. A single binucleated sporoplasm with a round iodinophilous vacuole was present. Mucous envelope was not seen.

Molecular findings: The direct sequencing of the PCR product amplified from the DNA content of the kidney sample detected contamination; therefore, the purified PCR product was subjected to molecular cloning, and eight positive clones were sequenced. DNA sequence data revealed that the kidney sample contained a mixture of the DNA of two different myxozoan species. One of them was identified as the 18S rDNA of *M. hakyi* sp. n., while no identity match was found to the other DNA sequence. BLASTn search showed, however, that the unknown DNA sequence belong to a myxozoan species, the 18S rDNA of which is 95.1% similar to *Henneguya ictaluri* (AF195510). Considering that only a single kidney sample was involved in the molecular analysis and the sample was contaminated with *M. hakyi* sp. n., we set aside the molecular characterization of *H. pulvinatus* sp. n. in the present study.

Remarks: Spores of *H. pulvinatus* sp. n. resemble most other *Hoferellus* spp. like *Hoferellus cyprini*, *Hoferellus carassii*, or *Hoferellus gilsoni*, but differ from them by the pillow-like attachment on the caudal ends of spores. The development of *H. pulvinatus* also differs from the above species as in most cases spores are already formed inside the renal glomeruli, and only aged spores are found inside renal tubules.

Histology: Spores and sporogonic stages were found inside the Bowman capsule in renal glomeruli (Fig. 10). The original structure of the glomerulus in infected

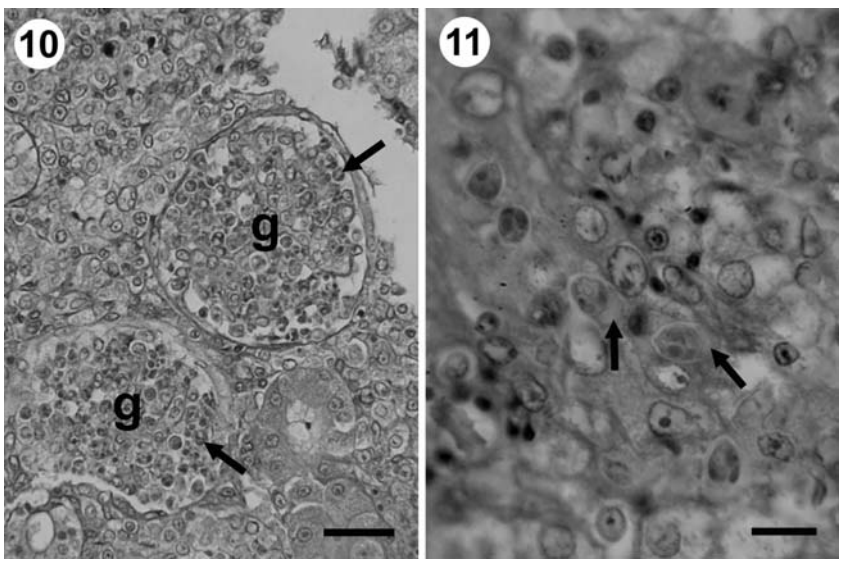

Fig. 10 *H. pulvinatus* sp. n. spores (*arrows*) and developmental stages in renal glomeruli (*g*) inside the Bowman's capsules. H&E. *Bar*=20 μm

Fig. 11 *H. pulvinatus* sp. n. spores in renal glomeruli bear a pillow-like structure on their caudal ends (*arrows*). H&E. *Bar*=10 μm

catfishes was hardly discernible. Some of the spores were found free in the urinary channels. The characteristic pillow-like attachment was easily discernible even in fixed spores (Fig. 11). The destruction of only the affected glomeruli was detected. Other histopathologic lesions of kidneys were not observed.

Discussion

Culture of ornamental aquarium fishes in Southeast Asia is a fast-developing branch of national economies. Cultured fishes and those harvested in natural waters are very quickly translocated to pet shops of Europe and North America. Despite preventive measures, some of the parasites infecting these fishes, first of all those infecting fingerlings, can easily accompany their hosts to their destinations. In prevention of disease transfers, ornamental fishes usually represent a less thoroughly inspected category in animal health control. Although the sutchi catfish is an extensively cultured, important food fish in Asian–European relation, their fingerlings are regarded as ornamental fish only. Sutchi catfish from its original Cambodian biotope was introduced to several South-Asian countries, where it transmitted several species of its original parasite fauna. Due to this fact, in the latter countries, where the fish has been intensively cultured, a study on its parasites, e.g., on myxosporeans, has an intrinsic economical value. Myxosporeans belong to parasites, which cannot be treated by the any existing preventive bath methods, and the infected fishes also show symptoms at their destination sites. Translocation of fishes from the original biotope often represents a threat to the original fish population of importing countries. Several pathogenic species (like the eel nematode *Anguillicola crassus* and the monogenean *Pseudodactylogyrus* spp., or a great number of parasites of the Asian wild carp) were introduced to Europe by fish import and caused severe losses in local fish populations (Bauer and Hoffman 1976; Molnár 1984; Taraschewski et al. 1987). Most likely, the import of sutchi catfish does not endanger the European fish populations as it has no closely related fish species in Europe, and this catfish species cannot endure the climate of the temperate zone. Finding new myxosporean spp. in imported material bears, therefore, mainly a scientific interest, but also provides useful data to experts working on the parasite fauna of the fish in the Southeast Asian region and worldwide. From the sutchi catfish, a great number of parasites have been recorded by Te et al. (1991; cit. by Arthur and Te 2006) in Vietnam, but the proper identification of several species requires further confirmation. Myxosporeans specific to the sutchi catfish seem to be relatively large in number (Molnár et al. 2006; Székely et al. 2009b). Some of the known myxosporeans cause severe symptoms and might be regarded as pathogens in the future. Although fish mortality caused by *M. hakyi* infection has not been recorded, papules appearing on the fish, producing easily observable skin changes, prohibit selling the fish and might cause difficulties in fish hatcheries. The whitish spots may complicate the differential diagnosis because with naked eyes, the approximately 1-mm-large white nodules could be regarded as trophonts of *Ichthyophthirius multifiliis*, the causative of the white spot disease. *H. pulvinatus* also seems to cause serious damages in renal glomeruli but further studies are necessary to properly evaluate its pathogenicity.

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