

**MYXIDIUM SHEDKOAЕ SOKOLOV, 2013
(MYXOZOA: MYXIDIIDAE), A PARASITE OF THE
GALLBLADDER OF *PERCCOTTUS GLENII* DYBOWSKI, 1877
(ACTINOPTRYGII: ODONTOBUTIDAE): SUPPLEMENTARY
DATA ON MORPHOLOGY AND PHYLOGENETIC POSITION
BASED ON 18S rDNA SEQUENCE ANALYSIS**

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(Received 17 January 2018; accepted 16 April 2018)

This paper is the first report on the molecular characterisation of myxozoan parasites from the odontobutid fish Chinese (Amur) sleeper (*Perccottus glenii* Dybowski, 1877). The authors determined the partial 18S rDNA sequence of *Myxidium shedkoeae* Sokolov, 2013 from the gallbladder of the fish. Phylogenies reconstructed using maximum likelihood and Bayesian inference analysis revealed that *M. shedkoeae* belongs to the hepatic biliary group of myxozoans (after Kristmundsson and Freeman, 2013) as a member of the clade consisting of *Zschokkella* sp. KLT-2014, *Myxidium truttiae* and *Zschokkella nova*. Some new morphological features of the parasite are also presented.

Key words: Myxozoa, *Myxidium shedkoeae*, *Perccottus glenii*, Chinese sleeper, odontobutid fish, phylogeny

The genus *Myxidium* Bütschli, 1882 comprises more than 200 species of myxozoans parasitic in kidneys, urinary and biliary tracts mostly of freshwater and marine fishes, but also amphibians, reptiles and birds (e. g. Eiras et al., 2011). As a rule, the genus *Myxidium* includes species with linear or slightly curved fusiform spores. The spores have one more or less pyriform polar capsule at each end with a binucleate sporoplasm between the polar capsules. The suture line of *Myxidium* spores is longitudinal, straight or slightly curved. The capsular foramina are situated in the sutural plane, at or near the end of the spore and, as a rule, open in opposite directions (Shulman, 1966; Lom and Dyková, 2006).

However, the data on the partial 18S rDNA sequences, as well as the morphology of the actinosporidian stage, show evidence that the genus *Myxidium* is

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polyphyletic (Jirků et al., 2007; Freeman et al., 2008; Bartošová et al., 2011; Jirků et al., 2011; Jones et al., 2011; Hartigan et al., 2011; Kalavati et al., 2013; Kristmundsson and Freeman, 2013; Heiniger and Adlard, 2014; Whipps et al., 2015; Li et al., 2016; Aguiar et al., 2017).

Myxidium shedkoe Sokolov, 2013 is one of two species of the genus *Myxidium* recorded in the Chinese (Amur) sleeper (*Perccottus glenii* Dybowski, 1877), an invasive species of odontobutid fishes. The parasite infects the gallbladder of the fish with no apparent pathological changes (Sokolov, 2013). Up to now, *M. shedkoe* has been found only in Primorsky Krai, Russia – in the native range of its host *Perccottus glenii*. In this paper, the morphological and molecular characterisation and the phylogeny of *M. shedkoe* are presented.

Materials and methods

Myxozoans were recovered from the gallbladder of *P. glenii* caught in Lake Khanka (Primorsky Krai, Russia: 44°31'N, 132°19'E) in August, 2015. Twenty-five fish were examined. For morphological examination, the plasmodia were fixed in 70° ethanol and processed to glycerol-gelatin (without colouring) and Canada balsam (stained with acetocarmine). The morphology of the spores was studied using glycerol-gelatin slides with an Axio Imager A1 microscope (Zeiss AG, Oberkochen, Germany). Species identification was performed according to key features: plasmodium localisation, size and shape of spores, and ornamentation of spore shell valves (see Sokolov, 2013).

