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ORIGINAL ARTICLE

Metchnikovella dobrovolskiji sp. nov. (Microsporidia: Metchnikovellida), a parasite of archigregarines *Selenidium pygospionis* from the polychaete *Pygospio elegans*

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Summary

Spore sacs and free spores of a metchnikovellid were found in several specimens of the archigregarine *Selenidium pygospionis* isolated from polychaetes *Pygospio elegans*, collected from the littoral area of the Kandalaksha Gulf of the White Sea. The morphology of spore sacs suggests that this species belongs to the genus *Metchnikovella* (*sensu* Caullery and Mesnil, 1914). The length of the spore sacs varied from 5.6 to 9.2 μm, the width of the spore sacs was 3.3–5 μm, each of them contained up to 12 oval spores. Both spore sacs and free spores were enclosed in vacuoles. The combination of morphological features and host range distinguishes the studied isolate from any known species. Results of the phylogenetic analysis based on the SSU rRNA gene showed that the sequence of this species had a significant difference from all available sequences of metchnikovellids and usually grouped close to the species *Metchnikovella incurvata* or formed a clade with it. Here we describe the studied isolate as a new species, *Metchnikovella dobrovolskiji* sp. nov. (Microsporidia: Metchnikovellida).

Key words: Microsporidia, Metchnikovellida, gregarines, Apicomplexa, polychaetes, hyperparasitism, White Sea

Introduction

Microsporidia (phylum Microsporidia Balbiani, 1882) are unicellular spore-forming eukaryotic parasites inhabiting a variety of multicellular and some unicellular hosts (Weiss and Becnel, 2014). The parasites belong to the holomycotan branch of Opisthokonta (Karpov et al., 2014). The synapomorphic feature of microsporidia is the highly elaborated invasion apparatus. It is a complex of organelles intended for the extrusion of the infectious sporoplasm from the spore into the host cell. In typical microsporidia, it consists of a polar sac, an anchoring disk, a polaroplast, a coiled polar filament, and a posterior vacuole (Vávra and Larsson, 2014).

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Metchnikovellids are hyperparasitic microsporidia. They parasitize in the gregarines inhabiting the intestines of marine invertebrates, mostly polychaetes. Recent phylogenetic and phylogenomic analyses robustly placed metchnikovellids as a basal branch to the clade, embracing all typical microsporidia (Mikhailov et al., 2017; Galindo et al., 2018; Nassonova et al., 2021). The invasion apparatus of metchnikovellids lacks an anchoring disk, a polaroplast, and a coiled polar filament. Instead, they have got a structure called "manubrium", which is believed to be a primitive form of the polar filament of typical microsporidia (Vivier, 1975). The manubrium is covered with a membrane, which extends into lamellar folds associated with a tubulovesicular network (Vavra and Larsson, 2014; Sokolova et al., 2013). There are two types of sporogony in the life cycle of metchnikovellids. Free spores are formed directly in the cytoplasm of the host (sometimes within the vacuoles), while sac-bound sporogony results in the formation of thick-walled spore sacs, enclosing the spores. The structures called 'spore sacs' by Larsson (2000, 2014) were recognized as 'cysts' by Caullery and Mesnil (1897, 1914, 1919) and Vivier (1975).

The general morphology of the spore sac is a key feature in classification of metchnikovellids developed over 100 years ago. To date, this group includes about 30 species, and it is evident that the diversity of these hyperparasites remains poorly known. The configuration of the metchnikovellid clade in the phylogenetic tree is weakly resolved, and the taxon sampling in this group is still limited. To achieve further progress in this field, modern morphological and, especially, molecular studies of metchnikovellids are necessary.

