Parasitology Research
https://doi.org/10.1007/s00436-020-06976-x

GENETICS, EVOLUTION, AND PHYLOGENY - ORIGINAL PAPER

Evolutionary relationships of *Metchnikovella dogieli* Paskerova et al., 2016 (Microsporidia: Metchnikovellidae) revealed by multigene phylogenetic analysis

Elena S. Nassonova[1,2] · Natalya I. Bondarenko[1,2] · Gita G. Paskerova[2] · Magdaléna Kováčiková[3] · Ekaterina V. Frolova[1,2] · Alexey V. Smirnov[2]

Received: 18 May 2020 / Accepted: 12 November 2020

Abstract

The species *Metchnikovella dogieli* (Paskerova et al. Protistology 10:148–157, 2016) belongs to one of the early diverging microsporidian groups, the metchnikovellids (Microsporidia: Metchnikovellidae). In relation to typical ('core') microsporidia, this group is considered primitive. The spores of metchnikovellids have no classical polar sac-anchoring disk complex, no coiled polar tube, no posterior vacuole, and no polaroplast. Instead, they possess a short thick manubrium that expands into a manubrial cistern. These organisms are hyperparasites; they infect gregarines that parasitise marine invertebrates. *M. dogieli* is a parasite of the archigregarine *Selenidium pygospionis* (Paskerova et al. Protist 169:826–852, 2018), which parasitises the polychaete *Pygospio elegans*. This species was discovered in samples collected in the silt littoral zone at the coast of the White Sea, North-West Russia, and was described based on light microscopy. No molecular data are available for this species, and the publicly accessible genomic data for metchnikovellids are limited to two species: *M. incurvata* Caullery & Mesnil, 1914 and *Amphiamblys* sp. WSBS2006. In the present study, we applied single-cell genomics methods with whole-genome amplification to perform next-generation sequencing of *M. dogieli* genomic DNA. We performed a phylogenetic analysis based on the SSU rRNA gene and reconstructed a multigene phylogeny using a concatenated alignment that included 46 conserved single-copy protein domains. The analyses recovered a fully supported clade of metchnikovellids as a basal group to the core microsporidia. Two members of the genus *Metchnikovella* did not form a clade in our tree. This may indicate that this genus is paraphyletic and requires revision.

Keywords Microsporidia · Metchnikovellids · Phylogeny · Phylogenomics

Introduction

Microsporidia (phylum Microsporidia Balbiani, 1882) are eukaryotic, unicellular spore-forming parasites of animals and some protists (Weiss and Becnel 2014). They belong to the holomycotan branch of opisthokonts. Microsporidia are known for their extreme genomic and metabolic simplification. They lack many typical eukaryotic organelles (Issi and Voronin 2007; Vávra and Lukeš 2013; Keeling et al. 2014; Weiss and Becnel 2014). The synapomorphic feature of microsporidia is the invasion apparatus, a highly elaborated complex of organelles essential for the extrusion of the infectious sporoplasm from the spore directly into the host cell. It consists of a polar sac-anchoring disk complex, a polaroplast, a coiled polar tube, and a posterior vacuole (Vávra and Larsson 2014). There are few groups of microsporidia in which the structure of the invasion apparatus deviates far from that described above. Among them is the family Metchnikovellidae Caullery and Mesnil 1914. The spores of its representatives do not possess a coiled polar tube, polaroplast or posterior vacuole; instead, they have a structure

Handling Editor: Julia Walochnik

✉ Elena S. Nassonova
nosema@mail.ru; e.nassonova@spbu.ru

[1] Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS, Tikhoretsky ave. 4, St. Petersburg, Russia 194064

[2] Department of Invertebrate Zoology, Faculty of Biology, St. Petersburg State University, Universitetskaya nab. 7/9, St. Petersburg, Russia 199034

[3] Department of Botany and Zoology, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

Published online: 08 January 2021

called a manubrium, which expands into a manubrial cistern (a lamellar fold in the terminology suggested by Larsson (2014)). These organelles, together with a polar cap, a probable homologue of the polar sac-anchoring disk complex, are believed to represent a primitive form of the extrusion apparatus of higher microsporidia (Vivier 1975). There are two types of sporogony in the life cycle of metchnikovellids: free and sac-bound (Larsson 2014). Sac-bound sporogony results in the formation of thick-walled spore-containing sacs ('cysts' sensu Caullery and Mesnil (1897, 1914, 1919) and Vivier (1975)). The general morphology of the spore sacs is a key feature in the classification system for metchnikovellids developed 100 years ago. All known metchnikovellids are hyperparasites of gregarines inhabiting the gut of polychaetes (Annelida, Polychaeta) and some other marine invertebrates (Vivier 1975; Schrével and Desportes 2013; Larsson 2014). Metchnikovellids are rarely detected and remain poorly studied; how they infect host cells remains unknown, as the extrusion has not been documented. Until recently, they remained among the few groups of organisms for which no molecular data are available. The first SSU rRNA and beta-tubulin phylogeny reconstructed for this group demonstrated relationships of metchnikovellids with microsporidia (Nassonova et al. 2016). Genomic studies of *Amphiamblys* sp. WSBS2006 (Mikhailov et al. 2017) and *Metchnikovella incurvata* Caullery & Mesnil, 1914 (Galindo et al. 2018) robustly showed that metchnikovellids diverged before typical ('core') members of Microsporidia; therefore, their specific ultrastructure probably retains ancestral characters of the latter group. In the present study, we obtained SSU rRNA gene sequence and genomic data on the species *Metchnikovella dogieli* Paskerova et al., 2016 and performed molecular phylogenetic and phylogenomic analyses. This species was recently described as inhabiting *Selenidium pygospionis* Paskerova et al., 2018, a gregarine that parasitises the gut of the polychaete *Pygospio elegans* Claparède, 1863 (Paskerova et al. 2016, 2018). The results supported the monophyly and previously established position of the metchnikovellid clade at the base of the phylogenomic tree of core microsporidia but cast doubt on the monophyletic status of the genus *Metchnikovella*, the most abundant and diverse genus of Metchnikovellidae.

