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Nephrocystidium pickii Weissenberg, 1921 belongs to Myxozoa (Cnidaria) but is not conspecific with *Myxidium lieberkuehni* Bütschli, 1882 (Myxozoa: Bivalvulida: Variisporina: Myxidiidae): molecular-genetic evidence

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Abstract We isolated and re-investigated *Nephrocystidium pickii* Weißenberg, 1921 (Myxozoa: Bivalvulida: Variisporina) using light microscopy and phylogenetic analysis of the small-subunit (SSU) ribosomal RNA gene. This species is a parasite of the northern pike *Esox lucius* L. (Actinopterygii: Esocidae) which localizes in the endothelial cells of the glomerular capillary. The results of the phylogenetic analysis including this species clarify its taxonomic status and show that although it is the closest relative to *Myxidium lieberkuehni* Bütschli, 1882, the two organisms are not conspecific, contrary to the earlier hypotheses. The data obtained highlight the necessity of a profound taxonomic revision of the Myxozoa and the need to clarify species affiliation of extrasporogonic developmental stages of these organisms that occur in different organs of fish.

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Introduction

Nephrocystidium pickii Weißenberg, 1921 is an intracellular parasite of the northern pike *Esox lucius* L. (Actinopterygii: Esocidae) which forms assemblies of 1–4 cellular trophozoites in hypertrophied endothelial cells of the glomerular capillaries. For the first time ever these trophozoites were described by Debaisieux (1919, 1920) who interpreted them as special intracellular developmental stages of *Myxidium lieberkuehni* Bütschli, 1882, a species of myxozoan with sporogonic stages (plasmodia) parasitising the urinary bladder of the northern pike. For two years Weißenberg (1921, cited after Weißenberg, 1922) was not aware of P. Debaisieux's publication, hence he described these trophozoites as a new species, *N. pickii*. Later, having read the paper and done additional research, Weißenberg (1922, 1923) came to a conclusion that *N. pickii* was a representative of the myxozoans but its conspecificity with *M. lieberkuehni* was not evident. Jírovec (1940) considered this point

of view on the systematic position of this parasite more reasonable.

Lom et al. (1989) have described in detail the morphology of the trophozoites of *N. pickii* using the data from transmission electron microscopy. According to these authors, these organisms are extrasporogonic developmental stages of myxozoans. According to the modern views, myxozoans are extensive group of aberrant parasitic cnidarians having complex life cycle including actinosporean and myxosporean phases of development (Nesnidal et al., 2013; Holzer et al., 2018). The development at the myxosporean phase occurs in the vertebrate host and includes two sequential stages: extrasporogonic one which precedes or occurs parallel to, the development of the sporogonic one. The initial stages of development of extrasporogonic myxosporeans occur inside the host cells specific for the parasite species (e.g. Molnár, 1994; Okamura et al., 2015). Doubtless myxozoan nature of *N. pickii* is characterized with such morphologic features as the presence of bundles of microtubules and numerous free ribosomes in the inner cells, presence of glycogen in the primary cells, the lack of centrioles and the cell-in-cell arrangement (Lom et al., 1989). Based on the regular co-occurrence of *N. pickii* with *M. lieberkuehni*, Lom et al. (1989) consider these organisms as conspecific. This point of view of the authors is generally accepted (Feist, 1997; Molnár, 2007).

Molecular methods are reliable tools for identifying the cryptic stages of life-cycle of myxozoans (e.g. Holzer et al., 2004, 2013; Grabner & El-Matbouli, 2008). In many cases they do not support traditional views on species and genus affiliation of the stages (Holzer et al., 2013). We decided to focus again on *N. pickii* as this organism is the parasite of economically important species of fish and it is pathogenic for its host (e.g. Weißenberg, 1923; Lom et al., 1989; Feist, 1997). The aim of this study is to verify the hypothesis by Debaisieux (1919, 1920) and Lom et al. (1989) about systematic position of *N. pickii* using the data on the primary structure of its small-subunit (SSU) rRNA gene.