The polychaete *Pygospio elegans* is a common species at the littoral of northern seas. The worm populations observed at the littoral zone of the Kandalaksha Gulf of the White Sea host two gregarine species, the eugregarine *Polyrhabdina pygospionis* (Paskerova et al., 2021) and the archigregarine *Selenidium pygospionis* (Paskerova et al., 2018). Each gregarine species can harbor two different metchnikovellids. *P. pygospionis* is the host for *Metchnikovella incurvata* (Caullery and Mesnil, 1914, 1919; Sokolova et al., 2013) and *M. spiralis* (Sokolova et al., 2014; Frolova et al., 2021). *S. pygospionis* could serve as a host for *M. dogieli* (Paskerova et al., 2016) and the new metchnikovellid species, characterized in the present paper. Based on the results of morphological studies and SSU rRNA sequence, here we describe this microsporidium as a new species *Metchnikovella dobrovolskiji* sp. nov.

Material and methods

Polychaetes *Pygospio elegans* Claparède, 1863 (Annelida: Spionidae) were collected from the sand-silt littoral zone in Kruglaya Bay, Chupa Inlet, Kandalaksha Gulf of the White Sea (66°20'17.2"N; 33°38'09.1"E) in June 2019. The worms were gathered from a single sampling site and further transported to the Department of Invertebrate Zoology, St. Petersburg University. Polychaetes were maintained in small containers at +10 °C with seawater; the water was changed once in two-three days. For examination, an individual polychaete was pressed between a coverslip and an object slide to release the content of the gut. The obtained squashed preparations were examined under a Leica M205C dissection microscope equipped with Rottermann contrast. Specimens showing the presence of presumably infected gregarines were examined using a Leica DM 2500 microscope equipped with differential interference contrast (DIC) and photographed with a Nikon DS-Fi3 digital camera operated with Nikon AR software. In case the presence of metchnikovellids in gregarines was proved with light microscopy, a large amount of Millipore-filtered seawater was added under the coverslip, which resulted in detaching the cells from the object slide. The gregarines containing free spores and spore sacs were collected from the slide individually 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 controlled for the presence of a gregarine using a Leica M205C dissection microscope.

DNA extraction from infected gregarines was performed using Arcturus® PicoPure® DNA Extraction Kit (Thermo Fischer Scientific, Waltham, MA, USA). Further, 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 by PCR using 1:10 diluted product of MDA reaction as a template with microsporidia-specific primers: 18F, 530R (Weiss and Vossbrinck, 1999) and 1353TnR (Nassonova et al., 2021). 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 1246 bp.

For the phylogenetic analysis, we built an alignment containing all available sequences of metchnikovellids and a selection of "core microsporidia". A set of "short-branch microsporidia" (*sensu* Bass et al., 2018) was used as an outgroup. Sequences were aligned using MAFFT v. 7.490 (Katoh and Standley, 2013) with two different preset modes – "favor accuracy" mode and "consider secondary structure" mode, as implemented in CIPRES portal (Miller et al., 2010). The mask was created for each alignment in two ways: by G-blocks algorithm (as implemented in SeaView v. 4.6.1 – Gouy et al., 2010) and manually, in order to include the maximal possible number of nucleotide positions. In addition, we prepared a so-called hand-made alignment with the same taxon set, which was made using MUSCLE algorithm (Edgar, 2004), as implemented in SeaView v. 4.6.1, and further polished manually. In this analysis, we used the 'extended mask' based on G-blocks selection of sites and further manually expanded to include the maximal possible number of nucleotide positions. The parameters of the masks used are listed in the Table 1.

The maximum likelihood (ML) phylogenetic analysis was performed using RAxML-HPC2 v. 8.2.12 (Stamatakis, 2014) at CIPRES portal. GTR + γ model of evolution and correction for intersite rate variation with 25 substitution rate categories were applied; the 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 5 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 IQ-Tree v. 2.1.2 was launched at CIPRES portal. All parameters for the run were estimated by the program. Best-fit model was found to be TIM3+F+I+G4, chosen according to Bayesian information criteria. It was the same for all alignments. The tree was tested using non-parametric bootstrapping (1000 pseudoreplicates).

