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Experimental and molecular biological examination of the host-specificity of fish parasitic myxozoans (Myxozoa)

Brief Summary of the Ph.D. Thesis

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Introduction

Myxozoans are common endoparasites of fish. More than 2000 species belonging to 62 genera are known nowadays, several of them are highly pathogenic. One of the best known species is *Myxobolus cerebralis*, the causative agent of the whirling disease in salmonids, and is responsible for high mortality in fish populations in Western Europe and in the United States. Well-known infections caused by myxozoans in Hungary include the malignant anaemia of the common carp evoked by *Myxobolus cyprini*, the gill infection caused by *M. pavlovskii* and the cranial connective tissue condition of the silver carp by *M. drjagini*, the gill sphaerosporosis of the common carp by *Sphaerospora molnari*, and the swimbladder inflammation by *S. renicola* whose aetiology and pathogenesis were clarified by Hungarian researchers. Besides the apparent pathological importance, the research on myxozoans is aimed at their complicated life cycle, taxonomy and phylogeny. All of the known myxozoan life cycles are associated with a vertebrate (mostly fish) and an invertebrate (usually oligochaete, rarely bryozoan) host, as well as with two spore types with significantly different morphologies, namely the myxospore developing inside fish and the actinospore in oligochaetes. As most of the myxozoans are highly host-specific, host recognition and invasion play extremely important roles in their life cycle. In the course of the research on host–parasite interactions, mainly the invasion mechanism of fish-infecting actinospores was examined so far, and little attention was paid to the host invasion and intraoligochaete development of myxospores. Results of the host recognition of fish-infecting actinospore stages suggest that chemical and mechanical stimuli, that are general characteristics of the host, cause the ‘invasive’ behaviour of actinospores. Later on, host specificity typical of myxozoans prevails via the specific immune response of fish. Such examinations have not been performed in case of the worm-infecting myxospores. In the Ph.D. work we planned to clarify the development of the myxozoans in oligochaete hosts using experimental and molecular biological methods. In the course of our project we performed oligochaete infection with *Myxobolus pseudodispar* myxospores originating from roach (*Rutilus rutilus*) in an *in vivo* experimental setup. The species composition of the oligochaete cultures, the effect of the species composition on infection prevalence and the susceptibility of different worm species were examined. We tracked the route of the parasite in the oligochaetes using *in situ* hybridisation (ISH). In addition, we clarified the life-cycle of *Myxobolus pavlovskii*, using experimental and molecular biological methods.

Materials and methods

Maintenance of the experimental animals and parasites

To keep up the myxozoan life-cycle in the laboratory, parasite-free (SPF) fish stocks and oligochaete cultures were reared and maintained. Four different oligochaete stock cultures were used in our experiments. Two of them were collected in the temperate water fish hatchery (TEHAG) in Százhalombatta. One of them was from the sediment of a fish pond, the other from the fish bed where the fish are sorted after the drainage of the ponds in autumn. Another oligochaete stock was obtained from an outflow of the fisheries council of the district of Upper Franconia (FFB) in Aufseß, Germany, and the fourth one originated from a fish wholesaler. Water from these oligochaete cultures was regularly filtered using 20-μm nylon mesh to check for a naturally occurring myxozoan infection. These regularly monitored stock cultures served as negative control in the infection experiments. Bulk infections were carried out to maintain the life cycle of *Myxobolus pseudodispar*. Approximately 10 g worms were placed in 5-L plastic tanks containing autoclaved mud and sand, and 10^5-10^6 myxospores were used for the infection of these test cultures. The exposed oligochaete populations were checked regularly after six weeks p.e. for the presence of actinospores. Photomicrographs and the measurements of the actinospores were taken. Actinospores were collected for molecular biological identification in 1.5-ml microtubes and stored at –20℃ until further use. Before the infection of the SPF roaches, the actinospores were checked and their number was evaluated. After three month post infection *Myxobolus pseudodispar* myxospores developed in the muscle of the fish. These myxospores were collected and used for infection of subsequent oligochaete populations.

