

## Ribosomal RNA of Metchnikovellids in Gregarine Transcriptomes and rDNA of Microsporidia *Sensu Lato* in Metagenomes

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**Abstract**—Numerous nucleotide sequences of microsporidia *sensu lato*, mainly belonging to the “Cryptomy-cota” (Rozellida, Rozellomycota, Rozellosporidia, treated here as synonyms), are found in metagenomes, transcriptomes, and amplicon libraries used for metabarcoding. In this study, we describe rDNA sequences of hyperparasitic metchnikovellid microsporidia found in the transcriptomes of unicellular protists belonging to Apicomplexa (Alveolata). The transcriptome of the eugregarine *Polyrhabdina* sp. (GenBank SRX6640468) contains the cDNA of *Metchnikovella incurvata*, the transcriptome of the archigregarine *Selenidium pygospionis* (GenBank SRX6640459) contains the cDNA of *Metchnikovella dogieli*, and in the transcriptome of the blastogregarine *Siedleckia* cf. *nematoides* (GenBank SRX6640464) we find cDNAs originating from a yet undescribed species representing a novel metchnikovellid family. We have modeled the secondary structure of the “ITS2” region of identified and unidentified metchnikovellids taking into account the covariant nucleotide substitutions. Based on the predicted secondary structure of rRNA, mapping of reads from cDNA libraries, and the absence of the endoribonuclease Las1 (PF04031), we conclude that there is no ITS2 processing in metchnikovellids, and the mature “5.8S”- and “28S”-like (LSU) rRNA are covalently fused, similarly to the LSU rRNA in the other microsporidia *sensu stricto*. We discuss several previously proposed (*Chytridiopsis typographi*, BAQA065) and new candidates for the sister group of microsporidia *sensu stricto*, and compare the reduced rRNA genes of microsporidia and the lengthened rRNA genes with group I introns of parasitic and lichen fungi in the context of neutral and adaptive evolutionary processes.

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### INTRODUCTION

The influx of novel molecular genetic data on eukaryotic microorganisms that present particular interest from a phylogenetic point of view comes from several sources. The most important source is genomics and transcriptomics of new strains introduced into cultures or sampled using the “single-cell” method, which allows to isolate and sequence individual cells of uncultivated species (Seenivasan et al., 2013; Burki et al., 2013, 2016; Janoušek et al., 2017; Gawryluk et al., 2019; Strassert et al., 2019; Tikhonenkov et al., 2020a, 2020b; etc.). However, there are also other sources of data. The studies of marker genes in DNA samples isolated from plankton, soil, bottom sediments, and

other substrates (Semenov, 2019), oftentimes reveal nucleotide sequences that cannot be assigned to the known phyla or kingdoms. According to the empirical rule (Kim et al., 2016), such phylogenetically isolated groups are represented by species that are rare in nature. This is evidenced by the small number of readings in the libraries and the rarity of repeated findings in independently constructed libraries. What these rare organisms might look like is largely unknown; therefore, the perspectives for their rapid introduction into the culture or capture by a micromanipulator for the application of single-cell genomics are obscure. Metagenomics and metabarcoding data, as well as analysis of genome and transcriptome contaminations

(Borner and Burmester, 2017) currently remains the only source of information about these organisms.

The diversity of nucleotide sequences in amplicon libraries obtained from natural samples exceeds the diversity visible with a microscope. It is as if the dark matter of life (microorganisms not cultivated in laboratory conditions) is present in natural ecosystems (Filée et al., 2005; Marcy et al., 2007). Unicellular parasites and parasitoids are a significant part (sometimes more than 50%) of the hidden eukaryotic diversity (Lefèvre et al., 2007, 2008; Lepère et al., 2008; Mahé et al., 2017), judging by the few identified relatives. Thus, the groups of parasitoids (López-García et al., 2001; Moon-van der Staay et al., 2001; Christaki et al., 2017) called Marine Alveolata (MALV) Groups I and II (Guillou et al., 2008) predominate in the picoplankton of the world ocean, from the surface to the abyssal, judging by the predominance of rDNA in the amplicons. The MALV groups were identified as ichthyodinium and sindinium dinoflagellates, and their diversity and ecological role were previously unappreciated. The rRNA genes of fungi and their relatives usually predominate in libraries obtained from freshwater and soil samples (Lepère et al., 2006; Monchy et al., 2011; Nakai et al., 2012; Ishida et al., 2015; Rojas-Jimenez et al., 2019). Initially, the largest group of fungal relatives was named LKM11 (Hannen et al., 1999; Lara et al., 2010) and then “cryptic fungi” or Cryptomycota (Jones et al., 2011a, 2011b). It gradually became clear that representatives of the genus *Rozella* Cornu 1872 belong to LKM11 (cryptomycots). They have been known since the 19th century and include intracellular parasites of fungi, oomycetes, and unicellular algae (Gleason et al., 2014; Letcher and Powell, 2018). Not long before the introduction of Cryptomycota, the genus *Rozella* was excluded from chytrid fungi and united with microsporidia based on molecular data (James et al., 2006). Besides *Rozella*, the cryptomycots also include intranuclear and cytoplasmic parasites of lobose amoebae, algae, and arthropods (daphnia, insects). It is noteworthy that the feeding cells of the *Rozella* species phagocytize the host cytoplasm like amoeboid organisms, while their zoospores are similar to chytrid zoospores (in that they do not feed and that they carry a single posterior flagellum with a second centriole adjacent to the basal body). The fungi include mycelial and yeast-like forms that feed osmotrophically, and if the members of *Rozella*, which feed via phagocytosis, were included in the taxon Fungi, then the taxon would be deprived of any nonmolecular diagnostic traits. Another group of initially unidentified rDNA sequences was assigned to aphelids (Karpov et al., 2013; Letcher et al., 2013), which have been known since the 19th century and were also relatively recently transferred from the chytrid fungi (in this case, to Rhizopoda) with the assignment of a rank of the class (Gromov, 2000). Aphelids are intracellular parasitoids similar to *Rozella*: they have trophic stages feeding via phagocytosis and non-

feeding uniflagellate or amoeboid zoospores (Karpov and Paskerova, 2020). Similar lifestyles and appearances of the closest relatives of fungi (aphelids and rozellids) raises the question of their similarity to the ancestors of true fungi and their status as parasites or parasitoids of terrestrial unicellular algae (Aleshin et al., 2015).

Fragments of the marker genes (rDNA) of thousands of unidentified cryptomycots have accumulated in electronic databases over the two decades of metabarcoding. These electronic databases also contain aphelids and holomycotans lacking any taxonomic association, e.g., members of GS01 clade (Tedersoo et al., 2017), but their sequences are orders of magnitude rarer. Zoosporic fungi (chytrids, blastocladales, and etc.), cryptomycots, aphelids, and other microscopic relatives of fungi currently constitute the dark matter of terrestrial communities, often overlooked by the studies (Grossart et al., 2016). Taxonomic descriptions exist only for a handful of cryptomycots (besides *Rozella*): a resurrected genus *Nucleophaga* Dangeard 1895 (Corsaro et al., 2014a) and the new genera *Mitosporidium*, *Paramicrosporidium*, and *Morellospora* (Haag et al., 2014; Corsaro et al., 2014b, 2020). All characterized cryptomycots are intracellular parasites.

Microsporidia in a traditional sense (Issi and Voronin, 2007; Issi, 2020; Wadi and Reinke, 2020) are obligate, intracellular parasites of vertebrate and invertebrate animals and different unicellular organisms (ciliates, gregarines, paramixids). This is a species-rich group (1500 species were described, and this is a small part of the diversity) that is clearly distinguished by morphological and ultrastructural traits: the losses of flagella, centrioles, and canonical mitochondria, a modification of the Golgi apparatus at the cytological and biochemical levels (loss of vesicular and clathrin transport), and the presence of spores equipped with a complex apparatus for infection of the host cell. After the exclusion from sporozoans in the old sense (which combined microsporidia, myxosporidia, haplosporidia, and partially modern Apicomplexa), microsporidia were considered a phylum (Sprague, 1977; Weiser, 1977), and various hypotheses on its phylogenetic relationship were put forward. At present, the hypothesis about the affinity of the phylum Microsporidia and the genus *Rozella*, which was originally suggested on the basis of a phylogenetic analysis of six genes (James et al., 2006), received strong support. Moreover, it was established that many cryptomycots are phylogenetically even closer to microsporidia than *Rozella* spp. Thus, cryptomycots are paraphyletic relative to microsporidia. If the aphelids are excluded from consideration, then the clade combining microsporidia and cryptomycots is sister to fungi. After several mutually exclusive initiatives on taxonomy that tried to balance the kingdom Fungi, the phylum Microsporidia, the genus *Rozella*, and other cryptomycotan genera, it was proposed to expand the volume of

microsporidia to all cryptomycots, with the exception for the genus *Rozella* and other phylogenetically early lines (Bass et al., 2018). Tedersoo et al. (2018) went further and suggested a common name for the group uniting microsporidia and rozellids (subkingdom Rozellomyceta) in a recent revision of the fungi system. The problem of the name for such phylogenetically unified but structurally heterogeneous large groups requires a separate consideration. In particular, the inclusion of all cryptomycots in Microsporidia gives a common name to a large monophyletic group, but there is simultaneously a cost in the form of a loss of diagnosis by the taxon Microsporidia in a new sense, since it unites plesiomorphic flagellate and phagotrophic forms, transitional forms, and highly specialized typical microsporidia. The use of the name Rozellomyceta has a cost in the form of the loss of diagnosis by fungi. A common problem of plesiomorphic and specialized species in the same clade was formulated by Hennig (1966) in the deviation rule and by Chupov (2002) in the concept of hidden, closely related (cryptaffine) taxa and is inevitable within the phylogenetic system. Within the framework of our work, we consider the group containing microsporidia *sensu lato* together with *Rozella*, NCLC1, and the XI clade (Lazarus and James, 2015), while we will use the name cryptomycots as an everyday term for nonranking paraphyletic residue after the isolation of a monophyletic group of typical microsporidia.

To describe the history of specialization of typical microsporidia (*sensu stricto*), hidden, closely related cryptomycots (cryptaffine) should be ranked by the degree of their affinity to microsporidia. At present, Chytridiopsida are considered a candidate for the position of the closest relative of typical microsporidia (Corsaro et al., 2019). Chytridiopsida were previously attributed to typical microsporidia, sometimes within the class Metchnikovellidea (Weiser, 1977). Another approach consists of the study of the least specialized of the typical microsporidia, which include metchnikovellids (Issi and Voronin, 2007) (hyperparasites known from gregarines parasitizing polychaetes). Metchnikovellids were described at the end of the 19th century, but their close relationship to microsporidia was proved only with electron microscopy (Vivier, 1965); since then, they are considered to be primitive members of microsporidia at the rank of the class Rudimicrosporea (Sprague, 1977) or the class Metchnikovellidea (Weiser, 1977). They differ from other typical microsporidia: they have less specialized spores with a short polar tube, and no polaroplast, anchoring disk, or posterior vacuole. In addition to sporogony, which produces free spores, their life cycle also includes the production of spores within spore sacs or cysts (they share these traits with Chytridiopsida). The hypothesis about the initial divergence of microsporidia *sensu stricto* into metchnikovellids, and all other microsporidians was confirmed with genomic data (Mikhailov et al., 2017; Galindo et al., 2018; Nas-

sonova et al., 2021). However, metchnikovellids are not the only group of primitive microsporidians. Other such microsporidians include *Buxtehudea*, *Jiroveciana*, *Burkea*, *Hessea* (Issi and Voronin, 2007; Larsson, 2014). There are yet no molecular data for them, and we will not discuss them here further, focusing only on the available molecular data.

## MATERIALS AND METHODS

**Databases and search procedure.** Nucleotide sequences were extracted from the databases of the National Center for Biotechnology Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>). In addition to the nonredundant nucleotide (*nr*) database (GenBank; Benson et al., 2013), we use original transcriptome data (Janouškovec et al., 2019), Sequence Read Archive (SRA) (ERX2404075–ERX2404077), databases containing long contigs assembled from the results of high-throughput amplicon sequencing (Jamy et al., 2019) and metagenomic data from Whole Genome Shotgun (*wgs*) projects (Mitchell et al., 2018; Sanchez and Cao, 2019; Cotto et al., 2020; Sharrar et al., 2020; Thornton et al., 2020). BLAST instruments were used to search for target sequences (Altschul et al., 1997), and 18S rRNA sequences from the members of the main groups of cryptomycots detected by previous studies were used as an input query.

**Phylogenetic analysis.** Nucleotide sequences of rRNAs were added to the previously prepared alignment using profile mode of MUSCLE (Edgar, 2004). The regions with problematic alignment were manually removed prior to tree construction. Bayesian inference was performed with MrBayes 3.2.5 (Ronquist et al., 2012) with a General Time-Reversible (GTR) model of nucleotide substitutions that take into account the unevenness of sites by the rate of evolution according to gamma distribution approximated by 10, 12, or 16 categories, and applying a covariance model of evolution. The number of independent launches, the number of Markov chain Monte Carlos (MCMCs) in each launch, and the number of generations varied. The trees were visualized with FigTree (Rambaut, 2010) and MEGA software (Kumar et al., 2016).