The SSU rRNA gene sequence obtained in this study was deposited with the GenBank under the accession number OP225322.

Table 1. The size of mask used in the phylogenetic analyses with different variants of alignment.

The variant of alignment	*G-blocks mask*, bp	*Extended mask*, bp
MAFFT, "accuracy" mode	1121	1323
MAFFT, "secondary structure" mode	1106	1309
Hand-made	–	1344

Notes: MAFFT, "accuracy" mode – the sequences were aligned with MAFFT v. 7.490 (Katoh and Standley, 2013) using "favor accuracy" mode. *MAFFT, "secondary structure" mode* – the sequences were aligned with MAFFT using "consider secondary structure" mode, as implemented in CIPRES portal (Miller et al., 2010). *Hand-made* – the alignment made using MUSCLE algorithm (Edgar, 2004), as implemented in SeaView v. 4.6.1 (Gouy et al., 2010) and further polished manually. *G-blocks mask* – the mask created by G-blocks algorithm as implemented in SeaView. *Extended mask* – the mask based on G-blocks selection of sites and further manually extended to include the maximal possible number of nucleotide positions.

Results

Among 58 examined polychaetes, 43 specimens (74%) were infected with the archigregarines *Selenidium pygospionis*. Of them, 35 polychaetes were co-infected with two gregarine species (*S. pygospionis* and *Polyrhabdina pygospionis*). Gregarines were either attached to the intestine epithelium or resided freely in the gut lumen. There were from one to about 100 parasites per host. Four polychaetes (6.9%) harbored archigregarines *S. pygospionis* with visible signs of metchnikovellid infection. While non-infected gregarines had homogenous cytoplasm, the infected ones possessed inclusions well-visible even at low magnification (Fig. 1, A). The movement of infec-ted gregarines did not differ in any way from the movement of healthy individuals (Suppl. video). When infected gregarines were slightly pressed with the coverslip, numerous spore sacs and free spores of the metchnikovellid became visible inside the cells (Fig.1, B–E).

