University of South Bohemia in České Budějovice
Faculty of Science

PHYLOGENY AND HOST SPECIFICITY OF KIDNEY INFECTING *MYXIDIUM* SPECIES

Bachelor thesis

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ANNOTATION

The focus of this Bachelor thesis was to determine the phylogenetic position of *Myxidium rhodei* Leger 1905, a common kidney-infecting myxosporean parasite of Eurasian cyprinid fish. Host specificity of *M. rhodei* was investigated by light microscopy and PCR screening with subsequent DNA sequencing.

DECLARATION

I hereby declare that I have worked on my bachelor's thesis independently and used only the sources listed in the bibliography. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

České Budějovice, 15.5.2017

Dariya Baiko

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1. INTRODUCTION

1.1. **Myxozoa,** Grassé 1970

Myxozoa is a subphylum of microscopic, nevertheless, multicellular endoparasitic Cnidarians. Their two-host life cycle involves an invertebrate (bryozoan, annelid) as a definitive host, and a vertebrate (fish, amphibian, reptile, bird, small mammal) as an intermediate host. Fish, though, remains to be the focus of interest in myxozoan research as numerous cases of infections are reported from all habitats all over the globe. Myxozoan infections are frequently inconspicuous, nevertheless, some, such as Whirling Disease and Proliferative Kidney Disease in salmonids, cause severe damage to fisheries (Eszterbauer *et al.* 2015, Hartikainen & Okamura 2015).

To date, the described species represent almost a fifth (18%) of all known Cnidaria (Figure 1). Due to peculiar nature of these animals the biodiversity of myxozoans is largely understudied, however, the estimations predict this group of animals is by far the largest among Cnidaria (Okamura *et al.* 2015a)

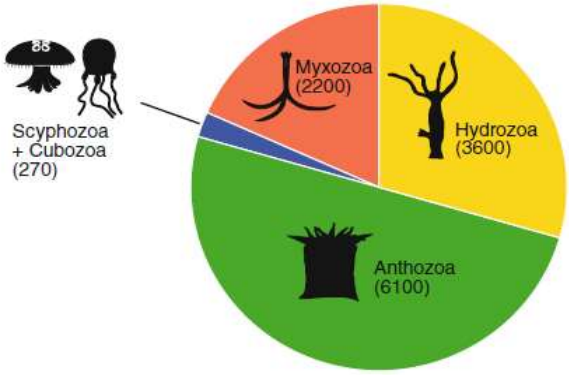

Figure 1. Pie chart showing the proportions (and numbers) of described species within Cnidaria. Data for the number of the number of described species from Zhang (2011). From Okamura et al. 2015a.

Myxozoa have come a long way to have their cnidarian origin recognized. Primarily, because from the first observation of a myxozoan by Jurine in 1825 and up till the 1990s these animals were classified as Protozoa, and only after the first phylogenetic analysis of SSU rRNA gene was performed, it was suggested to include this taxon to Metazoa (Smothers *et al.* 1994).

The most prominent feature of Myxozoa linking them to their jelly relatives — polar capsules — had not been linked to nematocysts of Cnidaria when the polar capsules were firstly described by Balbiani in 1860s. Thereafter, only in 1938 Weill noticed this homology, and thus suggested their relativity. The ultrastructural observation of polar capsules (Desser *et al.* 1983) and combined analysis of morphological and molecular features (Siddall *et al.* 1995)

has proved this connection. With the development of molecular biology and subsequent availability of sequencing methods, the morphological evidence had been supported by molecular data of several independent studies with cnidarian-specific genes and eventually suggested Myxozoa to be a sister taxon to Medusozoa (Jimenez-Guri *et al*. 2007, Holland *et al.* 2011, Shpirer & Chang 2014) (Figure 2).

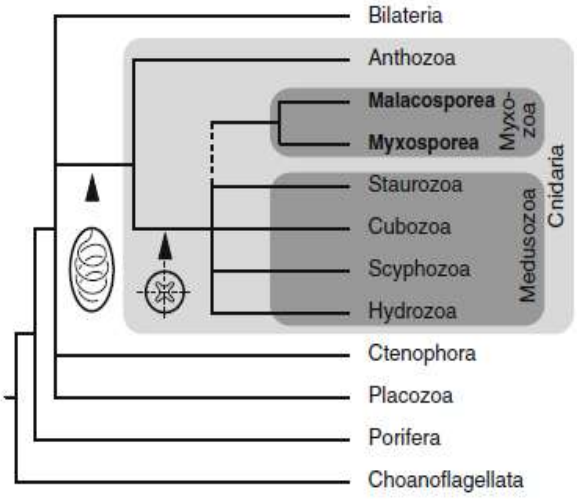

Figure 2. Consensus tree of basal metazoan relatioships and proposed position of Myxozoa. From Okamura et al. 2015b

Presently, there are two myxozoan classes: Malacosporea and Myxosporea. The representatives of Malacosporea (4 species described, Lom & Dyková 2006) are characterized by the macroscopic worm- or sac-like sexual stage in freshwater bryozoans and unicellular pseudoplasmodia in fish hosts. However, the majority of known myxozoan diversity (2310 species described, Morris 2010) belong to Myxosporea.

Myxosporeans exhibit a substantial simplification of their body plan to microscopic level. Myxosporean spores ('myxospores') are comprised of external valve cells that enclose the amoeboid infective sporoplasms as well as special cells with polar capsules. These relatively long-living multicellular spores are being produced in plasmodia or pseudoplasmodia in a range of intermediate hosts.

The definitive hosts of Myxosporea have been described to be annelids (oligochaetes and polychaetes). In the definitive host, a less studied short-living 'actinospore' stage develops. Very little is known about actinosporean stages in comparison to myxosporean ones. Actinospore stage was primarily considered as a separate class Actinosporea until it was united with Myxosporea by Markiw and Wolf in 1980s as two-host lifecycle of myxozoans was deduced. Nowadays, only 50 complete life cycles are known out of around 2300 discovered species of myxozoans (E. Eszterbauer *et al*. 2015)

The two-host lifecycle of Myxozoa corresponds to alternation of myxospore and actinospore stages (Figure 3). The transmittance between definitive and intermediate host occurs through water column. The spore anchors itself in the host using the special organelles — polar capsules. These intracellular structures, now affirmed as homologous to cnidarian nematocysts, discharge an extrudable filament when in contact with the possible host and get firmly attached to the tissue, thus facilitating the sporoplasm to infect the host.

Benthic definitive hosts (annelids) produce buoyant short-living actinospores that soon enough find their intermediate hosts (fish) by floating in the water column. Buoyancy is achieved with long peculiar appendages that allow them to get easily transported with water column movements. In the intermediate host (fish), relatively sturdy compact myxospores develop: they sink to the bottom to find a definitive host (annelid) and start the cycle over again.

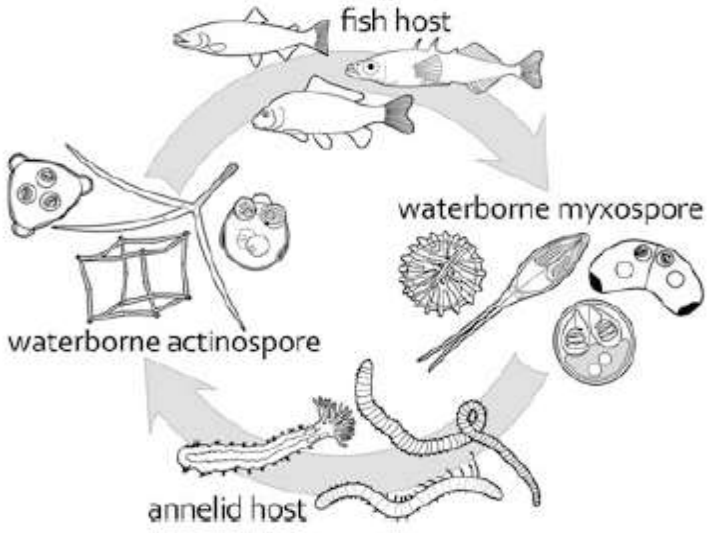

Figure 3. Generic two-host myxozoan life cycle. From Eszterbauer et al. 2015

1.2 Tissue & Host specificity of myxozoans

The extensive simplification of body plan associated with endoparasitic lifestyle of myxozoans combines features like absence of cilia and centrioles as well as lack of apparent embryonic and larval development along with reduction of tissues completely in myxosporeans and significantly in malacosporeans (Lom 1990, Canning *et al.* 2000, Canning & Okamura 2004). Endoparasitic lifestyle has allowed myxosporeans to reduce their own tissues and organs and develop very effective ways to exploit particular tissues of the host. Various myxozoan infections can be observed in practically all fish tissues and organs. After

penetration into the host body, parasite finds its way to the site where it can proliferate. Even though some myxosporeans can be found in a broad range of fish tissues, normally, the infection is precisely targeted to a specific tissue of the host where sporogony occurs. Tissue tropism can be selective enough to allocate the site of plasmodial development even to the particular region of the given organ or tissue (Molnár 1994, Molnár 2002).

According to the site of plasmodial development, the parasites can be histozoic – developing between the cells of tissues (epithelium, kidney, nerves, etc.), or coelozoic – developing in cavities of body organs (urinary and gall bladder and ducts, blood vessels lumen, etc.). Along with descriptions of typically histozoic (*Myxobolus* spp.*)* and typically coelozoic species (*Ceratomyxa* spp.), for many myxozoan parasites observations allow to draw conclusions that both coelozoic and histozoic stages occur over their lifecycle. For example, *Sphaerospora dykovae* have its extra-sporogonic stages travelling in the bloodstream in addition to sporogony in urinary tracts and renal tubules (Csaba 1976, Molnár 1984), and *Tetracapsuloides bryosalmonae* dwell in epithelial tissues in a vegetative stage before developing mature spores in renal tubules (Morris & Adams 2008).

