

Research Article

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Molecular phylogeny and new light microscopic data of *Metchnikovella spiralis* (Microsporidia: Metchnikovellidae), a hyperparasite of eugregarine *Polyrhabdina* sp. from the polychaete *Pygospio elegans*

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Abstract

Metchnikovellids are a deep-branching group of microsporidia, parasites of gregarines inhabiting the alimentary tract of polychaetes and some other invertebrates. The diversity and phylogeny of these hyperparasites remain poorly studied. Modern descriptions and molecular data are still lacking for many species. The results of a light microscopy study and molecular data for *Metchnikovella spiralis* Sokolova et al., 2014, a hyperparasite of the eugregarine *Polyrhabdina* sp., isolated from the polychaete *Pygospio elegans*, were obtained. The original description of *M. spiralis* was based primarily on the analysis of stained preparations and transmission electron microscopy images. Here, the species description was complemented with the results of *in vivo* observations and phylogenetic analysis based on the SSU rRNA gene. It was shown that in this species, free sporogony precedes sac-bound sporogony, as it occurs in the life cycle of most other metchnikovellids. Spore sacs are entwined with spirally wound cords, and possess only one polar plug. Phylogenetic analyses did not group *M. spiralis* with *M. incurvata*, another metchnikovellid from the same gregarine species, but placed it as a sister branch to *Amphiacantha*. The paraphyletic nature of the genus *Metchnikovella* was discussed. The taxonomic summary for *M. spiralis* was emended.

Introduction

Metchnikovellidae is a family of microsporidia belonging to the monotypic class Rudimicrosporea Sprague, 1977. It unites intracellular parasites of gregarines inhabiting the intestine of polychaetes and some other marine invertebrates. At present, approximately 30 species of metchnikovellids are known. However, only eight of them were studied by modern methods, including transmission electron microscopy (TEM) (Vivier and Schrével, 1973; Hildebrand, 1974; Desportes and Théodoridés, 1979; Ormières et al., 1981; Larsson, 2000; Larsson and Køie, 2006; Sokolova et al., 2013, 2014). These studies showed that the spores of metchnikovellids have distinctive structures. Unlike typical microsporidia, they do not possess a coiled polar filament, anchoring disk, polaroplast and posterior vacuole. Instead, they have short, straight and wide polar filaments, the so-called manubria, which expand into manubrial cisterns (Sokolova et al., 2013; Larsson, 2014). Merogony was never observed in these organisms, and it is assumed that it is absent in the life cycle (Larsson, 2000; Larsson and Køie, 2006). There are two types of sporogony. One results in the formation of so-called free spores, and the other produces the spore-containing sacs ('cysts'). The shape, size and structure of spore sacs are believed to be genus-specific traits (Vivier, 1975; Larsson, 2014).

Recent phylogenomic analyses have robustly placed metchnikovellids as a basal group for 'core' microsporidia (Mikhailov et al., 2017; Galindo et al., 2018; Nassonova et al., 2021) confirming the earlier hypothesis that these organisms represent a primitive group of Microsporidia (Vivier, 1975; Sprague, 1977; Weiser, 1977; Larsson, 2014). However, the configuration of the metchnikovellid tree remains poorly resolved, and taxon sampling is limited. To achieve progress in this field, further morphological and molecular studies of metchnikovellids are necessary.

The species, currently called *Metchnikovella spiralis* Sokolova, Paskerova, Rotari, Nassonova et Smirnov, 2014, was discovered in 1984 in the eugregarines *Polyrhabdina* sp. (Apicomplexa: Eugregarinida) inhabiting the intestine of polychaetes *Pygospio elegans* Claparède, 1863 (Polychaeta: Spionidae) from the White Sea (Rotari and Paskerova, 2007). The microsporidia possessed a unique structure – a cord spirally wrapped around the spore sac. This feature was initially suggested to be a genus-level characteristic. However, in 2010 this species was reisolated and studied by TEM (Sokolova et al., 2014). The obtained data showed that this organism, accord to its basic characteristics, fits the diagnosis of the genus *Metchnikovella*. Hence, it

was described as *M. spiralis*. Since 2010, this species has never been seen in the original sampling site despite annual monitoring, but in 2019 it was isolated from another location in the White Sea at a distance of ca. 9 km from the original site. The description of *M. spiralis* was completed with new morphological data and with the results of phylogenetic analyses based on the sequence of the SSU rRNA gene.

Materials and methods

In 2019, *P. elegans* bristle worms were collected at the Podpahta channel (66°18.108'N, 33°37.775'E), located in the Chupa Inlet, Kandalaksha Bay, White Sea. The animals were maintained at +10°C under room light in 500 mL containers with seawater that was periodically changed. For examination, the polychaetes were individually pressed between a coverslip and an object slide to release the content of the gut under a Leica M205C dissection microscope equipped with Rottermann contrast. The obtained squashed preparations were examined using a Leica DM 2500 microscope equipped with DIC optics and a Leica DFC295 digital camera. In cases when a gregarine showing indications of infection (unusual heterogeneity of the cytoplasm, visible individual spores or spore sacs) was found, a series of optical sections from each specimen were recorded using a 100× PlanApo objective lens to collect images of the cell at different focal planes. In some cases, to increase the focal depth, we applied Z-stacking of two images made at different focal planes using Adobe Photoshop software (www.adobe.com/ru/products/photoshop.html).