**Structural analysis.** Raw reads were mapped to measure the coverage along the metchnikovellid transcripts with Bowtie2 (Langmead and Salzberg, 2012) using parameters `–end-to-end`, `–no-mixed`, `–no-discardant`. The coverage data were extracted from the bam files of read alignments with BEDTools (Quinlan and Hall, 2010) with the parameters `-pc` to calculate the total coverage by fragments (i.e., from direct to reverse reading) and the “`–5-strand +`” to count the start points of fragments. In the control experiment, the SRA: SRR10440982 library was mapped to the *Saccharomyces cerevisiae* S288C rRNA operon (NC\_001144.5: 451786–457732). The known nomenclature was used in models for the secondary structures of large and small subunits of rRNA (Wuyts et al., 2001);

the structures of helices 19–21 were corrected according to the model ciliate species *Tetrahymena thermophila* and yeast *S. cerevisiae* (Lee and Gutell, 2012). Noncanonical pairs, including those confirmed via X-ray analysis and cryoelectron microscopy of ribosomes in model species, were not taken into account; conversely, complementary pairs at the ends of helices were included in the predicted helices, even if such helix expansion is not typical for most species. Local RNA folding was predicted with an Mfold server (Zuker, 2003) and accounting for suboptimal structures (15%). Both the current version, which predicts the structures at 37°C, and the version 2.3 with a temperature-selection option (in this case, 15°C was selected) were used. A four-domain model with typical elements in the helices II and III was used in the prediction of the ITS2 structures whenever possible (Joseph et al., 1999; Schultz et al., 2005; Coleman, 2007, 2015). The RNAalifold program (Bernhart et al., 2008) as a part of ViennaRNA web service (Gruber et al., 2015), was used for analysis of the region corresponding to the reduced ITS2 in metchnikovellids, taking into account information on the alignment of primary structures. The models were visualized with RnaViz 2 (Rijk et al., 2003).

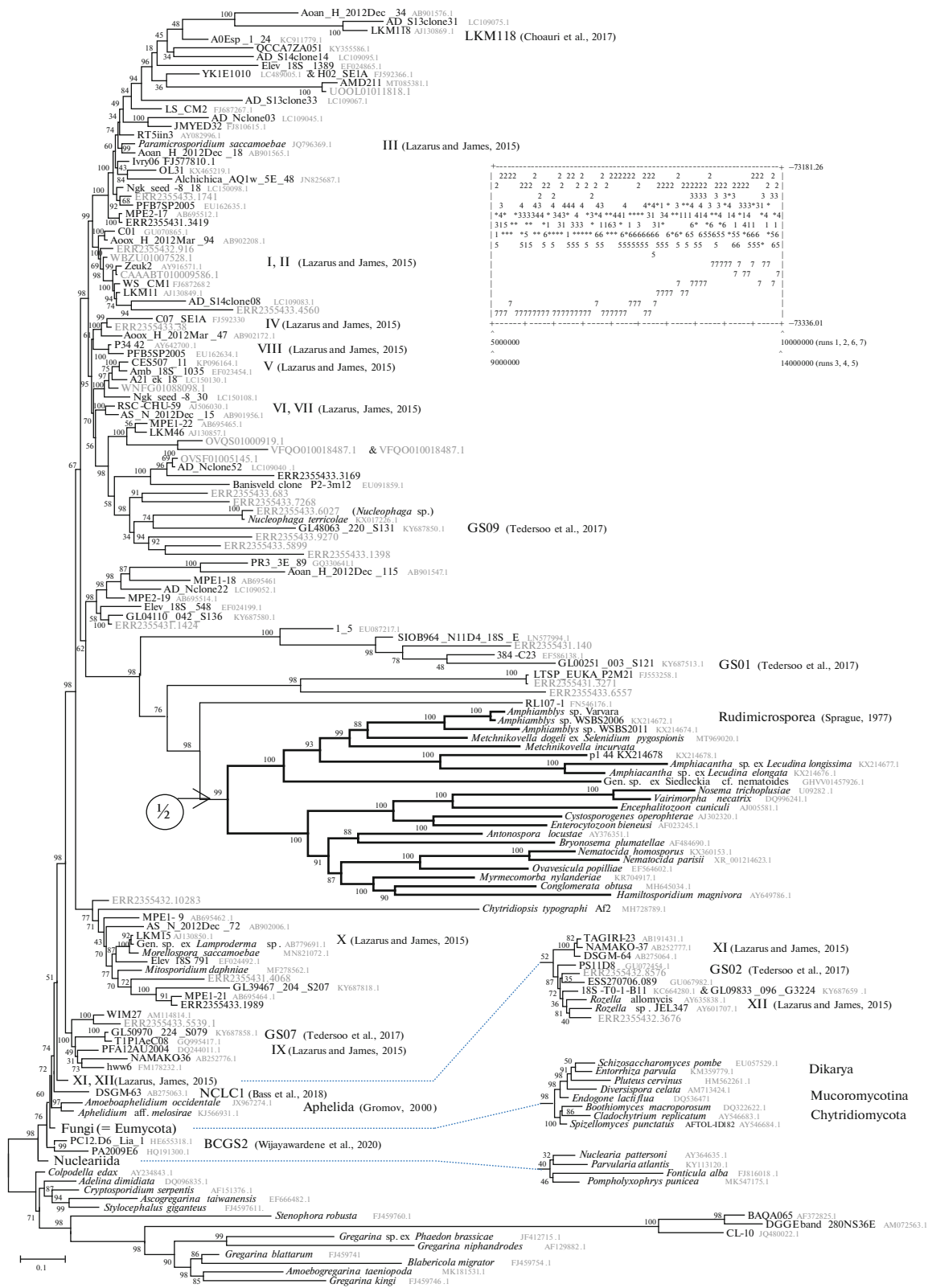
## RESULTS AND DISCUSSION

**Metchnikovellids.** In the analysis of the results of high-throughput cDNA sequencing of eugregarines, archigregarines, and blastogregarines (Janouškovec et al., 2019), sequences belonging to microsporidia (metchnikovellids) were found in three libraries. Here, we describe the rRNA contigs, although many protein-coding transcripts of metchnikovellids can be also assembled from these libraries. Based on the similarity of nucleotide sequences (GenBank QXFS01001040, QXFS01000707; Galindo et al., 2018), metchnikovellids from eugregarine *Polyrhabdina* sp. are assigned to the *Metchnikovella incurvata* Caullery et Mesnil 1914 species known from this host (Sokolova et al., 2013; Rotari et al., 2015). Based on the similarity of the nucleotide sequences, metchnikovellids from archigregarine *Selenidium pygospionis* are assigned to the *Metchnikovella dogieli* Paskerova et al., 2016 species (common parasite of *S. pygospionis*) (GenBank MT969020; Paskerova et al., 2016, 2018; Nasonova et al., 2021). No metchnikovellids were previously found in blastogregarines; nucleotide sequences found in the cDNA library (GenBank GHVV01457926, GHVV01457913, GHVV01457924) are the first such finding. The RNA for cDNA synthesis was isolated from several dozen blastogregarine cells extracted from the intestines of marine polychaetes *Scoloplos* cf. *armiger* and formally identified as *Siedleckia nematoides*. However, the polymorphism of protein-coding transcripts in the library indicates that the sample of blastogregarine cells is represented by a mixture of similar species. Therefore, the host should be more

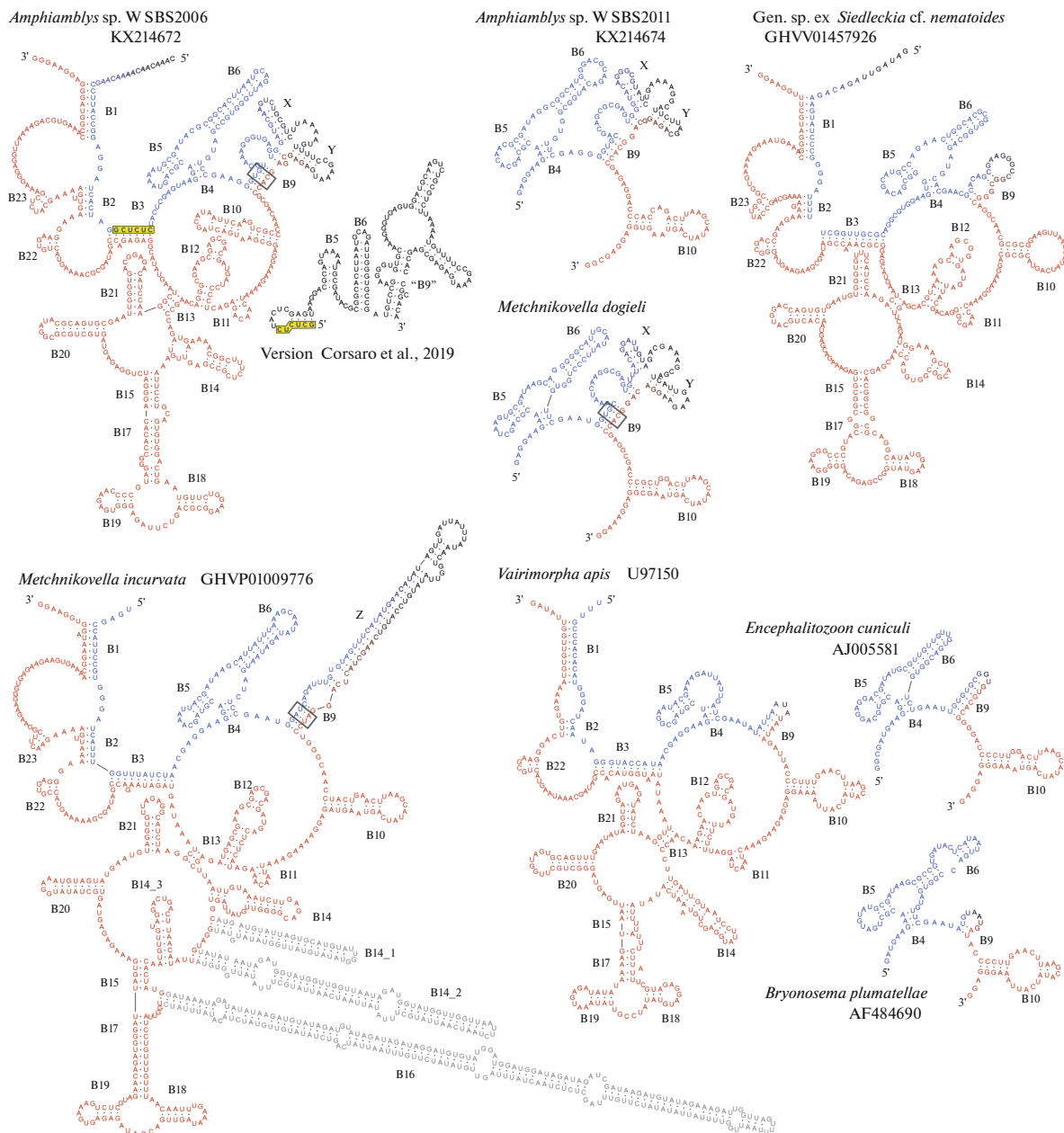
accurately referred to as *S. cf. nematoides*. No close similarity was found between the rRNA of metchnikovellid from *S. cf. nematoides* and any known nucleotide sequences, while its affinity to metchnikovellid group is justified by the constructed phylogenetic tree. No metchnikovellid rRNA sequences were found in the libraries from natural substrates (except for contaminant, GenBank no. KX214678). Conversely, metchnikovellids were found in three of five gregarine species studied with a high-throughput sequencing method (Janouškovec et al., 2019). Apparently, the diversity and abundance of metchnikovellids remains undetected by studies of rDNA amplicons obtained from samples of marine sediments.

Metchnikovellids form a monophyletic group sister to other typical microsporidia in the phylogenetic tree of the small subunit ribosomal RNA (Fig. 1). This result is consistent with previously published results (Mikhailov et al., 2017; Bass et al., 2018; Galindo et al., 2018; Corsaro et al., 2019, 2020; Nasonova et al., 2021). The *Amphiacantha* species are separated from *Metchnikovella* spp. and *Amphiamblys* spp. (the *p*-distances calculated for the aligned rRNA regions are about 0.3); based on this, it is possible to accept the allocation of two separate families for them: Amphiacanthidae and Metchnikovellidae (Larsson, 2000, 2014). The parasite from blastogregarine forms the third branch of metchnikovellids on the phylogenetic tree isolated from Amphiacanthidae and Metchnikovellidae.