Material and methods

The material used for the present study originated from the same isolate that was used for the description of *M. dogieli* (Paskerova et al. 2016). Approximately one hundred individuals of the polychaete *P. elegans* (Annelida: Spionidae) were collected at one site (approximately 9 m^2) in the silt littoral zone in the vicinity of the White Sea Biological Station of M. V. Lomonosov Moscow State University (Velikaja Salma, Kandalaksha Bay, White Sea, 66° 33.200′ N, 33° 6.283′ E) in August 2016. The polychaetes were dissected at the field station using manually sharpened thin needles, hand-drawn Pasteur pipettes and an MBS-10 stereomicroscope (LOMO, Russia). Individuals of the gregarines *S. pygospionis* that were densely populated with *M. dogieli* free spores and spore sacs were fixed in molecular-grade absolute ethanol (Amresco, USA) and stored at − 20 °C.

To obtain high-quality light-microscopy images, several polychaetes were transported to the laboratory at the Department of Invertebrate Zoology of Saint Petersburg State University. Worms were dissected in a similar manner as before using a Leica M205C dissection microscope. Infected gregarines were placed on slides and investigated using a Leica DM 2500 microscope equipped with DIC optics and Plan-Apo objective lenses and photographed using a DFC 295 digital camera (Leica, Germany).

Prior to DNA extraction, ethanol from the fixed samples was removed using a tapering Pasteur pipette; the remnants of ethanol were desiccated using a ScanVac vacuum evaporator (LaboGene, Denmark) at 2000 rpm during 15 min at room temperature. Total DNA extraction and whole-genome amplification were performed as described in Galindo et al. (2018). A sample with 2 heavily infected gregarine cells containing the spore sacs and numerous free spores of the hyperparasite was used for DNA isolation with the Arcturus® PicoPure® DNA Extraction Kit (Thermo Fischer Scientific, USA).

The SSU rRNA gene of *M. dogieli* was amplified by PCR using microsporidia-specific primers: 18F (Zhu et al. 1993), 530R (Weiss and Vossbrinck 1999) and 1353TnR (a modification of the primer V1492R) 5′-GCAGCCTTGTTACG ACTT-3′. PCR programme parameters were as follows: 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 the Cleanup mini Purification Kit (Eurogene, Russia). The Sanger sequencing reactions were carried out using the Applied Biosystems™ BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer Scientific, USA) and analysed with an Applied Biosystems™ 3500xL Genetic Analyzer (Thermo Fischer Scientific, USA).

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 a representative set of sequences of described species of the core microsporidia from five identified clades (Vossbrinck et al. 2014), all available sequences of metchnikovellids, a representative set of sequences of early diverging microsporidia, rozellids and nucleariids (the latter was used as an outgroup). The maximum likelihood (ML) phylogenetic analysis was performed using RAxML v.8.2.12 (Stamatakis 2014) run 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 randomised 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) using GTR model with gamma correction for intersite rate variation (8 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 less than 0.01); the first 25% of generations were discarded for burn-in.

To obtain a sufficient amount of DNA for the multigene analysis, the whole-genome amplification was performed by multiple displacement amplification (MDA) using the Repli-g Single Cell Amplification Kit (Qiagen, Germany), according to the manufacturer's protocol. The MDA product was checked for the presence of target DNA using PCR with microsporidia-specific primers for amplification of SSU rDNA as described above.

Verified MDA product was used to prepare a library with the Ion Torrent® DNA Library Preparation Kit (New England BioLabs® Inc., USA) and sequenced on the IonTorrent PGM platform (Life Technologies—Thermo Fischer Scientific, USA). Library preparation, sequencing and primary treatment of the data were performed according to the manufacturer's instructions using the services provided by the Core Facility Centre 'Biobank' of the Research Park of St. Petersburg State University (https://researchpark.spbu.ru/en/biobank-eng). The resulting dataset contained 1.7 million reads with maximal length 530 bp. Quality control of raw sequence data was performed using the FastQC tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/); reads were trimmed using the Trimmomatic tool (http://www.usadellab.org/cms/?page=trimmomatic). De novo genome assembly was performed using the SPAdes assembler v.3.13.0 (Bankevich et al. 2012) with settings for IonTorrent single end reads with initial k-mer of 21 and a maximum k-mer size of 127. The quality of assemblies was evaluated using QUAST (Gurevich et al. 2013). The distribution of the sequencing depth at each position was calculated with SAMtools to assess the completeness of the assembled genome (Li et al. 2009). The size of the assembly was 5.09 Mb in 11,579 scaffolds with N50 of 13,185 bp and assembly coverage × 37.