Furthermore, a large amount of Millipore-filtered seawater was added under the coverslip, which was detached from the object slide. The gregarines containing free spores and spore sacs of *M. spiralis* were collected under the coverslip using a hair-thin tapered-tip Pasteur pipette, washed in a fresh portion of Millipore-filtered seawater and placed in 200 µL PCR tubes with 1–2 µL of water. Each tube was manually controlled for the presence of gregarine using a Leica M205C dissection microscope at high magnifications. DNA extraction in these tubes was performed using Arcturus® PicoPure® DNA Extraction Kit (Thermo Fischer Scientific, Waltham, MA, USA). The purified DNA was amplified by Multiple Displacement Amplification (MDA) using Repli-g Single Cell Amplification Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. SSU rRNA gene was amplified using 1:10 diluted product of MDA reaction as a template with microsporidia-specific primers: 18F, 530R (Weiss and Vossbrinck, 1999) and 1353TnR (a modification of the primer V1492R) 5'-GCAGCCTTGTTACGACTT-3'. PCR program parameters were the following: initial denaturation (5 min at 95°C) followed by 35 cycles of 30 s at 95°C, 50 s at 50°C and 90 s at 72°C, followed by 7 min at 72°C for final extension. Amplicons were purified using Cleanup Mini Purification Kit (Eurogen, Moscow, Russia) or with ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The Sanger sequencing reactions were carried out using the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific, Waltham, MA, USA) and sequenced using Applied Biosystems™ 3500xL Genetic Analyzer (Thermo Fischer Scientific, Waltham, MA, USA). The final length of the assembled contig was 1238 bp.

The SSU rRNA gene sequence alignment was created using MUSCLE algorithm (Edgar, 2004) implemented in SeaView v. 4.6.1 (Gouy *et al.*, 2010), followed by manual polishing. The alignment included the representative set of sequences of core microsporidia from five identified clades (Vossbrinck *et al.*, 2014), all available sequences of metchnikovellids, and a representative set of sequences of early diverging microsporidia, rozellids, apheleids and nucleiids to form a proper set of outgroups. The

maximum likelihood (ML) phylogenetic analysis was performed using RAxML-HPC2 v. 8.2.12 (Stamatakis, 2014) at CIPRES portal (Miller *et al.*, 2010). GTR + γ model of evolution with 25 substitution rate categories was applied. A total of 100 independent ML inferences with distinct randomized MP starting trees were performed; the best-scoring tree was tested using non-parametric bootstrapping (1000 pseudoreplicates). Bayesian analysis was performed with MrBayes v. 3.2.6 (Ronquist *et al.*, 2012) at CIPRES portal using GTR model with γ correction for intersite rate variation (eight categories) and the covarion model. Trees were run as two separate chains (default heating parameters) for 15 million generations, by which time they had ceased converging (final average standard deviation of the split frequencies was <0.01), the first 25% of generations were discarded for burn-in.

The SSU rRNA gene sequence obtained in this study was deposited with the GenBank under the accession number: MW344837.

Results

Occurrence and prevalence

Among the 108 examined polychaetes, 87 specimens (80.6%) contained eugregarines *Polyrhabdina* sp. The number of gregarines per worm varied from 1 to 60. In 26 polychaetes (24.1%), the gregarines were infected with metchnikovellids. Of them, in 13 polychaetes (12%), the gregarines contained only the early stages of metchnikovellid proliferation, and no free spores or spore sacs were found. Hence, it was impossible to identify the infecting organism in these gregarines. The spore sacs of *M. incurvata* were present in gregarines from 11 polychaetes (10.2%). Two worms were parasitized with gregarines containing spore sacs of *M. spiralis*, which represented 1.85% of all examined polychaetes. In one case, there was a mixed infection. Gregarines containing spore sacs of either *M. incurvata* or *M. spiralis* were found in the same polychaete. However, both species have never been observed in one gregarine cell.

Light microscopy

The following variants of the infected gregarine cells were observed: (1) with free spores only, (2) with numerous free spores and a small number of spore sacs and (3) with numerous spore sacs and few clusters of free spores.

Free spores were oval, 2.5–3.5 × 2.1–2.3 µm (av. 3.1 × 2.1 µm, $n = 30$) in maximal dimension. They form clusters residing in parasitophorous vacuoles (Fig. 1). Spore sacs of *M. spiralis* were oval, 10.3–16.5 µm (av. 14.6 µm, $n = 45$) in length and 5.4–7.1 µm (av. 6.1 µm, $n = 45$) in width. The number of spore sacs varied from one to nine per gregarine cell. Each sac was enclosed individually in a spacious parasitophorous vacuole (Fig. 2A). Spore sacs were twined with a cord, which was coiled as a tightly packed spiral around them. The spiral cord had approximately 50 coils and formed a kind of outer carcass around the sac (Fig. 2B and C). The wall of the spore sac never contacted the membrane of the parasitophorous vacuole. The sacs ornamented with the spiral cord were termed 'spiral sacs' in Sokolova *et al.* (2014). In the squashed preparation, parasitophorous vacuoles were sometimes squeezed out of the host cell through rupture in the gregarine pellicle. In such cases, the vacuoles were significantly increased in size, and the distance between the coils of the spiral cord was increased as well (Fig. 2D). Sometimes, in isolated parasitophorous vacuoles pressed with a coverslip, the spiral cord was relaxed and slipped out of the spore sac (Fig. 2E–G). If the vacuole containing a spore sac was destroyed, the spiral arrangement of the cord immediately relaxed completely (Fig. 2H). It was impossible to detect any contact of the cord with the surface of the sac.