The fusion of mature 5.8S- and 28S-like rRNA in a single molecule is one of the features of microsporidia *sensu stricto* (Vossbrinck and Woese, 1986). There are no conservative ITS2 elements (which are considered important for processing) in typical microsporidia (Coleman, 2015). In other eukaryotes, B7 and B8 hairpins (conservative 5.8S rRNA elements) border ITS2. B7 and B8 hairpins are absent in typical microsporidia, and B6 hairpin is also absent in some of them (Fig. 2) (Rijk et al., 1998; Peyretailade et al., 1998; Peer et al., 2000). In metchnikovellids, we predict B6 hairpin and find no B7 and B8 hairpins, while Corsaro et al. (2019, Fig. 5) homologize *Amphiamblys* sp. B6 and *Chytridiopsis typographi* B8 helices, naming them the “GC-rich stem.” The question arises: which reconstructions are more reliable? The possibility of alternative variants that are, at the same time, close in the values of the free energy cast doubt on predictions for individual species, while the variability of the considered region makes it difficult to rely on distant species to identify matching elements. In general, however, three pairs of compensatory nucleotide substitutions in B4 stem relative to *Vairimorpha apis* and two pairs of compensatory substitutions in B9 stem in *Metchnikovella incurvata* relative to *M. dogieli* and *Amphiamblys* spp. can be noted for metchnikovellids (Fig. 2, rectangle). These compensatory substitutions allow us to determine the possible positions of B4 and B9 helices. In the metchnikovellid



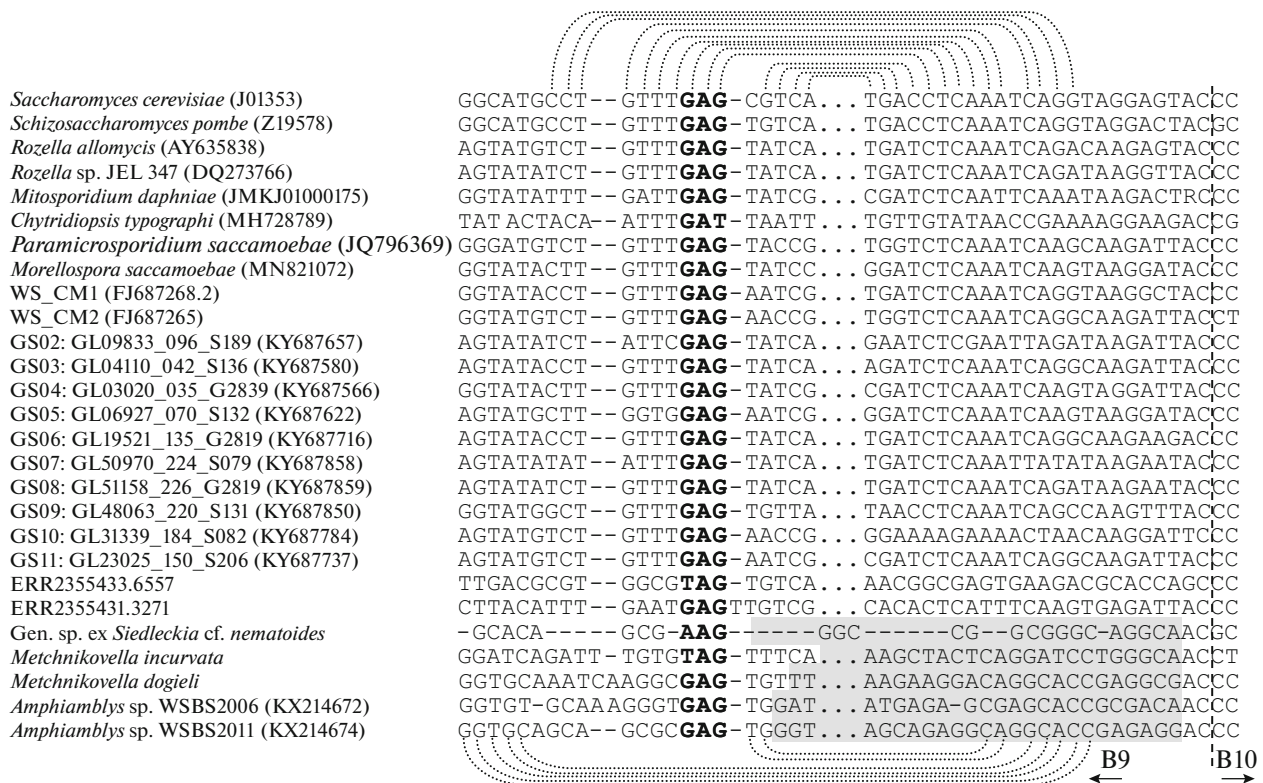
**Fig. 1.** Bayesian tree of small subunit ribosomal RNA from microsporidia. The result of the better launch “run2” is presented (ngen = 10000000, nchains = 8, nst = 6, ngammacat = 10, covarion = yes). The log-likelihood values of the top seven launches (out of 14) are presented on a scatterplot. The branch length in the subtree of typical microsporidia is halved (wide lines). Posterior probabilities are given as a percentage values. The topology convergence was not achieved; in particular, some clades with a high posterior probability in independent launches are absent in the presented tree (and vice versa). Probable artifacts of the position of BAQA65 and GS01 clades are discussed in the text.



**Fig. 2.** Predicted secondary structure of fused 5.8S- and 28S-like rRNA of typical microsporidia. The regions of sequences homologous to 5.8S rRNA are highlighted in blue; 28S rRNA is in red; the borders of 5.8S and 28S rRNA fall on the helix section B9, and the uncertainty of the border position is marked with a color gradient. The helices specific for metchnikovellids are designated *x*, *y*, and *z*. Two pairs of compensatory substitutions in B9 between *Metchnikovella incurvata*, *M. dogieli*, and *Amphiamblys* spp. are highlighted with a gray rectangle. The part of B3 helix contact attributed by Corsaro et al. (2019, Fig. 5) to the intramolecular helix within 5.8S rRNA is highlighted with yellow; we added the designations of B5, B6, and B9 helices to the Corsaro model. The structure for *Encephalitozoon cuniculi* and *Vairimorpha apis* (the generic name according to latest revision (Tokarev et al., 2020)) is given according to early models (Peyretailade et al., 1998; Rijk et al., 1998; Peer et al., 2000) with minor modifications. The rRNA helix nomenclature is given according to Wuyts et al., 2001.

from blastogregarine, as well as in other typical microsporidia, the B9 stem terminates in a simple loop. In other metchnikovellids, however, this position of ITS2 is occupied by specific structural elements that do not fit into the four-domain model. The RNAalifold instrument can fit the predicted struc-

tures of three species to their common model in the form of a garden scarecrow (Fig. 2), while the expansion of nucleotide sequences for *M. incurvata* into this site, as well as into many other rDNA sites of this species, can be assumed. It is not clear how the model generated by RNAalifold correlates with the four-



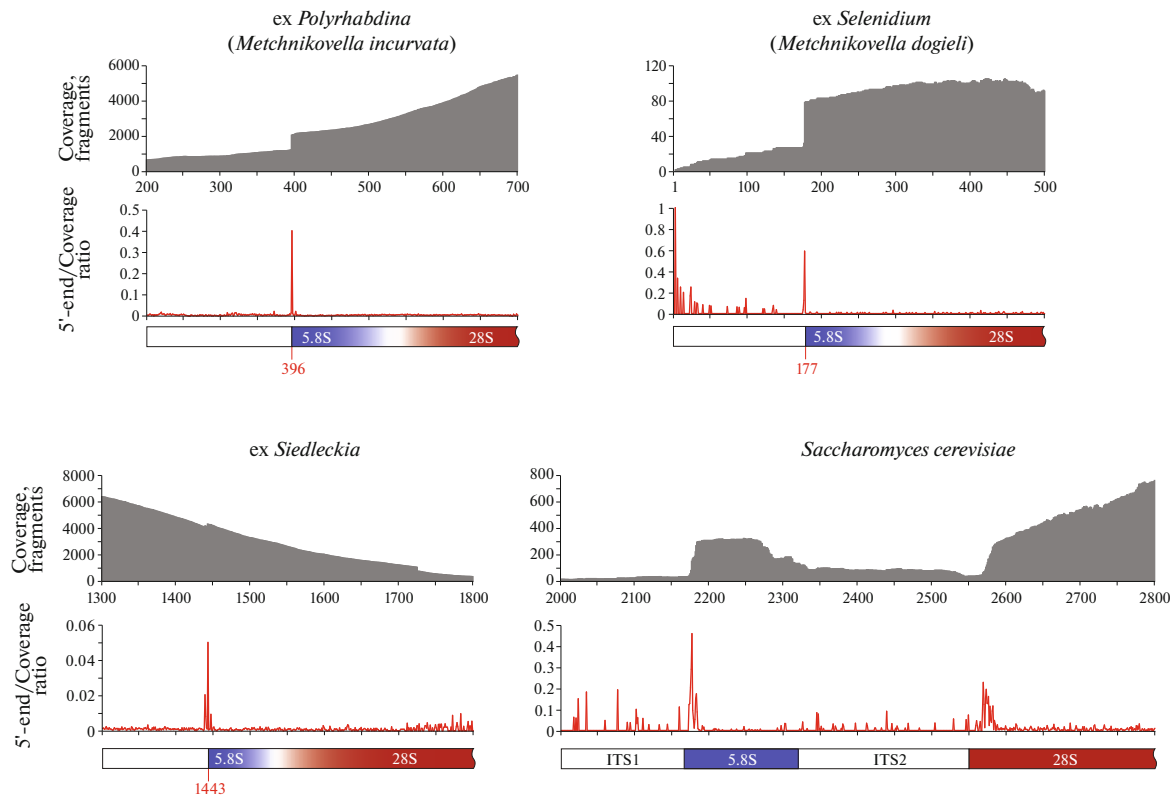
**Fig. 3.** Alignment of nucleotide sequences forming the base of the helix B9. The missing nucleotides of ITS2 and distal part of B9 are replaced with ellipsis. The compensatory nucleotides of B9 helix in yeasts and *Amphiblysis* sp. are connected by dotted arcs. The unaligned region is given on a gray background.

domain model and how sustainable it will be for new data.

Metchnikovellides preserve a modified form of the conservative motif ggyryrygtttGAGtrtcr near the 3'-border of 5.8S rDNA (Fig. 3). This region is usually complementary to the 5'-end region of 28S rRNA, and together they form the base of B9 helix. However, metchnikovellids simply have no complementary nucleotides in the immediate vicinity. In both the garden scarecrow model and the model of Corsaro et al. (Fig. 2), the conservative sequence of metchnikovellids homologous to 5.8S rRNA 3'-end is predicted not as a part of the helix (as in other species) but partially in the form of single-stranded loop. In addition to metchnikovellids, the considered region, as it seems, occupies a nonstandard position in the secondary structure of rRNA in some other species, e.g., *Chytridiopsis typographi*. Apparently, the function of 3'-terminal 5.8S rRNA region is not limited to 28S rRNA binding to form B9 helix; it also is suggestive of binding to an alternative (unknown) ligand. Otherwise, paired compensatory substitutions would gradually destroy the conservative motif, leaving only the complementarity of B9 strands due to compensatory substitutions in the same way as in variable ITS2 helices. In other typical microsporidia, this (unknown) function is also lost along with the corresponding nucleotide

sequence. A dual nature is probably common to many rRNA helices, which alternately act as either a double helix or a specific nucleotide sequence. This allows not only compensatory substitutions in evolution but also the long-term preservation of primary structures.

A question arises as to whether the peculiar elements at the junction of 5.8S and the rest of the large subunit of ribosomal RNA allow pre-rRNA processing in metchnikovellids. The ITS2 region of three species of metchnikovellids with available cDNA libraries is covered by individual readings at a level close to the coverage of adjacent structural elements, while a decrease in the amount of cDNA and the number of reads in this region is observed during ITS2 processing, as seen in the example of a control (yeast cDNA library) (Fig. 4). Metchnikovellids show no evidence of read termination at the ITS2 region, while such abrupt termination is seen at the junction of ITS1 and 5.8S, and is accompanied by a sharp change in the level of coverage at the border (Fig. 4) and the presence of technical sequences (adapters) in a significant portion of readings (Fig. 5). The metchnikovellid *Amphiblysis* sp. also has no homolog of Las1 endoribonuclease (PF04031), which is known to be involved in ITS2 processing. This gene is not found in the known genomes of microsporidia *sensu stricto*, but



**Fig. 4.** High-throughput cDNA read coverage of the junction regions of 5.8S- and 28S-like rRNA of metchnikovellid microsporidia and yeast *Saccharomyces cerevisiae* with processed ITS1 and ITS2 regions. The top diagrams for each organism show the coverage with reads from transcriptome libraries; the lower diagrams show the ratio of the start points of reads to the total coverage; the predicted regions of 5.8S (blue) and 28S (red) rRNA genes are shown in color below the diagrams.