A BLASTx search (Altschul et al. 1997) with maximum *e*-value of 1e-05 for the protein-coding genes was performed against every gene included in the 56-gene alignment of conserved single-copy protein domains (Torruella et al. 2012), kindly provided by Guifré Torruella (Université Paris-Sud, AgroParisTech, Université Paris-Saclay, Orsay, France); it was the same alignment that was used by Galindo et al. (2018). Gene sequences identified by BLAST were added to the individual gene alignments using SeaView v.4.6.1. For every single gene, we built an ML tree using PhyML v.3.0 software (Guindon et al. 2010). We applied the LG model; the programme estimated all other parameters automatically. The obtained trees were manually examined. We removed from the analysis several genes that are missing in microsporidia or provided evidently abnormal grouping of other taxa, differing significantly from generally accepted views on eukaryote phylogeny.

After this analysis, we selected 46 single-gene alignments, 40 of them contained sequences of *M. dogieli*, 36 contained sequences of *M. incurvata*, and 41 contained sequences of *Amphiamblys* sp. We concatenated single-gene alignments using SeaView v.4.6.1. For masking, G-blocks software with relaxed settings (Talavera and Castresana 2007) was used; the mask was further manually refined. The masked alignment used for the analysis was 15,814 positions in length; the total length of *M. dogieli* sequences was 10,210 positions.

Phylogenomic analyses were performed using RAxML v.8.2.12 and PhyloBayes v.1.4f (Lartillot and Philippe 2004), both run at the CIPRES portal. For the ML analysis, PROTGAMMA model with LG4M substitution matrix was applied. One hundred of the most parsimonious starting trees were built, and 100 non-parametric bootstrap pseudoreplicates were performed on best-scoring ML tree. The Bayesian analysis was performed on the same dataset with the site-heterogeneous CAT-GTR model and a gamma distribution of rates across sites approximated by four categories. Two independent chains were run; the maxdif value at the end of the calculation was 0.088. Also, the ML tree was calculated using PhyML with LG matrix and GTR model with 4 discrete gamma rate categories to assess SH-aLRT branch supports.

The dataset used in this study was deposited with the GeneBank under the accession numbers: MT969020 (SSU rDNA), MT951446, MW052334-MW052379 (protein-coding genes).

Results

The sample selected for molecular work contained 2 gregarines isolated from the same worm. The gregarines were densely populated with the spore sacs and numerous free spores of *M. dogieli* (Fig. 1a). The spore sacs had the characteristic morphology of *M. dogieli*: oval with rounded ends and a single plugging structure on one pole (Fig. 1b, c). One gregarine contained on average 12 sacs, with a maximum number of 24 sacs, plus an unknown number of free spores (Paskerova et al. 2016). Every sac contained on average 12 spores, so we can assume that the starting material for the whole-genome amplification comprised no fewer than 288 sac-bound spores plus an unknown number of free spores from the cytoplasm of the gregarines. This amount of material should be sufficient to yield a representative sample of starting DNA for whole-genome amplification, thereby minimising bias related to possible unrepresentative genome variants in a single spore.

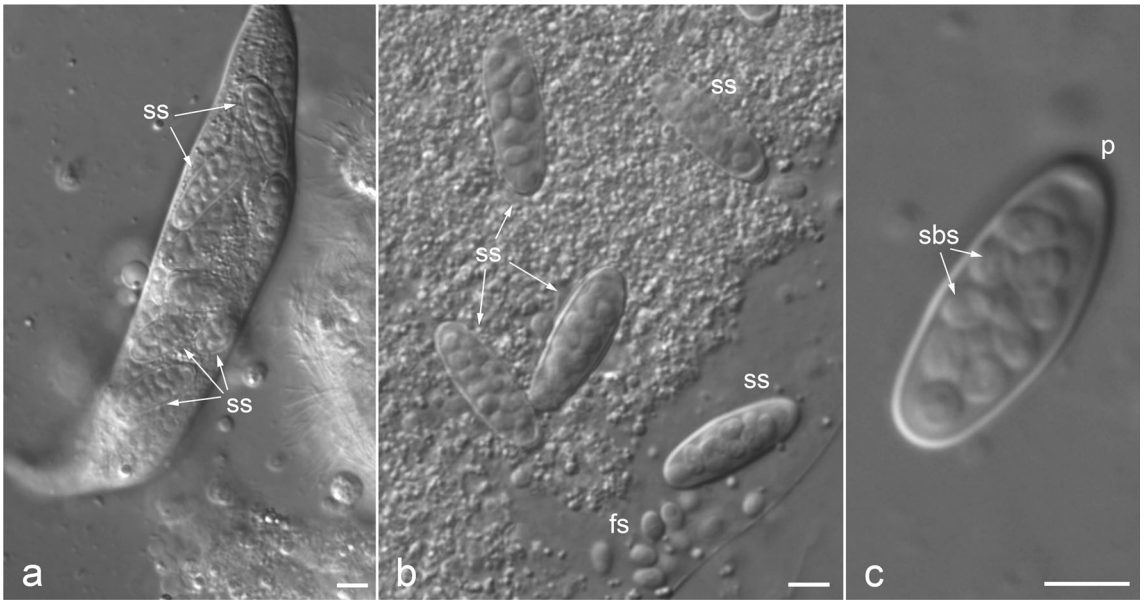

Fig. 1 Spore sacs and free spores of *Metchnikovella dogieli*, a parasite of the archigregarine *Selenidium pygospionis* from the polychaete *Pygospio elegans*. **a** Spore sacs and free spores in the cytoplasm of an intact archigregarine. **b** Spore sacs and free spores released from the ruptured host cell. **c** An isolated spore sac. fs free spores, p polar plug, ss spore sac, sbs sac-bound spores. Scale bar: 5 μm

The metchnikovellid SSU rRNA gene sequences were found to be highly divergent. The sequence of *M. dogieli* was only 39% identical to that of *M. incurvata*, and shared 48–49% identity with the sequences of *Amphiamblys* spp. and 37–40% identity with the sequences of *Amphiacantha* spp. (Supplementary Table S1). In contrast to those of *M. incurvata*, the variable regions of the SSU rRNA gene sequence of *M. dogieli* did not contain long AT-rich simple tandem repeats.