A part of the material was fixed in 96° ethanol for molecular study. Genomic DNA was extracted from one plasmodium with QIAprep Spin Miniprep Kits (Qiagen, Germany) according to the manufacturer's instructions. The PCR and sequencing settings, as well as the primers were carried out according to Li et al. (2012). PCR amplification of two overlapping fragments of 18S ribosomal DNA (18S rDNA) was amplified using the primers Eurib1 (5'-ACCTGGTTG ATCCTGCCAG-3') and reverse Eurib2 (5'-CTTCCGCTGGTTCACCTACGG-3'). PCR was conducted with a final reaction volume of 25 µl: 2 µl of template DNA (10–50 ng/µl), 1.25 U of Taq DNA Polymerase, 0.50 µl of dNTPs (10 mM), 0.50 µl of each primer (10 pmol), 2.5 µl of 1× Taq DNA Polymerase buffer, 1.25 µl of MgCl₂ (1.5 mM) and 17.5 µl of ultrapure water. All reagents were from Siberian Enzyme (SibEnzyme Ltd., Academtown near Novosibirsk, Russian Federation).

The PCR cycling protocol was 2 min at 95 °C, then 35 cycles of 1 min at 95 °C, 1 min at 48 °C, and 90 s at 72 °C, followed by a final extension at 72 °C for 7 min.