Even though the examples of myxozoan parasites infecting distantly related fish species occurs, it is more common to detect presence of a certain myxozoan in closely related fish species. To the latest knowledge, however, myxozoans tend to demonstrate a significant level of host specificity, namely infecting only a particular host species (Shul'man 1966, Hedrick *et al*. 2001, Urawa *et al*. 2011, Molnár & Eszterbauer 2015).

1.3 Phylogeny of myxozoans

Before molecular biology was routinely used for taxonomic analysis, the identification and assignment of myxozoans was performed only by morphological features of myxospores and plasmodia. Phylogeny of myxozoans is in great disagreement with the classical taxonomy (Fiala 2006). Fast evolutionary rates of myxosporeans lead to high spore plasticity and multiple examples of convergent evolution. Similar spore morphotypes evolved several times in known myxozoan history and thus species with similar morphology classified into the same genus are in fact distantly related (Fiala & Bartošová 2010). Even though myxospores possess a great number of criteria that gave rise to the detailed taxonomic differentiation, genera like *Myxidium*, *Zschokkella*, *Elipsomyxa* and *Sigmomyxa* are extremely challenging to discern. Moreover, all these genera are now known to be polyphyletic, therefore, even distinguishing between these four would fail to describe their evolution (Fiala *et al.* 2015b).

SSU rDNA sequence has been proven to be the reliable phylogenetic marker for analysing myxozoan relationship (e.g. Kent *et al.* 2001, Holzer *et al*. 2004, Fiala 2006, Burger & Adlard 2011, Bartošová *et al.* 2013, Bartošová-Sojková *et al*. 2014, Hartikainen *et al*. 2014). The relevance of using SSU rRNA genes for deducing phylogenetic relationship of myxozoans has been confirmed by analysis of LSU rRNA gene as well as EF2 (Elongation factor 2) gene (Whipps *et al*. 2004, Bartošová *et al.* 2009, Fiala & Bartošová 2010). Although LSU showed itself to be more informative for phylogenetic investigations (Bartošová *et al.* 2009), SSU-based analysis stays more relevant due to larger amount of already available data. As there are conserved and variable regions in SSU rDNA, discrimination at different taxonomic levels is possible. However, variable regions of myxozoan SSU rDNA experience high substitution rates that double the evolutional rate of myxozoans compared to other Radiata (Kent *et al.* 1996, Cavalier-Smith *et al*. 1996, Saulnier *et al*. 1999; Evans *et al.* 2010). This arises the problem of long branch attraction — a common artifact of phylogenetic analysis. High variability in sequences results in emergence of long branches in phylogenetic trees (Anderson & Swofford 2004). An eminent example of such a high variability is the representatives of marine urinary clade and, in particular, genus *Ceratomyxa*. Elucidating the phylogeny of long branching sequences is a challenging task as the nodes are unstable and the topology of the tree changes depending on the type of analysis (Bartošová *et al.* 2011, Fiala *et al.* 2015c)

The phylogenetic tree of Myxozoa based on SSU rDNA is split into two main lineages that correspond to the established classes: Malacosporea and Myxosporea. Myxosporeans, in turn, are represented by three lineages: basal sphaerosporid lineage as well as further radiated

marine and freshwater lineages. *Sphaerospora sensu stricto* clade (sphaerosporid lineage) is a monophyletic clade comprised of species that have unusually long SSU rDNA sequences (Jirků *et al*. 2007, Holzer *et al.* 2007, Holzer *et al.* 2013a, Holzer *et al.* 2013b, Bartošová *et al*. 2013, Eszterbauer *et al.* 2013). Freshwater and marine lineages, as the names suggest, group myxozoans roughly according to their environment. However, as numerous exceptions from freshwater/marine rule occur, separation according to the definitive host (oligochaete or polychaete) is, perhaps, more descriptive. Within the myxosporean lineages, the tendency for clustering according to tissue tropism in the intermediate host can be observed. Thus, freshwater and marine lineages each have histozoic and coelozoic groups. Further detailing within the groups allows to distinguish clades that collect myxosporeans infecting certain organ systems (e.g. urinary and gall bladder clades) or those with morphologically distinctive features (e.g. *Kudoa* and *Ceratomyxa* clades) (Fiala *et al.* 2015a).

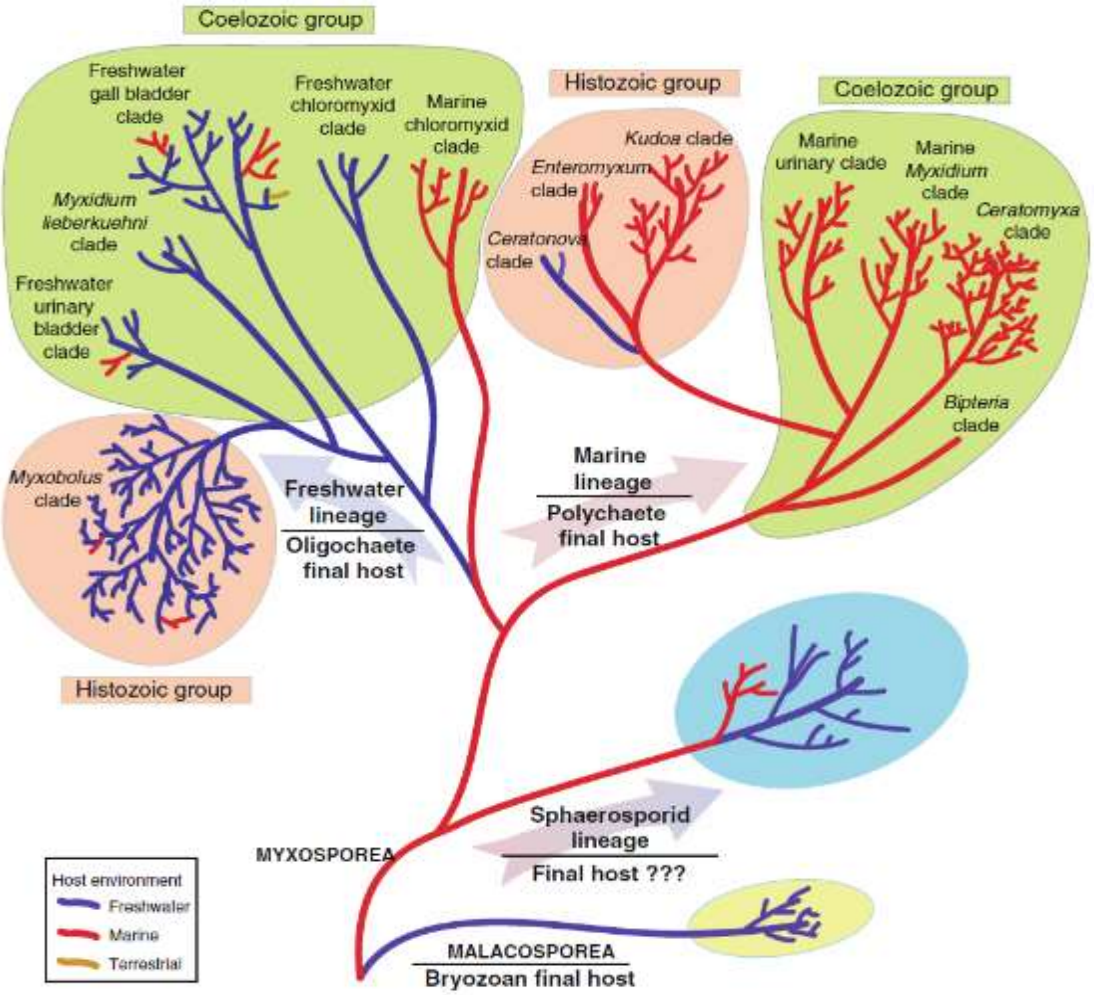

Figure 4. Summary of hypothetical evolution of the Myxozoa inferred from molecular data. From Fiala et al. 2015a

Interestingly, there is a prominent clade combining sequences of only actinosporean origin (not indicated in Figure 4). These 10 sequences of four morphotypes (aurantiactinomyxon, echinactinomyxon, raabeia, and synactinomyxon) from freshwater annelids branches as a sister group to *Myxidium lieberkuehni* clade (see Coelozoic group of Freshwater lineage in Figure 4). Myxosporean counterparts of these actinospores are unknown, therefore, they cannot be identified as life stages of any described genus. Thus, these sequences are given collective names as they were formerly described in Actinosporea before this taxon was suppressed (e.g. Raabeia sp. type 4) (Lom *et al.* 1997).

1.4 *Myxidium* spp. Bütschli, 1882

Myxidium is a polyphyletic myxosporean genus of typically coelozoic (rarely histozoic) parasites, numbering over 205 species in marine and freshwater fishes, 8 species in amphibians and 4 in reptiles (Lom & Dyková 2006). The representatives of this genus can be found in both freshwater and marine lineages of myxozoan phylogenetic tree. For example, in the freshwater lineage there is a number of *Myxidium* spp. in the urinary clade and the *M. lieberkuehni* clade, whereas in marine lineage *Myxidium* spp. are branching within *Enteromyxum* clade (Fiala *et al.* 2015b).

Morphologically the genus is described by spindle-shaped myxospores that can be straight, slightly crescent-shaped, or sigmoid. Two pyriform polar capsules are located at the somewhat pointy poles of the spore. Sututal line divides the spore into two equal shell valves: smooth or ridged. (Lom & Dyková 2006). Genus *Myxidium* is morphologically very similar to *Zschokkella*, *Elipsomyxa* and *Sigmomyxa,* therefore it is very problematic to discern the delicate differences between these genera (Fiala *et al.* 2015b).

