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COMPARATIVE STUDIES OF MYXOZOAN PARASITES OF WILD AND CULTURED FRESHWATER FISHES IN INDIA AND HUNGARY

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Science is the systematic classification of experience

-George Henry Lewes

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Table 2 Numbers, Locality and range of length of examined fishes in Hungary

ABBREVIATIONS

°C	Celsius
µm	micrometer
ml	milliliter
g	gram
ssrDNA	small subunit ribosomal DNA
DNA	deoxyribonucleic Acid
PCR	Polymerase chain reaction
mM	millimole
SPF	Specific parasite free
H. & E.	Hematoxylin and Eosin
ML	Maximum Likelihood

CHAPTER 1

INTRODUCTION

Diseases are a major threat to both wild and farmed fish. The pathogen-induced alterations in the viability and growth of wild fish stocks can have implications on the diversity and ecological status of aquatic ecosystems. Fish are the main component of aquatic communities, and pathogens can directly affect the health and production of wild and farmed fish (Eiras et al. 2008).

Myxozoan (Grassé, 1970) infestations are common in freshwater fishes. Most of the myxozoan cause mild infection but heavy infestations can become fatal, especially in young fish (Pote et al., 2000). Typically, myxozoan infections occur in the organ cavities and tissues of fishes, without causing any apparent pathological changes on the fish body. However, certain species (*Chloromyxum truttae, Henneguya ictaluri*) are known to cause severe tissue reactions and occasionally the mortality of the host (Bartholomew et al. 1989; Lom and Dyková 1992). Several myxozoan species had been described from different geographic areas (Landsberg and Lom 1991; Lom and Dyková 1992).

Myxozoans were classified as protozoans for a long time. After several years of debates on their taxonomic position, it has been proved that they belong to metazoans, within the phylum Cnidaria (Kent et al. 1994; Siddall et al. 1995).

Wolf and Markiw (1984) revealed that myxozoan development takes place in a two-host life cycle, where myxosporeans and actinosporeans are representing the separate life stages of the same species. Most myxozoans are transmitted from their invertebrate host to the vertebrate host in actinospore form and they are infecting the invertebrate host back from the vertebrate host as myxosporean. In the class of Myxosporea, the invertebrate hosts are annelids, in the class of Malacosporea, the invertebrate hosts are bryozoans.

Some of the most pathogenic species of myxozoans are *Myxobolus cerebralis, Myxobolus encephalicus, Sphaerospora (renicola) dykovae* causing whirling disease in salmonids, locomotory disturbance in carp fingerlings, and swim bladder inflammation in common carp (*Cyprinus carpio*), respectively (Molnar 1980). There are many other species such as

Tetracapsuloides bryosalmonae, Sphaerospora molnari, Thelohanellus nikolskii, Ceratomyxa shasta, Myxobolus pseudodispar etc., which are causing serious losses in fish stock.

The parasitosis due to the myxozoan is very significant, because effective treatment is lacking, and in consequence, very costly preventive measurements have to be done. A chemical therapeutic agent, called fumagillin was used successfully against some of the myxozoans (Molnar et al., 1987), however, its application was not affordable for fish farmers. Whereas, the plant-derived treatments are very cheap and manageable, though these are not well studied in the case of myxozoan. However, plant-based treatments can be an easy replacement for chemical drugs.

In India, the biodiversity of myxozoan has been less thoroughly described, with a lack of important characteristics and molecular data. The fish fauna is rich in myxozoan species in India. Of them, fish parasitologists, based on the morphology of spores (Kaur and Singh, 2012) described more than 300 myxozoan species mostly from the *Myxobolus, Henneguya* and *Thelohanellus* genera.

In Hungary, myxozoan biodiversity is well described but more efforts are necessary for studying the pathological and economical importance of myxozoan, including the mode of transmission and life cycle. During my master's research work, I had worked on the nematode parasites of lizards and rats (*Hemidactylus brooki* and *Rattus rattus* respectively) in India and at the same time also assisted in another project in the Indian laboratory (Molecular Taxonomy laboratory) for myxozoan parasites collection. Then, I found out that the myxozoans are not well studied in India. The lack of knowledge in identification of myxozoans and in treatment strategies against myxospores and actinospores inspired me to continue my research in Hungary.

This dissertation gives new information on the diversity of myxozoans found in Indian and Hungarian freshwater fish. The principle aspect of this dissertation emphasizes finding myxozoan infection on both wild and cultured fishes, and congregate the essential information of intrapiscine development, pathological aspects, particularly focus on their genetic analysis for identification and mitigation against actinospores by applying plant-derived products.

In the course of the morphological and genetic assessment of myxozoans, this study specifically embarks on the following objectives:

1. Identification of myxozoan species inhabiting freshwater fishes of India and Hungary.

2. Studies on the morphology and taxonomic status of the above myxozoans.

3. Determination of the pathogenicity of the myxozoans to their hosts.

4. Studies on their phylogeny by using ssrDNA genetic analysis.

5. Treatment trials against actinospore stages of the myxozoans by applying herbal drugs.

CHAPTER 2

LITERATURE REVIEW

Myxozoans have been found to infect lacustrine, riverine, marine wild and cultured fishes as well in India and abroad. They include several genera *viz., Leptotheca, Chloromyxum, Myxobolus, Henneguya, Unicauda, Thelohanellus, Myxidium, Zschokkella, Sphaerospora,* etc. Among them, *Myxobolus* is the most studied genus from this phylum. The number of species in this genus still keeps increasing all over the world (Carriero et al. 2013; Székely et al. 2015; Cech et al. 2015; Liu et al. 2016; Rosser et al. 2017; Zhang et al. 2017; Marcotegui and Martorelli 2017; Atkinson and Banner 2017).

Myxozoan can infect all organs and tissues of fish such as gallbladder, kidney, gills, intestine, muscles, skin, scales, etc. These researchers reported the myxozoans from organs and tissues in host fish from worldwide (Fujita, 1912; Ishii, 1915; Kudo, 1920; Nakai, 1926; Chakravarty, 1939, 1943; Sarkar, 1946; Tripathi, 1952; Hoshina, 1952; Schulman, 1966; Sanders et al. 1970; Hoffmam and Meyer, 1974; Markiw and Wolf, 1974; Kent and Hedrick, 1985; Maheshwari, 1987; Moser and Kent, 1994; Ram et al. 1994; Das and Das, 1995).

Recent findings on diversity, ecological and pathological aspects of the myxozoans grab the attention of significant research in this area. The present study of this dynamic group represents the importance of diversity, pathological changes in their respective host and infection control strategies against them.

2.1 Myxozoan classification

Myxozoans are microscopic metazoan parasites characterized by a two-host life cycle, which typically involves invertebrates and vertebrates as definitive and intermediate hosts, respectively and evolved as endoparasitic radiation of cnidarians exploiting invertebrate and vertebrate hosts in freshwater and marine environments.

Since the recognition of Myxosporea as a separate class by Jurine (1825) and proper observation by Müller (1841), myxozoan classification under the phylum Cnidaria had gone through many debates. Kudo (1933) and Tripathi (1948) proposed the first classification of

myxozoan and, after that, Shulman (1959, 1966) followed the same and made some changes. Previously, Myxozoan classification was based on the morphology of myxospores, which consist of valvogenic, capsulogenic and sporoplasmogenic cells. After the discovery, that a myxozoan life cycle contains both actinospore and a myxospore phase has changed the entire classification (Wolf and Markiw 1984). At that time, the phylum Myxozoa Grassé, 1970 was classified into the classes Myxosporea Bütschli 1881 (fish parasites, myxospores) and Actinosporea Noble, 1984 (worm parasites, actinospores), but getting the information that these classes actually represent morphologically distinct phases of single species, the Actinosporea was suppressed by Kent et al. (1994). Cavier-Smith et al., (1996) and Siddall et al., (1995) suggested that myxozoans showed the affinity with the phylum Cnidaria due to the presence of nematocysts like polar capsules.

There are two clades presently recognized: the Malacosporea (Canning, Curry, Feist, Longshaw, and Okamura, 2000) and the Myxosporea Bütschli, 1881. According to Canning and Okamura (2004), fundamental findings of origin and evolutionary aspects remained uncertain of the phylogenetic position of the myxozoans. Later on, Lom and Dyková (2006) constructed the classification scheme of the myxosporeans, based on spore morphology and fragmentary data resulting from SSU rDNA sequence analyses and assigned the 4 malacosporean and 2,180 myxosporean species to 62 myxozoan genera.

The shared characters of myxospores morphology have occurred in each genus of myxosporean (Fiala and Bartosova 2010). Fiala et al. (2015) presented a revised myxozoan classification, where myxozoa is considered as unranked subphylum under phylum Cnidaria. They suggested a combination of morphological, biological and molecular data for characterizing lineages at various levels (e.g. species, genus) and to evolve phylogenetically informative taxonomies.

The class Myxosporea consists of two orders: The Bivalvulida and Mutivalvulida. This classification is also considered based on the number and configuration of shell valves and the number and position of polar capsules to the suture plane. The Bivalvulida composes three suborders and twelve families while the Multivalvulida consists of two families.

Class Myxosporea Bütschli, 1881

Order Bivalvulida Schulman, 1959

Suborder Platysporina Kudo, 1919

Family Myxobolidae Thélohan, 1892, with thirteen genera.

Suborder Sphaeromyxina Lom and Noble, 1984

Family Sphaeromyxiidae Lom and Noble, 1984 with one genus

Suborder Variisporina Lom and Noble, 1984

Family Alatosporidae Schulman et al., 1979, with three genera

Family Auerbachiidae Evdokimova, 1973, with two genera

Family Ceratomyxidae Doflein, 1899, with four genera

Family Chloromyxidae Thélohan, 1892, with three genera

Family Fabesporidae Naidenova and Zaika, 1969, with one genus

Family Myxidiidae Thélohan, 1892, with four genera.

Family Ortholineidae Lom and Noble, 1984, with six genera

Family Parvicapsulidae Shulman 1953, with two genera

Family Sinuolineidae Shulman 1959, with eight genera

Family Sphaerosporidae Davis, 1917, with six genera

Order Multivalvulida Schulman, 1959

Family Kudoidae Meglitsch, 1947, with two genera

Family Trilosporidae Shulman, 1959, with two genera

Class Malacosporea Canning, Curry, Feist, Longshaw and Okamura, 2000

Order Malacovalvulida Canning et al., 2000

Family Saccosporidae Canning, Okamura and Curry, 1996, with two genera

2.2 Morphology and Life cycles of Myxozoan

Myxozoans are either histozoic (within tissues) or coelozoic (inhabiting cavities) and most organs in the fish hosts can be infected. The life cycle of myxozoans typically involves invertebrates and vertebrates as definitive and intermediate hosts, respectively (Fig.1).

Grassé (1960) initiated the ultrastructural studies of myxozoan. Multicellular origin and ultrastructural findings of myxozoa helped Wolf and Markiw (1984) discover the two-host life cycle of the myxozoans, which was considered a taxonomic milestone, and identified the Actinosporea as the part of the life cycle of Myxosporea. Therefore, Kent et al. (1994) suggested that new species should not be described solely based on actinosporean and proposed only a single class Myxosporea in the phylum Myxozoa.

For Myxosporea, annelids act as definitive hosts. Besides fish, amphibians and homeotherms can also act as intermediate hosts for myxozoans (Lom and Dykova 2006). Myxospore stages of myxozoan produced in fish hosts possess thickened walls and can remain infectious to annelids for months to years meanwhile actinospores produced in annelida hosts are relatively short-lived and morphologically complexed with expanded caudal appendices for buoyancy (Lom et al. 1989).

Ultrastructural evidence helps to understand the interaction of myxozoans with host cells (Lom et al. 1989) or tissues (Current and Janovy, 1976, 1977, 1978) and hence the myxozoan pathogenicity. The myxozoans morphology has high variations. The number of polar capsules varies from one to four and exceptionally unto 13 including one or more sporoplasm, the actual infectious germs. The sporoplasm and polar capsules are surrounded by a hard shell, which consists of two to seven valves and they adhere together by one or more suture line(s) (Fig. 2).

The formation of the spores discovered at the target site as sporogonic stages and the sporogenesis proceed by several cycles of presporogonic proliferation. Moreover, the division of pre-and extrasporogonic stages takes place by endogenous division into inner secondary cells, which may produce tertiary and quaternary cells. These purely proliferative cycles may take place in tissues and organs other than the target sites and their cells may be intercellular in tissues (or bloodstream) and or intracellular, irrespective of whether the sporogonic stage in the target site is histozoic or coelozoic. Moreover, the light microscopy studies made by Moser and Kent (1994) confirmed that trophozoites are truly multicellular. In developing trophozoites, early differentiation could be observed into somatic and generative elements. The generative elements are visible as discrete cells in the multinucleate somatic plasmodium.

The establishment of myxozoan life cycles under experimental conditions and understanding the fundamental transmission requirements of myxozoans will facilitate the development of

methods for avoidance of the infective stage of the parasite and of potential therapeutics or vaccines.

Lom and Dykova (1997) suggested that the life cycle of myxozoan is complicated. The actinosporean stage formation takes place in the oligochaete host and they eventually release actinospores into the water column, which are exposed to the fish to adhere chemotactically to it (Yokoyama et al. 1995a). For the attachment to the host tissue, sticky polar filaments act as attachment organs, and after the opening of the spore shell multinucleate sporoplasm (numerous small uninucleate) oozes out, which penetrate the host tissue and start the development (Daniels et al. 1976; Markiw, 1989) (Fig.1). Inside the oligochaetes, development occurs through the presporogonic stage, followed as primary, secondary, and tertiary cell, and continue as cell growth and proliferation (El-Matbouli et al. 1995). In addition to oligochaetes, some polychaetes have shown to act as the alternate host for *Ceratomyxa shasta* (Bartholomew et al., 1997). For Malacosporea bryozoans are definitive hosts, e.g. for *Tetracapsuloides bryosalmonae,* the causative agent for Proliferative Kidney Disease (PKD) in salmonid hosts (Longshaw et al., 1999, 2002; Feist et al., 2001). However, not all myxozoan life cycles have elucidated, though there is a consensus that most will undergo a two-host life cycle. It is, however, recognized that at some fish species sporogonic developmental stages can be transmitted directly (Diamant, 1997; Redondo *et al.,* 2002; Yasuda *et al.,* 2002) from one fish to other, although the possibility of additional invertebrate hosts has not been excluded.

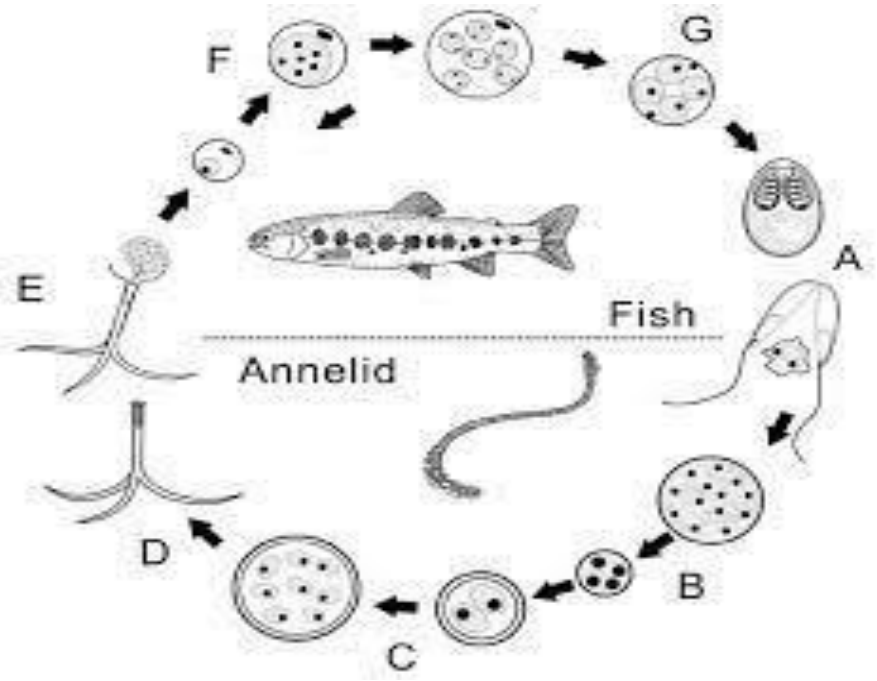

Fig.1 Generic two-host myxozoan life cycle showing alternate vertebrate and invertebrate hosts **A:** The polar filaments are extruded to anchor the spore to the gut epithelium, followed by opening of shell valves of myxospore. **B:** Gametogony. **C:** Sporogony of actinosporean phase. **D**: Mature actinospore stages develop in a pansporocyst, and actinospores are released into the water. **E:** Upon contact of actinospores with the skin or gills of the fish host, polar filaments extrude to anchor the spore to the skin or gills, facilitating invasion of the sporoplasms into the fish. **F:** Presporogonic multiplication in a cell-in-cell state. **G:** Sporogony of myxozoan phase (Adopted from Yokoyama et al. 2012)

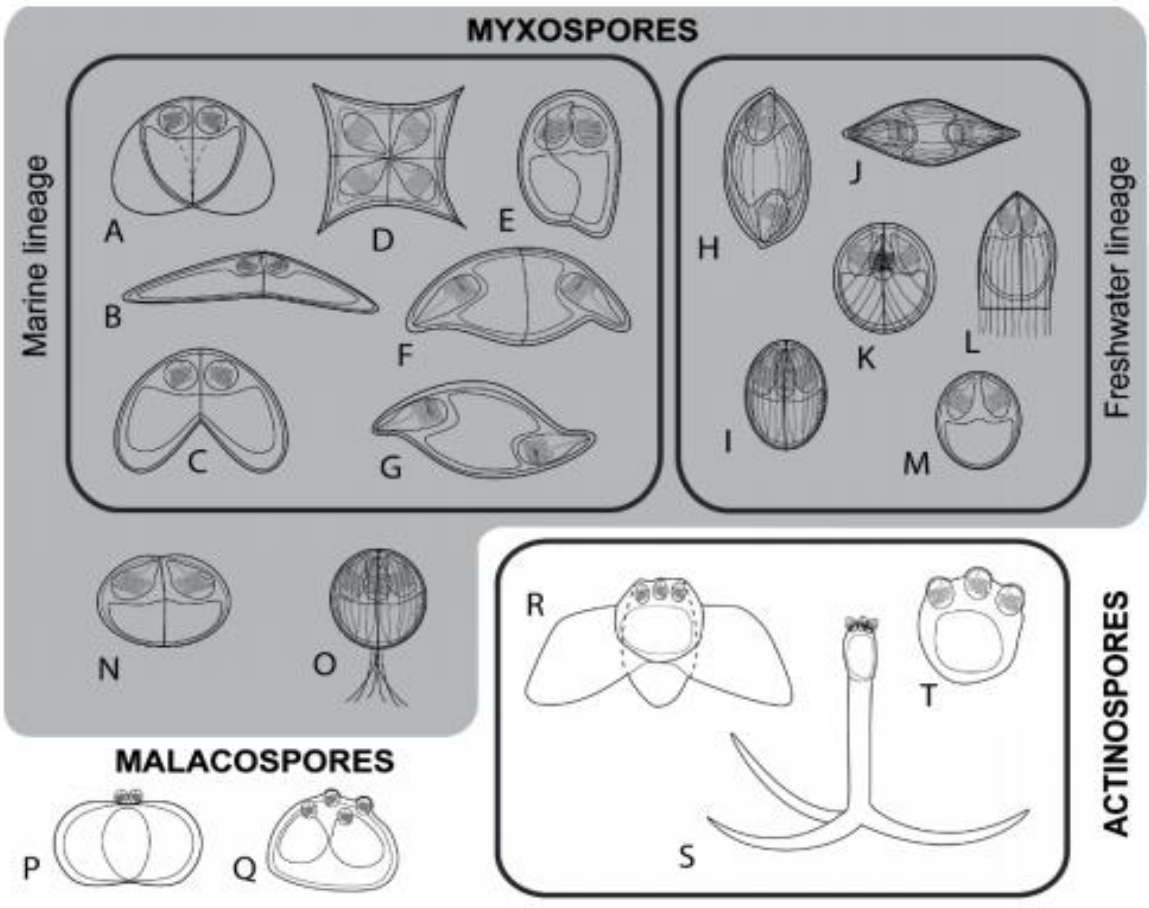

Fig.2 Myxozoan morphotypes. (A-O) Myxospore morphotypes with one selected morphotype per each phylogenetic clade. (A) Bipteria, (B) *Ceratomyxa*, (C) *Ceratonova*, (D) *Kudoa*, (E) *Parvicapsula*, (F) *Enteromyxum*, (G) marine *Myxidium*, (H) freshwater *Myxidium*, (I) freshwater *Chloromyxum*, (J) *Myxidium lieberkuehni*, (K) *Chloromyxum careni*, (L) *Hoferellus*, (M) *Myxobolus*, (N) *Sphaerospora*, (O) marine *Chloromyxum*. (P-Q) Malacosporean morphotypes, (P) fish malacospore of *Tetracapsuloides*, (Q) malacospore of *Tetracapsuloides* from bryozoan host (R-T) Common actinospore morphotypes, (R) aurantiactinomyxon, (S) triactinomyxon, (T) tetractinomyxon (Kodadkova, 2014)

2.3 Effects of host and ecological importance

Myxozoan parasites are normally present both in wild and captive fish. They do not cause any problem to their hosts when there is equilibrium between fish and the environment. When any kind of stress to the host occurs, such as handling, poor water quality, or overpopulation, the parasites come to power and several kinds of diseases arise (Lom and Noble, 1984).

The ecological role of myxozoan lies in their effect on fish populations. Most species are found in a balanced state with their hosts; striking epizootics have been rather exceptional. In fish, the pathogenic potential of some species can be fully unrevealed in captivity. Depending on the parasite species and body organs, the pathogenic action may be extremely varied; for example pressure atrophy in tissues exerted by parasite masses (plasmodia of *Myxobolus* species), irritation of epithelia of organ cavities (*Sphaerospora, Ceratomyxa*), enzymatic lysis of muscle tissue (species of the order Multivalvulida, like the genera *Hexacapsula*, *Kudoa* or *Unicapsula*), destruction of tissues (*Ceratomyxa shasta* or *Enteromyxum*) or *anaemia* (*Tetracapsuloides*). A special case of effect caused by parasites when in extensive marine fisheries, heavy infections with muscle-invading multivalvulid species can make a large part of the catch unmarketable because the flesh is unsightly and degraded. Lom and Dykova (1995) have reviewed the pathogenicity. The best-known example for the pathogenesis of a fish myxozoan is the development of the myxospore stage of *M. cerebralis* in the salmonid host, which gives rise to whirling disease. The disease was first observed among introduced, farmed-raised rainbow trout, *Oncorhynchus mykiss* by Bruno Hofer of the University of Munich in 1898 (Hofer, 1903). The most obvious clinical sign is tail-chasing behaviour. Other characteristic signs of the disease include cranial deformities due to interference with osteogenesis (Hoffman et al., 1972; and Hoffman, 1970), deformities of the jaws and opercula (Havelka and Volf, 1970), the disintegration of the fins (Havelka and Volf, 1970) and opercular cysts (Taylor and Haber, 1974).

Among the recently emerged myxozoan pathogens in maricultures are the species, *Kudoa thyrsites*, a cosmopolitan parasite infecting muscle tissue of many species of fish. It is the cause of 'soft flesh', muscle tissue degradation and produces unmarketable flesh quality in pen-reared *Salmo salar*.

Species of the genus *Enteromyxum* develop as small sporogonic pseudo-plasmodia in the intestinal tissue causing severe enteritis with high rate of mortalities. *Enteromyxum scophthalmi* is the cause of acute enteritis, starvation and death in cultured *Scophthalmus maximus* (Redondo

et al., 2002). Cultured marine fishes also suffer from infections with *Ceratomyxa sparusaurati*, a myxozoan parasite that lives in the gallbladder and causes its swelling, sloughing of the epithelial cells and trickling mortalities (Palenzuela et al. 1997). Among cyprinid fishes, swim bladder-inflammation caused by *Sphaerospora dykovae* is the most deleterious disease in common carp fry populations (Csaba et al. 1984).

The challenge for future research of myxozoans are to assess the pathogenicity of new intruders as parasites into the aquacultures and especially to discover that what allows myxosporea to follow the pattern of alternation in the life cycle between fish and invertebrate host.

2.4 Phylogenetic markers for myxozoan phylogeny

Previously, the identification of myxozoans was exclusively based on morphological characters of myxospores and plasmodia. Phylogeny of myxozoans with the classical taxonomy is not significantly approved, due to their rapid evolutionary rates consequences to high spore plasticity and several examples of convergent evolution. Similar spore morphological characters evolved several times in the myxozoans history and thus species with similar morphology classified into the same genus are in fact distantly related (Fiala 2006; Fiala and 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) (Fig. 3).

To reconstruct the phylogeny of myxozoan diversity, conservative phylogenetic markers were required. The small subunit ribosomal RNA gene (SSU) is a universal gene found all over the earth and has been used successfully in higher-level phylogenetic analyses of many eukaryotic taxa (Avise 2004). The first myxosporean SSU sequences were used to ascertain the position of myxozoans in the eukaryotic tree of life (Smothers et al. 1994; Sidall et al. 1995; Schlegel et al. 1996). Since then myxozoan SSU sequences have been used regularly to clarify the relationships among myxozoan species (Andree et al. 1999b; Kent et al. 2001; Holzer et al. 2004, Hartikainen et al. 2014; Fiala 2006, Burger and Adlard 2011; Bartošová et al. 2013; Bartošová-Sojková et al. 2014; Hervio et al. 1997). However, with limitations, this marker has repeatedly proved to be sufficiently informative to estimate the phylogenetic relationships among myxozoan species (Kent et al. 2001; Holzer et al. 2004; Fiala 2006; Burger and Allard

2011; Bartošová et al.2013; Hartikainen et al. 2014; Bartosova -Sojkova et al. 2014). Moreover, the universal genetic marker, the SSU is useful for myxozoan phylogenetic because its heterogeneity (conserved and variables regions) facilitates discrimination at different taxonomic levels.

The conserved region allows the development of general primers and alignment of DNA sequences, while the variable region informs about the diversification. The LSU is another genetic marker that is used for myxozoan phylogenetic, because of its conserved and variable regions. It is first used to assess the multivalvulidan relationship as a supporting marker with SSU-based phylogenies (Whipps and Kent 2006; Whipps et al. 2004a). Although, SSU is usually insufficient to distinguish differences at the intraspecies level. To overcome this restriction, the internal transcribed spacer region 1 (ITS 1) has been successfully used. This is also a relevant marker for large-scale phylogeographical studies of various myxozoans species eg: *Kudoa thyrsites* (Whipps and Kent 2006). Due to intra-genomic variation, it may cause a problem for characterizing strain variation. In addition, ITS1 and ITS2 were used to expose the cryptic species of amphibian myxospores (Hartigan et al. 2011) However, the high number of myxozoan SSU records in NCBI database still makes the first choice of the marker when analysing the myxozoan phylogeny.

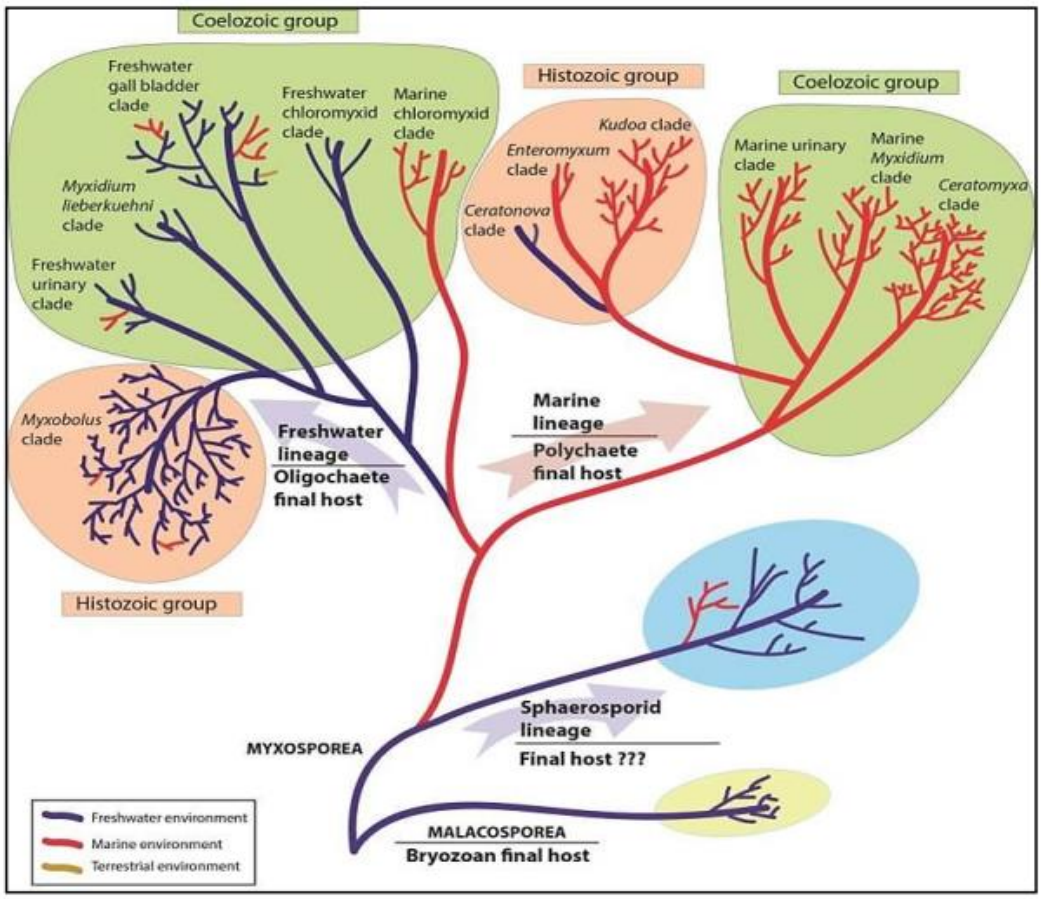

Fig. 3 Schematic evolution of the Myxozoa based on ssrDNA data. (Fiala et al. 2015b)

2.5 Treatment against myxozoan by using Herbal drugs

For ages, fish farmers have been using conventional treatments for parasites such as anti-parasitic and chemical treatments to control the diseases in fish. However, chemical treatments often have a negative impact on the fish as chemical residues accumulate in the fish tissues and have negative consequences on the environment and other water bodies (Bulfon et al. 2015; Caipang et al. 2015; Saika 1999). Therefore, plant-derived compounds such as plant extracts (e.g. *Mentha spp*. and *Allium sativum*), aqueous solution of plant stems and leaves (eg. *Azadirachta spp*.) and essential oils (eg. *Origanum sp*., *Eucalyptus sp.* and *Lippia spp*.) have been utilized as efficient mitigation against parasites in freshwater, brackishwater and marine aquaculture systems (Karagouni et al. 2005; Soares et al. 2016).