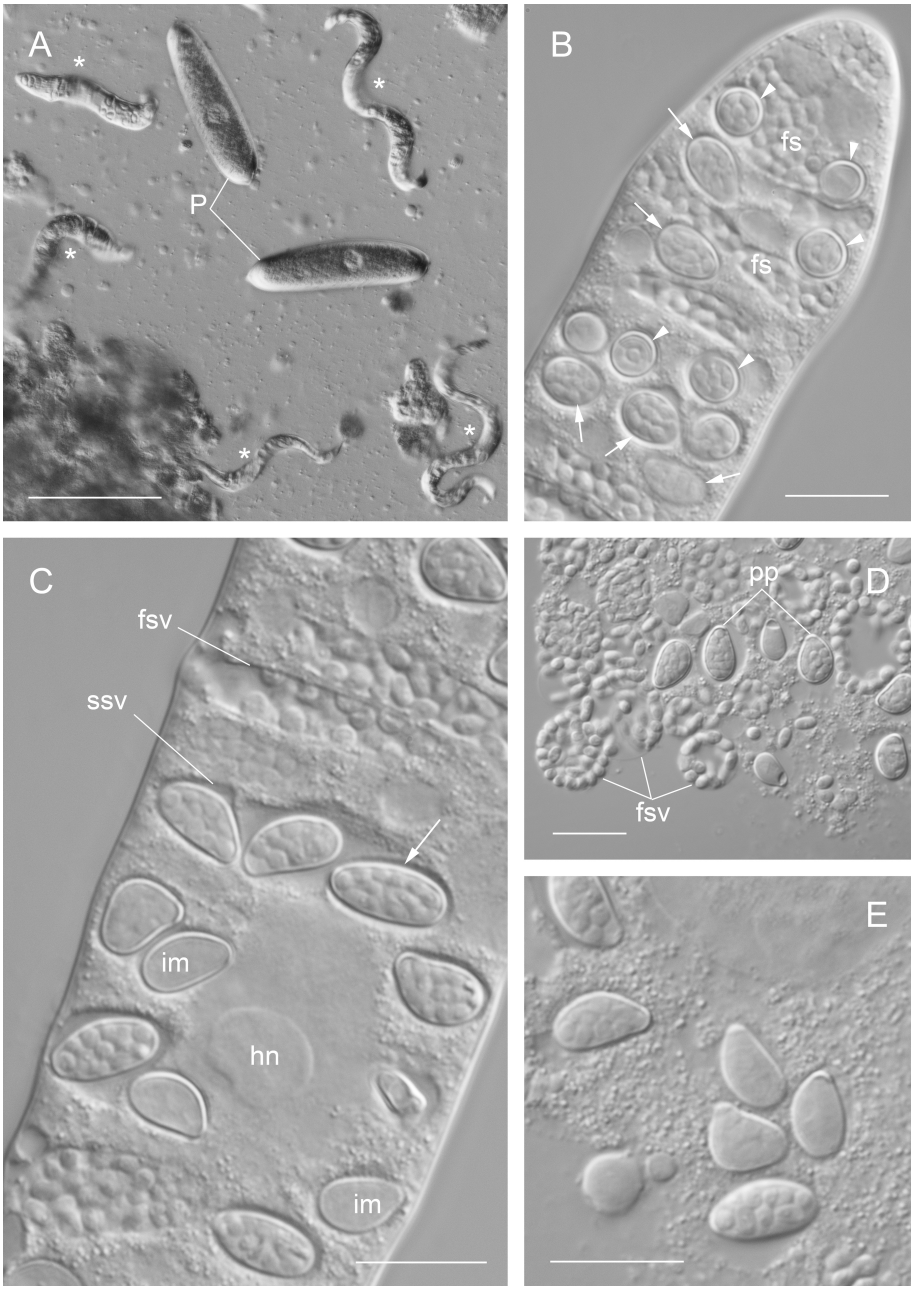

Fig. 1. Microsporidia *Metchnikovella dobrovolskiji*, a parasite of the archigregarine *Selenidium pygospionis* isolated from the polychaete *Pygospio elegans* (DIC). A – Infected *S. pygospionis* and uninfected eugregarines *Polyrhabdina pygospionis* from the same worm host; B – infected *S. pygospionis*, posterior end (the cytoplasm is filled with free spores and oval spore sacs, oriented parallel and transverse to the longitudinal axis of the host cell); C – the same gregarine, nucleus-containing region of the cell (free spores are encased in the vacuoles, up to several dozens of spores in each. Immature and mature spore sacs are individually enclosed in the vacuoles); D–E – spore sacs and free spores bound in the vacuoles are released from the crashed host cell (note a polar plug at one pole of each spore sac). *Abbreviations*: fs – free spores, fsv – vacuole with enclosed free spores, hn – host nucleus, im – immature spore sacs, P – *P. pygospionis*, pp – polar plug, ssv – vacuole with a spore sac. *Arrows* point at the spore sacs; *arrowheads* mark the spore sacs located across the longitudinal axis of the host cell; *asterisks* mark the infected archigregarines *S. pygospionis*. Scale bars: A – 50 μm, B–E – 10 μm.

Table 2. Groupings of metchnikovellids observed in SSU rRNA trees reconstructed using three different variants of alignment and two types of mask.

