Myxozoan hidden diversity: the case of Myxobolus pseudodispar Gorbunova, 1936

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Abstract: Myxobolus pseudodispar Gorbunova, 1936 (Myxozoa) was originally described as a parasite of common roach, Rutilus rutilus (Linnaeus), with developing stages in muscles and spores disseminated in macrophage centres of different organs and tissues. Later, this parasite was described from several other cyprinids, but with relatively large intraspecific differences based on SSU rDNA gene sequences. Within our long-term study on myxozoan biodiversity, we performed a broad microscopic and molecular screening of various freshwater fish species (over 450 specimens, 36 species) from different localities. We investigated the cryptic species status of M. pseudodispar. Our analysis revealed four new unique SSU rDNA sequences of M. pseudodispar as well as an infection in new fish host species. Myxobolus pseudodispar sequence analysis showed clear phylogenetic grouping according to fish host criterion forming 13 well-recognised clades. Using 1% SSU rDNA-based genetic distance criterion, at least ten new species of Myxobolus Bütschli, 1882 may be recognised in the group of M. pseudodispar sequences. Our analysis showed the paraphyletic character of M. pseudodispar sequences and the statistical tests rejected hypothetical tree topology with the monophyletic status of the M. pseudodispar group. Myxobolus pseudodispar represents a species complex and it is a typical example of myxozoan hidden diversity phenomenon confirming myxozoans as an evolutionary very successful group of parasites with a great ability to adapt to a new hosts with subsequent speciation events.

Keywords: Myxozoa, phylogeny, PCR screening, cryptic species, host specificity.

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The Myxozoa is a diverse group of endoparasites with a worldwide distribution numbering more than 2,600 described species, which represents approx. 20% of cnidarians (Okamura et al. 2015, 2018). However, much of myxozoan biodiversity appears to be hidden (Bartošová-Sojková et al. 2014, Hartikainen et al. 2016). Their complex life cycle involves two hosts: invertebrates (Oligochaeta, Polychaeta and Bryozoa) as a definitive host, and vertebrates (mainly fish, rarely amphibians, reptiles, birds or mammals) as an intermediate host (Okamura et al. 2015). Myxozoan infections in fish are frequently inconspicuous; however, several myxozoans are highly pathogenic for their hosts causing whirling disease or proliferative kidney disease in salmonid fish (Hedrick et al. 1993, Gilbert and Granath 2003).

Myxobolus Bütschli, 1882 is the largest genus of the Myxozoa with more than 850 described species (Eiras et al. 2005, 2014). Species of Myxobolus have been reported from fish worldwide (e.g., Carriero et al. 2013, Lövy et al. 2018, Folefack et al. 2019). They infect mostly freshwater fish but they have been found also in brackish and marine hosts (e.g., Liu et al. 2019, Rocha et al. 2019). The diversity success of the Myxobolus is assumed to be caused by the shape of spores with lateral flattening that enabled easier invasion of tissues from precursors that lived in body liquids and cavities. Therefore, they could colonise many types of tissues (Fiala et al. 2015). Species of Myxobolus constitute the largest clade in the phylogenetic tree branch within the oligochaete-infecting lineage of myxozoans (Holzer et al. 2018). However, it is a paraphyletic taxon with Henneguya Thélohan, 1892; Thelohanellus Kudo,
The SSU rDNA sequence differences among these clades suggested cryptic species status of *M. pseudodispar*. Cryptic species phenomenon was also reported for other *Myxobolus* ssp. (e.g., Rocha et al. 2019) and for other myxozoan genera e.g., *Chloromyxum* Mingazzini, 1890; *Sphaerosporea* Thélohan, 1892; *Zschokkela* Auerbach, 1910 (see Bartošová and Fiala 2011, Holzer et al. 2013). Moreover, the study of Molnár et al. (2002) indicated that morphologically similar species of *Myxobolus* from different fishes seem to be different species. In contrast, Guo et al. (2018) suggested that SSU rDNA difference as low as 2.4% does not necessarily define conspecificity.

In the present study, we aimed to analyse data from a large-scale screening of freshwater fishes to resolve the cryptic species status of *M. pseudodispar*. We assess the host specificity of each *M. pseudodispar* clade and trace the myxospore morphology of *M. pseudodispar* and related species on the evolutionary tree.

**MATERIALS AND METHODS**

**Sample collection and light microscopy**

During our large-scale research on the biodiversity of myxozoan parasites of freshwater fish in Europe, overall, 452 fish (36 species from different fish orders) were collected in ponds, dams and rivers in the Czech Republic (408) and in the Danube River (44) in Bulgaria during the years 2016–2018. Host species numbers are summarised in Supplementary Table 1.

Fish from the Czech Republic were parasitologically examined using light microscopy (Olympus BX51 microscope) at 400× magnification to detect the presence of spores, plasmodia or other development stages of Myxozoa in gills, kidney and gill bladder. Other host tissues were not included in the examination, and therefore myxosporean plasmodia in muscles or skin could not be formed. Digital photos of fresh spores were taken at 1000× magnification using an Olympus DP70 camera. Spores were measured from digital images using ImageJ 1.48q (Wayne Rasband, http://imagej.nih.gov/ij). Gills, kidneys and gill bladders of fish from Bulgaria were fixed only for molecular processing.

