New data on *Thelohanellus nikolskii* Achmerov, 1955 (Myxosporea, Myxobolidae) a parasite of the common carp (*Cyprinus carpio*, L.): The actinospore stage, intrapiscine tissue preference and molecular sequence

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**ABSTRACT**

*Thelohanellus nikolskii*, Achmerov, 1955 is a well-known myxozoan parasite of the common carp (*Cyprinus carpio* L.). Infection regularly manifests in numerous macroscopic cysts on the fins of two to three month-old pond-cultured carp fingerlings in July and August. However, a *Thelohanellus* infection is also common on the scales of two to three year-old common carp in ponds and natural waters in May and June. Based on myxospore morphology and tissue specificity, infection at both sites seems to be caused by the same species, namely *T. nikolskii*. This presumption was tested with molecular biological methods: SSU rDNA sequences of myxospores from fins of fingerlings and scales of older common carp were analysed and compared with each other and with related species available in GenBank. Sequence data revealed that the spores from the fins and scales represent the same species, *T. nikolskii*. Our study revealed a dichotomy in both infection site and time in *T. nikolskii*-infections: the fins of young carp are infected in Summer and Autumn, whereas the scales of older carp are infected in Spring. Myxosporean development of the species is well studied, little is known, however about the actinosporean stage of *T. nikolskii*. A previous experimental study suggests that aurantiactinomyxon actinospores of this species develop in *Tubifex tubifex*, Müller, 1774. The description included spore morphology but no genetic sequence data (Székely et al., 1998). We examined ~9000 oligochaetes from Lake Balaton and Kí-Balaton Water Reservoir searching for the intraoligochaete developmental stage of myxozoans. Five oligochaete species were examined, *Isochaetides michaelensi* Lastochin, 1936, *Branchiura sowerbyi* Beddard, 1892, *Nais* sp., Müller, 1774, *Dero* sp. Müller, 1774 and *Aelosoma* sp. Ehrenberg, 1828. Morphometrics and SSU rDNA sequences were obtained for the released actinospores. Among them, from a single *Nais* sp., the sequence of an aurantiactinomyxon isolate corresponded to the myxospore sequences of *T. nikolskii*.

1. Introduction

*Thelohanellus nikolskii* Achmerov, 1955 is a common, pathogenic myxosporean parasite specific to common carp. Originating in the Far East, it was inadvertently introduced to Europe in 1979 where it subsequently caused heavy infection in fingerlings of common carp cultured in fish ponds (Jeney, 1979; Molnár and Kovács-Gayer, 1981–1982; Molnár, 1982; Cirkovic et al., 2013). Achmerov (1955, 1960) was the first to describe four *Thelohanellus* species (*Thelohanellus nikolskii*, *T. amurensis*, *T. dogieli* and *T. hovorkai*) from Amur wild carp (*Cyprinus carpio haematopterus*) in the Amur Basin. Of these, *T. nikolskii* infected the fins, while *T. amurensis* Achmerov, 1955, *T. dogieli* Achmerov, 1955 and *T. hovorkai* Achmerov, (1960) parasitised the liver, skin and abdominal cavity, respectively. Two other *Thelohanellus* species showed high morphological similarity to *T. nikolskii*: *Thelohanellus cyprini* Hoshina and Hosoda (1957) infects the fins and *Thelohanellus kitauei* Egusa and Nakajima (1981) infects the intestines and skin of common carp (Zhai et al., 2016). Recently,
were collected from the Kis-Balaton Water Reservoir in May and June
fections of Thelohanellus spp., Molnár and Kovács-Gayer (1981, 1982)
and Molnár (1982) later identified the parasite as T. nikolskii. Histo-
logical study of plasmodia developing in the cartilaginous tissue of the
fin rays was done by Molnár (1982), while the ultrastructure on the
sporogenesis of this species was studied by Desser et al. (1983). In the
synopsis of Zhang et al. (2013), 11 other Thelohanellus species, some
of them with questioned validity, have been reported from different organs
of the common carp: T. acuminatus Achmerov, 1955; T. callilporis, Ky,
1971; T. chuhainensis Ma, Dong and Ma, 1999; T. hokaiagensis Ma, Dong
and Wang, 1999; T. kyi (Ky 1971) Zhang et al., (2013); T. leshanensis
Zhao and Ma, 1992; T. paraquisiturus Chen and Ma, 1998; T. pekingensis
Chen and Ma, 1998; T. quinghoensis Li and Wen, 1992; T. sagittarius Lie
and Nie, 1973 and T. wananensis Lei, 1988.