1.5 *Myxidium rhodei* Léger, 1905

Myxidium rhodei is a kidney-infecting myxosporean that has been well described morphologically. *M. rhodei* was first described by Léger from kidney tissue of the European bitterling (*Rhodeus amarus*) in 1905. Since then the recordings of *M.* cf. *rhodei* were documented from more than 10 cyprinid species using morphological analysis (Shul'man 1984, Dyková 1987, Alvarez-Pellitero 1989, Saraiva 2000, Longshaw 2005, Dzika 2006, Kirjušina 2007, Al-Jawda 2014, Batueva 2015). According to Longshaw (2005), such wide host range suggests that it is preferable to refer to the *Myxidium* spp. in these hosts as *M.* cf.

rhodei until the data is reliably confirmed. Roach (*Rutilus rutilus*) is considered as the most common host for *M.* cf. *rhodei*.

Spores of *M.* cf. *rhodei* are spindle-shaped with somewhat pointy poles. Pyriform polar capsules with 5-turn coiled filament are located at the poles. Mature spores usually are slightly narrowed equatorially (Athanassopoulou & Sommerville 1993a). Spores mature in plasmodia in Bowman capsules without invoking the immune response. In cases of heavy infections, plasmodia spread into interstitial kidney tissue; however, those plasmodia are normally actively attacked by fish immune system (Dyková 1987, Batueva 2015).

Additionally to infecting kidney tissue, *M.* cf. *rhodei* has been recorded from urinary duct, liver, muscles, spleen, heart, swimbladder and gonads (Kepr 1991, Brummer-Korvenkontio 1991, Athanassopoulou & Sommerville 1993a, Athanassopoulou & Sommerville 1993b, Saraiva 2000, Dzika 2006). Such spreading of the parasite was observed only in severely infected specimens. Kidney is the only confirmed site where sporogonic development occurs (Dyková 1987).

Despite the fact that *M.* cf. *rhodei* is regarded as one of the most common parasites of cyprinid fish in Eurasia, the molecular data of it hasn't been assessed yet, thus, the phylogenetic position of *M. rhodei* is still unknown.

OBJECTIVES

- To determine the phylogenetic position of *Myxidium rhodei,* parasite of cyprinid fish with unavailable molecular data.

- To ascertain *M. rhodei* host specificity using newly developed specific primers for this species.

- To screen the fish kidney DNA samples for the presence of putative kidney infecting myxozoan species of the phylogenetic clade of only actinosporean stages (i.e. hunting for missing myxosporean counterparts)

2. MATERIALS AND METHODS

2.1. Sampling

The majority of samples were taken from the laboratory collection of DNA and preserved tissue collected over the period from 2012 to 2016 from different localities all over Czech Republic as well as abroad (Poland). Some of the fish kidney samples of *Rutilus rutilus, Scardinius erythrophthalmus* and *Tinca tinca* were collected and dissected personally by me at Ruda field station in November 2014. Some kidney and gall bladder samples of *Carassius carassius* and *Misgurnus fossilis* were collected during the trip to Belarus in April 2015. The full list of localities and fish species investigated is presented in Table 1.

A total of 260 fish samples of 20 species was analyzed: 252 samples of 17 cyprinid species (order *Cypriniformes*) as well as 8 samples of 3 non-cyprinid fish species (orders *Perciformes, Siluriformes, Anguilliformes*) resulting in 265 kidney samples, 9 gall bladder samples and 1 gills sample.

The fish of smaller size/younger age was preferred for sampling to increase the chance of obtaining a better image of infection due to overall smaller kidney size. Moreover, the juvenile fish is less likely to have a secondary infection of other myxozoan and non-myxozoan parazites. According to Brummer-Korvenkontio *et al.* (1991), in case of *M. rhodei* in roaches, there is a tendency for decreasing prevalence with increasing age of the fish.

Table 1. List of localities for fish sampling with the corresponding fish species and their count. Note: CZ – Czech Republic, BY – Belarus, PL – Poland

Habitat	Locality	Fish species		Order	Number of individuals
Malše river	Plav, CZ	common dace	*Leuciscus leuciscus*	*Cypriniformes*	27
		common roach	*Rutilus rutilus*		9
		gudgeon	*Gobio gobio*		19
		chub	*Leuciscus cephalus*		35
		white bream	*Blicca bjoerkna*		1
		common nase	*Chondrostoma nasus*		1
		European eel	*Anguilla anguilla*	*Anguilliformes*	2
Malše river	Římov, CZ	common dace	*Leuciscus leuciscus*	*Cypriniformes*	11
		asp	*Aspius aspius*		2
		common bleak	*Alburnus alburnus*		11
		common bream	*Abramis brama*		12
pond	Northern Moravia, CZ	Grass carp	*Ctenopharyngodon idella*	*Cypriniformes*	2
		tench	*Tinca tinca*		1
		rudd	*Scardinius erythrophthalmus*		2
Holicky pond	Jindřiš, CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	7
		rudd	*Scardinius erythrophthalmus*		4
		common bleak	*Alburnus alburnus*		2
		European bitterling	*Rhodeus amarus*		21
Vožralý pond	Slavonice, CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	3
Horusicky pond	Veselí nad Lužnicí, CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	10
Záblatský pond	Záblatí (Ruda) CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	20
		Prussian carp	*Carassius gibelio*		1
		tench	*Tinca tinca*		5
		rudd	*Scardinius erythrophthalmus*		3
Velký Tisý pond	Lomnice nad Lužnicí (Ruda), CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	5
		tench	*Tinca tinca*		2
		rudd	*Scardinius erythrophthalmus*		2
		Eurasian ruffe	*Gymnocephalus cernuus*	*Perciformes*	5
		wels catfish	*Silurus glanis*	*Siluriformes*	1
Máchovo lake	Česká Lípa, CZ	common roach	*Rutilus rutilus*	*Cypriniformes*	1
Bohdanečský pond	Lázně Bohdaneč, CZ	European bitterling	*Rhodeus amarus*	*Cypriniformes*	3
		Spined loach	*Cobitis elongatoides*		1
Iput' river	Gomel, BY	European weather loach	*Misgurnus fossilis*	*Cypriniformes*	9
		Crucian carp	*Carassius carassius*		14
river	PL	European bitterling	*Rhodeus amarus*	*Cypriniformes*	5

2.2. Dissection

Fish were euthanized using an overdose of a clove oil. Prior to dissection, each fish was measured with a ruler and weighed. Fresh preparates of kidney samples as well as at times gall bladder contents and gills were examined under the *Olympus BX51* light microscope. The observed plasmodia and spores were photographed with the *Olympus DP70* digital camera.

All dissected specimens' kidney samples were taken for molecular analysis despite if they were microscopically positive for myxozoan presence. For molecular analysis, the small portion of kidney of each specimen was placed into 400 μL TNES urea buffer (10 mM Tris-HCl pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) (Asahida *et al.* 1996).

All animal manipulations were performed in accordance with Czech legislation (section 29 of Act No. 246/1992 Coll., on Protection of animals against cruelty, as amended by Act No. 77/2004 Coll.).

2.3. DNA extraction (Phenol-chloroform extraction)

All DNA samples were extracted from tissues by phenol-chloroform extraction.

The tissue samples dissolved/suspended in TNES urea buffer (pH 8) were digested with Proteinase K (Serva, Germany) at 55 ˚C overnight. After that, equal amount of phenol (pH 8) was added and the test tube contents were vigorously mixed for 5 minutes to ensure efficient extraction. Later, same amount of chloroform was added and the contents were mixed by overend turning of the tubes. Centrifugation at 4 ˚C and 15000g for 5 min allowed phase separation. The top aqueous layer containing DNA was transferred into a fresh test tube and triple amount of ice-cold 92% ethanol was added in order to precipitate DNA. Formed precipitate was centrifuged down at 4 ˚C and 15000g for 20 min. After decantation of ethanol from the tube, the DNA pellet was twice washed with 70% ethanol. After each addition of ethanol, centrifugation at 15000g for 5 min and decantation followed. The remainder of ethanol was dried at room temperature overnight or in thermoblock at 50 ˚C until no more liquid was observed in the tubes.

The DNA pellet was dissolved in nanopure water (50 μL - 500μL depending on the size of DNA pellet visually assessed) at room temperature. The samples were stored at 4˚C in case of immediate further analysis, or in freezer at -18 ˚C in case if long term storage was necessary.

2.4. Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used for amplification of the myxozoan DNA.

The amplification was performed by Taq purple polymerase (Top-Bio, Czech Republic) for most reactions. Titanium Taq polymerase (Clonitech Laboratories, USA) was also used in cases of low yield when utilizing Taq purple. Each polymerase was used with the corresponding buffer from the manufacturer: 10x Taq purple Buffer complete (Top-Bio, CR); 10x Titanium Taq buffer complete (Clonitech Laboratories, USA). The reactions with Taq purple were performed in volume of 25 μL, while those with Titanium Taq were in volume of 10 μL. A mixture of dNTPs from Promega (USA) was used.

The composition of the reaction mixtures for both polymerases is presented in Table 2.