In the recent past, few studies have been published to control myxosporean species such as *Myxobolus spp*. and *Enteromyxum spp.* (Athanassopoulou et al. 2004; Karagouni et al. 2005).

Origanum essential oil has revealed different degrees of protection against myxozoan infection in the sharp snout sea bream (*Abramis brama*) and gilthead (*Sparus aurata*). Using this oil have shown the reduction in the prevalence of *Enteromyxum leei* in *Sparus aurata* **(**Karagouni et al. 2005**)**

Cojocaru et al. (2007) revealed the 40 to 20% reduction in the prevalence of the infestation of the *Enteromyxum leei* in *S. aurata* after a month of oral and bath treatments using several essential oils.

Some other medicinal plant extracts have also shown the potential as anti-myxozoan agents. One such medicinal plant, called neem (*Azadirachta indica* A. Juss 1830) is also used as a biological control against several parasites. Neem cake has also been used in the livestock industry in animal feed (Bawa et al. 2006; Uko and Kamalu 2007). *Azadirachta indica* (neem) has been reported to exhibit immunomodulatory, anti-inflammatory, antihyperglycemic, anticarcinogenic, nematicidal, antiparasitic, antiviral, insecticidal and antioxidant properties (Khan et al. 2001; Salehzadeh et al. 2003; Wandscheera et al. 2004; Anthony et al. 2005). However, there is no knowledge about their biological control against myxozoans.

Some studies also suggested that using non-toxic chemicals and crude extracts of medicinal plants are manageable and cheap options (Wunderlich et al. 2017). Applying them in aquaculture, the conventional parasiticides could be replaced.

CHAPTER 3

MATERIAL AND METHODS

3.1 Myxozoan collection in India

3.1.1 Fish sampling

Samples were collected in freshwater from River Ganges tributaries and fish farms in Uttar Pradesh (27.7996° N, 78.2932° E) and West Bengal (22.9868° N, 87.8550° E), India. In Uttar Pradesh, different locations such as Meerut (28.9845° N, 77.7064° E), Parikshitgarh (28.9793° N, 77.9310° E), Hastinapur (29.1569° N, 77.9938° E), Bijnor (Bairaj) (29.3732° N, 78.1351° E) and also from the nearby market were chosen (purportedly, they were caught from River Ganga) (Fig. 4). In West Bengal, fishes were obtained from Diara Fish pond (Hooghly District) and hatchery (22°28′31″N 88°09′54″E), East Kolkata Wetlands 22.5263°N 88.4716°E (District South 24 parganas, Baranagar Block). Furthermore, two markets near Kalyani like Naihati, Battala area (22.89°N 88.42°E), Kakinara, Bhatpara area (22.51°N 88.23°E) (Table.1). Fish were collected with the help of local anglers. The exact locality and the number of examined fish species are presented in Table 1.

Table 1. Numbers, Locality and range of length of examined fishes in India

Fish species	**Number of fishes**	**Fish size (Length)**	**Locality**
Notopterus notopterus	20	20–25 cm	River Ganga, Hastinapur, Uttar Pradesh
Channa gachua	56	14-15 cm	Meerut Uttar Pradesh
Mystus vittatus	48	8–10 cm	Meerut Uttar Pradesh
Ompok pabda	27	10–16-cm	Sotiganj, Meerut, U.P.
Labeo rohita	25		Diara Fish Farm, Fish market Naihati, Battala market, West Bengal
Labeo rohita	13	26-33 cm	Naihati, Battala market, W.B.
Gibelion catla	9	38-46 cm	Kakinara, Bhatpara market, W.B.
Labeo rohita	12	26-33 cm	Naihati, Battala market, W.B.
Cirrhinus mrigala	10	32-46 cm	East Kolkata Wetlands, W.B.

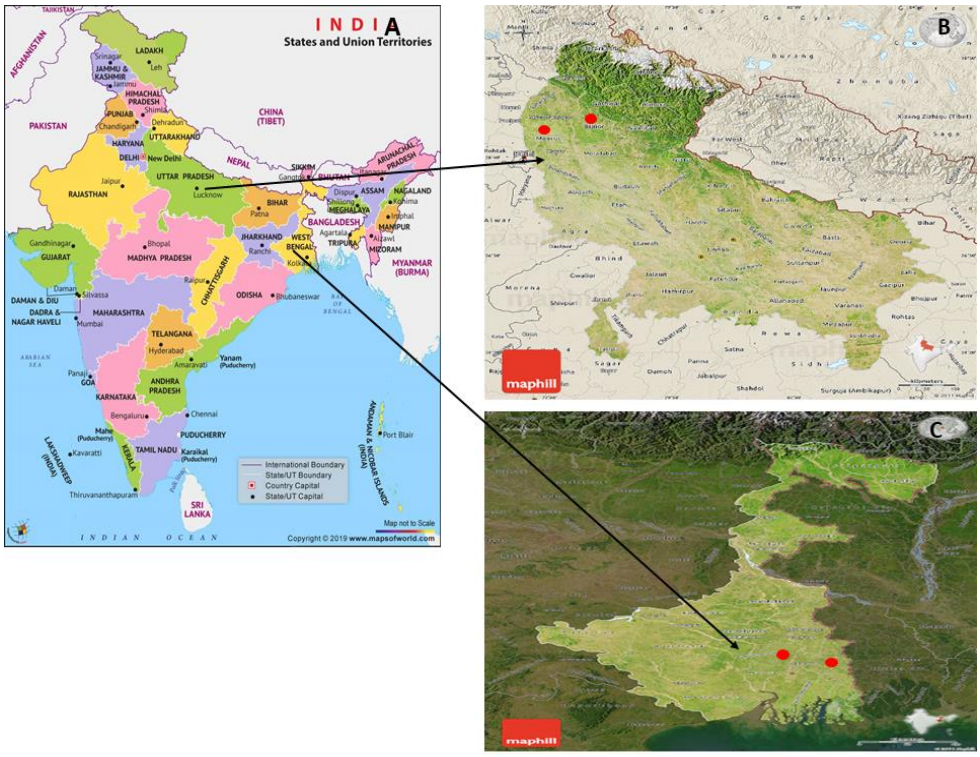

Fig. 4 Locations for myxozoan sampling in India, B: in Uttar Pradesh, C: in West Bengal

The River Ganges is the largest river in India and flows through five states. The River is the lifeline of over 500 million people from 11 basin states and provides habitat for numerous flora and fauna. The Ganga River harbours a unique assemblage of biodiversity due to its passage through three distinct biogeographic zones, namely Himalaya (West Himalaya - 2B), Gangetic Plain (Upper Gangetic Plain – 7A and Lower Gangetic Plain – 7B) and Coasts (East Coast-8B). A smaller stretch of the River Ganges, in Jharkhand state, also borders the Deccan Peninsula (Chhota Nagpur – 6B) (Rodgers and Panwar, 1988). The habitat mosaic of the River nurtures more than 140 non-piscine aquatic species including a wide variety of primitive phytoplankton to highly developed vertebrates like the Gangetic River dolphin (*Platanista gangetica*), Smooth-coated otter (*Lutrogale perspicillata*), Gharial (*Gravialis gangeticus*), Gangetic softshell turtle (*Nilssonia gangetica*) and several species of endemic freshwater crabs (Bashir et al. 2010). The River Ganges is also known to support more than 143 species of fish

that belong to 11 orders, 72 genera and 32 families (Sarkar et al. 2012), which accounts for 20% of the total freshwater fishes reported in India. In Uttar Pradesh, collected fishes were brought to the Molecular Taxonomy laboratory of the Department of Zoology, Chaudhary Charan Singh University and maintained in an aerated aquarium.

Whereas in West Bengal, fishes were directly purchased from the fish markets and the fish farmers of selected areas and brought them in live condition to the laboratory, Department of Zoology, University of Kalyani for further investigation. The fishes were sedated with clove oil and were killed with a cervical cut. After the fish sampling, procedures were followed for myxospores collections for thorough checking of the infected fish. The infected tissue and myxospores from the host fish were collected and preserved separately for morphology, histology and molecular characterization.

3.1.2 Myxozoan collection

Fresh fishes were dissected and examined within one or two days of capturing. The presence of myxozoans was detected by using the dissecting microscope at different magnifications, appropriate for the analysis of parasites. The whole body organs like gills, fins, intestine, kidney, gall bladder as well as muscles were examined thoroughly. Plasmodia from the scales were removed by a needle. Fins were cut from the fish body and checked for the presence of plasmodium. A portion of plasmodia was preserved for morphology, others were opened and spores obtained. Infected organ/s with myxozoan plasmodia were observed under the microscope. After examinations of hosts, plasmodia were carefully removed from tissues with a fine needle and dissected on a slide. Fresh preparations of ruptured plasmodia containing the spores were further studied by using a light microscope. Plasmodia in filaments, lamellae or in the gill arches were differentiated according to Molnár (2002) into epithelial, vascular, muscular and chondroid locations. Spores were measured according to the guidelines of Lom and Arthur (1989). For the suitable photo of the spores, were preserved in 4% formalin and for histological preparations, tissues were preserved in 10% formalin. Spores were preserved in 70% ethanol for molecular biology analysis. All the examinations were carried out in Veterinary Medical Research Institutes (VMRI) CAS, Budapest Hungary. Voucher samples of myxozoan spores were deposited in the collection of the Zoological Department of the Hungarian Natural History Museum of Budapest, Hungary. Measurements and drawings of

spores were compared to available literature reported from the same hosts as well as to species that were congeners to on the phylogenetic tree.

3.2 Myxozoans collection in Hungary

3.2.1 Fish sampling

In Hungary, samples were collected from Lake Balaton (46.8303° N, 17.7340° E), and River Danube (45°13′3″N, 29°45′41″E) and their tributaries. The sampling point comprises the following province; Keszthely (46.7655° N, 17.2480° E), Tihany (46.9129° N, 17.8880° E), Siófok (46.9091° N, 18.0746° E), Zala channel (46.7059°N 17.2646°E), Egerviz Creek (46°48'24.08"N 17°27'31.70"E) and Kis Balaton Reservoir (46.6393° N, 17.1412° E) (Fig. 5). The information of collected fish species is in Table. 2.

Table 2. Numbers, Locality and range of length of examined fishes in Hungary

Fish species	**Number of fishes**	**Fish size(Length)**	**Locality**
Lepomis gibbosus	21	0.8-15 cm	Sió channel, and Egerviz Creek, Hungary
Tinca tinca	7	29cm	Lake Balaton, Hungary
Chondrostoma nasus	6	20-40 cm	River Danube, City Nagymaros, Hungary
Rutilus rutilus	55	4-13 cm	Lake Balaton, Hungary

With a surface area of 594 sq. km, Lake Balaton is the largest freshwater body in Central Europe. It is one of the most significant natural treasures of Hungary and a unique ecological asset of the Central European region. For its large surface area, Lake Balaton is an extremely shallow lake - its average depth is a mere 3.2 m. Its main tributary, Zala River drains the western half of the watershed of 5,770 sq. km and discharges into the lake from the south-west. The excess water is discharged through the sluice in Siófok in a controlled manner to the Sió River, a canalized waterway connecting the Lake with River Danube. The lake and its environment

accommodate a rich and diverse flora and fauna. Consequences of continued efforts in environmental protection and nature conservation have preserved a rich diversity of species. There are 41 indigenous species of fish living in Lake Balaton and its tributaries.

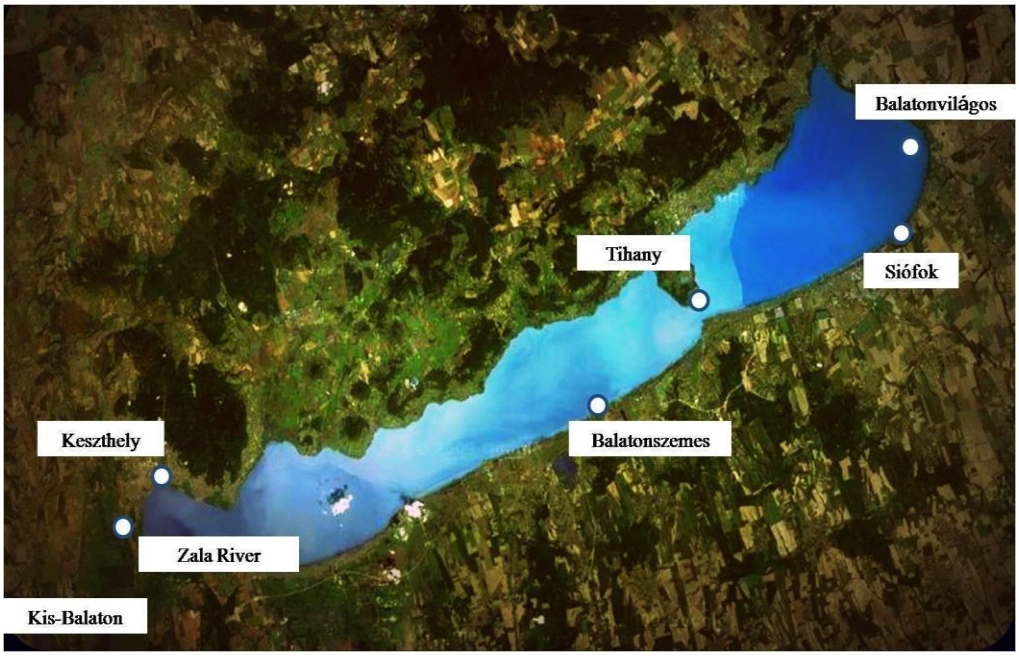

Fig.5 Locations for myxozoan and actinospores sampling in Hungary

3.2.2 Myxozoans collection

Plasmodia collected from fishes followed the same procedure as described in section 3.1.2. After the thorough examination of fish organs and tissues under the dissecting microscope, myxozoan samples were collected separately for histology and molecular processing in 10% formalin and 70% alcohol respectively. The myxozoan samples were collected from the muscles of roaches (*Rutilus rutilus*) in order to infect the oligochaetes (*Tubifex tubifex)* for actinospores production for plant-derived drug treatment experiments.

3.2.3 Inducing and maintaining myxozoan infection in oligochaetes

Specific Pathogen Free (SPF) oligochaetes (*Tubifex tubifex*) were purchased from Veresi Díszállat Üzlet (Ökomester Bt.) Veresegyház. Oligochaetes were harvested in two aquaria with sterilized mud and filtered water. Mud was collected from Lake Balaton then kept air-dried for seven months. After this procedure, the sterilized mud was used in aquaria. Approximately 800

oligochaetes (*Tubifex tubifex*) were placed in each aquarium. The water temperature was maintained between 21-22°C. The oligochaetes were fed complete nutrition fish pellet food.

Induced myxozoan infection was conducted with *Myxobolus pseudodispar* based on the description of Székely et al. 2001 trials at Veterinary Medical Research Institutes (VMRI) CAS, Budapest Hungary. *M. pseudodispar* plasmodia (filled with mature myxospores) were collected from the naturally infected fish muscles of the roach (*Rutilus rutilus*) (Fig. 6). The plasmodia were separated from fish tissue and were given to the oligochaetes (laboratory-cultured parasite-free *Tubifex tubifex*) to induce myxozoan infection.

To maintain the infection in *T. tubifex*, freshly collected *M. pseudodispar* plasmodia were added regularly two to three fish per week (depending on infection intensity) to the aquaria. After two months of the first infection, aquaria water was checked for released actinospores (Triactinomyxons). The water of the aquaria was filtered with a nylon mesh cone (21 μm) and actinospores were separated from the remaining debris (Fig. 7). Viable, intact Triactinomyxons were collected in 48- well microplates for herbal drug treatment experiments.

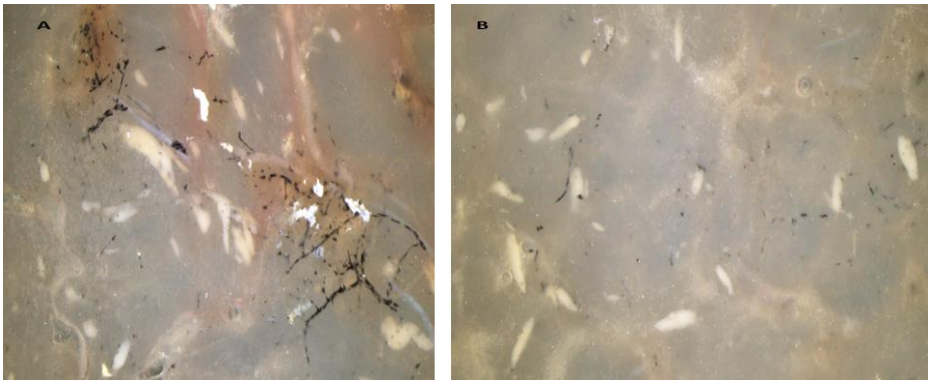

Fig. 6 A piece of muscle of Roach (*Rutilus rutilus*) filled with plasmodia of *Myxobolus pseudodispar* (all the white spots)

Fig.7 Procedures for actinospores collection from filtered debris of infected

3.2.4 Viability test for actinospores

Significant production of actinospores was observed and then active actinospores were collected for viability test at two different temperatures: 20°C and 4°C. The actinospores were collected in 48 well microplates separately, and kept on different temperatures and the mortality was observed per hour.

3.2.5 Treatment trials against actinospores

3.2.5.1 Neem, Turmeric and Garlic derivatives for Treatment

Neem (*Azadirachta indica*) bark extract is aqueous, propylene glycol containing plant extract from neem bark and marketed by Sigma-Aldrich (Lot: BCBF6855V), Germany, was used as the commercial product which is registered in the European Union. To optimize the exact concentration, trials were performed with different concentrations of solutions and which were

prepared by using filtered water and the stock solutions. Several experiments were performed with this extract, in concentration ranges narrowing in the 2 – 100 fold dilution range. In addition, working concentrations were selected based on the rationale that was not unrealistic and which could have an effect on the actinospores.

In order to identify other effective water-soluble plant components, aqueous extracts of some herbs and spices (garlic (*Allium sativum)*, turmeric (*Curcuma longa)*) were also tested. Turmeric and garlic were commercially purchased from the grocery stores in powder form (Szilasfood kft. Lucullus brand). To prepare the working solution, 1g of a commercially available powder was dissolved in 10 ml of distilled water at room temperature (20 ° C) with shaking for 1 hour in falcon tubes, then tubes were placed in a 65 ° C water bath for 4 hours and allowed to cool at room temperature overnight to allow the insoluble particles to settle to the bottom. The plant solution was separated from the insoluble particles by centrifugation (5000 g, 15 min). The supernatant was filtered through a 0.45 μm filter and the filtrate was kept at 4 ° C until use.

3.2.5.2 Experiment Setup

The experiment was performed in flat-bottomed, 48-well microplates under laboratory conditions. Using a dropper, 20 actinospores in 100 μL filtered water were placed into each well of the microplate followed by the different dilution of neem stock solution by adding 100 μL of each with a micropipette and the total volume of 200 μL distilled water with actinspores served as control. Three replicates of each dilution and control were applied. The microplates were closed and incubated at room temperature. The wells were observed under the microscope for morphological changes and counting of dead actinospores at 30 minutes, 1 hour, and 2 hours of the time exposure period. The same procedure was followed for turmeric and garlic treatments as well.

3.2.5.3 Data analysis

The mortality data of actinospores was processed in an excel sheet and the mean of the three replicates were calculated for each trial at the given time interval. Graphical representation of the viability of the actinospores was made in Microsoft Excel 2016 program.

3.3Histology

The highly infected tissues of host fishes were fixed in 4% formalin and processed for histology using paraffin wax as an embedding medium. Histological sections measuring 5 to 8 μm in

thickness were stained in haematoxylin and eosin (H. & E.). Sections were examined and photographed under a phase-contrast of Olympus BH-2 microscope equipped with a DP-20 digital camera, to determine the location of spore development.

3.4 Genomic DNA isolation and Sequencing

In the case of Indian myxozoan samples, the genomic DNA was isolated using the DNeasy™ Blood and Tissue kit (Qiagen, Germany). Then nested polymerase chain reaction (PCR) was performed. The paired primers listed in Table 3 were used. The first reaction mixture consisted of 2 µl of genomic DNA, 5 µl of 1 mM deoxyribonucleotide triphosphates (dNTPs, Biotools, Spain), 0.45 µl of each primer, 2.5 µl of 10 × Taq buffer (Biotools), 0.40 µl of Taq polymerase (1 U; Biotools) and 14.20 µl of distilled water with a total volume of 25 µl. The PCR cycle consisted of an initial denaturation step at 95 °C for 3 min, followed by 35 cycles at 95 °C for 40 s, 56 °C for 1 min, 72 °C for 1 min, completed with a final extension step at 72 °C for 7 min. The second round of the nested PCR reactions were performed by a volume of 50 µl, consisting of 1 µl of amplified DNA, 10 µl of 1 mM dNTPs (Biotools), 0.90 µl of each primer, 5 µl of 10 × Taq buffer (Biotools), 0.80 µl of Taq polymerase (1 U; Biotools) and 31.40 µl of distilled water. Second-round amplifications were carried out as follows: 95 °C for 3 min, followed by 35 cycles at 95 °C for 50 s, 56 °C for 50 s, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. Both PCR cycles were performed in a Mastercycler personal-2231 (Eppendorf, Germany).

For Hungarian samples, *Myxobolus dechtiari and Thelohanellus* species, genomic DNA was isolated from the obtained pellet using the Genaid Tissue Genomic DNA Mini kit, following the manufacturer's recommended protocol for animal tissue. ssrDNA was amplified by semi-nested PCR: The paired primers listed in Table 3 were used. The reaction mixture for the first round PCR consisted of 14.4 µl nuclease-free water, 2.5 µl of 10× DreamTaq buffer (Thermo Scientific, Vilnius, Lithuania), 0.1 µl of DreamTaq Polymerase (1 U; Thermo Scientific), 0.2 mM dNTPs (Thermo Scientific), 0.325 µM of each primer and 2 µl of the extracted DNA in a final volume of 25 µl. The following profile was used for amplification: an initial denaturation step at 95 °C for 3 min followed by 40 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and completed with a terminal extension step at 72 °C for 7 min. This was followed by the second round of semi-nested PCR reactions. The reaction mixture contained 31.8 µl nuclease-free water, 5 µl of 10× DreamTaq buffer (Thermo Scientific), 0.2 µl of DreamTaq polymerase (2 U; Thermo Scientific), 0.2 mM dNTPs (Thermo Scientific), 0.325 µM of each primer and 1

µl from the first round PCR product in a final volume of 50 µl. The amplification conditions were: 95 °C for 3 min, followed by 35 cycles at 95 °C for 50s, 55 °C for 50s, 72 °C for 1 min 40s, and terminated with an extension step at 72 °C for 7 min. The PCR cycles were run by Applied Biosystems SimpliAmp Thermal Cycler (Life Technologies). The primer's sequences are listed in Table 4.

Table 3: Pairs of PCR primers used to amplify the ssrDNA gene

Fish host	**Parasite**	**PCR primer pairs**	**Application**
Notopterus notopterus	*Henneguya ganapatiae*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Mystus vittatus	*Henneguya mystasi*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Channa gachua	*Myxobolus cylindricus*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Ompok pabda	*Myxobolus ompok*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Labeo rohita	*Myxobolus dermiscalis*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Labeo rohita	*Myxobolus bandyopadhyayi*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Gabelioncatla	*Myxobolus chakravartyi*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Cirrihinus mrigala	*Myxobolus rewensis*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Labeo rohita	*Thelohanellus caudatus*	ERIB1/ERIB10, Myx1/SphR	Genomic DNA, Nested PCR
Lepomis gibbosus	*Myxobolus dechtiari*	ERIB1/ERIB10, ERIB1/CR1R, ERIB10/CR1F	Genomic DNA, Semi-nested PCR
Tinca tinca	*Thelohanellus pyriformis*	ERIB1/ERIB10, Myx1/ ERIB10	Genomic DNA, Semi-nested PCR
Chondrostoma nasus	*Thelohanellus fuhrmanni*	ERIB1/ERIB10, Myx1/ ERIB10	Genomic DNA, Semi-nested PCR

Table 4: Primers sequences used for PCR and sequencing

Primer	Sequences	References
ERIB 1	5'- ACCTGGTTGATCCTGCCAG - 3'	Barta et al. (1997)
ERIB 10	5'- CTTCCGCAGGTTCACCTACGG - 3'	Barta et al. (1997)
Myx1F	5' – GTGAGACTGCGGACGGCTCAG – 3'	Hallett and Diamant, (2001)
SphR	5' – GTTACCATTGTAGCGCGCGT – 3'	Eszterbauer and Székely, (2004)
ACT1fr	5' – TTGGGTAATTTGCGCGCCTGCTGCC – 3'	Hallett and Diamant, (2001)
MC5	5' – CCTGAGAAACGGCTACCACATCCA – 3'	Molnár et al. (2002)
MC3	5' – GATTAGCCTGACAGATCACTCCACA – 3'	Molnár et al. (2002)
MB5	5' – ACCGCTCCTGTTAATCATCACC – 3'	Eszterbauer, (2004)
MB3	5' – GATGATTAACAGGAGCGGTTGG – 3'	Eszterbauer, (2004)
CR1 R	5'-CTAGGACGGTATCTGATCGTCTTCG– 3'	Székely et al. (2015)
CR1 F	5' – CGA AGA CGA TCA GAT ACC GTC CTAG– 3'	Székely et al. (2015)
ACT3f	5'-CAT GGA ACG AAC AAT-3'	Hallett and Diamant, (2001)
MB5r	5′-ACCGCTCCTGTTAATCATCACC-3′	Eszterbauer, (2004)
MB3f	5′-ATGATTAACAGGAGCGGTTGG-3′	Eszterbauer, (2004)
MB5f	5′-GATGATTAACAGGAGCGGTTGG-3′	Eszterbauer, (2004)
ACT1r	5′-AATTTCACCTCTCGCTGCCA-3′	Hallet and Diamant (2001)
MYXGEN 4f	5′-GTGCCTTGAATAAATCAGAG-3′	Diamant et al. (2004)

The PCR products were analysed by using the 1% agarose gels in Tris–Acetate–EDTA buffer, gel was stained with ethidium bromide. The PCR products were excised from the gel, purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid Tawai city, Taiwan) and sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) with an

ABI PRISM 3100 Genetic Analyzer (Life Technologies), using the amplification and inner primers.