**DNA isolation, PCR, cloning and sequencing**

All obtained fish tissue samples were kept in 400 μl of TNES urea buffer (10 mM Tris-HCl with pH 8; 125 mM NaCl; 10 mM EDTA; 0.5% SDS and 4M urea). Genomic DNA extraction was performed by standard phenol-chloroform extraction method with four-hour digestion with proteinase K (50 μg ml-1 180; Serva, Heidelberg, Germany) at 55°C and final elution in 100 μl of DNase-free water (Holzer et al. 2004).

We screened all obtained DNA samples for myxozoan infection using nested PCR by general myxozoan primer combination sets for partial SSU rDNA: 18S+18Sg (Hillis and Dixon 1991) in the first run; MyxGP2 F (Kent et al. 1998) + Act1R (Hallett and Diamant 2001) in the second run. The PCR cycling parameters were set up for first run: denaturation 95°C for three min, followed by 35 cycles of three steps: 95°C for one min, 62°C for one min, 72°C for two min, and after cycles a final extension 72°C for ten min; second run: denaturation 95°C for three min, followed by 35 cycles of three steps: 95°C for 40 s, 56°C for 40 s, 72°C for one min.
50 s, 72 °C for one min 40 s with a final incubation 72 °C for ten min (details in Table 1). Determination of the host species was verified by PCR and sequencing if the host identification was doubtful. We used CobF424 + CobR876 primers (Boore and Brown 2000) for amplification of partial mitochondrial cytochrome b gene. The PCR cycling parameters were set up as follows: denaturation 95 °C for three min, followed by 30 cycles of three steps: 95 °C for one min, 50 °C for one min, 72 °C for one min with a final incubation 72 °C for five min.

PCRs of the partial SSU rDNA of the parasite and cytochrome b gene of the host were performed using an AmpOne HS-Taq premix (GeneAll Biotechnology Co., Ltd., Seoul, South Korea) with 10 μl of AmpOne HS-Taq premix, 0.5 μl of each primer (25 pmol), 8.5 μl of water and 0.5 μl of extracted DNA (100–300 ng/μl). Obtained PCR products were purified by Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei, Taiwan) and sequenced directly by Sanger sequencing (Seqme).

PCR products were cloned if the direct sequencing revealed myxozoan coinfections. PCR fragments were cloned into the pDrive vector using a PCR Cloning Kit (Qiagen, Hilden, Germany) and then transformed into competent Escherichia coli cells (Life Technologies, Prague, Czech Republic). Cloned DNA plasmids were extracted and purified by the High Pure Plasmid Isolation Kit (Roche Applied Science, Penzberg, Germany) and three colonies of each plasmid were sequenced by Sanger sequencing (Seqme).

Alignments and phylogenetic analyses
Two alignments were constructed to reveal phylogenetic relationships of myxobolid SSU rDNA sequences. The first alignment composed of 117 myxosporean partial SSU rDNA, from which 81 sequences were newly obtained in this study and 36 sequences were retrieved from GenBank. The second alignment consisted of the selected sequences representing the groups of very closely related sequences with identical or almost identical sequences. Sequences in both datasets were aligned in MAFFT v7.017 (Katoh et al. 2005) using E-INS-i multiple alignment method implemented in Geneious v11.0.3 (Kearse et al. 2012). The alignments were manually edited and ambiguously aligned regions were removed.

Phylogenetic analysis was done by Bayesian inference (BI) and maximum likelihood (ML) methods. ML analysis was performed with RAxML v7.2.8 (Stamatakis 2006) with a GTR + Γ model selected by jModelTest2 (Posada 2008). Bootstrap supports were calculated from 1,000 replications. BI was performed in MrBayes v3.0 (Ronquist and Huelsenbeck 2003) with the GTR + Γ model of evolution. MrBayes was run to estimate posterior probabilities over one million generations via two independent runs of four simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Species-specific divergences were identified from proportional distances (in %) which were calculated in program Geneious based on the SSU rDNA dataset of all sequences under study.

**Fig. 1** Myxobolid spores obtained from the kidneys of different fish hosts. A – spore of *Myxobolus* sp. from *Rutilus rutilus* (Linnaeus) from Rájský pond, CZ; B – spore of *Myxobolus* sp. from *Phoxinus phoxinus* (Linnaeus) from Hostačovka brook, CZ; C – spore of *Myxobolus* sp. from *Gobio gobio* (Linnaeus) from Hostačovka brook, CZ.

**Table 1.** List of primers used for PCR screening including sequence of primers.

| Primer name | Sequence (5’→3’) | Annealing temperature, °C | References |
|-------------|------------------|---------------------------|------------|
| 18e         | TGTTGATCCTGCCAGT  | 64                        | Hillis and Dixon (1991) |
| 18g         | GGTAGTAACGGGCGGGTGTG | 58                        | Kent et al. (1998)    |
| MyxGP2F     | WTGGATAACGGGAA    | 50                        | Boore and Brown (2000) |
| Act1R       | AATTTCACCTCTGGGCA | 60                        | Hallett and Diamant (2001) |
| CobF424     | GGWTAYGTTYTCTGGWCCARAT | 50                      |            |
| CobR876     | GCRTAWGCAWARRAARTACAYTCWG | 50                    |            |

50 s, 72 °C for one min 40 s with a final incubation 72 °C for ten min (details in Table 1).
Abbreviation of locality

