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ORIGINAL PAPER

Discovery of a novel microsporidium in laboratory colonies of Mediterranean cricket *Gryllus bimaculatus* (Orthoptera: Gryllidae): *Microsporidium grylli* sp. nov.

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Abstract
A microsporidium was found in a Mediterranean cricket *Gryllus bimaculatus* from a pet market in the UK and a lab stock at the Moscow Zoo (originating from London Zoo). The spores were ovoid, uninucleate, 6.3 × 3.7 μm in size (unfixed), in packets by of 8, 16, or 32. The spores were easily discharged upon dessication or slight mechanical pressure. The polar tube was isofilar, with 15–16 coils arranged in 1–2 rows. The polaroplast was composed of thin lamellae and occupied about one third of the spore volume. The endospore was 200 nm thick, thinning over the anchoring disc. The exospore was thin, uniform, and with no ornamentation. Phylogenetics based upon small subunit ribosomal RNA (Genbank accession # MG663123) and RNA polymerase II largest subunit (# MG664544) genes placed the parasite at the base of the *Trachipleistophora/Vavraia* lineage. The RPB1 locus was polymorphic but similar genetic structure and identical clones were found in both isolates, confirming their common geographic origin. Due to in insufficient ultrastructural data and prominent divergence from described species, the parasite is provisionally placed to the collective taxon *Microsporidium*.

Keywords Mediterranean cricket · Microsporidia · Molecular phylogeny · Infection · Laboratory culture

Introduction

Microsporidia are ubiquitous parasites of animals, most abundant in arthropods and fishes. The presence of these parasites in insect colonies may lead to serious losses of sericulture, apiculture, and insect rearing for feed. Crickets are considered as an important diet constituent for insectivorous reptiles and mammals and are used for food in traditional cuisine of some countries as well as an alternative protein source in modern industry (Ayieko et al. 2016). As hosts of various parasites and carriers of pathogens, edible insects, including crickets, deserve attention regarding biosafety due to the presence of bacteria harmful to humans (Vandeweyer et al. 2017) and insect viruses pathogenic to reptiles (Just et al. 2001) and mammals (Lorbacher de Ruiz et al. 1986). Moreover, microsporidia from orthopteran insects can also be infectious to humans, e.g., *Tubulinosema acridophagus* from the locust *Schistocerca americana* (Choudhary et al. 2011; Meissner et al. 2012) and this fact augments the necessity for examination of reared orthopterans for microsporidial infections.

A microsporidium *Paranosema* (*Nosema*) *grylli* was discovered in 1995 in a laboratory population of Mediterranean crickets *Gryllus bimaculatus* (Sokolova et al. 2003) and no new isolates of this species have been recovered since, either from natural or laboratory populations. Screening of *G. bimaculatus* was carried out using artificially bred insects in two independent studies, one intended for identification of a cricket disease observed in the insect breeding facility at the Moscow Zoo and another being an attempt to find novel isolates of *P. grylli* in the UK for genetic polymorphism studies of this parasite. No *P. grylli* isolates were found, but an unknown microsporidium was discovered infecting the muscle and fat body of *G. bimaculatus*. In the present paper, a preliminary description of this parasite is given.

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Materials and methods

Cricket handling for rearing and analyses

The research of cricket microsporidia was initiated independently in the UK and Russia. In the UK, approximately 50 crickets were bought from a pet food supplier: Livefoods UK Ltd. Insects were maintained at 28 °C with 12-h light and 12-h dark period in plastic fish tanks with mesh lids. Crickets were fed a mixture of rolled oats and Ready brek®. Damp cotton wool was maintained in cultures as an egg-laying substrate and water source. Cardboard egg trays and tissue towels were used to provide a substrate. Damp cotton wool containing eggs was removed, placed in covered dishes, and kept moist until egg hatching. Newly hatched offspring were then kept in small covered containers, before transfer to covered fish tanks.

In Russia, crickets were routinely reared under similar conditions in the insect rearing facility at the Entomology Department at the Moscow Zoo. A particular stock of *G. bimaculatus* was sent to our Russian team for parasitological analysis due to suspicion of an infectious disease. As indicated by the head of Entomology Department of the Moscow Zoo Mikhail V. Berezin, the stock originated from the London Zoo.