Jeney (1979) supposed that Thelohanellus infection of the common
carp was introduced to Hungary through importation of Cyprinus carpio
haematopterus (Amur wild carp, koi carp), a subspecies of the common
carp, from the Far East. Besides Hungary, T. nikolskii infection in the fins
of carp fingerlings became a wide ranging disease in several European
countries, e.g., in Serbia (Cirkovic, 1986; Hacmanjek, 1985), in Czechia
(Dyková and Lom, 1988), and in Moldova (Trombítsky et al., 1983,
1990; Moslu, 1993). Thelohanellosis on the scales of older carp speci-
mens were observed first by Moshu and Molnár (1997) in Moldova and
soon after the infection was observed also in Hungary (Molnár and
Szekely, 1997). Based on morphological similarity of myxosporans and
cysts and development of plasmodial in the cartilaginous tissue of the
scales and fins, the above authors identified the species developing in
the scales also as T. nikolskii.

Known life cycles of myxosporans include development in an
invertebrate host. The intraoligochaete development of T. nikolskii was
studied first by Székely et al. (1998). These authors experimentally
infected the oligochaetes Branchiura sowerbyi Beddard, 1892 Tubifex
tubifex Müller, 1774 and Limnodrilus hoffmeisteri Claparède, 1862 with
T. nikolskii myxosporans, and detected waterborne aurantiactinomyxon
type actinospores released from Tubifex tubifex, however this connection
has not been confirmed with molecular genetic data.

Our study confirms the identity of Thelohanellus nikolskii-like cysts
developing in the fins and scales of common carp, using a combination of
morphological, histological and molecular genetic methods. More-
over, the oligochaete host and the actinospore type of T. nikolskii was
determined by studying actinosporeans released by oligochaetes from
Lake Balaton and Kis-Balaton Reservoir.

Common carp (Cyprinus carpio) in Hungary is cultured in ponds ac-
cording to a three year system. After artificial propagation in a hatchery,
carp fry are raised in a series of nursery ponds, whereas two or three
year-old stocks are reared in large, often over 100 ha, ponds. Natural propagation of carp in natural waters is negligible, due to the absence of
suitable spawning grounds. Natural waters are resupplied with two and
three year old carp year after year from fish farms.

2. Materials and methods

2.1. Collection of fish and myxospore samples

Two month-old common carp fingerlings with fin infections with T.
nikolskii cysts were collected in July 2015 from a fish pond where
about 70% of the stock was infected by this parasite. Earlier in the
same year, seven specimens of three-year-old common carp with scale in-
fections of Thelohanellus plasmodia were also collected. Three specimens
were collected from the Kis-Balaton Water Reservoir in May and June
while four specimens were obtained from fish farms throughout the
country in the same period (Table 1). Infected fish were transported to the
laboratory alive in plastic sacs filled with oxygen. Fingerlings in the