Alignment, mask	Clade / group of sequences				
	***Amphiacantha* clade: *Amphiacantha sp.* + *Metchnikovella spiralis* + KX214678**	***Amphiamblys* clade: *Amphiamblys* sp. + *M. dogieli***	***Metchnikovella incurvata* + *M. dobrovolskiji***	***Amphiacantha* clade + (*M. incurvata* + *M. dobrovolskiji*)**	***Amphiamblys* clade + (*M. incurvata* + *M. dobrovolskiji*)**
MAFFT "accuracy" mode, G-blocks mask	100/100/1.0	73/83/0.98	–/–/–	–/–/–	–/–/–
MAFFT "accuracy" mode, extended mask	100/100/1.0	76/87/1.0	–/–/–	–/–/–	–/–/–
MAFFT "secondary structure" mode, G-blocks mask	100/100/1.0	71/80/0.99	42/47/0.36	28/41/0.47	–/–/–
MAFFT "secondary structure" mode, extended mask	100/100/1.0	77/83/1.0	46/51/0.47	45/50/0.78	–/–/–
Hand-made alignment, extended mask	100/100/1.0	65/73/0.98	52/64/0.69	30/42/–	–/–/0.46

Notes: The different variants of alignment and the types of applied masks are described in the caption to Table 1. The support values are indicated in the same manner as in the phylogenetic tree in Figure 2. Left to right: the support values from RAxML/IQ-Tree/MrBayes (BS/BS/PP). The tree based on the alignment made in MAFFT "consider secondary structure" mode, with extended mask was selected as a primary one in Figure 2, A, as showing maximal support for most clades.

Spore sacs were oval, sometimes irregularly oval (pear-shaped) or ovoid, with rounded ends and a thin polar plug ("plugging thickening") at one end. The spore sacs measured 3.3–5×5.6–9.2 μm in maximal dimension (n=79). Their maximal diameter when viewed from the top was about 4.5 μm. Spore sacs were enclosed individually in the vacuoles and were dispersed cha-otically in the cytoplasm of the gregarine. Some of them were located parallel to the longitudinal axis of the host cell, others – across this axis, the latter in the images looked rounded (Fig. 1, B). Some spore sacs were immature. They had almost the same shape and size as the mature ones, but did not contain clearly visible spores with developed spore walls (Fig. 1, C). The host cells contained a variable number of spore sacs (11–41, n=5), and each spore sac had up to 12 spores. Sac-bound spores were oval and measured 1.3–2.4×0.9–1.6 μm (n=39). In addition to spore sacs, a large part of the infected gregarine cell was occupied by free spores. When the gregarine was broken under the pressure of the coverslip, we observed the release of free spores and spore sacs. Both were bounded with vacuoles of unknown origin, traditionally termed "parasitophorous vacu-oles" (Fig.1, D). Each vacuole contained one spore sac or up to several dozens of free spores. The free spores were oval and measured 1.2–3.1×1.1–1.7 μm (n=44).

In the reconstructed SSU rRNA trees (Fig. 2), metchnikovellids together with the sequence of morphologically unidentified parasite from *Siedleckia* cf. *nematoides* (Mikhailov et al., 2022) formed a fully supported group branching as a sister to 'core' microsporidia. The latter sequence was found to be the most basal among the Metchnikovellida clade. Among the rest of metchnikovellids, the configuration of the tree was not stable (Table 2). Two clades were always recovered. One was fully supported with all methods and comprised two sequences of *Amphiacantha* sp., environmental clone p1_44 (GenBank KX214678) and the sequence of *Metchnikovella spiralis*, as it was previously shown by Frolova et al. (2021). The second clade had lower support and united two sequences of *Amphiamblys* sp. and *Metchnikovella dogieli*. The sequence of the studied isolate either formed a weakly supported clade with *M. incurvata* (Fig. 2, A) or formed an independent lineage next to *M. incurvata* (Fig. 2, B). When it formed a clade with *M. incurvata*, this clade was a sister group either to *Amphiacantha* plus *M. spiralis* clade or (in one case only) to *Amphiamblys* plus *M. dogieli* clade (Fig. 2, C). In no case, either of these positions in the tree was properly supported (Table 2).