Table 2: Composition of the PCR mixture

Reaction component	Taq purple polymerase, total volume: 25 μL		Titanium Taq polymerase, total volume: 10 μL	
	Stock concentration	Volume per reaction	Stock concentration	Volume per reaction
10x buffer	-	2.50 μL	-	1.00 μL
dNTP	250 μM	2.00 μL	10 mM	0.20 μL
Forward primer	10 μmol	1.00 μL	10 μmol	0.20 μL
Reverse primer	10 μmol	1.00 μL	10 μmol	0.20 μL
Polymerase	1U/1μL	1.00 μL	1U/1μL	0.05 μL
ddH_2O	-	16.50 μL	-	7.35 μL
DNA	-	1.00 μL	-	1.00 μL

In order to ensure successful myxozoan DNA amplification, in all cases the samples were pre-amplified with universal primers for eukaryotic organisms (erib1–erib10, 18e–18g). Consequently, the nested PCR with myxozoan-specific primers (MyxospecF–MyxospecR, MyxGP2F–ACT1R, Myxgen4F–ACT1R) or specifically designed primers (Mrhod511F–Mrhod953R, Mgia511F–Mgia953R, Mrhodei_sstricF1–Mrhodei_sstricR1) were performed. The data concerning the above-mentioned primers is presented in Table 3. For some primers, higher annealing temperatures were used instead of those mentioned in the source literature. The optimal annealing temperatures for newly designed primers were determined by gradient

PCR in *LifePro Thermal Cycler* (Bioer, China). The newly designed primers were manufactured by Generi Biotech (Czech Republic)

Table 3: Primers used for PCR

Primer name	Sequence (5'→3')	Annealing temperature, °C	References
erib1	ACCTGGTTGATCCTGCCAG	60	Barta *et al.* 1997
erib10	CTTCCGCAGGGTTCACCTACGG		Barta *et al.* 1997
18e	TGGTTGATCCTGCCAGT	64	Hillis & Dixon 1991
18g	GGTAGTAGCGACGGGCGGTGTG		Hillis & Dixon 1991
MyxospecF	TTCTGCCCTATCAACTTGTTG	54	Fiala 2006
MyxospecR	GGTTTCNCDGRGGGMCCAAC		Fiala 2006
MyxGP2F	WTGGATAACCGTGGGAAA	58	Kent *et al*. 1998
ACT1R	AATTTCACCTCTCGCTGCCA		Hallet & Diamant 2001
Myxgen4F	GTGCCTTGAATAAATCAGAG	58	Kent *et al.* 2000
ACT1R	AATTTCACCTCTCGCTGCCA		Hallet & Diamant 2001
Mrhod511F	GTTTCCTATATGGATAATCATACTGG	52	Present study
Mrhod953R	CATCTCATAAGACATAATGGTCAAC		Present study
Mgia511F	GTTTGTGACAAACATGCGATACCGG	63	Present study
Mgia953R	CACTCCGCAAACACAACACACGACC		Present study
Mrhodei_sstric_F1	AAGGATTCGACACTGGAATG	60	Present study
Mrhodei_sstric_R1	TGCGGGTATATACATAGCGCC		Present study

The amplification with Taq purple polymerase and universal eukaryotic primers (18e–18g) was carried out in a *Tpersonal* cycler (Biometra, Germany), and consisted of 30 cycles of 95 ˚C for 1 min, 64 ˚C for 1 min and 72 ˚C for 2 min, followed by 10 min incubation at 72 ˚C. Amplification with universal eukaryotic primers erib1–erib10 consisted of 30 cycles of 95 ˚C for 1 min, 60 ˚C for 1 min and 72 ˚C for 2 min, followed by 10 min incubation at 72 ˚C. One microliter of PCR amplicons (either 18e-18g or erib1-erib10) was used as DNA template for nested PCR with myxozoan or specifically designed primers.

The temperature programs are summarized in Table 4.

Table 4: PCR temperature program for Taq purple polymerase

	Temperature, ˚C	Duration	
	95	3 min	
denaturation	95	1 min	30 cycles
annealing	*60	1 min	
elongation (Taq purple/Titanium Taq)	72 / 68	**45 s/1 min/1 min 30 sec/2 min	
final incubation (Taq purple/Titanium Taq)	72 / 68	10 min	

(*) Annealing temperatures were set according to the optimal working temperature of the primers mentioned in Table 3.

(**) Elongation times were adjusted according to the expected product length considering the polymerase working speed as 1000 bp/min.

2.5. Gel electrophoresis

PCR amplification products were analyzed by gel electrophoresis on 1% agarose gel in TAE buffer (0.04 M Tris acetate, 1 mM EDTA). Appropriate amount of agarose (Serva, Germany) was weighed and suspended in appropriate volume of TAE buffer. Agarose was dissolved by heating the flask in the microwave oven for approximately 2 minutes on medium high power (500 W). Then, agarose solution was cooled to approximately 50 ˚C by placing the flask under running tap water. Ethidium bromide (Top-Bio, Czech Republic) was then added, resulting in the final concentration of 0.5 μg/mL. Thereafter, the mixture was poured into the appropriate tray with a comb. Gel solidified in approximately 30 min. After removing the comb and placing the gel into the electrophoresis chamber filled with TAE buffer, PCR products were loaded onto the gel (20 μL per well). An appropriate DNA ladder (Promega,

Czech Republic) was also loaded on each gel for the comparative estimation of product length. The electrophoresis was run at 90 V (MP-300V, Major Science, USA) for approximately 40 min. The results were visualized by illumination of the gels with UV light (Vilber Lourmat, France). Bands of interest were cut out and extracted from the gel fragments.

2.6. PCR product purification

The desired amplicons were extracted from the gel fragments with the commercial kit *Gel/PCR DNA Fragments Extraction Kit* (Geneaid, Taiwan) according to the manufacturer's instructions. The amplicons were eluted with 50 μL of nanopure water. The extracted DNA fragments were used for sequencing and/or cloning and were stored in the freezer (-18 ˚C).

2.7. Cloning

Cloning was performed in order to obtain single species sequences in cases when mixed infection was suspected and therefore direct sequencing resulted in chromatograms with overlapping peaks.

PCR Cloning Kit (Qiagen, Germany) was used for cloning according to the following procedure. First, the ligation of the desired PCR product with the cloning vector was performed by gently mixing 0.5 μL Cloning vector, 2.0 μL PCR product and 2.5 μL Ligation Master Mix and incubating at 14 ˚C for 2 hours in *Tpersonal* thermocycler (Biometra, Germany). Afterwards, the tubes were placed on ice to finish the ligation step. Freshly thawed *Escherichia coli* DH5α competent cells (50 μL) were gently added to the ligation mixture and incubated on ice for 8 min. The transformation of vector with the desired DNA fragment into the bacterial cells was done by exposing the mixture to a heat shock (water bath, 42 ˚C, 30 s) and consequent incubation on ice for 2 min. Next, the cells were incubated at 37 ˚C for 1 hour in 200 μL of SOC medium while being shaken horizontally (210 rpm). Meanwhile, LB medium agar plates were prepared by spreading 40 μL X-Gal with ampicillin onto each plate and incubating them at 37 ˚C for 1 hour. Ampicillin addition ensured that only competent cells could grow, while X-Gal provided indication of transformation success. Cell suspension was spread on the prepared LB agar plates and those were incubated at 37 ˚C overnight (16 h). The following day, blue and white colonies could be observed on the plates. The latter contain the desired DNA fragment in their plasmids. From each plate, 5 random white colonies were picked with a plastic tip and each was placed in 30 μL of nanopure water. After shaking the

suspension for 10 minutes, it was ready to use as a template for PCR screening with universal plasmid primers M13F (5'-GTTTTCCCAGTCACGAC-3') and M13R (5'-AACAGCTATGACCATG-3'). The rest of the colonies suspensions were stored in fridge until the PCR screening showed results. The composition of the PCR mixture in presented in Table 5. Amplification with Taq purple polymerase consisted of primary denaturation at 95 ˚C for 10 min, followed by 20 cycles of 30 seconds of denaturation at 95 ˚C, 1 minute of primer annealing at 54 ˚C, and 1 minute elongation at 72 ˚C. The amplification was completed by final extension at 72 ˚C for 10 min. The PCR products were separated by gel electrophoresis as described above.

Table 5: Composition of the plasmid screening PCR mixture

Component	Stock concentration	Volume per reaction
10x buffer	-	1.30 µL
dNTP	250 µM	1.00 µL
Primer M13F	10 µM	0.50 µL
Primer M13R	10 µM	0.50 µL
Polymerase (Taq purple)	1U/µL	0.50 µL
ddH_2O	-	7.20 µL
Bacterial cell suspension	-	2.00 µL
	Final volume	13.00 µL

The colonies suspensions whose PCR products showed higher molecular weight on the gel were transferred into the glass test tubes with 3 mL of LB liquid medium and 12 µL of ampicillin. The colonies were shaken overnight at 37 ˚C. The following day, plasmid DNA extraction was performed with the *High Pure Plasmid Isolation Kit* (Roche, Switzerland) according to the manufacturer's instructions.

2.8. Sequencing

Sequencing was performed by the company SEQme s.r.o. (Czech Republic) using the Sanger sequencing method. The samples were prepared according to the company's requirements, namely, 9 µL of purified PCR product or plasmid DNA were mixed with 1 µL of one of the primers (forward or reverse) used for amplification. The concentrations of DNA in PCR products and plasmid isolates required for sequencing were: 10 ng/µL for PCR

products, 50 ng/μL for plasmids. The concentrations were measured on the *Biochrom Libra S12* spectrophotometer. Samples that exceeded the required concentration were diluted accordingly.