| Name of the sample | Age of the carp | Organ | Sampling date (dd.mm. yyyy) | Sampling site | Location in Hungary |
|--------------------|----------------|-------|-----------------------------|---------------|---------------------|
| TU1                | Fingerling fins | 14.07.2015 | Tiszagyála | North-East Hungary |
| TU2                | Fingerling fins | 14.07.2015 | Tiszagyála | North-East Hungary |
| TK4                | 3 years old scales | 19.05.2015 | Kis-Balaton Reservoir | West Hungary |
| TK5                | 3 years old scales | 01.06.2015 | Tiszavasvári | North-East Hungary |
| TK6                | 3 years old scales | 02.06.2015 | Kőrösladány | South-East Hungary |
| TK7                | 3 years old scales | 03.06.2015 | Kis-Balaton Reservoir | West Hungary |
| TK8                | 3 years old scales | 04.06.2015 | Rückevé | Central Hungary |
| TK9                | 3 years old scales | 10.06.2015 | Kis-Balaton Reservoir | West Hungary |
| TK10               | 3 years old scales | 12.06.2015 | Paks | South Hungary |

Table 1

Summary of the data about Thelohanellus spp. infected fish samples examined with molecular methods in this study.

2.2. Collection of oligochaetes

Sediment from Lake Balaton and Kis-Balaton Reservoir with pre-
vious T. nikolskii records was collected near water vegetation at about
0.5–1 m depth with a net in 2010–2015. On each sampling occasion, as
much as 40–60 L of mud was sieved in-situ through a 100 µm mesh net.
that removed clay particles. Oligochaetes trapped together with debris, vegetation roots and decayed particles were then transferred to the laboratory with minimal lake water. Additional dechlorinated tap water and aeration were supplied in the laboratory for the collection. Oligochaetes were hand-sorted from the retained material in trays filled with tap water up to the level of mesh of the sieve for several hours or overnight. This encouraged the oligochaetes to make their way into the water in the trays through the mesh of the sieves. Oligochaetes were identified according to the key to Timm (1999). Some questionable specimens were identified by Tarmo Timm (Vörstijarv Limnological Institute, Estonia) through photo or couriered specimens. Oligochaetes were collected throughout the year except during Winter (November to March).

2.3. Morphological investigation of actinospore types released

Oligochaetes were separated into cell-well-plates according to the methods of Yokoyama et al. (1991). They were placed individually in each well with 200 μl dechlorinated tap water. Plates were then kept at room temperature (23–25 °C) and stored at 4 °C. Each well was scanned for released actinospores using a Zeiss Trewal 3 inverted microscope. Released actinospores were pipetted, examined with a compound microscope and several preserved in 80% ethanol for molecular identification. Photomicrographs were taken from fresh actinospores under both bright and phase contrast field, using a DP-20 digital camera mounted on an Olympus BH-2 microscope. Subsequently, line drawings of actinospores were made based on the photos.

Measurements of the morphological characteristics were taken from a variable number of spores (depending on availability) from one infected oligochaete if possible. Measurements of actinospores were made according to the guidelines of Lom et al. (1997). The number of secondary cells and turns of tubules in polar capsules is given for certain actinospores where the number could be confidently determined by light microscopy. Prevalence of infection of the T. nikolskii actinospore type was calculated based on the percentage of infected Nais sp. A sample of released actinospores from each infected oligochaete was preserved in 80% ethanol.

2.4. Molecular identification of the collected spores

Myxo- and actinospore samples preserved in ethanol from fish and infected oligochaetes were centrifuged at 10,000 × g for 10 min, then the ethanol removed. The genomic DNA was extracted from the pelleted spores using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The partial SSU rDNA region was amplified using a nested PCR described in detail by Cech et al. (2015); universal eukaryotic primers ERIB1 and ERIB10 (Barta et al., 1997) were used in the first round PCR and myxozoan-specific primers Myx1F and SpH (Hallett and Diamant, 2001; Eszterbauer and Székely, 2004) were used in the second round PCR.

The amplicons were analysed by electrophoresis in a 1% agarose gel. All the appropriate PCR products were excised from the gel, purified with the Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan) and sequenced directly using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) with an ABI PRISM® 3100 Genetic Analyser (Life Technologies). The following primers were used for the sequencing reaction to generate overlapping fragments and coverage in both directions: ACT1F, MC3, MC5, MB5R, MB3F, SpH and CR1 R (Hallett and Diamant, 2001; Molnár et al., 2002; Eszterbauer and Székely, 2004; Székely et al., 2015).