3.5 Phylogenetic Analysis

Obtained sequences were assembled in MEGA 6.0 (Tamura et al. 2013) and MEGA X (Kumar et al. 2015) and manual adjustments were performed by eye to correct the alignment and remove ambiguous positions from the dataset. Assembled sequences were verified as myxozoan by GenBank BLAST search. The sequences were aligned using Clustal W (Thompson et al. 1994) within MEGA 6.0 and MEGA X. Phylogenetic analysis was performed using maximum likelihood (ML) analysis. The data set was tested using MEGA 6.0 and MEGA X. for the nucleotide substitution model of best fit, and the model shown by the Akaike Information Criterion (AIC) as the best-fitting one was chosen. Bootstrap values based on 1000 resampled datasets were generated. BI phylogenetic tree was computed by Topali 2.5 (Milne et al., 2008). Sequences of species related to spore isolates were downloaded from GenBank for the phylogenetic analysis. Taxa were selected on the basis of BLAST matches.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Myxozoan samples from India

4.1.1 Results

The present study revealed different myxozoan infections of Indian fish fauna. Two *Henneguya* species (*H. ganapatiae* and *H. mystasi*) were redescribed from *Notopterus notopterus* and *Mystus vittatus*. Our re-description included microscopic photos on spores, histological preparations with data on tissue location and molecular genetic examinations. *Myxobolus* species (*M. cylindricus, M. ompok* n. sp., *M. dermiscalis, M. bandyopadhyai* n. sp., *M. chakravartyi* and *M. rewensis*) were detected in *Channa gachua, Ompok pabda, Labeo rohita, Gibelion catla* and *Cirrhinus mrigala* respectively. In our study, *M. cylindricus, M. rewensis* and *M. chakravartyi* were described with their first sequence data and with well-illustrated morphology. *Myxobolus ompok* n. sp. and *Myxobolus bandyopadhyayi* n. sp. were described by our research team as new species, based on morphometrical and sequencing results. One *Thelohanellus* species, *Thelohanellus caudatus* was determined from *Labeo rohita*. Summary of the detected myxozoan species from India is presented in Table 5. Detailed results with the morphology of spores, plasmodial development site, tissue and site-specificity of cysts location and possible pathological changes caused by these parasites and the sequencing results are presented in the following sub-chapters.

Table 5. Descriptive data of collected samples from India

Host Fish	**Myxozoan infection**	**Infection site**	**Infection intensity**	**GenBank Accession number**
Notopterus notopterus	*Henneguya ganapatiae*	Gill filaments	75%	MT365528
Channa gachua	*Myxobolus cylindricus*	Gill lamellae	78.5%	MH424126
Mystus vittatus	*Henneguya mystasi*	Gill lamellae	81.2%	MH300136
Ompok pabda	*Myxobolus ompok n. sp.*	Kidney	70%	MG760575, MG760574
Labeo rohita	*Thelohanellus caudatus*	Fins	28%	MZ230375, MZ230376 and MZ230379

Labeo rohita	*Myxobolus dermiscalis*	Scales	25%	MZ230378
Gibelion catla	*Myxobolus chakravartyi*	Fins	44%	MZ230377
Labeo rohita	*Myxobolus bandyopadhyayi n. sp.*	Scales	25%	MZ230380
Cirrhinus mrigala	*Myxobolus rewensis*	Fins	30%	MZ230381

4.1.1.1 Redescription of *Henneguya ganapatiae* Quadri, 1970

Morphological records

Bronze featherback (*Notopterus notopterus*) (N = 20; 20–25 cm total length) were collected from River Ganga, Hastinapur, Uttar Pradesh, India. Mature myxospores of *H. ganapatiae* were observed in gills of 15/20 (75%) fish.

Description of Myxospore

Myxospore body ellipsoidal in both frontal and sutural views, with two slightly curved caudal processes. Spore valves have thin walls, surface smooth, without ridges (Fig. 8A, B, C). Myxospore body length 9.7 ± 0.4 (9.3–10.0 μm), width 4.5 ± 0.5 (4.0–4.8 μm). Two polar capsules, pyriform, approximately equal size, length 3.3 ± 0.2 (2.6–3.2 μm) and width 1.6 ± 0.1 (1.4–1.8 μm). Polar tubules not observed. Length of caudal processes 23.7 ± 1.4 (22.0–25.0 μm).

Type host: Bronze featherback, local name “patra”, *Notopterus notopterus* (Pallas, 1769) (Notopteridae).

Site of infection: Gill filaments

Reference materials: Digitized photos of syntype spores retained in the collection of Fish Pathology and Parasitology Group, Centre for Agricultural Research, Institute for Veterinary Medical Research, Budapest, Hungary.

Prevalence: 75% (15/20)

Molecular and Phylogenetic analysis: Sequence data of the ssrDNA of *H. ganapatiae* (1660 bp) from a single host fish, was deposited in NCBI GenBank (accession number

MT365528). Pairwise comparisons revealed that the most similar myxozoans were *H. chaudharyi* (89.4%; from spotted snakehead fish *Channa punctata*; KT279402), and *H. setiuensis* (90.7%; MH743111), *H. calcariferi* (90.8%; MH743109) and *H. voronini* (90.4%; MH743110) described from barramundi *Lates calcarifer* from Malaysia. *Henneguya ganapatiae* clustered with other *Henneguya* species that parasitize fresh and brackish water fish hosts (Fig. 9).

Remarks: The parasite species observed in bronze featherback had morphology and measurements that correspond to *Henneguya ganapatiae* Quadri (1970).

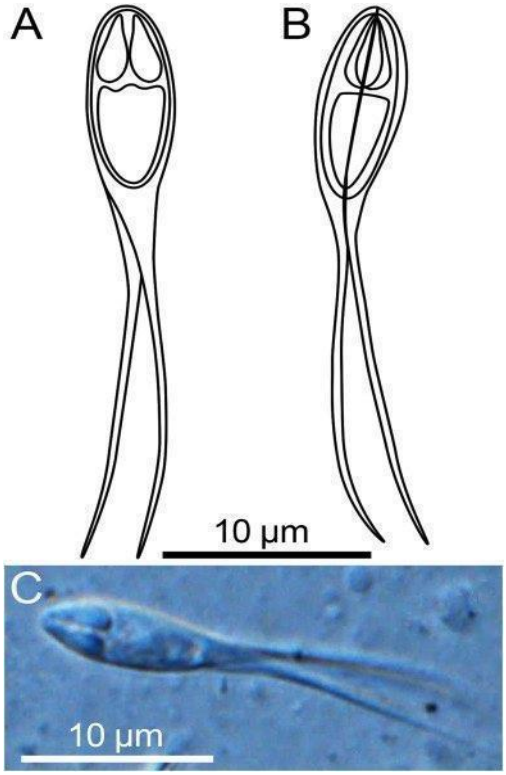

Fig. 8. Myxospores of *Henneguya ganapatiae*. A–B: Line drawings of mature myxospores in frontal and valvular view showing polar capsules and caudal processes. C: Fresh, unstained myxospore in frontal view showing the two pyriform polar capsules and caudal processes.

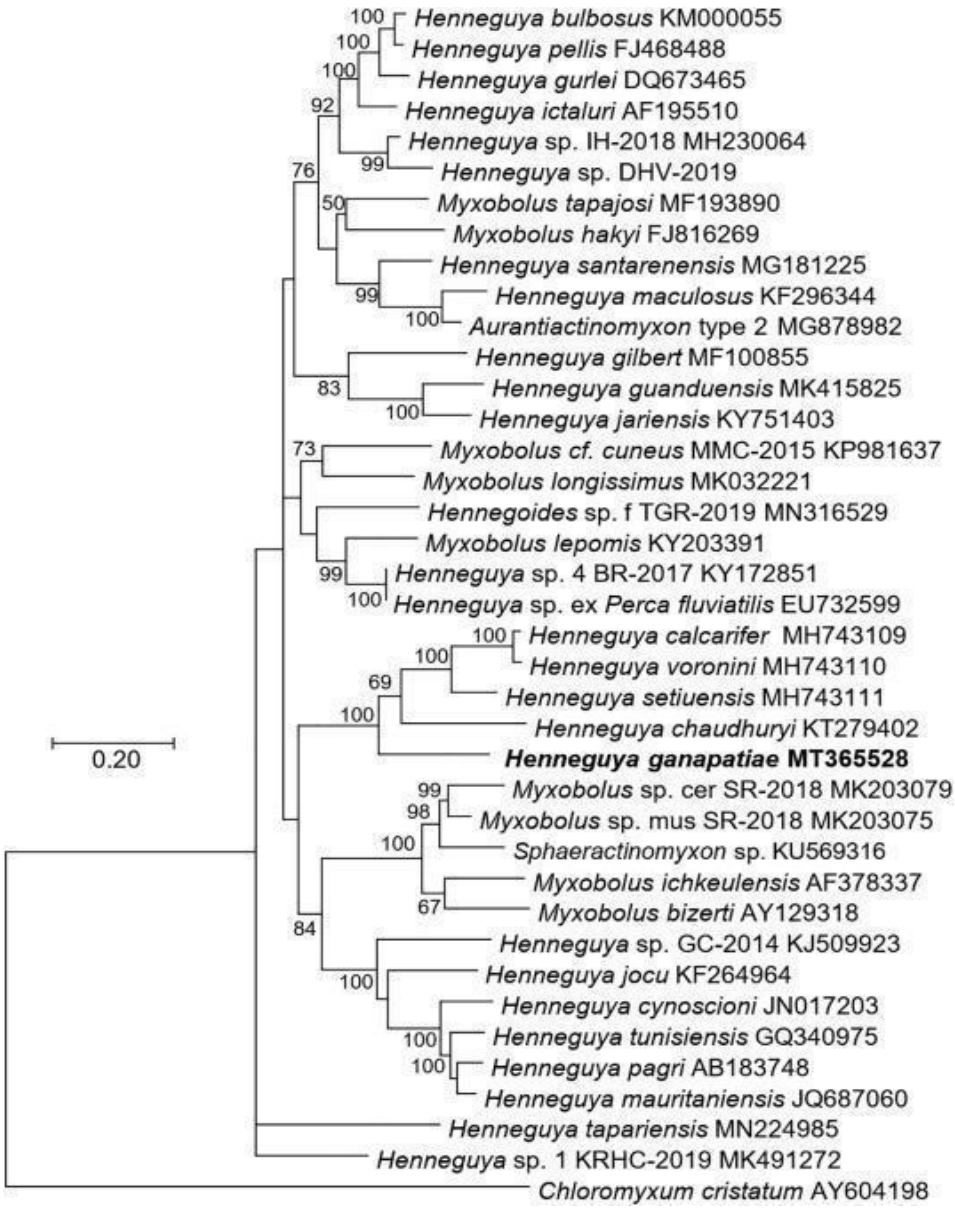

Fig. 9 Phylogenetic tree generated by maximum likelihood analysis of ssrDNA sequences of *Henneguya ganapatiae* and other closely-related myxozoan species; GenBank accession numbers shown after the species name. Novel data are in bold. Numbers at nodes indicate the bootstrap confidence values >50 (ML). *Chloromyxum cristatum* was used as an outgroup.

4.1.1.2 *Henneguya mystasi (*Haldar, Samal and Mukhopadhyay, 1997)

Morphological Records

In the gill filaments of *Mystus vittatus* small plasmodia of oval or ellipsoidal shape were found developing inside the capillary network of the gill lamellae. Plasmodia in the infected lamellae emerged somewhat over the surface of the lamellar rows. Thirty-nine out of 48 fish (81.2%) proved to be infected. The intensity of infection was moderate to high, 30–200 cysts per hemibranchia. In most cases, matured plasmodia containing matured spores were found.

Description of myxospores

Myxospores elongated with two straight caudal appendages, and with elongated polar capsules located side by side (Fig. 10). Myxopore wall thin and smooth composed of two equal valves. The oral end of the myxospore body blunt in frontal view. The caudal end rounded, but continued into the caudal appendages. The total length of the myxospores (from the anterior end of the spore body to the end of caudal appendages) 26.4–36 (30.6 ± 2.7) (N = 50) (Fig. 11 a, b). The spore body 12–14 (12.6 ± 0.76) (N = 50), the width of the myxospore body 3.8–4 (3.9 ± 0.1) (N = 50), the thickness of the myxospore body 2.4–3.6 (3.1 ± 0.34) (N = 8) (Fig. 11 a, b). The two polar capsules elongated, opening at the anterior end of the myxospore body. One of the capsules slightly larger. The larger polar capsules measure 5.6–6.4 (6 ± 0.35) (N = 50) in length, 1.1–1.3 (1.2 ± 0.31) (N = 50) μm in width and 1–1.3 (1.2 ± 0.1) (N = 8). Coils of the polar filaments not seen. The lengths of the caudal appendages 14.4–22 (18 ± 1) (n = 22) (Fig. 11 a, b). The suture not seen. A large round iodinophilous vacuole in the sporoplasm present.

Remarks

Up to now, only three species of *Henneguya* have recorded from different *Mystus spp*. from India (*H. bleekeri* Haldar and Mukherjee, 1985 from *M. bleekeri* Day; *H. mystusia* Sarkar, 1985 from *Mystus spp.* and *H. mystasi* Haldar et al. 1997 from *M. gulio* Hamilton) (Kalavati and Nandi, 2007). The descriptions of all three species lacked important characteristics and presented only the shape and size of the spores (Kalavati and Nandi, 2007). Based on morphological similarities and the close genetic relations of *Mystus spp.*, we regard *M. vittatus* as a suitable host for *H. mystasi*. The present species was identified as *H. mystasi* by having elongated myxospores with two straight caudal appendages, elongated polar capsules located side by side, one of the capsules somewhat larger, spore body blunt in shape anteriorly while

the caudal end was rounded and continuing into the caudal appendages. *Henneguya bleekeri* Haldar and Mukherjee, 1985 clearly differs from *H. mystasi* by its short bifurcated recurving caudal attachment that bifurcates at some distance from the caudal end and is curved in the form of two unequal caudal appendages like a 'U' shape. The spores of *H. mystusia* resemble those of *H. mystasi,* but have a posteriorly tapering end of the spore body and straight, longer caudal attachments.

Molecular data

The ssrDNA sequences of *H. mystasi* collected from the gill filaments showed the highest similarity (95.5–96%) to different *Henneguya* species of the same clade (*H. bicaudi* and *H. pellucida* Adriano et al., 2005), but were positioned separately from other species well supported by the bootstrap values and posterior probabilities (98% and 1.00) (Fig. 12). This species had the closest similarity to *H. bicaudi*, a species described from an Indian cyprinid fish (92.5%). Surprisingly, it was also similar (95%) to *H. pellucida*, a parasite infecting a characid fish known from South America. A BLAST search using ssrDNA of *H. mystasi* showed that this species could be distinguished from all *Henneguya* sequences available in the GenBank database. Both ML and BI analyses produced similar tree topologies; therefore, only the ML tree is presented here, along with the posterior probabilities of the BI tree.

Taxonomy Summary

Host: local common name 'tengara', *Mystus vittatus* (Bl.) (Siluriformes: Bagridae).

Locality: Meerut (29°01′N, 77°45′E), Uttar Pradesh, India.

Site of tissue development: Gill lamellae.

Voucher material: Digitised photos of spores were deposited in the Parasitological Collection of the Zoological Department, Hungarian Natural History Museum, Budapest (Coll. No. 71845).

Prevalence of infection: 81.2% (39 out of 48 fish of the 8–10 cm size group infected).

Intensity of infection: moderate to high.

Representative DNA sequence: The ssrDNA sequence of *H. mystasi* was deposited in GenBank under the accession number MH300136.

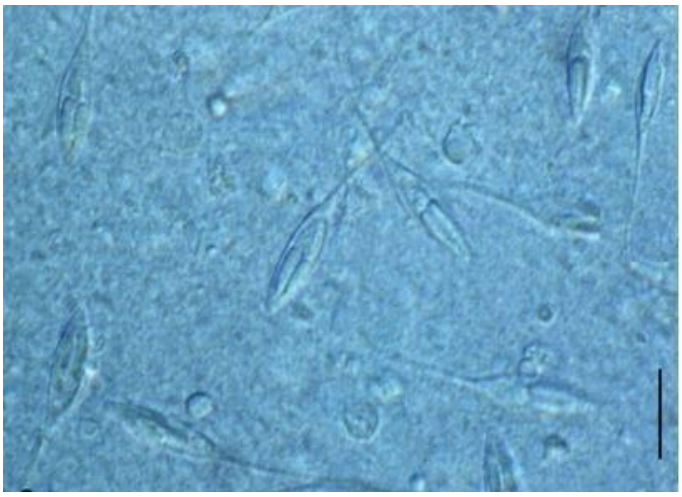

Fig. 10 Ethanol-fixed spores of *Henneguya mystasi* from the gills of *Mystus vittatus*. Bar= 10 µm

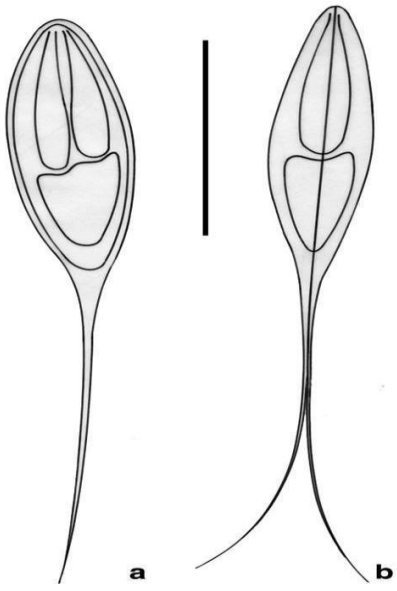

Fig. 11 Schematic drawing of a myxospore of *Henneguya mystasi* infecting *Mystus vittatus*. **a** Myxospore in frontal view; **b** spore in sutural view. Bar =10 µm

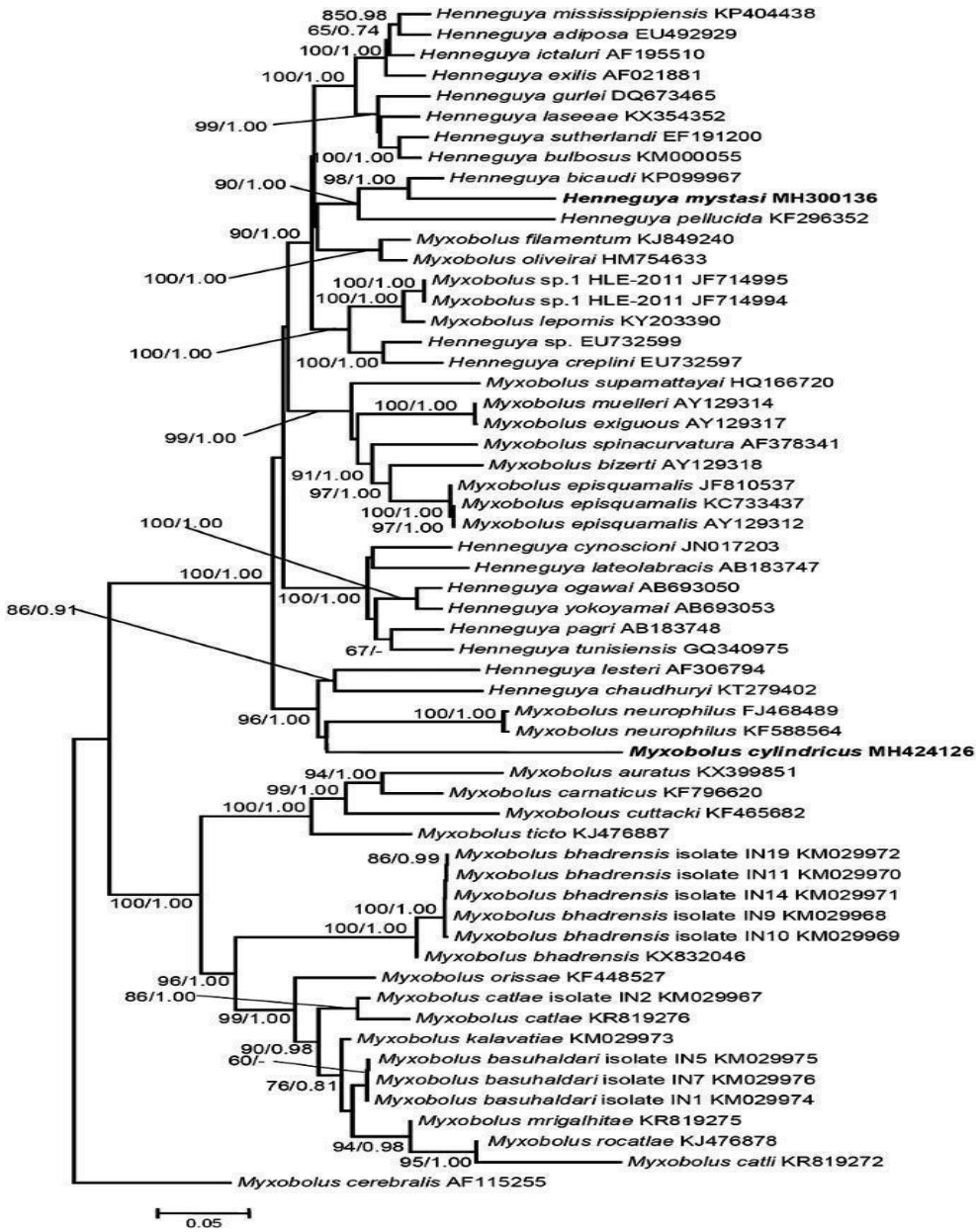

Fig. 12 Phylogenetic tree generated through maximum likelihood analysis of the ssrDNA sequences of *Myxobolus cylindricus*, *Henneguya mystasi* and selected species. Numbers at nodes indicate the bootstrap values (ML) and posterior probabilities (BI). Unsupported nodes by BI are marked with a hyphen. GenBank accession numbers of all selected species are listed adjacent to species names. *Myxobolus cerebralis* was used as outgroup. *Myxobolus cylindricus* and *Henneguya mystasi* examined in this study are indicated in bold

4.1.1.3 *Myxobolus cylindricus* (Sarkar, Mazumdar and Pramanik, 1985) emend.

During the study, myxosporean plasmodia were found only in the gills of *Channa gachua*. By the shape and size of the spores and the specific hosts, the species was identified as *M. cylindricus* Sarkar, Mazumdar and Pramanik 1985.

Morphological Records

In the gill filaments of *C. gachua* small plasmodia of oval or ellipsoidal shape were found, located between two gill lamellae. These plasmodia only rarely emerged over the level of lamellae. Forty-four out of 56 fish (78.5%) proved to be infected. The intensity of infection was moderate to high, 30–200 cysts per hemi-branchia. In most cases, matured plasmodia containing matured myxospores were found.

Description of myxospores

Myxospores were relatively large, ellipsoidal in frontal and elongated ellipsoidal in sutural view (Fig. 13). They were 12.8–14.9 (13.5 ± 0.59) μm long (N = 50), 5.6–6.4 (5.9 ± 0.35) μm wide (N = 50), and very thin, 3.2–4.4 (3.8 ± 0.31) μm (N = 14) (Fig. 14 a, b). Polar capsules were equal in size, elongated pyriform, slightly converging anteriorly, 3.6–4.8 (4.3 ± 0.39) μm long (N = 50) and 0.7–1.2 (0.9 ± 0.21) μm wide (N = 50) (Fig. 14 a, b). They had a large sporoplasm with a similarly large iodinophilous vacuole filling the space of the myxospore. The polar capsule was relatively short compared to the length of the myxospore (Fig. 13). Coils of polar filaments were not seen. A sutural protrusion, sutural edge markings, mucous envelope and intercapsular appendix were not observed. The ssrDNA sequence of *M. cylindricus* was deposited in GenBank under the accession number MH424126.

Remarks

By its elongated ellipsoidal shape and relatively short polar capsules, the species presented here is differed in shape and size from most of the *Myxobolus* species described from *Channa spp.* but, corresponded to *M. cylindricus* Sarkar et al., 1985, and despite the fact that the original authors reported on finding myxospores in the kidney, we identified our species with the above species. The morphological data observed by us and those given by Sarkar et al. (1985) are very similar. *Myxobolus aligarhensis*, *M. andhrae* and *M. channai* clearly differ from *M. cylindricus* by having unequal polar capsules. The spores of *M. maruliensis* differ from those of *M. cylindricus* by having a posterior truncated end and very long polar capsules. Moreover, *M.*

noblei differs from *M. cylindricus* by its short elliptical shape and by its short, blunt posterior extension of the myxospore valve.

Molecular and Phylogenetic analysis

The ssrDNA sequences of *M. cylindricus* generated highly similar topologies in both ML and BI analyses and the phylogenetic position of *M. cylindricus* was identical in both phylograms. The sequences of *M. cylindricus* differed from most of the *Myxobolus* species described in India from cyprinid fishes, and it showed the closest similarity (92.0%) to the sequences of *M. neurophilus* (KF588564 and FJ468489), a parasite of a perciform host (Fig. 12). *Myxobolus cylindricus* did not show close similarity to any other *Myxobolus spp.* from India due to the lack of available molecular sequences from this region of India. It had only a relatively close similarity (82%) to *Henneguya chaudhuryi*, a species belonging to a different genus but described from a closely related channid host. It also showed some similarity (82%) to another *Henneguya* species, *H. lesteri*, a species described by Hallett and Diamant (2001) from *Sillago analis*, a coastal fish of Australia.

Taxonomy Summary

Host: local common name ‘chanaga’, *Channa gachua* (Ham.) (Perciformes: Channidae).

Locality: Meerut (29°01′N, 77°45′E), Uttar Pradesh, India.

Site of tissue development: Gill lamellae.

Voucher material: Digitized photos of spores were deposited in the Parasitological Collection of the Zoological Department, Hungarian Natural History Museum, Budapest, (Coll. No. 71844).

Prevalence of infection: 78.5% (44 out of 56 fish of the 10–14 cm size group were infected).

Intensity of infection: moderate to high.

Representative DNA sequence: The ssrDNA sequence of *M. cylindricus* was deposited in GenBank under the accession number MH424126.

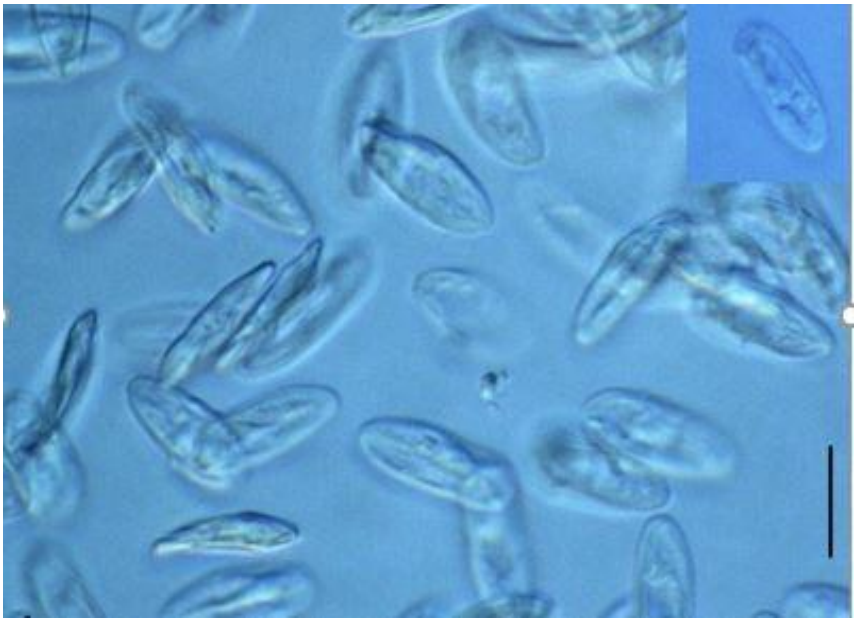

Fig. 13 Ethanol-fixed myxospores of *Myxobolus cylindricus* from the gill of *Channa gachua*. Bar =10 µm

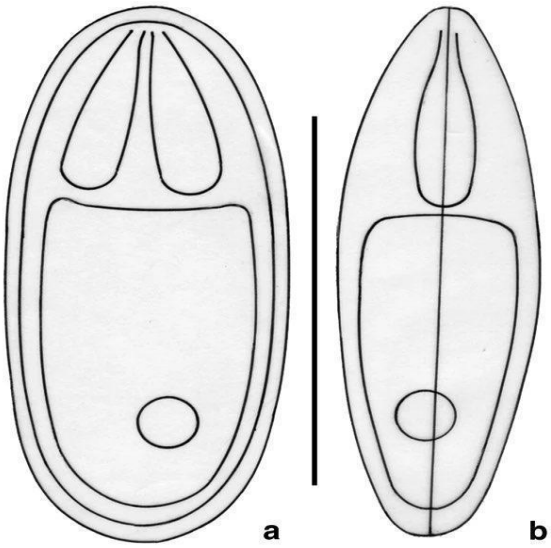

Fig. 14 Schematic drawing of a myxospore of *Myxobolus cylindricus* infecting *Channa gachua*. **a.** Myxospore in frontal view; **b.** spore in sutural view. Bar= 10 µm

4.1.1.4 *Myxobolus ompok n. sp.*

Morphological records

Infection was found only in the kidney. The detailed examination of Pabdah catfish (*Ompok pabda*) specimens did not demonstrate any signs of abnormalities such as lesions, bruising, or deformities, but in some of the fish, heavy infection of the kidneys with an unknown species of *Myxobolus* Bütschli, 1892 was found.

Description of myxospores

Myxospores are large, elongated pyriform both in frontal view and sutural view (Fig. 15). Myxospores are 13.6–14.48 (14.4 ± 0.42) long, 5.6–6.4 (6.5 ± 0.33) wide, and 5.2–6.4 (5.9 ± 0.43) thick. Two polar capsules were elongated, narrowing anteriorly, and equal in size. The length of the polar capsule is 8.0–8.5 (8.2 ± 0.2), and the width is 1.5–2.4 (1.8 ± 0.33) (Fig. 16). Polar filaments were coiled with six turns within the polar capsule. They are situated perpendicularly to the longitudinal axis of the capsule. Myxospores are without intercapsular appendix. Sutural line is indistinct. Valves are smooth and relatively thick, measuring 0.6–0.9 (0.8). Sporoplasm nuclei are indiscernible. Iodinophilous vacuole in the sporoplasm or mucous envelope was not found.