2.9. Phylogenetic analysis

Alignment was constructed from the obtained SSU rDNA sequences and selected sequences retrieved from GenBank covering all phylogenetic clades of the freshwater myxosporean lineage. Three representatives of the marine myxosporean clade were used as outgroups (*Auerbachia pulchra*, *Myxidium gadi* and *Schulmania aenigmatosa*). Sequences were aligned using MAFFT v7.017 (Katoh *et al*. 2005) with the E-INS-i multiple alignment method and gap opening penalty set to 1.0 and gap extension penalty to 0.0. Phylogenetic trees were calculated using maximum likelihood (ML), maximum parsimony (MP) and Bayesian inference (BI) in Geneious 8.0.5 (Biomatters Ltd., New Zealand). ML analysis was performed using RAxML v7.2.8 (Stamatakis 2006) with a GTR + Γ model in Geneious 8.0.5. MP was done in PAUP* v4.0b10 (Swofford 2001) with a heuristic search, Ts:Tv = 1:2 and random addition of taxa. Bootstrap support was calculated from 500 replicates in ML and 1000 replicates in MP analysis. BI was done in MrBayes v3.0 (Ronquist & Huelsenbeck 2003) with the GTR + Γ model of evolution. MrBayes was run to estimate posterior probabilities over 1 million generations via 2 independent runs of 4 simultaneous Markov Chain Monte Carlo (MCMC) algorithms with every 100th tree saved. Tracer v1.4.1 (Rambaut & Drummond 2007) was used to ascertain the length of burn-in period.

2.10. Spore measurements

Spore measurements were recorded from digital photographs of fresh tissue preparates using program ImageJ 1.50i (National Institutes of Health, USA). Spore length, spore width as well as polar capsule length and polar capsule width were noted. The guidelines for measurements were taken from Lom & Dyková (1992).

Statistical evaluation was performed by Student t-test function implemented in MS Excel. Principal component analysis (PCA) was performed using a web tool ClustVis (BETA) (Metsalu & Vilo 2015). Singular Value Decomposition (SVD) with imputation was used to calculate principal components.

3. RESULTS

3.1. PCR screening for *Myxidium* cf. *rhodei*

Myxidium cf. *rhodei* cysts containing spores of similar morphology were observed microscopically in fresh preparates of kidney tissues of roach (*Rutilus rutilus*), common dace (*Leuciscus leuciscus*), chub (*L. cephalus*), common bleak (*Alburnus alburnus*) and European bitterling (*Rhodeus amarus*).

At the early stage of the project, by means of PCR amplification with primers universal for all myxozoans, the partial sequence of *M.* cf. *rhodei* was obtained from roach that contained spores of *M.* cf. *rhodei* morphology. Identical sequences were obtained from dace and chub in which *M.* cf. *rhodei* spores were also microscopically observed. As these primers were specific for all myxozoans and the sequence analysis did not reveal mixed infections, the obtained sequences were assigned to be of the observed myxospores. Moreover, phylogenetically the sequence clustered with other known urinary infecting myxosporeans of freshwater fish. This supported the assumption that the analyzed sequence is indeed a desired sequence of *M.* cf. *rhodei*.

The sequence of *M.* cf. *rhodei* from roach was used as a template for designing primers that would have specifically amplified only *M.* cf. *rhodei* sequence (Mrhod511F – Mrhod953R, see Table 3). Screening through 255 kidney samples and 9 bile samples of 20 fish species (including 3 species of non-cyprinids) with the newly designed primers detected the presence of *M.* cf. *rhodei* in 43 kidney specimens of 5 cyprinid species: 33 in roach, 5 in rudd, 2 in dace and chub each, and 1 in bleak (Table 6). Interestingly, microscopically positive European bitterlings, the type host of *M. rhodei*, as well as some other specimens of bleaks and daces were PCR negative with this pair of primers.

Analyzing the sequence from the DNA sample of microscopically positive bitterling kidney after amplification with myxozoan universal primers showed that it is not similar to that of *M.* cf. *rhodei* from roach.

Table 6. PCR screening with primers specific for M. cf. rhodei from roach

Fish species	Number of screened samples	Number of positive samples
Rutilus rutilus	55	**33**
Scardinius erythrophtalamus	7	**5**
Leuciscus leuciscus	37	**2**
Leuciscus cephalus	35	**2**
Rhodeus amarus	29	**0**
Alburnus alburnus	13	**1**
Tinca tinca	8	0
Gobio gobio	19	0
Carassius carassius	20	0
Misgurnus fossilis	13	0
Ctenopharyngodon idella	2	0
Anguilla anguilla	2	0
Chondrostoma nasus	1	0
Aspius aspius	2	0
Abramis brama	12	0
Cobitis elongatoides	1	0
Carassius gibelio	1	0
Blicca bjoerkna	1	0
Gymnocephalus cernuus	5	0
Silurus glanis	1	0
total	264	43

At the next stage of the project, the new set of primers designed from the partial sequence of bitterling-borne *M.* cf. *rhodei* was used for another round of screening. From 173 kidney and 9 bile samples of 13 species (including 1 non-cyprinid species) 20 samples of 4 cyprinid species were recorded as positive: 10 bitterlings, 5 daces, 3 weather loaches and 2 bleaks (Table 7). Noteworthy is that none of roach samples were positive with this set of primers. Daces and bleaks that were positive with roach-deduced primers and those positive with bitterling-deduced were different fish individuals.

Table 7. PCR screening with primers specific for M. rhodei from bitterling

Fish species	Number of screened samples	Number of positive samples
Rhodeus amarus	31	**10**
Leuciscus leuciscus	27	**5**
Misgurnus fossilis	9	**3**
Alburnus alburnus	2	**2**
Rutilus rutilus	27	**0**
Scardinius erythrophtalamus	5	0
Leuciscus cephalus	35	0
Tinca tinca	2	0
Gobio gobio	19	0
Carassius carassius	18	0
Ctenopharyngodon idella	2	0
Gymnocephalus cernuus	3	0
Silurus glanis	1	0
Cobitis elongatoides	1	0
total	182	20

3.2. Phylogenetic analysis of *Myxidium* cf. *rhodei*

Roach-infecting *M.* cf. *rhodei* sequences clustered within the freshwater urinary clade (highlighted blue in Figure 5Figure 6Figure 7). All these sequences showed a high degree of similarity, however, some sequences had slightly longer branches ([LC P9 DB54] and [RR P16 DB48]/[RR P1 DB110] pair) that could indicate some genetic variability. Tree topology of this group was almost identical in ML and BI. In MP consensus tree, slightly long-branching pair of sequences [RR P16 DB48]/[RR P1 DB110] was allocated at the out-most position in this group with moderate bootstrap support. Within the group, moderately supported separation into pond/river clusters could be noticed in ML and MP trees (see Figures 5, 6 and 7 and Table 8); in BI tree this separation was unsupported.

Phylogenetically closest relatives to roach-infecting *M.* cf. *rhodei* were sequences of *Hoferellus cyprini* from kidney and urinary bladder of *Cyprinus carpio* and *H. carassii* from

urinary bladder of *Carassius auratus* but this node was exhibiting very low support in all three trees.

Bitterling-infecting M. rhodei sequences were identical (highlighted orange in Figure 5, 6 and 7) and clustered within the freshwater gall bladder clade. These sequences were obtained from different hosts from distant localities: *Rhodeus amarus* from Poland, *Alburnus albrnus* from the Czech Republic and *Misgurnus fossilis* from Belarus. The closest relatives to the obtained sequences were *Zschokkella* sp. from *Amerius nebulosus* and *Sphaerospora* sp. from kidney of *Oncorhynchus nerka* with moderate support in MP and very high support in ML and BI. The node to the next related group of *Zchokkella* spp. sequences did not get congruent support from three analyses: it experienced strong support in BI, moderate in ML and low support in MP. The obtained sequences together with the ones of *Zschokkella* spp. clustered as a sister group to kidney-infecting *Myxidium* parasites of turtles (*M. chelonarum* and *M. hardella*) with high nodal support in ML and BI.

Table 8. Legend to sequences coding in Figures 5, 6 and 7. First pair of letters indicating the fish host is followed by habitat code with the specimen number(s); 'DB' followed by number(s) is the unique internal code of the sequence.

Fish host		Habitat	
Code	Interpretation	Code	Interpretation
AA	*Alburnus alburnus*	G	Gomel, river
CC	*Carassius carassius*	J	Jindřiš, pond
EEL	*Anguilla anguilla*	M	Máchovo, lake
LC	*Leuciscus cephalus*	P	Plav, river
LL	*Leuciscus leuciscus*	Po	Poland, river
MF	*Misgurnus fossilis*	Ri	Římov, river
RA	*Rhodeus amarus*	Ru	Ruda, pond
RR	*Rutilus rutilus*		
SE	*Scardinius erythrophthalmus*		