The sequence fragments were assembled using MEGA 7 software (Kumar et al., 2016). The contiguous SSU rDNA sequences and the most similar myxozoan sequences from GenBank based on BLAST matches were aligned with the software CLUSTAL W (Thompson et al., 1994).

DNA pairwise distances were calculated with the MEGA 7 software using the Maximum Composite Likelihood model. Phylogenetic analysis was performed on a 1704 bp final alignment via Maximum Likelihood (ML) with Myxobolus cerebralis as the outgroup. The dataset was tested using MEGA 7 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 (GTR + G + I model). Bootstrap values based on 1000 resampled datasets were generated.

3. Results

3.1. General observations of Thelohanellus nikolskii infections in Hungary

Thelohanellus nikolskii infection was observed in carp fry cultured in ponds from the second half of July to September (Summer) (Fig. 1). The first external sign of infection was darkening of the fin and appearance of dark colour nodules in the fin-rays of 3–4 cm long carp. In some fish the fins were eroded (see Molnár and Kovács-Gayer, 1981–1982; Molnár, 1982). Mature plasmodia filled with myxospores appeared at the end of July and early August (Fig. 2). By the end of August most myxospores were released from opened plasmodia and in the autumn months only distortions of the fins marked past infections. Less frequently, late formation of cysts were recorded also in September in 8–16 cm long fingerlings. The ultrastructure of the spores corresponded to T. nikolskii spores described by Desser et al. (1983). In two-year-old carp mostly scale infections were observed but less frequently fin infections also occurred. In the three-year-old carp Thelohanellus plasmodia were found only in the scales. These plasmodia infected series of scales causing roughness on the surface of fish (Fig. 3). Concurrent infections on the scales and fins were not recorded. The earliest scale-thelohanelliosis was observed at the beginning of May (spring) and the latest one was recorded in the middle of June. In these infections, plasmodia were located at the outer periphery of the scales, in the non-overlapping region. The plasmodium was surrounded by a very thin cartilaginous layer. The original compact cartilaginous plate of the scale was damaged and only calcified islands could be recognised (Fig. 4A). The ultrastructure of the plasmodia and the myxospores obtained from the scales (Fig. 4AB) corresponded to those described by Moshu and Molnár (1997). The myxospores obtained from fin and scale cysts had a similar shape and overlapped in measurements (Table 2).

3.2. Actinospore stage of Thelohanellus nikolskii in oligochaetes

Altogether 9452 oligochaetes were collected, belonging to the five most common oligochaete species in the Lake: Isochaetides michaelensi...
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Lastochin, 1936, Branchiura sowerbyi Beddard, 1892, Nais sp., Müller, 1774, Dero sp. Müller, 1774 and Aelosoma sp. Ehrenberg, 1828. Thirteen different actinospore morphotypes were identified from I. michaelseni, B. sowerbyi and Nais sp. However, no actinospores were found from Dero and Aelosoma spp. Actinospores could be assigned to the aurantiactinomyxon (5), neoactinomyxon (1), raabeia (2), synactinomyxon (1), and triactinomyxon (4) collective groups.

Aurantiactinomyxon types were the most diverse form while triactinomyxon types were the most prevalent (Table 3). One Nais sp. specimen out of the 1200 examined released an undescribed aurantiactinomyxon type actinospore (Fig. 5), which corresponded to T. nikolskii based on the SSU rDNA sequence (Fig. 6). Only these spores were the subject of this study and are described as follows:

Aurantiactinomyxon type

Description: Mature actinospores (n = 10) triangular to almost circular in apical view, diameter 10.3 (9.3–12.0) μm. Caudal processes of equal length, finger-like with rounded tips, and curved downwards, 14.6 (12.7–16.0) μm and 6.5 (5.3–7.3) μm wide, with largest span of 36.9 (33.5–40.2) μm. Polar capsules, three equally sized, round in apical view, 3.3 × 2.6 μm, with 3–4 rounds of polar tubule. Sporoplasm contains 8 germ cells.