Infection experiments

Experimental infections of Myxobolus pavlovskii

For the infection trial, eight parasite-free silver carp (Hypophthalmichthys molitrix) fry specimens (3−4 cm total length) were used. The fish were infected with actinospores by a two week cohabitation with the spore-releasing oligochaete culture before they were placed in a 30-litre aquarium. After 94 days, the fish were dissected, and the gills were examined for the presence of myxospores. Photomicrographs were taken of the infected gill filaments, and gills were fixed for histological examination. Whole gill arches were fixed in 10% neutral buffered formalin, embedded in paraffin, cut approximately in 5 μm thin sections, and stained with haematoxylin and eosin. Detected plasmodia were isolated from gill tissues and myxospores were collected for morphological and molecular identification. Myxospores

collected from the gills of five silver carp specimens infected experimentally in the present study were used to infect oligochaetes. The infection experiments were carried out as mentioned above by the bulk infections with *Myxobolus pseudodispar*. 10^6 Myxobolus pavlovskii were used for the infection. After one month p.e., the water from the exposed cultures and the control was regularly checked for the presence of actinospores by filtration for a period of 6 months.

Experimental infections of Myxobolus pseudodispar

Besides the bulk infections individual exposures were carried out to evaluate the susceptibility of the oligochaetes. Each oligochaete was exposed to a dose of 10^3-10^4 myxospores. The homogenized and concentrated myxospore suspension was mixed with autoclaved mud. One worm was placed in each 2-mL tube with 1.5 mL mud containing myxospores. After 1 h, 4 h, 8 h, 24 h, 1 week, 4 weeks, 3 months p.e., five oligochaetes of either origin were sampled. To observe the infection, besides checking for the actinospore release in cell-well plates, a *M. pseudodispar*-specific PCR assay was performed on worm specimens as well. For molecular characterization of the worms, the posterior part of oligochaetes were used, and the anterior parts of the worms were fixed for morphological identification and *in situ* hybridisation.

Molecular biological examinations

For the amplification of the 18S ribosomal DNA of the myxozoan parasites (myxospores and actinospores), a nested PCR assay were used. In the first round, primers 18e and 18r were used, followed by a second round PCR with the primer pair SphF and SphR. To amplify the entire sequence of the 18S rDNA of *Myxobolus pavlovskii*, the primer combinations ERI-B1 – MB3 and Myx4rF– ERIB-10 were used. For the detection of *M. pseudodispar,* a parasite-specific PCR assay was developed. For the identification of the oligochaetes, a part of the mt 16S rDNA were amplified using the primer pair Tub16SF–Tub16SR. The PCR products were sequenced.

Phylogenetic analysis

For the phylogenetic analysis of the examined oligochaetes, maximum likelihood (ML) algorithm and Bayesian inference analysis were performed.

In situ hybridization (ISH)

To track the route of *M. pseudodispar* in the oligochaete host, *in situ* hybridisation method was used. For the ISH, oligochaetes fixed in 10% NBF were embedded in paraffin, and 5 µm longitudinal sections were cut. The *M. pseudodispar*-specific oligonucleotides: MpF1 and PseudoR, designed for the *M. pseudodispar*-specific PCR assay and the also species-specific primers: MpR and PseudoF were labelled with biotin and used. The biotin-streptavidin-alkaline phosphatase-BCIP/NBT complex, which binds the parasite DNA specifically, signalled the presence of the parasitic developmental forms with a dark blue colour in the worm's tissues. On sections fixated at various time points of development, the parasite's location was examined by light microscopy, and photos were taken by a digital camera. The slides were examined under a light microscope, and photomicrographs were taken with a camera.

Vital staining

We planned to study the invasion mechanism of the parasite into the oligochaete host using vital staining. For the staining, fluorescein-diacetate (FDA) and propidium-iodide (PI) were used. FDA is a vital stain, which dye the cytoplasma of the cells dark green, while PI permeates only in the nucleus of dead cells, and emits red fluorescence.

Results

Life-cycle of *Myxobolus pavlovskii*

In the oligochaete population, originated from TEHAG fish ponds, water-borne echinactinomyxon-type actinospores were detected. From the examined oligochaete specimens only two released echinactinomyxon-type actinospores. One worm specimen of the two died due to the microscopical examination, the other were morphologically identified as a *Limnodrilus* species. In the molecular biological identification, a 335-bp-long sequence of the mt 16S rDNA was successfully amplified from one echinactinomyxon-releasing worm (HM991165), and a BLASTn search revealed a 95.59% sequence similarity to *L. udekemianus* (AF325986).

