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Infection of the Carpathian brook lamprey (*Eudontomyzon danfordi* Regan, 1911) with a dermocystid parasite in the Tisza River Basin, Hungary

Boglárka Sellyei[1] | Gábor Cech[1] | Ádám Varga[1] | Kálmán Molnár[1] | Csaba Székely[1] | Dóra Somogyi[2,3] | Krisztián Nyeste[2,3] | László Antal[2]

[1]Centre for Agricultural Research, Institute for Veterinary Medical Research, Budapest, Hungary

[2]Department of Hydrobiology, University of Debrecen, Debrecen, Hungary

[3]Pál Juhász-Nagy Doctoral School of Biology and Environmental Sciences, University of Debrecen, Debrecen, Hungary

Correspondence
László Antal, Department of Hydrobiology, University of Debrecen, Debrecen, Hungary.
Email: antal.laszlo@science.unideb.hu

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Abstract

The Carpathian brook lamprey (*Eudontomyzon danfordi* Regan, 1911) is an endemic protected species of Cephalaspidomorphi in the Carpathian Basin. No parasites have become known from these jawless vertebrates to date. Here, the authors describe an infection from a single specimen manifesting in protuberant skin cysts 7–10 mm in diameter, scattered on the body surface. Similar dermal infection was observed in 25 of the 274 lampreys recorded in the population survey. Skin cysts filled with round spore-like structures of a dermocystid parasite were found. These particles measured 8–14 µm in diameter and had an about 0.5-µm-thick wall, and containing mainly a granular mass and a relatively scarce plasma. No hyphae were recorded. Despite conspicuous morphological changes in the skin, no inflammatory reactions were found. The molecular analysis of 18S rDNA showed similarity to dermocystid species of several fish species but differed from them approximately by 2%. This is the first record of a dermocystid parasite infecting a jawless vertebrate.

KEYWORDS
18S rDNA, carpathian brook lamprey, dermocystid parasite, histology, skin cysts

1 | INTRODUCTION

Up to now, three lamprey species have been recorded from the Carpathian Basin (Kottelat & Freyhof, 2007). The Ukrainian brook lamprey *Eudontomyzon mariae* (Berg, 1931) is known from the drainage of the Danube River, the range of Carpathian brook lamprey *Eudontomyzon danfordi* Regan, 1911 is restricted to the Upper Tisza region and its tributaries while the Danubian brook lamprey *Eudontomyzon vladykovi* Oliva & Zanandrea, 1959 is distributed in the drainage area of the upper and middle Danube region (Kottelat & Freyhof, 2007). During their larval stage they burrow in fine sediment and feed on detritus and microorganisms. After undergoing metamorphosis, the adults of *E. danfordi* become parasitic and attach to the body of live or dead fish.

Little is known about the parasitic infections caused in different genera of lampreys. Eight parasites, all of them helminths, were recorded from river lamprey *Lampetra fluviatilis* (Linnaeus, 1758) (Bikhovskaya-Pavlovskaya, 1964). Sobecka, Moskal, and Więcaszek (2009) collected data on 4 species of lampreys, and 10 helminth species were recorded from Ukrainian brook lamprey (*E. mariae*). Up to now, no parasitic protozoan species has been reported from *Eudontomyzon* spp.

Dermocystid parasites are worldwide common organisms. They inhabit a wide range of invertebrate and vertebrate animals (Glockling, Marshall, & Gleason, 2013), including fishes, amphibians, birds, mammals and even humans, for example *Rhinosporidium seeberi* (Breitschwerdt and Castellano, 1998; López, 2006; Lupi, Tyring, & McGinnis, 2005; Mendoza, Taylor, & Ajello, 2002).

In fishes, the occurrence of *Dermocystidium* spp. is the most common. The systematic position of the genus *Dermocystidium* Perez, 1907 was a subject of debate for a long time. Some authors, like Dyková and Lom (1992), argued that due to formation of hyphae, this organism is in phylogenetic relation with fungi. By phylogenetic analysis, Ragan et al. (1996) stated that this clade diverges near the animal–fungal dichotomy, and it is a type organism from which metazoa and fungi may have evolved. Mendoza et al. (2002) classified *Dermocystidium* species into the class Mesomycetozoea, as a group of microorganisms at the boundary between animals and fungi and this system remained accepted since then.