Type host: Nais sp.

Type locality: Kis Balaton.

Prevalence of infection: 1/1200 (0.08%)

Site of infection: Body coelom.

Molecular data: An SSU rDNA amplicon of 477 bp was generated (GenBank accession # MT569892). A BLAST search indicated highest similarity (99.8%) with T. nikolskii (DQ231156), differing in one nucleotide.

Remarks: Actinospores were found floating in the water of the cell-well plate in which the Nais worm was kept.

Differential diagnosis: This Aurantiactinomyxon type differs from other aurantiactinomyxons by having shorter caudal processes, about half to one-third of the length of known types. The caudal processes dimensions were most similar to the aurantiactinomyxon type ‘B2’ of Eszterbauer et al. (2006).

3.3. Molecular biological data

Partial SSU rDNA sequences were analysed from myxospores from the fins of fingerlings (two myxospore samples TU1, TU2), from myxospores from the scales of three year old common carp specimens (seven samples TK4, TK5, TK6, TK7, TK8, TK9, TK10) and from actinospore samples released by I. michaelseni, B. sowerbyi and Nais sp. Results of the released actinospores by the first two oligochaete species have been already published (Borkhanuddin et al. 2014; Zhao et al., 2016). The Aurantiactinomyxon morphotype released by the Nais sp. became a part of this study (AUM5). The sequence lengths and GenBank accession numbers with pairwise distances to T. nikolskii (DQ231156) are

Fig. 2. A: Section of an infected fin, containing T. nikolskii cysts, stained with hematoxilin-eosin. Cartilage of finray (cf) is next to the cyst. Plasmodium (p) is in the achromatic tegument, mature myxospores (s) are in the middle, sporoblasts (sb) are at the edges. Around the plasmodium, there is a thick connective tissue (ct) layer, containing cartilaginous elements (c). Multilayer epithelium (e) is the outer layer. B: T. nikolskii myxospores from the plasmodium.

Fig. 3. Thelohanellus cysts on the scales of an aged common carp specimen.

Fig. 4. A: Cross section of infected scales, stained with hematoxilin-eosin. The plasmodia (p) are filled with myxospores (s) and are surrounded by cartilaginous tissue (c) of the scales, covered by the epithelium layer (e). B: T. nikolskii myxospores from a plasmodium in the scale.
presented in Table 4. SSU rDNA sequences >1000 bp were generated from samples TU1, TU2, TK4, TK5, TK6, TK7, TK8 and TK9, and shorter sequences were determined from TK10 and the actinospore sample (AUM5) which were used to display the phylogenetic positions of the examined myxozoans on the Maximum Likelihood tree (Fig. 6). All of the aligned sequences were 99.0%–100% similar to the previously deposited SSU rDNA sequences of T. nikolskii (DQ231156 and GU165832) in GenBank. Based on the final 1704 bp alignment, the conserved region was 1070 bp, the variable region was 573 bp and the parsimony informative region was 344 bp. The mean distance between the fin (2 samples) and scale (7 samples) sample groups was 0.4%. Within the groups, the overall mean distances were 0.4% in scale samples and 0.1% in fin samples.

4. Discussion

Most myxosporeans, among them Thelohanellus spp. are host, organ and tissue specific (Molnár, 1994; Molnár and Eszterbauer, 2015). For example, Achmerov (1960) reported four different Thelohanellus species from the Amur wild carp each of them infecting a different organ.