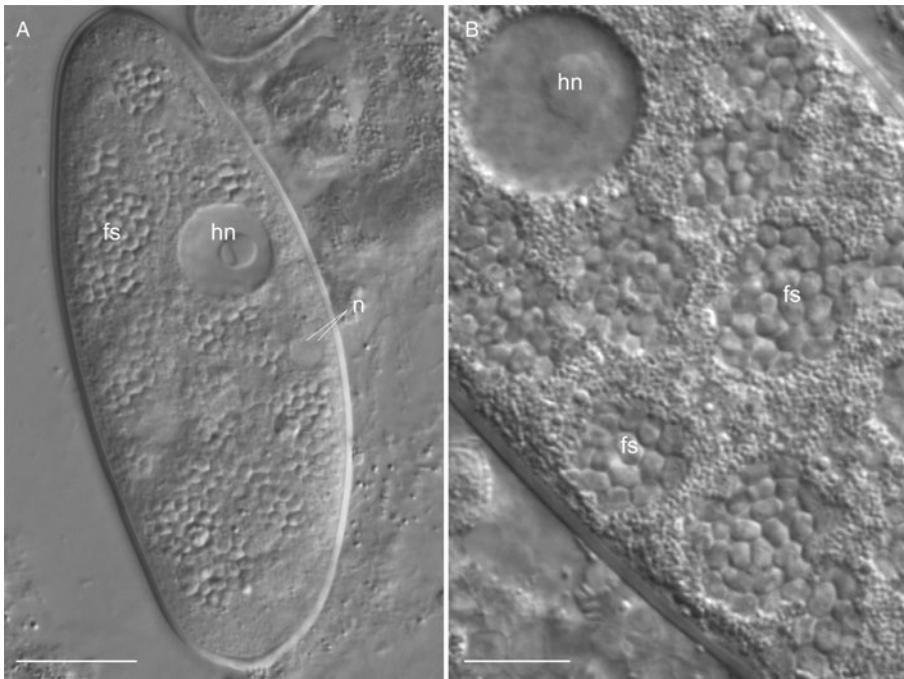


Fig. 1. Living microsporidia *Metchnikovella spiralis*, early stages of infection in the eugregarine *Polyrrhabdina* sp., differential interference contrast (DIC). (A) Host cell filled with free spores of the hyperparasite; (B) clusters of free spores. fs, free spores of the metchnikovellid; hn, host nucleus; n, nucleus of sporont mother cell. Scale bars: 20 μm (A), 10 μm (B).

Each spore sac contained eight oval spores, arranged in two rows and measuring $2.4\text{--}3.5 \times 2.4\text{--}2.9 \mu\text{m}$ (av. $2.9 \times 2.6 \mu\text{m}$, $n = 30$) in length and width, respectively (Fig. 2E–G). Spore sacs had one polar plug, appearing as a thickening at one pole of the sac (Fig. 2E–I). In contrast, the spore sacs of *M. incurvata* found in the gregarines with mixed infection were elongated, slightly bent and had two polar plugs (Fig. 2I).

A schematic drawing of the spore sac of *M. spiralis* is shown in Fig. 3.

Molecular phylogeny

In the reconstructed SSU rRNA tree (Fig. 4), all main clades of ‘core’ microsporidia (sensu Vossbrinck *et al.*, 2014, Fig. 6.3a) were robustly recovered, excluding clade 2. The latter was split into two groups. The group *Paranosema/Antonospora* was retrieved as a sister to clade 3 with rather high support, whereas the group *Nematocida/Ovavesicula* constituted a highly supported basal lineage of the core microsporidia. This topology is congruent with recently published phylogenies, where the splitting of clade 2 into two distinct lineages was also observed (Mikhailov *et al.*, 2017; Williams *et al.*, 2018; Nassonova *et al.*, 2021). The position of the *Hamiltosporidium/Neoflabelliforma* group within clade 3 (as was shown in Vossbrinck *et al.*, 2014) was not recovered, which is congruent with the results of Mikhailov *et al.* (2017) and Nassonova *et al.* (2021). With rather high support in Bayesian analysis (but with low support in the ML trees), this group was retrieved as a sister to a well-supported assemblage uniting clade 4 and clade 5.

Metchnikovellids constituted a robustly supported group branching at the base of ‘core’ microsporidia. The metchnikovellid sequences formed two sister clades. One was highly supported (1.00/100%) and comprised the sequences of *Amphiacantha* spp., environmental clone p1_44 (GenBank KX214678) and *M. spiralis*. The second clade was only moderately supported (0.89/67%) and encompassed available sequences of *Amphiamblys* spp. and two other *Metchnikovella* species. This topology is congruent with the hypothesis of Larsson (2014), who proposed the transfer of the representatives of the genus *Amphiacantha* from a single family Metchnikovellidae into a separate family Amphiacanthidae.

Metchnikovella spiralis did not form a sister branch to either *M. incurvata* or *M. dogieli*, as might be expected, and the sequences of *Metchnikovella* spp. did not form a monophyletic clade.

The positions of *Chytridiopsis typographi* and *Nucleophaga* spp. were unstable in the tree, which is consistent with our previous analysis (Nassonova *et al.*, 2021) and with the results obtained by Mikhailov *et al.* (2021). In the Bayesian analysis, *Nucleophaga* spp. had a more basal position than *C. typographi*, yielding the topology demonstrated by Corsaro *et al.* (2018, 2020). However, in the ML trees, the pattern of branching was inverted [in some ML trees the grouping of *C. typographi* within clade X (sensu Lazarus and James, 2015) was observed]; in either case, the support was low (PP 0.60–0.74; BS 40–66%) and the backbone of the tree in the intermediate zone between long-branch and short-branch microsporidia (sensu Bass *et al.*, 2018) was not resolved, which may have been caused by the rapid evolutionary rate and highly derived character of the *C. typographi* SSU rRNA sequence.

The topology of the lower part of the SSU rRNA tree corresponded to that observed in recent studies (Lazarus and James, 2015; Corsaro *et al.* 2016, 2018, 2020; Grossart *et al.*, 2016; Mikhailov *et al.*, 2017; Stentiford *et al.*, 2017; Bass *et al.*, 2018; Williams *et al.*, 2018; Nassonova *et al.*, 2021). The main clades of rozellids, including those classified as short-branched microsporidia by Bass *et al.* (2018), were recovered mostly with their usual composition.

Discussion

Identification and occurrence of *M. spiralis*

The unique structure of the spore sacs, including the presence of the spiral cord (‘spiral sacs’), the size of the sacs and of the individual spores and the number of spores in each sac left no doubt that for the first time since 2010 the species *M. spiralis* was isolated albeit from a different sampling site in the White Sea (the distance between Levin Reach, the site of isolation in 2010, and Podpahta channel, the site of isolation in 2019, is approximately 9.33 km by sea).