Discussion

The order Metchnikovellida Vivier, 1975 comprises two families, the monotypic family Amphiacanthidae Larsson, 2000 with the genus *Amphiacantha* Caullery et Mesnil, 1914 and the family

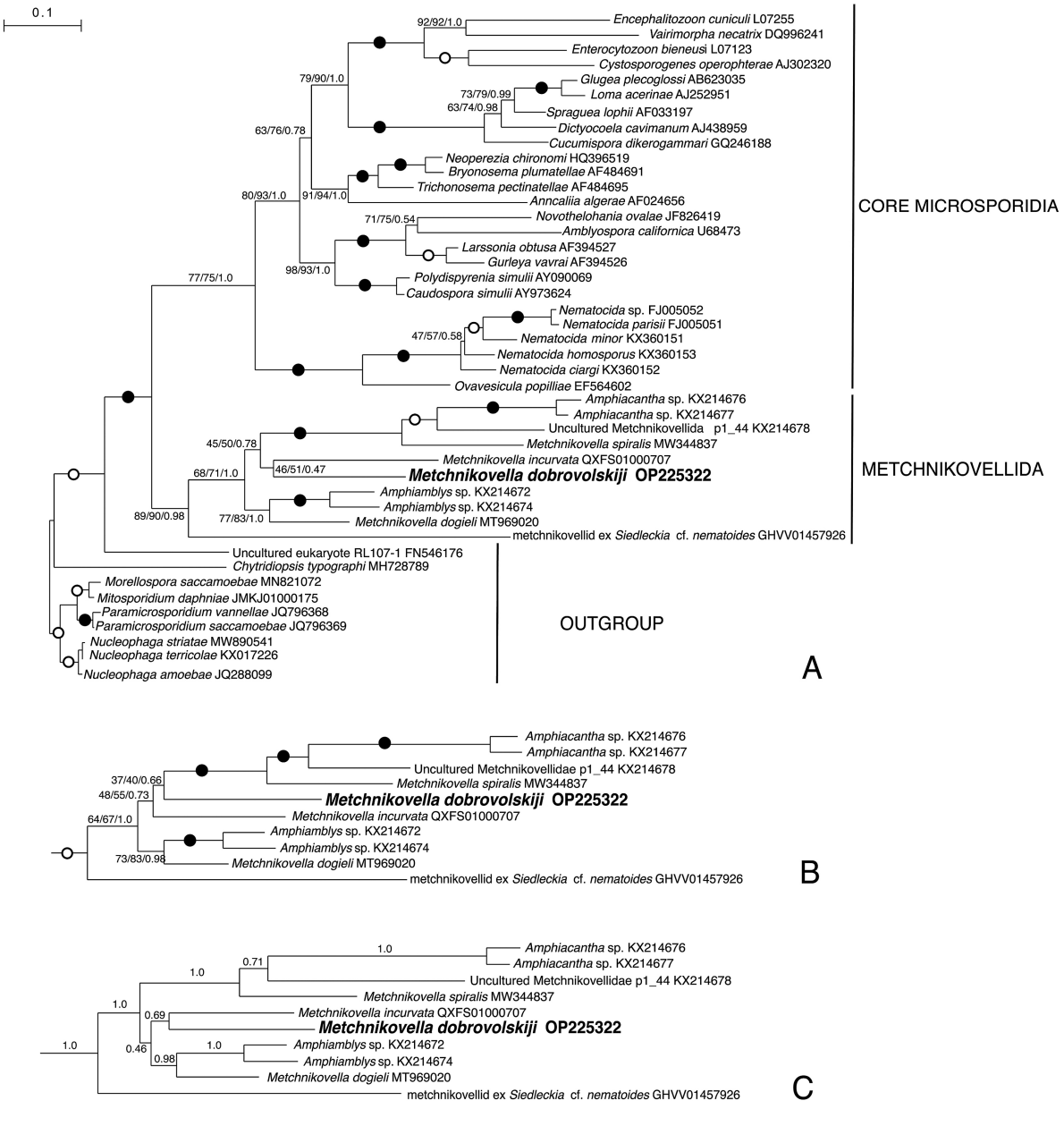

Fig. 2. SSU rRNA phylogeny of Microsporidia and related lineages including the sequence of *Metchnikovella dobrovolskiji* retrieved in this study (in bold). Black blobs indicate nodes, fully supported by all three methods used (100/100/1.0; RAxML/IQ-tree/MrBayes, respectively). White circles indicate highly supported nodes (all kinds of support over 95% for ML methods and 0.95 for Bayesian method). A – The tree based on the alignment made in MAFFT "consider secondary structure" mode, with extended mask, selected as a primary one as showing maximal support for most clades; 1309 nucleotide positions. Support values are indicated for RAxML (GTR+γ+I) / IQ-tree (TIM3+F+I+G4) / MrBayes (GTR+γ+covarion); B – a fragment of the tree showing an alternative configuration for Metchnikovellida clade. The tree is based on the alignment made by MAFFT in "favor accuracy" mode and G-blocks mask; 1121 nucleotide positions; support values are indicated as above; C – a fragment of the tree built by MrBayes (GTR+γ+covarion) on hand-made alignment with the extended mask showing alterative grouping of clades within Metchnilkovellida; 1344 nucleotide positions.

Metchnikovellidae Caullery et Mesnil, 1914 with the genera *Metchnikovella* Caullery et Mesnil, 1897 and *Amphiamblys* Caullery et Mesnil, 1914. The major difference between these two families is the morphology of their spore sacs. According to this 'classical' definition, representatives of the family Amphiacanthidae have spindle-like spore sacs with thread-like ends. They do not have polar plugs, and demonstrate the presence of paired nuclei at least at one stage in the life cycle (Larsson, 2014). The family Metchnikovellidae comprises parasites possessing spore sacs with rounded ends, and may have one or two polar plugs or not to have them at all (Larsson, 2014). Within this family, members of the genus *Amphiamblys* have long, rod-like spore sacs without polar plugs at both ends. The length of their spore sacs exceeds ten times the width. The species of the genus *Metchnikovella* have oval spore sacs, much shorter in length, with rounded thick ends, terminated with polar plugs at one or both ends (Sokolova et al., 2014).