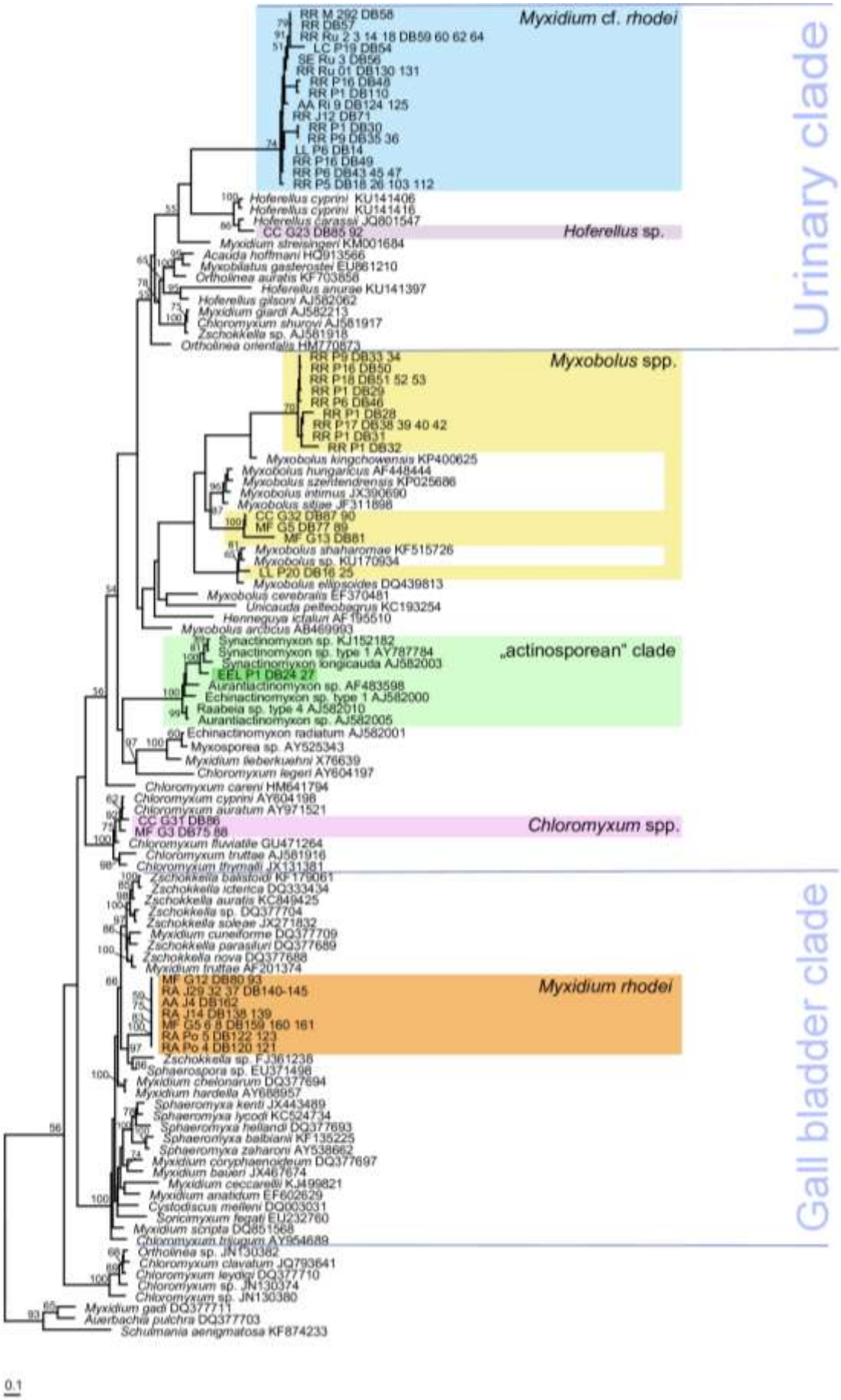

Figure 5. Maximum Likelihood phylogenetic tree based on SSU rDNA. Nodal support is indicated by bootstrap values

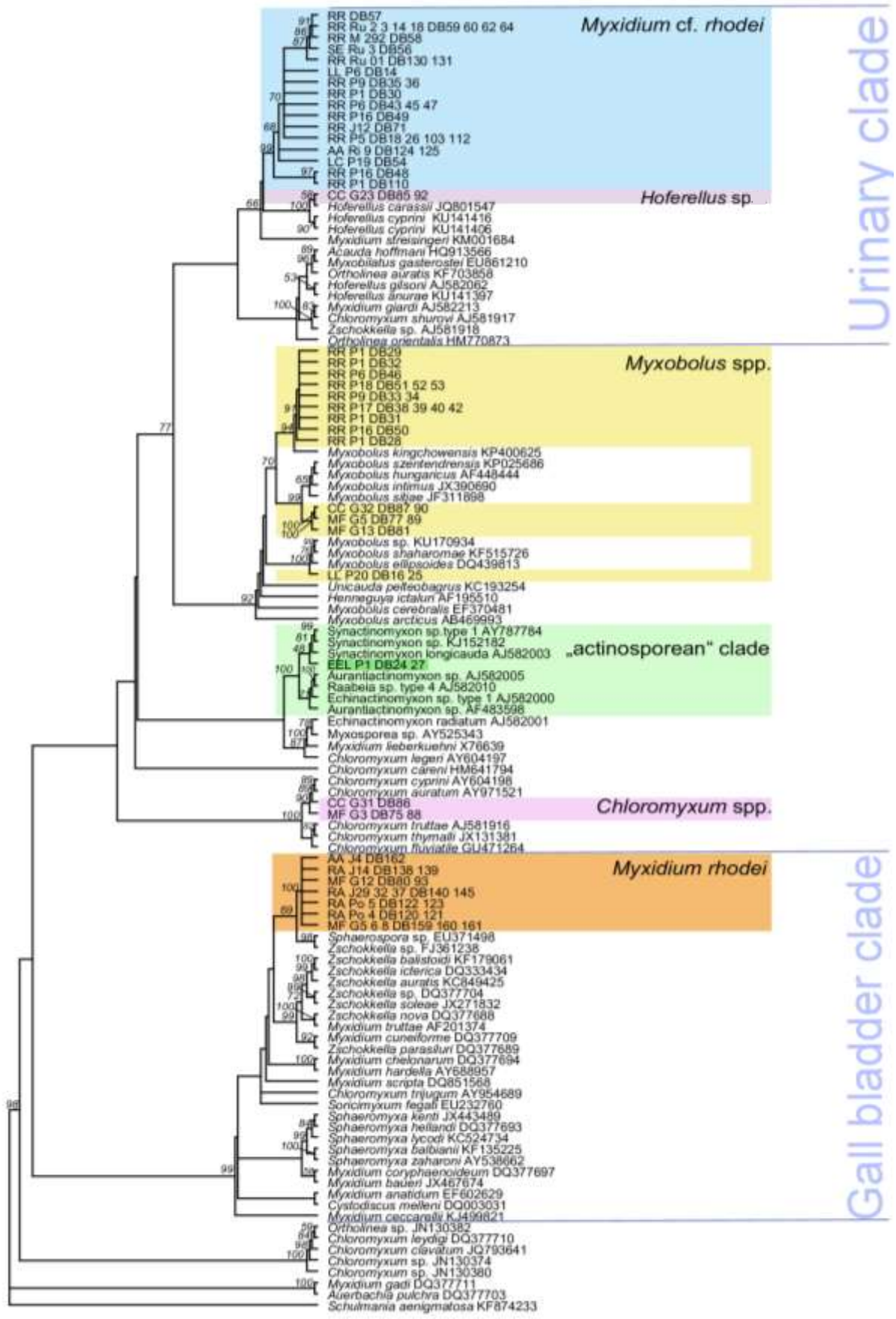

Figure 6. Maximum Parsimony consensus tree based on SSU rDNA. Nodal support is indicated by bootstrap values

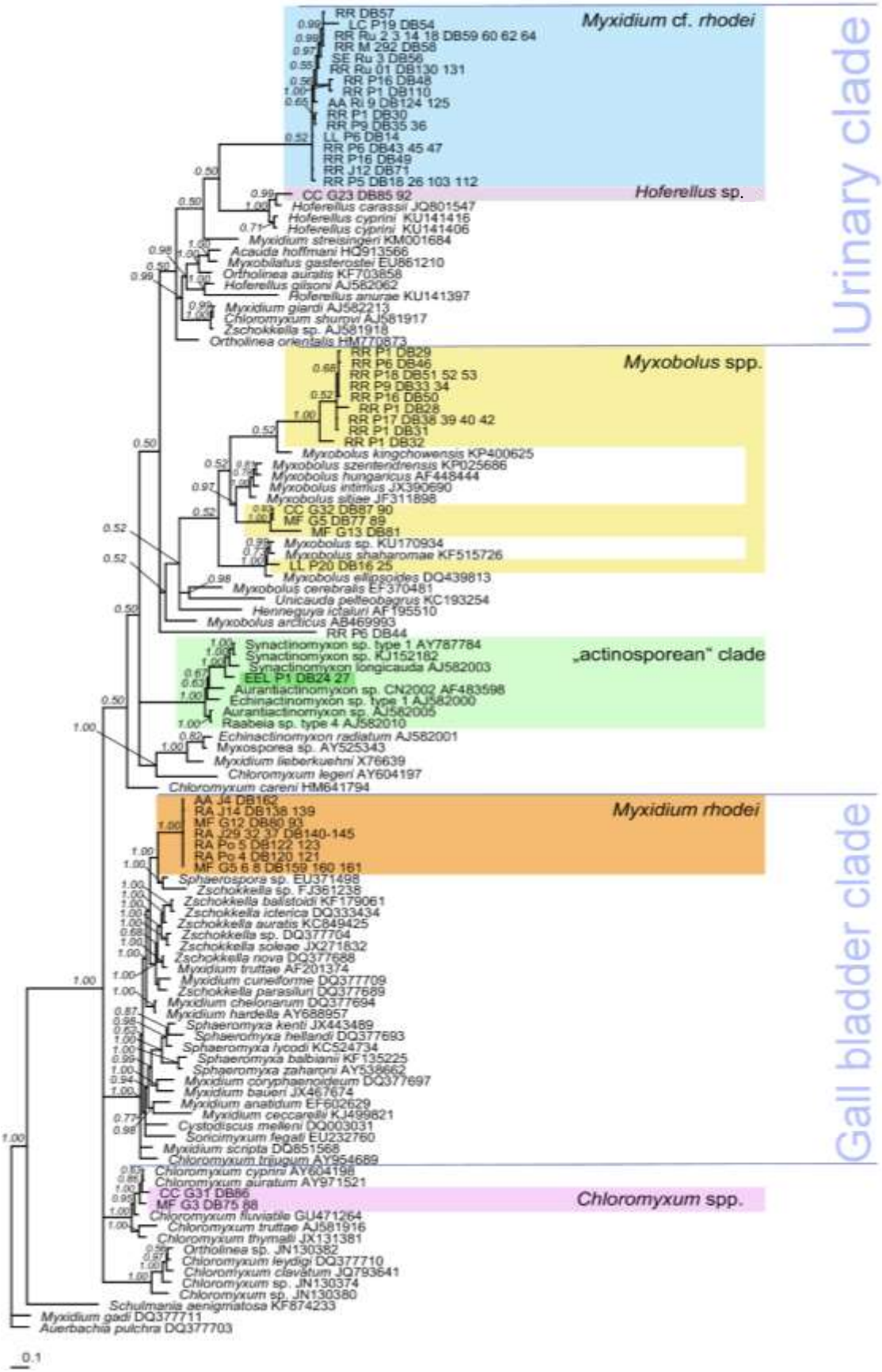

Figure 7. Bayesian Interference phylogenetic tree based on SSU rDNA. Nodal support is indicated by posterior probabilities.