According to a survey in 2010, when 333 worms were examined, only three polychaetes contained gregarines with *M. spiralis*



Fig. 2. Living microsporidia *Metchnikovella spiralis*, late stages of infection in the eugregarine *Polyrrhabdina* sp., differential interference contrast (DIC). (A) Infected gregarine with spore sacs within the individual parasitophorous vacuoles (arrows) and numerous free spores, stacked image; (B) infected gregarine slightly pressed with the coverslip for better visualization of the intracellular parasites, arrowheads point to 'spiral sac' with enclosed spores. (C) The same as (B), another focal plane of the same site showing striation corresponding to the cord spirally entwined around the sac; (D) the squashed preparation showing the elongated parasitophorous vacuoles (arrows), which are releasing from the host cell; (E, F) spore sac at different focal planes, showing the coiling of the spiral cord (arrowheads); (G) an isolated parasitophorous vacuole enclosing the spore sac with spiral cord slightly relaxed and slipped out of the sac (arrowheads); (H) spore sacs with completely relaxed and unwound spiral cord and an empty spore sac, asterisk marking the place, where a detached polar plug used to be; (I) spore sacs of two species of metchnikovellids isolated from one specimen of the worm: *M. spiralis* with one polar plug, *M. incurvata* with two plugs. It is a case of mixed infection in the population of *Polyrrhabdina* sp. fs, free spores; hn, host nucleus; mi, *M. incurvata*; ms, *M. spiralis*; pp, polar plug; sc, spiral cord; ss, spore sac. Scale bars: 20 μm (A, D), 10 μm (B, C, G–I), 5 μm (E, F).

(0.9%), while 11 polychaetes contained *M. incurvata*-infected gregarines (3.3%) (Rotari et al., 2015). In the current study, *M. spiralis* was detected in 1.85% of the studied polychaetes, while *M. incurvata* was found in 10.2% of worms. This supports the suggestion that *M. incurvata* (as a species probably introduced from the Atlantic region) turned out to be more competitive and invasive than *M. spiralis* (Rotari et al., 2015).

The mixed invasions of two metchnikovellids were found in host populations, but gregarine cells infected with both hyperparasites at once have never been observed. This may be a result of the low occurrence of metchnikovellids or suppression of the development of one hyperparasite by another. For example,

M. incurvata may inhibit *M. spiralis* development. It might be dealing with post-invasion modifications of the gregarine pellicle resulting in its resistance to the penetration of the second species or with fast proliferation of the first ('pioneer') parasite resulting in a rapid depletion of resources of the host cell and inhibition of any further parasite proliferation.

Parasitophorous vacuole and spiral cord

According to Sokolova et al. (2014), the space around the spore sac in the parasitophorous vacuole of *M. spiralis* is filled with fine filamentous material. The spiral cord seems to consist of

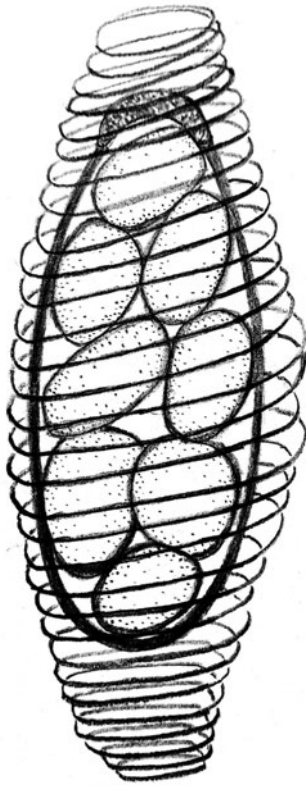


Fig. 3. A schematic drawing of the *Metchnikovella spiralis* spore sac. The number of spiral cord coils in the diagram is deliberately halved to show the contents of the spore sac.

the same compacted and twisted filamentous material and is suggested to be a derivative of the external layer of the spore sac wall. The spiral cord probably serves as an external skeleton of the sac, which may help to maintain the volume of the parasitophorous vacuole in the gregarine cell (Sokolova *et al.*, 2014). The elongation of vacuole and uncoiling of the spiral cord observed under a light microscope occurred due to coverslip pressure and can be considered an artefact; however, it remains unclear how this cord behaves under natural conditions when the spore sac is released in the environment.

To date, the spiral cord of *M. spiralis* remains a unique example of such a complex spore sac structure among metchnikovellids. To a certain extent, it may be possible to compare it with the ornamentation of sporophorous vesicles of 'core' Microsporidia; however, this kind of structure is also rare. For example, *Trichodubosqia epeori* possesses sporophorous vesicles with needle-like appendages approximately 20 µm long 'with internal material similar to collagen' (Batson, 1982). In contrast to rare external structures, different kinds of structures and inclusions are often observed in the episporontal space of many species of 'core' microsporidia (Vávra and Larsson, 2014). For instance, *Trichoctosporea pygopellita* and *Agglomerata volgensae* have fibrous projections of sporoblasts, connecting the exospore with the envelope of the sporophorous vesicle (Larsson, 1994; Larsson and Voronin, 2000). The functional significance of such structures is also unclear.

Possible relationships with the genus *Amphiacantha*

Phylogenetically, the sequence of the SSU rRNA gene *M. spiralis* turned out to be closer to those belonging to the representatives of the genus *Amphiacantha* rather than that of *Metchnikovella* spp. This is surprising to a certain extent because these two genera are very different in morphology and biology. Members of the

genus *Amphiacantha* have spindle-shaped spore sacs, terminating as thread-like prolongations. They do not have polar plugs. The length of sacs may exceed the width more than ten times, and they contain approximately 50 roundish spores (Caullery and Mesnil, 1914; Larsson, 2000). Species of the genus *Amphiacantha* develop directly in the host cytoplasm, and parasitophorous vacuoles have never been observed in the course of their development and around the mature spore sacs. Therefore, differences are numerous and basic, and it is impossible to suggest that *M. spiralis* might be a member of the genus *Amphiacantha*.

