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Research Article

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A novel myxozoan parasite of terrestrial mammals: description of *Soricimyxum minuti* sp. n. (Myxosporea) in pygmy shrew *Sorex minutus* from Hungary

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Abstract: As part of a biodiversity study in northwestern Hungary, we conducted a parasitological survey of small mammals. In both common shrews (*Sorex araneus* Linnaeus) and pygmy shrews (*Sorex minutus* Linnaeus), we found myxospores of a species of *Soricimyxum* Prunescu, Prunescu, Pucek et Lom, 2007 (Myxosporea) and plasmodia in the bile ducts within the liver. Spores from both species of shrewswere morphologically and morphometrically indistinguishable, but differed in their SSU rRNA gene sequences by 3.3%. We identified spores and developmental stages from the common shrew as *Soricimyxum fegati* Prunescu, Prunescu, Pucek et Lom, 2007, based on morphometric data and DNA sequence similarity. Spores from the pygmy shrew were only 96.7% similar to *S. fegati*, hence we identified them as a novel myxosporean *Soricimyxum minuti* sp. n. This is only the second myxosporean parasite species described from mammals.

Keywords: Myxozoa, *Soricimyxum* spp., bile ducts, liver, mammalian hosts, *Sorex* spp., Hungary

Myxosporeans are generally known as parasites of aquatic vertebrates, primarily fishes, but also amphibians and reptiles (Laveran 1897, Eiras 2005). Rare infections have also been described from the bile ducts and hepatic parenchyma of aquatic birds (Lowenstine et al. 2002, Bartholomew et al. 2008). The first suggestion that Myxozoa can infect terrestrial mammals was from Friedrich et al. (2000) who reported myxosporean-like presporogenic stages in the brain of moles (*Talpa europeae* Linnaeus).

More recently, Prunescu et al. (2007) described unambiguous myxozoan developmental stages and mature *Myxidium/Zschokella*-type myxospores from the parenchyma and the intrahepatic bile ducts in the common shrew *Sorex araneus* Linnaeus, in Poland. This novel parasite, *Soricimyxum fegati* Prunescu, Prunescu, Pucek et Lom, 2007, was classified into a new genus to reflect the heretofore atypical vertebrate host. Subsequent survey work in the Czech Republic showed the host range of the parasite included not only common shrews, but also pygmy shrews *Sorex minutus* Linnaeus, and the lesser white-toothed shrew, *Crocidura suaveolens* (Pallas) (Dyková et al. 2007, 2011).

Dyková et al. (2007) amended the original description with more rigorous morphological description and SSU rDNA sequence data. In the framework of the National Biodiversity Monitoring Program we had the opportunity to examine shrews in Hungary for myxozoan infections. We found infections in two shrew species, common shrew *S. araneus* and pygmy shrew *S. minutus* and herein we describe a new species of *Soricimyxum* Prunescu, Prunescu, Pucek et Lom, 2007, *S. minuti* sp. n. from pygmy shrew.

MATERIALS AND METHODS

Shrew samples were collected within the framework of the National Biodiversity Monitoring Program in the Hanság Area, North-West Hungary. Animals that died during trapping were studied for parasite infections. From May 2007 to September 2010, we examined 21 common shrews *Sorex araneus* and 3 pygmy shrews *S. minutus*. Shrews were kept at 4 °C until necropsy. Bile, gall bladder scrapings and liver pieces were examined as fresh squash preparations, at 400× with an Olympus BH2 microscope under Nomarski differential interference contrast illumination. Digital images were captured with an Olympus DP20 camera and spores measured from these images. We measured spore length, width and thickness, and polar capsule diameter, from mature, unfixed myxospores. All measurements are in micrometres unless otherwise indicated. We followed the nomenclature guidelines of Lom and Arthur (1989). Liver tissue that included both mature spores and developmental stages was stored in 80% ethanol for later molecular studies.