Up to now, 19 species of *Metchnikovella* have been described (see Table 1 in Paskerova et al., 2016). In size and shape of the spore sacs, *M. dobrovolskiji* is significantly different from *M. dogieli*, described from the same gregarine host. The spore sacs of *M. dobrovolskiji* are much smaller in length and width (3.3–5×5.6–9.2 μm against 9.5–34×4.8–9.2 μm in *M. dogieli*). The shape also differs, as *M. dogieli* spore sacs are longer and sometimes bent. Moreover, spore sacs of *M. dobrovolskiji* are enclosed in vacuoles as well as the free spores of this species. The new parasite is most similar in morphology to *M. brasili*, *M. hovassei* and *M. mesnili*. All these species have oval spore sacs with one polar plug. Their spore sacs are also measured in length up to 10 μm. In *M. hovassei*, both free spores and spore sacs are enclosed in vacuoles (Vivier and Schrével, 1973). This feature brings it closest to our isolate. Nevertheless, all these metchnikovellids parasitize different hosts: *M. brasili* lives in eugregarines *Polyrhabdina brasili* from *Spio martinensis* (Caullery and Mesnil, 1919), *M. hovassei* is a parasite of *Lecudina pellucida* from *Perinereis cultrifera* (Vivier and Schrével, 1973), and *M. mesnili* parasitizes archigregarines *Selenidium* sp. that live in polychaetes *Travisia forbesii* (Awerinzew, 1908). The species described in the present paper lives in archigregarines *Selenidium pygospionis* from the polychaete *Pygospio elegans*. This is a new host combination, not reported for any of the species listed above. *M. dobrovolskiji* produces oval spores, while other mentioned species form roundish ones. Therefore, the new isolate of metchnikovellids shows a unique combination of individual characters that are used for the species identification within the genus *Metchnikovella* Caullery and Mesnil, 1914: the host range, the super-host range, the size and shape of the spore sacs and the spores. Hence, we cannot consider it as co-specific with any of the previously described species.