Table 2
Comparison of infection site, size of plasmodia and myxospore dimensions of Thelohanellus nikolskii collected from the fins and scales of common carp. All measurements except the size of plasmodia are in micrometer (μm).

| Localisation | Size of plasmodia (mm) | Spore length | Spore width | Spore thickness | Capsule length | Capsule width | Polar tubule turns | Thickness of valves |
|--------------|------------------------|--------------|-------------|----------------|---------------|---------------|------------------|-------------------|
| Fins Up to 2 | 16.5 (15.0–19.0)       | 10.0         | 8.7–12.5    | 6.8 ± 1.1      | 6.8 ± 0.6     | 5.3 ± 0.7     | 6–7              | 0.7–1             |
| Fins Up to 2 | 16.2 ± 1.0             | 9 ± 1.6      | 6.6 ± 0.6   | 6.3 ± 0.4      | 5.4 ± 0.8     | 6–7           | 0.7–1            |
| Scales Up to 3 | 17.5 (16.2–18.7) | 10.0         | 7.5 (6.2–7.5)| 7.5 (6.2–7.5) | 5.6 (5.0–6.5) | 6–7           | 1.2–1.5          |

Table 3
Summary of actinospore morphotypes detected by the authors in the sampling period from 2010 to 2015.

| Morphotype            | Host          | Prevalence | Reference             |
|-----------------------|---------------|------------|-----------------------|
| Aurantiactinomyxon 1  | Isochaetides  | 10/7818    | Borkhanuddin, 2013    |
|                       | michaelesi   |            | (dissertation)        |
| Aurantiactinomyxon 2  | Isochaetides  | 6/7818     | Borkhanuddin, 2013    |
|                       | michaelesi   |            | (dissertation)        |
| Aurantiactinomyxon 3  | Branchiura   | 1/434      | Borkhanuddin, 2013    |
|                       | sowerbyi     |            | (dissertation)        |
| Aurantiactinomyxon 4  | Branchiura   | 1/434      | Zhao et al. (2016)    |
|                       | sowerbyi     |            |                       |
| Aurantiactinomyxon 5  | Nais sp.      | 1/1200     | this study            |
| Neactinomyxon 1       | Isochaetides  | 2/7818     | Borkhanuddin et al. 2014 |
|                       | michaelesi   |            |                       |
| Raabeia 1             | Isochaetides  | 5/7818     | Borkhanuddin et al. 2014 |
|                       | michaelesi   |            |                       |
| Raabeia 2             | Isochaetides  | 2/7818     | Borkhanuddin et al. 2014 |
|                       | michaelesi   |            |                       |
| Synactinomyxon 1      | Isochaetides  | 1/7818     | Borkhanuddin et al. 2014 |
|                       | michaelesi   |            |                       |
| Triactinomyxon 1      | Isochaetides  | NA         | Székely et al., 2014  |
|                       | michaelesi   |            |                       |
| Triactinomyxon 2      | Isochaetides  | NA         | Székely et al., 2014  |
|                       | michaelesi   |            |                       |
| Triactinomyxon 3      | Isochaetides  | NA         | Székely et al., 2014  |
|                       | michaelesi   |            |                       |
| Triactinomyxon 4      | Isochaetides  | NA         | Borkhanuddin, 2013    |
|                       | michaelesi   |            | (dissertation)        |

Fig. 5. Microphotograph of fresh, unstained actinospore of Aurantiactinomyxon type (AUM5) from Nais sp. Insert – apical view of spore with protruding polar capsules.