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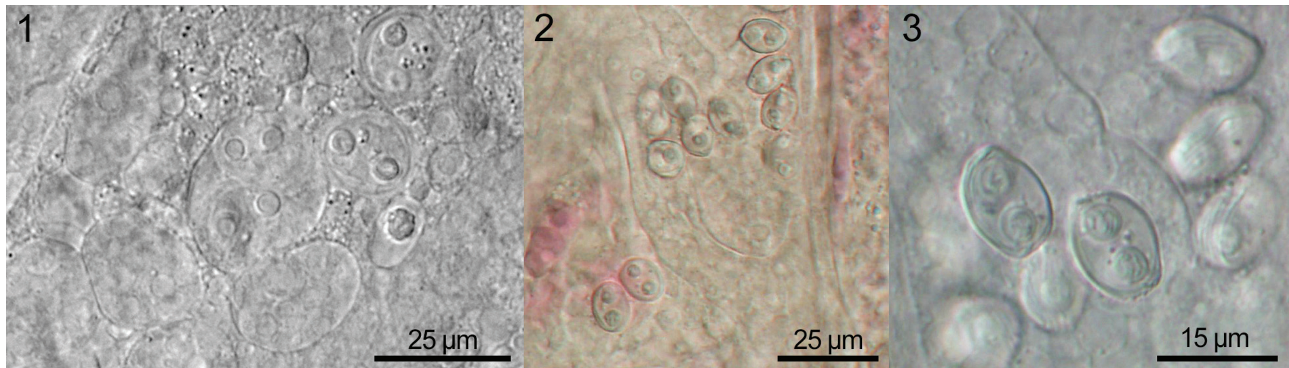
Table 1. Primers used for PCR and sequencing.

Primer	Sequence (5'–3')	Use	Reference
ERIB1	ACC TGG TTG ATC CTG CCA G	1st round PCR	Barta et al (1997)
ERIB10	CTT CCG CAG GTT CAC CTA CGG	1st round PCR	Barta et al. (1997)
Myx1F	GTG AGA CTG CGG ACG GCT CAG	2nd round PCR	Hallett and Diamant (2001)
SphR	GTT ACC ATT GTA GCG CGC GT	2nd round PCR and sequencing	Eszterbauer and Székely (2004)
ACT1fr	TTG GGT AAT TTG CGC GCC TGC TGC C	sequencing	Hallett and Diamant (2001)
MC5	CCT GAG AAA CGG CTA CCA CAT CCA	sequencing	Molnár et al. (2002)
MB5r	ACC GCT CCT GTT AAT CAT CAC C	sequencing	Eszterbauer (2004)
SFOR3	TGT GCG CGA GAG GTG AAA TTC	sequencing	Present study
SREV3	TTT CAG CCT TCG AAC CAT ACT	sequencing	Present study



Figs. 1–3. *Soricimyxum minuti* sp. n. in the bile duct of the pygmy shrew *Sorex minutus* Linnaeus. **Fig. 1.** Pansporoblasts with immature spores. **Fig. 2.** Polysporous plasmodia with mature spores. **Fig. 3.** Mature myxospores showing ridges on valve cell surface ridges.

DNA sequencing

DNA was extracted from ethanol-preserved tissue by first centrifuging at 5000× *g* for 5 min to pellet the myxospores, then removing the ethanol. Total DNA was extracted using a QIAGEN DNeasy Blood and Tissue kit (animal tissue protocol; Qiagen, Hilden, Germany) and eluted in 50 µl AE buffer.

Parasite SSU rDNA was amplified in two rounds. The first round used primers ERIB1 and ERIB10 (Table 1) in a 25 µl reaction mixture, which comprised 1 µl genomic DNA, 0.2 mM deoxyribonucleotide triphosphates (dNTPs, Thermo Scientific, Vilnius, Lithuania), 1 µM each primer, 1× DreamTaq buffer (Thermo Scientific), 1 unit DreamTaq polymerase (Thermo Scientific) and distilled water. PCR consisted of an initial denaturation step of 95 °C for 3 min, followed by 35 cycles of 94 °C for 50 s, 56 °C for 50 s, 72 °C for 80 s, and was finished with terminal extension at 72 °C for 7 min, then rested at 4 °C.