B = Bolevecky pond, CZ
GJ = Obecnik pond, CZ
H = Hostačovka brook, CZ
N = Novoselo, Danube river, BG
RA = Rájský pond, CZ
RD = Rímov Reservoir, CZ
V = Vodňany, CZ
ZD = Zelzka Reservoir, CZ
Z = Zlatý brook, CZ

Abbreviation of sampled tissue

G = Gills
K = Kidney

ML = ML bootstraps (0–100)
BI = Bayesian inference (0–1.00)

Topological test

We used TreeGraph v2.0.47-206 beta (Stöver and Müller 2010) to generate constrained alternate tree topology from the phylogenetic analysis. Designed alternate topology in Newick format was specified in the assumption block of the PAUP* program (Swofford 2001). The dataset with selected ML parameters was executed in PAUP* to generate likelihood scores of the best topology and the constrained tree. Resulted per-site log likelihood scores were analysed for significant differences in CONSEL v6.1 (Shimodaira and Hasegawa 2001), using three likelihood-based tests: approximately unbiased (AU), Kishino-Hasegawa (KH), and Shimodaira-Hasegawa (SH).

RESULTS

Morphology of Myxobolus pseudodispar

Myxozoan spores corresponding to the morphological diagnosis of Myxobolus pseudodispar were observed microscopically in fresh tissue preparations of four cypriniform fish: Abramis brama, Gobio gobio (Linneaus),...
Fig. 3. The phylogenetic tree based on the restricted alignment, in which a representative sequence of each individual clade was included. Each taxon is provided with fish host species the names and comparison of the morphology of myxozoan spores (if available) schematically drawn in the same scale including spore of original description of *Myxobolus pseudodispar* Gorbunova, 1936 (morphometric data are not available for *Leuciscus* and *Blicca/Vimba* clades). *Myxobolus bhadrensis* Seenappa et Manohar, 1981 was used as outgroup. Newly identified clades and host species are in blue and bold. Maximum likelihood/Bayesian inference nodal supports are shown at every node by colour square according to the appropriate scale shown in the legend. Clamps connect taxa with the sequence similarities higher than 99% - indicated by the number in the left rectangle next to clade name. The number in the right rectangle shows the sequence similarity of the *M. pseudodispar* clade (or clade assemblages with similarities higher than 99%) with the closest related clade. GenBank acc. numbers are given behind the species names.