Mesomycetozoea (including the order Dermocystida) are an emerging and important parasites in fish and amphibians causing declines in the host populations, as the review by Rowley et al. (2013) presents vast number of examples from the past decades. In Hungary, *Dermocystidium* infections in fish have already been found in perch *Perca fluviatilis* Linnaeus, 1758, eel *Anguilla anguilla* (Linnaeus, 1758), common carp *Cyprinus carpio* Linnaeus, 1758 and crucian carp *Carassius carassius* (Linnaeus, 1758) (Csaba & Láng, 1991; Molnár, 1979; Molnár, Müller, Lefler, & Csorbai, 2008; Molnár & Sövényi, 1984).

Dermocystidium infection is a less known but common parasitosis in fish. The best-studied subject of fish dermocystidiosis is *D. percae* Reichenbach-Klinke, 1950 causing infection in perch (Morley, Campbell, & Lewis, 2008; Pekkarinen & Lotman, 2003; Pronin, 1976). Additionally, there are reports on devastating infections among salmonid fishes (Bruno, 2001; Olson & Holt, 1995) and infections of cyprinids are also common (Červinka, Vitovec, Lom, Hoška, & Kubů, 1974; Csaba & Láng, 1991; Dyková & Lom, 1992; Gjurčevič, 2008; Molnár et al., 2008). Studies have been published on infection of the eel (Molnár & Sövényi, 1984; Wootten & McVicar, 1982). Moreover, there are publications from Africa and South America about dermocystidiosis as an emerging problem among cultured native and introduced fishes (Eiras & Silva-Souza, 2000; El-Mansy, 2008; Fujimoto et al., 2017; Steckert, Cardoso, Tancredo, Martins, & Jeronimo, 2019). More recently, Mahboub and Shaheen (2020) provided useful data on the prevalence, diagnosis and experimental challenge of *Dermocystidium* sp. infection in Nile tilapia (*Oreochromis niloticus)* in Egypt, while in Australia, Shamsi et al. (2020) reported on a heavy infection caused by a *Dermocystidium* sp. in Murray cod. No dermocystid infection has been known from the members of Cephalaspidomorphi until now.

In this paper, we report infection by a dermocystid parasite causing cysts on the skin of the Carpathian brook lamprey, which was collected from a small stream in the Tisza River basin.

2 | MATERIALS AND METHODS

2.1 | Sampling

Lampreys were collected with electric sampling equipment (Hans Grassl GmbH) as part of a sampling for a phylogenetic study on 18 April 2017 from the Kemence stream (48.429306°N 21.447389°E) in the Zemplén Mountains on the territory of Aggtelek National Park, Hungary. During the fishing, some specimens with skin cysts were noticed. Two hundred and seventy-four lampreys of a length from 15 to 20 cm were caught. Each lamprey was observed for a while in a portable fish tank and then released back into the stream. As the Carpathian brook lamprey is a strictly protected species in Hungary, we have killed only a single 16-cm-long (at least 4 years old) mature animal. The specimen was killed by severing the spinal cord (Addis et al., 2012) then cut into two pieces, and the anterior portion of the body was preserved for further investigations in 70% ethanol, and the posterior ones was in 10% buffered formalin.

2.2 | Morphological and histological methods

Pathological studies were performed in the laboratory of the Fish Parasitological and Pathological Team of the Institute for Veterinary Medical Research, Centre for Agricultural Research. A lamprey specimen having protuberant cysts in the skin was investigated under a preparation microscope, including examination of inner organs. A single cyst from the posterior part fixed in 10% buffered formalin was opened from which a small amount of white, tiny granular material was extracted. Thousands of spore-like particles found in this smear were studied under a light microscope.

Photographs and digital images were taken using an Olympus BX53 research microscope equipped with cellSens Entry image archiving software. The spore-like particles were measured on the basis of digital images.

A cross-sectional slice from the portion of the body segment fixed in 10% buffered formalin was routinely processed for histopathology. 4- to 5-μm-thick sections were cut and stained with haematoxylin and eosin.

Histological description of tissues, where cysts were formed, was based upon the guidelines of Elliott (2011).