In our SSU rRNA tree, clade 1 and clades 3–5 (sensu Vossbrinck et al. 2014, Fig. 6.3a) of the core microsporidia were robustly recovered, whereas clade 2 was split into two groups (Fig. 2). The group *Paranosema*/*Antonospora* was recovered as a sister to clade 3 with rather high support, whereas the group *Nematocida*/*Ovavesicula* formed a highly supported basal lineage of the core microsporidia. This topology of the Microsporidia tree 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; Corsaro et al. 2018). The position of *Hamiltosporidium* spp. within clade 3 (as it was shown in Vossbrinck et al. 2014) was not recovered, which is congruent with the results of Mikhailov et al. (2017). In our SSU rRNA tree, a lineage of *Hamiltosporidium* spp. was recovered with moderate support as a sister to a well-supported assemblage uniting clade 4 and clade 5.

In the SSU rRNA phylogeny, the metchnikovellids constituted a highly supported clade at the base of the core microsporidia. The metchnikovellid sequences formed two sister clades. One was highly supported and comprised the sequences of *Amphiacantha* spp. and the only available metchnikovellid environmental sequence p1_44 (KX214678), which originated from a freshwater sample. The second clade was moderately supported and comprised available sequences of *Amphiamblys* spp. and *Metchnikovella* spp. This topology is congruent with the hypothesis of Larsson (2014), who proposed splitting the family Metchnikovellidae into two families: Amphiacanthidae, encompassing the representatives of the genus *Amphiacantha*, and Metchnikovellidae, encompassing *Amphiamblys* + *Metchnikovella*.

The sequences of species nominally belonging to the genus *Metchnikovella* did not form a monophyletic group. Instead, they branched hierarchically. *M. dogieli* was recovered as sister to the fully supported subclade consisting of two *Amphiamblys* sp. sequences, whereas *M. incurvata* was the earliest diverging lineage in the clade *Amphiamblys* + *Metchnikovella*. This configuration was robust across all our trees but had adequate support only in the Bayesian analysis; in the ML analysis, it had low support.

In our SSU rRNA tree, the closest group to the metchnikovellids was a clade composed of two environmental sequences: BAQA65, obtained from brackish anoxic sediment (AF372825), and CL-10, obtained from soda lake sediment (JQ480022). This clade appears sister to the core members of Microsporidia + Metchnikovellidae, which is congruent with the findings of Mikhailov et al. (2017) and Corsaro et al. (2020).

Further towards the base of the tree is a divergence of *Chytridiopsis typographi* and *Nucleophaga* spp. The position of *Chytridiopsis* was unstable across our SSU rRNA trees. In the Bayesian analysis, *Nucleophaga* spp. had a more basal position than *Chytridiopsis*, yielding the same topology