Classification:

Family: Myxobolidae Thélohan, 1892

Genus: Myxobolus Bütschli, 1882

Taxonomic summary

Type-host: *Ompok pabda* (Hamilton, 1822).

Local name of the host: Pabdah.

Type-locality: Sotiganj, Meerut (28° 59′ 0″ N, 77° 42′ 0″ E), UP, India.

Site of infection: Kidney.

Prevalence of infection: 19/27 of the 10–16-cm size group.

Intensity of infection (calculated by Kaur and Attri 2015): High.

Type-material: Digitized photos of spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest.

Representative DNA sequence

The ssrDNA sequence of *Myxobolus ompok n. sp.* was deposited in GenBank under the accession numbers MG760574 and MG760575 collected from two infected hosts.

Histology

In histological sections stained by hematoxylin and eosin, round-shaped plasmodia of 150 to 200 μm in cross section were found. These plasmodia located in the renal interstitial of the kidney were bordered by a thin connective tissue layer and contained 1000 to 2000 matured spores. Accumulation of macrophages was not observed around plasmodia (Fig. 17).

Molecular and Phylogenetic analysis

Phylogenetic analysis of the ssrDNA sequence of *M. ompok n. sp.* placed the two isolates (1704 and 1708 bp) sequenced within a clade of myxozoan parasites of siluriform fishes that represent a single novel species. The isolates in BLAST search shared significant sequence similarity only with *Myxobolus miyarii* (91.5%) from China infecting the Amur catfish, *Silurus asotus* Linnaeus. Besides this, *M. ompok n. sp.* indicated that it was not identical with any *Myxobolus sp.* sequences available in GenBank but was also found to be closely related to some *Henneguya* species (90–91%), including *H. pellis* (FJ468488), *H. bulbosus* (KM000055), *H. sutherlandi* (EF191200), *H. gurlei* (DQ673465), *H. laseeae* (KX354352), *H. exilis* (AF021881), *H. ictaluri* (AF195510), *H. adiposa* (EU492929), and *H. mississippiensis* (KP404438) described from siluriform fishes. The generated ML and BI analyses produced an identical topology; therefore, only the ML phylogenetic tree is presented here. The phylogenetic tree showed a strong support for clade (90% and 1.00) that belongs to *M. miyarii* and *M. ompok n. sp.* and apparently placed *M. ompok n. sp.* in a clade of freshwater *Henneguya–Myxobolus* parasites that infect fish of Siluriformes (Fig. 18).

Remarks

Of the *Myxobolus* species known from Indian catfishes, none is reported from *O. pabda*. *Myxobolus ompok n. sp.* differs from them in its morphological characteristics. In addition to myxospore morphology, the ssrDNA sequences of two isolates deposited in the GenBank database indicate that *M. ompok n. sp.* should be regarded as a new species. Despite severe infection with plasmodium in the kidney, no disease signs were recorded externally on the fish examined in this study.

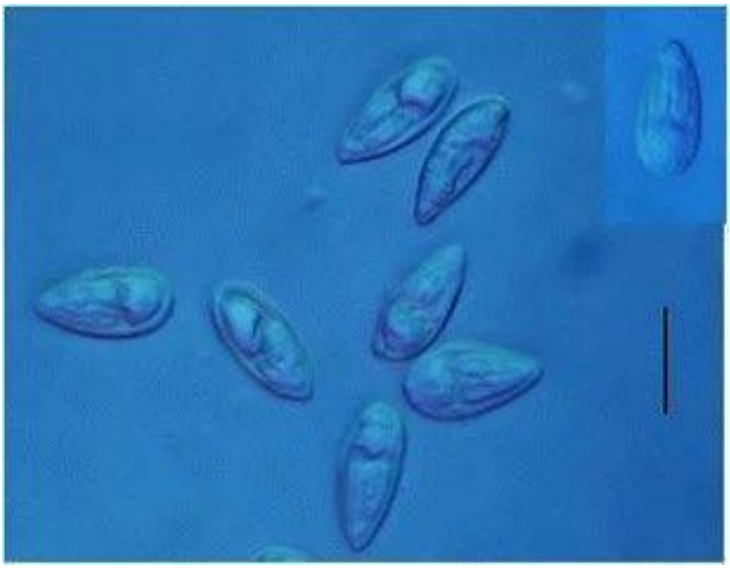

Fig. 15 Myxospores of *Myxobolus ompok n. sp.* from the kidney of *Ompok pabda*. Scale bar = 10 μm

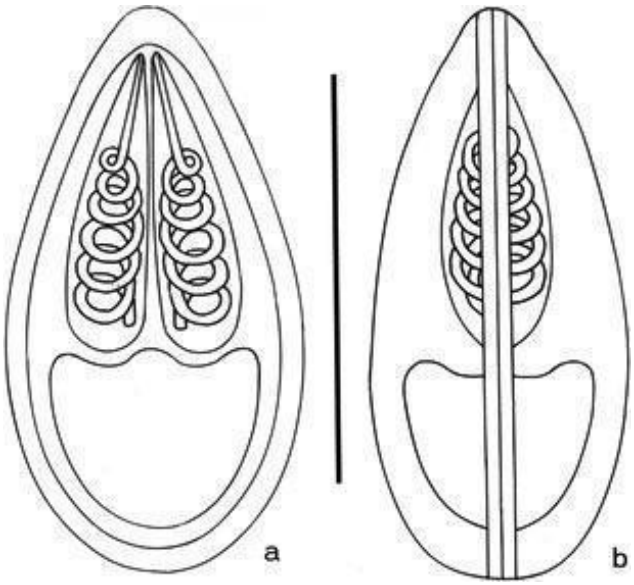

Fig. 16 Schematic drawing of myxospores of *Myxobolus ompok n. sp.* infecting *Ompok pabda*. **a** Myxospore in frontal view. **b** Myxospore in sutural view. Scale bar = 10 μm

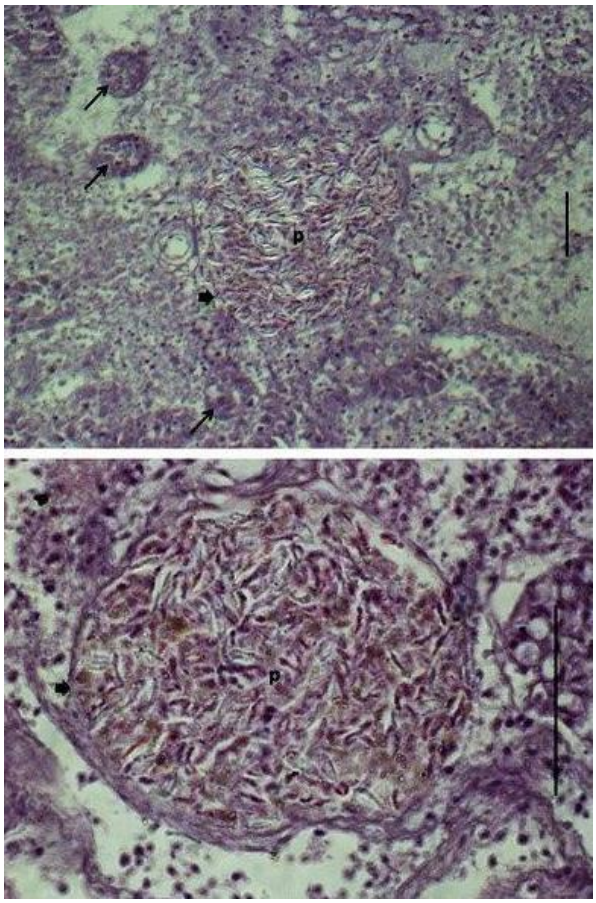

Fig.17 Plasmodium (p) filled by matured myxospores and bordered by thin connective tissue (arrowhead) in the interstitial tissue of the kidney. Around the plasmodium, fragments of cross-sectioned renal tubules (arrows) are seen. H. & E. staining. Bar = 50 μm

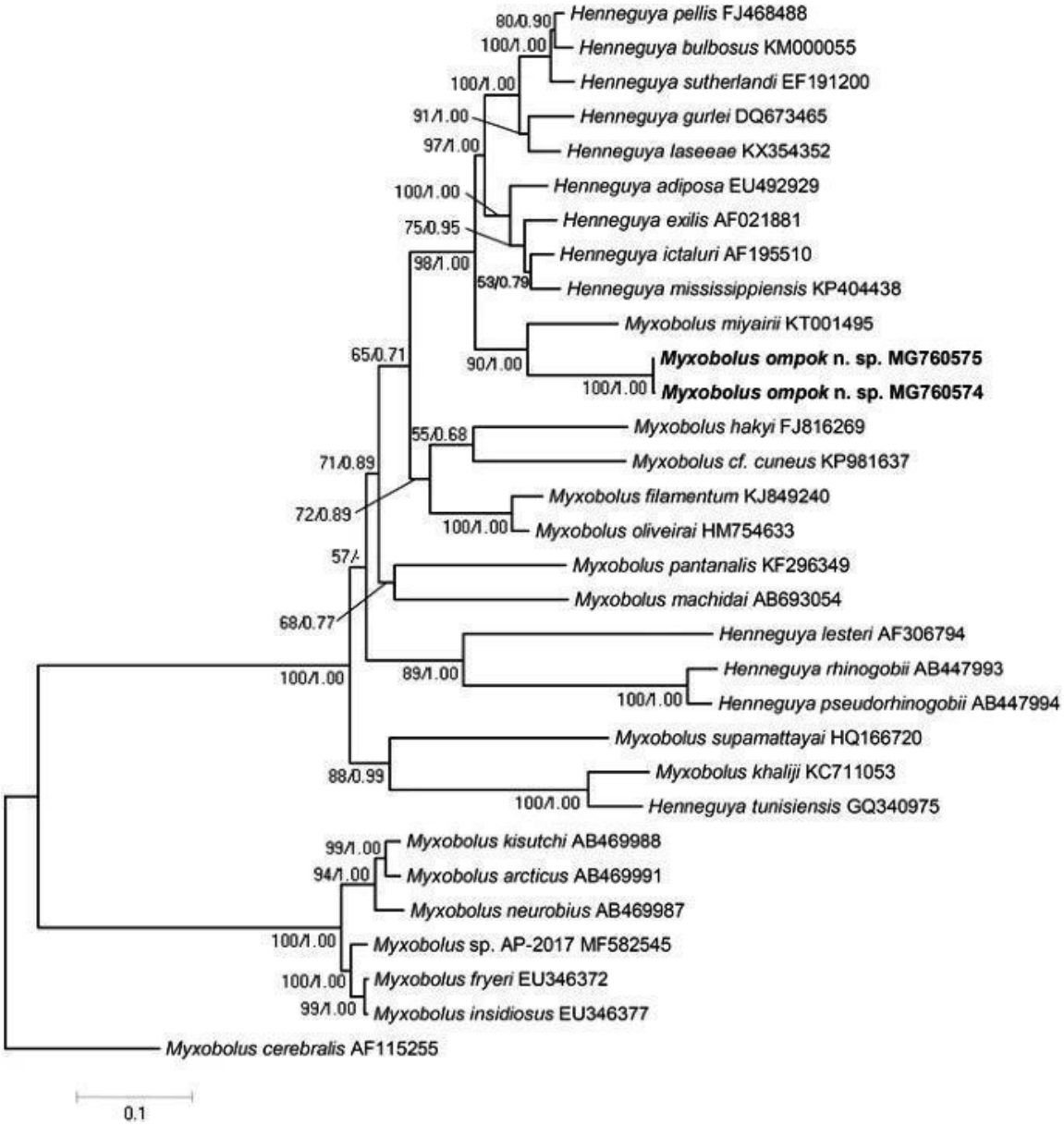

Fig. 18 Phylogenetic tree generated through maximum likelihood analysis of the ssrDNA sequences of *Myxobolus ompok n. sp*. and selected species. Numbers at nodes indicate the bootstrap values (ML) and posterior probabilities (BI). Unsupported nodes by BI are marked with a hyphen. The GenBank accession numbers of all selected species are listed adjacent to the species names. *Myxobolus cerebralis* was used as outgroup. *Myxobolus ompok n. sp.* examined in this study is indicated in bold

4 .1.1.5 *Myxobolus dermiscalis* Kaur et al. 2016

Morphological records

Round shape plasmodia (Fig. 19) with a diameter of 700 to 1500 were located in the superficial tissues of the scale. They occurred in 4 of 13 rohu specimens from the fish farm. Plasmodia contained 800 to 2000 myxospores.

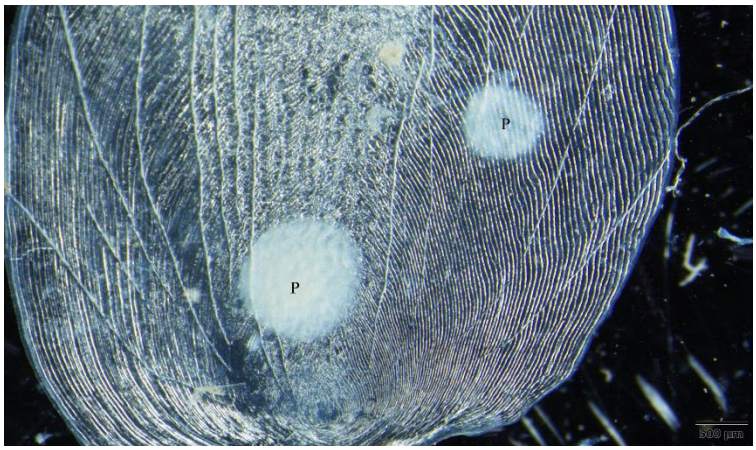

Fig. 19 *Myxobolus dermiscalis* plasmodia (P) in the scale of a rohu. Mount picture. Bar = 500 µm

Description of myxospores

Myxospores (Fig. 20 a,b,c) short ellipsoidal in frontal view and lemon shaped in sutural view. Myxospores 10.6 ± 0.44 (9.9-11.2) long, 8.5 ± 0.3 (8-8, 8) wide and 6-6.2 thick. Two polar capsules elongated pyriform, uniform in size, 5.4 ± 0.3 (5-5.8) long, and 2.5 ± 0.22 (2.2-2.7) wide. Polar tubules not seen. The proximal end of the myxospore has a short-flattened plate. Nuclei of the sporoplasm and polar capsules not seen. Iodinophilous vacuole and mucous envelope not found. The thickness of the myxospore wall 0.7-0.8.

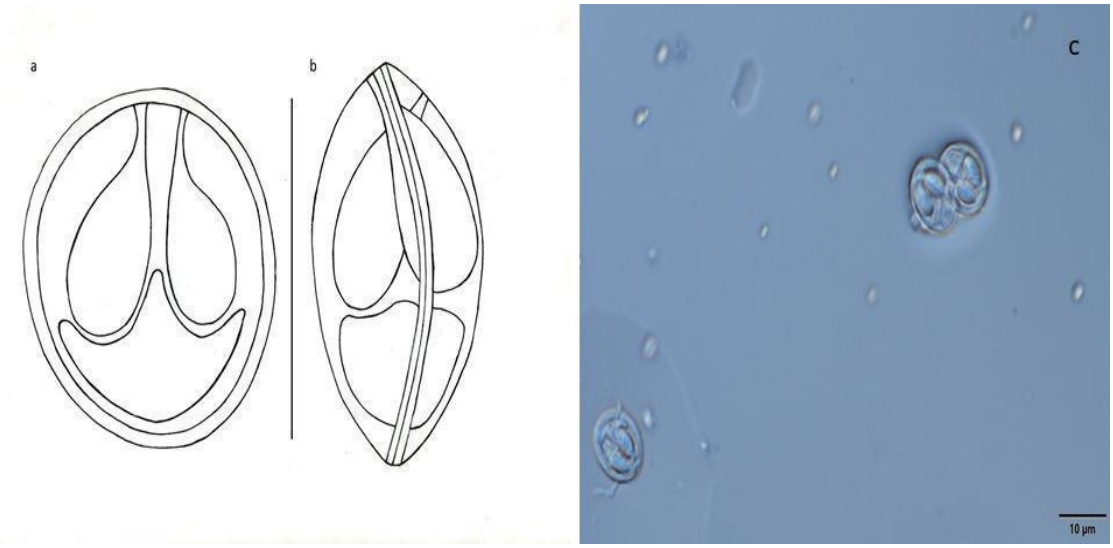

Fig. 20 Schematic drawing of myxospore of *Myxobolus dermiscalis*, a: frontal view, b: sutural view. Bar = 10 µm, c: *M. dermiscalis* myxospores in frontal view. Mount picture. Bar = 10 µm

Host: Rohu, *Labeo rohita* Hamilton

Locality: Naihati, Battala market (22.89°N 88.42°E) (on kalyani expressway)

Site of infection: scales

Prevalence of infection: 4 specimens from 13 fish

Type material: Photo-types and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *M. bandyopadhyayi* was deposited in the GenBank under accession number MZ230378

Molecular data: ssrDNA sequence (GenBank accession number: MZ230378) of the parasite was 99.4% similar to *Myxobolus dermiscalis* (KM092529) and 98.8% similar to a *Myxobolus* sp. sample (KM401439) which was also isolated from the scales of rohu. These three sequences formed a monophyletic clade with maximum bootstrap. As the closest relatives on the phylogenetic tree, the sequence of *Myxobolus rewensis* (MZ230381,) showed 91.8% and *Thelohanellus* sp. (KM401440) 91.6% similarity, respectively (Fig. 21).

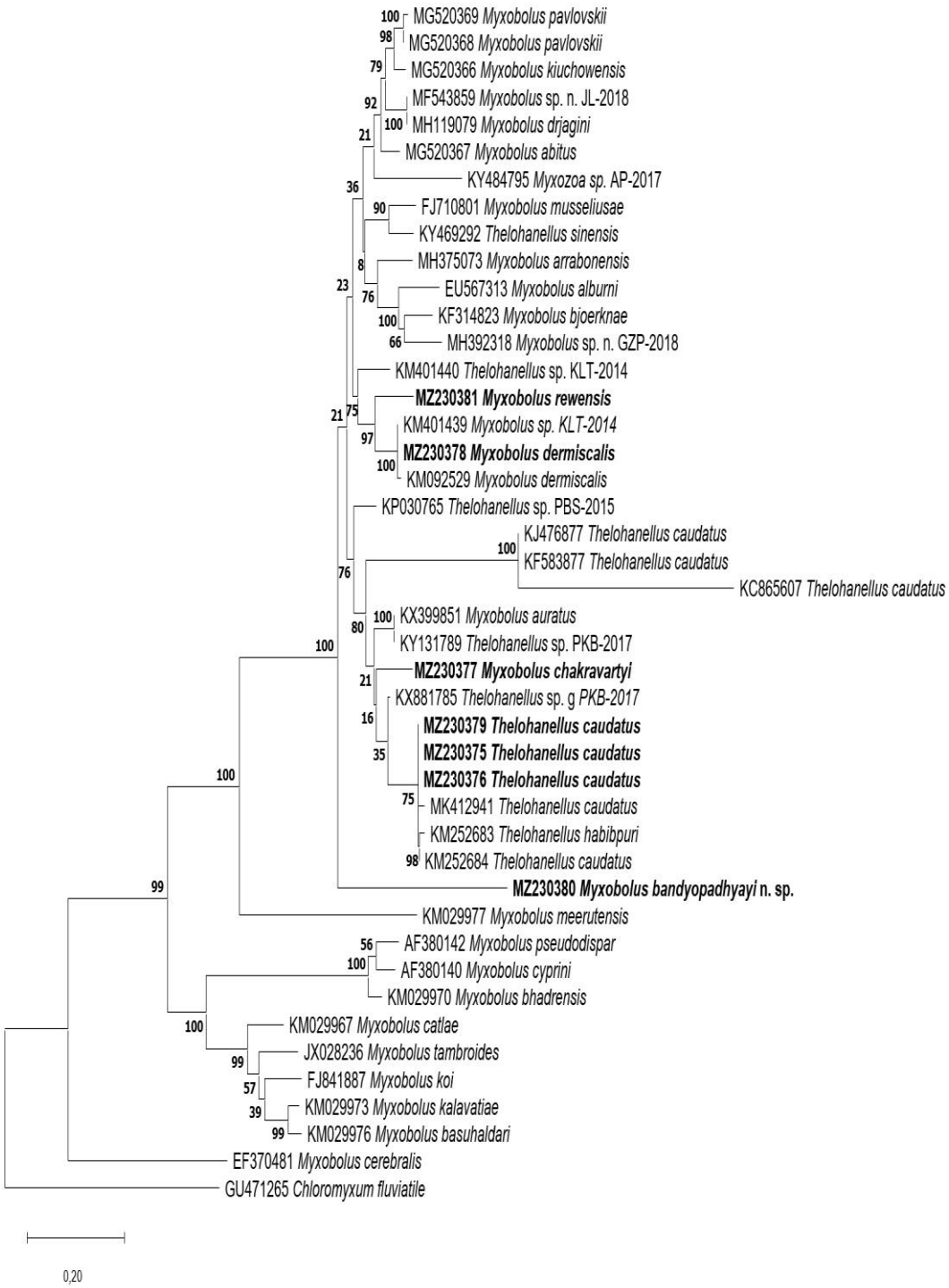

Fig. 21 Phylogenetic analysis of the studied myxozoan species based on ssrDNA sequences with Maximum Likelihood algorithm. *Chloromyxum fluviatile* was used as the outgroup. Bootstrap values are given at the nodes. The scale-bar indicates the number of expected substitutions per site

4.1.1.6 *Myxobolus bandyopadhyayi* n. sp.

Morphological records

Round shape, plasmodia (Fig. 22) with a diameter of 700 to 1500 were located in the superficial tissues of the scale. They occurred in 3 rohu specimens out of the 12 from the fish market. Plasmodia contained 4000 to 10000 myxospores.

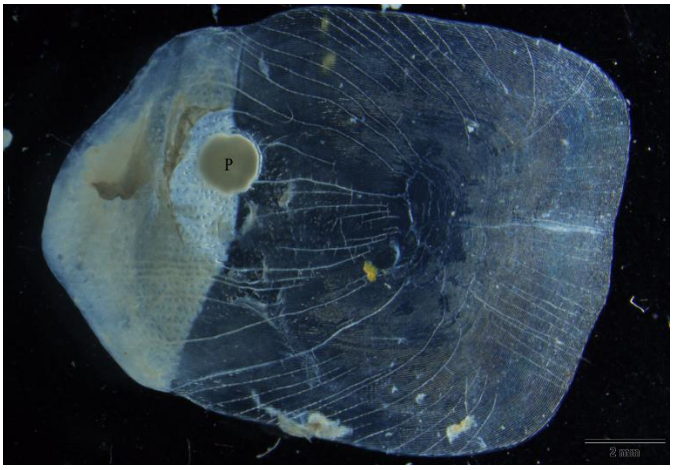

Fig. 22. *Myxobolus bandyopadhyayi* n. sp. plasmodium (P) in the scale of a rohu. Mount picture. Bar = 2 mm

Description of myxospores

Myxospores (Fig. 23 a, b, c) small sized, roundish or short ellipsoidal in frontal view and lemon shaped in sutural view. Myxospores 8.4 ± 0.19 (8.3-8.8) long, 6.6 ± 0.54 (5.2-7.5) wide and 4.6 ± 0.4 (4.3-5.4) thick. Two polar capsules short pyriform, different in size. The larger 4.2 ± 0.38 (3.7-4.5) long, and 2.5 ± 0.18 (2.2-2.7) wide, the smaller 2.3 ± 0.4 (1.7-2.8) long and 1.65 ± 0.05 (1.6-1.7) wide. Polar tubules not seen. Myxospore with approximately 1.2 long, triangular, eccentrically located intercapsular appendix. Sporoplasm nuclei indiscernible. A small distinct iodinophilous vacuole present in the sporoplasm. Mucous envelope not found. The thickness of the myxospore wall 0.65 ± 0.05 (0.6-0.7). Nuclei of the polar capsules distinct.

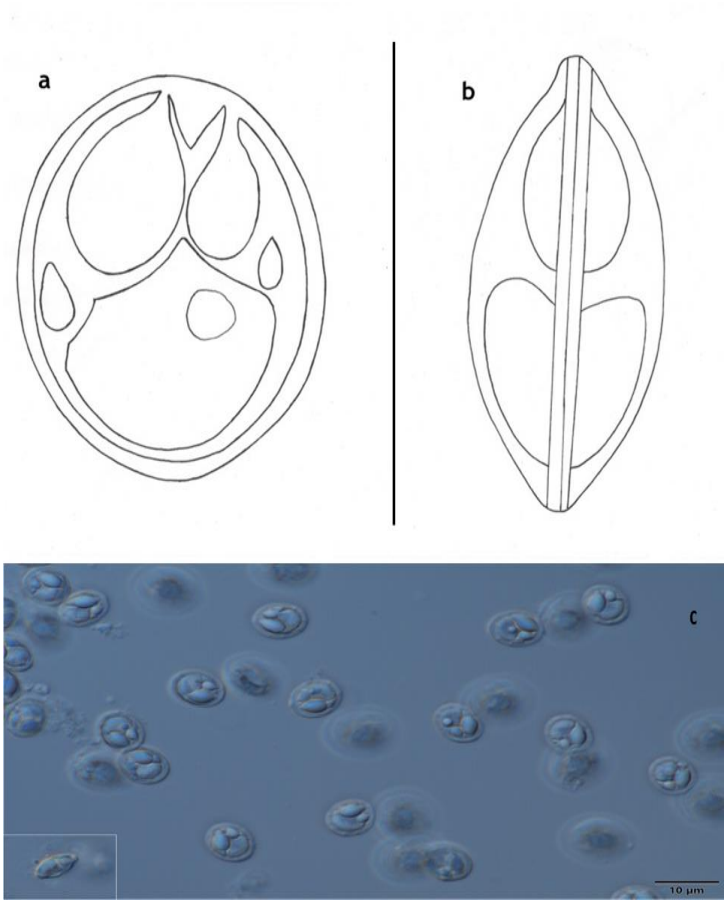

Fig. 23 Schematic drawing of spore of *Myxobolus bandyopadhyayi*, a: frontal view, b: sutural view. Bar = 10 µm, c: *M. bandyopadhyayi* n. sp. spores of a rohu in frontal view. Inset: A spore in sutural view. Mount picture. Bar = 10 µm

Type host: Rohu, *Labeo rohita* Hamilton

Type locality: Naihati, Battala market (22.89°N 88.42°E) (on kalyani expressway)

Site of development: Scale

Type material: Photo-types and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *M. bandyopadhyayi* was deposited in the GenBank under accession number MZ230380.

Prevalence of infection: 3 specimens from 12 fish.

Molecular data: ssrDNA sequences of *M. bandyopadhyayi* (MZ230380) collected from the scales of rohu significantly differed from any myxozoan species deposited in the GenBank. This species was located as a single species in a distinct clade, the highest similarity to other myxozoan species was 83.9% in the relation of *Myxobolus chakravartyi* (Fig. 21).

Remarks: By the short oval shape of its spores, by the different sized but short ellipsoidal polar capsules, this species differed from most species known from major Indian carps. Of the *Myxobolus* species with two different sized polar capsules *M. buccoroofus* Basu and Haldar, 2004 had dissimilar myxospore shape. Polar capsules of *M. calbasui* Chakravarty, 1939, *M. chilkensis* Kalavati et al., 1992 and *M. bhadrensis* Seenappa and Manohar, 1980 had elongated shape. Myxospores of *M. edellae* Sarkar, 1999 are also nearly spherical, but the difference between its two polar capsules is only moderate.

Etymology: The species is named after Prof. Probir K. Bandyopadhyay, the well-known fish parasitologist, who managed this recent cooperation from the Indian side and actively worked until his death in 2021 January.

4.1.1.7 *Myxobolus chakravartyi* Haldar, Das, Sharma, 1983

Morphological records

Out of the 9 catla specimens purchased in the fish market 4 were infected with small ellipsoidal shape plasmodia reaching a size of 200-300 in length and 150-200 in width and attaching to the cartilaginous fin rays (Fig. 24). Plasmodia contained 2000 to 8000 myxospores.