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RRR9888053.22006858.2 ▲ CAGAGT
RRR9888053.29646036.2 ▲ CAGAGTGAACGAAGTGAAGCGACGCCA
RRR9888053.4725979.2 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTG
RRR9888053.6492720.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAG
RRR9888053.30068891.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTC
RRR9888053.14088610.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTC
RRR9888053.25564814.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAA
RRR9888053.6492720.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTC
RRR9888053.25970309.2 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAA
RRR9888053.26206329.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACG
RRR9888053.3764031.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.27230621.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.28045731.2 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.24667357.1 ▲ CAGAGTGAACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.14411721.2 ▲ AACGAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.24769792.2 ▲ GAAGTGAAGCGACGCCAGTAAAAACGCACTGAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.24341515.2 ▲ GAGCGACGCTGGAAAAATTCGAGGAGAAAGAGAAAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.28700024.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.28039401.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.22685274.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.6858908.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.29284160.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.29157972.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.19281674.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.9363190.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.28086587.1 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.26474371.1 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.6703882.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.29157972.1 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.12689680.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT
RRR9888053.29758326.2 ▲ AAGCACTGGTATCAA-CG-CAGAGTACGG-AAACGCCATCAGTGGATCACTTGGTTTCTACGTCGAGGAAAGAACGCACT

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**Fig. 5.** Adapters at 5'-end of 5.8S rRNA of metchnikovellid *Metchnikovella dogieli*. The adapters indicate a termination of insert at 5'-end of 5.8S rRNA in many reads (as a result of ITS1 processing). Variability of 5'-end (AA or AAA or AAAA) is seen. No similar massive breaks at the junction of 5.8S- and 28S-like rRNA of metchnikovellids are observed, which indicates the absence of ITS2 processing.

it is present in the genomes of cryptomycots that possess an extended ITS2 region (*Mitosporidium daphniae* and *Paramicrosporidium saccamoebae*). All this points toward the absence of ITS2 processing in metchnikovellids and the fusion of mature 5.8S and 28S-like

rRNA into a single molecule in a manner similar to that in other microsporidia *sensu stricto*.

**Problem of *Chytridiopsis typographi*.** The genus *Chytridiopsis* Schneider, 1884 is known since the 19th century and includes the parasites of the intesti-



nal epithelium of insects (beetles, caddis flies). It was traditionally considered to be part of microsporidia; in some systems, they were brought closer to the metchnikovellids (Weiser, 1977; Larsson, 1993). Along with metchnikovellids, Chytridiopsida are distinguished by spores with a short polar tube and an undeveloped polaroplast. However, the tube is longer than that in metchnikovellids; it forms several turns and is covered with a honeycomb structure. There are two stages of sporogony in the life cycle that lead either to spores lying free in the parasitophorous vacuole or spores enclosed in a common cyst.

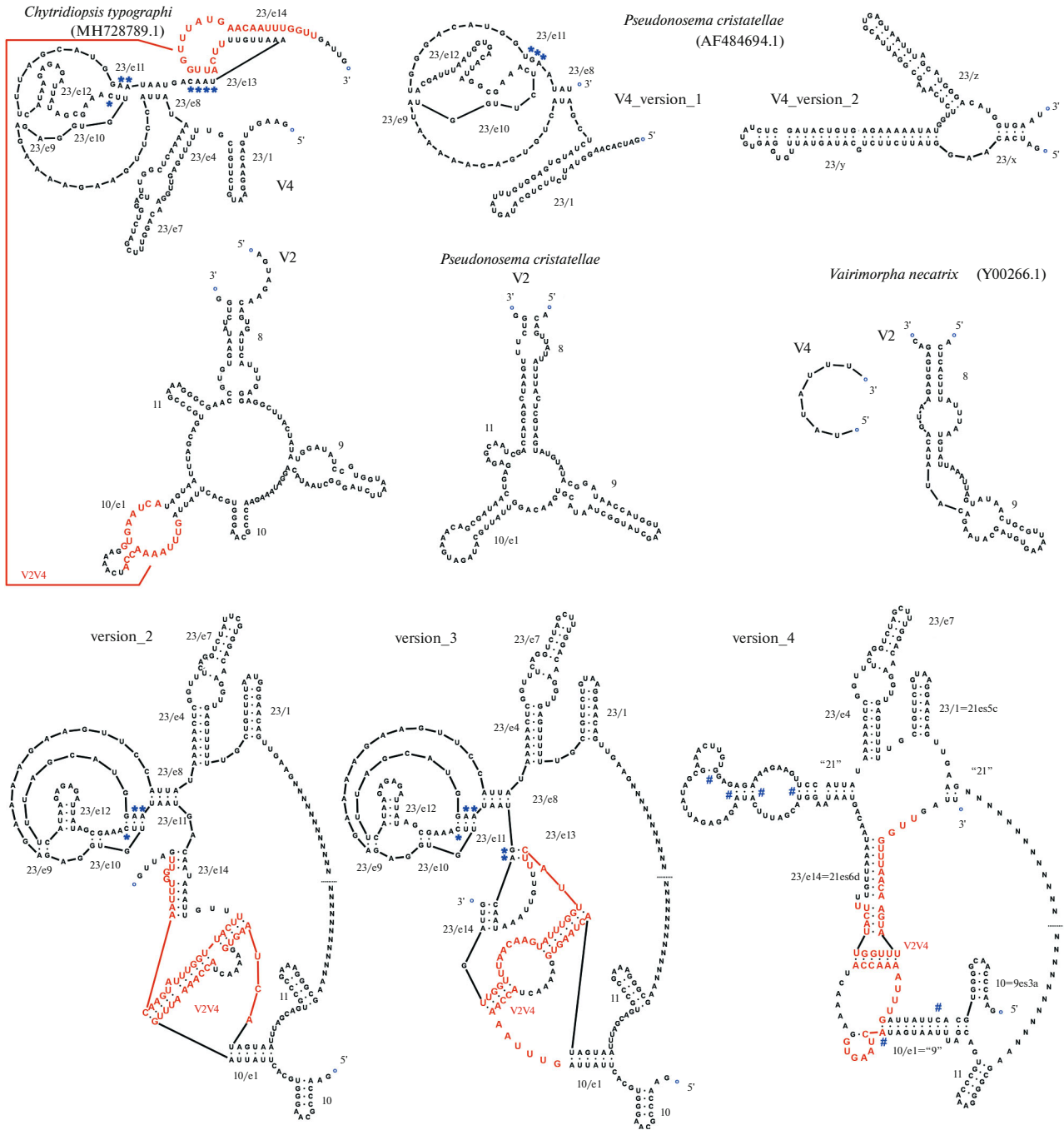
Analysis of the 18S rRNA nucleotide sequences of the first studied member of the genus, *Chytridiopsis typographi* (a parasite of *Ips typographi* bark beetle), placed it outside the limits of typical microsporidia (including metchnikovellids) and established it as a sister group (Corsaro et al., 2019). However, *C. typographi* 18S rRNA is more similar to typical eukaryotic rRNA in its general architecture. The V2, V3, V4, V7, V8, and V9 regions in *C. typographi* rRNA are not shortened as significantly as in typical microsporidia (Barandun et al., 2019). In typical microsporidia, the region of complementarity with V2 region (23/e13 and 23/e14 helices) is lost even in the least reduced state. The eukaryotic contact V2–V4 is predicted in *C. typographi* 18S rRNA (Fig. 6) (Alkemar and Nygård, 2003). As in most eukaryotes, the primary structure of *C. typographi* V4 region allows migration of branches between the 23/e13 and 23/e14 helices (Wuyts et al., 2000). Depending on this migration, the formation of the V2–V4 pseudoknot is another possibility (even a series of possibilities) of a “breathing” structure, as opposed to the “frozen” state, which can be assumed from crystallized ribosomes or cryoelectron microscopy data (Lee and Gutell, 2012; Petrov et al., 2014). The extended area of imperfect complementarity between regions V2 and V4 (Fig. 6, highlighted with the font) strongly indicates in favor of in vivo transition between several (more than two) alternative states than exclusively kissing-loops between small terminal loops of two stable 21es6d and 9 hairpins (Fig. 6, version 4).

Unlike typical microsporidia, *C. typographi* has B7 and B8 hairpins in the 5.8S rRNA 3'-region, and the size and structure of its ITS2 are close to typical. A structure with three hairpins can be selected from the spectrum of predicted ITS2 structures that are similar in the free energy of formation; among the three hairpins, the second (in order and length) has an internal U–U-loop, as should the canonical hairpin II (see different versions in Fig. 7 and in Corsaro et al. (2019, Fig. 5)). Hairpin IV is absent, as it is apparently in *Paramicrosporidium*. The absence of hairpin IV does not interfere with ITS2 processing in yeasts *S. cerevisiae* (Coleman, 2015). Information on related species and compensatory substitutions in ITS2 is required to verify the models of ITS2 secondary structures of cryptomycots. In general, it can be assumed that

*C. typographi* has an ITS2 structure that is close to the typical ITS2 structure, and, consequently, has ITS2 processing and separate 5.8S and 28S rRNA. The pleiomorphies of *C. typographi* in 18S rRNA, 5.8S rRNA, and ITS2 do not reject the hypothesis of a sister relationship with microsporidia *sensu stricto*, but evidence in the form of synapomorphies is required for its justification, and no such synapomorphies have been revealed.

We found no synapomorphies with typical microsporidia in the rRNA structure of *C. typographi* at the level of loops and helices, but we note multiple autapomorphies: deletions in helix regions 23, 24, 27, 28, 29 in 18S rRNA. They were partially indicated previously (Corsaro et al., 2019). Some of them affect one branch of the helix (23, 24) while preserving the typical size and nucleotide sequence of the other branch. Such violations should be confirmed by an independent study of other members of Chytridiopsida. In addition, many nucleotide substitutions in the primary structure of *C. typographi* rRNA lead it to be one of the longest branches in the microsporidian rRNA phylogeny, next in length after the branches of microsporidia *sensu stricto*. In such cases, long branch attraction artifacts can be suspected in erroneously uniting these sequences (Felsenstein, 1978; Hendy and Penny, 1989), and solid grounds are needed to exclude such possibility in relation to *C. typographi* and microsporidia *sensu stricto*. We were unable to achieve the chain convergence and stable topology of the microsporidian rRNA tree. Only some of tree elements found stable support, and the integration of *C. typographi* with typical microsporidia was not among them. We observed the inclusion of *C. typographi* into the clade previously designated by number X (Lazarus and James, 2015) in individual chains (MCMC) and even whole runs in the construction of the Bayesian tree of 18S rRNA. We obtained similar results when analyzing the 28S rRNA gene sequences. The same group X includes *Mitosporidium daphniae*, *Morellospora saccharomycetozoa* and other cryptomycots with immotile spores with polar tube and without flagellated zoospores in the life cycle. We did not observe inclusion of *C. typographi* in other groups with immotile spores—III (*Paramicrosporidium*) and *Nucleophaga*. Thus, we found no confirmation of a sister relationship between *C. typographi* and typical microsporidia and consider this issue requiring further study. It also raises a question of the place of other genera (*Intexta*, *Nolleria*) currently assigned to Chytridiopsida (but not yet studied with molecular-genetic methods) on the phylogenetic tree.

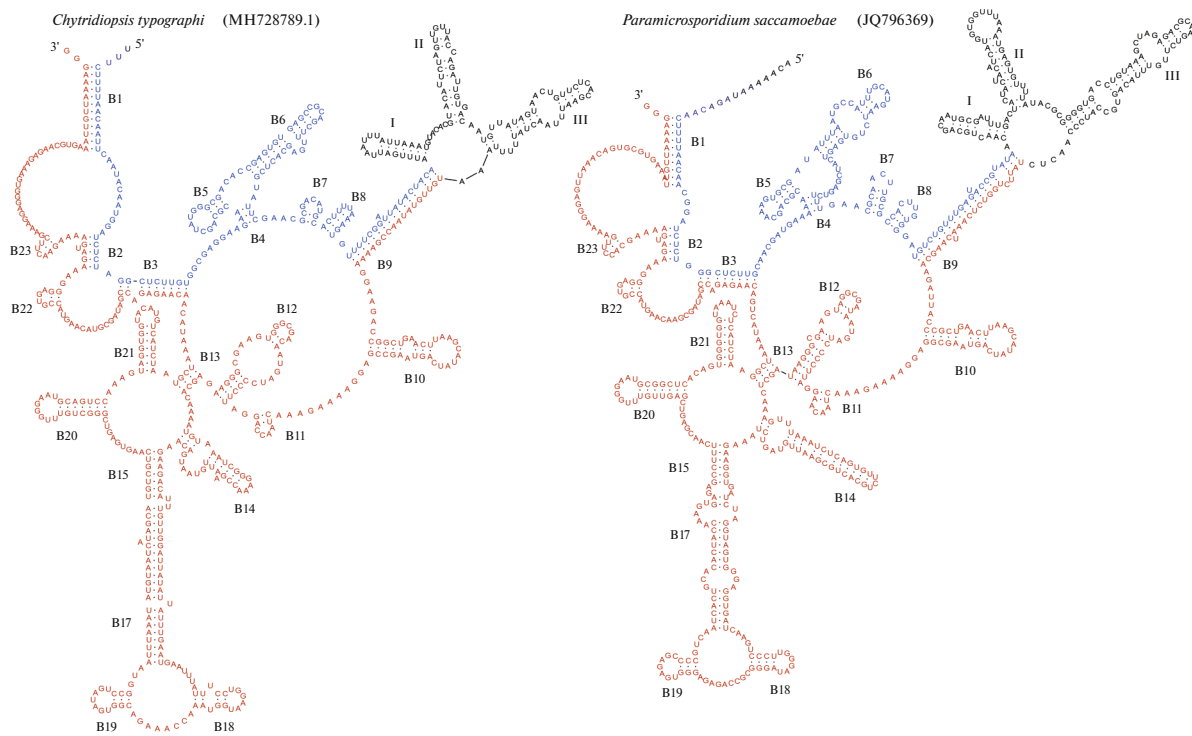
The *Chytridiopsis typographi* species was traditionally included in microsporidia, yet phylogenetically it belongs to one of the clades of the Cryptomycota grade. This illustrates the problematic differentiation of microsporidia *sensu stricto* and cryptomycots at the level of morphological and ultrastructural traits.