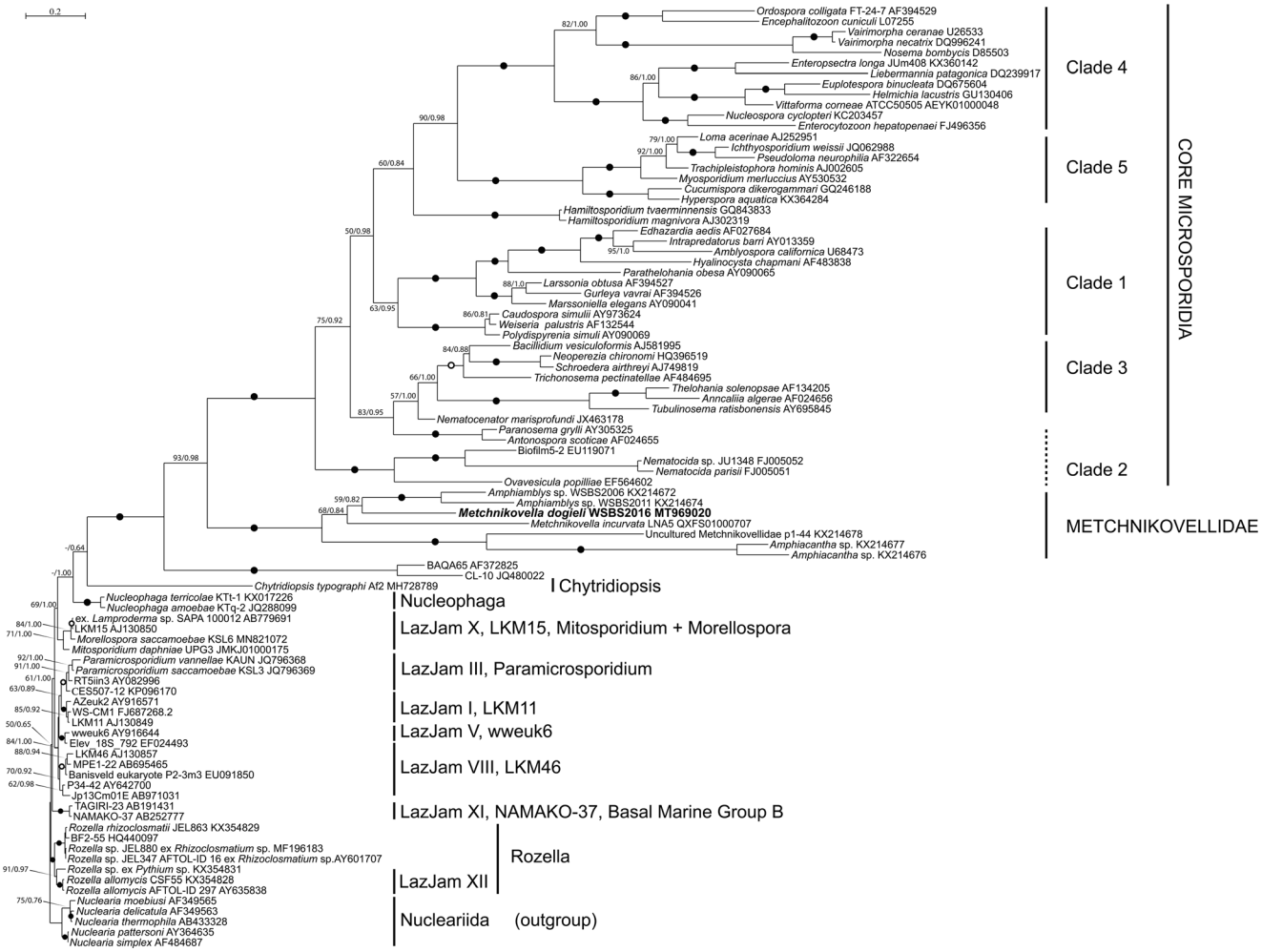

Fig. 2 SSU rRNA phylogeny of Microsporidia and related lineages, including the sequence of *Metchnikovella dogieli* retrieved in this study (in bold). Black dots indicate support values ≥99% ML bootstrap (BS) and ≥0.99 Bayesian posterior probability (PP). Open circles correspond to BS ≥95% and PP ≥0.95. BS and PP values are indicated above the branches; support values BS <50% and PP ≤0.63 are not shown; '–' indicates that the pattern of branching was not recovered by one of the reconstruction methods used. The major microsporidian clades established by Vossbrinck et al. (2014, Fig. 6.3a) are labelled. The dotted line shows clade 2, which was resolved as paraphyletic. The clades with environmental sequences are marked as LazJam × with Latin numerals according to the designations introduced by Lazarus and James (2015); clade designations according to Corsaro et al. (2016, 2020) are also used. Isolated and described taxa from these lineages are indicated

shown by Corsaro et al. (2018, 2020). However, in some of our ML trees, the pattern of branching was inverted; in either case, the support was low. It may have been caused by the fast evolutionary rate and derived character of the *Chytridiopsis* SSU rDNA sequence. Anyway, the position of *Nucleophaga* spp. close to Microsporidia was consistent with the observations of Corsaro et al. (2016, 2018), Grossart et al. (2016), Mikhailov et al. (2017), Stentiford et al. (2017), and Bass et al. (2018). The main clades of rozellids (including those classified as short-branched microsporidia by Bass et al. (2018)) revealed in the previous studies (Lazarus and James 2015; Corsaro et al. 2016, 2018, 2020; Grossart et al. 2016; Stentiford et al. 2017; Williams et al. 2018) were recovered in usual composition, including the clades containing morphologically described representatives: the LazJam X (LKM15) clade, harbouring *Mitosporidium daphniae* and *Morellospora saccamoebae*, and the LazJam III clade, containing *Paramicrosporidium* spp. Sequences of *Rozella* spp. formed the most basal clade, closest to the outgroup (Fig. 2).

The SSU rRNA gene phylogeny was not able to resolve the relationships within the Metchnikovellidae clade with full support. To better recover these relationships, multigene phylogenetic analysis was performed. It produced a robust and highly supported tree, revealing metchnikovellids as a fully supported clade at the base of the core microsporidia (Fig. 3). The nearest outgroup to this assemblage was *Mitosporidium daphniae*, further followed by *Paramicrosporidium saccamoebae* and *Rozella allomycis*. Together, they formed a fully supported clade 'Microsporidia + Rozellida'. This topology was congruent with previously published data by