The spore body of the echinactinomyxon was pyriform, and the sporoplasms of the actinospores contained 32 (30−34) secondary cells (n = 6). Polar capsules were located anteriorly, deep beneath the valve. The caudal projections are attached to the spore body without style, tapering towards the pointed distal ends.

In the molecular biological examinations the entire 2,004-bp-long consensus sequence of the 18S rDNA was successfully amplified (HM991164). This shared a 99.87% similarity with the

1,578-bp-long, partial 18S rDNA sequence of *M. pavlovskii* available in GenBank (AF507973).

In all successful infected fish specimen, a massive infection was attained. The plasmodia were located in the epithelium of the gill lamellae and could easily be scraped off during the dissection. The myxospores detected were morphologically similar to those of *M. pavlovskii* described by Akhmerov (1954), just slight differences were in the range of their measurements.

The entire 18S rDNA was successfully amplified and sequenced from two myxospore samples collected from different fish hosts. These DNA sequences were identical and found to be also congruent with the DNA sequence of the echinactinomyxon examined. They differed only in a single nucleotide (0.13%) from that of *M. pavlovskii* available in GenBank (AF507973).

The subsequent trial to infect the laboratory oligochaete stock with these *M. pavlovskii* myxospores failed, as no actinospores were detected in the water of the exposed worm container for 6 months upon regular checks.

Oligochaete identification

The mt 16S rDNA of 156 oligochaete specimens was genetically analysed from the 4 oligochaete test cultures. Morphologically, 19 oligochaete specimens were identified; 7 were identified as *Tubifex tubifex*, 4 as *Limnodrilus claparedeanus*, 2 as *Limnodrilus hoffmeisteri*, 2 as *Psammoryctides barbatus,* 2 as *Psammoryctides moravicus,* 1 as *Potamothrix bavaricus* and 1 as *Potamothrix hammoniensis*. The results of the morphological identifications confirmed the molecular findings in most cases, and in some cases, they supplied further details concerning the identity of the worm specimens. Two specimens genetically identified as *Psammoryctides* sp. were found to be *P. moravicus,* and *Potamothrix* sp. was identified as *P. hammoniensis* using morphology. Molecular and morphological examinations led to inconsistensy for a specimen identified as *Limnodrilus cervix* based on DNA sequence data, while it was found to be *L. claparedeanus* by morphological identification.

Phylogenetic analysis

The results of the two different methods were similar. Phylogenetic analyses used maximum likelihood algorithm clustered the *Psammoryctides* spp. and *Limnodrilus* spp. in one clade, and *T. tubifex* lineages and *Potamothrix* spp. in another. The six *T. tubifex* lineages were clearly distinct from each other. In the case of Bayesian method, the *Psammoryctides spp.* clustered in a different clade. In both cases, the six *T. tubifex* lineages were clearly distinct from each other. Lineage I and VI grouped together while lineage II, III, IV and V formed a

separate group. There were 2 worm samples (JF783970) which relate closely to *T. tubifex* lineage V, while only 94.7-95.3% sequence similarity was found. In the phylogenetic tree, the *T. tubifex* lineages susceptible to *M. pseudodispar* located in the *T. tubifex* branch, along with the non-susceptible lineages. The genetic variation among lineages varied between 6.6-15.0%. Among *L. hoffmeisteri* samples, notable genetic differences were observed. In the sequences of the 35 specimens examined, the greatest difference detected was 15.5%.

Experimental infections with *M. pseudodispar*

The life-cycle maintenance in the laboratory has been succesful since 2007. The parasite-specific PCR results derived from the bulk and individual exposures revealed that the test cultures from the TEHAG fish ponds and from Aufseß, Germany, seemed to be the most susceptible, as infection prevalence in the experimental setup was 60.5% and 55.4% respectively. The least-susceptible test culture was the one originating from TEHAG fish bed with 33.3% infection prevalence. The susceptibility of the various *T. tubifex* lineages to *M. pseudodispar* differed considerably. Worms from *T. tubifex* lineage II were most susceptible for *M. pseudodispar* as 90.5% of the examined specimens released TAMs. Of the *T. tubifex* lineage III, 22.2% of worms released actinospores. The oligochaetes of lineage V were less susceptible, only 18.8% of the examined specimens released TAMs. None of the examined worms of lineage VI were infected. *P. barbatus* and *P. moravicus* were found also suitable hosts for *M. pseudodispar* by PCR, and they also released actinospores.