A fully-nested, second round PCR used primers Myx1F and SphR (Table 1) in a PCR scaled-up to 50 µl. The second-round PCR program was 35 cycles of 94 °C for 50 s, 56 °C for 50 s, 72 °C for 60 s, with a terminal extension at 72 °C for 10 min, then rest at 4 °C. PCRs were performed in an Applied Biosystems® (Foster City, California, USA) 2720 Thermal Cycler. The PCR products were electrophoresed in 1.0% agarose gels in Tris-Acetate-EDTA buffer, stained with 1% ethidium bromide, and then purified using a EZ-10 Spin column PCR Purification Kit (Bio Basic Inc., Markham, Ontario, Canada).

Purified PCR products were sequenced in both directions with the primers listed in Table 1, using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit with an ABI 3100 Genetic Analyser.

Unless otherwise noted, sequence assembly and phylogenetic analyses were performed using MEGA 6.06 (Tamura et al. 2013). Forward and reverse sequence segments were assembled by eye and ambiguous bases clarified with reference to the corresponding ABI chromatograms. Nucleotide sequences were aligned with CLUSTAL W (Thompson et al. 1994) and then adjusted manually. DNA pairwise distances were calculated using a Kimura-2 substitution model, then tested to determine the best-fit nucleotide substitution model shown by the Akaike Information Criterion using Mega 6.06. *Chloromyxum leydigi* Mingazzini, 1890 was chosen as an outgroup, consistent with previous analyses (Dyková et al. 2007).

Maximum Likelihood (ML) analyses used the GTR+G+I model, with bootstrap values based on 1000 resamples. The ML tree was visualised using Tree Explorer within MEGA.

RESULTS

Soricimyxum minuti sp. n. Figs. 1–4

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Description: Myxozoan plasmodial stages (Fig. 1) or mature spores (Figs. 2, 3) were observed in bile ducts in 8/21 (36%) common shrew *Sorex araneus* and 1/3 (33%) pygmy shrew *S. minutus*. Mature spores from both hosts were morphologically and morphometrically indistinguishable from *S. fegati* (Dyková et al. 2007). Mature spores sigmoidal in side/sutural view, ellipsoidal in front/valvular view (Fig. 4) length 12.3–13.3 (12.6 ± 0.3), width 8.4–9.6 (9.2 ± 0.5), thickness 7.4–8.4 (8.0 ± 0.4). Relatively thick suture running length of spore and protruding ~0.5 from valve surface; appears as a thick spore margin in valvu-

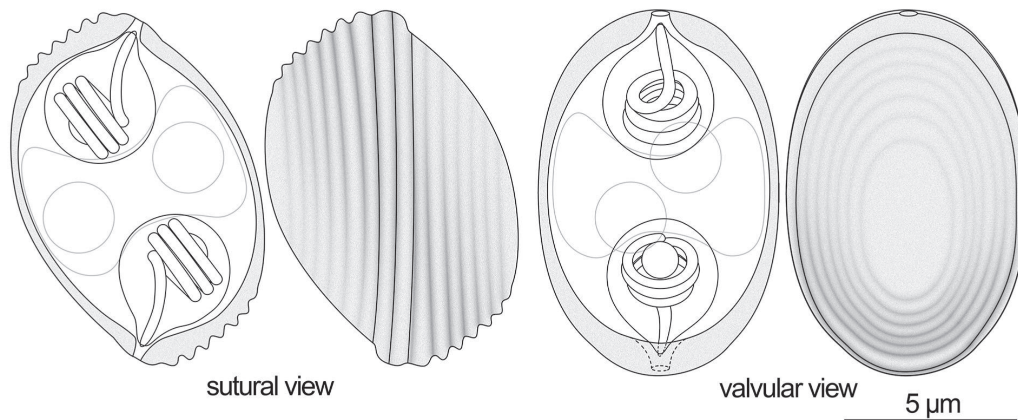

Fig. 4. Drawing of *Soricimyxum minuti* sp. n. from *Sorex minutus* Linnaeus in sutural and valvular views, showing cross-section and surface features for each view.