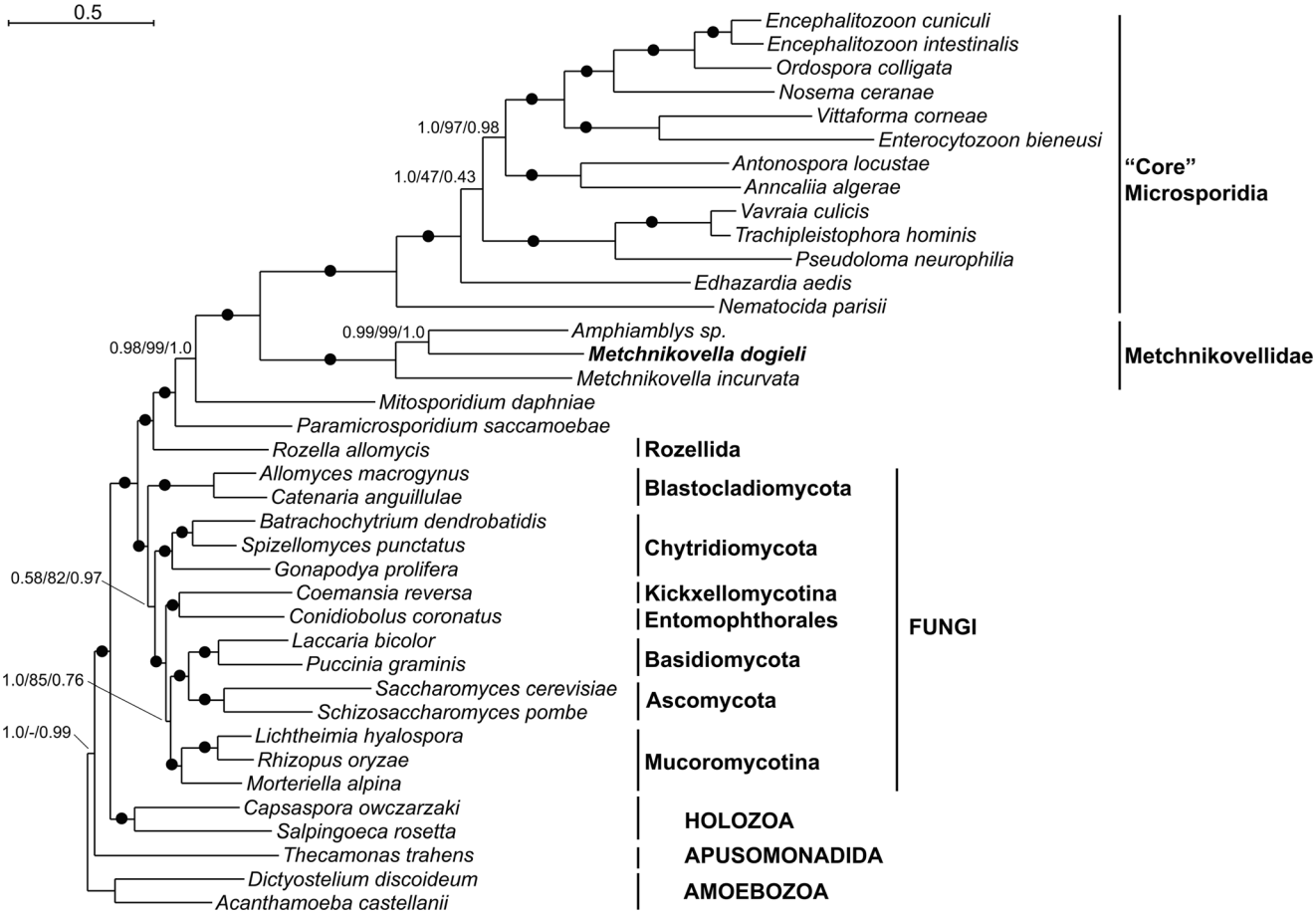

Fig. 3 Phylogenomic tree of Holomycota showing the position of metchnikovellids and the paraphyly of the genus *Metchnikovella*. The tree was reconstructed using a concatenated alignment of a dataset of 46 single-copy protein domains for 33 representatives of the Holomycota clade and 5 other Amorphea species used as an outgroup. The support values are as follows: posterior probability (PhyloBayes), bootstrap (RaxML), and aLRT (PhyML). Clades sharing full support gained with all three methods are indicated by black dots

Mikhalov et al. (2017) and Galindo et al. (2018). All other groupings in the tree were fully or highly supported and corresponded to the widely accepted views on the phylogeny of relevant taxa.

As in the SSU rRNA tree, in the multigene tree, two species of *Metchnikovella* did not form a single clade. *M. dogieli* grouped with *Amphiamblys* sp. with high (almost full) support, whereas *M. incurvata* formed an outgroup to this assemblage. Therefore, the genus *Metchnikovella* was revealed as paraphyletic in both our analyses.

Discussion

The genera of metchnikovellids are distinguished based on the structure and morphology of their spore sacs ('cysts' in the terminology applied by Caullery and Mesnil (1897, 1914, 1919) and Vivier (1975)). Three robustly defined genera were described based on the overall shape and length-to-width ratio of the spore sacs (Caullery and Mesnil 1914). The first established genus was *Metchnikovella* Caullery & Mesnil 1897, which was later defined as the genus characterised by cylindrical or fusiform sacs with rounded thick ends and a length under 10 times the width. The number of spores in a sac varied from 8 to 32. Microsporidia of this genus parasitise gregarines from a wide range of genera (mostly *Lecudina*, *Selenidium*, and *Polyrhabdina*), which infect predominantly polychaetes, as well as sipunculids and oligochaetes (reviewed by Larsson 2014; Paskerova et al. 2016). The genus *Amphiamblys* Caullery & Mesnil 1914 was defined as a genus forming cylindrical cysts with a length exceeding 10 times the width. The number of spores in a sac varied from 20 to 40. These organisms are parasites of gregarines of the genera *Ancora*, *Bhatiella*, and *Lecudina*, which infect polychaetes and echiurids (Caullery and Mesnil 1914, 1919; Reichenow 1932; Desportes and Théodoridès 1979; Ormières et al. 1981). The genus *Amphiacantha* Caullery & Mesnil, 1914 is characterised by fusiform cysts with long filament extremities that contain up to hundreds of spores. Metchnikovellids of this genus occur in gregarines of the genus *Lecudina*, which inhabit the polychaetes *Lumbrineris* spp. (Caullery and Mesnil 1914; Stubblefield 1955).