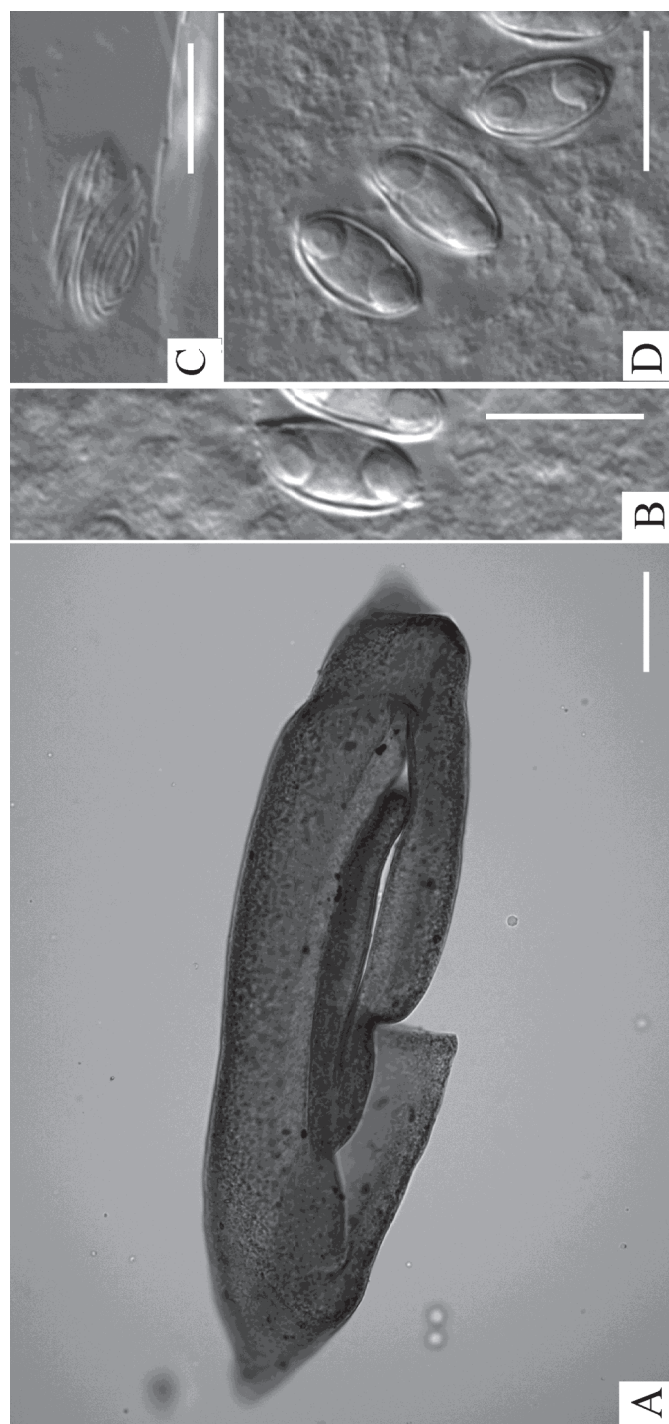


Fig. 1. Micrographs of *Myxidium shedkoae* from the gallbladder of *Perccottus glenii* from Lake Khanka: A = transversal section of plasmodium; B = spore, sutural view; C = ridges on spore shell valves; D = spores, valvular view. Scale bars A = 100 µm; B-D = 10 µm

Table 1
List of species used in the phylogenetic analysis

GenBank accession no.	Parasite species	Host species	Locality	References
AB530261.1	<i>Myxobolus spirosulcatus</i>	<i>Seriola quinqueradiata</i>	Japan: Ehime	Yokoyama et al., 2010
KT625442.1	<i>Myxidium amazonense</i>	<i>Corydoras melini</i>	Brazil: Amazonas state, Rio Negro river	Mathews et al., 2015
DQ377709.1	<i>Myxidium cuneiforme</i>	<i>Cyprinus carpio haematopterus</i>	China	Fiala, 2006
DQ851568.1	<i>Myxidium scripta</i>	<i>Trachemys scripta elegans</i>	USA: Louisiana	Roberts et al., 2008
MG748712.1	<i>Myxidium shedkoeae</i>	<i>Perecoptus glenii</i>	Russia: Primorsky Krai, Khanka Lake	Present study
AF201374.1	<i>Myxidium truttae</i>	<i>Oncorhynchus kisutch</i>	Canada: British Columbia, Big Qualicum	Kent et al., 2000
AJ582061.2	<i>Myxidium truttae</i>	<i>Salmo trutta</i>	United Kingdom: Northern Scotland	Holzer et al., 2004
KC849425.1	<i>Zschokkella auratis</i>	<i>Sparus aurata</i>	Portugal: Portimão, Alvor estuary	Rocha et al., 2013
DQ377688.1	<i>Zschokkella nova</i>	<i>Ctenopharyngodon idella</i>	Czech Republic	Fiala, 2006
GU471279.1	<i>Zschokkella nova</i>	<i>Carassius gibelio</i>	Hubei Province, Nuishan Lake	Bartošová and Fiala, 2011
DQ377689.1	<i>Zschokkella parasiluri</i>	<i>Pseudobagrus fulvidraco</i>	China	Fiala, 2006
JX271832.1	<i>Zschokkella oleae</i>	<i>Solea solea</i>	Tunisia: Ghar El Melh lagoon	Yemmen et al., 2013
DQ118776.1	<i>Zschokkella</i> sp. SA-2005	<i>Carassius auratus</i>	USA	Zielinski et al., 2005 (unpublished)
KM401441	<i>Zschokkella</i> sp. KLT-2014	<i>Labeo rohita</i>	Myanmar	Tun et al., 2014 (unpublished)
DQ377704.2	<i>Zschokkella</i> sp. of Fiala, 2006	<i>Haemulon sciurus</i>	Mexico: Caribbean Sea	Fiala, 2006
Outgroup EF602629.1	<i>Myxidium amatidum</i>	<i>Anas platyrhynchos</i>	USA	Bartholomew et al., 2008

PCR products were sequenced with the two PCR primers mentioned above and two additional primers NSF1179/18 (5'-AATTTGACTCAACACGGG-3') and NSR581/18 (5'-TCTCAGGCTCCCTCTCCGG-3').

Sequence fragments were assembled using MEGA V6.06 (Tamura et al., 2013). In addition, ambiguous bases were clarified using the corresponding ABI chromatograms of BioEdit (Hall, 1999). One partial 18S rDNA sequence (1875 bp) was deposited in GenBank with accession number MG748712.