*Myxobolus pseudodispar* according to the original description by Gorbunova (1936)
| Host fish          | Nr of fish | Coordinates         | Positive in total | PCR screening | Prevalence in total | Spores/ Plasmodia observed | Clade       |
|-------------------|------------|---------------------|-------------------|---------------|---------------------|----------------------------|-------------|
| Abramis brama     | 3          | 49°48′46.440″N; 15°28′28.920″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   | 36% (13/36) |
|                   | 3          | 48°49′58.400″N; 14°29′0.960″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   | 36% (13/36) |
|                   | 29         | 49°40′27.480″N; 15°9′48.600″E | 45% (13/29)       | gills 3/29; kidney 1/2 | 2/29 | Spores in kidney     |             |
|                   | 1          | 49°9′22.320″N; 14°40′28.200″E | 0% (0/1)          | gills 0/1 | 0% (0/1) | NA                   |             |
| Blicca bjoerkna   | 6          | 49°40′27.480″N; 15°9′48.600″E | 0% (0/6)          | gills 0/6; kidney 0/6 | 0% (0/6) | NA                   |             |
|                   | 5          | 44°09′46.0″N; 22°47′17.5″E | 60% (3/5)         | gills 3/5; kidney 3/5 | 27% (3/11) | 6% (1/16) |             |
|                   |            |                      |                   |               |                     |                            |             |
| Gobio gobio       | 6          | 49°48′56.707″N; 15°31′18.786″E | 33% (2/6)         | gills 0/6; kidney 0/6 | 0% (0/6) | Spores in kidney     |             |
|                   | 3          | 49°40′27.480″N; 15°9′48.600″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   | 17% (2/12) |
|                   | 2          | 50°4′11.640″N; 13°55′48.000″E | 0% (0/2)          | gills 0/2; kidney 0/2 | 0% (0/2) | NA                   |             |
|                   | 1          | 49°1′24.514″N; 15°56′44.319″E | 0% (0/1)          | gills 0/1; kidney 0/1 | 0% (0/1) | NA                   |             |
| Leuciscus idus    | 10         | 49°50′51.720″N; 15°29′44.160″E | 0% (0/10)         | gills 0/9; kidney 0/10 | 0% (0/10) | NA                   |             |
|                   | 2          | 49°40′27.480″N; 15°9′48.600″E | 0% (0/2)          | gills 0/2; kidney 0/2 | 0% (0/2) | NA                   |             |
|                   | 1          | 49°3′14.760″N; 14°45′46.800″E | 0% (0/1)          | gills 0/1; kidney 0/1 | 0% (0/1) | NA                   |             |
|                   | 1          | 44°09′46.0″N; 22°47′17.5″E | 100% (1/1)        | gills 1/1; kidney 1/1 | 100% (1/1) | NA                   |             |
|                   | 1          | 43°56′27.395″N; 22°50′21.820″E | 0% (0/1)          | gills 0/1; kidney 0/1 | 0% (0/1) | NA                   |             |
| Leuciscus leuciscus | 2          | 49°1′24.514″N; 15°56′44.319″E | 0% (0/2)          | gills 0/2; kidney 0/2 | 0% (0/2) | NA                   | 20% (1/5) |
|                   | 3          | 48°49′58.400″N; 14°29′0.960″E | 33% (1/3)         | gills 1/3; kidney 1/2 | 33% (1/3) | NA                   |             |
| Perca fluviatilis  | 3          | 49°50′51.720″N; 15°29′44.160″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   |             |
|                   | 10         | 50°3′14.040″N; 13°56′52.440″E | 0% (0/10)         | gills 0/10; kidney 0/10 | 0% (0/10) | NA                   |             |
|                   | 4          | 49°48′46.440″N; 15°28′28.920″E | 0% (0/4)          | gills 0/4; kidney 0/4 | 0% (0/4) | NA                   |             |
|                   | 3          | 49°49′45.840″N; 15°0′5.800″E | 0% (0/4)          | gills 0/4; kidney 0/4 | 0% (0/4) | NA                   |             |
|                   | 1          | 49°48′46.440″N; 14°29′0.960″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   |             |
|                   | 2          | 49°40′27.480″N; 15°9′48.600″E | 14% (1/7)         | gills 0/7; kidney 0/7 | 14% (1/7) | NA                   |             |
|                   | 1          | 49°9′36.188″N; 13°34′43.040″E | 17% (1/6)         | gills 1/6; kidney 1/6 | 17% (1/6) | Plasmodia in gills   |             |
|                   | 1          | 49°2′22.320″N; 14°40′28.200″E | 0% (0/1)          | gills 0/1 | 0% (0/1) | NA                   |             |
| Phoxinus phoxinus  | 14         | 49°48′56.707″N; 15°31′48.786″E | 71% (10/14)       | gills 4/14; kidney 7/14; muscle=1/1 | 71% (10/14) | Spores in kidney and gills; Plasmodia in kidney | 65% (11/17) |
|                   | 2          | 48°43′44.528″N; 13°35′60.745″E | 50% (1/2)         | gills 0/2; kidney 0/2; skin=1 | 50% (1/2) | Spores on skin       |             |
|                   | 1          | 49°50′51.720″N; 15°29′44.160″E | 0% (0/1)          | gills 0/1; kidney 0/1 | 0% (0/1) | NA                   |             |
| Rutilus rutilus   | 15         | 49°40′27.480″N; 15°9′48.600″E | 81% (13/16)       | gills 6/16; kidney 6/16 | 81% (13/16) | Spores in gills and kidney; Plasmodia in gills (Rutilus clade 2); Spores in kidney (Rutilus clade 1) | 21% (9/42) |
|                   | 5          | 49°48′56.707″N; 15°31′48.786″E | 0% (0/5)          | gills 0/5; kidney 0/5 | 0% (0/5) | NA                   |             |
|                   | 12         | 49°49′45.840″N; 15°28′5.800″E | 83% (10/12)       | gills 7/12; kidney 5/8 | 83% (10/12) | Spores in gills and kidney; Plasmodia in gills (Rutilus clade 2) |             |
|                   | 3          | 48°49′58.400″N; 14°29′0.960″E | 0% (0/3)          | gills 0/3; kidney 0/3 | 0% (0/3) | NA                   |             |
|                   | 2          | 49°9′36.188″N; 13°34′43.040″E | 100% (2/2)        | gills 0/2; kidney 2/2 | 100% (2/2) | Spores in gills (Rutilus clade 1); gills 1/3 (Rutilus clade 2) |             |
|                   | 1          | 49°2′22.320″N; 14°40′28.200″E | 0% (0/1)          | gills 0/1 | 0% (0/1) | NA                   |             |
Phoxinus phoxinus (Linnaeus) and Rutilus rutilus (Fig. 1). Plasmodia of the Myxobolus origin were observed in gills of *A. brama*, in kidney and gills of *R. rutilus* and in kidney of *P. phoxinus* and in gills of Scardinius erythrophthalmus (see Table 2).

| Host species spectrum and coinfections |
|--------------------------------------|
| We screened using PCR 452 fish from several localities in the Czech Republic and two localities in Bulgaria and subsequently sequenced positive PCR products (Supplementary file 1). The highest prevalence of *M. pseudodispar* was detected in vimba bream, *Vimba vimba* (Linnaeus) (86%; 6/7), common roach, *R. rutilus* (67%; 28/42) and common minnow, *P. phoxinus* (65%; 11/17). Lower prevalence has been found in other cyprinids: freshwater bream *A. brama* (36%; 13/36) white bream, *Blicca bjoerkna* (27%; 3/11) gudgeon, *G. gobio* (17%; 2/12) ide, *L. idus* (65%; 1/16) common dace, *L. leuciscus* (Linnaeus) (20%; 1/5), *S. caurinus* (Linnaeus) (20%; 1/5), and common rudd, *S. erythrophthalmus* (3.8%; 1/26). Myxozoans were detected in two perciform fish: European perch, *Perca fluviatilis* Linnaeus (5%; 2/38) and pikeperch, *Sander lucioperca* (Linnaeus) (29%; 5/17) (Table 2). One sample of the gall bladder from *P. phoxinus* was PCR positive for *M. pseudodispar*, which may represent detection of pre-sporengonic stages of this parasite since we did not observe any spores and plasmodia in the bile. Myxobolus diversicapsularis Slukhrai in Shulman, 1966; *M. elegans* Kashkovski in Shulman, 1966; *M. parviformis* Kallert, Eszterbauer, Erseus, El-Matbouli et Haas, 2005; *Myxidium spp.; Henneguya* sp. and *Chloromyxum* sp. were morphologically and molecularly identified in coinfections with *M. pseudodispar*.