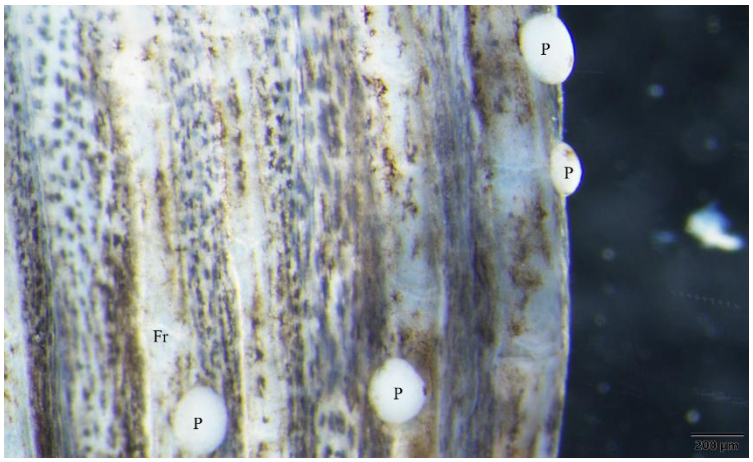

Fig. 24 *Myxobolus chakravartyi* plasmodia (P) in the fin of a catla in close contact with fin rays (Fr). Mount picture. Bar = 200 µm

Description of myxospores

Myxospores (Fig. 25 a, b, c) medium sized, roundish or short ellipsoidal in frontal view and lemon shaped in sutural view having a small conical, 0.5-1 long protuberance at the anterior end. Spores 12±0.38 (11.6-12.6) long, 10 ±0.45 (9.4-10.7) wide and 6 ± 0.36 (4-6.2) thick. Two polar capsules short pyriform, different in size. The larger 5.7 ± 0.36 (5.4-6.2) long, and 4.1 ± 0.61 (3.5-5) wide, the smaller 3.6 ± 0.15 (3.5-3.8) long and 2.2 ± 0.1 (2.1-2-3) wide. Polar tubules not seen. Myxospore with a large, triangular, eccentrically located intercapsular appendix. Sporoplasm nuclei indiscernible. A small iodinophilous vacuole found in the sporoplasm. Mucous envelope not found. The thickness of the spore wall (which corresponds to the emerging collar of the suture) 1 ± 0.16 (0.8-1.2). Some crippled myxopores (Fig.) bear *Henneguya* like tails of 4 to 5.

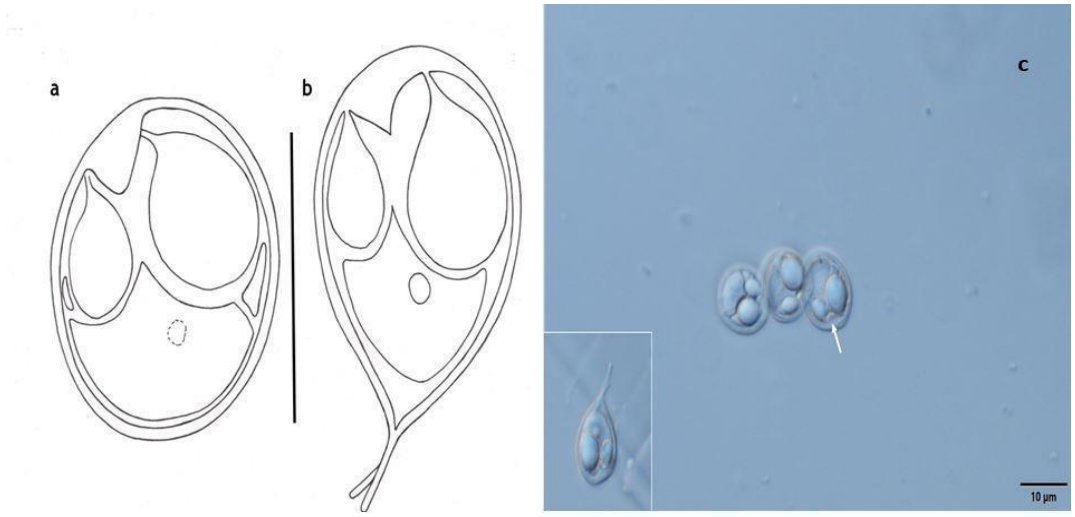

Fig. 25 Schematic drawing of spore of *Myxobolus chakravartyi*, a: frontal view, b: frontal view of deformed spore with caudal extension. Bar = 10 µm, c: *M. chakravartyi* myxospores from the fin of a catla. Arrow is indicating the charasteristic intercapsular appendix. Inset: deformed myxospore with the *Henneguya* like caudal extension. Mount picture. Bar = 10 µm

Host: Catla *Gibelion catla* (Hamilton)

Locality: Kakinara, Bhatpara market (22.51°N 88.23°E), on Kalyani expressway

Site of infection: fins.

Prevalence of infection: 4 specimens from 9 fish

Type material: Photo-types and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *M. bandyopadhyayi* was deposited in the GenBank under accession number MZ230377.

Molecular data: ssrDNA sequence of *Myxobolus chakravartyi* (GenBank accession number: MZ230377) was closest to the sequences of *Myxobolus auratus* (KX399851) and several *Thelohanellus* sp. samples (KP030765, KX881785, KY131789). The sequence similarities fell within the range between 93.4 and 94.7%. Sequences of *Thelohanellus caudatus* showed a similarity between 92.9-94.1% (Fig. 21)

4.1.1.8 *Myxobolus rewensis* Srivastava, 1979

Morphological records

3 fish of the 11 mrigal specimens purchased in the fish market were infected with small ellipsoidal shape plasmodia reaching a size of 1-2.2 mm in length and 0.5-0.7mm in width, which were located in the fins between two fin rays close to the base of the fin. (Fig. 26). Plasmodia contained 2000 to 10000 myxospores.

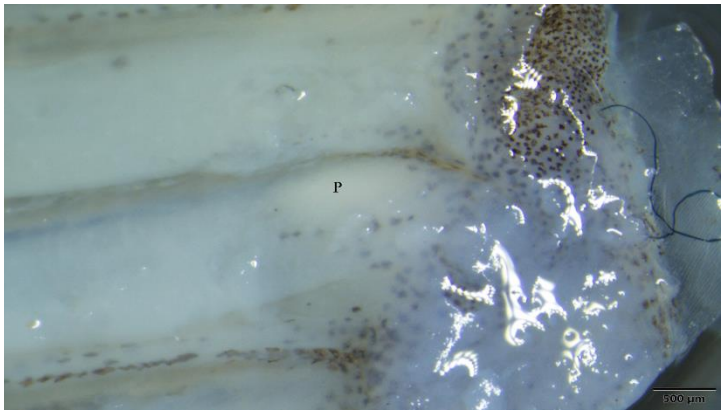

Fig. 26 *Myxobolus rewensis* plasmodium (P) in the fin of a mrigal. Mount picture. Bar = 500 µm

Description of myxospore

Myxospores (Fig. 27 a,b,c) small sized, ellipsoidal in frontal view and lemon shaped in sutural view, 8.8±0.16 (8.5-9.1) long, 7.3 ±0.27 (6.8-9.7) wide and 5.2 ± 0.22 (5-5.4) thick. Two oval polar capsules equal, 3.8 ± 0.19 (3.4-4) long, 2.1 ± 0.14 (2.1-2.3) wide. Polar tubules not seen. Nuclei of the polar capsules distinct, measuring 1.7x0.6 in the mean. Myxospore with a rounded, about 0.9-1 thick intercapsular appendix in which a bright globule with a diameter of 0.8 located. Sporoplasm nuclei indiscernible. A small iodinophilous vacuole present in the sporoplasm. Mucous envelope not found. The myxospore wall (which corresponds to the emerging collar of the suture) has 8 sutural edge markings. Its thickness, 0.66 ± 0.05 (0.6-0.7).

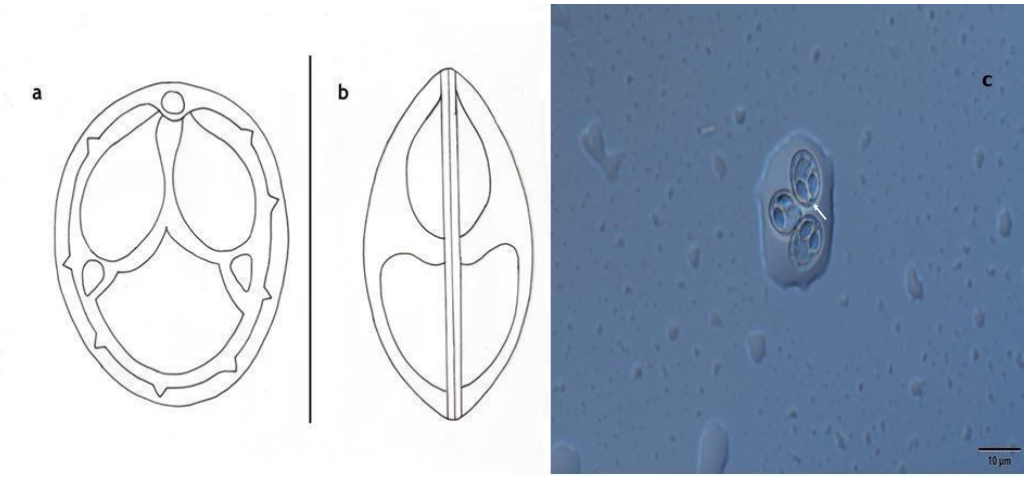

Fig. 27 Schematic drawing of myxospore of *Myxobolus rewensis*, a: frontal view, b: sutural view. Bar = 10 µm, c: Myxospores of *M. rewensis* from the fin of mrigal. Arrow is indicating the characteristic globule inside the intercapsular appendix. Mount picture. Bar = 10 µm

Host: Mrigal, *Cirrhinus mrigala* Hamilton

Locality: East Kolkata Wetlands 22.5263°N 88.4716°E (District South 24 parganas, Baranagar Block).

Site of infection: Fins

Type material: Photo-types and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *M. rewensis* was deposited in the GenBank under accession number MZ230381.

Prevalence of infection: 3 specimens from 10 fish.

Molecular data: ssrDNA sequence of *Myxobolus rewensis* was a sister group of *Myxobolus dermiscalis* showing a 91.9% similarity. There was also a 90.1% similarity to a *Thelohanellus* sp. sample (KM401440) from the skin and gill arch of rohu (Fig. 21).

4.1.1.9 *Thelohanellus caudatus* infection of Rohu

Morphological records

Thelohanellus infection on the fins was found in 4 rohu specimens from 13 fish obtained from the Diara fish farm, while 3 infected specimens were observed among the 12 fish purchased on the fish market. Plasmodia (1 to 14 cysts) were recovered from the tail fins and the anal fins. Relatively small elliptical shape plasmodia (Fig. 28) of 1 to 2 mm long and 0.5-1 mm thick, locating at the proximal parts of the fins, close to the body attached to the cartilaginous fin rays.

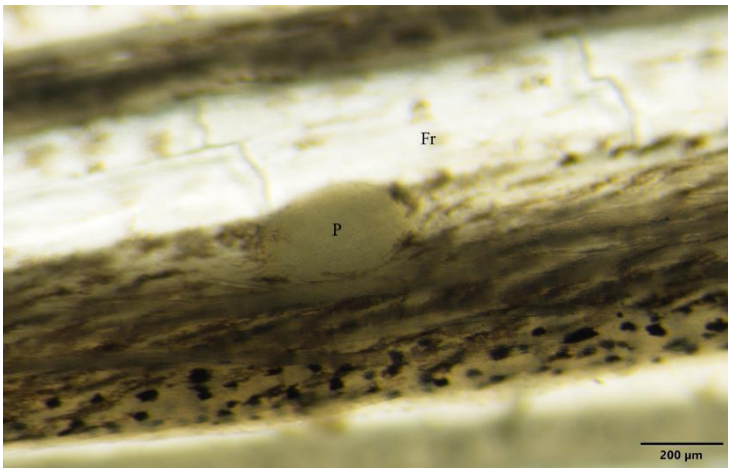

Fig. 28 A plasmodium (P) of *Thelohanellus caudatus* in the fin of a rohu in close contact with the cartilaginous fin ray (Fr). Mount picture. Bar = 200 µm

Description of myxospore

Mature myxospore (Fig. 29 a, b, c) pyriform, with slightly tapering anterior and round posterior end, 13.7 ± 0.93 (13.3-14.6) long (n = 25), 9± 0.82 (7.5-9) wide (n = 25) and 6.8 ± 0.41 (6.2-7.3) thick (n = 15). The wall relatively thick 1±0.2 (0.7-1.1). Single short ellipsoidal polar capsule present close to the apex of myxospore, 6.5±04 (6.2-6.9) (n = 25) long, 4.7 ± 0.47 (4.2-5) (n =25) thick. Polar tubules not seen. At one side between the polar capsule and the sporoplasm a bright nucleus of the capsulogenic cell, about 1x1.5 present. No iodinophilous vacuole or mucous envelope seen.

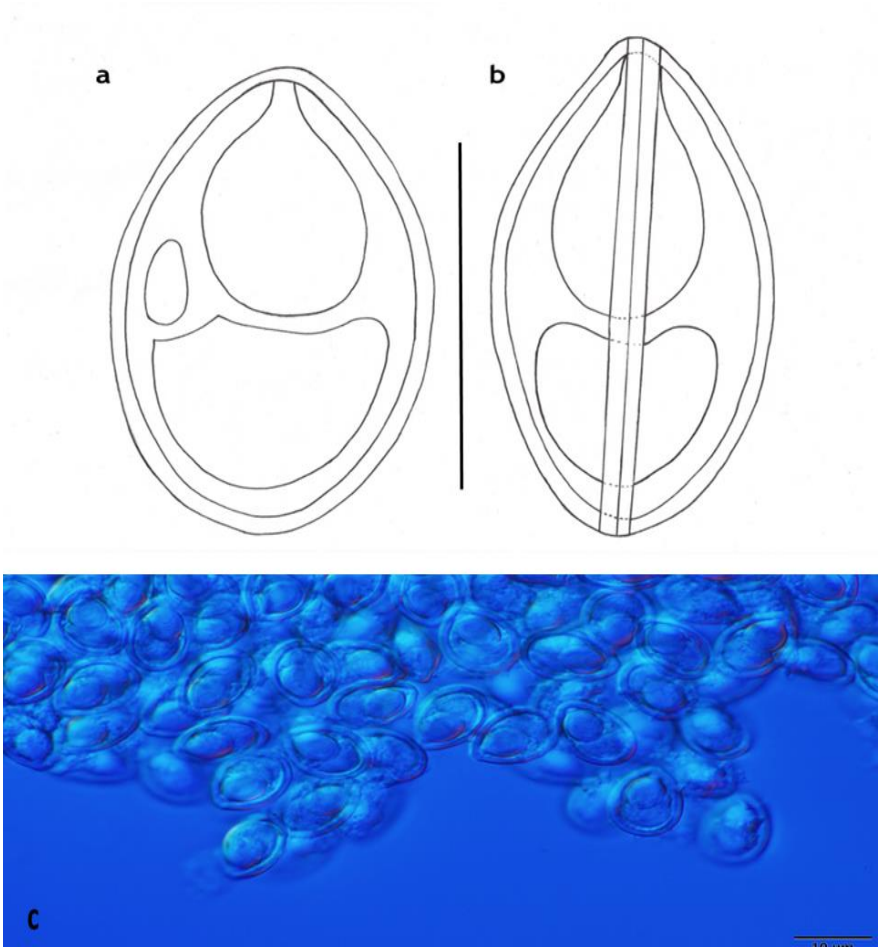

Fig. 29 Schematic drawing of myxospore of *Thelohanellus caudatus*, a: frontal view, b: sutural view. Bar = 10 µm, c: Myxospores of *T. caudatus*. Mount picture. Bar = 10 µm

Host: Rohu, *Labeo rohita* Hamilton

Locality: Diara Fish Farm (Hooghly District) Fish pond and hatchery (22°28′31″N 88°09′54″E) and Fish market Naihati, Battala market (22.89°N 88.42°E) (on kalyani expressway)

Site of infection: Fins

Material: Photo-types were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *T. caudatus* was deposited in the GenBank under accession numbers MZ230375, MZ230376 and MZ230379.

Prevalence of infection: 7 specimens from 25 fish.

Molecular data: ssrDNA sequences of *T. caudatus* (MZ230375-6 and MZ230379) collected from the fins of rohu were identical with each other and showed a 99.8 % similarity with *T. caudatus* sample deposited in the GenBank as KM252684. However, with the other deposited sequences (MK412941) only 98.8% similarity was found. Interestingly enough also 99.0% similarity was received with the sequence (KM252683) of *T. habibpuri* Acharia and Dutta, 2007. Interestingly, there is also another *T. caudatus* clade (KC865607, KF583877, and KJ476877) with maximum bootstrap distinctly from the above mentioned sequences. Genetic similarities from our samples are 87.3% in the case of two sequences (KF583877, KJ476877) and 71.1% in the case of KC865607 (Fig.21).

4.1.2 Discussion

The myxozoan species were exclusively identified based on the morphology and size of myxospores for a long time. Some authors including Lom and Dyková (1992), Molnár (2002), Dyková and Lom (2007) and Molnár and Eszterbauer (2015) suggested that the examination of host, organ and tissue specificity would be a useful tool in the appropriate identification of the given species. In 1999, Andree et al., suggested using molecular methods for the identification of myxozoan species and for describing new species, DNA sequences (especially ssrDNA) have become a very important addition to the taxonomy of Myxozoa. Unfortunately, molecular sequences and histological data are not available for several myxozoan species described in the past. Descriptions very often lack the location of plasmodial stages and only the shape and size of the myxospores are available. Moreover, it is known that spores are often carried by the bloodstream to different organs, therefore, the origin of myxospores can be misleading regarding the place of infection (Molnar, 2007; Molnar et Eszterbauer 2015)

Concerning studies on Indian *Myxobolus* and *Henneguya* species, Fish Pathology and Parasitology Research Team in Hungary was among the first to use molecular techniques for complete identification and this research in each case was supported by histological evidence.

Among the most studied myxozoan genus, *Henneguya* spp. are important disease agents in both wild and cultivated fish, with a considerable increase in the number of novel *Henneguya* species identified recently (Eiras and Adriano, 2012). Kalavati and Nandi (2007) reported 23 *Henneguya* species from Indian fishes. Five myxobolids are known from the host, *Notopterus* spp.: three *Henneguya* spp. (*H. ganapatiae, H. notopterae, H. singhi*) and two *Myxobolus* (*M. meglitschus* and *M. notopterum).* As we only encountered one of the *Henneguya* spp. recorded

from gills of the bronze featherback, we were unable to test the validity of the other two species with either a redescription of morphology or the addition of molecular data. We confirmed that the species we found, *H. ganapatiae*, shows morphometric differences to *H. notopterae* and *H. singhi* in polar capsules and spore body. However, no sequence data were available from *Henneguya* spp. from *N. notopterus* in India, for comparison. Phylogenetic analysis showed that *H. ganapatiae* was most similar (90.7%) to three *Henneguya* species from gill filaments, gill lamellae and muscles, respectively, of *Lates calcarifer* from Malaysia, and *Henneguya chaudharyi* (89.4%) from gill filaments of *Channa punctatus* from India.

Due to difficulties obtaining live infected *N. notopterus*, we were unable to prepare histological sections and thus could not study developmental stages or the specific site preference of *H. ganapatiae* on the gills; both of which are important non-molecular characters for species descriptions. We identified the myxospores as, *H. ganapatiae* Quadri, 1970 on the basis of the host, geographic locality, tissue tropism and similarity of myxospore morphology and morphometry.

Up to this time, three *Henneguya* species are recovered from the genus *Mystus* in India *viz. H. mystusia* Sarkar, 1985, from the gills of *Mystus sp., H. mystasi* Haldar, Samal and Mukhopadhyay, 1997 and *H. bleekeri* Haldar et Mukherjee, 1985 from the gills of the *M. gulio* and *M. bleekeri* respectively. The sequences of two *Henneguya* species are known from Indian fishes, *H. bicaudi* recovered from *Cirrhinus mrigala*.(Kaur and Attri, 2015) and *H. chaudharyi* recovered from *Channa punctatus* (Chaudhary et al., 2017). Presented *Henneguya* species, based on morphological characters and the close phylogenetic relationship of fishes within the genus *Mystus*, myxospores collected from the gills of *M. vittatus* were identified as *H. mystasi* known before only from the gills of *M. gulio* Hamilton 1822. It is known that several myxozoan species are host-specific. A few studies, however, indicate that some *Myxobolus* species might also infect closely related hosts belonging to the same genus or members of a tribe, including closely related fishes (Cech et al. 2012; Molnar et al., 2011). *H. mystasi* had the closest similarity to *H. bicaudi*, a species described from an Indian cyprinid fish (92.5%). Surprisingly, it was also similar (95%) to *H. pellucida*, a parasite infecting a characid fish known from South America. Hence, *H. mystasi* can be distinguished from other available *Henneguya* sequences by its ssrDNA data on Genbank.

Myxobolus Bütschli, 1882 is the largest genus in the phylum Myxozoa (Eiras et al. 2005, 2014). Kaur and Singh (2012) described 130 species in the synopsis of *Myxobolus* from Indian fishes. Consequently, the number of described *Myxobolus* species has been increasing. (Basu et al.

2009; Kaur et al. 2008; Kaur et al. 2012; Rajesh et al. 2014; Szekely et al. 2015; Ghosh et al.2017). Six *Myxobolus* species have been identified from the genus *Channa* in India, *M. cylindricus* Sarkar, Mazumdar, Pramanik, 1985 emend. (Gupta and Khera, 1988) from *C. gachua* Hamilton, *M. maruliensis* Sarkar, Mazumdar, Pramanik, 1985 from *C. marulius* Hamilton; *M. noblei* Sarkar, 1982 from *C. striata* Bloch and *M. aligarhensis* Bhatt and Siddiqui, 1964; *M. andhrae* Lalitha Kumari, 1969 and *M. channai* Kalavati, Sandeep and Narasimhamurti, 1981 emend. Gupta and Khera (Kalavati and Nandi, 2007) from *C. punctata* Bloch (Gupta and Khera, 1988; Kaur et Attri, 2015). In our study, *Myxobolus* spores were identified as myxospores of *M. cylindricus* in the gills of *Channa gachua*, although the original authors detected the spores of this parasite in the kidney of the host. Due to the lack of molecular analysis in the original species description, sequence comparison could not be done but based on the morphological similarities of the myxospores and the identical host species, we are regarding the parasite as *M. cylindricus*. Plasmodial developmental site is not described by Sarkar et al. 1985, and regarding, that myxospores migration to the kidney by bloodstream can easily occur from different organs (Molnar 2007; Molnár and Eszterbauer 2015), our findings are not inconsistent with the taxonomical important organ or tissue specificity of the myxozoans.

Currently, there is no data available for myxozoan parasites of *Ompok pabda* catfish in India. The present study is the first record for a *Myxobolus* species infecting the Pabda catfish. *Myxobolus ompok* n. sp. infecting the kidney of the *O. pabda*, differs in spore morphology from the already known Indian species, and the ssrDNA sequences available in the nucleotide sequence database are also different. Molnár (2007) differentiated two major types of kidney infection caused by myxozoans: In the first case, plasmodia containing several hundreds of spores develop in the renal interstitium, more probably inside the lumen of a capillary. Similar cysts might, however, be formed inside the melano-macrophage centers, where macrophages accumulate spores carried there by the bloodstream. In the latter case, the presence of macrophages among spores and the formation of melanin pigments can be observed regularly. Although in the infection detected by us, young developmental plasmodia were not seen, the lack of macrophages and the accumulation of pigments suggested that plasmodia developed in the renal tissues, and thus, the kidney should be regarded as a major location of *M. ompok* n. sp. The location of plasmodia in the interstitial tissue of the kidney resembles those of *M. erythrophthalmi* and *M. shaharomae* (Molnár et al. 2009). These latter species, however,

formed plasmodia in the liver and testis and the intestine as well. More studies are required for this species whether it is kidney-specific.

The molecular analysis of two isolates of the present species suggests that *M. ompok*, together with *M. miyarii*, form a distinct clade of *Myxobolus*. The clade, composed of *M. ompok* n. sp. and *M. miyarii* is a sister to some *Henneguya* species described from genetically closely related fishes. This type of phylogenetic affinity between *Myxobolus* and *Henneguya* parasites have also been reported by previous workers (Adriano et al. 2012; Carriero et al. 2013; Moreira et al. 2014). The taxonomic division between the genera of *Myxobolus* and *Henneguya* was based on the presence of the caudal process in *Henneguya* species, which is absent from *Myxobolus* species. The phylogenetic analysis studied here grouped both genera together, which gives support to the work done previously (Kent et al. 2001; Liu et al. 2010).

The *Myxobolu*s spp. infecting major carps in India (*Labeo, Cirrhinus* and *Catla*) are well represented by 20 species from the genera *Labeo* and *Cirrhinus* and by 10 species from the genus *Gibelion* (Catla). Since their synopsis, this number increased at least with 20 new species (Eiras et al. 2021). Unfortunately, the majority of these species has been poorly described, they are characterized only by measurements and by the shape of spores and polar capsules, providing less information on the location of plasmodia in infected tissues. The validity of a few species is recently supported with molecular biological data (Szekely et al. 2015; Chaudhary et al., 2018, 2019; Gupta et al., 2018).

Ahmad and Kaur (2018) studied the prevalence, site, and tissue preference of fingerlings of major carps in the same fish farm and concluded that the 10 investigated, *Myxobolus* species developed in three different fish species (*C. mrigala, L. rohita, G. catla*) and in each case showed a typical site preference producing in most cases intralamellar gill infections. Our present survey is in agreement with their results as host, organ and tissue specificity is observable in all cases.

The myxospore species collected for this study was placed next to already sequenced species like *Myxobolus dermiscalis* or *Thelohanellus caudatus* from *Labeo rohita.* In the case of *Myxobolus dermiscalis,* the morphology and sequence data from previous studies (Kaur et al., 2016) corresponded to our results, its identification raised no doubts at all.

Other species, like *Myxobolus chakravartyi* from *G. catla* and *M. rewensis* from *C. mrigala* were placed separately from other species, as these species had no sequence data up to this point. *M. chakravartyi* is mostly surrounded by *Thelohanellus* sequences with the exception of *Myxobolus auratus* (KX399851), however the annotation of this *Myxobolus* sample lacks a lot

of details like host species, isolation source etc., Moreover publication data are also missing. The position of M. *chakravartyi* between *Thelohanellus* samples was not unusual as *Thelohanellus* and *Myxobolus* species do not form distinct groups in phylogenetic analyses, *Thelohanellus* species are scattered between the *Myxobolus* species (Shin et al., 2014).

Myxobolus rewensis was placed next to *Myxobolus dermiscalis* on the phylogenetic tree. Other known parasites of mrigal, like *M. catlae* and *M. kalavatiae* are placed in a different clade. These latter species infect the gill lamellae, unlike *M. rewensis* which occurs on the fins. Interestingly, *Myxobolus bandyopadhyayi* n. sp. from *Labeo rohita* occupied a separate position on the phylogenetic tree, there were no other species in close relation to it. *M. bandyopadhyayi n. sp.* could be a representative of a separate lineage inside the myxobolid clade, further molecular data of Indian myxozoans might reveal closer relatives of this species.

Kaur et al. (2017) reported 52 species of genus *Thelohanellus* Kudo, 1933 from freshwater fishes in India. *Thelohanellus* species possess very strict host and tissue specificity, moreover, some species occur exclusively in carp fishes (Akhmerov 1955, 1960). Various species of *Thelohanellus* were recovered from *Labeo rohita* in India such as *T. anilae*, *T. dykovi*, *T. filli*, *T. imphalensis*, *T. rohitae*, *T. bifurcate* from gills*; T. caudatus* from between the rays of caudal fin and anal fin*; T. chilkensis* from gall bladder*; T. endodermitus* from under the surface of scales*; T. habibpuri* from pectoral fin*; T. rohi* from caudal fin*; T. avijiti* from dorsal fin; *T. muscularis* from head muscles (Kaur et al. 2017*).* The present study found that the *T. caudatus* samples were morphologically similar to the originally described species by Pagarkar and Das in 1993.

However, phylogenetic analysis of *Thelohanellus caudatus* samples are grouped into two distinct clades. Unfortunately, some of the sequences forming one clade, do not have available publications under the annotation, therefore the morphology of those samples and their species identity in regard cannot be verified. The other clade included our samples (MZ230375, MZ230376 and MZ230379) and *Thelohanellus caudatus* samples (KM252684, MK412941) and *Thelohanellus habibpuri* (KM252683). The identity of MK412941 seems to be well supported as the related article by Mondal et al. (2014) includes morphological observations besides the sequence data. The other *T. caudatus* sample in this clade (KM252684) together with *T. habibpuri* (KM252683) lack a published research article, however, KM252684 (*T. caudatus*) having the same species identity raises no question to be answered. On the other hand, the presence of *T. habibpuri* (KM252683) can be explained by the assumption that this

species can be referred to as a junior synonym of *T. caudatus*. The incomplete description of *T. habibpuri* by Acharia and Dutta (2007) resembles morphological characteristics highly similar to *T. caudatus*.

More than 150 species of the family Myxobolidae have been described in India from fishes up to now; however, only a small percentage of them have sequence data, and numerous species even lack important morphological characteristics such as the location and size of plasmodia. In recent years, molecular analyses for myxozoa have become more and more widely used in India and an increasing trend can be observed in this field. Therefore, several species re-descriptions, revisions, and new descriptions can be expected in the coming years. These sequence data and additional morphological observations, such as histological methods will improve our knowledge about the Indian myxozoan fauna (Chaudhary et al. 2019).

4.2 Myxozoan samples from Hungary

4.2.1 Results

During the study of myxozoans in Hungary, *Myxobolus dechtiari* was detected in its natural fish host, with North American origin, *Lepomis gibbosus*. This was the first detection of this parasite in Hungary, and we provided the first sequencing data about it. Two *Thelohanellus* species (*T. pyriformis* and *T. fuhrmanni*) were determined from *Tinca tinca* and *Chondrostoma nasus* respectively. This study revealed the first molecular genetic data of both species. Moreover, *Myxobolus pseudodispar* spores were collected from the muscles of *Rutilus rutilus* to perform the infection trial by applying plant-derived drugs treatment experiments on the actinospores from the parasite. The summary of the samples examined in Hungary are listed in Table 6. The detailed descriptions of the species are presented in the following subchapters.

