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Thesis of the PhD Dissertation

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

BY

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INTRODUCTION AND OBJECTIVES

As the human population continues to grow, finding sources to feed those people is one of the most important challenges faced around the globe. Even in troubled economic times, men, women, and children need to eat. A healthy diet, high in protein is necessary to ensure that the growing population does not suffer from sickness and disease. Fish and other aquatic organisms are healthy sources of protein but parasitic diseases are one of the most serious problems in fishes, much concern among the wild fish stock because in most cases they can cause significant harm. Moreover, parasites often cause serious disease outbreaks among farmed fish.

Myxozoans are cnidarian multicellular parasites more than 2,400 species have been described, parasitizing most commonly fish and aquatic invertebrates. This group received extensive attention not only due to the pathogenic potential of many species and damages that it may cause to its hosts in both wild and in aquacultures but also by the complexity of its life cycle, morphological characterization, and its cryptic evolution from the free-living cnidarians. The advances in molecular biology tools and their uses in the systematics of Myxozoa in the past few years have revealed inconsistencies between molecular-based phylogenies and traditional morphology-based analyses, which often lead to the revision of the current taxonomy. Freshwater fishes of India possess extensive diversity of myxozoans, but the detailed taxonomy of myxozoans is still in its initial stage. Most of the studies performed on myxozoa in India were based only on comparative morphology so far. As far as the concern of Hungarian myxozoan species are well documented but still lacking behind in the other aspects such as host- parasites interaction, immunological interaction between host-parasites, seasonal distribution, etc. The present Ph.D. work is about first to detect the myxozoan parasites infection in freshwater fishes in India and Hungary. In the context of the mitigation against them, there is no effective plant -derived drug available. The available chemical drugs are not affordable for fish farmers and have adverse effects on the other aquatic organisms. The second part of the Ph.D. dissertation includes the trials against the actinospore stages of myxozoan by applying plant-derived products.

The research was focused on the following aspects of myxozoans:

- Identification of myxozoan species inhabiting freshwater fishes of India and Hungary.
- Studies on the morphology and taxonomic status of the above myxozoans.
- Determination of the pathogenicity of the myxozoans to their hosts.
- Studies on their phylogeny by using ssrDNA sequence analysis.
- Treatment trials against actinospore stages of myxozoans with the application of plant-derived herbal drugs.

MATERIALS AND METHODS

Samples collection from India:

Samples were collected during 2017- 2019 from two states in India: Uttar Pradesh and West Bengal. Fishes were collected near from River Ganges, cultured fish farms, and nearby fish markets in Uttar Pradesh and fishes were collected from cultured farms and markets near Kalyani expressway in West Bengal. The collected fishes were: *Notopterus notopterus, Channa gachua, Mystus vittatus, Ompok pabda, Labeo rohita, Cirrhinus mrigala, Gibelion catla.*

Samples collection from Hungary

Fishes were collected during 2017- 2019 from Lake Balaton, River Danube and its tributaries, Keszthely, Tihany, Siófok, Zala channel, Egerviz Creek, and Kis Balaton Reservoir. The common nase (*Chondrostoma nasus*), tench (*Tinca tinca*), and pumpkinseed (*Lepomis gibbosus*) were checked for myxozoan infection. The roaches (*Rutilus rutilus*) were also collected in order to sample *Myxobolus pseudodispar* from Lake Balaton.

Morphological analysis

First necropsy was performed with a complete parasitological examination of the different organs of the fish host. The plasmodium or myxospores were detected and morphological parameters were recorded. The spores were fixed in 80% ethanol for molecular investigations, the rest of the spores were fixed in 10% formalin for morphological measurements and microphotographs. For histological sections, the plasmodium or the spores were preserved in the 4% formalin and processed for histology using paraffin wax as an embedding medium.

DNA isolation and Phylogenetic analysis

The 80% alcohol-fixed myxospores were used for DNA isolation with a DNA extraction kit, from the collected spores and organs, following the manufacturer instructions. Using the extracted DNA

as a template, PCRs were performed with different primer sets. Minimum 1500 bp long fragment of ssrDNA gene was amplified from myxozoan samples with nested/semi-nested PCRs. General eukaryotic primers were used in the first round and Myxozoa-specific primers were used in the second round to enhance the specificity of the reaction.

The PCR products were visualized with agarose gel-electrophoresis, the appropriate products were purified with commercial purification kits. After sequencing, MEGA 6.0 and MEGA X were used for phylogenetic analysis with Maximum Likelihood algorithm. Bayesian Inference for phylogenetic trees was computed by Topali 2.5. The reliability of the computed trees were tested by bootstrap and posterior probability values.

Oligochaete rearing and actinospores production

The plasmodia of *M. pseudodispar* were collected from the muscles of common roaches (*Rutilus rutilus*) and the host fishes were collected from Lake Balaton to establish the life cycle in the laboratory for actinospores production. The aquaria were prepared with sterile mud and distilled water in which the parasite-free; SPF oligochaetes (*Tubifex tubifex*) were reared in the laboratory. The infected muscles were added to the aquaria of oligochaetes and checked after two months for actinospores.

Plant-derived solutions

The neem (*Azadirachta indica*) bark extract marketed by Sigma-Aldrich (Lot: BCBF6855V), Germany, was used as the commercial product which is registered in the European Union. The water-soluble plant components: Turmeric (*Curcuma longa*) and Garlic (*Allium sativum*) were commercially purchased from the grocery stores in powder form (Szilasfood kft., Lucullus brand). Different stock solutions were prepared using filtered water in concentration ranges narrowed by the 2 – 100 fold dilution and the exact concentration was optimized by performing experiments at different dilutions such as 5%, 10%, 20%, 60%, 80%, 100%. The working concentrations were prepared from the stock accordingly.