ISH

The results of the ISH showed that the development of *M. pseudodispar* took place in the gut epithelium. As soon as a few hours p.e., developmental stages of *M. pseudodispar* could be seen in sections of the gut epithelia. In the first week of development, stages were detected in the basal lamina of the gut epithelium. One month p.e., developmental stages were seen along the whole length of the gut epithelium. After three months p.e., numerous pansporocysts containing four folded TAMs each were detected. An interesting finding was that in the first days of development, worm amoebocytes located in the coelom showed a dark-blue colouration as well, as they had incorporated parasite cells.

Results of the fluorescent staining of actino- and myxospores

The staining method worked well with actinospores, the dyes easily entered the cells, and after a brief staining of 10-15 minutes, they could be examined by fluorescent microscopy. Unfortunately, staining was insufficient in myxospores, because due to the more compact

build of the spore, the dye could not enter the cell in sufficient amounts, leading to a very weak staining, hindering us in examining the parasite's entry into the worm this way.

Discussion

In our study, an echinactinomyxon-type actinospore, detected as natural infection in an oligochaete population originated from THEHAg fish ponds, was identified as the transmission stage of *Myxobolus pavlovskii*, a common parasite of bighead carp and silver carp. The 18S rDNA sequence of the echinactinomyxon of this study was found to be identical except 1 nucleotide with the *M. pavlovskii* myxospore sequence published previously (HM991164). Although *M. pavlovskii* frequently occurs in fish, in the course of surveys focused on the detection of the actinospore stages of myxozoan species in Hungarian waters, an echinactinomyxon-type has never been found.

The development of *M. pavlovskii* has formerly been studied by German researchers. Their results disagree with ours as we found an echinactinomyxon in contrast with the hexactinomyxon-type actinospore they designated as the actinospore stage of this parasite. In our study, the myxospore morphology and the histology of experimental infections and the 18S rDNA sequences confirmed that the species at hand was indeed *M. pavlovskii,.* and refuted the previous results of the German researchers.

Effort was also made to complete the life cycle of *M. pavlovskii* by infecting a parasite-free oligochaete culture with myxospores collected from the gills of the experimentally infected silver carp fry, but this attempt remained unsuccessful. This failure reveal the problem that the species composition of the used oligochaete culture can be an important factor in similar infection experiments. Our results suggest that the species composition of an oligochaete culture may change over time. So the failure of the experiment can be explained by the a changed composition of the worm culture which at the time of the trial might no longer be adequate for the development of the parasite. The species composition of the oligochaete populations used for infection experiments, due to the absence of the susceptible host, can be a crucial factor in the infection experiments with the myxozoans.

The main goal of the Ph.D. study was to clarify the intraoligochaete development of the myxozoans, and to detect their species-specificity. *Myxobolus pseudodispar*, the common parasite of cyprinids were used for these studies.

Besides the previously detected suitable oligochaete hosts (i.e. *Tubifex tubifex* and *Limnodrilus hoffmeisteri*), the susceptibility of further worm species was demonstrated by

experimental exposure: *Psammoryctides barbatus* and *P. moravicus*. Although the number of examined worms were low in some cases, our findings seem to demonstrate that, similarly to the vertebrate host range of *M. pseudodispar*, several oligochaete species can serve as invertebrate hosts of the parasite.

According to our studies to detect the susceptibility of the worm species and lineages for *M. pseudodispar, T. tubifex* lineages I and III were susceptible hosts not only for *M. cerebralis*, but also for M. pseudodispar. Additionally, *T. tubifex* lineage II could also be infected with *M. pseudodispar* myxospores and produced actinospores. *T. tubifex* lineage VI appeared to be non-susceptible as is the case for *M. cerebralis*, since no infection by both parasites has been detected so far. However, just a few specimens were examined from *T. tubifex* lineage IV, VI and *P. hammoniensis*, thus we cannot draw a definite conclusion concerning their susceptibility to *M. pseudodispar*.