Table 6. Descriptive data of collected samples from Hungary

Host Fish	**Myxozoan infection**	**Infection site**	**Infection intensity**	**GenBank Accession number**
Lepomis gibbosus	*Myxobolus dechtiari*	Cartilagenous gill rays of filaments	19%	MW588907, MW588908
Tinca tinca	*Thelohanellus pyriformis*	Arteria brachialis efferents	14%	Not obtained yet
Chondrostoma nasus	*Thelohanellus fuhrmanni*	Under the skin of snout	16%	Not obtained yet

4.2.1.1 *Myxobolus dechtiari* Cone and Anderson, 1977

Morphological records:

Plasmodia were observed (Fig. 30) from cartilageous gill rays of the *Lepomis gibbosus*. The observed myxospores corresponded in shape and size to myxospores described by Cone and Anderson (1977a, b). Following these findings, pumpkinseeds were more carefully studied also in Hungary, but only a single specimen of two examined fish proved to be infected in July 2009. This specimen, which was collected from the Egervíz Creek, was infected with some plasmodia containing matured myxospores. Further plasmodia were found only in three fishes of the examined 20 specimens collected from the Sió channel in 2018. No myxospore or plasmodia were found in pumpkinseed specimens collected in different sectors of Lake Balaton. Plasmodia

were located at the tips of the gill filaments (Fig. 30 B). In the infected specimens, 20 to 44 plasmodia were counted. At the site of plasmodial development, gill filaments was observed distorted. Myxospores found in this study corresponded in shape and size to the species *Myxobolus dechtiari* described from pumpkinseeds in Canada.

Description of myxospores

Matured plasmodia elongated in shape measuring 1.5-2 mm in length and 0.5-0.7 mm in diameter located typically at the distal regions of gill filaments (Fig. 30 (A, B)). Myxospores ellipsoidal in frontal view (Fig. 31 A, B, C) and lemon-shape in sutural view (Fig. 31 (D) inset) measured 12.5 ± 0.46 (12-13.4) (N=50) in length, 10 ± 0.37 (9.6-10.4) (N=50) in width, and 7.4 ± 0.37 (7-8) (N=10) in thickness. Polar capsules pyriform, equal in size, slightly converging anteriorly, 5.6 ± 0.21 (5.3-6) (N=50) in length, 3.2 ± 0.16 (3-3.6) (N= 50) in width. Seven to eight polar tube coils arranged perpendicular to the capsule length wounded densely in the polar capsule. A small, round, 0.4 ± 0.1 (0.3-0.5) (N=50) intercapsular appendix present in the myxospores. Sutural protrusion formed a circular rim around the myxospore emerging about 0.7 over the surface of the myxospore (Fig. 31). In sutural view both at the anterior and posterior end a 0.9 (0.8 to 1), protrusion seen. The thickness of the rim in sutural view measured about 0.5-0.7. The fresh myxospores showed 5 to 7 clear sutural edge markings. A single binucleated sporoplasm with a large iodinophilous vacuole present. Mucous envelope absent.

Host: Pumpkinseed, *Lepomis gibbosus* (L.) (Centrarchidae).

Locality: Sió channel, Hungary, City Siófok, (46° 54' 46.3" N, 17° 53' 16.8' E)

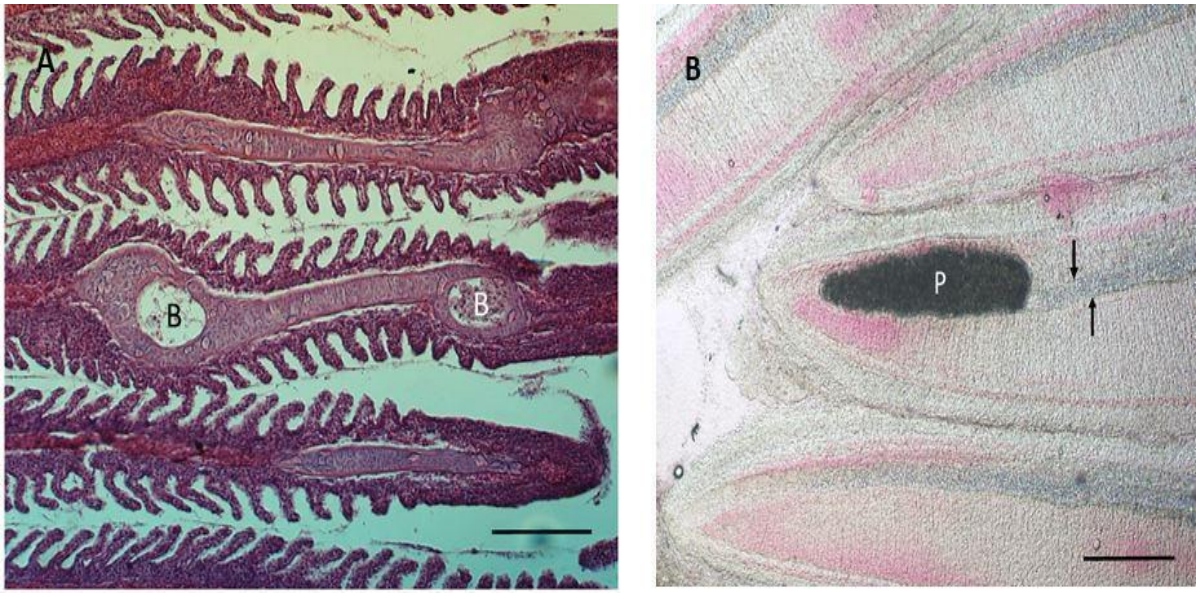

Fig. 30 Figures illustrating location of *Myxobolus dechtiari* plasmodia on gill filaments (A, B) and morphology of its myxospores. **(A)** Spore batches (B) of *Myxobolus dechtiari* enclosed into the distorted cartilaginous gill ray in a pumpkinseed H. & E., Bar =100 μm. **(B)** Close to the tip of the filament, a plasmodium (P) in continuation of the cartilaginous gill ray (arrows) is located. Fresh mount. H. & E., Bar =100 μm.

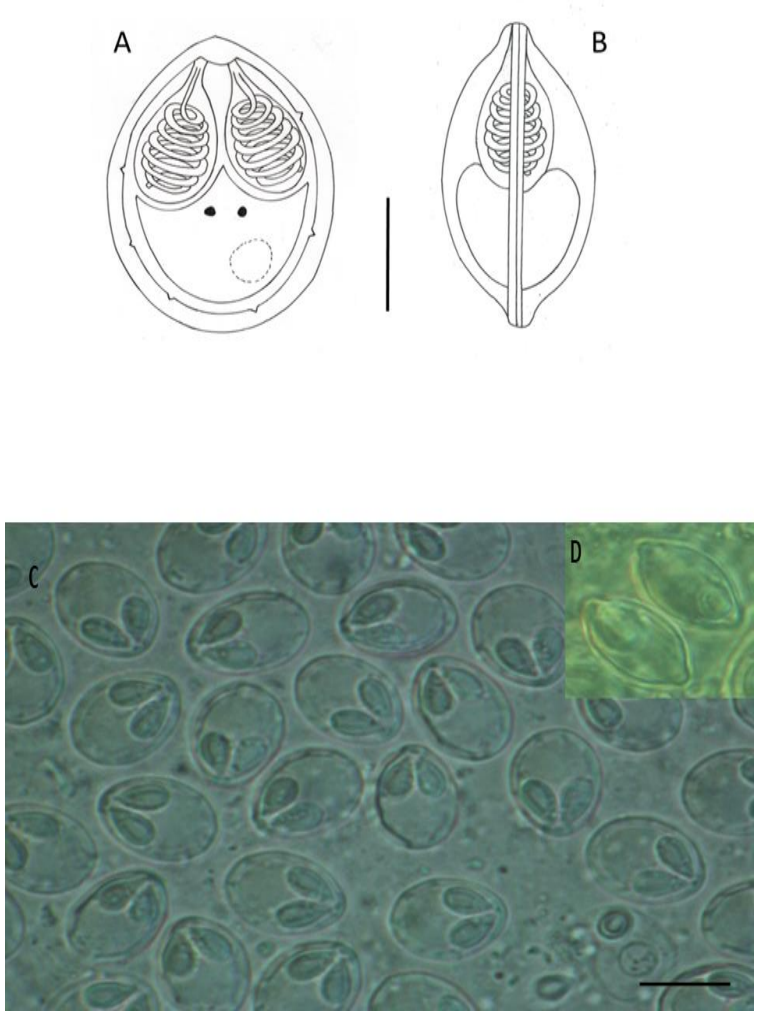

Fig. 31 Myxospores of *Myxobolus dechtiari*. A–B: Line drawings of mature myxospores in frontal and valvular view showing polar capsules with coiled polar tubules and the longitudinal grooves. Bar = 10 µm, C: Fresh, unstained myxospores in frontal view, Inset (D): myxospores of *M. dechtiari* in sutural view. Bar = 10 µm.

Additional locality: Neiva River, Portugal and Egervíz Creek, Hungary

Site of tissue development: Cartilaginous gill rays of filaments

Material: Syntype myxospores in 70% ethanol were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest, Coll. No. HNHM-PAR-20891. The ssrDNA sequence of *M. dechtiari* was deposited in the GenBank under the accession number MW588907 and MW588908.

Molecular and Phylogenetic analyses: Two isolates of ssrDNA sequences of *M. dechtiari* collected in Hungary and proceeded for molecular analysis. They were similar to each other with 99.6% similarity. BLASTn search for successfully sequenced samples against the GenBank did not reveal any known species with a similarity higher than 94% above the entire sequence lengths with the highest similarity with two actinospores species and one *Myxobolus* species: *Raabeia* sp. type 1 (94.2-94.8%, KJ152184) from *Isochaetides michaelseni, Triactinomyxon* sp. (91.3-91.8%, AF378351) from *Lumbriculus hoffeisteri* and *Myxobolus osburni* (91.1-91.3%, AF378338) from the same host fish **(**Fig. 32).

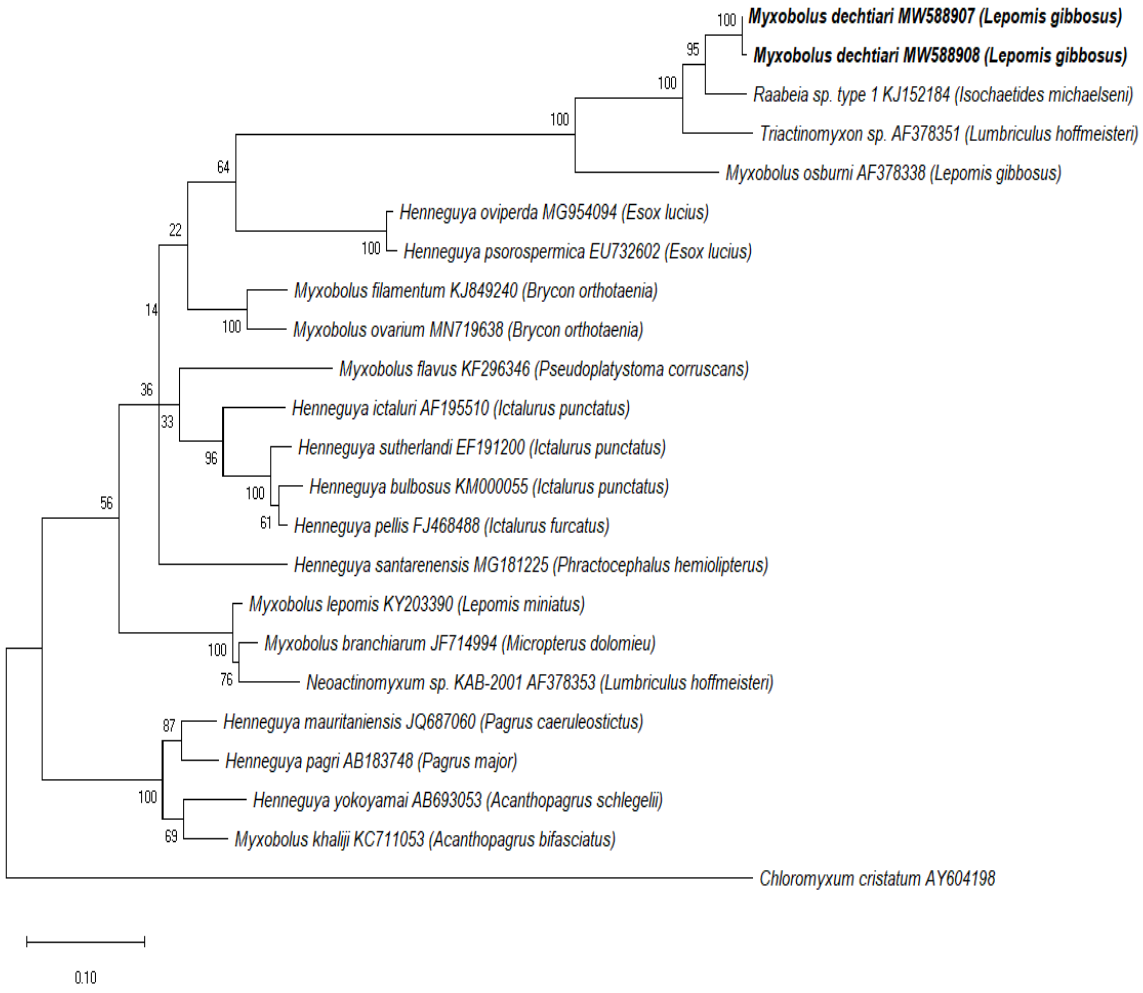

Fig. 32 Phylogenetic tree generated by maximum likelihood analysis of ssrDNA sequences of *Myxobolus dechtiari* and other closely related myxozoan species identified by BLAST; GenBank accession numbers and their host name shown after the species name. Numbers at nodes indicate the bootstrap confidence values (ML). *Chloromyxum cristatum* was used as an outgroup

Histology

Elongated shape plasmodia filled with myxospores (Fig. 33, (5)) located in the gill filaments as a continuation of the cartilaginous gill arches. At this part of the filament, the cartilaginous gill ray was not observable. In a similar way in most cases no cartilaginous elements were seen around plasmodia cross-sectioned at the central part of the plasmodia, though the cartilage of the gill rays of the uninfected filaments was clearly seen (Fig. 33, (6)). At this part, the plasmodium was surrounded by a double contoured thick eosinophil capsule. At its anterior and posterior end, the plasmodium joined to the cartilaginous gill ray so that in a short part the gradually thinning cartilage cells surrounded the plasmodium. (Fig. 33, (7 and 8)). Cross-section at the central part of the plasmodium showed that the cartilage sheet around the cyst is continuous. At larger magnification of the attenuate plasmodium wall, a three-layered structure was recognized. Covering the ectoplasm of the plasmodium, the flattened nuclei of cartilage cells were well observed (Fig. 33), while the outer wall was composed of the connective tissue sheet of the cartilaginous gill ray, indicating that only the inner wall belongs to the plasmodium. In adult cases, when plasmodia were not observed anymore, small batches of myxospores enclosed in the cartilaginous gill rays were observed. (Fig. 30). Gill rays, around the myxospore batches were thickened and became distorted. At these infected parts, gill lamellae became shorter (Fig. 30). No signs of other host reactions were recorded. The myxospores were delimited from the chondrocytes only by a thin eosinophil ectoplasm.

Remarks: The size and shape of the collected myxospores correspond to that of *M. dechtiari* as described by Cone and Anderson (1977a) and to myxospores found by Lom (1969) and tentatively identified as *M. karelicus*. We agree with Moshu (2012) who identified this parasite as *M. dechtiari*. Lom (1969) reported on the presence of a small intercapsular appendix in the myxospores. In descriptions made by Cone and Anderson (1977a) and Cone (2001), no data was presented about this appendix. The other major difference between the original description and our findings is that we identified a kind of histotropism to cartilaginous elements. We suppose that Cone and Anderson (1977b) saw a similar chondrophil location when they stated that plasmodia were among the basal cells at the distal ends of gill lamellae. Cross-sections made on plasmodia show that the plasmodium located in all parts of the filament inside the cartilaginous gill ray, but due to compression of the thick plasmodium the cartilage cells became destroyed and the plasmodium remained covered by the wall of the gill ray. ssrDNA sequences of this species prove that this species differs from sequences of all studied species. Unfortunately, no molecular data are available on *Myxobolus* species infecting centrarchid

fishes in America, but future comparisons with sequences of *M. dechtiari* parasitizing *L. gibbosus* from its original region will be important to settle taxonomic doubts.

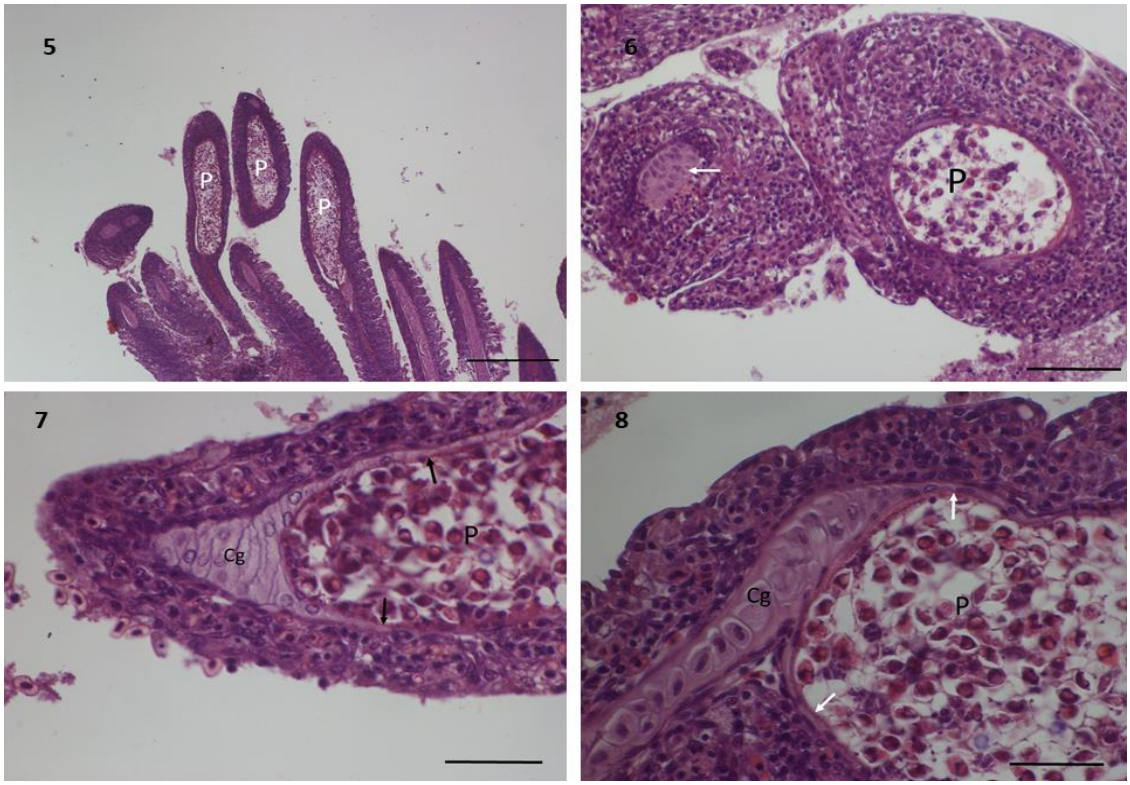

Fig. 33 Relation of plasmodia and cartilage of the gill ray. (5) *M. dechtiari* plasmodia (P) in the tip of gill filaments of the pumpkinseed. H. & E., Bar = 200 µm (6) Plasmodium (P) in a cross-section of gill filament. In the neighboring uninfected filament, the cartilaginous gill ray (arrow) is well seen. H. & E., Bar =100 µm (7) Posterior end of *M. dechtiari* plasmodium (P) close to the tip of filament. The gradually thinning gill ray surrounds the plasmodium (arrows). H. & E., Bar = 50 µm (8) Cross-section of *M. dechtiari* plasmodium (P) at its border region. Between the double membrane of the plasmodial wall some chondrocyte are seen (arrow) H. & E., Bar = 100 µm

4.2.1.2 *Thelohanellus pyriformis* Thélohan, 1892

Morphological records

Thelohanellus infection in tench was found in June 2019 in a fish of 29 cm in length caught in Lake Balaton at Balatonszemes (46°48'36.4"N 17°45'55.9"E). By inspecting hemibranchia of this fish under a dissecting microscope, small dark nodules were found in the arteria efferent, which proved to be spore-filled plasmodia of a *Thelohanellus sp.* When studying the gill filament under a compound microscope, round or oval plasmodia were located in the lumen of the efferent arteria (Fig. 35). Each of the eight filaments was infected with 2 to 8 plasmodia. By the shape and size of the spores and owing to this specific location, the species found in the type host was identified as *T. pyriformis* Thélohan, 1892.

Description of myxospore

Mature myxospore pyriform, with slight tapering anterior and round posterior end, 19 ± 0.6 (18-19.5) long (n = 50), 8.2 ± 0.5 (7.5-9) wide (n = 50) and 7.3 ± 0.3 (7-7.5 thick (n = 25). In some myxospores, the anterior end slightly bent (Fig. 34). Myxospore wall formed by 2 shell valves of equal size separated by a sutural ridge, and light microscopically showing about 0.5 in thickness. The wall at the posterior pole of the myxospore is thickened showing some nodules on its surface. Single pyriform polar capsule present close to the apex of the spore, 8.4 ±05 (7.5-9) (n = 50) long, 4.6 ± 0.3 (4-5) (n = 50) thick (Figs. 34). Polar tubules closely coiled with 9, less frequently with 10 turns, arranged perpendicular to the longitudinal axis of the capsule (Figs. 34 c). At one side of the polar capsule close to the blunt end a bright nucleus of the capsulogenic cell, about 1 in diameter present. Sporoplasm located at the posterior pole of the spore contains two round nuclei. No iodinophilous vacuole seen. At the anterior end of the spore besides the thickened polar capsule one or two bright round globules (probably the nuclei of the valvogenic cells), are located. Some of the myxospores are surrounded by a mucous envelope.

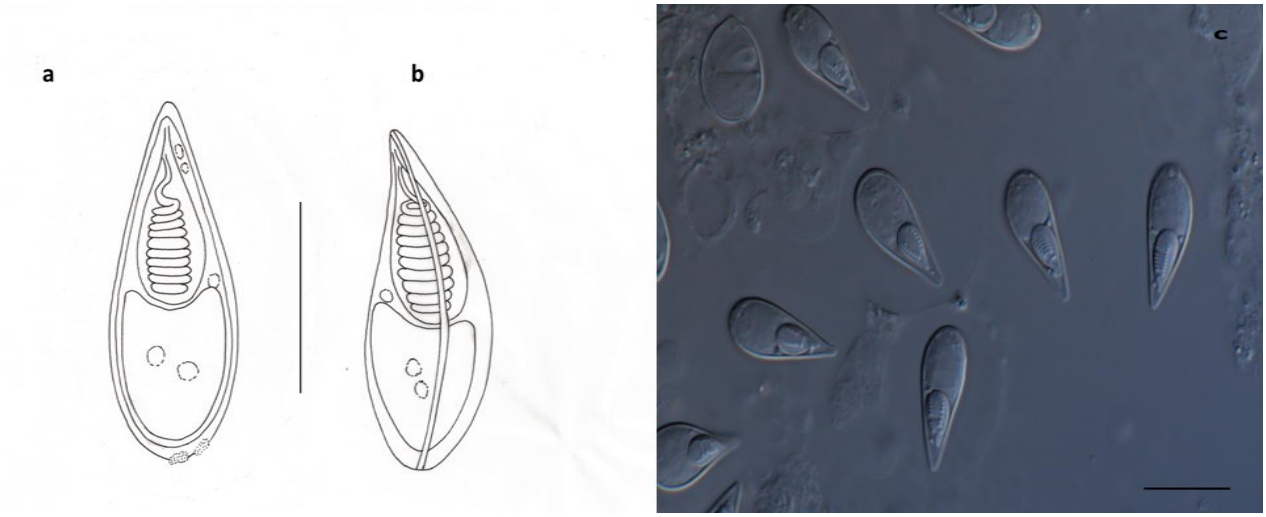

Fig. 34 Schematic drawings of the myxospores of *Thelohanellus pyriformis.* a) Myxospore in frontal view, b) Myxospore in sutural view. Bar=10 µm, c) Myxospores of *Thelohanellus pyriformis.* Mucus envelope around myxospore Bar =10 µm

Host: Tench, *Tinca tinca* L.

Locality: Lake Balaton, Hungary

Site of tissue development: Arteria brachialis efferents.

Material: Photo-types and histological preparations were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence of *T. pyriformis* will be deposited in the GenBank.

Prevalence: 1 specimen from 7 fish.

Molecular and Phylogenetic analysis

The phylogenetic analysis revealed the closest relative of this species is *T. cf. fuhrmanni* with 96.4% nucleotide identity. 95.1% similarity was found with *T. magnacysta* and 91.6% similarity was observed to a *Thelohanellus* species-2018 (MK053786) from *Cobitis paludica* (Fig. 37).

Remarks: Nodules at the thickened wall at the posterior end were regarded by Dyková and Lom (1987) as sutural notches, and seem to be better nodular growings on the wall.

Histology:

Plasmodia of *Thelohanellus pyriformis* were observed in the lumen of arteria brachialis afferents (Fig. 35). Round cysts measured 25 x 25 mm and elongated ones 25 x 160 mm. They contained from 500 to 3000 spores. Infection was detected only in arteria brachialis afferents while Arteria brachialis efferents were uninfected (Fig. 35).

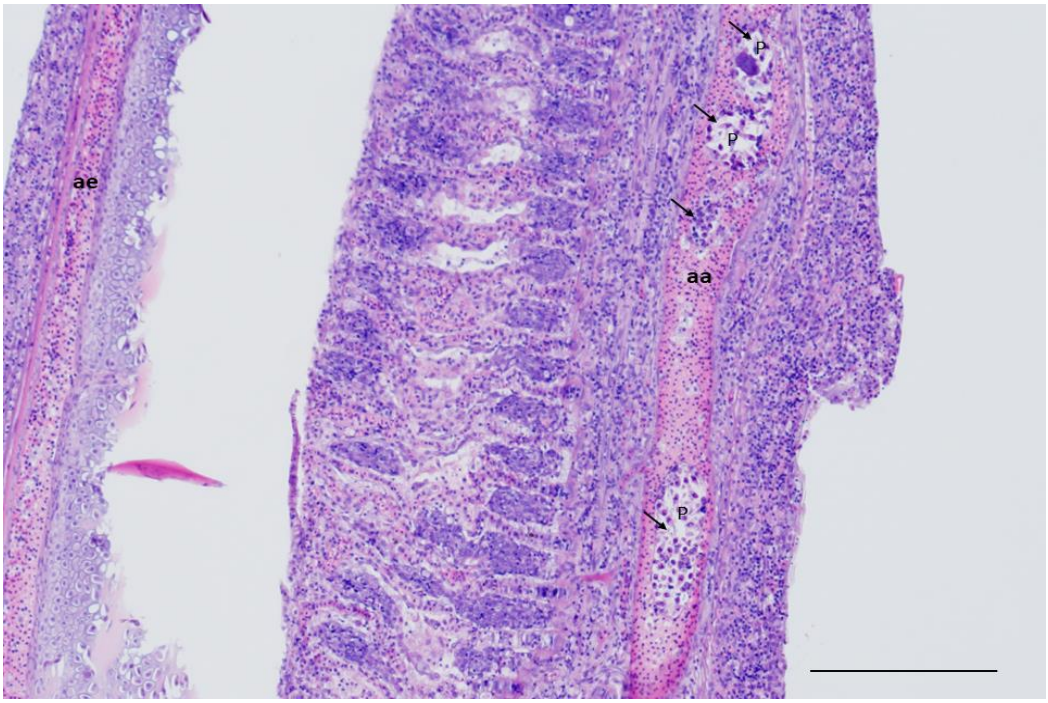

Fig. 35 Plasmodia (arrows, P) of *Thelohanellus pyriformis* in the lumen of arteria brachialis afferent (aa). Arteria brachialis efferent (ae) is uninfected. H. & E. staining. Bar=10 μm

4.2.1.3 *Thelohanellus fuhrmanni* Auerbach, 1909

Morphological records

During our long-term examinations on myxozoan infections of the common nase, *Thelohanellus* infection was found only in two cases. First, it was found in 2004 in a 34 cm long nase collected in the Danube close to the city Győr (46°48'36.4"N 17°45'55.9"E). Another case was found in 2019 when *Thelohanellus* infection was found in one of the seven nase of 31-33 cm long caught from the Danube at Nagymaros (47° 47' 16.9368" N 18° 57' 14.9256" E). In both cases, a single large, flat 4 x 4 mm size cyst was found on the snout of the specimens. The cysts were visible to the naked eye. Inside the cyst, 5-6 global shape plasmodia were located filled by about 10000 myxospores. The spores of the species resembled *T. fuhrmanni* described by Auerbach (1909). The type species of *T. fuhrmanni* located around the mouth in the connective tissue under the mucous membrane was originally found in roach (*Rutilus rutilus*).

