FINAL REPORT

Investigation of fish infecting trematodes and their developmental stages by molecular methods

During the research, Hungarian natural fresh waters and aquacultures were monitored for Digenean trematodes, which are well known agents of several fish diseases. The study included parasites of the body surface of the fish as well as inner organs, gills and the musculature. Fish are infected by the larval stage metacercaria, there for the emphasis was put mostly on them, however we also tried to collect adult worms from water birds or get them from chicken infection experiments. Usually, fresh water snails were also collected on the sampling, because those are the hosts of the first larval stage of the Digeneans, the so-called cercariae. We also tried to gather information about potentially zoonotic species. Throughout the four years research various members of families Echinostomatidae, Heterophyidae, Opistorchiidae and Diplostomatidae were investigated, as it is detailed below.

During the first year, echinostomatid metacercariae with collar spines characteristic of species of the genera *Petasiger* and *Paryphostomum*, were found in the lateral line scales of a roach (*Rutilus rutilus*), an apparently unique site. In a subsequent examination of numerous fishes from 20 different species, similar infections were found in 11 species. The infection was virtually restricted to the lateral line scales, other scales being infected only incidentally. Two types of metacercariae were found. The first type had three central dorsal spines that were larger than the remainder and tended to resemble the angle spines. The second type had all 19 dorsal spines of a similar size. ITS region of the metacercariae were amplified and sequenced.The second type of metacercaria (five parallel samples) exhibited a 100% similarity to *Petasiger phalacrocoracis* deposited in the GenBank database. Interestingly, one sample of the second type metacercariae differed from the *Petasiger phalacrocoracis* specimens based on ITS sequences. ITS results obtained from two metacercariae of the first type showed a 2.8–2.9 % difference from sequences of the second type of metacercaria and were not identical with any *Petasiger* or *Paryphostomum* sequences available in the Genbank. Adult specimens of *Petasiger phalacrocoracis* (two samples), *Petasiger exaeretus* (two samples) and *Paryphostomum radiatum* (three samples) were collected from the gut of cormorant (*Phalacrocorax carbo*). Sequences of adult trematodes confirmed that the second type of metacercaria belongs to *P. phalacrocoracis*, however the first type remained unidentified at species level. Metacercariae of *Paryphostomum radiatum* were not found yet. The analyses of the genes 28S rDNA and nadI (nicotinamide adenine dinucleotide dehydrogenase subunit 1) also did not show any results with the

published *Petasiger* and *Paryphostomum* sequences in the Genbank. Overall, the results confirmed that second type of metacercaria is identical with *P. phalacrocoracis*, but the first type does not belongs to any of the already published species based on the 28S rDNA and nadI sequences.

The result were presented in Folia Parasitologica (DOI: 10.14411/fp.2015.017) and Acta Veterinaria Hungarica (DOI: 10.1556/004.2017.020).

In parasitic surveys made on fishes of Lake Balaton and its tributaries in Hungary, an infection with metacercaeriae supposedly belonging to *Echinochasmus* genus (Trematoda: Echinostomatidae) was found in 7 species of fishes belonging to 5 families. In ruffe (*Gymnocephalus cernua*), malformations of the gill filaments were also recorded due to this infection. Malformation appeared in duplication of the filaments at about their half-length. At the point where filaments divided, there was found always a metacercaria incorporated into the cartilaginous gill rays of the filament. Bifurcation was found in all of the studied ruffe specimens, but on the same fish specimens several other metacercariae of the same type were found which caused only local distortions. In the other 6 infected fish species only local symptoms were found in the cartilage at the attachment sites of the metacercarians. In the course of molecular examination ITS region of two metacercariae from ruffe and one from stone moroko (*Pseudorasbora parva*) were amplified and sequenced. These sequences showed 93.4 % similarity to ITS sequence of an *Echinochasmus* sp. sample (FJ756940), a cercaria collected from gravel snail (*Lithoglyphus naticoides*). Other members of the family Echinostomatidae, like *Echinostoma, Echinoparyphium, Paryphostomum* and *Petasiger* species showed only a moderate similarity, averaging between 82-84%. Interestingly, one of our cercaria samples (a gymnocephala type cercaria) collected from *Lithoglyphus naticoides* was identical with the above mentioned metacercaria samples, and its sampling area (Lake Balaton, Keszthely) matched with the collection site of a part of the metacercariae. Due to the few molecular data of genus *Echinochasmus*, we were not entirely certain that these malforming metacercariae belong to genus *Echinochasmus*, even the morphological studies of metacercariae did not result an unquestionable proof to it. Therefore, experimental infections in chicks (force-feeding them with metacercariae) are used to obtain adult specimens of the parasite, and the sequences of the ITS region are employed to link life-history stages and help confirm the identification of the metacercaria at the generic level. The morphological characteristics of these adult specimens confirmed them to belong to the genus *Echinochasmus*. Moreover, their ITS sequences (4 parallel samples) were identical to the sequences from metacercariae.