Our phylogenetic analyses supported the heterogeneity of the genus *Metchnikovella* demonstrated in our previous studies (Nassonova et al., 2021; Frolova et al., 2021). Despite the increment in the number of obtained sequences, the phylogenetic tree of metchnikovellids based on SSU rRNA gene sequences remains not stable. Almost all species form very long branches in the tree, while the distances at the basal part of the tree are much shorter, so the tree has low stemminess, which complicates recovery of correct topology (Smith, 1994). Its configuration depends a lot on the alignment quality, mask used and the algorithm of the analysis.

Larsson (2014) proposed to transfer *Metchnikovella* species possessing the oval spore sacs with a single polar plug to the genus *Caulleryetta* Dogiel, 1922. However, there is no modern data on the type species of these two genera. According to our data, mechnikovellids with *Caulleryetta*-like morphology do not group together in the phylogenetic trees. Therefore, despite the obvious need to revise the family Metchnikovellidae, we believe that this should be done only after a robust and representative phylogenetic tree is constructed. First of all, it is necessary to reisolate and study the type species of both genera – *Caulleryetta* (*Metchnikovella*) *mesnili* Dogiel, 1922 and *Metchnikovella spionis* Caullery et Mesnil, 1897.

Taxonomic summary

Phylum Microsporidia Balbiani, 1982
Class Rudimicrosporea Sprague, 1977
Order Metchnikovellida Vivier, 1975
Family Metchnikovellidae Caullery and Mesnil, 1914
Genus *Metchnikovella* Caullery and Mesnil, 1897, *sensu* Caullery and Mesnil (1914)
Metchnikovella dobrovolskiji sp. nov.

Diagnosis. Free spores are oval (1.2–3.1×0.9–1.6 μm), encompassed in the vacuoles. Spore sacs are oval (5.6–9.2×3.3–5 μm), sometimes ovoid, with rounded ends and a polar plug at one end, enclosed in individual vacuoles. The number of sac-bound spores varies from 10 to 12. Sac-bound

spores are oval (0.9–1.6×1.3–2.4 μm).

Differential diagnosis. The species differs from the congeners and other metchnikovellids by the combination of characters: the size and shape of the spore sacs, the number of spores per sac, occurrence of spore sacs and free spores in the vacuoles, the super-host and host range.

Type locality. Kandalaksha Gulf of the White Sea, 66°20'17.2"N; 33°38'09.1"E. Littoral zone.

Type habitat. Marine.

Type host and super-host. Archigregarine *Selenidium pygospionis* (Apicomplexa: Selenidiidae) from the polychaete *Pygospio elegans* (Annelida: Spionidae).

Location in host. Gregarine cytoplasm.

Type material. Images of the live gregarines are stored in the image collection of the Department of Invertebrate Zoology, St. Petersburg University under the following numbers: 72_90-15000 – 72_90-15155, video materials are stored under the names Sample013 – Sample019. Frozen purified genomic DNA of the infected gregarines as well as the individual infected gregarine cells fixed in 96% ethanol are stored at the same department.

Etymology. This species was named in honor of the outstanding Russian zoologist, parasitologist, pedagogue and teacher, Andrej A. Dobrovolskij (1939–2019).

Gene sequences: SSU rDNA gene sequence of *M. dobrovolskiji* has been deposited in the GenBank under the accession number OP225322.

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Supplementary material

Video S1. Movement of the infected *Selenidum pygospionis*. Infected gregarines demonstrate typical movement characteristic of this species. In the cytoplasm of actively moving gregarines, the metchnikovellid spore sacs are clearly visible.