Phylogenetic analyses

The phylogenetic tree based on SSU rDNA revealed that all newly obtained sequences clustered in close relation to *M. pseudodispar* sequences from GenBank (Fig. 2). They clearly grouped according to fish host species preferences forming 13 well-recognised clades. Part of the newly obtained sequences are identical or almost identical to already known sequences that clustered in clades named according to their predominant hosts: *Abramis, Blicca/Vimba, Rutilus* I and *Rutilus* II. Four new clades were explored by our new sequence data: *Gobio, L. leuciscus, L. idus and Phoxinus*. None of our newly obtained sequences clustered to five already identified *M. pseudodispar* sequences forming clades *Alburnus, Rutilus* III and IV, *Scardinius* I and II (Fig. 2). Sequence similarities among most of the clades in the *M. pseudodispar* group ranged from 90.1% to 99.8%, whereas only three clades exceeded 99% of sequence similarity between their members (Fig. 3).

Our analysis suggested that not all phylogenetic clades of *M. pseudodispar* shared a common ancestor. The paraphyletic character of *M. pseudodispar* group is caused by the clustering of Myxobolus bartai Salim et Desser, 2000 (AF1866835), *M. klamathellus* Atkinson et Banner, 2017 (KX261616), *M. ladogensis* Runyangovtsev et Shulman, 1997 (KU160629), *M. ridouti* Easy et Cone, 2009 (GQ292745), *M. stani* Iwanowicz et Iwanowicz, 2013 (DQ779995) and *Myxobolus* sp. (AY591531) within this group. Particularly, *Alburnus* and *Gobio* clades are sister related to the above-mentioned species of *Myxobolus* except for *M. ladogensis*, which is a sister taxon to *Rutilus* III, *Rutilus* IV and *Scardinius* I clades. We performed statistical tests to assess the possible monophyletic status of *M. pseudodispar* sequences. The best topology resulted from the
Table 3. Details of all species of *Myxobolus* Bützchli, 1882 included in the phylogenetic analyses names of hosts, fish organs or tissues, GenBank accession numbers for parasite taxa and spore dimensions (length × width).

| Myxozoan species | Host | Fish organ or tissue | GenBank Acc. No. | Spore dimensions (length × width) | Reference |
|------------------|------|----------------------|------------------|-----------------------------------|-----------|
| *M. artus* Akhmerov, 1960 | *Cyprinus carpio* | Skeletal muscle | FJ710799 | NA | NA |
| *M. bartaei* Salim et Desser, 2000 | *Notropis cornutus* | Body wall musculature | AF186835 | 11.0 ± 9.3 (10.3–11.4) × 10.8 (10.0–11.3) | Salim and Desser (2000) |
| *M. bhadreensis* Seenappa et Manohar, 1981 | *Laheo rohitu* | Kidney | KX832046 | 10.0 ± 9.1 (9.2–10.4) × 6.6 ± 0.37 (6.0–7.2) | Zeng et al. (2018) |
| *M. cyprini* Dolleijn, 1898 | *Cyprinus carpio* | Muscle | AF380140 | NA | Molnár et al. (2002) |
| *M. kingchowensis* Chen et Ma, 1998 | *Carassius gibelio* | Muscle | KX640624 | 11.21 ± 9.64 (8.63–12.20) × 8.43 ± 0.32 (7.83–9.14) | Zeng et al. (2018) |
| *M. klamathellus* Atkinson et Banner, 2017 | *Gila coerulea* | Kidney | KX61616 | 14.3 ± 9.4 (13–15) × 9.7 ± 0.4 (9–10) | Atkinson and Banner (2017) |
| *M. ladogensis* Rumyantsev et Shulman, 1997 | - | Subcutaneous and kidney | KX650629 | NA | Unpublished |
| *M. musculi* Keisselitz, 1908 | *Luciobarbus bocagei* | Muscle | KJ388981 | NA | Molnár et al. (2002) |
| *M. pseudodispar* Gorbunova, 1936 | *Abramis brama* | Muscle, kidney, gills, skin | AF466648–9 | NA | Present study, Molnár et al. (2002) |
| *M. pseudodispar* | *Alburnus alburnus* | Muscle | KU340981 | NA | Forró and Eszterbauer (2016) |
| *M. pseudodispar* | *Rutilus rutillus*, *Abramis brama* | Gills, kidney | EF466088 | 10.34 ± 0.61 (9.4–11) × 6.95 ± 0.32 (6.4–7.3) | Present study |
| *M. pseudodispar* | *Rutilus rutillus*, *Sander lucioperca*, *Scardinius erythrophthalmus* | Gills, kidney | KU340991 | 10.06 ± 0.62 (9.3–10.8) × 7.59 ± 0.96 (6.3–8.6) | Present study |
| *M. pseudodispar* | *Rutilus rutillus* | Muscle | AF380145 | NA | Molnár et al. (2002) |
| *M. pseudodispar* | *Rutilus rutillus* | Muscle | KU340988-90 | NA | Forró and Eszterbauer (2016) |
| *M. pseudodispar* | *Scardinius erythrophthalmus* | Muscle | KU340997 | NA | Forró and Eszterbauer (2016) |
| *M. pseudodispar* | *Scardinius erythrophthalmus* | Muscle | KU340976–8 | NA | Forró and Eszterbauer (2016) |
| *M. ridouti* Easy et Cone, 2009 | *Pimephales notatus* | Muscle | GQ292745 | 9.9 ± 0.3 (9.5–10.5) × 10.1 ± 0.4 (9.4–10.9) | Easy and Cone (2009) |
| *M. stanii* Iwanowicz et Iwanowicz, 2013 | *Campostoma oligolepis* | Muscle | DQ779995 | 10.0 ± 0.7 (7.5–11.0) × 8.8 ± 1.5 (6.3–11.3) | Iwanowicz et al. (2013) |
| *M. terreggiansensis* Székely, Shanhorn-Harrison, Cech, Ostoros et Molnár, 2009 | *Osteochilus vittatus* | Muscle | EU643629 | 12.7 ± 0.51 (12–13.4) × 7.4 ± 0.53 (6.7–8.3) | Székely et al. (2009) |
| *Myxobolus* sp. | *Gobio gobio* | Kidney | MN401328 | 11.75 ± 0.24 (11.5–12.1) × 8.28 ± 0.79 (7.3–9.5) | Present study |
| *Myxobolus* sp. | *Leuciscus idas* | Gills, kidney | MN401333 | NA | Present study |
| *Myxobolus* sp. | *Leuciscus leuciscus* | Gills, kidney | MN401336 | NA | Present study |
| *Myxobolus* sp. | *Perca fluviatilis* | Gills | MN401331 | NA | Present study |
| *Myxobolus* sp. | *Phoxinus phoxinus* | Gills, kidney, skin, bile, muscle | MN401332 | 10.85 ± 0.92 (9.2–11.86) × 8.93 ± 0.14 (8.8–9.2) | Present study |
| *Myxobolus* sp. | *Ptychocheilus oregenensis* | Muscle | AV591531 | NA | Kent et al. (2004) |
| *Myxobolus* sp. | *Salmonevia bulgarica* | Gills | MN401330 | NA | Present study |
| *Myxobolus* sp. | *Sander lucioperca* | Gills, kidney | MN401335 | NA | Present study |
| *Myxobolus* sp. | *Scardinius erythrophthalmus* | Gills | MN401334 | NA | Present study |
| *Myxobolus* sp. | *Vimba vimba* | Gills, kidney | MN401329 | NA | Present study |