2.3 | Molecular methods

Genomic DNA was extracted from 80% ethanol fixed material obtained from the cysts using the Geneaid™ DNA Isolation Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan) according to the manufacturer's instructions. Amplification and sequencing of the 18S rDNA were conducted using the primers (AmgF 5′-GTAGTCATATGCTTGTCTC; AmgR 5′-TATTGCCTCAAACTTCCAT) described by González-Hernández et al. (2010). PCR was performed in 25-μl total volumes containing <1 μg DNA, 0.2 μM of each primer, 200 μM dNTPs (Thermo Fisher Scientific) and 1 unit of DreamTaq (Thermo Fisher Scientific) in a SimpliAmp Thermal Cycler with a PCR program including 3 min of initial denaturation at 95°C and 7 min of the final elongation at 72°C, then 35 cycles at 95°C for 30 s, 49°C for 30 s and 72°C for 90 s. The PCR products were electrophoresed in 1.0%

FIGURE 1 Carpathian brook lamprey (*Eudontomyzon danfordi* Regan, 1911) infected by large cysts of a dermocystid parasite. (×2) [Colour figure can be viewed at wileyonlinelibrary.com]

agarose gels in Tris-acetate-EDTA (TAE) buffer gel, stained with 1% ethidium bromide and then purified with an EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Markham, Canada). The purified PCR product of the 18S rDNA was sequenced with the PCR primers. ABI BigDye Terminator v3.1 Cycle Sequencing Kit was used for sequencing, and the sequences were read by an ABI 3100 Genetic Analyser.

The sequence fragments were assembled using MEGA 6.06 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The contiguous 18S rDNA sequences and the most similar dermocystid sequences from the GenBank based on BLAST matches were aligned with the CLUSTAL W software (Thompson, Higgins, & Gibson, 1994). DNA pairwise distances were calculated with the MEGA 6.06 software using the p-distance model. Phylogenetic analysis was performed via maximum likelihood (ML), and *Sphaerothecum destruens* and *S. caipira* were used as out-group. The data set was tested using MEGA 6.06 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 1,000 resampled data sets were generated.

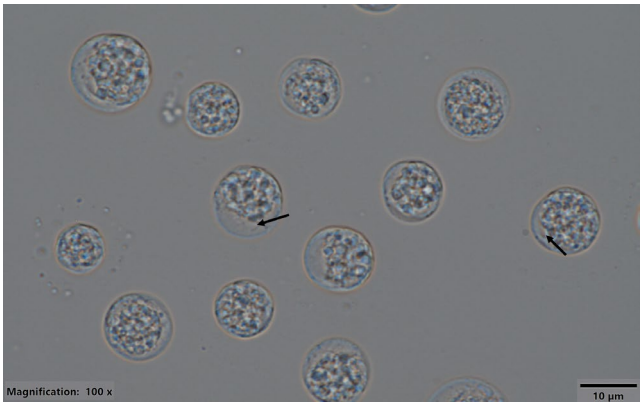

FIGURE 2 Light microscopic image of spore-like particles in smear extracted from the ruptured cyst [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

Twenty-five out of the collected 274 Carpathian brook lamprey specimens (9.1%) showed protuberant cysts on the skin. Scattered on the body surface of the infected specimens, 1–10 (3.5 ± 3.4) cysts were found in the skin (Figure 1). On the single animal killed for further examination, 10 protuberant cysts 7–10 mm in diameter were situated in different parts of the body. In the smear excreted from the ruptured cyst, thousands of round spore-like particles were observed and 50 of them were measured 8–14 (10.49 ± 2.41) µm in diameter based on digital images (Figure 2). They had an approximately 0.5-µm-thick wall and a relatively scarce cytoplasm filled with granular mass. In some spore-like particles, the nucleus (arrows) could be identified among the granules.

In histological sections, the cyst emerges over the body surface, located in the hypodermis of the skin (Figures 3 and 4). The layers of the skin are observable in order, an epithelial layer, a thin basal lamina, a dermis composed of dense collagenous tissue, a thin brown pigmented layer which separates the dermis from the hypodermis and hypodermis (Figures 4 and 5). The cyst wall was mostly detached from the hypodermis, and close contact can be noticed at the basal part of the cyst over the skeletal muscle. The adipose tissue, a frequent component of the hypodermis, is only partly visible. In the present case, hyphal forms characteristics to numerous *Dermocystidium* sp. infections were not detected. Around the cyst, no inflammatory reaction was observed.