However, Borzák et al. (2018) pointed out that some myxosporeans can develop also in different organs if the organ is composed of the same tissue. The occurrence of Thelohanellus plasmodia with myxospores in scales of older common carp (2–3 year olds), corresponding in size and shape to T. nikolskii described originally from fin rays of carp fingerlings, suggested that this myxosporean species might develop in two different sites in common carp depending on host age. Notably, the tissue preference of plasmodia in the two different locations was the same, namely plasmodia started their development in the cartilaginous elements of the fins and scales built up from collagenous material (Molnár and Molnár, 1997). Consideration of the formation of the cartilage in fin rays and in scales provides insight into this apparent dichotomy of infection site. In general it is accepted that fin rays are made from cartilage. However, there are no chondrocytes in the fin rays contrary to the rays in gill lamellae. Collagenous material of fin rays is produced by perichondral (periosteal) cells (Molnár, 1982; Desser et al., 1983). As the fish gets older the collagenous fin ray becomes rigid, losing its soft structure due to ossification and in this process becomes less suitable for plasmodial development. On the other hand, histology of scale infection with myxosporeans is relatively less studied. In scale infections with T. nikolskii serious scale deformation was observed. The cartilaginous scale plate fragmented into amorphous cartilage islands, and the plasmodia developed in the cartilaginous elements. The plasmodia on the scales were located in non-overlapping regions which are particularly exposed to external effects. After minor injuries the scales start
regenerating quickly, however the calcification process depends on external conditions, like temperature (Ghods et al., 2020), calcium and phosphate content, and salinity of the water (Ogawa et al., 2010). Until calcification is complete, the regenerating scale also has a soft structure, which might facilitate the infection with *T. nikolskii*.

During the development of *T. nikolskii*, beside the different organ location, there is also an apparent temporal dichotomy. Myxospores in older carp individuals developed in the springtime while those in fingerlings were formed during the summer or autumn. A similar phenomenon was observed by Circovic et al. (2013) who studied the prevalence and different forms of *T. nikolskii* in Serbia. One of the two possible explanations for the different sporulation times is that fingerlings and older fishes are cultivated in separate ponds in Hungary where the chance of cross-infections seems to be excluded. However, ponds receive water from the same channels which may carry actinospores. Moreover, different age groups of carp are often moved between ponds even in the same year which could provide relatively constant infection of oligochaetes and fish. Carp spawning in nature is usually from the end of April through May in Hungary. The larvae and the fry are very fragile so a parasite infection on the fin can easily cause host death, before any clinical signs appear. Usually in a month, they become fingerlings, when *T. nikolskii* cysts can be observed on the fins. Plasmodium development

![Phylogenetic position of *Thelohanellus nikolskii* spores from the fins and scales of common carp based on SSU rDNA analysis by the Maximum Likelihood algorithm. *Myxobolus cerebralis* was used as the outgroup. Bootstrap values are given at the nodes. The scale-bar indicates the number of expected substitutions per site.](image)

**Table 4**
Details of myxosporean samples sequenced (SSU rDNA) and similarity to *Thelohanellus nikolskii*.

| Sample name | Spore type | Source | Sequence length (bp) | GenBank accession number | Similarity to *T. nikolskii* (DQ231156) |
|-------------|------------|--------|----------------------|--------------------------|----------------------------------------|
| TU1         | myxospores | fins of carp fingerlings | 1618                | MT535575                 | 100%                                   |
| TU2         | myxospores | fins of carp fingerlings | 1271                | MT535576                 | 100%                                   |
| TK4         | myxospores | scales of 3 year old carp | 1618              | MT535578                 | 99.6%                                  |
| TK5         | myxospores | scales of 3 year old carp | 1618                | MT535580                 | 99.6%                                  |
| TK6         | myxospores | scales of 3 year old carp | 1618                | MT535581                 | 99.4%                                  |
| TK7         | myxospores | scales of 3 year old carp | 1618                | MT535577                 | 99.0%                                  |
| TK8         | myxospores | scales of 3 year old carp | 1618                | MT535579                 | 99.4%                                  |
| TK9         | myxospores | scales of 3 year old carp | 1618                | MT535582                 | 99.4%                                  |
| TK10        | myxospores | scales of 3 year old carp | 592                 | MT535583                 | 99.3%                                  |
| AUM 5       | actinospores | oligochaete - Nais | 477                 | MT569892                 | 99.8%                                  |
in older fish can happen earlier because they are already present in the ponds and the infection is not lethal.