However, theoretically, it is possible to consider a homology of the spiral cord in *M. spiralis* with the thread-like prolongations of the spindle-shaped spore sacs of *Amphiacantha*. Logically, we can speculate that the spiral cord in *M. spiralis* originated as a result of the further development of long terminal threads seen in *Amphiacantha* spore sacs, which start to coil around the sac. The similarity of the fine structure of the cord with the material of the external sac wall, noted by Sokolova *et al.* (2014), and the putative site of contact of the cord with the envelope of the spore sac shown in the same study (Sokolova *et al.*, 2014; Fig. 4C) may be an indication in favour of this suggestion. Thus, molecular phylogeny probably indicates the relationships between the species, showing cord-like structures as a part of the spore sac. However, the sequences of SSU rRNA gene in metchnikovellids are highly divergent, and unambiguous alignment of some regions of the SSU gene is almost impossible in these organisms. The number of available sequences of metchnikovellids is very low, and all of them represent relatively long branches in the phylogenetic tree. This might result in the underestimation of genetic distances and complicates the task of the phylogenetic reconstruction. The involvement of additional markers (e.g. phylogenomics data) and/or expanding the taxon sampling is highly desirable to further progress towards a reconstruction of robust phylogeny of metchnikovellids and the taxonomical conclusions.

Taxonomic issues

Metchnikovella spiralis was found in 1984 in the eugregarine *Polyrhabdina* sp. from the gut of the polychaete *P. elegans*. The species, due to the unusual spiral ornamentation around the spore sac (as was visible in the LM optics of that time), was presumed to belong to a new genus, which was provisionally named 'Vivierus spiralis' (Rotari, 1988). Later, this name (as 'Vivieria spiralis') appeared in the abstract of the Proceedings of VII European Congress of Protistology, published in the journal 'Protistology' (Rotari and Paskerova, 2007). Because neither of these names was published according to the International Code of Zoological Nomenclature rules (ICZN, 4th edition, Chapter 3, Article 9), the genus name 'Vivierus' ('Vivieria') cannot be considered validly published.

Sokolova *et al.* (2014) argued that the species studied was identical to one previously described under the name 'Vivieria spiralis'. However, this study showed that it fits the formal diagnosis of the genus *Metchnikovella* based on the following characteristics: (1) length of the spore sacs does not exceed ten times the width; (2) a small number (eight) of sac-bound spores per sac; and (3) oval sac-bound spores (Hildebrand and Vivier, 1971; Vivier and Shrével, 1973; Sokolova *et al.*, 2013, 2014). Taking into account the similarity of several ultrastructural characteristics with *Metchnikovella* (oval sac-bound spores, horseshoe-shaped nucleus, ornamentation of the exospore of free spores, cysts with polar thickening), it was decided that there is no reason to establish a new genus for this isolate (Sokolova *et al.*, 2014, p. 1118).