For phylogenetic analysis, the sequence newly obtained in this study and 16 additional sequences of closely related species retrieved from the GenBank database were aligned using ClustalW in MEGA 6.0 (Tamura et al., 2013). The accession numbers of the sequences analysed in this study are given in Table 1 and Fig. 2 showing the phylogenetic tree.

Phylogenies were reconstructed using maximum likelihood (ML) and Bayesian inference (BI) analyses. Maximum likelihood analysis was performed using MEGA 6.0. Bayesian analysis was performed in MrBayes 3.2.2 (Ronquist and Huelsenbeck, 2003). In addition, jModelTest version 0.1.1 (Posada, 2008) was used to estimate the best nucleotide substitution model for the dataset. In both analyses the GTR+G+I model was used. Branch support was estimated by bootstrap analysis with 1000 replicates. BI analysis was performed with 10,000,000 generations. All myxozoans analysed, including the outgroup – *Myxidium anatum* Bartholomew, Atkinson, Hallett, Lowenstine, Garner, Gardiner, Rideout, Keel et Brown, 2008 – belong to the hepatic biliary group (after Kristmundsson and Freeman, 2013).

Results

Morphology

Plasmodia of *M. shedkoae* were found in 16% of 25 *P. glenii* specimens examined for parasitic infection. The plasmodia are flat (Fig. 1A), circular and polysporous. Spores (n = 15) are fusiform in valvular view, with protrusive, truncated-cone-shaped poles, 12–13.4 × 6.2–7.6 µm; thickness of the spores is 5.2–5.7 µm. Shell valves have longitudinal ridges, some of which merge with each other. The suture line is longitudinal and rectilinear, more or less oblique, occasionally almost median. Polar capsules are subspherical with tapering ends, 3.8–5.0 × 3.1–3.9 µm; the ratio of polar capsules based on length is 1 : 1–1.2. There are 5 coils of polar filament within each of them. A sporoplasm is located between the polar capsules (Fig. 1B).

Sequence and phylogenetic analysis

One sequence of *M. shedkoae* (1875 bp in length) is distinct from all other sequences in the GenBank database. A BLAST search revealed that *M. shedkoae*

is most closely related to *Zschokkella* sp. KLT-2014 (KM401441; 88% similarity over 1898 bp), *Zschokkella auratis* Rocha, Casal, Rangel, Severino, Castro, Azevedo et Santos, 2013 (KC849425, 88% of similarity over 1902 bp), *Myxidium truttae* Léger, 1930 (AF201374, 91% of similarity over 1139 bp).