Materials and methods

One specimen of the northern pike was caught in the northern part of the Lake Ladoga (North-Western Russia, 61°30′28″N, 30°16′34″E) in October 2016. There were multiple granulomas found in the fish specimen kidneys. Living trophozoites from granulomas were observed and photographed using the Olympus CX-41 (Olympus Corporation, Japan) light microscope with phase contrast. For histological analysis, a fragment of the kidneys was fixed in 10% buffered formalin. The fixed fragment of the kidneys was embedded in paraffin using STP-120 (Thermo Scientific, USA). Paraffin blocks were made using EC-350 Embedding Centre (Thermo Scientific, USA) and cut using the microtome HM-440 (Thermo Scientific, USA) at a thickness of 5–6 μm. The sections were stained with Mayer's haematoxylin and eosin. Sections were observed using the Axio Imager A1 (Zeiss AG, Germany) light microscope. Following Lom et al. (1989) we define a hypertrophic host cell together with the mass of parasite cells filling its cytoplasm as xenoma.

For the molecular study, material was fixed with 96% ethanol in the field. Total genomic DNA was isolated from three preserved capsules. A separate DNA sample was isolated from each capsule. In the laboratory, the capsules were dissected using sterile tools under the microscopic control, and the cells were transferred immediately into a portion of guanidine isothiocyanate buffer for preservation and homogenization. Further procedures followed a standard protocol of guanidine isothiocyanate DNA extraction (Maniatis et al., 1982). Small-subunit ribosomal RNA was amplified using PCR with three pairs of universal eukaryotic primers: RibA, sAF and A10s1 were used as alternative forward primers and RibB as a reverse primer (Kudryavtsev et al., 2009, 2011; Medlin et al., 1988). The PCR program utilized a recombinant Taq DNA polymerase (Thermo Scientific) and a standard program of 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 60 s and elongation at 72°C for 120 s. The amplicons of correct length were purified on agarose gel and tested by direct sequencing with PCR primers. To obtain the full sequences, PCR products were cloned in a pTZ57R cloning vector using InsTAclone PCR cloning kit (Thermo Scientific) following manufacturer's protocol. Colonies were tested by PCR amplification using vector-specific M13 primers and ScreenMix HS PCR premix (Evrogen). Amplicons of appropriate size were sequenced in the Core Facility Center "Development of Molecular and Cell Technologies" (Saint-Petersburg State

University) using BigDye Terminator cycle sequencing kit and ABI Prism 3500xl Genetic Analyser automatic sequencer (Applied Biosystems). Three molecular clones obtained from the two DNA samples were sequenced completely using vector-specific and conserved internal primers.

The sequences were aligned with the database of previously published SSU rRNA gene sequences from different genera of Myxozoa. Sequences were initially automatically aligned using MUSCLE algorithm (Edgar, 2004) implemented in Seaview v. 4.7 (Gouy et al., 2010). The alignment was further adjusted manually. The final alignment used for the phylogenetic tree reconstruction consisted of 76 sequences of different myxozoans with 1,483 unambiguously aligned nucleotide positions. Phylogenetic tree was reconstructed using maximum likelihood and Bayesian algorithms implemented in RaxML v. 8.2.10 (Stamatakis, 2014) and MrBayes v. 3.2.6 (Ronquist et al., 2012). The maximum likelihood analysis used a default algorithm with 100 independent searches for the best tree from the random starting trees and GTRGAMMAI substitution model. The analysis was followed by non-parametric bootstrap with 1000 pseudoreplicates, and the bootstrap results were mapped on the best tree. Bayesian analysis used a GTR substitution model, gamma model for among-site rate variation (8 rate categories) with a proportion of invariable sites. Markov chain Monte-Carlo analysis was performed in 2 runs of 4 simultaneous chains for 15,000,000, sampled every 100 generations. A quarter of the samples were discarded as a burn in. Convergence was evaluated using Tracer v. 1.6 (Rambaut et al., 2014). All analyses were performed using the CIPRES portal (Miller et al., 2010). Sequences obtained during this study are deposited in the GenBank database under the accession numbers MH734525-MH734527.