Description of myxospore

Mature myxospore pyriform, with slight tapering anterior and round posterior end, 16.3 ± 0.39l (15.5-16.5) long (n = 50), 6.5 ± 0.55 (6.3-7) wide (n = 50) and 6.3 ± 0.53 (5.8-7) thick (n = 25) (Fig. 36 a, b). Myxospore wall formed by 2 shell valves of equal-size separated by a thick and fairly well marked sutural ridge. Light microscopically in the sutural plane the myxospore wall is about 0.5-0.6 in thick but at the posterior end, it thickens and bears some nodules on its surface. Single flask-shaped polar capsule present close to apex of myxospore, 6.5 ±08 (5.5-7) (n = 50) long, 3 ± 0.1 (3-3.2) (n = 20) thick (Figs. 36). Polar tubules coil in a single layer of 6 turns arranged in most cases obliquely to the longitudinal axis of capsule (Figs. 36). At one side, close to the blunt end of the polar capsule, a bright nucleus of the capsulogenic cell present. Sporoplasm located close to the posterior pole of the spore, contains two round nuclei. At the anterior end of the spore besides the thickened polar capsule one or two bright round globules (probably the nuclei of the valvogenic cells), are located. Sutural ridge thickened and fairly well marked (Fig. 36 c). Most of the myxospores are surrounded by a pale, oval shape on average 13.5 x 9 mm mucous envelope, which attaches to the myxospore wall at the anterior pole.

Host: Common nase, *Chondrostoma nasus* L.

Locality: River Danube, City Nagymaros, Hungary

Site of tissue development: Plasmodium under the skin of the snout.

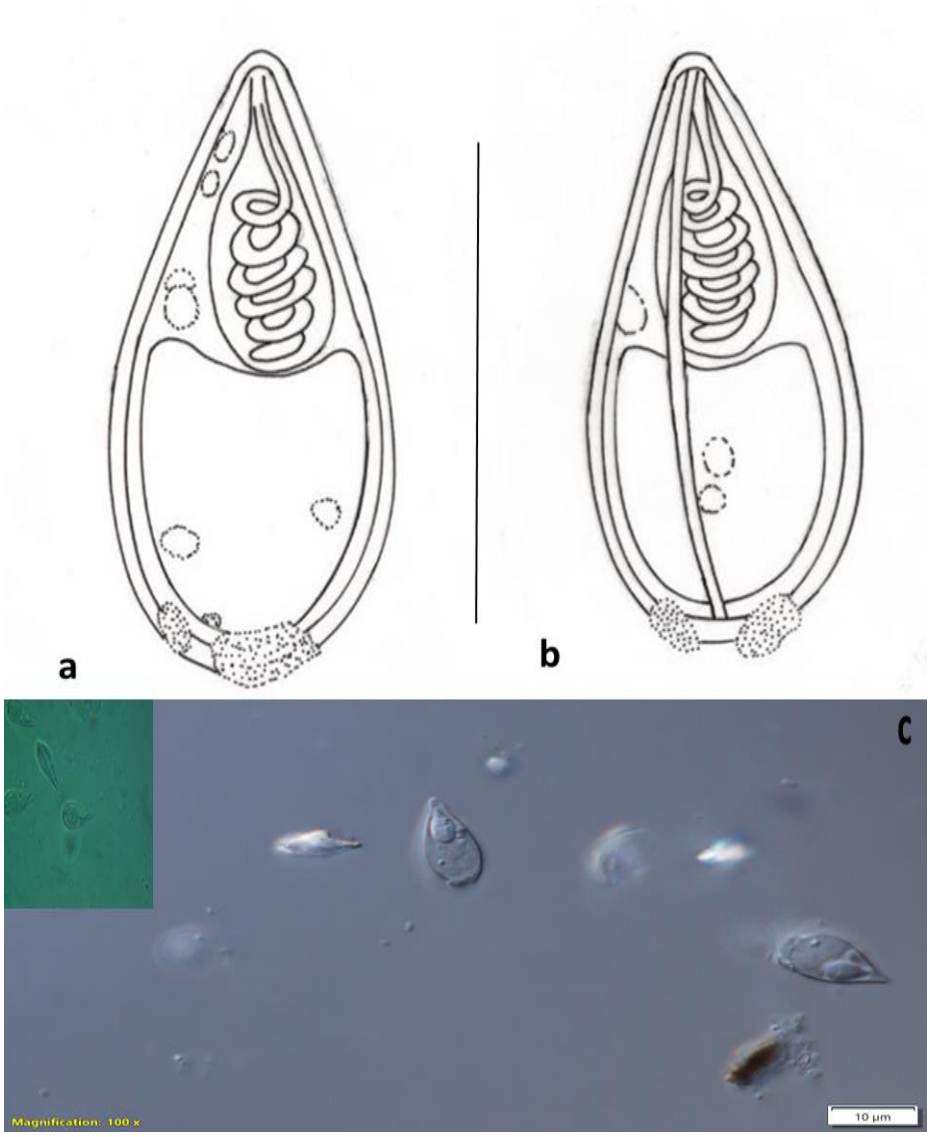

Fig. 36 Myxospores of *Thelohanellus fuhrmanni*. **a-b**: Line drawings of mature myxospores in frontal and valvular view showing polar capsules with coiled polar tubules and the longitudinal grooves. Bar = 10 µm, **c**: Fresh, unstained myxospores in frontal view, Inset: myxospores of *T. fuhrmanni* in sutural view. Bar = 10 µm.

Material: Photo-types were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest. The ssrDNA sequence will be deposited in the GenBank.

Prevalence: 1 specimen of 6 nases of 3 to 4 years old.

Molecular data: This species showed 97.6% with *Thelohanellus magnacysta* (Ksepka et al 2018) described from the somatic muscles of *Cyprinella venusta* Girard, 1856 and 96.4% similarity was found to *Thelohanellus pyriformis* sequenced by us in this study from the gill of *Tinca tinca*. 93% similarity was observed with a *Thelohanellus* sp.-2018 (MK053786), obtained from the intestinal epithelium of *Cobitis paludica* (Fig. 37).

Remarks: Nodules at the thickened wall at the posterior end found also at *T. pyriformis* are more emphasized at this species.

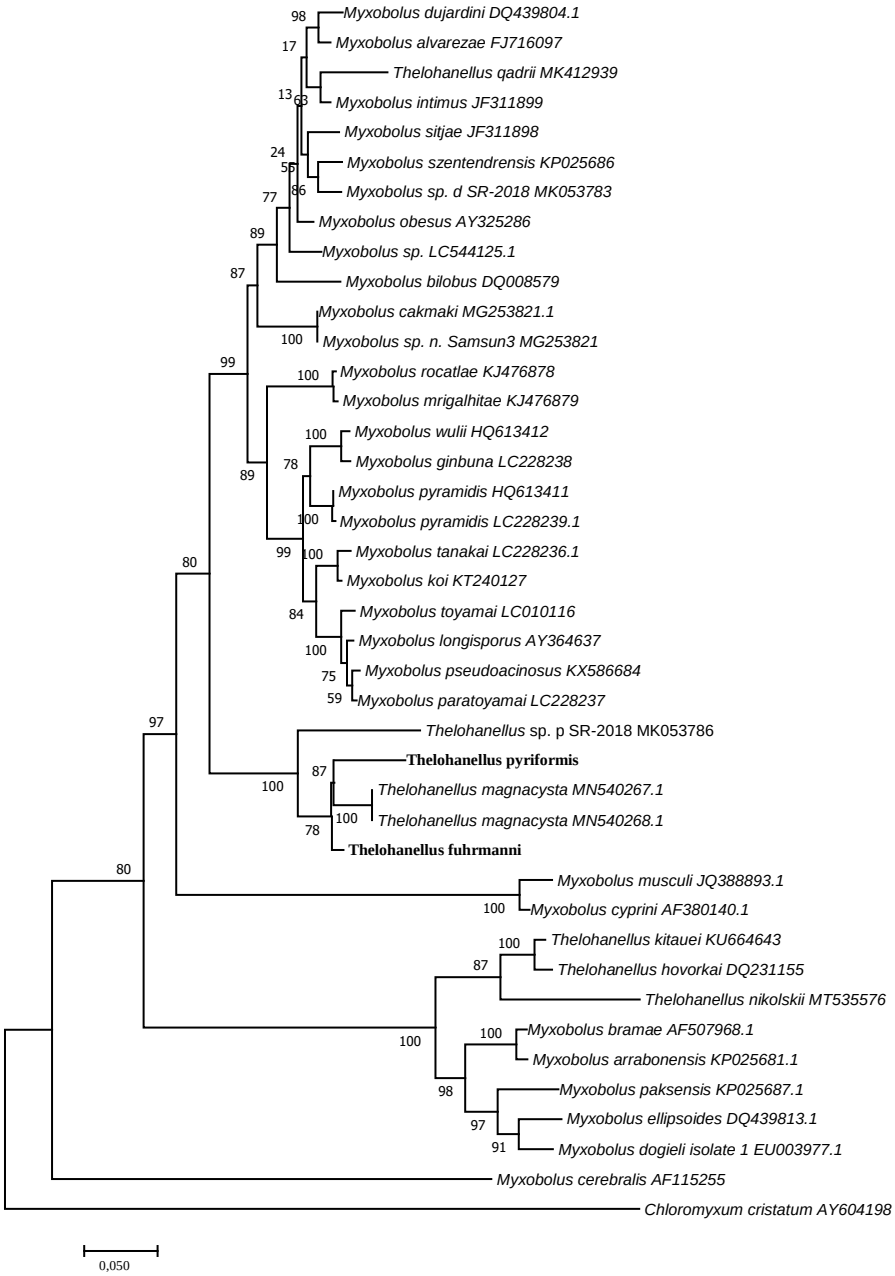

Fig. 37 Phylogenetic tree generated by maximum likelihood analysis of ssrDNA sequences of *Thelohanellus pyriformis* and *Thelohanellus fuhrmanni* and other closely related myxozoan species identified by BLAST; GenBank accession numbers. Numbers at nodes indicate the bootstrap confidence values (ML). *Chloromyxum cristatum* was used as an outgroup

Histology

Plasmodia of this species were located inside the loose connective tissue of the snout. Five to six-round shape plasmodia with a diameter of 1-1.3 mm developed in close vicinity within an about 4.3-4.5 mm long and 3-3.2 mm wide cyst formed by the host (Fig. 38 (1)). The thickness of the cyst was not measured. Plasmodia were surrounded by a relatively thin dense connective tissue wall of the host built up from three layers (Fig. 38 (2)). This layer wrapped plasmodia from outside and separated plasmodium from each other also in the interplasmodial space. Plasmodia were separated from the host capsule by their own very thin eosinophil staining ectoplasm. Only sporogonic stages were found in plasmodia. Dark staining early developmental stages are located in a single layer at the periphery. Centrally it was followed by a layer of sporoblasts and forming myxospores, while the centre of the plasmodium was filled by young and matured myxospores (Fig. 38 (3)).

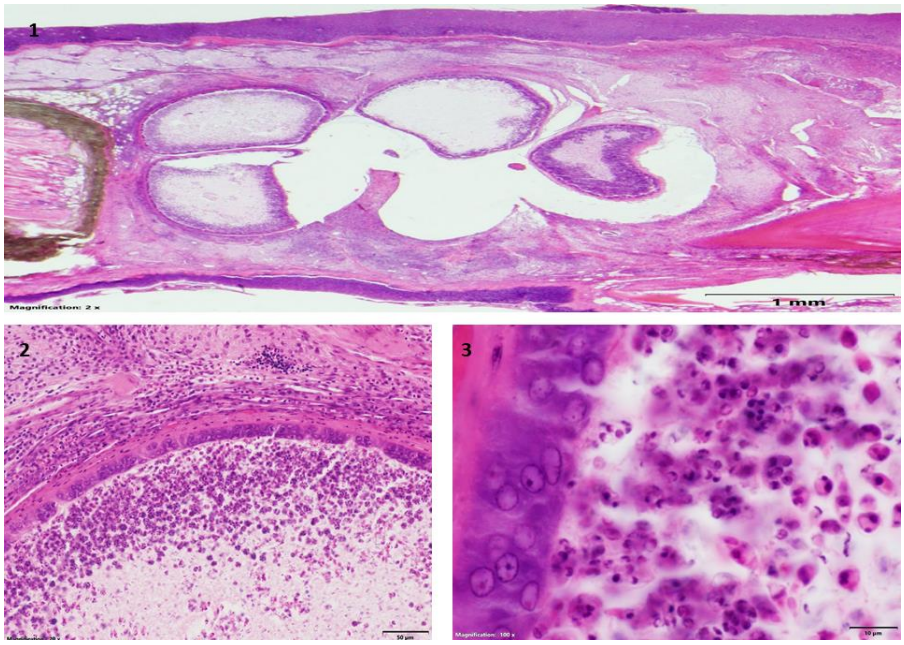

Fig. 38 Hisotlogical sections: **(1)** Plasmodia (p) of *Thelohanellus* cf. *fuhrmanni* enclosed in a cyst inside the loose connective tissue (ct) in the snout of the common nase. Empty holes (h) show the place of plasmodia fell out during histological processing or damaged by syringe at sampling myxospores. H. & E. staining, Bar = 1mm, **(2)** A part of *T.* cf. *fuhrmanni* plasmodium. Inside the connective tissue capsule (arrow) and the thin ectoplasm a dark staining raw of young developmental stages is located, followed toward the centre by a zone of sporoblast (spb) and young myxospores (ys). The centre of the plasmodium is filled by matured spores. H. & E. staining, Bar = 50 µm **(3)** A part of the peripheral layer of the plasmodium. yds= young developing stages, spb= layer of sporoblasts, fs= forming myxospores, Bar = 10 µm

4. 2.2 Discussion

The myxozoan fauna of Hungarian fishes are well studied, although there are still many species to be discovered and resolve their complex morphology and molecular characterization. Especially the parasites of invasive fish species deserve special attention. The pumpkinseed sunfish (*Lepomis gibbosus*) is a North American freshwater fish, which was introduced to Europe as ornamental fish more than a hundred years ago. In 1905, the fish was introduced also to Hungary into the pond system of Iharos. Unfortunately, the fish escaped and reached the Drava, Lake Balaton, and then spread very quickly throughout the Danube water system. Nowadays it has become widespread; it can occur everywhere in the vegetated, muddy or sandy bed sections of clear, stagnant, or slow-flowing Hungarian waters (Vutskits, 1913).

Li and Desser (1985) and Cone and Anderson (1977a, 1977b) described nine *Myxobolus* species from *L. gibbosus* in America. After the revision of Cone (2001), only six species were validated *viz. M. dechtiari* Cone and Anderson, 1977, *M. gibbosus* Herrick, 1941, *M. magnaspherus* Cone and Anderson, 1977, *M. osburni* Herrick, 1936, ***M. paralintoni***

Li and Desser (1985) and *M. uvuliferis* Cone and Anderson, 1977. These three species: *M. gibbosus, M. uvuliferis,and M. dechtiari* were recovered from gills. Lom (1969) identified the first *Myxobolus* infection in *L. gibbosus* as *Myxobolus* cf. *carelicus* Perushewsky, 1940 in Europe Subsequently; Moshu (1912) validated this species as *M. dechtiari* Cone and Anderson, 1977.

M. dechtiari, is characterized by specific histotropism to cartilage, especially to the cartilaginous gill rays. We suppose that Cone and Anderson (1977) noticed a similar chondrophil location when they stated that plasmodia were among the basal cells at the distal ends of gill lamellae. The latter location seems to be more common in fish, but up to this time, relatively few papers concerning this problem. Molnár et al. (2008) found that *M. feisti* in roach (*Rutilus rutilus* (L)) and *M. susanlimae* in bleak (*Alburnus alburnus* (L.)) cause similar type infections. Lom (1969) reported on the presence of a small intercapsular appendix in the myxospores, but in descriptions by Cone and Anderson (1977a) and Cone (2001), no data was presented about this appendix.

Histological studies made on the plasmodia of *M. dechtiari* seems to prove that this species develops in the cartilaginous gill rays of the filaments. For a closer identification of the location of *Myxobolus* spp. developing in the gill filaments Molnár et al. (2018) suggested the preparation of cross-sections of plasmodia. In the case of preparing only longitudinal sections of the gill filament, it may be supposed that plasmodia obscure only the cartilage. Here, both

longitudinal and cross-sections might indicate that the whole development took place inside the cartilage. However, in the present study, no observation was made in the early developmental stages. In the case of *M. feisti* and *M. susanlimae* spp. myxospores enclosed in the filament cartilage were found commonly (Molnár et al., 2008), but after a detailed study on young plasmodial stages, Molnár et al. (2012) concluded that *M. feisti* starts its development close to the cartilage and myxospores are enclosed by cartilage cells only by a secondary reaction. According to *M. dechtiari* description, further studies needed on early stages, to confirm the cartilaginous development even in the early stages. Molnár (1994) states that *Myxobolus* species have a relatively strict host-, organ- and tissue specificity. Besides the morphology of myxospores, these features offer immense help in species identification. Most species develop in a specific organ of the fish; therefore, tissue tropism is one of the most decisive factors for differentiating species with morphologically similar myxospores.

The phylogenetic analysis reveals that the sequence of *M. dechtiari* differs from other species available in the GenBank. This observation is consistent with the general tendency of myxozoan parasites to diverge according to the fish host and the location (Ferguson et al., 2008; Naldoni et al., 2011; Adriano et al., 2012; Carriero et al., 2013; Molnár and Eszterbauer 2015). Consequently, *M. dechtiari* shows the highest similarity to the North-American *M. osburni* described from the same fish species. *M. dechtiari* shows also a somewhat close relationship to a triactinomyxon type actinospore from North America and a raabeia type from Hungary. The result does not provide any support to decide which type of actinospore could be a possible developmental stage for *M. dechtiari*; *Myxobolus* species have most commonly triactinomyxons as actinospore, however other morphotypes could also exist, like raabeia (Yokoyama et al., 1995; Molnár et al., 1999; Eszterbauer et al., 2006) and echinoactinomyxon (Marton and Eszterbauer 2011). According to the Andree et al., 1999, molecular data (preferably ssrDNA) are required for description, comparison, and identification of Myxozoan species, but our results could not be compared to most of the myxozoan species from pumpkinseed (only to *M. osburni*) due to the lack of sequence data in the Genbank.

The genus *Thelohanellus* Kudo, 1933 is one of the myxozoan genera most numerous in species and are typically histozoic (rarely coelozoic) bivalvulidan myxozoans. Currently, *Thelohanellus* comprises 108 nominal species throughout the world, most of which are plasmodia-forming parasites infecting various tissues of freshwater fishes, and some of which are pathogenic, causing economic losses in aquaculture (Zhang et al., 2013). Identification of several *Thelohanellus* sp. is made difficult because the description of the old known species

was relatively poor and most species were originally described as *Myxobolus* with one polar capsule (Kudo, 1920).

Common nase (*Chondrostoma nasus*) is the most common fish in European rivers; it belongs to the Leuciscinae subfamily of cyprinid fishes. However, its myxozoan fauna is studied poorly. So far only four *Myxobolus* species (*M. chondrostomi* Donec, 1962, *M. arrabonensis* Cech et al. 2015, *M. szentendrensis* Cech et al. 2015 and *M. paksensis* Cech et al. 2015) were described from the muscle, gill filaments, gill lamellae and swim bladder of the common nase as type host, respectively. However, Donec and Shulman (1984) reported several known species from *C. nasus* which were originally described from other cyprinid fishes: *M. albovae* Krasilnikova 1966; *M. bliccae* Donec and Toziyakova 1984; *M. bramae* Reuss 1906; *M. carassii* Klokacewa 1914; *M. caudatus* Gogebashvili 1966; *M. circulus* Akhmerov 1960; *M. cyprini* Doflein 1898; *M. dispar* Thélohan 1895; *M. donecae* Kashkovsky 1969; *M. ellipsoides* Thélohan 1892; *M. exiguus* Thélohan 1895; *M. lobatus* Dogiel and Bychowsky 1934; *M. macrocapsularis* Reuss 1906; and *M. musculi* Keysselitz 1908 (Donec and Shulman 1984). In this study, we detected a *Thelohanellus* species from the snout of the common nase. According to the shape of the myxospores and the location of the plasmodium, we identified the species as *Thelohanellus fuhrmanni* Auerbach, 1909, although the size of the spores and the type host (*Rutilus rutilus*) was different from in the original description. Due to the observed differences in some of the key taxonomic elements, we tend to suppose that the species found in the nase represents a new unknown sp. However, the shape of the spores and the site of infection, written by Auerbach (1909) as "connective tissue under the mucous membrane of the mouth" fairly corresponds to our data, so until further studies we designated this species as *T. cf. fuhrmanni*. Our study is the first, which revealed the ssrDNA of this *Thelohanellus* species described from nase. Unfortunately, no sequence data are available from this well-known parasite from its type host. Therefore, the phylogenetic analysis could not help in the identification in this case. Despite the increasing sequence data of myxozoa, only a few species were closely related to *T. cf. fuhrmanni*. 97.6% nucleotide identity was observed with *Thelohanellus magnacysta* (Ksepka et al 2018) described from the somatic muscles of *Cyprinella venusta* Girard, 1856. 96.4% similarity was found to *Thelohanellus pyriformis* sequenced by us in this study from the gill of *Tinca tinca*. 93.3% nucleotide similarity was shown with *Myxobolus intimus* isolated from the capillary network of gill lamellae of *Aspius aspius*. 93% similarity was observed with a *Thelohanellus* sp.-2018 (MK053786), obtained from the intestinal epithelium of *Cobitis paludica*.

Tench (*Tinca tinca* L.) is considered to be one of the original European cyprinid species. It is most likely to have evolved from primitive Tertiary Paleoleuciscus in large lake systems of Central Europe. Many species of myxozoan have been reported from tench. Two species are known to occur in tench from the Sphaerosporidae Davis, 1917 family, namely *Sphaerospora galinae* in the renal tubule lumens and *S. tincae* in the anterior kidney (Lom et al., 1985; Lom and Dykova, 1992). Two species are described from the Myxidiidae Thelohan 1892 family, *Myxidium rhodei* from the kidney of the tench and *Zschokkella nova* from the gall bladder of cyprinids, including tench (Lom and Dykova, 1992). Among species of Myxobolidae, the following ones are described in tench: *M. baueri*, *M. bramae* (gills), *M. crassus*, *M. cyprini* (muscle), *M. cycloides* (gills, skin and other organs), *M. dispar* (gills, skin and other organs), *M. dogieli*, *M. donesae*, *M. dujardini*, *M. ellipsoides* (connective tissue cells of gas bladder and gills), *M. gigans*, *M. karelicus*, *M. muelleri* (gills and fins), *M. musculi*, *M. oviformis*, *M. shulmani* (Lom and Dykova, 1992) and *Thelohanellus pyriformis* Thelohan, 1892 (Svobodova and Kolarova, 2004). *Myxidium rhodei* and *Thelohanellus pyriformis* infections are reported most often from the tench.

T. pyriformis was originally reported from the gills, kidneys, and spleen of tench. Molnar (1979) described this parasite from the gills and skin of *Tinca tinca* and *C. nasus* from Hungary as well. Subsequently, Schulman (1984) had reported *T. pyriformis* also from other hosts and most varied body organs. Dykova and Lom (1987) described plasmodia of this parasite from the vessels of gills and concluded that the infection could lead to hypertrophy of endothelial cells in gills. In our recent study, we identified the *Thelohanellus* species detected in the gills of tench as *T. pyriformis* based on the correspondence of morphometrical characteristics of the spores, the location of the plasmodia and the type host species identity with the original description. In histological preparations, plasmodia were clearly seen to be located inside the arteria efferent obstructing partially its lumen. The microscopic picture corresponded in all respect to those described by Dyková and Lom (1987), however, having only histological preparations we could only strongly suppose that the plasmodium wall was composed of endothelial cells as the above authors stated.

Moreover, we provided the first ssrDNA sequence data of this parasite. The phylogenetic analysis revealed the closest relative of this species is *T. cf. fuhrmanni* with 96.4% nucleotide identity. 95.1% similarity was found with *T. magnacysta* and 91.6% similarity was observed to a *Thelohanellus* species-2018 (MK053786) from *Cobitis paludica*.

4.3 Collection of Actinosporean stages and Plant-based treatment trials

4.3.1 Results

The oligochaetes were kept in laboratory conditions, which are explained in section 3.2.3
Of them, Triactinomyxon stages of *Myxobolus pseudodispar,* developed in *Tubifex tubifex* after infecting them with myxospores of *M. pseudodispar* collected from the muscles of the roaches were closely studied (Fig.39). Produced triactinomyxons from oligochaetes (*Tubifex tubifex*) were collected for herbal drug treatment initial trials (Fig. 40). Active Triactinomyxons were collected in 48 well microplates for a treatment experiment. Several experiments were performed at different dilutions of herbal drugs.

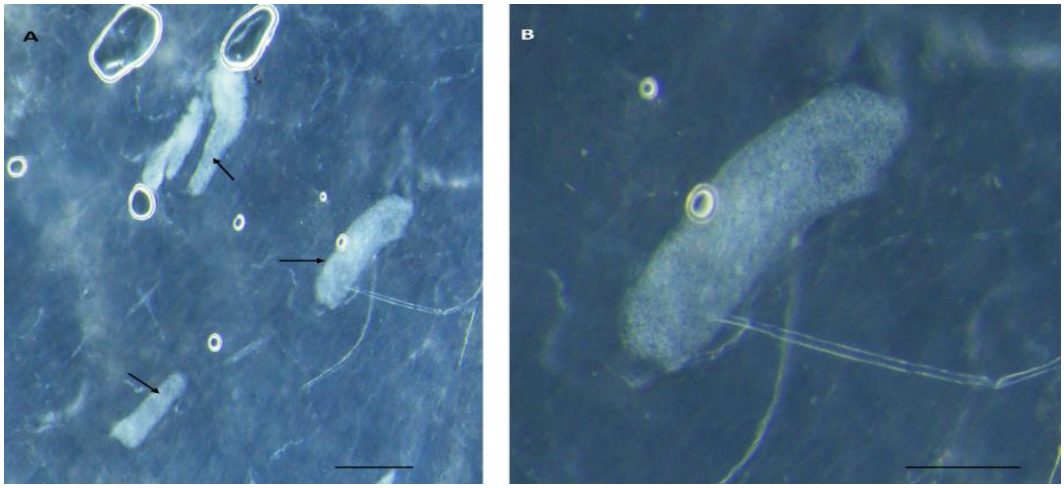

Fig. 39 A: Crushed muscle of Roach infected with *Myxobolus pseudodipar* plasmodia (arrow showing), B: Single plasmodium. Bar=10 μm

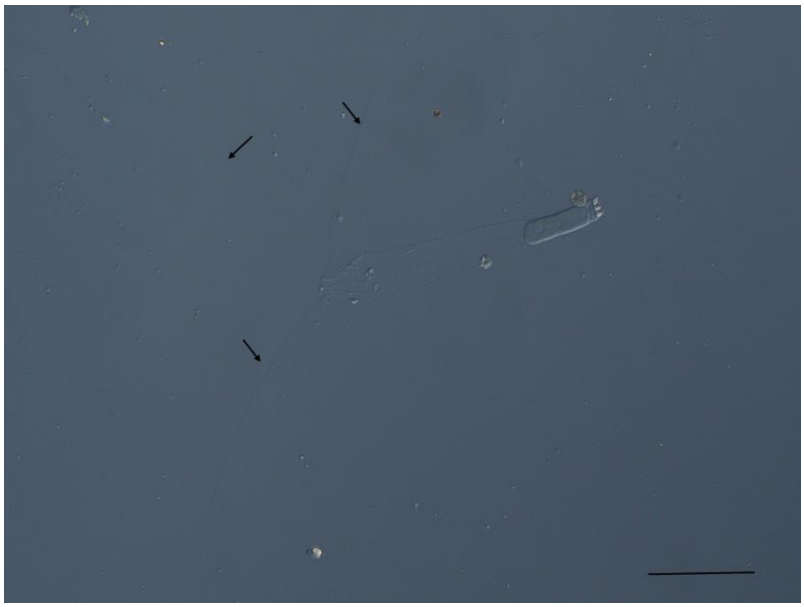

Fig. 40 Microphotograph of Triactinomyxon *of Myxobolus pseudodispar* from *Rutilus rutilus* (arrow showing three arms) Bar = 10 µm

4.3.1.1 Viability test outcome

In the experiment for the viability of actinospores different results were detected on the two different temperatures. The actinospores were active, intact and viable at 20°C for 48 hours while on 4°C, intact actinospore forms were found even after one week (Fig. 41). Actinospores were determined active or infectious when at least one of their polar capsules was intact.