The results of this study was published in the Journal of Fish Diseases (DOI:10.1111/jfd.12469).

We put emphasis on collecting *Apophallus* sp. (Digenea: Heterophyidae), which are known to cause the black-spot disease in many different fish species. It has generally been thought that metacercariae of *A. muehlingi* infect cyprinid fishes, whereas those of *A. donicus* develop in percids. During the present work, 99 black-spotted specimens out of 150 individuals of three cyprinid species, common

bream *Abramis brama* (L., 1758), white bream *Blicca bjoerkna* (L., 1758) and rudd *Scardinius erythrophthalmus* (L., 1758), and 18 specimens out of 52 individuals of two percid species, ruffe *Gymnocephalus cernua* (L., 1758) and perch *Perca fluviatilis* (L., 1758), were collected from three regions of Lake Balaton in Hungary, namely Keszthely, Siófok and Tihany In addition, 23 nase *Chondrostoma nasus* (L., 1758) and 5 chub *Squalius cephalus* (L., 1758) were collected from the River Danube close to the city of Szentendre. Additionally, cercariae were collected from about 50 gravel snails *Lithoglyphus naticoides* from Lake Balaton at the city of Keszthely. Due to the difficulties in identifying metacercariae morphologically, experimental infections in chicks were used to obtain adult specimens for study. 28 *Apophallus* samples were analysed for the ITS region and COI genes, including cercarial, metacercarial and adult developmental stages. Generally, the results support Odening's (1973) hypothesis that metacercariae of *A. muehlingi* infect cyprinid fishes, whereas those of *A. donicus* infect percid fishes. Unexpectedly, our molecular studies also revealed a third species of *Apophallus*. Samples of metacercariae from chub, nase and rudd resulted in a well-defined phylogenetic clade; this was indicated by both genes as being distinct from both *A. muehlingi* and *A. donicus*. Later, the morphology of adult worms gained in the chicken experiments confirmed the separateness of this third species.

The results were published in Parasitology Research (DOI: 10.1007/s00436-017-5617-5) except the description of the third *Apophallus* species due to the lack of good quality histological sections.

Opistorchiid species, like *Opistorchis* and *Metorchis* genera (Digenea: Heterophyidae) are regarded as potentially zoonotic species. They could be found in the musculature of fish, and consuming raw or uncooked fish meat could cause for example metorchiasis, as it is a frequent problem in the Far East. Therefore, during the third year Opisthorchiid trematodes (Platyhelminthes: Digenea) of common carp (*Cyprinus carpio*) were monitored in Hungarian aquacultures. Four geographically distinct and methodologically different fish farms were chosen for monitoring, 258 one year old fingerlings were investigated from each farm. The fish were anesthetized and decapitated and left and right sections of the musculature were examined under stereomicroscope. Muscle filets were digested in pepsin-solution to free the metacercariae from the tissue to be counted and for further morphological analysis. A few specimens were selected to analyse the sequence of the ITS region to confirm the morphological results. Opisthorchiid metacercariae were not found until now, however a non-zoonotic muscle parasite species occurred in abundance (36 infected individuals of 258 fish) in one of the four fish farms. This species was supposed to be *Holostephanus* sp. based on its morphological characteristics, which was confirmed by the molecular data (only ITS so far, there are no available COI sequences from *Holostephanus* in the Genbank). Another fish farm was also infected by Digenean trematodes, however the present metacercariae belonged to the ectoparasite *Posthodiplostomum* sp. but the rate of infection was very low (only two out of the 258 specimen were infected by 6 metacercariae). The rate of infection showed remarkable difference between the four fish

farms. Two of them were infected by metacercariae, while the other two farms proved to be negative. The infected fish stocks were cultured in ponds close to natural habitats, where rich bird fauna (final hosts) is present and freshwater snails (first intermediate hosts) are in abundance. A second round survey was carried out in the infected farm. In this study 21 one-summer-old, 27 two-summer-old and 30 three-summer-old carp specimens from a Northern Hungarian Plains fish farm were investigated. After the artificial digestion and the filtartion, the contact metacercariae could be collected and counted. As results, all of the one-summer-old carps (21 specimens) were infected, 27 two-summer-old fish showed 77,7% infection prevalence while 90% of the 27 three-summer-old specimens were positive. *Holostephanus* species are known to be non-zoonotic, however infection experiment of mices were executed to test if the metacercariae could develop in mammals. In the infection experiment, no adult worms were found in the guts of the mice.