3.3. Other kidney-infecting species

PCR with myxozoan universal primers with subsequent sequencing showed a variety of myxozoan infections in kidneys of studied cyprinid fish. Along with the desired sequence of *Myxidium* cf. *rhodei*, several *Myxobolus* spp. were detected as well as two *Chloromyxum* spp. and one *Hoferellus* sp. (highlighted in yellow, pink and purple respectively in Figure 5Figure 6Figure 7).

The obtained sequence [CC G23 DB85 92] from kidney of *Carassius carassius* from Belarus was closely related to *H. carassii* from urinary bladder of *C. auratus auratus* from the Czech Republic.

Two sequences [CC G31 DB86] and [MF G3 DB75 88] were clustering with *Chloromyxum* spp. The first one, from gall bladder of *Carassius carassius,* was closely related to *Chloromyxum auratum* and *Chloromyxum cyprini* from gall bladders of *Carassius auratus* and *Ctenopharyngodon idella* respectively. The other obtained sequence ([MF G3 DB75 88]) was branching as a sister sequence to the above mentioned ones with high bootstrap support in ML and MP trees and significant posterior probability in BI. Together with *Chloromyxum fluviatile* these *Chloromyxum* spp. from cyprinids formed a sister group to salmonid-infecting *C. truttae* and *C. thymalli.* The relationship within this gall bladder infecting *Chloromyxum* cluster was well supported in all three trees.

A sequence [LL P20 DB16 25] from kidney of *Leuciscus leuciscus* grouped with *Myxobolus* sp. from *Pelecus cultratus* and actinospore sequence of *Myxobolus shaharomae* as well as with *M. ellipsoides* from *Leuciscus cephalus* according to ML and BI trees with moderate and high nodal support. In MP tree, [LL P20 DB16 25] occupied the sister position to the other three sequences with maximum bootstrap support.

Three sequences ([CC G32 DB87 90], [MF G5 DB77 89], [MF G13 DB81]) from kidneys of *C. carassius* and *Misgurnus fossilis* branched as a sister cluster to gill infecting *Myxobolus* spp. with very high support in all three trees. Whereas the first two sequences were almost identical, [MF G13 DB81] separated aside with longer branching in ML and BI trees; this node was fully justified in all three trees.

A whole cluster of obtained sequences ([RR P1 DB29], [RR P6 DB46], [RR P18 DB51 52 53], [RR P9 DB33 34], [RR P16 DB50], [RR P1 DB28], [RR P17 DB38 39 40 42], [RR P1 DB31], [RR P1 DB32]) isolated from kidney samples of *R. rutilus* represent, perhaps, a novel species of genus *Myxobolus* as the cluster exhibited long branching with regard to the closest

sequence (kidney infecting *M. kingchowensis* from *Carassius auratus auratus)*. Moreover, the node was unsupported in ML and BI trees.

3.4. Screening for actinosporean clade

Universal myxozoan primers detected the presence of a myxosporean from 2 kidney samples from European eel (*Anguilla anguilla*). The partial SSU rDNA sequence (EEL P1 DB24 27) was homologous to those of the clade in which cluster actinosporean stages only (highlighted green in Figure 5, Figure 6 Figure 7). This was the first record of myxozoan sequence from the fish host that fell into this clade. As the microscopic data from these eel kidney samples was uncertain, it was impossible to connect this sequence to any known morphologically described species. Though, some *Myxidium*-like spores were observed in one of the PCR positive samples.

Specific primers were designed to attempt getting more sequences similar to those in “actinosporean” clade. However, among 104 fish samples of 8 cyprinid species screened with the newly designed primers, none showed positive results (Table 9).

Table 9. Summary of "actinosporean" clade PCR screening

Fish species	Number of screened samples	Number of positive samples
Rutilus rutilus	46	0
Scardinius erythrophthalmus	3	0
Leuciscus cephalus	9	0
Leuciscus leuciscus	8	0
Tinca tinca	5	0
Carassius carassius	19	0
Misgurnus fossilis	11	0
Anguilla anguilla	2	**2**
Chondrostoma nasus	1	0
total	104	2

3.5. Spore comparison of *Myxidium* cf. *rhodei* from roach and *M. rhodei* from bitterling

Measurements of spores from *Rhodeus amarus* (n = 20) and spores from *Rutilus rutilus* (n = 21) exhibited a noticeable difference in spore dimensions (Table 10). Two-tailed homoscedastic Student t-test indicated that there was a significant difference between the sets of spore length, spore width and polar capsule width measurements (Figure 8). Principal component analysis (PCA) supported the t-test as it indicated separation of data sets with regard to principal component 1 and principal component 2 that explained 60.3% and 22.7% of the total variance, respectively (Figure 9).

Table 10. Measurements of Myxidium cf. rhodei spores from roach and bitterling. Mean ± standard deviation (minimum–maximum). Measurements expressed in µm.

		Rhodeus amarus	*Rutilus rutilus*
Spore	Length	13.7±0.62 (12.5 – 14.8)	11.6±0.54 (10.6 – 12.6)
	Width	5.4±0.38 (4.6 – 5.9)	4.4±0.49 (3.4 – 5.2)
Polar capsule	Length	4.0±0.39 (3.2 – 4.9)	3.7±0.38 (2.9 – 4.4)
	Width	3.1±0.28 (2.6 – 3.6)	2.9±0.26 (2.3 – 3.4)

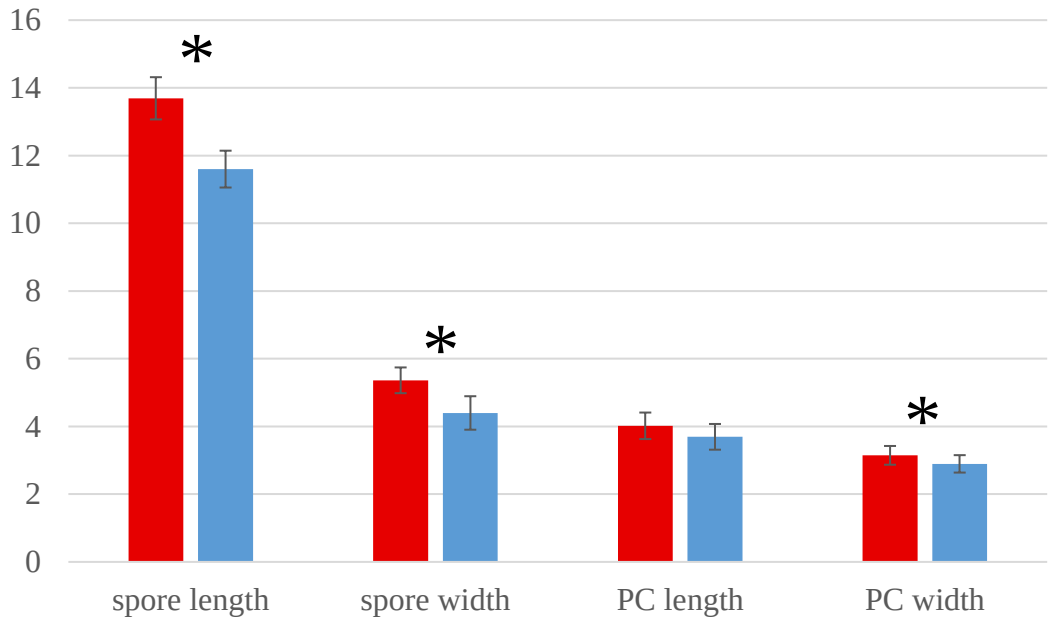

Figure 8. Bar chart of Myxidium cf. rhodei spore measurements means; red - spores from Rhodeus amarus, blue - spores from Rutilus rutilus. Vertical axis in µm. PC = polar capsules. Error bars: ±standard deviation. Asterisks indicate p<0.05.