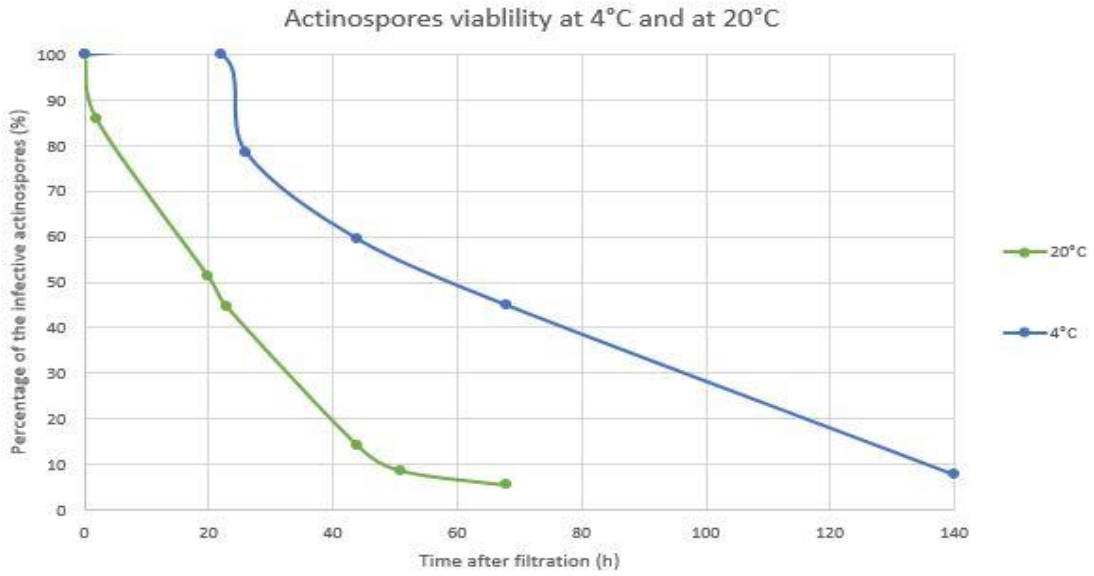

Fig. 41. Temperature effect on the viability of actinospores

4.3.1.2 Treatment Trials outcome

4.3.1.2.1 Neem-derivative treatment outcome

In order to perform further larger-volume experiments, we sought to determine different drug efficacy thresholds. The LD50, LD90 (LD: lethal dose, the median lethal dose that kills 50% and 90% of the organisms, respectively) projected on actinospores were determined at 10- (LD 90) and 5-fold dilution (LD 50) (10- 20% dilution) thresholds of neem solution (Fig. 44).

Based on our previous observations, the efficacy of the extract was evaluated by direct incorporation of the drug with actinospores, followed by 1 and 2 hours, respectively. Based on our observations, the effect of the applied plant extract is immediate, but no later than 1 hour after treatment, major morphological deformities were seen in actinospores (Fig. 42, 43) such as polar coil filaments were extruded out completely and arms of actinospores were shrunk and broken.

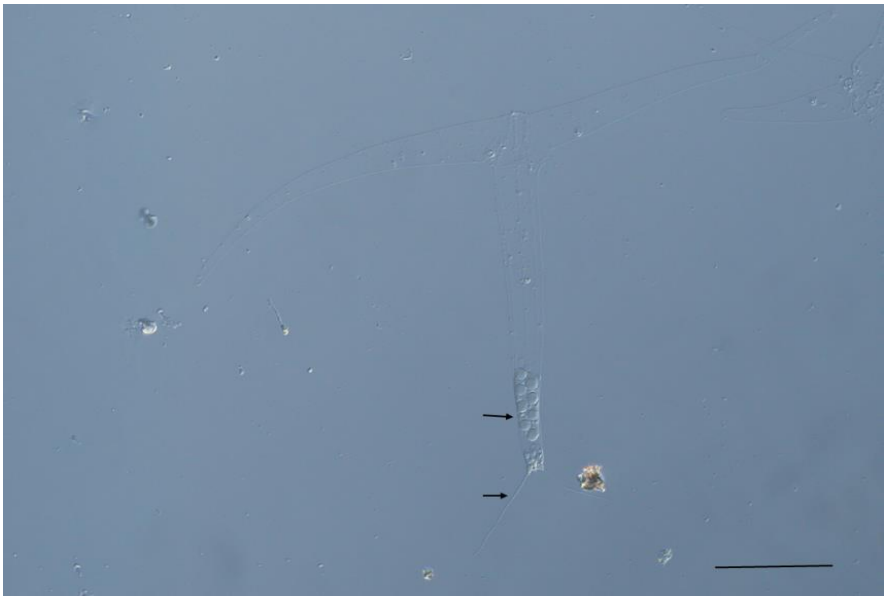

Fig. 42 Microphotograph of Triactinomyxon after neem treatment, arrow showing mature sporoplasm and extruded filament Bar = 10 µm

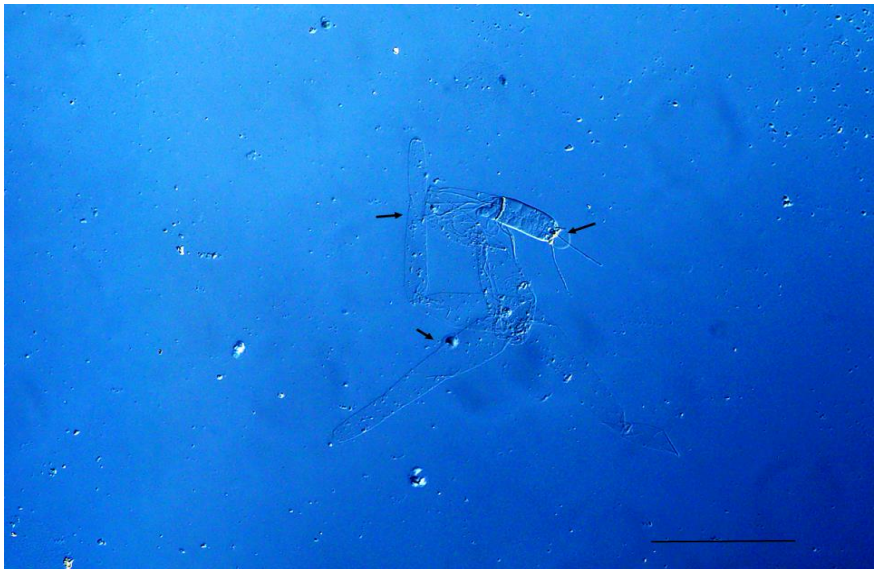

Fig. 43 Microphotograph of Triactinomyxon after neem treatment, arrow showing damaged arms and out filaments Bar = 10 µm

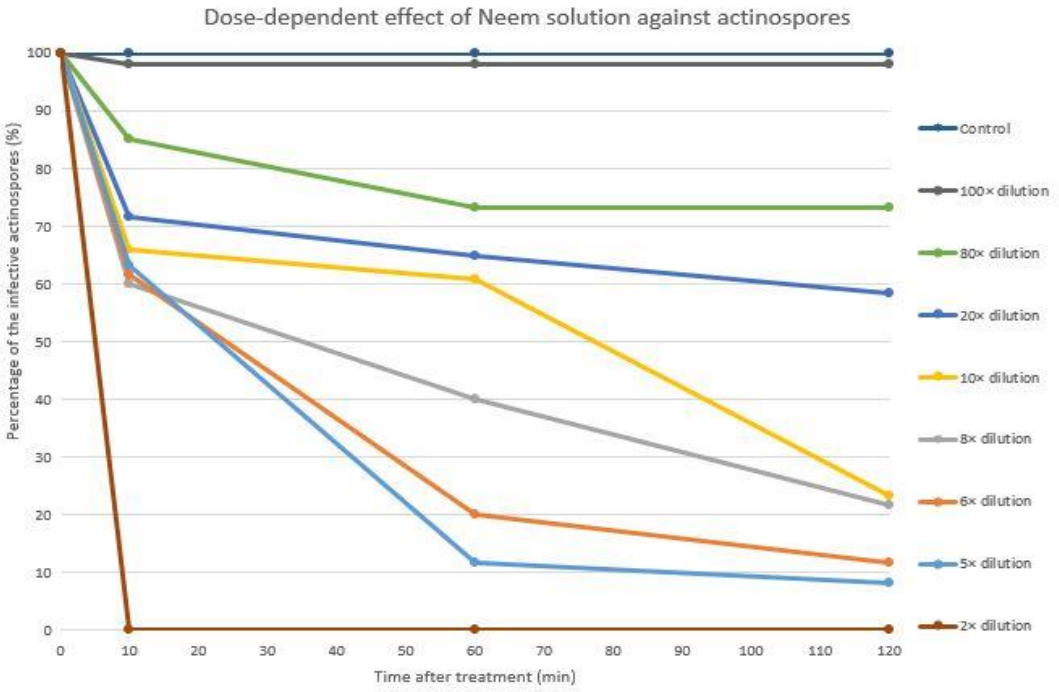

Fig. 44 Effect of Neem bark Extract on the infectivity of actinospores at different concentrations

4.3.1.2.2 Preliminary outcome of Turmeric and Garlic treatment

Preliminary results with the plant solutions suggested that turmeric contains active ingredients that affect the viability of actinospores. Both the plant solutions were applied at different dilutions but could not notice visible changes except at very high concentrations; 50% of the stocking solution. The turmeric treatment provided some visible effects after 2 hours while garlic showed less noticeable changes after 2 hours. No drastic changes were noticed after adding lower concentrations of the solutions.

4.3.2 Discussion

One of the objectives of my dissertation was to perform treatment experiments against actinospores. I have learned the techniques for collecting actionsporean stages and performing experiences with them, and I am planning to utilize this knowledge in India. In India, studies on actinosporean stages of myxozoan are a neglected research field. Up to this time, no paper appeared in this respect. After returning to India based on Hungarian results, I might have a chance to record a series of actinosporean stages, probably from a new oligochaete host.

Methods used for isolation of actinosporean stages in our laboratory showed that several unidentified actinosporean types could be isolated from oligochaetes. Of them at present Triactinomyxon stage of *Myxobolus pseudodispar* (Székely et al. 1999) is an excellent tool for studying its life cycle in the laboratory. The species *Myxobolus pseudodispar* is a common myxozoan parasite of the roach in Hungary (Baska 1987). The development of *Myxobolus pseudodispar* Gorbunova 1936, an intracellular myxozoan muscle parasite of the roach *Rutilus rutilus* L., was studied in experimentally infected oligochaetes. Besides its common occurrence, little is known about its pathogenicity. In 1999 by Szekely et al., *M. pseudodispar* was successfully developed in *Tubifex tubifex*. The intraoligochaete development of the parasite was described and the triactinomyxon-type actinospores were detected.

In the past, plant-derived medicines have long been used traditionally as cures for many diseases (Duke,1987). In fish farming, the compounds of several plants have been used and the effective immune response and protective abilities against pathogens were observed (Harikrishnan et al. 2011). Many studies have proved that essential oils, extracts and compounds from plants might have potential effects against parasites in aquaculture (Valladao et al. 2015; Bulfon et al. 2015).

Regarding the chemical control against myxozoa, little progress has been achieved in the development of chemotherapeutics. Acetarsol (Stovarsol) was used against the whirling disease by Scolari (1954) and Baur (1959) although partial improvement had been observed. Alderman (1986) repeated the same treatment but was unable to record the same results.

Rhee et al. (1990a, b) demonstrated the therapeutic effect of fumagillin dicyclohexylamine salt for the treatment and prevention of *T. kitauei*. The efficacy of fumagillin against *Sphaerospora renicola* causing renal sphaerosporosis in common carps (*Cyprinus carpio*) has been reported

(Haberkorn et al. 1983, 1987, 1988; Melhorn et al. 1984), whereas infections caused by *Myxobolus cyprini* Doflein, 1898, and *Thelohanellus nikolskii* Akhmerov, 1955, were not substantially affected by fumagillin (Molnar et al, 1987). The drug has a limited safety margin and is known to affect the erythropoietic system of the host (Rajendran, 2003).

Control against myxozoan by using herbal drugs is not a well-studied area. Some researchers revealed the potential effect of plant-derived drugs such as essential oils, aqueous solutions of plants and plant excrudes (Wuherlisch et al. 2007). A treatment for myxoboliosis in carps by a traditional herbal mixture consisting of garlic and salt was suggested by Dey and Chandra (1994). The mixture was found effective in checking fish mortality, decomposing and breaking of parasitic cysts in the gills, inducing healing and regeneration of damaged gill tissue.

In our study, treatment trials were carried out with turmeric, garlic, and neem solutions in different concentrations against the actinospores of *M. pseudodispar* in vitro. Neem treatment was the most promising against actinospores. According to the turmeric and garlic solutions, less visible effects on actinospores were observed. However, these plant solutions treatments require more study to check the infectivity of the treated actinospores on fish, and test if the effective solutions have any toxic effects on the aquatic ecosystem.

These initial trials against actinospores suggested that herbal drugs could be strong and affordable treatments for farmers. Thus, more efforts and concerns are required in this less studied field.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The first objective of my dissertation was to describe myxozoan species from India and Hungary, with their morphological characters and molecular phylogeny.

In India, I have detected several previously morphological described species, such as *Henneguya ganapatiae*, *Henneguya mystasi, Myxobolus cylindricus, Myxobolus rewensis, Myxobolus dermiscalis, Myxobolus chakravartyii and Thelohanellus caudatus.* However, the original description of these species often lacks information about key taxonomic properties, like histological description, the exact location of the plasmodia, type of the host tissue, which made the identification really challenging. In our redescriptions we included these missing characteristics, and also provided microscopic photos of spores, histological preparations and ssrDNA sequence data. Two new species were also detected, *M. ompok* n. sp. from *Ompok pabda* and *M. bandyopadhyayi* n. sp. from *Labeo rohita*. Our study clearly shows the importance of molecular examination and detailed morphological description of discovered myxozoan species in taxonomy.

However, the Hungarian myxozoan fauna is well documented, parasites of invasive fish species are needed to monitor regularly. This study revealed the presence of *M. dechtiari* introduced along with its ornamental host fish *Lepomis gibbosus* to Europe and to Hungary. Two less studied *Thelohanellus* species *T. pyriformis* and *T. fuhrmanni* were recovered from *Tinca tinca* and *Chondrostoma nasus* respectively, whereas the morphological deatails were supplemented with ssrDNA results as well.

The second objective was to perform the treatment trials against actinospores infection with the application of herbal drugs.

The treatment trials concluded that neem could be a potential drug against actinospores of *Myxobolus pseudodispar* infection. Whereas the other plant solutions: turmeric and garlic have shown less promising effects on that parasite. These plant solutions are requisite to optimizing effective concentrations. However, there is scarcity of research on using herbal drugs treatment against myxozoan given the necessity to perform these trials at a larger scale to get information

about the efficiency of these treatments in the aquaculture industry against other parasites as well.

5.2 Recommendations

The taxonomic study of the myxozoan species of freshwater fishes and the information about the infection site, the seasonal intensity of the major metazoan parasites, a vital field of research in India. The life cycle of the myxozoans has not been elucidated from India up to this time, which is essential for a vast future investigation. The knowledge of the life cycle and other infection information of myxozoan parasites of freshwater fishes will make possible the therapeutic treatment and control of these parasites as well as it would prevent the economic loss in the fishery industry. Whereas in Hungary, myxozoan research should pay attention to more investigation on pathogenicity and immunological aspects in fish and annelids hosts. The wide host range of these parasites and geographical distribution from different countries will explore the route of migration of fishes as well as the parasites, which needs more concern to mitigate future outbreaks.

Currently, there are no effective plant-derived drugs available for the treatment of myxozoan infections. Myxospores are often resistant to many common disinfectant regimens, making disinfection and quarantine challenging. Oligochaete (e.g., *Tubifex* and *Tubifex*-like worms) and polychaete (marine worms, including bristle worms) management is an important aspect for controlling these parasites. The plant-based treatment could be a more affordable and environmentally friendly option for farmers and water bodies as well. However, plant-based treatment research on myxozoans needs more attention in countries worldwide

SUMMARY

The Myxozoa comprise over 2,000 species of microscopic obligate parasites that use both invertebrate and vertebrate hosts as part of their life cycle. These parasites are the causative agents of some serious diseases in freshwater, estuarine and marine habitats. Objectives of the present study were: Morphological characterization of myxozoan parasites from freshwater fishes from India and Hungary and perform the treatment trials against actinospores by using herbal drugs.

The identification of the parasites was well supported by the small ribosomal subunit (ssrDNA) data. Phylogenetic analysis and histological data were also presented for the described species to give a strong clarification of taxa identification.

During 2017-2019, freshwater fishes were collected in India from Uttar Pradesh and West Bengal for the detection of myxozoan infection. In Uttar Pradesh, we have found *Henneguya ganapatiae* species from *Notopterus notopterus* and *Henneguya mystasi* from *Mystus vittattus.* Plasmodia were recovered from gill filaments and gill lamellae in both cases respectively. From the pabda catfish (*Ompok pabda),* a new species of M*yxobolus* was detected from the renal tubules of the kidney: *Myxbolus ompok* n.sp. and *M. cylindricus* was found from gill filaments of the snake-headed fish (*Channa gachua*) . Besides that, Indian major carps (*Catla catla, Cirrhinus mrigala, Labeo rohita*) were also studied for myxozoan infection in West Bengal: *Myxobolus dermiscalis* and *Thelohanellus caudatus* plasmodia were found on the scales and fins from *Labeo rohita* respectively. A new species of *Myxobolus* from scales of *Labeo rohita* was also detected, which is named *Myxobolus bandyopadyayi* n. sp. Two other *Myxobolus* species were reported from the fins of *C. mrigala* and *Gibelion catla*: *M. rewensis* and *M. chakravartyi* respectively.

The myxozoan fauna of freshwater fishes in Hungary was also studied. We have confirmed the *Myxobolus dechtiari* infection from *Lepomis gibbous* in Europe. The occurrence of *M. dechtiari* proved the cross-continent infection transfer with its specific host. The presence of the infection was confirmed with the well-described histological analysis of the infected gill cartilaginous rays and ssrDNA molecular data. Long ago described, two *Thelohanellus* species were recorded. *Thelohanellus pyriformis* from the arteria brachialis of the gills of *Tinca tinca* and *T. fuhrmanni* infection was found under the skin of the snout of *Chondrostoma nasus.* The description was supported with the first molecular data for both the *Thelohanellus* species.

The treatment trials were performed against actinospores by applied of herbal drugs: Neem-derivative and plant solutions of turmeric and garlic. These herbal products were purchased commercially for the laboratory treatment trials. Specific Parasite Free oligochaetes (*Tubifex tubifex)* were kept in laboratory condition with sterile mud and filtered water in aquaria. *Myxobolus pseudodispar* infection was collected from the muscle tissue of the roach (*Rutilus rutilus*) from Lake Balaton. Infected muscle pieces were added to the aquaria of *T. tubifex*, and after the observed interval, actinospores were collected from the filtered water. Collected actinospores were treated with the above-described herbal drugs in 48-well microplates, in vitro.

According to the treatment results, solutions containing at least 10-20 % of neem derivative was the most effective against the actinospores of *M. pseudodispar* in one hour time period. Actinospores deformities and the extrusion of polar filaments from polar capsules were also noticed. In the case of turmeric and garlic, after 2 hours some visible changes were observed, but only in very high herb concentrations (50 %). Treatments with these plant solutions require more study to optimize the effective doses also in ecotoxicological aspects.

ÖSSZEFOGLALÁS

A nyálkaspórások csoportja több mint 2000 mikroszkopikus méretű, obligát parazitafajt tartalmaz, melyek életciklusuk során gerinctelen és gerinces gazdaszervezeteket egyaránt megfertőznek. Ezek a paraziták okozzák az édesvízi és a tengeri élőlények néhány súlyos betegségét. Jelen tanulmány célkitűzései a következők voltak: Az indiai és magyarországi édesvízi halak nyálkaspórás parazitáinak morfológiai jellemzése, és növényi eredetű hatóanyagokkal végzett kezelési kísérletek kivitelezése az aktinospórák ellen.

A paraziták azonosítását a kis riboszomális alegység RNS-ének részleges szekvencia (ssrDNS) adatai jól alátámasztották. A taxonok azonosításának egyértelmű tisztázása érdekében filogenetikai elemzéseket és szövettani eredmények is bemutatásra kerültek a leírt fajokról.

2017-2019 során édesvízi halakat gyűjtöttünk Indiában Uttar Pradeshből és Nyugat-Bengálból nyálkaspórás fertőzés kimutatására. Uttar Pradeshben a *Notopterus notopterus*-ból leírtuk a *Henneguya ganapatiae* fajt és a *Mystus vittattus*-ból a *Henneguya mystasi*-t. A plazmódiumokat mindkét esetben kopoltyúlemezekből és kopoltyúlemezkékből azonosítottuk. A pabda harcsa (*Ompok pabda*) vese tubulusaiból egy új *Myxobolus* fajt mutattunk ki: *Myxbolus ompok* n.sp. néven. A kígyófejű hal (*Channa gachua*) kopoltyúlemezeiből a *M. cylindricus*-t azonosítottuk. Emellett a főbb indiai pontyféléket (*Catla catla, Cirrhinus mrigala, Labeo rohita*) is vizsgáltuk nyálkaspórás fertőzések után kutatva Nyugat-Bengálban: *Myxobolus dermiscalis* és *Thelohanellus caudatus* plazmódiumokat azonosítottunk a *Labeo rohita* pikkelyeiről és az uszonyáról. A *Labeo rohita* pikkelyéről egy új *Myxobolus* fajt is kimutattunk, amelyet *M. bandyopadyayi* n. sp.-ként írtunk le. Két másik *Myxobolus* fajt is azonosítottunk a *C. mrigala* és *Gibelion catla* uszonyaiból: *M. rewensis*-t és *M. chakravartyi*-t.

Tanulmányoztuk továbbá a magyarországi halak nyálkaspórás faunáját is. *Myxobolus dechtiari* fertőzést azonosítottunk az Európába behurcolt naphalból (*Lepomis gibbosus*). A *M. dechtiari* jelenléte jól bizonyítja a kórokozók együttes migrációját a gazdafajjal együtt akár kontinensek között is. A fertőzés jelenlétét a kopoltyú porcos sugarainak szövettani vizsgálatával és az ssrDNS szekvencia vizsgálatával igazoltuk. Továbbá két, már régóta ismert *Thelohanellus* fajt azonosítottunk: a compó (*Tinca tinca*) kopoltyújának artéria branchiálisából a *Thelohanellus pyriformis*-t, a paduc (*Chondrostoma nasus*) orrának bőre alól a *T. fuhrmanni* fertőzést írtuk le és szekvencia adatokkal egészítettük ki.

Az aktinospórák elleni kezelési kísérleteket növényi eredetű hatóanyagok alkalmazásával végeztük: kurkuma, fokhagyma és az indiai orgona (*Azadirachta indica*) vizes oldatai. Ezeket a növényi készítményeket kereskedelmi forgalomban is kapható alapanyagokból készítettük el a laboratóriumi vizsgálatokhoz. Specifikus parazitáktól mentes *Tubifex tubifex*-et laboratóriumi körülmények között steril iszapban, szűrt vizes átfolyással tartottuk akváriumokban. A *Myxobolus pseudodispar* fertőzést okozó myxospórákat Balatonból származó bodorkák (*Rutilus rutilus*) izomszövetéből gyűjtöttük ki. A fertőzött izom-darabkákat hozzáadtuk a *T. tubifex*-et tartalmazó akváriumokhoz, és a férgek sikeres fertőzése után aktinosporákat gyűjtöttünk a leszűrt vízből. Az összegyűjtött aktinosporákat a fent leírt növényi gyógyszerekkel kezeltük in vitro módon, 48 lyukú sejttenyésztő lemezeken.

A kezelés során kapott eredmények alapján a legalább 10-20%-ban *neem*-származékot tartalmazó oldatok voltak a leghatékonyabbak a *M. pseudodispar* aktinosporái ellen, már egy óra letelte után is. Az aktinospórák deformációját és a poláris kapszulák kilökődését egyaránt megfigyeltük. A kurkuma és a fokhagyma esetében 2 óra elteltével már néhány esetben látható változás is észlelhető volt, de csak nagyon magas gyógynövénykoncentrációnál (50%). Ezekkel a növényi oldatokkal végzett kezelések további vizsgálatokat igényelnek a hatékony dózisok ökotoxikológiai szempontból történő optimalizálása érdekében, hogy a későbbiekben a haltermelési gyakorlat során is felhasználhatóak legyenek.

NEW SCIENTIFIC RESULTS

1. Detection of *Henneguya ganapatiae* from the food fish *Notopterus notopterus* and *Henneguya mystasi* from *Mystus vittatus* and *Myxobolus* and *Thelohanellus* infection from Indian major carps: *Myxobolus rewensis* from *Cirrihinus mrigala*, *Thelohanellus caudatus* from *Labeo rohita* and *Myxobolus chakravartyii*, supporting with first molecular data.

2. Description of two new *Myxobolus* species: *Myxobolus ompok* from pabda catfish *Ompok pabda* and *Myxobolus bandyopadhyayi* from *Labeo rohita.*

3. The occurrence of an American *Myxobolus* species in Europe: *Myxobolus dechtiari.*

4. Description of two *Thelohanellus* species with first molecular validity: *Thelohanellus pyriformis* from *Tinca tinca* and *Thelohanellus cf. fuhrmanni* from *Chondrostoma nasus.*

5. Treatment trials against actinospores by the application of herbal drugs proved that neem is a potential herbal drug against *M. pseudodispar* whereas turmeric and garlic solutions showed less effective results.

PUBLICATIONS

Articles published in peer-reviewed scientific journals

1. **U. Goswami,** K. Molnár, G. Cech, J.C. Eiras, P.K. Bandyopadhyay, S. Ghosh, I. Czeglédi, C. Székely (2021) Evidence of the American *Myxobolus dechtiari* was introduced along with its host *Lepomis gibbosus* in Europe: Molecular and histological data. International Journal for Parasitology: Parasites and Wildlife, https://doi.org/10.1016/j.ijppaw.2021.04.005

2. Borkhanuddin M.H*., **Goswami U*.,** Cech G., Molnár K., Atkinson S.D., Székely Cs., (2020)Description of myxosporeans (Cnidaria: Myxozoa) infecting the popular food fish *Notopterus notopterus* (Pisces: Notopteridae) in Malaysia and India. Food and waterborne parasitology, doi.org/10.1016/j.fawpar.2020.e00092 **(* *Shared first authorship*)**

3. Chaudhary A., Gupta A., **Goswami U.**, Cech G., Singh H.S., Molnár K., Székely Cs., (2019) Molecular Genetic Studies on *Myxobolus cylindricus* and *Henneguya mystasi* (Myxosporea: Myxobolidae) Infecting Two Indian Fish Species, *Channa gachua* and *Mystus vittatus*, Respectively. Acta Parasitologica, https://link.springer.com/article/10.2478/s11686-018-00014-8

4. Chaudhary A., **Goswami U.,** Gupta A., Cech G., Singh H.S., Molnár K., Székely Cs., Sharma B.,(2018) Morphological, histological, and molecular description of *Myxobolus ompok n. sp*. (Myxosporea: Myxobolidae), a kidney myxozoan from Pabdah catfish *Ompok pabda* (Hamilton, 1822) (Siluriformes: Siluridae) in India. Parasitology Research, https://doi.org/10.1007/s00436-018-5882

5. Székely C., Ghosh S., Borzák R., **Goswami, U.,** Molnár, K., Cech, G., (2021) The occurrence of known *Myxobolus* and *Thelohanellus* species (Myxozoa, Myxosporea) from Indian major carps with the description of *Myxobolus bandyopadhyayi n. sp*. in West Bengal. International Journal for Parasitology: Parasites and Wildlife. https://doi.org/10.1016/j.ijppaw.2021.07.008.

Abstracts published in conference handbooks/ Oral presentations

1. Description of some known and unknown species of myxozoans parasites from India". 9-13 September 2019, European Association of Fish Pathologists, Porto, Portugal Page no.179.

2. Description of new and known myxozoans infecting wild Indian Fishes in Uttar Pradesh, India. HAKI, Page no. 54-55, 29-30 May 2019, Szarvas, Hungary.

3. Molecular and morphological characterization of myxozoan parasites of Indian wild fishes. Academic presentation, Page no. 93, 21-24 January 2019. University of Veterinary Medicine, Budapest, Hungary.

4. Morphological and molecular studies on *Thelohanellus spp.* infecting cyprinids fishes in Hungary. Academic presentation, January 2020. University of Veterinary Medicine, Budapest, Hungary.

5. *Myxobolus* infection in the gill cartilage of the American pumpkinseed sunfish *Lepomis gibbosus* introduced to Europe. Academic presentation, January 2021. University of Veterinary Medicine, Budapest, Hungary

6. Preliminary investigation of effect of plant based drugs on the actinospores of *Myxobolus pseudodispar*. XLV Halászati Tudomanyos Tanácskozás, HAKI, Page no. 36-37, 8-9 September, 2021 Szarvas, Hungary.

Poster presentation-

1. "Host-parasite relationship and phylogeny of *Myxobolus ompok n. sp.* of *Ompok pabda* from India" in ZIBI Summer School on Pathogen-Host Interplay, 15-30 June 2018, Freie University, Berlin, Germany

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