The material containing spores and collected from the cysts was subjected to DNA extraction and molecular examinations. PCR amplification and sequencing resulted an 1348-bp-long product. For sequence analysis, 39 related sequences from the GenBank were downloaded and aligned. The final alignment of 18S rDNA sequences of dermocystid species was 1,411 bp long, 1,166 positions were conservative, 244 were variable, and 191 of them were parsim-informative. Dermocystid parasite from the Carpathian brook lamprey was positioned in a monophyletic group (supported

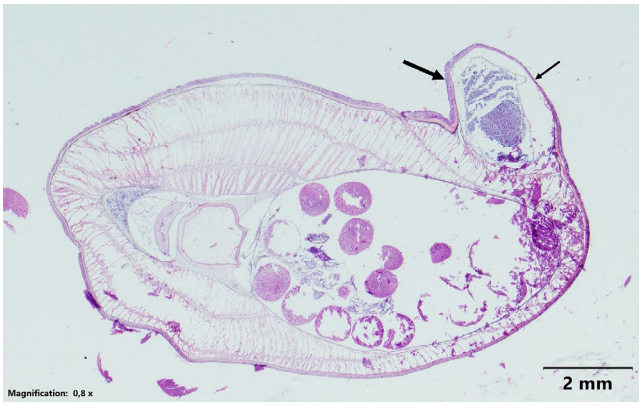

FIGURE 3 Histological cross section of a Carpathian brook lamprey with large cysts on its body surface (arrow) stained with haematoxylin and eosin (H&E). At the top of the cyst, the skin became thinner (small arrow) [Colour figure can be viewed at wileyonlinelibrary.com]

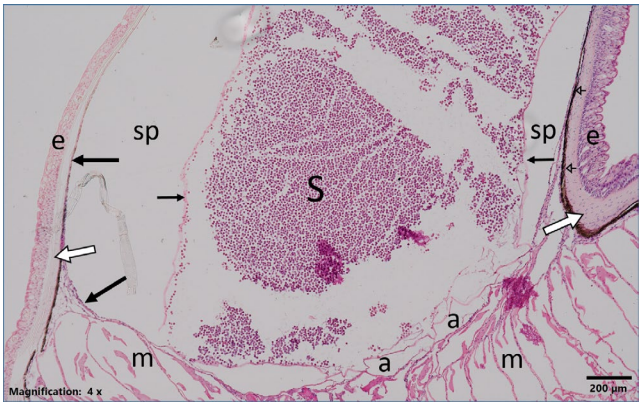

FIGURE 4 Histological section of the dermocystid cyst stained with H&E.; epidermis (e); dermis (white arrow); pigmented layer (blank arrow); hypodermis (black arrow); wall of the cyst (small arrow); spore-like particles in cyst (S); adipose tissue of the hypodermis (a); skeletal muscle (m) [Colour figure can be viewed at wileyonlinelibrary.com]

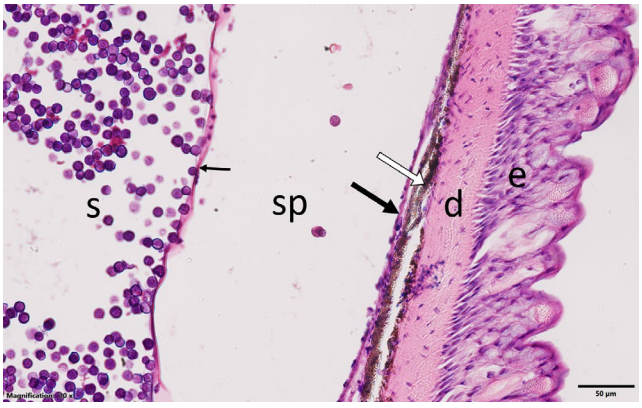

FIGURE 5 Enlarged section of Figure 4 showing the cyst and the skin layers of the fish. Spore-like particles in cyst (S); cyst wall (small arrow); space (SP) between the detached cyst wall and the hypodermis (black arrow); pigmented layer (white arrow); dermis (d); epidermis (e) [Colour figure can be viewed at wileyonlinelibrary.com]

by high bootstrap values) with several dermocystid species like *Dermocystidium salmonis*, *D. hylarum*, *Rhinosporidium seeberi*, *Valentines rwandae*, and *Amphibiocystidium* species. However, the best-known member of dermocystids, *Dermocystidium percae*, was branched outside this group (Figure 6). The most similar sequences were *Dermocystidium salmonis* isolate (MN245010) from cardinal tetra (*Paracheirodon axelrodi*) with 98.4%, *Amphibiocystidium* sp. *viridescens* isolates (EF493028 and EF493029) from red-spotted newt (*Notophthalmus viridescens*) with 98.3% *Dermocystidium* sp. (U21336) from brook trout (*Salvelinus fontinalis*) with 98.0% and *Dermocystidium* sp. isolate from perch (*Perca fluviatilis*) with 98.0% sequence similarity (p-distances in order: 0.016; 0.017; 0.020 and 0.020). Other sequences from the monophyletic clade showed similarities between 96.5% and 97.8%. Taxa outside the clade, such as *Dermocystidium percae*, had much lower similarities (below 95%).