In the infection trials, *T. tubifex* lineage V and *L. hoffmeisteri* specimens were positive for the presence of *M. pseudodispar* DNA, but in most cases, TAM-release could not be detected. In these cases, sporoplasms entered, but their development was likely aborted at some point. The formation of the fish-infecting actinospore stages might be interrupted by the immune system of the worm. This is also supported by our ISH findings that the amoebocytes involved in the cellular immune response of the oligochaetes phagocytised invading parasite cells. These oligochaetes may serve as disruptors of the life cycle (biological filter species) as they remove and inactivate myxospores from the sediment. Our results in the infection experiments using different oligochaete cultures show that the species composition of the oligochaete population, the proportion of the susceptible, resistant and biological filter species, is an important factor in the parasite transmission dynamics. The mt 16S rDNA sequences, amplified for the molecular identification of the worm species, were also used to study the releationships among the oligochaete species. The results of the phylogenetic analysis of the examined oligochaetes are congruent with previously published data. The clusters among the examined oligochate samples correspond to the species and lineages described previously. Besides the six aforementioned lineages, two examined *T. tubifex* specimens clustered next to *T. tubifex* lineage V, while the genetic difference was much higher than the intra-lineage variations detected so far, which suggests that there might be new separate lineages in *T. tubifex sensu lato*. Furthermore, the results of the phylogenetic analyses did not reveal correlation between the susceptibility of *T. tubifex* lineages to *M. pseudodispar* and their phylogenetic position.

The intraoligochaete development of *M. pseudodispar* were followed using *in situ* hybridisation. According to our results the gut epithelia seemed to be portal of entry for the sporoplasms, and the development of the parasite took place mainly in the gut epithelium as well. This finding confirmed the previous histological studies of *M. cerebralis.* In the first week

p.e., we detected intensive positive staining in the basal lamina of the gut epithelia, which indicated that this extracellular matrix may be involved in the longitudinal migration of the parasite along the gut epithelium. Another interesting finding was that the amoebocytes in the coelomic fluid of the oligochates' coelom were stained strongly in the first few weeks p.e. These free cells most likely play an important role in the cellular immune response of worms. Our results suggest that the worm's immune system reacts to the myxozoan infection.

As of this time, only a few studies dealt with the intraoligochaete development and oligochaete host specificity of myxosporeans, and they focused almost entirely on *M. cerebralis*. The Ph.D. study provides detailed information about the invertebrate host range of *M. pseudodispar,* and the susceptibility of the different oligochaete species and lineages to the parasite. Besides, we revealed that the species composition of the oligochaete cultures may influence the results of the myxozoan infection experiments significantly. To ascertain the route of the intraoligochaete development provided new detailes for the understanding of the infection mechanisms of myxozoans. In addition, the previous results in the developmental cycle of *Myxobolus pavlovskii* were disproved. We determined and confirmed, using infection experiments, histological and molecular methods, that the actinospore stage of *M. pavlovskii* is an echinactinomyxon.

New scientific results

1. Disproving the previously published results, with successful infection experiment we detected an echinactinomyxon-type actinospore stage for *Myxobolus pavlovskii*.
2. The results of the infection experiments were proved with molecular biological methods, and the entire 18S rDNA sequence of *M. pavlovskii* were determined.
3. The first *Myxobolus* sp. with an echinactinomyxon-type actinospore stage was detected. In addition, one of the oligochaete host of *M. pavlovskii*, the *Limnodrilus udekemianus* was first identified.
4. The possible susceptible oligochaete hosts of *Myxobolus pseudodispar* were examined, and susceptible, resistant and biological filter species and lineages were separated for the first time, and we revealed that the species composition of the oligochaete cultures can influence the results of the myxozoan infection experiments.
5. We confirmed that *Psammoryctides barbatus* and *Psammoryctides moravicus* are susceptible hosts for *M. pseudodispar*. The first mt 16S rDNA sequences from *Psammoryctides moravicus*, *Potamothrix hammoniensis*, *Limnodrilus claparedeanus* were published.
6. For the detection of *M. pseudodispar,* a species-specific PCR, and for the ISH specific probes and a short, 1-day-long method were developed and optimized. The route of the intraoligochaete development of *M. pseudodispar* was studied foremost. According to the results, the gut epithelia seemed to be the portal of entry for the sporoplasms, and the development of the parasite took place mainly in the gut epithelium as well.
7. The ISH results suggest that the worm’s immune system reacts to the myxozoan infection as the amoebocytes of the worm incorporated parasite cells.