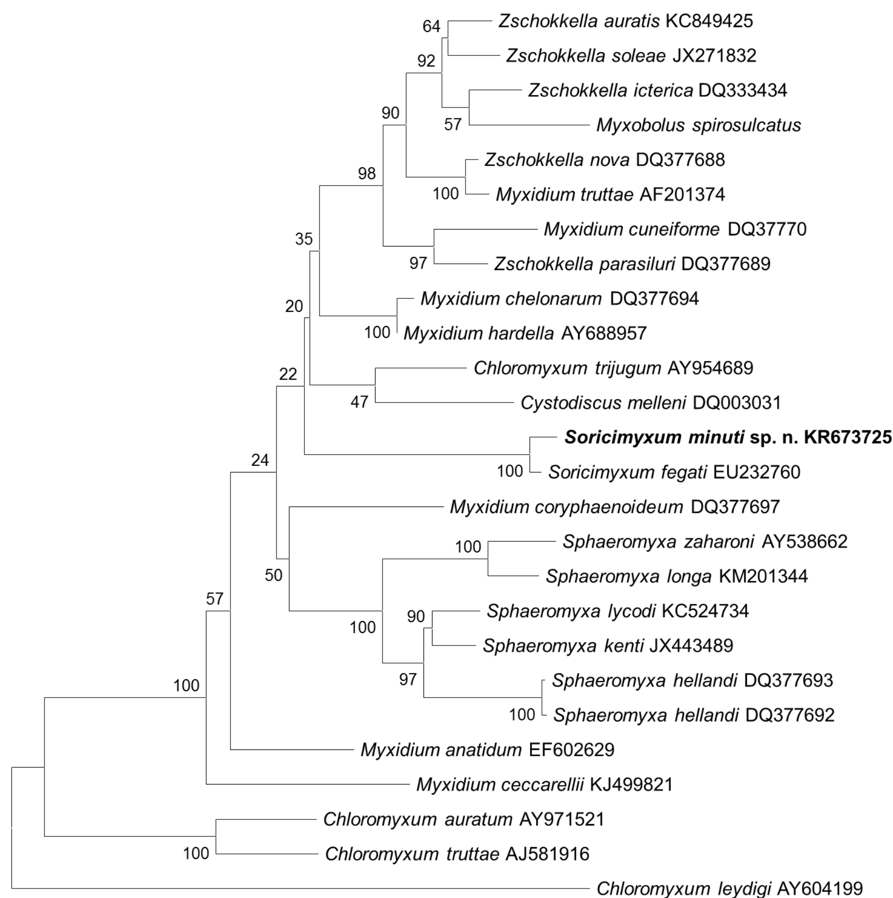

Fig. 5. SSU rDNA tree from Maximum Likelihood analysis, showing the phylogenetic positions of *Soricimyxum minuti* sp. n. and *Soricimyxum fegati* relative to selected myxozoans from other non-fish vertebrates. Bootstrap percentages are given at nodes; GenBank accession numbers are in parentheses.

lar view. Valve cells with fine, longitudinal surface ridges; parallel with the suture; distinctly visible in side view only, with greatest relief at ends of spore and very shallow to non-existent towards middle of spore (Fig. 4). Two polar capsules, situated at opposite ends of spore, near-spherical, equal sized, diameter 4.0–4.5 (4.3 ± 0.2). Polar filaments with 2–3 turns in one plane. In front view, filament discharge channels opened anteriorly and posteriorly, in side view they follow curve of spore.

Vegetative stages: Ellipsoidal 30–50 × 10–30, finely granular multicellular plasmodia that contain developing and mature myxospores (Fig. 1).

Type and only host: Pygmy shrew *Sorex minutus* (Linnaeus).

Type locality: Lipót, North-West Hungary (47°52'03"N; 17°27'24"E).

Site of tissue development: Bile ducts and gall bladder.

Type material: Digitised photos of syntype spores and histological sections were deposited in the parasitological collection of the Zoological Department, Hungarian Natural History Museum, Budapest (Coll. No. HNHM-18388). The SSU rDNA sequence was 1 629 bp (excluding primers) and was deposited in the NCBI GenBank database (accession number KR673725).

Prevalence of infection: 1/3 pygmy shrews.

Etymology: The species is named after the mammalian host *Sorex minutus*.