4 | DISCUSSION

Numerous different species of dermocystid parasites have been described worldwide, which infect freshwater and anadromous fishes produce gill infections, skin lesions, visceral diseases and eye infections (Feist, Longshaw, Hurrell, & Mander, 2004; Hassan, Osman, & Mahmoud, 2014; Mahboub & Shaheen, 2020; Molnár et al., 2008; Zhang & Wang, 2005). However, our study is the first finding of a dermocystid species as the first parasite ever recorded in the Carpathian brook lamprey belonging to the class of Cephalaspidomorphi.

Dermocystid infections are generally manifested as small round, oval or elongate cysts sometimes stuffed with long spore-producing hyphae (Dyková & Lom, 2007), with different locations and morphology of the spores, depending on the parasite species (Novotny & Smolova, 2006).

In the developmental cycle of several *Dermocystidium* sp., a small multicellular plasmodium grows and becomes confined within a distinct hyaline cyst wall, and then, the multinucleate cytoplasmic contents become segmented into uninucleate cells that are eventually transformed into a large number of spores (Bruno, 2001; Pekkarinen, Lom, Murphy, Ragan, & Dyková, 2003; Pekkarinen & Lotman, 2003). Mature spores contain a large central vacuole or refractile body the cytoplasm with the nucleus being restricted to a narrow peripheral layer (Dyková & Lom, 1992). The skin cyst that developed on the trunk of the Carpathian brook lamprey in the hypodermis differed from dermocystid infections found earlier in eel, common carp and crucian carp in Hungary (Csaba & Láng, 1991; Molnár et al., 2008; Molnár & Sövényi, 1984).

The wall of the cyst of this species was very thin, in contrast to the cyst walls of other species described by Molnár and Sövényi (1984) and Feist etal. (2004) from the eel and the bullhead *Cottus gobio* Linnaeus, 1758, respectively.

Within the cyst, only separate spore-like particles were found that inside structures did not resemble to mature *Dermocystidium* spores and hyphal forms were not present.

A possible reason for this might be that the infection was only in an early stage of infection. It is known from the work of Červinka et al. (1974) that in the early stages of development, the spores show a much more granular structure than the more mature ones that are characterized by a ring shape.

Pekkarinen and Lotman (2003) and Pekkarinen et al. (2003) who studied the development of *Dermocystidium percae* and *D. fennicum* found that developing spores have granulated structure similarly to the spore-like particles in present study. The lack of mature spores does not make well-grounded to identify the species found by us as a *Dermosporidium* sp.; therefore, we referring it as dermocystid parasite.

Sampling further lamprey specimens and conducting a more detailed study for finding the expected mature stages of the parasite is problematic. Carpathian brook lamprey species is a strictly protected animal, and due to its hidden lifestyle in the mud, the collection is limited to a few days during the spawning period.