Results

Morphology

Two types of xenoma were observed in histological sections of kidneys: early unencapsulated (Fig. 1A, B) and grown encapsulated (Fig. 1C). When studying the early xenoma, it is clearly seen that it represents a hypertrophied endothelial cell of a glomerular capillary by trophozoites of *N. pickii* (Fig. 1B). This hypertrophied cell contains one large nucleus. The longitudinal size of the studied early xenoma is 75 µm (Fig. 1A). Grown xenomas are much larger (their longitudinal size, without capsule, is 500–650 µm, n = 7) and contain a fragmented nucleus (Fig. 1C). These xenomas are isolated from the surrounding renal parenchyma by thick connective tissue capsule (Fig. 1C). The granulomas found by visual analysis of the kidneys are encapsulated grown xenomas of *N. pickii.*

Trophozoites in the early xenoma are visualised under light microscope as unicellular bodies, and in grown xenoma - as one- to three-cell bodies (Fig. 1D). Fixed trophozoites can be either rounded or irregularly polygonal, with smooth edges. The sizes of the fixed trophozoites range from 4.3 × 4.3 µm to 6.6 × 6.5 µm (n = 15). Live trophozoites in a drop of water during the mechanical destruction of xenoma had a spherical cell body with filose pseudopodia (Fig. 2).

Molecular phylogenetic analysis

SSU rRNA gene sequences of *N. pickii* were 2,094–2,095 base pairs long excluding PCR primers. Pairwise differences between molecular clones were in 0.4–0.5% of all nucleotide positions. Initial BLAST search of the sequences against NCBI database yielded a variety of myxozoan SSU rRNA genes, with sequences of *M. lieberkuehni* (X76638 and X76639) being always the best hits during searches. The latter isolates were also the closest relatives to *N. pickii* in all phylogenetic trees (Fig. 3). Molecular differences between *N. pickii* and *M. lieberkuehni* were always significant, and this clade was only well-supported in the Bayesian analysis (PP = 1), while its bootstrap support value was 77. The clade of *N. pickii* and *M. lieberkuehni* always belonged to an assemblage that contained also *Sphaerospora oncorhynchi* Kent, Whitaker & Margolis, 1993, unidentified myxosporean from the Waukwaas River (AY525343) (both are parasites of the excretory system of salmonids) and *Chloromyxum legeri* Tourraine, 1931 AY604197 (parasite of the gallbladder of cyprinids) (Fig. 3). This clade always received a full support in the phylogenetic tree and was constantly sister to a heterogeneous clade containing sequences of representatives of the genera *Hoferellus* Berg, 1898,