Molecular analysis. SSU rDNA gene sequences were obtained from two infections in common shrew and the one from pygmy shrew. Sequences from the two common shrew samples were identical to each other, and were 99.8% similar (3/1 391 nt differences) to *S. fegati* type sequence EU232760. The sequence from the pygmy shrew was 96.7% similar to *S. fegati* (45/1 414 nt differences), hence we identified this as representative of a novel myxosporean species.

Remarks. Morphologically, myxospores from the two shrew species appeared identical and matched previous reports of *S. fegati* (Dyková et al. 2007, Prunescu et al. 2007). We observed that spores from both hosts had two to three polar filament turns within the polar capsules, whereas *S. fegati* is reported to have one to two turns. Morphometrically, spores found in both shrew species were inseparable in all dimensions, and equivalent to measurements of *S. fegati* made by Dyková et al. (2007) who showed, by re-measurement of the type material, that the type spore dimensions (Prunescu et al. 2007) were erroneously small. The similarity in measurements between *S. fegati* and *S. minuti* sp. n. means that spore morphometry alone is insufficient to unambiguously identify species of *Soricimyxum*.

Phylogenetic analysis showed that *S. minuti* clustered most closely with the other known shrew myxozoan, *S. fegati*, and that these two species formed a distinct branch (albeit with low bootstrap support) within a clade of morphologically similar myxosporeans of the genera *Zschokkella* Auerbach, 1910, *Myxidium* Bütschli, 1882 and *Cystodiscus* Lutz, 1889 from fish, reptiles and amphibians (Fig. 5.; Dyková et al. 2007). Our data add weight to Prunescu et al.'s (2007) classification of these parasites into a new genus, *Soricimyxum*, with both morphological and molecular similarities supporting inclusion of this genus alongside *Myxidium*/*Zschokkella* of the family Myxidiidae Thelohan, 1892.

DISCUSSION

We discovered different myxozoan infections in two species of terrestrial mammals in Hungary – the shrews *S. araneus* and *S. minutus*. SSU rDNA sequence data provided the ultimate clarification of identities of myxospores from the two shrew species. The sequence from Hungarian common shrew was almost identical (99.8%) to *S. fegati* from the Czech Republic (Dyková et al. 2007). This small amount of variation may be due to genetic drift between the geographically distant common shrew populations. Although we could not distinguish spores from Hungarian common and pygmy shrews based on morphology or morphometric data, SSU rDNA sequences differed by 3.3% between the two hosts. Given that the myxozoans are sympatric, this amount of sequence variation is significantly more than that observed previously between distinct, but morphologically similar myxozoan species, which often have <2% sequence variation (e.g. Molnar et al. 2002, Whipps and Diggles 2006, Ferguson et al. 2008). Hence, we are confident to describe the infection in pygmy shrews as a novel myxozoan, *Soricimyxum minuti*, based on specific host and SSU rDNA identity. Additional infections with *S. minuti* are needed for histopathological studies to document spore development and assess any pathogenic effects of this myxozoan.

Our discovery of a relative of *S. fegati*, in a closely related host, shows that there exists at least two myxozoans that have radiated away from entirely aquatic habitats to parasitise terrestrial vertebrates. This radiation is extraordinary, as myxozoans are almost exclusively aquatic parasites. Infections in non-fish vertebrates are uncommon and cases of homeotherms serving as viable hosts are presently restricted to North American waterfowl and European shrews (Hallett et al. 2015). That very few semiaquatic or terrestrial myxozoan parasites have been discovered may indicate fundamental barriers exist to these parasites successfully switching from aquatic to non-aquatic hosts. We agree with Dyková et al. (2011) who speculated that, as with all known aquatic myxosporean life cycles, the life cycle of species of *Soricimyxum* involves an annelid worm definitive host. To surmount the obstacle of transmission between hosts in a non-aquatic environment, terrestrial Myxozoa could rely on trophic transmission between hosts, and hence vermivorous small mammals may be particularly suitable terrestrial hosts. Additional study to determine the parasites' definitive hosts and modes of terrestrial transmission would provide important insight into the gamut of myxozoan adaptations.

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