NA – not available

ML analysis with paraphyletic character tested against the constrained topology with monophyletic *M. pseudodispar* sequences. All statistical tests (AU, SH and KH) rejected the monophyly of *M. pseudodispar* at the significance level 0.05 (Supplementary Table 2).

Although *M. pseudodispar* SSU rDNA sequence clades reflect host species affinity, there are few exceptions to this trend. *Myxobolus pseudodispar* from *R. rutillus* (*Rutilus I* clade) was also identified in five specimens of *S. lucioperca* and in one individual of *S. erythrophthalmus*. This clade showed high nodal support in both ML and BI analyses (87/1.0). The *Rutilus II* clade with ML/BI nodal support (100/1.0) includes the sequence from one specimen of *A. brama* (KU340983). The *Abramis* clade with predominant sequences from *A. brama* also includes sequences from *B. bjoerkna*, *S. bulgarica* and the perciform fish *P. fluviatilis* (Fig. 3).

No phylogenetic trend according to the site of infection was observed except the *Phoxinus* clade that showed a clear pattern of kidney and gills associated sequence clusters. Similarly, there was no tendency of clustering of *M. pseudodispar* sequences from the same geographic region.

In order to assess the influence of the taxon sampling on phylogenetic distribution, nodal support and cryptic status of

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Myxobolus pseudodispar, we created a less comprehensive dataset for the phylogenetic analysis, in which a representative sequence of each M. pseudodispar clade was included (Fig. 3). The resulting tree was additionally supplied by information about the systematic classification of the hosts and the comparison of the morphology of myxozoan spores (if available). This restricted analysis suggested almost the same topology as the comprehensive phylogenetic analysis of all obtained sequences. The only difference is the switch of the branching of the Rutilus I and Rutilus II clades. Nodal support remained generally low, however, several nodes were better supported in the restricted dataset. The exclusion of the repetitive identical sequences in the restricted dataset caused the different topological arrangements of the taxa at the tree and in connection with that, the tree is more explicit with the clear paraphyletic character of the sequences assigned to M. pseudodispar.

SSU rDNA sequences of the M. pseudodispar group have so far been obtained only from cypriniform hosts, from the Leuciscinae (A. brama, B. bjoerkna, R. rutilus and S. erythrophthalmus) and Alburninae (Alburnus alburnus). We revealed identical sequences of M. pseudodispar recorded in GenBank from four additional hosts: S. bulgarica, V. vimba (both Cypriniformes: Leuciscinae), P. fluviatilis and S. lucioperca (both Perciformes) (Fig. 3). Four new unique sequences very similar to M. pseudodispar sequences from GenBank and clustering within the M. pseudodispar group were acquired from tissues of L. leuciscus, L. idus, P. phoxinus (Cypriniformes: Leuciscinae) and G. gobio (Cypriniformes: Gobioninae).