**Fig. 6.** V2 and V4 regions of 18S rRNA of *Chytridiopsis typographi* and two typical microsporidia. The probable contact zone V2–V4 (various versions) is marked in bold. \*, pairs predicted from covariation but not confirmed by the structures of model species; #, noncanonical pairs introduced to match the model (Lee and Gutell, 2012).

**Problem of BAQA065 (GenBank AF372825).** The BAQA065 sequence appeared in one of the first works on metabarcoding (Dawson and Pace, 2002), isolated from the bottom sediment of a shallow, brackish lagoon (Berkeley, United States). According to the tree reconstruction technique available at that time, BAQA065 was placed between the eukaryotic “crown” and long branches of microsporidia, diplo-

monads, and trichomonads (Archaezoa) on the constructed tree. Over the past 19 years, the BAQA065 5'-end was verified by two independent findings of similar sequences: in deep layers of coastal sediments of the North Sea and in a soda lake in India (DGGE band 280NS36E fragment, GenBank AM072563 (Wilms et al., 2006) and CL-10, GenBank JQ480022 (Antony et al., 2013)). The 3'-end has not yet been ver-

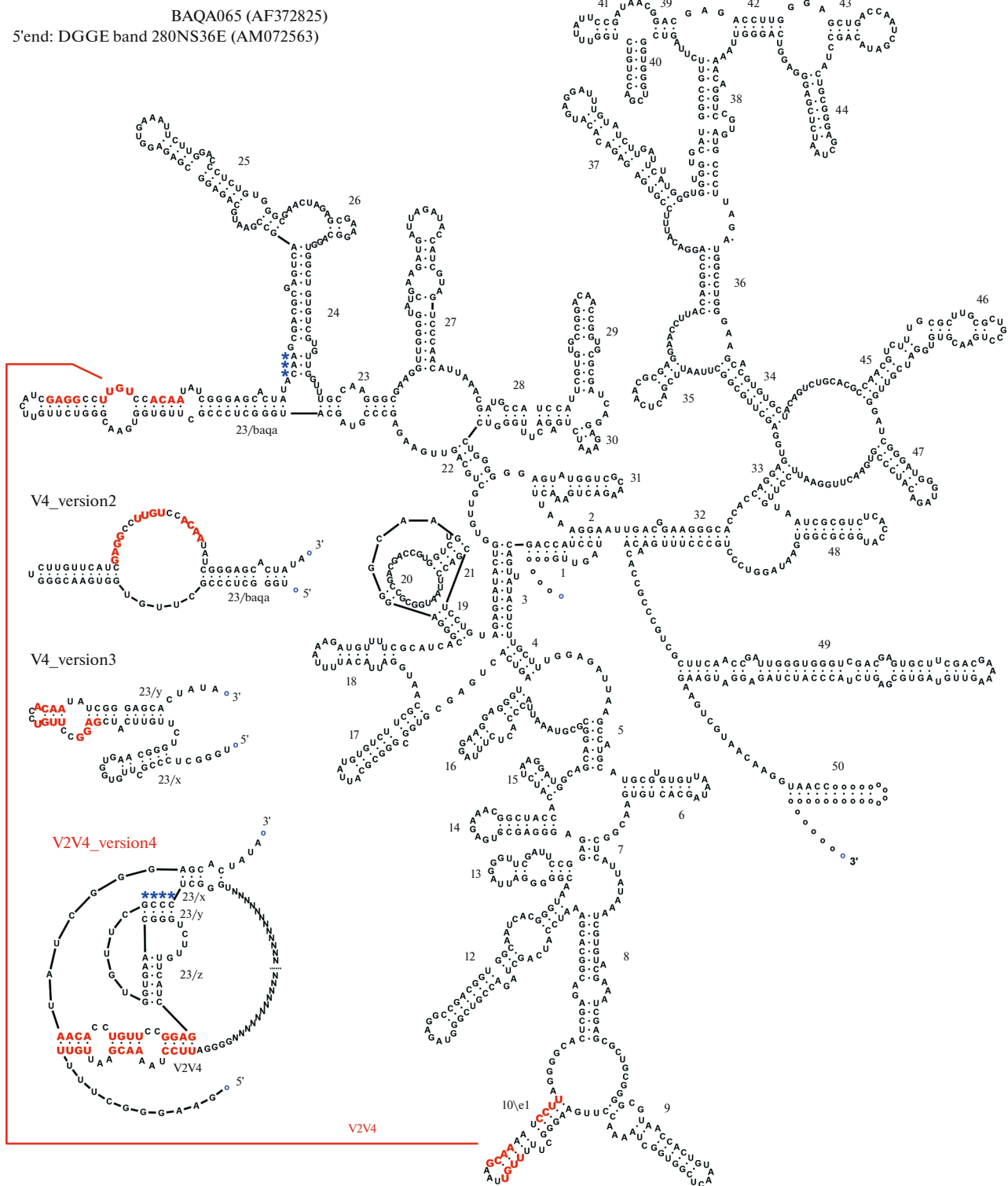


**Fig. 7.** Fragment of pre-rRNA (5.8S–ITS2–B domain of 28S rRNA) in two species of cryptomycots. The predicted hairpins I, II, and III correspond to a spacer (ITS2).

ified, which is consistent with the rarity of rRNA genes in amplicon libraries that have not been identified by phylum (kingdom) (Kim et al., 2016) (and, probably, the rarity of the cells carrying them). In later works, the clade BAQA065 appeared sister to typical microsporidia (Mikhailov et al., 2017, Fig. S1) and closer to microsporidia than *Chytridiopsis typographi* (Corsaro et al., 2019, 2020; Nassonova et al., 2021). Unlike *C. typographi*, the small subunit rRNA of BAQA065 has a size close to prokaryotic, with a mosaic of ancestral traits (uncharacteristic to typical microsporidia) and traits of typical microsporidia. The first includes the preservation of two long hairpins (17 and 18) in the V3 region and hairpin 46 in the V8 region. The second are sharply reduced V2, V4, and V7 regions—common features of typical microsporidia (Fig. 8). The V4 region is reduced by about a half as compared with those typical for eukaryotes. In the absence of similar sequences with compensatory substitutions, we were unable to recover such elements of the V4 region as pseudoknots (Wuyts et al., 2000). However, the large number of alternative folding variants indicates that the molecular dynamics predicted for the V4 region (Wuyts et al., 2000) are preserved in some form in BAQA065. For example, the weak pairs AU and GU make up 50% on the ambiguously folded helix region (Fig. 8, variants 1 and 2), whereas common regions contain 30% weak pairs and 70% strong GC pairs. The interaction V2–V4 cannot be excluded,

since there is an extended region with partial complementarity between them.

According to the primary structure of the conservative region at the base of the helix 49, the BAQA065 clone is similar to eugregarines of the Gregarinidae family (Fig. 9), rather than Opisthokonta (Cavalier-Smith and Chao, 2003; Aleshin et al., 2007). In addition to Gregarinidae, the same motif is found in the member of another family of eugregarines, *Ascogregarina taiwanensis* (Actinocephalidae). Other actinocephalid species are not similar to Gregarinidae. This example demonstrates that homoplasy in the considered region is a rare, but possible phenomenon. Thus, BAQA065 combines alternative signals: microsporidia (in the form of common apomorphic rRNA architecture) and eugregarines of Gregarinidae family (in the form of a short specific motif). In taxonomic sets without eugregarines, tree-constructing programs put BAQA065 as the closest branch to typical microsporidia on the constructed trees (Mikhailov et al., 2017; Corsaro et al., 2019, 2020; Nassonova et al., 2021). In the presence of Gregarinidae, BAQA065 occupies alternative positions in independent chains. Of the six independent runs (with four Markov chain Monte Carlo each), the clade BAQA065 grouped with typical microsporidia in two launches (a posteriori probability of 0.8 and 0.94) and with eugregarines of Gregarinidae family in four (a posteriori probability from 0.91 to 1.0). Naturally, the statistics of independent launches do not guide us toward the selection of a true tree in



**Fig. 8.** Predicted structure of small subunit of rRNA for BAQA065. The missing 5'-end was completed by the DGGE band 280NS36E clone; thus, the helices 2–6 are hybrid. The probable contact zone V2–V4 is highlighted by the font and connected by lines and is duplicated on the inset. \*, pairs predicted from covariation but not validated by the structures of model species (Lee and Gutell, 2012).

this case, but this result demonstrates a conflict of signals and erroneous reconstruction, at least in some launches. In the absence of BAQA065, related short sequences CL-10 and DGGE band 280NS36E lacking the problematic 3'-end occupy the place of a sister group of typical microsporidia with a posterior probability close to 1.0 (tree is not shown) and do not group with eugregarines. Despite the progress in the technique of rRNA phylogenetic analysis, the position of BAQA065 remains uncertain. A technical reason for the contradictions, such as chimeric nature of BAQA065, cannot be excluded (Berney et al., 2004). As noted above, the problematic 3'-end of BAQA065 is not yet verified. Some variant of molecular homoplasy is another possible reason. According to one scenario, evolutionary early microsporidia *sensu stricto* (BAQA065) changed the conservative motif in helix 49, along with many other autapomorphies. This scenario is consistent with the position of CL-10 and DGGE band 280NS36E in the absence of BAQA065, which is close to microsporidia *sensu stricto*, and raises questions regarding the reason for the evolutionary conservatism of a small element on the scale of all Opisthokonta and the circumstances of the termination of this state in certain species (BAQA065). According to another scenario, similar reductions of the same rRNA variable regions occurred in phylogenetically unrelated eukaryotes: Holomycota (typical microsporidia) and Gregarinidae (BAQA065). Here, a question is raised about the functional characteristics of ribosomes without these elements. It is necessary to note that a strong reduction of the V2, V4, V7 regions (and often also V3 and V9) is characteristic not only of microsporidia but also of unrelated parasitic species: diplomonads, parabasalids, ascetosporidia of *Mikrocytos* genus, fungi of *Neozygites* genus (Fig. 10). These regions also show a slight reduction in some other parasitic species (in archigregarines of genus *Selenidium*, nematode genus *Pelodera*). In most specialized parasites, mitochondria, the rRNA is maximally reduced (in multicellular animals, kinetoplastids, sporozoans by two to three times relative to microsporidia). Ribosomes with such rRNA are capable of protein synthesis, but the diversity of mRNA that they have to translate is thousands of times less than the diversity of mRNA in the cytoplasm.

**RL107-1 (GenBank: FN546176).** If the BAQA065 clade is not taken into account, the RL107-1 organism is the closest relative of typical microsporidia (Fig. 1). Its DNA was extracted from highly acidified water of flooded coal quarry in Germany with a very poor eukaryotic population (Huss and Bauer, 2011). According to the size and other traits, the small subunit of ribosomal RNA of the RL107-1 is intermediate between typical eukaryotic 18S rRNA and prokaryotic type microsporidian rRNA. Helices 46 and 17 are present (the latter is shortened). Helix 43 is shortened and helix 23/e4–23/e7 is completely lost in the V4 region, while helix 23/1 is reduced to 4 base pairs.

Despite the reduction of the V2 and V4 regions, the possibility of contact between them due to RNA complementarity site in RL107-1 is preserved (unlike typical microsporidia) (Fig. 11). Such contact is most likely realized not on a permanent basis but as a variant of intramolecular rRNA rearrangements (Wuyts et al., 2000), as in other eukaryotes. Thus, RL107-1 is closer both phylogenetically and by structural traits of rRNA to typical microsporidia than *Chytridiopsis typographi*.