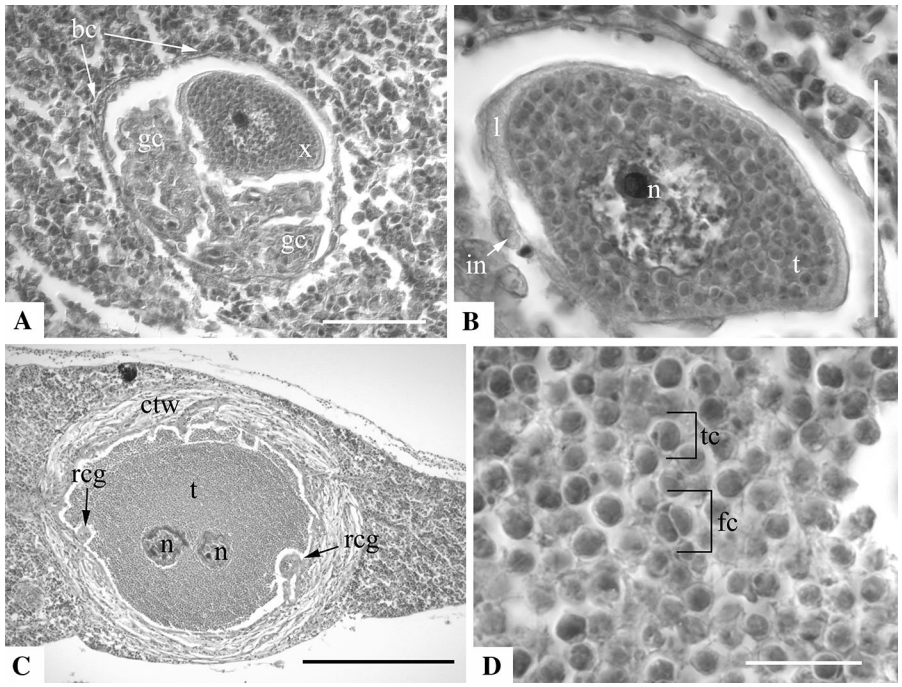

Fig. 1 Xenomas with trophozoites of *N. pickii*: A, B, Early xenoma; C, Encapsulated grown xenoma; D, Fixed trophozoites from grown xenoma. *Abbreviations*: bc, parietal leaf of Bowman's capsule; ctv, connective tissue capsule; fc, three-celled trophozoite; gc, glomerular capillary; in, nucleus of an intact endothelial cell; l, lumen of glomerular capillary; n, nucleus of an infected endothelial cell; rcg, rudiments of a glomerular capillary; t, trophozoites; tc, two-celled trophozoite; x, xenoma. *Scale-bars*: A, B, 50 µm; C, 300 µm; D, 15 µm

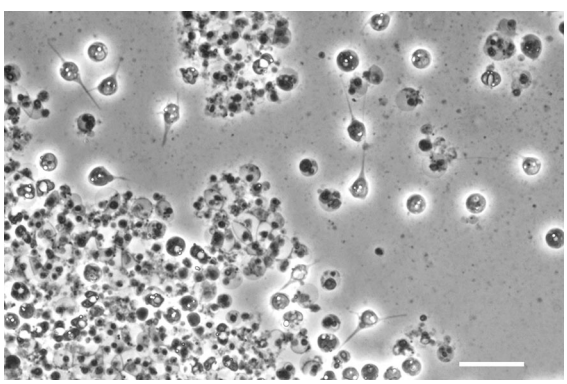

Fig. 2 Living trophozoites of *N. pickii* in a drop of water. *Scale-bar*: 20 µm

Myxidium Bütschli, 1882, *Myxobolus* Bütschli, 1882, *Myxobilatus* Davis, 1944, *Ortholinea* Shulman, 1962, *Sphaerospora* Thélohan, 1892, *Thelohanellus* Kudo, 1933, Stole, 1899, *Unicauda* Davis, 1944, *Zschokkella* Auerbach, 1909 and sequence KF263540 attributed to triactinomyxon actinosporean stage. The latter clade only received a good support in the maximum likelihood analysis but was not supported in the Bayesian tree. It is noteworthy, that most of the genera of myxozoans represented by multiple species in the tree were either para- or polyphyletic, and the resolution of deeper nodes of the SSU rRNA phylogenetic tree of Myxozoa was often low.

The clade containing *N. pickii*, in addition to the constant presence in the phylogenetic trees, was also supported by several unique nucleotide substitutions shared by all its sequences. In particular, these unique substitutions were seen in the positions (corresponding to *M. lieberkuehni* X76638): 563, 602, 621, 651–671, 1,137–1,141, 1,214, 1,230–1,235 and 1,430–1,431.

Discussion

Morphology of the studied xenomas and trophozoites of *N. pickii* fully corresponds to that known from literature (Debaisieux, 1920; Weißenberg, 1923; Jírovec, 1940; Lom et al., 1989). At the same time, assertion of Debaisieux (1919, 1920) and Lom et al.