The comparison of myxospore morphologies of M. pseudodispar and closely related Myxobolus spp. revealed that from an evolutionary point of view, there is a slight trend of change of myxospore morphology from an irregular oval shape to oval and finally to round spores (Fig. 3). The typical irregular shape was seen in three Rutilus clades (for the fourth Rutilus clade no morphology data are available) as documented in the original description of M. pseudodispar. Oval symmetrical spores of Myxobolus spp. were observed in G. gobio. Sequences of this species, forming the above-mentioned Gobio clade, is very closely related to M. pseudodispar (KU340981) from A. alburnus, for which no morphology data are available.

**DISCUSSION**

During a large host screening of various freshwater fish species, we focused on species identity of detected Myxobolus spp. morphologically resembling Myxobolus pseudodispar, a common myxosporean parasite of cyprinid fish with pleomorphic myxospore morphology and wide host spectrum. The large diversity of species of Myxobolus together with a very simple myxospore morphology as the main taxonomic criterion is the cause of difficulties in the identification species of Myxobolus. Some myxosporeans may display a certain degree of myxospore polymorphism in the shape and size (Guo et al. 2018), which makes the myxosporean taxonomy even more problematic.

Myxobolus pseudodispar and its closely related species represent a typical example of this problem. Pleomorphic character and irregular shape of spores make difficulties in distinguishing species according to morphology and it has been suggested that M. pseudodispar may be a cryptic species assemblage with affinities of individual species to specific hosts (Fórro and Eszterbauer 2016). Therefore, molecular taxonomy based on DNA sequences is indispensable for species determination in these cases. However, there are no exact rules for determining whether particular differences in morphology and molecular data can be used to discriminate intra- or interspecific variation in the Myxozoa.

Our SSU rDNA-based phylogenetic analysis showed differences in sequences of M. pseudodispar from different fish hosts and thus supported the cryptic character of different species of Myxobolus in the investigated group rather than congruence with a single species. Records of M. pseudodispar from different fish hosts represent very likely individual species of the genus Myxobolus with very similar myxospore morphology parasitising specific hosts or a specific group of fish. The SSU rDNA similarities within sequences of individual clades were more than 99%, whereas interclade similarities ranged from 90.1 to 99.8%, similarly as reported by Fórro and Eszterbauer (2016). To find the boundary for species delimitation in the percentage of sequence similarities is often problematic. The similarity of SSU rDNA of M. cyprini and M. artus, the most closely related species in our analysis, is 99%. Therefore, we assume that dissimilarity of the sequences around 1% may be the boundary to delimitate the species of Myxobolus. However, several clades have similarity higher than 99% with another clade (e.g., similarity between the Blicca/Vimba and Abramis clade), suggesting rather single species existence than existence of two independent species. Nevertheless, host specificity is still clearly evident in these clades. Using only the genetic distance criterion, at least ten new species of Myxobolus may be described in the future from the M. pseudodispar group.

Myxobolus pseudodispar was originally described as an endoparasite with developing stages in the muscle and spores scattered in different tissues of the common roach, Rutilus rutilus (see Gorbunova 1936). Phylogenetically, several different sequences identified as M. pseudodispar were documented from the common roach in previous studies (Molnár et al. 2002, Kallert et al. 2007, Fórro and Eszterbauer 2016). We supported these sequence differences in the present study and suggested four different phylogenetic clades of M. pseudodispar in R. rutilus. Morphology and dimensions of myxobolid spores of three phylogenetic clades from the common roach correspond to the original description of M. pseudodispar by Gorbunova (1936). No morphology data are available for the fourth Rutilus clade, but its SSU rDNA shows very high similarity with the sequence of the Rutilus clade III. Thus, we may predict similar morphology of myxospores of these two clades. We assume that M. pseudodispar firstly seen in R. rutilus by Gorbunova (1936) belongs to one of these four Rutilus clades, but because of the M. pseudodispar (and related species) myxospore pleomorphy and lack of additional morphological diagnostic features, it is not possible to decide which clade corresponds to the species in the original description.
Myxobolus pseudodispar has been supposed to have a relatively wide host spectrum. At least, five closely related fish species from the Cyprinidae were reported to be infected by this parasite (e.g., Gorbunova 1936, Baska 1987, Molnár et al. 2002, Forró and Eszterbauer 2016). Our fish screening revealed another six cyprinid and two perciform fishes to be infected by Myxobolus spp. with similar morphology to M. pseudodispar and phylogenetically closely related to M. pseudodispar sequences retrieved from GenBank. However, with the knowledge of the phylogenetic clade host specificity of these Myxobolus specimens and their genetic distances, it is obvious that the host spectrum of the originally described M. pseudodispar is rather narrow. As described above, we assume that the sequence of this species fits in one of the four clades according to the type host. Only three other hosts, Abramis brama, Sander lucioperca and Scardinus erythrophthalmus, were recorded as hosts for myxobolids in these four respective clades. Moreover, this myxozoan sequence was detected only once in A. brama (Baska 1987, Forró and Eszterbauer 2016) and once in Scardinus erythrophthalmus. Most of other M. pseudodispar related sequences studied in the present work have also a narrow host spectrum similarly as many other species of Myxobolus, e.g., M. bejeranoi Lövü, Smirnov, Brekhman, Ofek et Lotan, 2018, from hybrid tilapia of Oreochromis aureus (Steindachner) male × Oreochromis niloticus (Linnaeus) female, or M. pseudowulii Zhang, Zhai, Liu et Gu, 2017 from the yellow catfish Pylodictis olivaris (Rafinesque) (Zhang et al. 2017, Lövü et al. 2018). The Abramis clade is the exception of the narrow host spectrum with four hosts (Abramis brama, Blicca bjöerkerka, Perca fluviatilis and Sabanejewia bulgarica) detected to be infected by this myxobolid.