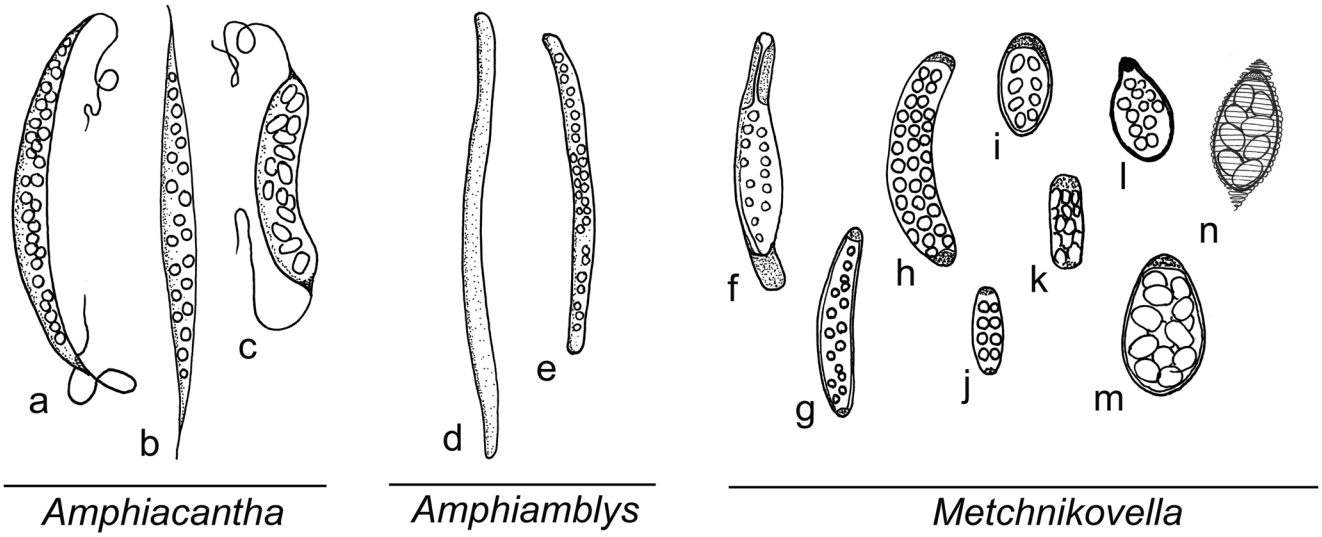

Fig. 4 Schematic drawing showing general morphology of spore sacs belonging to three genera of metchnikovellids: a—*Amphiacantha attenuata*; b—*A. ovalis*; c—*A. longa*; d—*Amphiamblys capitellae*; e—*A. capitellides*; f—*Metchnikovella spionis*; g—*M. incurvata*; h—*M. legeri*; i—*M. hovassei*; j—*M. nereidis*; k—*M. minima*; l—*M.* (*Caulleryetta*) *mesnili*; m—*M. dogieli*; n—*M. spiralis*; a, c–k are after Caullery and Mesnil (1919); b—after Vivier (1975); l—after Dogiel (1922); m, n are original drawings. Drawn not to scale because of considerable size differences between spore sacs of various species

Several authors have proposed the establishment of more genera based on the same set of characters. Among the characteristic features of *Metchnikovella*, there is a polar plug (also known as a plugging structure or thickening) that closes the spore sac from one or both poles. Members of the genus *Metchnikovella* described by Caullery and Mesnil have plugs on both sides of the spore sac. Dogiel (1922) suggested establishing the genus *Caulleryetta* for a new species of metchnikovellids possessing a pyriform sac with a single polar plug. This suggestion was not widely accepted; neither was the proposal by Schereschevsky (1924) to establish the genus *Microsporidyopsis*. The latter author provided a detailed description of observations made on a metchnikovellid isolated from the gregarine *Lecudina* sp. (formerly *Doliocystis* sp.) inhabiting *Nereis parallelogramma* Claparède, 1868; however, no justifications for establishing a new genus were provided, and no discriminating characters were described. According to Larsson (2014), the shape of the spore sacs, its possession of only one polar plug, and the small number of spores it contains justified its affiliation with the genus *Caulleryetta*.

Vivier (1975) merged both *Caulleryetta* and *Microsporidyopsis* back into *Metchnikovella*, considering both of them as junior synonyms of this genus. Sprague (1977) and Canning and Vávra (2000) shared this opinion and recognised only three genera of metchnikovellids: *Metchnikovella*, *Amphiamblys*, and *Amphiacantha*. Notably, Sprague et al. (1992) subsequently listed all five metchnikovellid genera in the 'checklist of available generic names' and noted that they should be considered valid until proven otherwise. These five genera were also listed by Becnel et al. (2014) and Cali et al. (2017). Issi (1986) suggested a new genus, *Desportesia*, for the species *Amphiamblys laubieri* Desportes & Theodorides, 1979 due to some unique ultrastructural features of this species (internal membrane folds, resembling the elements of polaroplast). However, this suggestion was not widely accepted, and this genus has not been adopted other than being listed, together with metchnikovellid genera such as *Metchnikovella*, *Amphiamblys*, *Amphiacantha*, and *Caulleryetta*, in a chapter dedicated to microsporidia by the same authors (Issi and Voronin 2007).