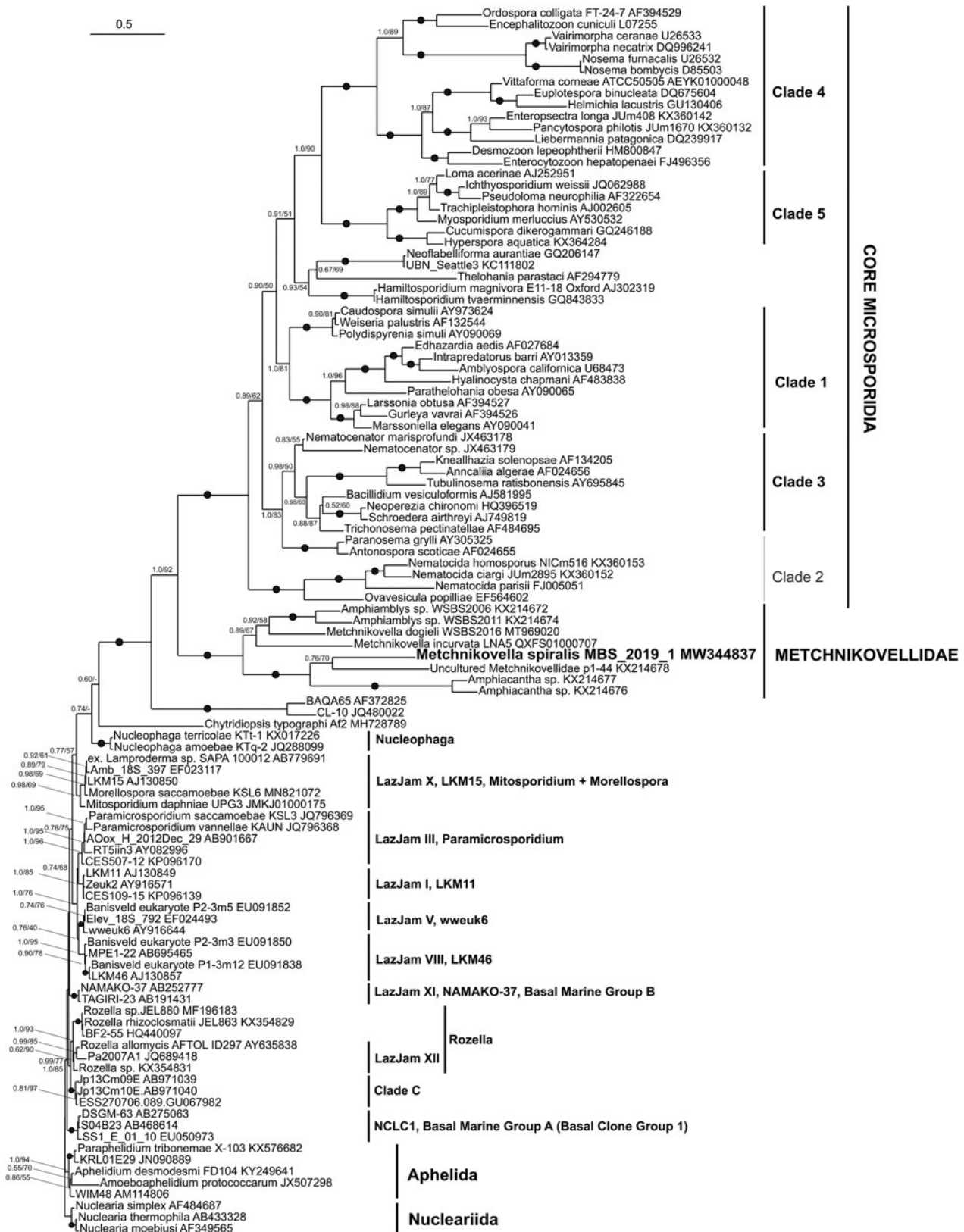


Fig. 4. SSU rRNA phylogeny of Microsporidia and related lineages including the sequence of *Metchnikovella spiralis* retrieved in this study (in bold). Black blobs indicate support values of ≥ 0.99 Bayesian posterior probability (PP) and $\geq 99\%$ ML bootstrap (BS). PP and BS values are indicated above the branches; support values PP < 0.50 and BS $< 50\%$ are not shown; ‘-’ indicates that the pattern of branching was not recovered by one of the reconstruction methods used. The major microsporidial clades established by Vossbrinck et al. (2014, Fig. 6.3a) are labelled. Dotted line shows the clade 2, which occurred to be paraphyletic. The clades with environmental sequences are labelled as LazJam x with Latin numerals according to the designations introduced by Lazarus and James (2015); the designations of the clades are according to Corsaro et al. (2016, 2020), Ishida et al. (2015), Nagahama et al. (2011), Richards et al. (2015) are also used. Isolated and described taxa from these lineages are indicated.

The definition of the genus *Metchnikovella* is very wide. In light of modern data, this genus looks rather heterogeneous compared with the relatively narrowly defined genera *Amphiamblys*

and *Amphiacantha* (reviewed in Nasonova et al., 2021). Dogiel (1922) suggested a new genus, *Caulleryetta*, for metchnikovellids whose spore sacs possess only one plug. Larsson (2014) supported

this suggestion, although the validity of the genus *Caulleryetta* was not justified by other researchers (e.g. Vivier and Schrével, 1973; Vivier, 1975).

The main difficulty in verifying the taxonomy of metchnikovellids is the lack of modern data on the type species *Metchnikovella spionis* Caullery & Mesnil, 1897, and *Metchnikovella* ('Caulleryetta') *mesnili* Dogiel, 1922. This is one of the main reasons why modern researchers prefer to utilize older systems based on the original classification by Caullery and Mesnil (1914, 1919) supported by Vivier (1975). Switching between different systems before obtaining molecular data for key representatives of the genus *Metchnikovella* seems to be premature.

Metchnikovella spiralis has oval spore sacs with only one polar plug, so based on this character, it could be classified as *Caulleryetta*. However, *M. dogieli* also has one polar plug but does not group with *M. spiralis* in the SSU rRNA tree. The latter species cannot be classified in the genus *Amphiacantha*; however, it shows some affinities with this lineage. Most likely, the suggestion to establish a new genus for *M. spiralis*, as proposed by Rotari and Paskerova (2007), is reasonable; however, we would prefer not to propose such a revision before phylogenomic data confirm this position of *M. spiralis*. The practical solution at the moment is to leave the paraphyletic genus *Metchnikovella* as it is defined by Vivier (1975) unless molecular data on the type species of this genus become available. For substantial revision of the genus, data on the type species are highly desirable.

Taxonomic summary of *M. spiralis* should be corrected as the single polar plug of the spore was not mentioned in the original description of *M. spiralis*. This feature is one of the important discriminating criteria and should be included in the diagnosis of this species. Sokolova *et al.* (2014) concluded that the free sporogony sequence occurred at the same time as the sac-bound sporogony, while our observations show that the free sporogony precedes the sac-bound one.

Taxonomic summary

Metchnikovella spiralis Sokolova *et al.*, 2014, *emend.*

This species differentiates from congeners and other metchnikovellids by the unique structure of the spore sac: a light-reflecting thick wall with one polar thickening and an external carcass composed of dense cord spirally coiled around the sac, visible in the light microscope as regularly arranged striations on the surfaces of the sac. On ultrathin sections, the external envelope can be seen as tangential or cross-sectioned filaments regularly arranged in the lumen of parasitophorous vacuole between the sac wall and membrane of the vacuole. Free sporogony occurs before sac-bound sporogony. Other details are described in Sokolova *et al.* (2014).

Type locality: Levin reach, Chupa Inlet, Kandalaksha Gulf of the White Sea, Russia (66°17.878'N, 33°27.774'E).

Type host: eugregarines *Polyrhabdina* sp. (Apicomplexa: Eugregarinida) from the polychaete *P. elegans* Claparède, 1863 (Polychaeta: Spionidae).

Locality and host used in the emended diagnosis: Podpahta Channel, Chupa Inlet, Kandalaksha Bay, White Sea (66°18.108'N, 33°37.775'E); *Polyrhabdina* sp. from *P. elegans*.

Type material: as designated in Sokolova *et al.*, 2014.

Deposition of materials used in the emended diagnosis. LM images of eugregarines infected with *M. spiralis* are in the image collection of the Department of Invertebrate Zoology, St Petersburg State University. Figures 1 and 2 (this publication) show some specimens. Frozen purified genomic DNA of the infected gregarines as well as the individual infected gregarine cells fixed in 96% ethanol are stored in the same department.

Gene sequences: SSU rDNA gene sequence of *M. spiralis* has been deposited in GenBank under accession number MW344837.