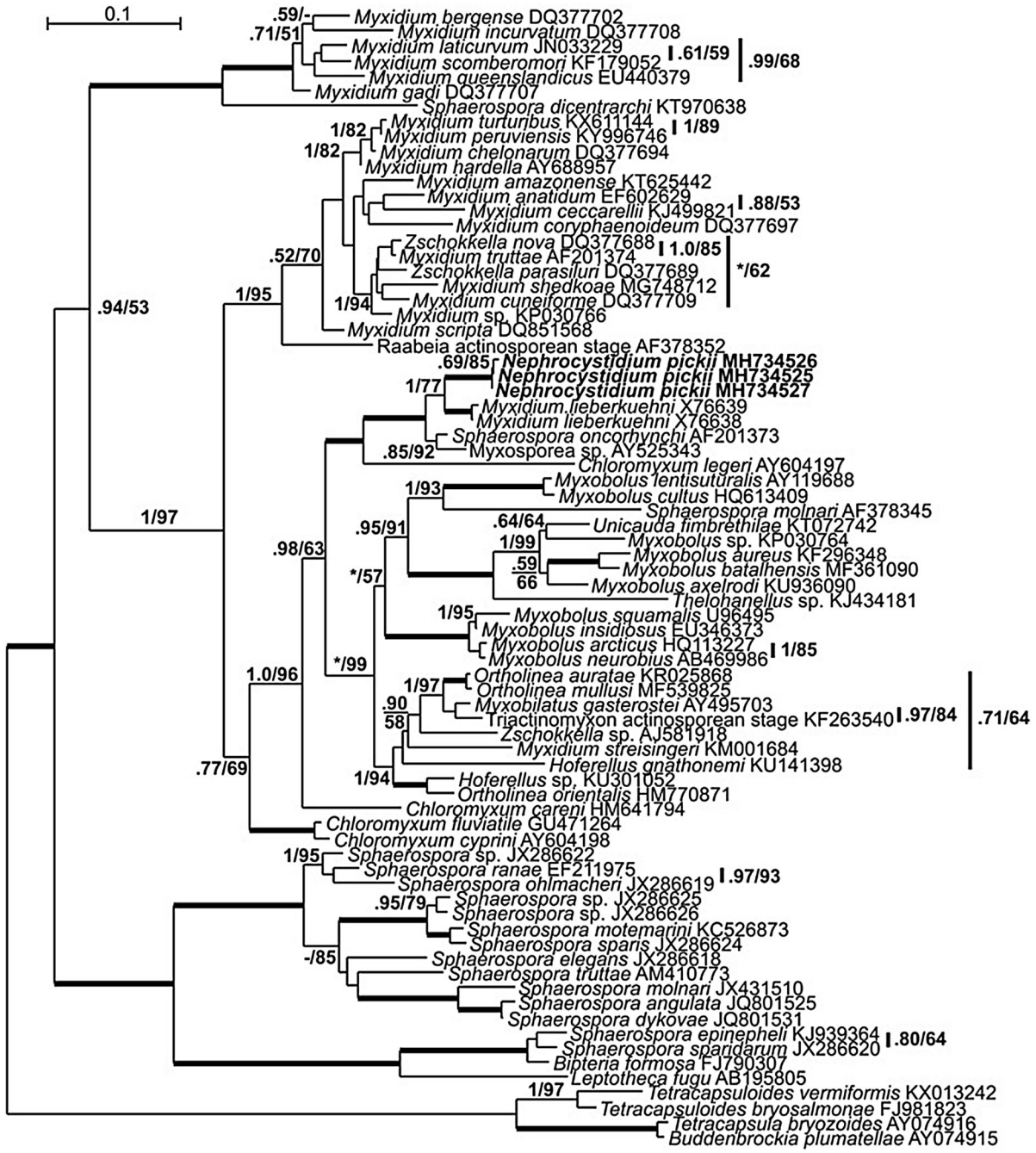

Fig. 3 Maximum likelihood phylogenetic tree for selected species of Myxozoa (76 sequences) based on the SSU rRNA gene (1,483 nucleotide positions). New sequences are in bold. The tree is rooted with Malacosporea; numbers at nodes indicate Bayesian posterior probabilities/bootstrap values if above 0.5/50; thick branches = 1/100. Asterisks indicate that the branch was not recovered in the Bayesian tree which had alternative topology in some nodes. *Scale-bar*: 0.1 substitutions/site