There is no information about the RL107-1 structure or way of life, or whether their DNA was obtained from cells vegetating in an acid lake or resting cells (brought from the coast). It is unknown whether the RL107-1 sequence originates from an undescribed organism or belongs to some known species of primitive microsporidia with an undetermined rDNA nucleotide sequence. No rDNA similar to RL107-1 was found in the libraries from other natural substrates.

#### **Other possible relatives of typical microsporidia.**

The reconstruction of the phylogenetic tree of microsporidia *sensu lato* is at the very beginning. This group is sister to fungi, which means that it is as ancient as the kingdom Fungi and similarly (if not more) differentiated at the level of primary structures. By the heterogeneity of molecular evolution rates, it seems to cover the entire known range for eukaryotic life, since it includes the most rapidly evolving typical microsporidia, the rRNA of which is so derived that it was previously considered separate from the eukaryotic type (Vossbrinck et al., 1987). An unambiguous alignment of some rRNA regions is impossible. The taxonomic sample of microsporidia is extremely rarefied, which leads to an underestimation of the genetic distances and additionally complicates reconstruction. Only rRNA gene fragments are known for their many groups of high-ranking microsporidia (other markers are not yet available). It is unlikely that rRNA genes will be enough for a reliable reconstruction of all nodes of the microsporidian phylogenetic tree, especially under such complicating circumstances.

Up to two dozen clades constitute the cryptomycota (Lazarus and James, 2015; Chouari et al., 2017; Matsubayashi et al., 2017; Tedersoo et al., 2017). Their phylogenetic relationships are not yet reconstructed reliably (with few exceptions). In particular, it is unknown which clades are closer to microsporidia *sensu stricto*. In the constructed trees, these are, generally, the longest branches (in different combinations): *Chytridiopsis typographi*, *Nucleophaga* spp., *Paramicrosporidium* spp., LKM118, LKM15, MPE1-21, clade VIII, clone Banisveld P2-3m12, GS03 (clone GL48063.220.S131), and etc. (Corsaro et al., 2016, 2019, 2020; Grossart et al., 2016; Stentiford et al., 2017; Tedersoo et al., 2017; Bass et al., 2018). In our reconstructions, most of previously identified candidates occupied a position close to typical microsporidia in individual runs of the MrBayes program.

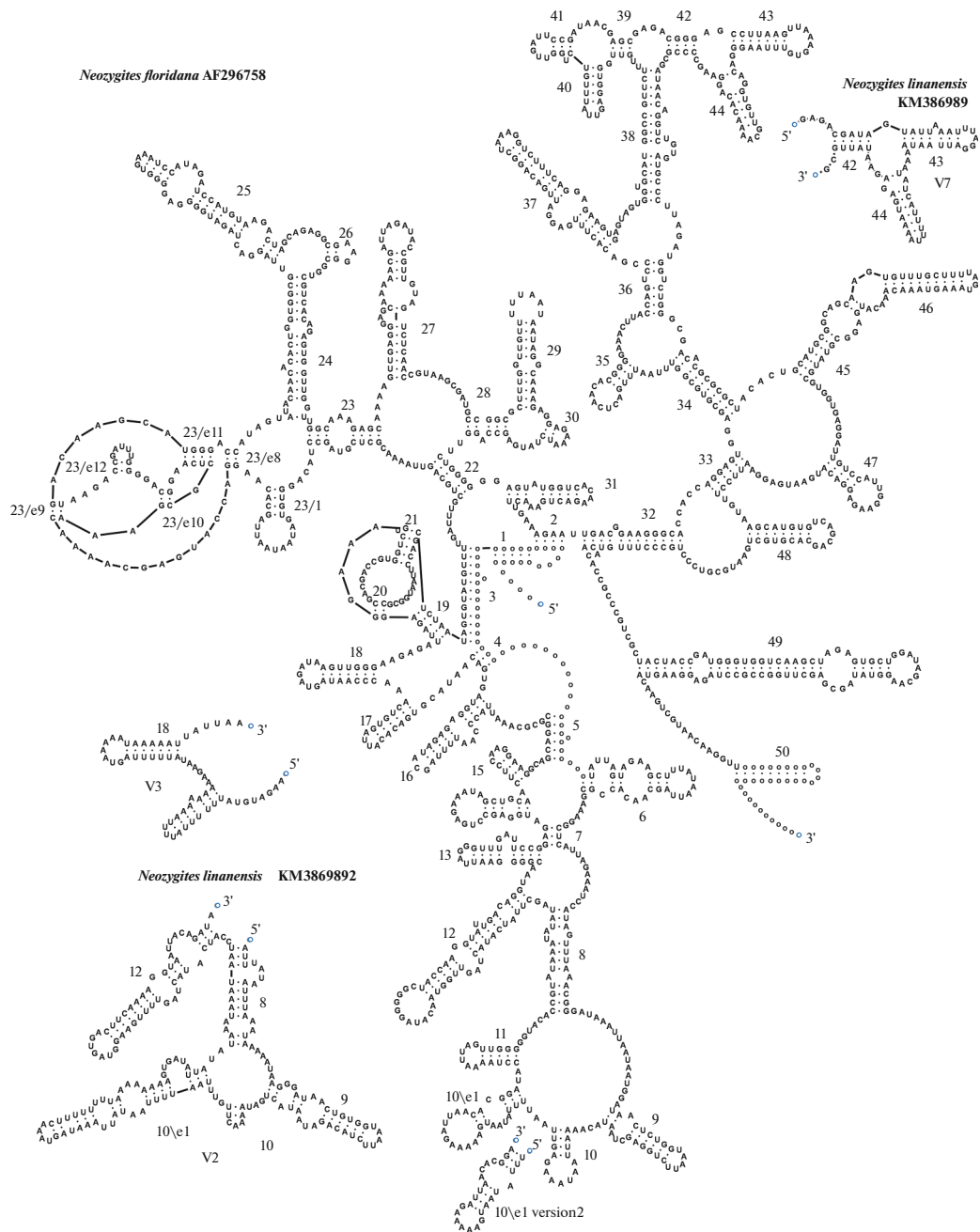
<i>Colpodella edax</i> (AY234843)	CCGTCGCTCCTACCGATT . . . AGAGGAAGGAGAAGT
<i>Adelina dimidiata</i> (DQ096835)	CCGTCGCTCCTACCGATT . . . AGAGGAAGGAGAAGT
<i>Cryptosporidium serpentis</i> (AF151376)	CCGTCGCTCCTACCGATT . . . AGAGGAAGGAGAAGT
<i>Stylocephalus giganteus</i> (FJ459761)	CCGTCGCTCCTACCGATT . . . AGAGGAAGGAGAAGT
<i>Ascogregarina taiwanensis</i> (EF666482)	CCGTCGCTTCAATCGACT . . . GAAGGATGAAAAAGT
<i>Gregarina niphandrodes</i> (AF129882)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
<i>Blabericola migrator</i> (FJ459754)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
<i>Amoebogregarina taeniopoda</i> (MK181531)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
<i>Gregarina ormierei</i> (KJ736741)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
<i>Enterocystis dorypterygis</i> (KY697695)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
BAQA065 (AF372825)	CCGTCGCTTCAACCGATT . . . AGAGGATGAGAAGT
RL107-1 (FN546176)	CCGTCGGTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Metchnikovella dogieli</i>	CCGTCGATACTACCGAT . . . AGAGGAAGTAAAAAGT
<i>Amphiamblys</i> sp. WSBS2006 (KX214672)	CCGTCGATACTACCGAT . . . AGAGGAAGTAAAAAGT
<i>Amphiacantha</i> sp. (KX214676)	CCGTCGATACTACCGGTT . . . CGAGGAAGTAAAAAGT
<i>Nematocida parisii</i> (GL501349)	CCGTCGCTATCTGAGAT . . . AGATTGGATAAAAAAGT
<i>Ovavesicula popilliae</i> (EF564602)	CCGTCGCTATCTAAGAT . . . AGATCGGATAGAAGT
<i>Antonospora locustae</i> (AY376351)	CCGTCACTACCTAAGAT . . . AGATAAGGTATAAGT
<i>Hamiltosporidium magnivora</i> (AY649786)	CCGTCGTTATCTAAGAT . . . AGATTGGATACAAGT
<i>Enterocytozoon bieneusi</i> (AF023245)	CCGTCGCTATCTGAGAT . . . AGATAAAGTACAAGT
<i>Vairimorpha necatrix</i> (DQ996241)	CCGTCGCTATCTAAGAT . . . AGATCTGATATAAGT
<i>Chytridiopsis typographi</i> (MH728789.1)	CCGTCGATACTACCGATG . . . AGCGGGTTTCCCCA
<i>Nucleophaga amoebae</i> (JQ288099)	CCGTCGATACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Paramicrosporidium vannellae</i> (JQ796368)	CCGTCGCTACTACCGAAT . . . AGCCAAAGTAAAAAGT
<i>Mitosporidium daphniae</i> (JMKJ01000175)	CCGTCGATACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Rozella allomycis</i> (AY635838)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Cladochytrium replicatum</i> (AY546683)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Allomyces arbuscula</i> (AY552524)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Mucor hiemalis</i> (FJ605511)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Saccharomyces cerevisiae</i> (J01353)	CCGTCGCTAGTACCGATT . . . AGAGGAAC TAAAAAGT
<i>Nuclearia simplex</i> (AF349566)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Monosiga brevicollis</i> (AF100940)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT
<i>Homo sapiens</i> (KY962518)	CCGTCGCTACTACCGATT . . . AGAGGAAGTAAAAAGT

**Fig. 9.** Alignment fragment of small subunit ribosomal RNA genes from Opisthokonta (including microsporidia), sporozoans (including eugregarines), and BAQA065 and RL107-1 sequences. The state specific to Opisthokonta is given on a gray background; the state specific to Gregarinidae is on a black background. The ellipsis symbolizes missing nucleotides.

However, in other, independent launches, all of these candidates would be placed far from typical microsporidia; moreover, the alternative positions received high values of a posteriori probability in specific launches. Thus, the convergence of topologies was not achieved in our phylogeny reconstructions based on rRNA genes, despite the large number of MCMC generations (more than 10000000). We used a large number of categories (10, 16) and sets of more than 150 operational taxonomic units to take into account the heterogeneity of evolutionary rates in rRNA. A large number of categories creates the danger of so-called overparametrization and poor convergence. However, the use of a small number of categories led to a low resolution (low values of a posteriori probabilities of many

clades). Variation of the *temp* parameter (in the range from 0.02 to 0.5) also did not make it possible to achieve convergence. Apparently, many tens of millions of MCMC generations are required for the convergence of 18S rRNA tree topology.