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Author contribution. E.V.F. and E.S.N. suggested an overall concept and design of the study, conducted molecular work, performed molecular phylogeny, drafted the manuscript; E.V.F. and G.G.P. performed the field work, dissection of polychaetes, isolation of the parasites; A.V.S. and E.V.F. did light microscopy and single-cell manipulations. All authors contributed to the improvement of the draft and accepted the final version of the manuscript.

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Conflict of interest. None.

Ethical standards. This article does not contain any studies with human participants or warm-blooded animals performed by any of the authors. The Educational and Research Station 'Belomorskaia' of Saint Petersburg University at the White Sea has permission to collect invertebrate animals for scientific work on its own territory and other sites situated around the station. The invertebrates of interest are neither endangered nor protected species within the region. Animal handling and dissecting was performed at cold temperature to avoid distress and unnecessary suffering.

References

- Bass D, Czech I, Williams BAP, Berney C, Dunthorn M, Mahé F, Torruella G, Stentiford GD and Williams TA (2018) Clarifying the relationships between microsporidia and cryptomycota. *Journal of Eukaryotic Microbiology* **65**, 773–782.
- Batson BS (1982) A light and electron microscopical study of *Trichoduboscqia epeori* Léger (Microspora: Duboscqiidae). *The Journal of Protozoology* **29**, 202–212.
- Caullery M and Mesnil F (1914) Sur les Metchnikovellidae et autres Protistes parasites des Grégarines d'Annélides. *Comptes Rendus de Seances de la Societe de Biologie, Paris* **77**, 527–532.
- Caullery M and Mesnil F (1919) Metchnikovellidae et autres Protistes parasites des Grégarines d' Annélides. *Annales de l'Institut Pasteur* **33**, 209–240.
- Corsaro D, Michel R, Walochnik J, Venditti D, Müller KD, Hauröder B and Wylezich C (2016) Molecular identification of *Nucleophaga terricolae* sp. nov. (Rozellomycota), and new insights on the origin of the Microsporidia. *Parasitology Research* **115**, 3003–3011.
- Corsaro D, Wylezich C, Venditti D, Michel R, Walochnik J and Wegensteiner R (2018) Filling gaps in the microsporidian tree: rDNA phylogeny of *Chytridiopsis typographi* (Microsporidia: Chytridiopsida). *Parasitology Research* **118**, 169–180.
- Corsaro D, Walochnik J, Venditti D, Hauröder B and Michel R (2020) Solving an old enigma: *Morellospora saccamoebae* gen. nov., sp. nov. (Rozellomycota), a *Sphaerita*-like parasite of free-living amoebae. *Parasitology Research* **119**, 925–934.
- Desportes I and Théodoridès J (1979) Étude ultrastructurale d'*Amphiambly laubieri* n. sp. (Microsporidie, Metchnikovellidae) parasite d'une Grégarine (*Lecudina* sp.) d'un Echiurien abyssal. *Protistologica* **15**, 435–457.
- Dogiel VA (1922) Sur un nouveau genre de Metchnikovellidae. *Annales de l'Institut Pasteur* **36**, 574–577.
- Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.
- Galindo LJ, Torruella G, Moreira D, Timpano H, Paskerova G, Smirnov A, Nassonova E and López-García P (2018) Evolutionary genomics of *Metchnikovella incurvata* (Metchnikovellidae): an early branching microsporidium. *Genome Biology and Evolution* **10**, 2736–2748.
- Gouy M, Guindon S and Gascuel O (2010) SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution* **27**, 221–224.