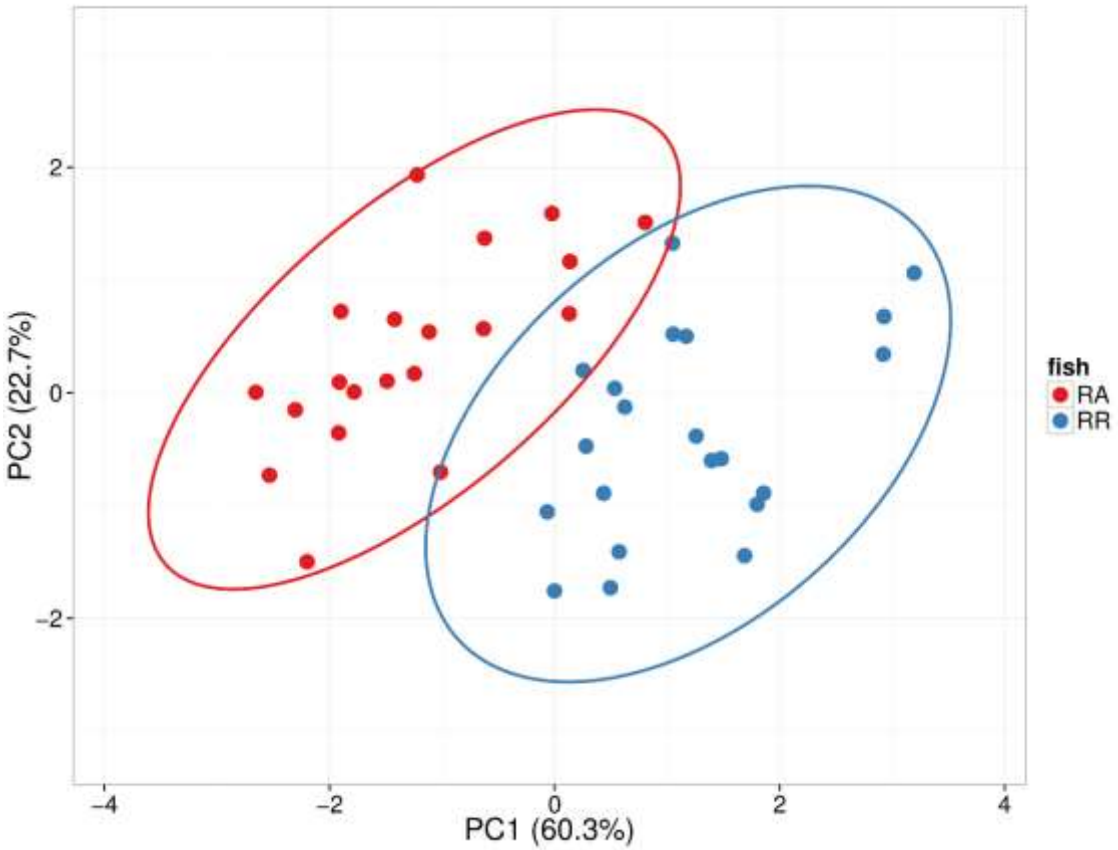

Figure 9. Principal component analysis (PCA) plot. X and Y axis show principal component 1 and principal component 2 that explain 60.3% and 22.7% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse. N = 41 data points.
RA = Rhodeus amarus, RR = Rutilus rutilus, PC = principal component.

DISCUSSION

This is the first report on molecular data assessment of *Myxidium rhodei*. Analysis of myxozoan SSU rDNA sequence from the type host European bitterling (*Rhodeus amarus*) and the typical host common roach (*Rutilus rutilus*) evidently indicated that the myxosporean from bitterling is not the same as the one from roach. Moreover, the phylogenetic relationships of these two were very distant: *M.* cf. *rhodei* from roach belongs to freshwater urinary clade, whereas *M. rhodei* from bitterling clusters within freshwater gall bladder clade. Even though the exact position of *M.* cf. *rhodei* from roach and *M. rhodei* from bitterling in the phylogenetic trees may be further cleared out, the general trend is more than apparent. Since the type host for *M. rhodei* as described by Léger (1930) is *Rhodeus amarus*, re-naming of roach-infecting *Myxidium* sp. is necessary.

With the knowledge that roach-infecting *M.* cf. *rhodei* is not the true *M. rhodei*, thorough revision of morphology of these myxosporeans should be performed in order to figure out if there are any differences that went unnoticed before. Our spore dimensions measurements indicated a significant difference between the spores of *M*. cf. *rhodei* from roach and *M. rhodei* from bitterling: the bitterling-borne spores were larger. Literature review on *M. rhodei* showed that after Léger's descriptions of *M. rhodei* from *Rhodeus amarus* in 1905 and 1930, no investigation of this myxosporean was done in its type host. Léger reported spores of 14 - 15 μm long and 3.8 - 4 μm thick — slightly longer and narrower than observed in bitterling in this study. In comparison, spore dimensions from roach kidney addressed by Dyková *et al.* (1987) were 10 - 15 μm by 4.6 – 5.4 μm and even smaller by Athanassopoulou and Sommerville (1993a) — 10.03±0.91 (9 – 12) μm by 4.00±0.83 (3 – 5) μm and 9.65±1.05 (9 - 13) μm by 3.6±0.66 (3 – 5) μm. Reports of *M*. cf. *rhodei* from *Leuciscus cephalus cabeda* and *Chondrostoma polylepis* by Alvarez (1989) and from *Chondrostoma polylepis* by Saraiva *et al.* (2000) give dimensions similar to those of Dyková *et al.* and Athanassopoulou and Sommerville. Our measurements of roach-borne *Myxidium* sp. were in range of that reported in the literature.

Roach-infecting *Myxidium* cf. *rhodei* had been also present in kidney samples of *Scardinius erythrophthalmus*, *Leuciscus leuciscus*, *L. cephalus* and *Alburnus alburnus*, whereas the true *M. rhodei* from bitterling was also detected in *L. leuciscus*, *Misgurnus fossilis* and *A. alburnus*. This suggests that none of these two *Myxidium* spp. is showing particular host specificity. Nevertheless, those fish species that were not confirmed as positive by microscopic investigation additionally to PCR positive results should not be identified as hosts

for *M.* cf. *rhodei* or *M. rhodei*. As the actinospores may penetrate any fish and only then are killed by immune system of the non-hosts, there is a chance of detection of myxozoan before it is eliminated from the fish. From the above mentioned PCR positive fish species only *M. fossilis* was not microscopically confirmed to develop myxospores.

It was proved many times that phylogeny of myxozoan species reflects their site of infection in fish host rather than their morphology (e.g. Fiala 2006). However, kidney infecting *Myxidium rhodei* from *Rhodeus amarus* clustered unexpectedly within the gall bladder clade that includes mostly species infecting the gall bladder. Nevertheless, the closest relatives of *M. rhodei* from *Rhodeus amarus* are *Sphaerospora* sp. (EU371498) and *Zschokkella* sp. (FJ361238) both from kidney of their fish host. It is an exceptional cluster of three myxosporeans infecting different tissue than the gall bladder within this clade. We can assume that the common ancestor of these three species was very likely a *Myxidium*/*Zschokkella*-type species infecting gall bladder even though *Sphaerospora* sp. with very different morphology is in that group – there is an unpublished prove that this myxozoan was incorrectly sequenced and sequence with AccNo EU371498 actually belongs to kidney infecting *Myxidium* sp. from *Oncorhynchus nerca* (Bartošová-Sojková, pers. com.).

Almost identical morphology of the spore as *Myxidium rhodei* is documented for *M. pfeifferi* a gall bladder parasite of cyprinid fish (Athanassopoulou and Sommerville 1993a). These two species were even questioned that they may represent a single species parasitizing both organs. Unfortunately, there are no molecular data that would help to resolve this issue. The position of *M. rhodei* within the gall bladder clade allow us to speculate that *M. pfeifferi* may be close relative of *M. rhodei* and represent a link between gall bladder infecting and kidney infecting species of the freshwater gall bladder clade.

Although the majority of samples were from Czech Republic, some foreign samples (Poland, Belarus) allows to get a glimpse into locality distribution of *M. rhodei*. Noteworthy is that sequences from *R. amarus*, *A. alburnus* and *M. fossilis* were almost identical even though fish was sampled in three distant localities (Poland and Czech Republic for *R. amarus*, Czech Republic for *A. alburnus* and Belarus for *M. fossilis*). Roach-infecting *M.* cf. *rhodei*, on the other hand, exhibited some minor genetic variability as well as some habitat separation. This may be questionable due to sequence quality and inconsistent length, therefore further investigation into this topic should be done.

Longer DNA sequence fragments were not possible to obtain due to some PCR inhibition as well as time limitation. Many samples showed mixed infections when amplified with

universal myxozoan primers, therefore much more cloning would have been needed to obtain clear and complete SSU rDNA sequences.

We tried to cover a wide fish species range, however, the main focus was on most commonly described *M.* cf. *rhodei* hosts. Additionally, the availability of fish tissue specimens played a large role in choosing samples for screening. Thus, many other fish species are represented by 1 or 2 specimens only.

Even though there is an immense number of available *Myxobolus* spp. sequences in GenBank and the extensive research on *Myxobolus* spp. from roach was conducted before, we obtained a novel sequence of *Myxobolus* sp. from kidney of *Rutilus rutilus*. This indicates once again that biodiversity of myxozoans is largely understudied.

CONCLUSIONS

- The phylogenetic position of *Myxidium rhodei* from its type host *Rhodeus amarus* was deduced to be within the freshwater gall bladder clade, whereas phylogenetic position of *Myxidium* cf. *rhodei* from its typical host *Rutilus rutilus* was deduced to be within the freshwater urinary clade.
- Neither *Myxidium rhodei* from its type host *Rhodeus amarus* nor *Myxidium* cf. *rhodei* from its typical host *Rutilus rutilus* exhibit a particular host specificity; *Myxidium rhodei* from its type host *Rhodeus amarus* was detected by PCR screening in 3 other cyprinid fish species: *Leuciscus leuciscus, Misgurnus fossilis* and *Alburnus alburnus; Myxidium* cf. *rhodei* from its typical host *Rutilus rutilus* was detected by PCR screening in 4 other cyprinid fish species: *Leuciscus leuciscus, Leuciscus cephalus, Scardinius erythrophthalmus* and *Alburnus alburnus.*
- *Myxidium* cf. *rhodei* from its typical host *Rutilus rutilus* exhibited some genetic variability depending on the habitat of the fish host (river/pond).
- Morphological comparison of the spores of *Myxidium rhodei* from its type host *Rhodeus amarus* and *Myxidium* cf. *rhodei* from its typical host *Rutilus rutilus* indicated a significant difference in spore dimensions.
- A novel species of genus *Myxobolus* from *Rutilus rutilus* was discovered; phylogenetically it was not closely related to any known myxozoan sequence in the GenBank.
- First myxosporean sequence that clustered within the actinosporean clade was obtained from *Anguilla anguilla.*

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