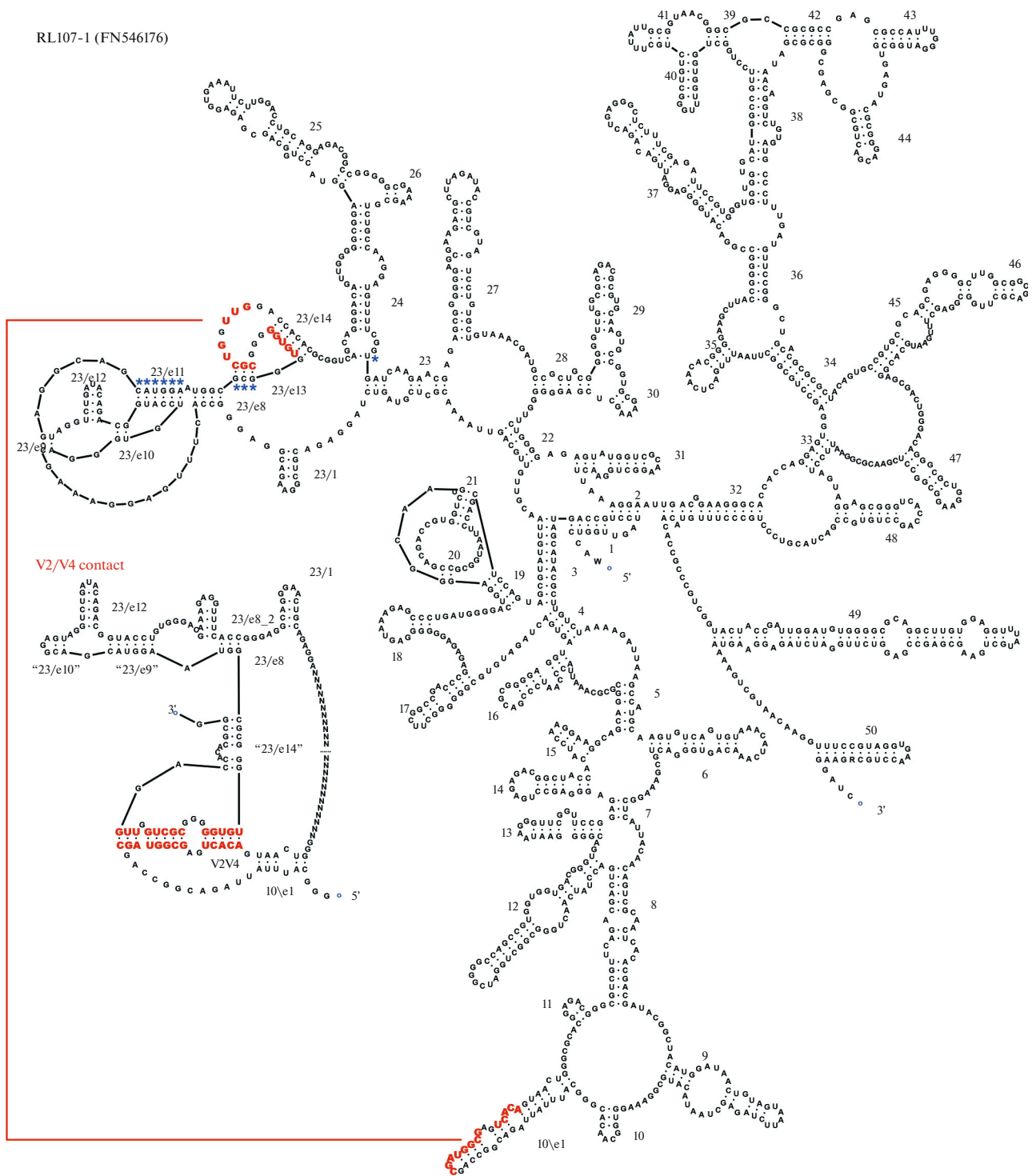
Large amounts of data (Mueller et al., 2014; Taylor et al., 2014; Timling et al., 2014) or single sequences (Smith et al., 2007; Taylor et al., 2007, 2008; Eichorst and Kuske, 2012; McGuire et al., 2013; Lipson et al., 2014; Wurzbacher et al., 2014; Page and Flannery, 2018) of ITS and fragments (less than 1 kb) of 28S rRNA gene of cryptomycots were obtained in some works on metabarcoding. In a Bayesian tree with a selection of these sequences (Fig. 12), the location of large groups of cryptomycots largely coincides with



**Fig. 10.** Predicted structure of small subunit rRNA of *Neozygites*. The parasitic fungi Neozygitomycetes (Entomophthoromycotina) are not related to microsporidia (White et al., 2006) but both have a similar (although not identical) reduction in rRNA variable regions (Freimoser, 2000).

those in 18S rRNA tree, which can be deduced by the amplicons overlapping both genes (Weber et al., 2009; Tedersoo et al., 2017; Jamy et al., 2019) or by long contigs from metagenomic assemblies (Mitchell et al., 2018; Sanchez and Cao, 2019; Cotto et al., 2020; Sharrar et al., 2020; Thornton et al., 2020). In the construction of the 28S rRNA tree, problems with the convergence of topology arise (as with 18S rRNA). Some especially long branches do not find the correct position. For example, one of the early Holomycota

clades, GS01 (Tedersoo et al., 2017), on the 28S rRNA tree branches off near the root of typical microsporidia (Fig. 12) instead of grouping with BCGS2, as observed in some of our launches and in published trees (Wijayawardene et al., 2019). The position of GS01 can be attributed to the long branch attraction artifact, which manifests itself in 18S rRNA and 28S rRNA trees; however, some other differences between 18S rRNA and 28S rRNA trees are probably caused by the additional phylogenetic signal, which allows better resolu-



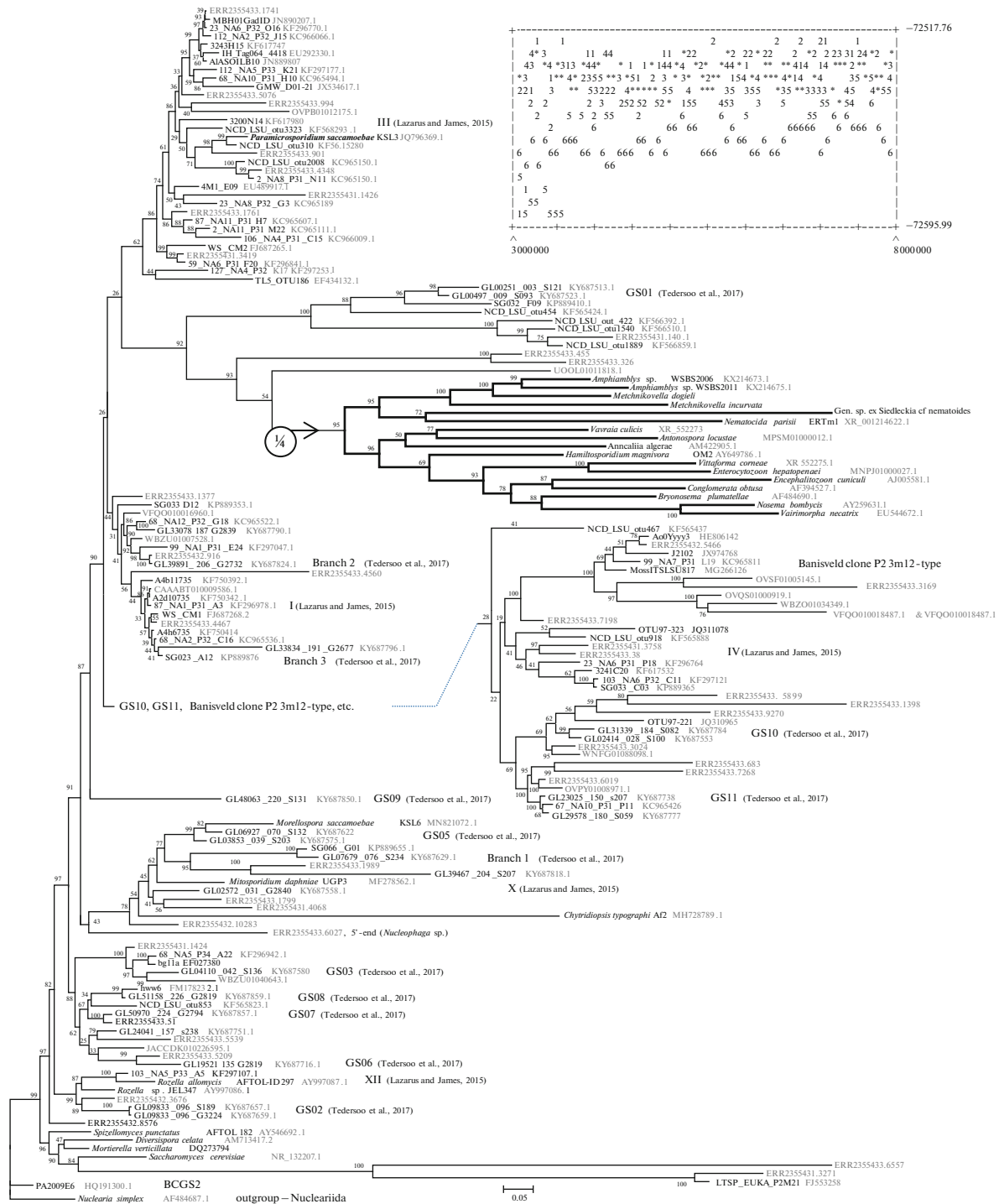
**Fig. 11.** Predicted structure of small subunit rRNA of RL107-1 clone. The probable contact zone V2–V4 is highlighted by the font and connected with lines and is duplicated on the inset. \*, pairs predicted from covariance but not validated by the structures of model species (Lee and Gutell, 2012).

tion of some nodes by 18S rRNA and others by 28S rRNA.

The adaptation of high-throughput sequencing methods to obtain the extended contigs and the assembly of metagenomes opens new prospects for

metabarcoding and phylogenetic analysis due to the better accounting for the diversity and the recruitment of data on 18S rRNA and 28S rRNA genes without the need for isolation of laboratory cultures. Dozens of operational taxonomic units of cryptomycots (repre-





**Fig. 12.** Bayesian tree of concatenated 5.8S and 28S rRNA of microsporidia, including cryptomycots (consensus of the top four runs, nst = 6, ngammacat = 10, rates = invgamma, temp = 0.1, ngen = 8000000). The log-likelihood values in the top six launches (out of 12) are on a scatterplot. The posterior probabilities are expressed as a percentage values. The branch lengths in the subtree of typical microsporidia are reduced by four times (wide lines).

senting most of clades previously known from rDNA) were discovered in a single study using high-throughput sequencing (Jamy et al., 2019), as well as some new, previously unencountered sequences. Some of the new clades in the 18S rRNA tree are formed by

especially long branches, of which ERR2355431.3271 and ERR2355433.6557 are closer to microsporidia *sensu stricto* than *Chytridiopsis typographi* and all other previously considered candidates (according to the results of at least some runs of MrBayes program),

with the exception of RL107-1 (Fig. 1). One of these sequences (ERR2355431.3271) coincides with earlier findings (GenBank nos. FJ553258.1, FJ553594.1), containing an internal transcribed spacer with short flanking gene regions (Hartmann et al., 2009). According to the general characteristics of rRNA structure and ITS regions (the size of V2, V3, V4, V7 18S rRNA regions and the B7 and B8 hairpins in the 5.8S rRNA and 3'-region, ITS2), these organisms maintain a plesiomorphic state. They do not share the autapomorphies of *C. typographyi* in rDNA organization and do not group with this species in the tree. Another candidate represented by a UOOL01011818 contig from the metagenome of activated sludge (Mitchell et al., 2018) shows a greater affinity to typical microsporidia in the tree with the large subunit rRNA sequences, than in one with the small subunit sequences (Figs. 1 and 12). It is possible that some of the current candidates are, in fact, close relatives of microsporidia *sensu stricto*, but a more rigorous testing of this hypothesis is needed for each candidate. The combination of data on two rRNA genes is promising for the phylogenetics, but it requires verification of the deposited contigs. For example, the ERR2355433.6027 contig in the region corresponding to 18S rRNA is 95% identical to *Nucleophaga terricola* KTt-1 and 94% identical to *N. amoeba* KTq-2 and apparently provides information about the 5'-end of the *Nucleophaga* sp. 28S rRNA gene (Fig. 12). However, approximately 1000 nucleotides of 28S rRNA gene at the 3' end of the contig most likely originate from one of the tardigrade species, judging by sequence similarity. The successful identification of such chimeras is limited by a lack of information about the nucleotide sequences of the 28S rRNA gene.

**Problem of molecular evolution rates.** The ribosomes of typical microsporidia differ greatly from the ribosomes of other eukaryotes. Significant differences from typical rRNA in the primary and sometimes in secondary structure were also found in some other taxa (in many or all studied members): Excavata (*Giardia lamblia*, *Trichomonas vaginalis* and many others), Ascetosporidia (*Mikrocytos mackini*, *Marteilia*, and etc.), Foraminifera, some Amoebozoa, haemosporidia, and some other sporozoans (*Plasmodium*, *Trichotokara*, *Trollidium*, Cephaloidophoroidea, etc.), some animals (myxosporidia, dicyemids, orthonectids, fan-wing insects, many nematodes, from which *Pelodera*, *Riouxgolvania* genera are distinguished), etc. (Leipe et al., 1993; Smothers et al., 1994; Pawlowski et al., 1994, 1996; Chalwatzis et al., 1995; Fitch et al., 1995; Katiyar et al., 1995; Whiting et al., 1997; Carnegie et al., 2003; Rueckert et al., 2011, 2013; Hasegawa et al., 2012; Wakeman, 2020). Many, though not all, of them are parasites. A question arises as to why the ribosomes are strongly changed in these groups. Although the ribosomes also perform some specific functions, protein synthesis is still their main and universal function, and it is difficult to imagine that nat-

ural selection would greatly modify the ribosomes of microsporidia or other organisms to perform some special function. The assumption of the adaptability of mass differences in rRNA does not fit into the basic concept of the prevailing role of neutral events in molecular evolution (Kimura, 1985). In species with highly modified rRNA, other universal molecular machines (evolutionary conservative structural proteins and enzymes, including elements of the cytoskeleton, DNA replication apparatus, metabolic enzymes, etc.) are usually also highly modified. Such a picture indicates an increased rate of molecular evolution of not only rDNA but of most of the genome in some groups of related species. There must be a common reason for the acceleration of their molecular evolution over a long period of time, which we would like to know.