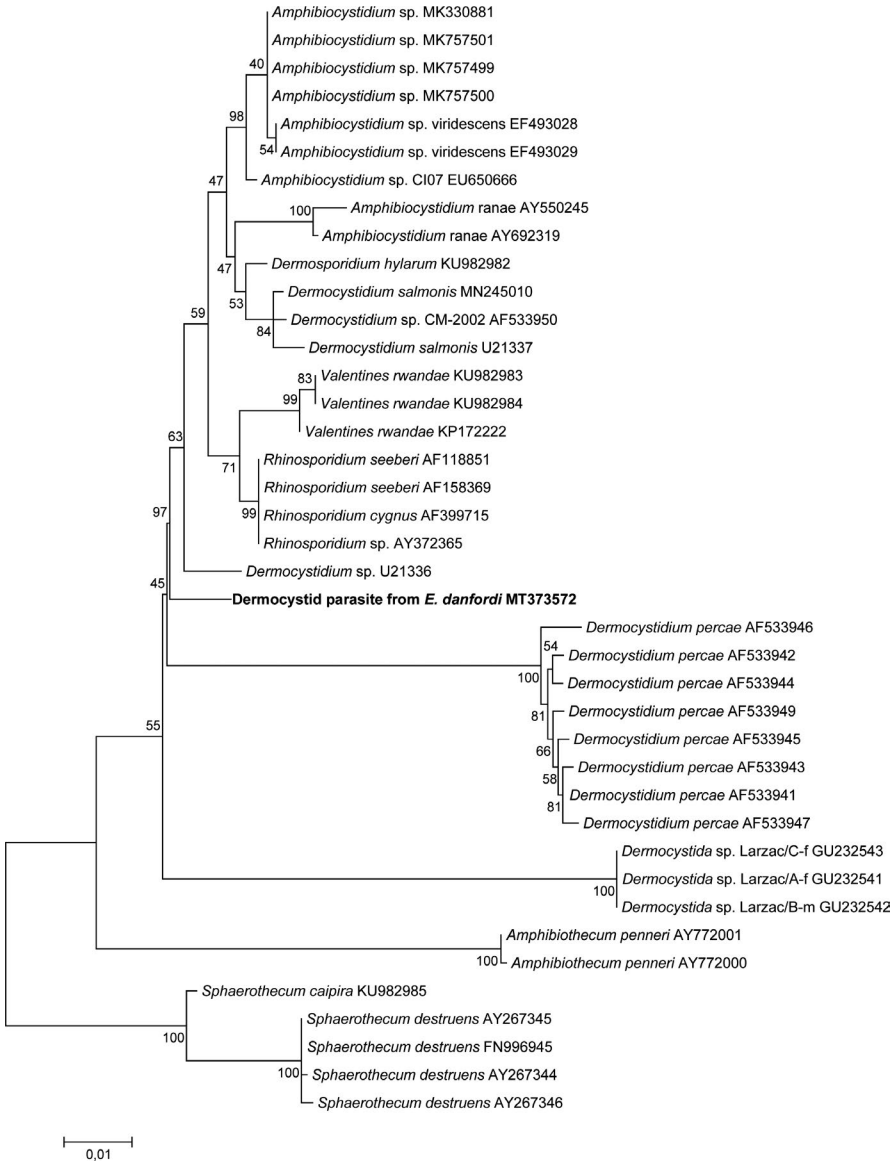

FIGURE 6 Phylogenetic tree of the partial 18S rDNA sequences of the dermocystid parasite detected in Carpathian brook lamprey (GenBank accession no.: MT373572) compared with reference isolates (with accession numbers) obtained from GenBank. The tree was derived by maximum-likelihood analysis (MEGA 6.06) of a 1411-bp region. Bootstrap support values (>75%), indicated at the nodes, were obtained from 1,000 bootstrap replicates and are reported as percentages. Scale bar indicates the number of nucleotide substitutions per site.

Despite these morphological differences, the molecular results clearly proved that our species is related to the *Dermocystidium* species known from salmonid and percid fishes, but the phylogenetic distance is remarkable. No other species could be regarded as a close relative; the phylogenetic position of the new species is distinct from that of other members of the *Dermocystidium* genus. The 18S rDNA sequences clearly define this species as a new member of dermocystids, which makes sense in view of the phylogenetic distances between the hosts.

However, it is remarkable that the studied parasite of the Carpathian brook lamprey is positioned among dermocystids of teleost fishes. Petromyzontiformes, as a members of the class Cephalaspidomorphi, have a basal and distinct phylogenetic position beside the infraphylum Gnathostomata (jawed vertebrates) including bony fishes.

However, tetrapods are phylogenetically much closer relatives to bony fish than to lampreys and their relatives. Considering this fact, it should be emphasized that this is the first record of a dermocystid parasite from a separate and phylogenetically basal vertebrate taxon, the Cephalaspidomorphi. Their presence in lampreys can be explained by the fact that pathogens in Mesomycetozoea are true generalists (Gozlan et al., 2014), and therefore, it is possible that dermocystid species can parasitize a broad range of taxa including Petromyzontiformes in addition to bony fishes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This study was carried out following relevant national and international guidelines pertaining to the care and welfare of fish. The collection and storage of the samples were approved by the National Inspectorate for Environment, Nature and Water, Hungary (permission number: OKTF-KP/3460-27/2016).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Boglárka Sellyei https://orcid.org/0000-0002-1926-8256
Gábor Cech https://orcid.org/0000-0003-4060-4522
Kálmán Molnár https://orcid.org/0000-0002-6138-7929
Csaba Székely https://orcid.org/0000-0001-8831-9099
Dóra Somogyi https://orcid.org/0000-0003-2486-1414
Krisztián Nyeste https://orcid.org/0000-0002-9848-7608
László Antal https://orcid.org/0000-0001-9831-1429

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