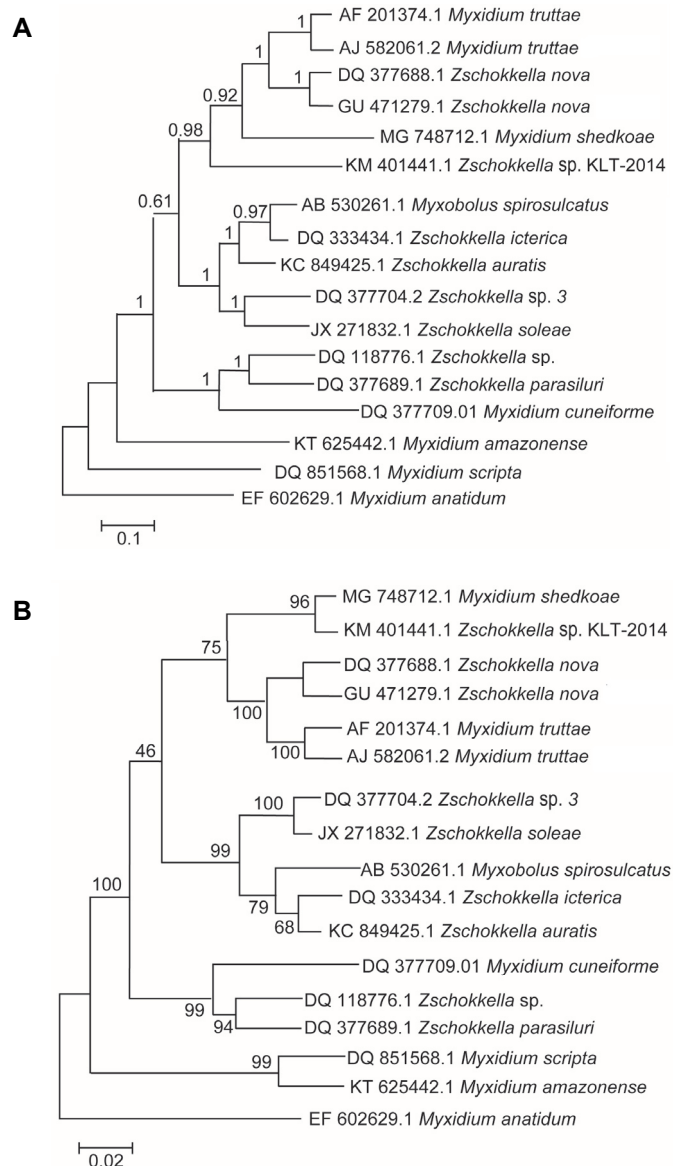


Fig. 2. Phylograms of *Myxidium shedkoe* position according to GTR + G + I substitution model for partial 18S rDNA sequence dataset: A = Bayesian inference; B = maximum likelihood.

The species *Myxidium anatum* is the outgroup in both trees

Both BI and ML analyses support *M. shedkoae* as a member of the clade also composed of *Zschokkella* sp. KLT-2014 *M. truttae* and *Zschokkella nova* Klokacewa, 1914 (Figs 2A and 2B). The topologies of the BI and ML trees differed in the position of *M. shedkoae* relative to *Zschokkella* sp. KLT-2014. The BI analysis with moderate support revealed that *M. shedkoae* is a sister to the *M. truttae* + *Z. nova* group, and placed the subclade of *M. shedkoae* + (*M. truttae* + *Z. nova*) as a sister branch to *Zschokkella* sp. KLT-2014 (Fig. 2A). The ML analysis showed that *M. shedkoae* and *Zschokkella* sp. KLT-2014 unite into a well-supported group, which is a sister to the *M. truttae* + *Z. nova* group (Fig. 2B).

The clade including *Zschokkella soleae* Yemmen, Marton, Bahri et Eszterbauer, 2013, *Zschokkella icterica* Diamant et Paperna, 1992, *Z. auratis*, *Myxobolus spirosulcatus* Maeno, Sorimachi, Ogawa et Kearns, 1995 and *Zschokkella* sp. of Fiala, 2006 from marine fishes is the nearest neighbour to the clade mentioned above. However, the sister relationship of these clades does not have high support in either of the two trees.

Discussion

Our study clarified the plasmodium morphology of *M. shedkoae*. According to Sokolov (2013), this species is characterised by a spherical plasmodium. However, in reality, the plasmodium of *M. shedkoae* is flat. Previous data were based on the observation of plasmodia that had been twisted during fixation. The morphology of the spores corresponds to the original description of Sokolov (2013).

Four other species of the genus *Myxidium* have been revealed as parasites of odontobutid fishes: *M. rimskykorsakowi* Schulman, 1962, *M. hyseleotris* Chen in Chen et Ma, 1998, *M. monstrosus* Schulman, 1962 and *M. odontobutis* Wu, Wang et Jiang, 1985 (see Sokolov, 2013). However, the morphological features of *M. monstrosus* and *M. odontobutis* do not fit to the morphotype of *Myxidium* spp. The two species probably belong to one of the other genera – *Triangula* Chen et Hsieh, 1984 or *Cardimyxobolus* Ma, Dong et Wang, 1982 (see Sokolov, 2013).