(1989) that this parasite is conspecific with *M. lieberkuehni* was by no means supported by the molecular genetic data (Fig. 3). We agree with the statement of Lom et al. (1989) that *N. pickii* represents the extrasporogonic developmental stage of myxozoans. However, the sporogonic stage of the parasite is

still unknown. The position of *N. pickii* in the phylogenetic tree as a sister species to *M. lieberkuehni* indicates that this species belongs to the suborder Variisporina. At the same time, the described topology does not allow us to make a priori conclusion about the spore morphotype of the parasite. It is impeded by the generally accepted polyphyly of the key genera of the suborder Variisporina that is demonstrated by consistent clustering of non-congeneric species (e.g. *Myxidium truttae* + *Zschokkella nova*, *Myxidium giardi* + *Chloromyxum schurovi* and *Myxidium incurvatum* + *Sinuolinea phyllopteryxa*, see Fiala, 2006; Bartošová et al., 2011; Jirků et al., 2011) and also low level of homology (82.9–83.7%) between sequences for *N. pickii* and *M. lieberkuehni*.

Close phylogenetic relationships of *M. lieberkuehni* with *S. oncorhynchi*, and of *S. oncorhynchi* with Myxosporea sp. from Waukwaas River, and also of *C. legeri* with the ancestor of these three myxozoans have already been described in literature (e.g. Fiala & Dyková, 2004; Jones et al., 2004; Jirků et al., 2011). We have further detailed the clade structure that unites all these species.

The leading factors for the formation of phylogenetic lineages within myxozoans are the host and tissue specificity, host phylogeny and environment inhabited by the host (e.g. Eszterbauer, 2004; Holzer et al., 2004; Fiala, 2006; Rocha et al., 2014). Influence power of these factors in different myxozoan groups is not equal (Carriero et al., 2013; Zatti et al., 2018). Within the Variisporina tissue tropism is a phylogenetic character with strong signal (Ferguson et al., 2008; Rocha et al., 2013). Considering phylogenetic proximity of *N. pickii* to *M. lieberkuehni*, we can assume that sporogonic stage of the latter species also parasitises in excretory system of fish. It is noteworthy that for the northern pikes, one more species of suborder Variisporina is known besides *M. lieberkuehni*, with a sporogonic stage localised in the excretory system, i.e. *Sphaerospora minuta* Konovalov, 1967. This species is recorded in the northern pike both in the Asian and European parts of its geographical range (Konovalov, 1967; Moshu & Trombitsky, 2007).

According to Holzer et al. (2013), more than 10% of extrasporogonic stages of myxozoans circulating in blood of the studied common carp *Cyprinus carpio* L. and goldfish *Carassius auratus* (L.) belonged to the species that were not specific for these hosts. Thus, *N. pickii* is not necessarily a parasite whose sporogonic stage parasitises in the northern pike. It is possible that the encapsulation of grown xenomas is the host's response to the presence of a parasite which is non-specific for it. The fate of encapsulated xenomas has already been discussed in literature. According to Lom et al. (1989), most of the trophozoites of *N. pickii* having reached the stage of one primary cell with one secondary and two tertiary cells eventually become nonviable and most of the xenomas are destroyed by the host tissue.

In conclusion, our data together with the results by Holzer et al. (2013) highlight the necessity of checking numerous previous hypotheses about species affiliation of extrasporogonic developmental stages of myxozoans that occur in different organs of fish (e.g. see Molnár, 2007).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed.

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