Intracellular parasites live “on everything ready”; they take many metabolites from the host instead of their independent biosynthesis. The genes responsible for the synthesis of these substances are exempt from selection. They change under the influence of mutations and eventually are completely lost from the genome. However, this circumstance does not seem to have a direct effect on the functions of ribosomes or the evolution rate. Other common factors do affect the parasites, e.g., those that reduce the effective population size ( $N_e$ ): a low population size limited by the host population size; periodic sharp declines in the population size according to Lotka–Volterra model; the limitation of panmixia due to inbreeding in subpopulations in the body of a single host; and the prevalence of asexual reproduction (adaptation of the parasite to a low intensity of invasion). A neutral drift should proceed at the same rate in small and large populations: the rate of fixation in some is compensated by a proportionally greater number of mutations in others (Kimura, 1985). However, mutations that are slightly harmful (exposed to selection) in large populations become effectively neutral in small populations (upon achievement of the selection coefficient threshold of  $|s| \leq 1/(2N_e)$ ), i.e., they are fixed with the dynamics of neutral ones (Kimura, 1985). They increase the pool of neutral mutations in small populations. A decrease in the frequency of sexual process due to asexual reproduction in the parasites slows the recombination, including the appearance of several slightly harmful mutations in one individual, and therefore, slows the purifying selection against slightly harmful mutations and increases the probability of their fixation. Microsporidia are a living example of the implementation of the Muller’s ratchet. They gradually and irreversibly destroy their conservative molecular structures, including ribosomes (Melnikov et al., 2018a); moreover, a decrease in the efficiency of the translation apparatus of microsporidia was confirmed by the experimentally detected decrease in the translation accuracy (Melnikov et al., 2018b). To correct defective proteins, additional costs for the synthesis of new mol-

ecules for replacement or ATP costs for the work of chaperones are required. Although the parasite draws resources from the host, it seems that, instead of correcting translation errors at their expense, it could direct these resources for the preparation of several additional spores and thus increase the number of descendants, i.e., increase its adaptability and win (with a certain ratio of  $s$  and  $N_c$ ) over competitors with a less-accurate translation apparatus.

If parasitism is the reason for the high rate of microsporidia evolution, two other questions are raised. All known microsporidia *sensu lato* are intracellular parasites. Although an indisputable reconstruction of the lifestyle of their closest common ancestor has not yet been completed, the hypothesis that it was an intracellular parasite is the most cost effective (based on available data) (Aleshin et al., 2015). Then why did the Muller's ratchet almost destroy the ribosomes of typical microsporidia and had little effect on the ribosomes of *Rozella* and other cryptomycots during the same time of evolution from a common ancestor? There is no answer to this question yet. The second question asks why typical microsporidia with optimized, almost destroyed ribosomes are in a state of biological progress.

**Reduced and lengthened rRNA genes.** Typical microsporidia possess reduced rRNA. Among distant relatives of microsporidia (true fungi), greatly shortened and extremely divergent rRNA are known only in *Neozygites* species (Entomophthoromycotina: Neozygitomycetes), which are similar to microsporidia in their lifestyle, and parasitize small arthropods (Fig. 10) (Freimoser, 2000; Delalibera et al., 2004; Zhou et al., 2017). However, many lichen and parasitic fungi have lengthened rRNA genes (DePriest and Been, 1992; Gargas et al., 1995). Gene elongation occurs due to self-splicing group-I introns. There is currently no evidence that group-I introns encode microRNAs, regulatory elements or genes that might be useful for the host, unlike some spliceosomal introns in mRNAs (review: Chorev and Carmel, 2012). Group-I introns are inserted only into the conserved rDNA regions. They are removed during rRNA maturation and do not enter the ribosome, and the primary structures of such rRNA are not characterized by an excessive evolutionary rate. Group-I introns are found in different eukaryotes (Roger, 2019), including free-living ones, but the intensity and extensiveness of their colonization of rRNA genes is not the same. For example, they are common in rRNA genes of lichen algae. Unlike lichen fungi, many algae live both in a lichen association and in a free state. The hypothesis of intron transfer (mutual re-infection of parasites and hosts with introns as a part of an association) was put forward, but it was not generally supported, since most algal introns are similar to other algal introns, while fungal introns are similar to introns found in other fungi (Bhattacharya et al., 1996, 2002; Karpov et al., 2019). The rRNA accounts for 50–80% of all transcription in

mammalian and yeast cells (Paule, 1998; Warner, 1999; Moss and Stefanovsky, 2002); the level of rRNA synthesis affects the cell physiology, growth rate (cell cycle duration), and division and differentiation (Warner, 1999; Russell and Zomerdijk, 2005; Noack Watt et al., 2016). At the same time, the level of rRNA synthesis (as was repeatedly shown) is limited by the copy number of rRNA genes (Stevenson and Schmidt, 2004; Roger, 2019; and etc.); consequently, it can be expected that fewer mature rRNAs will be produced per unit time from the same number of rRNA genes loaded with introns (there are no observations for group-I introns that their presence increases the expression level, as was repeatedly reported for spliceosomal introns in mRNA). In some species of lichen and parasitic fungi, group-I introns account for up to half of the length of rRNA genes (Gargas et al., 1995; Karpov et al., 2017), and, therefore, the transcription of introns takes up to 20–30% of all transcription costs. A question arises as to whether these energy costs are significant and whether the expansion of group-I introns can be attributed to the random drift of a neutral trait. Even when the luxury of the maintenance of group-I introns is transferred to the host, why should the parasite keep the transcription of useless DNA regions, instead of increasing the production of functional molecules and, eventually, the number of spores, i.e., the number of descendants, which is equivalent to an increase in the adaptability? There are approximately  $2 \times 10^5$  ribosomes in a *S. cerevisiae* yeast cell. With active growth, the yeast divides every hour and a half; thus, at least 33 rRNA copies per second per cell are synthesized (Warner, 1999), and this value is underestimated, since it does not take into account ribosome decay. Taking the price of the synthesis of ribonucleotide triphosphate in 12 macroergic bonds for a cell anaerobically fermenting glucose on a minimal medium (Wagner, 2005), we obtain the price of transcription of a typical group-I intron at  $12 \times 350 \times 33 = 1.4 \times 10^5$  macroergs per second per cell or approximately  $1.4 \times 10^5 / 1.34 \times 10^7 = 0.01$  of the total energy costs of the yeast cell for transcription (Wagner, 2005), which coincides up to an order of magnitude with the estimation obtained simply from the ratio of the lengths of one intron to the pre-rRNA and rRNA contribution to common transcription. These costs must be attributed to the total energy costs of the cell. According to available estimations, the costs for RNA synthesis account for 5–10% of the costs for protein synthesis (Wagner, 2005), and, together, the synthesis of RNA and protein accounts for up to 76.6% of the total ATP consumption of the cell (Förster et al., 2003). Thus, the transcription of one group-I intron consumes about  $3.8 \times 10^{-4}$  of the energy costs of yeast cells. When the growth rate is limited by the available energy (nutrients), this will slow the growth rate (production of descendants) by the same value,  $3.8 \times 10^{-4}$ . For wild *Saccharomyces* species, the effective popula-

tion size  $N_e$  is estimated as  $1.36 \times 10^7$  (Wagner, 2005); consequently, the trait cannot be neutral for a haploid population when it exceeds the threshold value of the selection coefficient  $|s| = 0.73 \times 10^{-7}$ , and its fate in the population will be determined primarily by selection, not by random drift. The price of even one group-I intron exceeds the threshold value  $s$  for yeast by about three orders of magnitude; consequently, the existence of introns in rDNA cannot be explained by a neutral trait drift, especially for a frequency of more than 0.1%. Applied to other species, other values of the  $s$  selection coefficient can be expected. For example, parasites importing nucleotides or their precursors from the host cytoplasm instead of ATP for complete synthesis can manage the cost of carriers, thus reducing the cost of translation by an order of magnitude, and this can already explain the survival of the owner of one intron in rRNA due to chance in 1% of species at the same  $N_e$  value. More importantly, the growth and reproduction of the parasite (and in general of any species) can be limited not by nutrients but by some particular metabolite (vitamin, essential amino acid, essential fatty acid, and etc.) or specific mineral substance. In this case, the proportionality between  $s$  and the energy consumption for the reproduction will be violated. Lastly, the threshold  $s$  values change at a low effective population size ( $N_e$ ), which can make the presence of introns not so improbable, even if the reproduction is limited by food.

Unlike the hypothesis of the initial morphological diversity (Mamkaev, 1968), the transformation of degrading organs or macromolecules into various remnants due to the destruction and modification of nonessential regions is intuitively clear. For example, a variety of eye defects are observed in blind species. Reduced and lengthened rRNA genes seem to be different variants of fixation of slightly harmful traits due to drift (Melnikov et al., 2018b). However, a wide distribution of modification of rRNA genes in the parasites raises the question of whether the adaptive component is hidden in these modifications. Based on the calculations above, we can assume that shortening of rRNA length in microsporidia gives them significant savings in transcription, which more than covers the cost of the reduction of the translation accuracy (a question then arises as to why other species do not optimize the ribosome). The parasites usually benefit from the production of large numbers of eggs or spores. However, there can be situations in which the spore loss cannot be covered by a one-time mass production out of season. The probability of infection is not always associated in a simple way with the season. Like 17-year cicadas, the hosts sometimes slow the passage of their life cycle in order to desynchronize it with the parasite cycles. Using the example of temperate bacteriophages, it is known that lysogeny can be a no less efficient strategy for the parasite than the productive infection. The reduced ribosomes of

microsporidia or the lengthened rRNA genes of parasitic fungi presumably slow down the development of the parasite, and stretch the period of spore production; the latter due to an excessive load on the transcription apparatus, and the former as a result of the reduced efficiency of translation (Melnikov et al., 2018b). We observed the continuous (long-term) coexistence in the laboratory tube of filamentous yellow-green alga *Tribonema gayanum* and a parasite (parasitoid) *Sanchytrium tribonematis* with highly intronized rRNA genes (up to six introns in 18S rRNA gene, up to nine introns in 28S rRNA gene), although the dependence of the rate of development on the number of group I introns was not studied. The presence of introns in rRNA genes of free-living species can be considered an argument against the association of the expansion of group I introns with a parasitic way of life. Although the introns in free-living species are not as frequent ( $\sim 10^{-3}$ – $10^{-2}$  at the average per species) and the number of introns rarely exceeds one to two per rRNA gene, there are examples, such as myxomycetes in Amoebozoa, which possess no fewer or even more introns than the parasitic fungi (both by the proportion of species carrying the introns and the number of introns per rRNA gene). It is difficult to think of a reason why myxomycetes would need to constitutively slow their life cycle. The proposed means of slowing development due to introns or ribosome defects are not regulated and do not have a noticeable benefit in the presence of subtle means for the regulation of rRNA synthesis (Warner, 1999; Moss and Stefanovsky, 2002; Engel et al., 2013; Torreira et al., 2017; Fernández-Tornero, 2018), including those at individual stages of growth or with quorum sensing (Najmi and Schneider, 2021) and with an accuracy that guides the cell differentiation in embryonic development (Noack Watt et al., 2016), the regulation of ribosome biogenesis (Chaker-Margot, 2018), and the regulation of the work of ready ribosomes (Usachev et al., 2020), including that in the known microsporidia (Barandun et al., 2019). Finally, a long spore viability can be an alternative or a good addition to the prolonged spore production period. A large diversity of rRNA genes of cryptomycots was found in metagenomes, but only a few of them are massive in specific libraries. It is possible that the diverse but sparse types of rRNA genes originate from dormant stages, which lie in wait for their host until they perish. However, the ideal invented organization is not necessarily realized in nature. The real ratio of destructive processes due to Muller's ratchet and adaptations to parasitism in microsporidia rRNA genes requires further study.

We know very little about the actual and effective size of microorganisms. According to metabarcoding of rDNA amplicons, the cryptomycot population in terrestrial and marine biotopes is abundant and diverse (Lepère et al., 2006; Monchy et al., 2011; Nakai et al., 2012; Grossart et al., 2016; Rojas-Jimenez et al., 2017, 2019; Arroyo et al., 2018), while

typical microsporidia are found much less frequently with this method. To what extent does the ratio of rRNA genes in the libraries reflect the real ratio of viable cells in nature, and what portion of the amplicons originate from the vegetative cells, spores, and DNA of dead cells? The conclusion about low population size of typical microsporidia in nature is contrary to the opinion about their biological progress, but it would explain the rapid degradation of their genomes due to Muller's ratchet. However, such a conclusion would be premature, since the currently employed metabarcoding methods can lead to the underestimation of typical microsporidia due to the selection of amplicons by size (which distinguishes rDNA of typical microsporidia, but not cryptomycots, from typical eukaryotes), as well as the high level of differences in nucleotide sequences, which reduces the efficiency of the annealing of universal primers and the recognition of certain sequences as rDNA. The adaptation of metabarcoding methods for typical microsporidia will help to better account for them (Trzebny et al., 2020). Conversely, the allelic polymorphism depends on the effective size (Kimura, 1985) and can be a simple and good method of its measurement in the era of the active accumulation of genomic data. Such estimations are required for a more substantive discussion of the reasons for the different evolution rates of cryptomycots and typical microsporidia.

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#### COMPLIANCE WITH ETHICAL STANDARDS

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

*Statement on animal welfare.* All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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