Myxidium shedkoae differs from *M. rimskykorsakowi* and *M. hyseleotris* in plasmodium localisation and ornamentation of the spore shell valves. The plasmodia of *M. rimskykorsakowi* parasitise the urinary bladder of *P. glenii*, while the plasmodia of *M. hyseleotris* the kidneys of *Micropercops cinctus* (Dabry de Thiersant, 1872) (see Shulman, 1962, 1966; Donec and Shulman, 1984; Chen and Ma, 1998). The spore shell valves of *M. rimskykorsakowi* and *M. hyseleotris* have only several non-intersecting longitudinal ridges (Shulman, 1962; Chen and Ma, 1998). In addition, *M. shedkoae* differs from *M. rimskykorsakowi* in spore size, and from *M. hyseleotris* in spore shape. Taking into account the data of the current and previous (Sokolov, 2013) studies, the mean length and

width of *M. shedkoe* spores are $13.1 \times 7 \mu\text{m}$. These figures fit to the maximum values of length and width of *M. rimskykorsakowi* spores – $13 \times 7 \mu\text{m}$ (Shulman, 1962; Chen and Ma, 1998). The spores of *M. hyseleotris* are wide oval, without protrusive poles (Chen and Ma, 1998; Sokolov, 2013).

The systematics of the genus *Myxidium* is artificial and does not reflect the real phylogenetic relationships of the constituent species. Species of the genus *Myxidium* in many phylogenetic reconstructions are distributed among different clades according to the site of infection and host ecology (Holzer et al., 2004; Fiala, 2006; Freeman et al., 2008; Fiala and Bartošová, 2010; Bartošová et al., 2009; Bartošová and Fiala, 2011; Hartigan et al., 2011; Jirků et al., 2011; Bartošová et al., 2013; Kristmundsson and Freeman, 2013). These facts support the opinion of Eszterbauer (2004) that the infection site specificity is an important factor in myxozoan phylogeny.

The monophyly of the *M. truttae* + *Z. nova* group, which is a sister to *M. shedkoe* according to BI analysis, is also supported by phylogenetic models of different authors (Fiala, 2006; Freeman et al., 2008; Bartošová et al., 2011; Bartošová and Fiala, 2011; Jones et al., 2011; Kalavati et al., 2013; Kristmundsson and Freeman, 2013; Heiniger and Adlard, 2014; Li et al., 2016; Aguiar et al., 2017). The species *Myxidium shedkoe*, *Z. nova* and *M. truttae* parasitise different systematic groups of freshwater fish – odontobutids, cyprinids and salmoniformes, respectively. The geographic ranges of these parasite species vary in extent, but they have a common overlap zone, which occurs in the Amur Transition Region (see Donec and Shulman, 1984; Sokolov, 2013). According to Bartošová and Fiala (2011), *Z. nova* is a complex of cryptic species, as evidenced by the high genetic distances between different populations of this parasite (Table 1, Fig. 2). It should be noted that the genus *Zschokkella* Auerbach, 1909, like some other genera of the suborder Variisporina, is a polyphyletic group and its members are clustered with *Myxidium* spp., congeneric species and, to a smaller extent, species of other myxozoan genera (Fiala, 2006; Freeman et al., 2008; Kristmundsson and Freeman, 2013; Heiniger and Adlard, 2014). There are no data on the morphology of *Zschokkella* sp. KLT-2014 placed in the closest position to *M. shedkoe* in the ML tree. Due to this fact we cannot discuss phylogenetic relationships and taxonomic position of this parasite.

This is the first report on the molecular characterisation of myxozoans specific to *P. glenii*, providing the baseline data for future research on the taxonomy and phylogeny of parasites of this fish species.

Acknowledgements

The authors are grateful to Semyon Yu. Bodrov ('Taxon' Research Resource Center, http://www.ckp-rf.ru/ckp/3038/?sphrase_id=8879024) for help in material sequencing. The research was supported by the state orders 0221-2014-0042 and 0109-2014-0028.

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