Viability Test

The actinospores were collected in 48- well microplates. First, the viability test was performed at two different temperatures: 4°c and 20°c. The mortality was observed per hour.

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 by adding 100 µL of each with a micropipette and the total volume of 200 µL distilled water with actinospores serving as control. Three replicates of each dilution and controls were applied. The microplates were incubated at room temperature. The wells were observed under a microscope for morphological changes and counting of dead actinospores at 30 minutes, 1 hour, and 2 hours of the time exposure period. The mortality data was observed and exported in the MS Excel program. Graphs were prepared in the MS Excel program.

RESULTS

Myxozoans infection

In India, from the state, Uttar Pradesh myxozoan infections were detected in many freshwater fishes. The *Henneguya* (75% prevalence) was recovered from the gill filaments in knife fish (*Notopterus notopterus*). *Myxobolus cylindricus* (78.5% prevalence) and *Henneguya mystasi* (81.2% prevalence) detected from gill lamellae in *Channa gachua* and *Mystus vittatus* respectively. A new myxobolus species *Myxobolus ompok n. sp.* (70% prevalence) was identified from the kidney of *Ompok pabda.*

From West Bengal, the Indian major cyprinids were examined: *Thelohanellus caudatus* (28% prevalence) from the fins, *Myxobolus dermiscalis* (25% prevalence) from scales, and a new species *Myxobolus bandyopadhyayi n. sp.* (25% prevalence) from scales were detected from *Labeo rohita*.

Myxobolus chakravartyi (44% prevalence) from fins of *Gibelion catla* and *Myxobolus rewensis* (30% prevalence) from the fins of *Cirrhinus mrigala* were identified.

In Hungary, two less-known *Thelohanellus* species were detected: *Thelohanellus pyriformis* (14% prevalence) was detected from the arteria brachialis efferents in *Tinca tinca* and *Thelohanellus fuhrmanni* (16% prevalence) from the under the skin of snout in *Chondrostoma nasus* was extracted.

The complex identification of American *Myxobolus dechetiari* (19% prevalence) was resolved. The plasmodia were detected from the cartilaginous gill rays of pumpkinseed. This myxozoan parasite was introduced in Europe with the ornamental host fish *Lepomis gibbosus*. The morphological data were supported by histopathology analysis of gill cartilaginous rays infected with *M. dechtiari* and with ssrDNA gene analysis.

Treatment trials with plant-derived drugs

The viability test was performed at two different temperatures. The actinospores were detected active and viable at 20° C for 48 hours. At 4°C, actinospores were active and viable for a week.

The neem treatment showed the 5 (LD 50) fold and 10 (LD 90) fold solutions were effective. The effect of the neem bark extract is immediate, but no later than 1 hour after treatment, major morphological deformities were found such as polar coil filaments were extruded out completely and arms of actinospores were shrunk and broken.

Turmeric and garlic solutions were applied at different dilutions but markable changes were not observed except at very high concentrations; 50% of the stocking solution. After the 2 hours of turmeric treatment, visible effects were seen while garlic showed less visible effects after 2 hours. No drastic changes were noticed after adding lower concentrations of the solutions.

Conclusion and Recommendation

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

Several myxozoan species were recovered from India, and those were redescribed with strong support by molecular and histological data. The collected myxozoan species were *Henneguya ganapatiae*, *Henneguya mystasi*, *Myxobolus cylindricus*, *Myxobolus rewensis*, *Myxobolus dermiscalis*, *Myxobolus chakravartyii*, and *Thelohanellus caudatus.* However, their original description did not include the key taxonomic properties, like histological analysis and immunological interaction with the host, location of the plasmodia, type of the host tissue, site-specificity, which made the identification really challenging. Two new species were described also, *Myxobolus ompok* n. sp. from *Ompok pabda* and *Myxobolus 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 myxozoa fauna is well documented but the parasites of invasive fish species should be monitored 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* where the morphological data were supplemented with ssrDNA results as well. Moreover, updating the morphological characteristics of some previously described species (*H. ganapatiae, T. fuhrmanni, M. chakravartyii etc.*) was conducted in order to make the available description more accurate.

The second objective was to perform the treatment trials against actinospore stages of myxozoan infection by applying 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 showed less promising effects on that parasite. These plant solutions require optimizing the effective concentrations.

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 requires more research in India. There is no data available on the life cycle of the myxozoans from India, which necessitates intensive future investigation. The biological information of these parasites of freshwater fishes will make possible the treatment and control against them, as well as it would prevent the economic loss in the fishery industry. In Hungary, myxozoan research should focus on more investigation of 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 requires more concern to mitigate future outbreaks.

Currently, there are no effective plant-derived drugs available for the treatment of myxozoan infections. The plant-based treatment could be a more affordable and environmentally friendly option for farmers and for water bodies as well. Therefore, plant-based treatment research on myxozoans requires more attention in countries worldwide.

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 n.sp*. from pabda catfish, *Ompok pabda*, and *Myxobolus bandyopadhyayi n.sp.* from *Labeo rohita*

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

4. Detection 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

Peer-reviewed Journal articles

1. **Goswami U.,** Molnár K., Cech G., Eiras J.C., Bandyopadhyay P.K., Ghosh S., Czeglédi I., Székely C. (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 C. (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 C. (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 C., 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 accepted in conferences booklet/ Oral presentation-

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. Priliminary investigastion 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