- Grossart H-P, Wurzbacher C, James TY and Kagami M (2016) Discovery of dark matter fungi in aquatic ecosystems demands a reappraisal of the phylogeny and ecology of zoosporic fungi. *Fungal Ecology* **19**, 28–38.
- Hildebrand HF (1974) Observations ultrastructurales sur le stade plasmodial de *Metchnikovella wohlfarthi* Hildebrand et Vivier 1971, Microsporidie hyperparasite de la Grégarine *Lecudina tuzetae*. *Protistologica* **10**, 5–15.
- Hildebrand H and Vivier E (1971) Observations ultrastructurales sur le sporoblaste de *Metchnikovella wohlfarthi* n. sp. (Microsporidies), parasite de la grégarine *Lecudina tuzetae*. *Protistologica* **7**, 131–139.
- Ishida S, Nozaki D, Grossart HP and Kagami M (2015) Novel basal, fungal lineages from freshwater phytoplankton and lake samples. *Environmental Microbiology Reports* **7**, 435–441.
- Larsson JR (1994) *Trichoctosporea pygopellita* gen. et sp. nov. (Microspora, Thelohaniidae), a microsporidian parasite of the mosquito *Aedes vexans* (Diptera, Culicidae). *Archiv für Protistenkunde* **144**, 147–161.
- Larsson JR (2000) The hyperparasitic microsporidium *Amphiacantha longa* Caullery et Mesnil, 1914 (Microspora: Metchnikovellidae) – description of the cytology, redescription of the species, emended diagnosis of the genus *Amphiacantha* and establishment of the new family Amphiacanthidae. *Folia Parasitologica* **47**, 241–256.
- Larsson JIR (2014) The primitive microsporidia. In Weiss LM and Becnel JJ (eds), *Microsporidia: Pathogens of Opportunity*. Hoboken, New Jersey: John Wiley and Sons, Inc, pp. 605–634. doi: 10.1002/9781118395264.ch24
- Larsson JR and Koie M (2006) The ultrastructure and reproduction of *Amphiambls capitellides* (Microspora, Metchnikovellidae), a parasite of the gregarine *Ancora sagittata* (Apicomplexa, Lecudinidae), with redescription of the species and comments on the taxonomy. *European Journal of Protistology* **42**, 233–248.
- Larsson JIR and Voronin VN (2000) Light and electron microscopic study of *Agglomerata volgensae* n. sp. (Microspora: Dubosquidae), a new microsporidian parasite of *Daphnia magna* (Crustacea: Daphniidae). *European Journal of Protistology* **36**, 89–99.
- Lazarus KL and James TY (2015) Surveying the biodiversity of the Cryptomycota using a targeted PCR approach. *Fungal Ecology* **14**, 62–70.
- Mikhailov KV, Simdyanov TG and Aleoshin VV (2017) Genomic survey of a hyperparasitic microsporidian *Amphiambls* sp. (Metchnikovellidae). *Genome Biology and Evolution* **9**, 454–467.
- Mikhailov KV, Nasonova ES, Shishkin YA, Paskerova GG, Simdyanov TG, Yudina VA, Smirnov AV, Janoušková J and Aleoshin VV (2021) Ribosomal RNA of the metchnikovellids in gregarine transcriptomes and rDNA of the microsporidia *sensu lato* in environmental metagenomes. *Zhurnal Obshchei Biologii* **82**. doi: 10.31857/S0044459621030040 (in Russian with English summary).
- Miller MA, Pfeiffer W and Schwartz T (2010) Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: 2010 Gateway Computing Environments Workshop (GCE). Presented at the 2010 Gateway Computing Environments Workshop (GCE), IEEE, New Orleans, LA, USA, pp. 1–8. doi: 10.1109/GCE.2010.5676129.
- Nagahama T, Takahashi E, Nagano Y, Abdel-Wahab MA and Miyazaki M (2011) Molecular evidence that deep-branching fungi are major fungal components in deep-sea methane cold-seep sediments. *Environmental Microbiology* **13**, 2359–2370.
- Nasonova ES, Bondarenko NI, Paskerova GG, Kováčiková M, Frolova EV and Smirnov AV (2021) Evolutionary relationships of *Metchnikovella dogieli* Paskerova et al., 2016 (Microsporidia: Metchnikovellidae) revealed by multigene phylogenetic analysis. *Parasitology Research* **120**, 525–534.
- Ormières R, Loubès C and Maurand J (1981) *Amphiambls bhatiellae* n. sp., Microsporidie parasite de *Bhatiella marphysae* Setna, 1931, Eugrégarine d'Annelide Polychète. *Protistologica* **17**, 273–280.
- Richards TA, Leonard G, Mahé F, Del Campo J, Romac S, Jones MD, Maguire F, Dunthorn M, De Vargas C, Massana R and Chambouvet A (2015) Molecular diversity and distribution of marine fungi across 130 European environmental samples. *Proceedings of the Royal Society B: Biological Sciences* **282**, 20152243.
- Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA and Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* **61**, 539–542.
- Rotari YM (1988) *Microsporidia-hyperparasites of the White Sea polychaetes* (MS Dissertation), Biological Science, Dept. Invertebrate Zoology, Leningrad State University, Leningrad.
- Rotari Y and Paskerova G (2007) New data on hyperparasitic microsporidians from polychaetes of the White Sea. *Protistology* **5**, 67–68.
- Rotari YM, Paskerova GG and Sokolova YY (2015) Diversity of metchnikovellids (Metchnikovellidae, Rudimicrosporea), hyperparasites of bristle worms (Annelida, Polychaeta) from the White Sea. *Protistology* **9**, 50–59.
- Sokolova YY, Paskerova GG, Rotari YM, Nasonova ES and Smirnov AV (2013) Fine structure of *Metchnikovella incurvata* Caullery and Mesnil, 1914 (Microsporidia), a hyperparasite of gregarines *Polyrhabdina* sp. from the polychaete *Pygospio elegans*. *Parasitology* **140**, 855–867.
- Sokolova Y, Paskerova G, Rotari Y, Nasonova E and Smirnov A (2014) Description of *Metchnikovella spiralis* sp. n. (Microsporidia: Metchnikovellidae), with notes on the ultrastructure of metchnikovellids. *Parasitology* **141**, 1108–1122.
- Sprague V (1977) Classification and phylogeny of microsporidia. In Bulla LA and Cheng TC (eds), *Comparative Pathobiology*, vol. 2. New York, USA: Plenum Press, pp. 1–30.
- Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics (Oxford, England)* **30**, 1312–1313.
- Stentiford GD, Ramilo A, Abollo E, Kerr R, Bateman KS, Feist SW, Bass D and Villalba A (2017) *Hyperspora aquatica* n.g.n., n.sp. (Microsporidia), hyperparasitic in *Marteilia cochillia* (Paramyxida), is closely related to crustacean-infecting microsporidian taxa. *Parasitology* **144**, 186–199.
- Vávra J and Larsson JIR (2014) Structure of microsporidia. In Weiss LM and Becnel JJ (eds), *Microsporidia: Pathogens of Opportunity*. Hoboken, New Jersey: John Wiley and Sons, Inc, pp. 1–70. doi: 10.1002/9781118395264.ch1.
- Vivier E (1975) The microsporidia of the protozoa. *Protistologica* **11**, 345–361.
- Vivier E and Schrével J (1973) Étude en microscopie photonique et électronique de différents stades du cycle de *Metchnikovella hovassei* et observations sur la position systématique des Metchnikovellidae. *Protistologica* **9**, 95–118.
- Vossbrinck CR, Debrunner-Vossbrinck BA and Weiss LM (2014) Phylogeny of the microsporidia. *Microsporidia: Pathogens of Opportunity*. Hoboken, New Jersey: John Wiley and Sons, Inc, pp. 203–220. doi: 10.1002/9781118395264.ch6.
- Weiser J (1977) Contribution to the classification of microsporidia. *Vestník Československe Spolecnosti Zoologicke* **41**, 308–321.
- Weiss LM and Vossbrinck CR (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. In Wittner M and Weiss L (eds), *The Microsporidia and Microsporidiosis*. American Society of Microbiologists. Washington, DC: ASM Press, pp. 129–171. doi: 10.1128/9781555818227.ch4.
- Williams BAP, Hamilton KM, Jones MD and Bass D (2018) Group-specific environmental sequencing reveals high levels of ecological heterogeneity across the microsporidian radiation: ecological heterogeneity in the microsporidia. *Environmental Microbiology* **10**, 328–336.