Another possible explanation for the observed temporal dichotomy is that two distinct periods may exist during the development of T. nikolskii. There is one developmental phase from May to September and a second one from September to May. Myxospores released from fin cysts in September sink to the sediment and are consumed by oligochaetes in which intraoligochaete development culminates in the production of actinospores in about April (Spring). These actinospores could start developing plasmodia in two or three year-old carp during May. On the other hand, myxospores that developed on scales during May infect oligochaetes releasing actinospores subsequently around June and initiate infection of carp fingerlings. These two cycles seem to be asynchronous. This is consistent with the dependence of intra-oligochaete development on water temperature and associated thermal temperature units or degree days (Szekely et al., 1996; Marton and Eszterbauer 2012).

Molecular biological examinations based on SSU rDNA sequences were carried out in the present study which revealed that myxospores from fin cysts and scale cysts are almost identical. There is only 0.4% nucleotide differences between the scale and fin samples, comparable to the variance of the scale sequences, for which 0.4% dissimilarity also was observed within that group. Only 0.1% difference was observed among the fin samples. SSU rDNA sequences were used for identification which is a commonly used region in phylogenetic studies for Myxozoa. Their repetitive arrangement within the genome provides multiple templates of DNA for PCR. Despite the several DNA repair mechanisms, polymorphisms between repeats can occur even at significant levels.

Mean distance within the group of fin samples was lower (0.1%) than the mean distance between groups (0.4%), and the overall genetic distance does not exceed the generally accepted distance values.

During our oligochaete survey, we isolated several actinospore morphotypes, of which a short SSU rDNA sequence of an aurantiactinomyxon type released by a single unidentified Nais sp. was 99.8% similar to a myxospore sample collected from the fins of common carp, identified as T. nikolskii (DQ231156) by Eszterbauer et al., (2006). Thus our molecular comparison suggested that this aurantiactinomyxon represents the alternate life stage of T. nikolskii (Fig. 7). No genetic analysis was performed on the host Nais worm collected in this study; its identification was based on morphology. Out of the 1200 individuals of the Nais sp. that were monitored, only one worm released actinospores. Low infection prevalences in oligochaete populations are common in actinospores studies (e.g. Hallett et al., 2001; Rocha et al., 2020).

Aurantiactinomyxon morphotypes are known developmental stages for several Thelohanellus species, including T. hovorkai and T. nikolskii (Szeke, 1998) and Thelohanellus kitaeki (Zha et al., 2016). The aurantiactinomyxon isolated in this study was morphologically distinct from that reported by Szekely et al. (1998). Additionally, we collected the actinospores from a Nais sp. rather than a Tubifex tubifex. Since the earlier aurantiactinomyxon was characterised phenotypically only, a comparison of the DNA sequence data between the two studies was not possible. Notably, different phenotypes that correspond to a single genotype have been reported for aurantiactinomyxon (Hallett et al., 2002). However, a feature common to both phenotypes was the number of sporoplasm nuclei, which was not the case for the two aurantiactinomyxons associated with T. nikolskii. We suggest that the developing phase of T. nikolskii may occur in a range of oligochaete species, for which more broad research is needed to determine this association. Apart from aurantiactinomyxon, Nais worms have been reported to be the host for triactinomyxon-type actinospores for several myxosporian species such as Triactinomyxon naidanum (Naidu 1956), Hoferellus cyprini (Großheider and Köring, 1992), Hoferellus carassii (Trouillier et al., 1996), and Myxobilatus gasterostei (Atkinson and Bartholomew, 2009).

Oligochaete species belonging to Naidinae undergo reproduction in which several daughter worms are produced through a double cleavage process. Because naidines can reproduce rapidly every few days, they are able to reach high population densities quickly (Bely and Wray, 2004). This breeding feature enables myxozoan parasites to be transmitted via both horizontal and vertical pathways (Atkinson and Bartholomew, 2009). However, this propagation was not observed during our study, suggesting that an experimental study is needed to confirm this transmission pattern.

Compliance with ethical standards

All experiments and handling of fish in the present study were conducted according to the animal welfare guidelines and recommendations (permission number PEI/001/1002–13/2015) under the Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary.

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