Black-spot disease can be caused also by *Posthodiplostomum* (Digenea: Diplostomatidae) metacercariae, however infection of inner organs are also frequent symptoms in case of *Posthodiplostomum* infections. During the second year an investigation has started on *Posthodiplostomum* species, where supposedly a North American *Posthodiplostomum* species was observed in the invasive fish, the pumpkinseed (*Lepomis gibbosus*), most of the their inner organs (liver, spleen, kidneys, guts) were susceptible for infection. Additional *Posthodiplostomum* samples were collected from native fish species (common bream, white bream and roach) for *Posthodiplostomum* metacercariae. Metacercariae were fed to chicks to get adult worms for morphological and molecular analysis. 19 samples (metacercariae and adult individuals) were identified based on the ITS sequences and 11 of them for COI gene to confirm the ITS results. Flukes (metacercariae and adults) originated from pumpkinseed were identified again as *Posthodiplostomum centrarchii* while the specimens from the cyprinid fishes differed remarkably from species *Posthodiplostomum centrarchii*, and they were identified as *Posthodiplostomum cuticola*, which is a native species of Europe. It should be noted that *P. centrarchii* was found until now only in one single site, the Maconka-water reservoir in Northern Hungary. The distribution of *P. cuticola* is much broader and includes the most of Hungarian natural waters. Surprisingly, three black-spotted specimens of common bream (*Abramis brama*) had *Posthodiplostomum centrarchii* infection. It was collected only on a single occasion from the Keszthely-area of Lake Balaton. Only molecular data (ITS sequences) support this fact, unfortunately samples were not put aside for morphological investigation as those black spots were presumed to be caused by *Apophallus* sp. At this point it cannot be stated without doubt that common breams can be infected by *P. centrarchii*, as more supporting findings are necessary for confirmation. Moreover, it would increase its possibilty if infected pumpkinseeds could be found in the Lake Balaton which has not happened yet.

Metagonimus Katsurada, 1912 (Digenea: Heterophyidae) species are well-known zoonotic parasites, which reach their mature stage in the small intestine of water birds, mammals and humans as well. Up

to this time, ten *Metagonimus* species are known mostly from Eastern and Southern Asia. The representatives of the genus *Metagonimus* have been reported from Eastern Europe too, but only the species *M. yokogawai* was documented in Serbia, Bulgaria, Hungary, Czech Republic and Spain. The identification of the European findings was based only on morphological characteristics. In the past decade sequence analysis of Digenean flukes became common and several studies of *Metagonimus* species involves molecular methods. Mostly the nuclear ITS region, 28S rDNA and the mitochondrial COI is the subject of these sequence analysis. During a recent survey 150 fish specimens of bleak (*Alburnus alburnus*), common nase (*Chondrostoma nasus*), ide (*Leuciscus idus*) and chub (*Squalius cephalus*) were investigated for *Metagonimus* infection in the river Danube. Experimental infections in chicks were carried out for correct species identification. The morphology (native microscopy and histology) of metacercariae and adult individuals was characterised and 14 specimens were analysed by sequencing the ITS region, 28S rDNA and the mithocondrial COI .The molecular results strongly support that *Metagonimus* specimens collected in Hungary differ remarkably from the already described *Metagonimus* species known from the Far-East and may represent *Metagonimus romanicus* (Ciurea, 1915). Although musculature of carp specimens from aquacultures was checked regularly for *Metagonimus* metacercariae, they were never found; therefore carps raised in Hungarian aquaculture seem to be free from zoonotic flukes.

Diána Sándor joined the research as an MSc student during the second year and participated in the collection of samples and the molecular work. Her MSc thesis included the study about the *Apophallus* species and it was defended successfully with summa cum laude.

Based on the results of the project further scientific papers are in preparation on the topics of description of the new *Apophallu*s and *Metagonimus* species and the Hungarian occurrence of *Posthodiplostomum centrarchii*.