Larsson (2014) provided a detailed review of the systematics and taxonomy of Metchnikovellidae. He noted that 'the spore sacs of *Metchnikovella* are the widest, and in contrast to the other genera, their shape varies in an exceptional way'. Caullery and Mesnil (1914) noted that the type species of *Metchnikovella*, *M. spionis*, differs from all other metchnikovellids in the presence of elongated tips of the 'cysts' forming the plug. They noted that the genus *Metchnikovella* should perhaps be restricted to this species only (Caullery and Mesnil 1919), pointing out that all other species in the genus were placed there provisionally. Sprague et al. (1992) cited this statement and noted that the genus *Metchnikovella* is 'almost certainly a heterogeneous assemblage'.

Ultrastructural studies have evidenced for some heterogeneity among *Metchnikovella* spp. in the structure of the spore-sac wall and in the pattern of free sporogony. Vivier and Schrével (1973) described the development of *M. hovassei*, a species with spore sacs possessing the single plug, and noted that its sacs possess a two-layered wall with a dense layer of fibrous material and a wider, reticulate surface layer; free

sporogony in this species takes place in parasitophorous vacuoles. In contrast, in *M. incurvata* spore sacs, which possess two plugs, the sac wall has several layers of amorphous material, and free spores develop freely in the cytoplasm and are not bounded (Sokolova et al. 2013). Larsson used these observations, among others, to argue for the restoration of the genus *Caulleryetta* and suggested that all *Metchnikovella* species possessing the single polar plug should be transferred to this genus (Larsson 2014, p. 621).

The results of our phylogenetic study support the presumed heterogeneity of the genus *Metchnikovella*. It appears that organisms currently assigned to this genus do not form a monophyletic clade. Hence, we conclude that this genus is an artificial assemblage. Even a brief analysis of the spore-sac morphology of metchnikovellid genera shows that while the spore sacs of the genera *Amphiacantha* and *Amphiamblys* appear largely homogeneous, the genus *Metchnikovella* shows a huge diversity of spore-sacs morphologies (Vivier 1975; Sokolova et al. 2013; Larsson 2014). Among the members of this genus, some species show elongate and slightly curved spore sacs with two plugs; some have rounded or oblong spore sacs with a single plug, and one species has spore sacs with no described plugs (Fig. 4). Certainly, sequences of more species are necessary, but it is reasonable to suggest that the genus *Metchnikovella* be revised when more molecular data become available. This problem requires further investigation.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-020-06976-x.

Acknowledgements The authors thank the staff of the White Sea Biological Station of M. V. Lomonosov Moscow State University for providing facilities for field sampling and material processing, as well as for their kind and friendly approach. The authors also thank Guifré Torruella, Luis Javier Galindo, Hélène Timpano, David Moreira, and Purificación López-García (Ecologie Systématique Evolution, CNRS, Université Paris-Sud, AgroParisTech, Université Paris-Saclay, Orsay, France) for the alignments of conserved single-copy protein domains used for the phylogenomic analysis and for sharing their expertise in performing of single-cell genomics. This study utilised equipment of the Core Facility Centres 'Biobank', 'Development of Molecular and Cell Technologies', and 'Culture Collection of Microorganisms' of the Research Park of Saint Petersburg State University.

Author contributions Elena Nassonova and Alexey Smirnov suggested an overall concept and design of the study, performed molecular phylogeny and phylogenomics, and drafted the manuscript; Gita Paskerova, Ekaterina Frolova, and Magdaléna Kováčiková performed the field work, dissections of polychaetes, and isolation of the parasites; Alexey Smirnov and Gita Paskerova did light microscopy and single-cell manipulations; Elena Nassonova conducted molecular studies; Natalya Bondarenko performed bioinformatic treatment of NGS data. All authors contributed to the improvement of the draft and accepted the final version of the manuscript.

Funding This study was financially supported by the Russian Science Foundation—project no. 19-74-20136 (phylogenetic analyses, bioinformatics and data treatment) and by the Russian Foundation for Basic Research—projects no. 18-04-01359 (light microscopy, molecular work, NGS sequencing). Financial support for fieldwork of GP was provided in part by St. Petersburg State University—grant no. 1.42.1099.2016. The fieldwork of MK was supported by the Czech Science Foundation, project no. GBP505/12/G112 (ECIP).

Data availability The dataset used in this study is deposited with the GeneBank under the accession numbers: MT969020 (SSU rDNA), MT951446, MW052334-MW052379 (protein-coding genes).

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or warm-blooded animals performed by any of the authors. The White Sea Biological Station of M. V. Lomonosov Moscow State University 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.

Consent for publication All the authors mentioned in the manuscript have agreed for authorship, read and approved the manuscript, and given consent for submission and subsequent publication of the manuscript.

Code availability (software application or custom code)

FastQC http://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Trimmomatic http://www.usadellab.org/cms/?page=trimmomatic
SPAdes v.3.13.0 http://cab.spbu.ru/files/release3.13.0/manual.html
QUAST http://cab.spbu.ru/software/quast/
SAMtools http://samtools.sourceforge.net
SeaView 4.6.1 http://doua.prabi.fr/software/seaview
BLASTx https://blast.ncbi.nlm.nih.gov/Blast.cgi?LINK_LOC=blasthome&PAGE_TYPE=BlastSearch&PROGRAM=blastx
RAxML-HPC2 on XSEDE (v.8.2.12) https://www.phylo.org
MrBayes v.3.2.6 http://www.phylo.org
PhyloBayes MPI on XSEDE (v.1.4f) http://www.phylo.org/tools/obsolete/phylobayes_xsede.html

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