SSU rDNA sequences of Myxobolus pseudodispar and its closely related Myxobolus spp. (e.g., Myxobolus bartai and M. musculi Keisselitz, 1908) were obtained previously only from cyprinid hosts (Salim and Desser 2000, Molnár et al. 2002, Kent et al. 2004, Easy and Cone 2009, Iwanowicz et al. 2013, Forró and Eszterbauer 2016, Atkinson and Banner 2017, Zhang et al. 2018), specifically, from the subfamily Leuciscinae (A. brama; B. bjöerkerka; Campos toma oligolepis Hubbbs et Greene; Gila coerulea [Girard]; Luxilus cornutus [Mitchell]; Ptychocheilus oreognensis [Richardson]; R. rutilus and S. erythrophthalmus), Cyprininae (Carassius gibelio [Bloch]; Cyprinus carpio Linnaeus); Labeoninae (Labeo rohita [Hamilton]; Osteochilus vitatus [Valenciennes]); Barbinia (Luciobarbus boagei [Steindachner]) and Alburninae (Alburnus alburnus). These myxobolids form a diverse group of muscle-infecting Myxobolus spp. within a large subclade VIII according to Liu et al. (2019), which is specific for the Cypriniformes. Among newly identified hosts for M. pseudodispar, there are mostly cyprinids from the subfamily Leuciscinae (S. bulgarica; Vimba vimba; Leuciscus leuciscus; Leuciscus idus; Phoxinus phoxinus) and newly from the subfamily Gobiolineae (Gobio gobioides). Interestingly, we detected, by PCR and identified by sequencing, these myxobolids from two perciform fish P. fluviatilis and S. lucioperca. Myxobolus pseudodispar identified in P. fluviatilis (identical with M. pseudodispar GenBank AccNo. AF466649) was also detected in other three cyprinid fish species suggesting a wide host spectrum for the M. pseudodispar group which was broadened by host switching and adaptation even to a phylogenetically more distant host from Perciformes. However, no spores or development stages of Myxobolus were microscopically determined in these two perciform hosts. Hence, the presence of M. pseudodispar in P. fluviatilis and S. lucioperca may be also explained by the occurrence of these species in accidental hosts and a blind at by of the parasite development.

The prevalence of M. pseudodispar was higher (67%; 28/40) in the type host (R. rutilus) in the present work in comparison with 41% recorded in Molnár et al. (2010) and much higher than 17%, 0.4% and 19%, respectively, which were recorded by Athanassopoulou and Sommerville (1993). At very high prevalence (94%) was documented by Forró and Eszterbauer (2016) after experimental exposure, which likely does not correlate with levels of natural infection. Discrepancies in different levels of prevalence can be explained by different localities under study, different age of screened fish or different sampling time of the year. The screening methodology is also very important for the prevalence analysis. PCR is very sensitive and detects also microscopically undetectable infections (Grossel et al. 2005).

In our study, we derived the prevalence from combination of light microscopy, PCR and sequencing, which is likely the reason for higher prevalence values we observed for compared to these based only on the microscopical examination. The highest prevalence, interestingly, was detected in vimba bream V. vimba (86%; 6/7) and in common minnow P. phoxinus (65%; 11/17), the hosts in which M. pseudodispar has never been observed.

Type of locality and geographic origin of the samples do not seem to have an influence on the phylogenetic relationships within the species of M. pseudodispar complex. Samples in the present study were obtained from three different locality types (pond, reservoir and river/brook) and from two different countries: Czech Republic (ponds, reservoirs, rivers) and Bulgaria (Danube River). Several sequences from identical hosts but different localities were found to be identical, e.g., Myxobolus sp. SSU rDNA sequences from V. vimba from the pond in Vořňany (CZ) and from the Danube River in Bulgaria revealed 100% sequence identity. In addition to that, several sequences from the samples from the same geographic locality and the same host, e.g., M. pseudodispar from R. rutilus from Švihov Reservoir (CZ), clustered in different lineages at the phylogenetic tree with similarity around 96%. This may be due to the ability of M. pseudodispar to infect several oligochaete host species (Marton and Eszterbauer 2012) and thus the observed diversity of Myxobolus may be the result of co-evolution of a definitive host and its parasite.

The evolutionary success of the species of Myxobolus diversity is obvious and has been documented many times (recently reviewed in Liu et al. 2019). We proved that M. pseudodispar is a complex of closely related species that parasitise not only in the different hosts but also in different host tissues as the spores were observed frequently.
out of muscles, where the development of plasmodia was documented (e.g., Gorbunova 1936, Gonzalez-Lanza and Alvarez-Pellitero 1985, Baska 1987). Myxobolus pseudodispar is a typical example of a great ability of myxozoans to speciate after adapting to new hosts either by host switching or by host-parasite coevolutionary processes.

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