Scientific publications

In peer-reviewed journals

1. Marton Sz., Eszterbauer E.: **The development of *Myxobolus pavlovskii* (Myxozoa: Myxobolidae) includes an echinactinomyxon-type actinospore**, Folia Parasitol., 58. 157–63, 2011. IF: 1,533
2. Marton Sz., Eszterbauer E.: **The susceptibility of diverse species of cultured oligochaetes for the fish parasite Myxobolus pseudodispar Gorbunova, (Myxozoa)**, J. Fish Dis., (in press) IF: 1,603
3. Marton Sz., Eszterbauer E.: **Hazai és nemzetközi eredmények a halparazita nyálkaspórások (Myxozoa) gazdafajlagosságának kísérletes vizsgálatában**, Magy. Állatorv. Lapja (in press) IF: 0.300

Abstracts and proceedings of International Congresses

1. Marton Sz., Kallert D., Eszterbauer E.: **The susceptibility of diverse species of cultured oligochaetes for the fish parasite, *Myxobolus pseudodispar***. 15th EAFP International Conference on Diseases of Fish and Shellfish, 12-16th September 2011, Split, Croatia. Abstract No. O-068.

2. Eszterbauer E., Marton Sz.: **The intraoligochaete development of *Myxobolus pseudodispar* (Ph: Myxozoa)**. 15th EAFP International Conference on Diseases of Fish and Shellfish, 12-16th September 2011, Split, Croatia. Abstract No. P-269.

3. Marton Sz., Eszterbauer E.: **The life cycle of *Myxobolus pavlovskii* (Myxozoa): a *Myxobolus* species with echinactinomyxon type actinospore.** 14th EAFP International Conference on Diseases of Fish and Shellfish, 14-19th September 2009, Prague, Czech Republic. p. 081.

Other publications

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4. Molnár K., Eszterbauer E., Marton Sz., Cech G., Székely Cs.: ***Myxobolus erythrophthalmi* sp. n. and *Myxobolus shaharomae* sp. n. (Myxozoa: Myxobolidae) from the inner organs of rudd, *Scardinius erythrophthalmus* (L.) and bleak, *Alburnus alburnus* (L.)**, J. Fish Dis., 32. 219–231, 2009. IF: 1,697

5. Molnár K., Marton Sz., Eszterbauer E., Székely Cs.: **Description of *Myxobolus gayerae* sp. n. and re-description of *Myxobolus leuciscini* infecting the European chub from the Hungarian stretch of the river Danube**, Dis. Aquat. Org., 78. 147–153, 2007. IF: 1,598

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7. Molnár K., Marton Sz., Eszterbauer E., Székely Cs.: **Comparative morphological and molecular studies on *Myxobolus* spp. infecting chub from the River Danube, Hungary, and description of *Myxobolus muellericus* sp. n.**, Dis. Aquat. Org., 73. 49–61, 2006. IF: 1,509

8. Eszterbauer E., Marton Sz., Rácz O.Z., Letenyei M., Molnár, K.: **Morphological and genetic differences among actinosporean stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification**, Syst. Parasitol., 65. 97–114, 2006. IF: 0,856

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1. Eszterbauer E., Rónai Zs., Marton Sz., Ursu K., Baska F., Láng M.: **Dissemination of *Mycobacterium* spp. via commercial fish food**. 15th EAFP International Conference on Diseases of Fish and Shellfish, 12-16 September 2011, Split, Croatia. Abstract No. P-126.

2. Marton Sz., Eszterbauer E., Székely Cs., Molnár K.: **Morphological and phylogenetic studies on *Myxobolus* spp. infecting chub in Hungary**. *4th Croatian Congress of Microbiology with International Participation*, 24th-27th of September 2008, Zadar, Croatia.

3. Marton Sz., Eszterbauer E., Székely Cs., Molnár K.: **Comparative morphological and phylogenetic studies on *Myxobolus* spp. infecting chub**. *ISFP7*, 24th-28th of September 2007, Viterbo, Italy. Parassitologia, 49, Suppl.2:160.

4. Eszterbauer E., Marton Sz., Letenyei M., Rácz O., Molnár K.: **Morphological and genetic differences among actinosporean developmental stages of fish-parasitic myxosporeans (Myxozoa): difficulties of species identification**. *EMOP IX*, 18-23 July, 2004. Valencia, Spain. Abstract No. 1120.