Crickets were killed prior to screening by placing in a container and cooling to − 20 °C in a conventional freezer. Each insect was then individually dissected, by first removing the head and gut, and then extracting softer tissues for DNA extraction or preparation of smears. Dead crickets were stored at − 20 °C. For transmission electron microscopy (TEM) studies, crickets were immobilized by cooling in a refrigerator or brief exposure to ether vapor (in a sealed bottle) after which they were killed by decapitation.

Light microscopy and spore purification

For light microscopy (LM), fresh tissue smears were prepared from midgut epithelium, fat body, Malpighian tubes, and thoracic muscles of examined crickets, which are the most frequent infection sites for diverse disease agents, including microsporidia in orthopteran hosts. Smears were examined using a Carl Zeiss Imager M1 light microscope with bright field and Nomarski contrast, Leica ATC 2000 light microscope and a M15c phase contrast microscope (Vickers Instruments). Positive samples containing spores of microsporidia were dried and either directly stained with 5 mM Calcofluor white or fixed with methanol and subsequently stained with 5 μM DAPI (Sigma Aldrich) by placing a drop of stain on the slide and applying a coverslip immediately prior to examination under epifluorescence. Calcofluor white-stained smears were examined using an Olympus BX51 fluorescence microscope equipped with a Nikon coolpix digital camera and DAPI-stained smears were examined using a Zeiss Axioplan Fluorescence microscope and fluorescent images were captured by an Orca digital camera (Hamamatsu).

For purification of spores of the microsporidia, infected tissues of insects were homogenized using a glass homogenizer with a Teflon pestle in ~ 1 ml of purified water. The homogenate was centrifuged at 500*g* for 5 min in a Microcentaur (Sanyo) or a Minispin Plus (Eppendorf) microcentrifuges. The spore layer was identified by its light creamy color and was confirmed by phase contrast LM examination, using a small sample.

Electron microscopy

For transmission electron microscopy, four samples of spore suspension were placed into Eppendorf tubes using a Pasteur pipette. All solution changes and washes were made by pelleting spore samples, removing the supernatant and resuspending the spores in the desired solution using a Pasteur pipette. Centrifugation and cell pelleting steps were performed using a microcentaur microcentrifuge (Sanyo) at 13,400*g* for 1 min. Pelleted cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 1 mg/ml calcium chloride for 2 h, washed three times for 15 min each in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer and 0.3 M sucrose for 2 h. After washing three times for 15 min with distilled water, tubes were placed in a water bath (50 °C) and spores were quickly resuspended in 1% agarose using a whirlmixer (Fisherbrand) and immediately centrifuged at 13,400*g* for ~ 45 s. This produced an agarose plug containing a pellet of spores. Each plug was cut from the tube and placed immediately into a fixation jar containing water (samples 1 and 2) or 25% ethanol solution in water (samples 3 and 4) to prevent the plug drying out. Excess agarose was then removed from each plug using a microscope slide. Encapsulated spores were dehydrated in an ascending ethanol series followed by one change of 100% dried acetone for 15 min. LV resin (Agar Scientific) was used for infiltration and embedding. Specimen blocks were trimmed with an LKB pyramitome and sectioned with glass knives using a Reichert-Jung Ultracut microtome. Sections were mounted onto 200 and 400 mesh copper grids and examined using a JEOL1010 transmission electron microscope operating at 80 kV, without staining.

Image brightness and contrast was adjusted using Quartz PCI, version 5.10 (Quartz imaging corporation). Measurements of micrographs were made using ImageJ (Schneider et al. 2012).

Molecular genetics and phylogenetics

In the UK, DNA extractions were made from 200 μl of spore suspension using a DNEasy® Blood and Tissue kit (QIAgen), following the manufacturer's protocol for DNA purification

from animal tissues using a microcentrifuge. The initial incubation with proteinase K was extended to 3–4 h, during which samples were mixed periodically. DNA extraction for the "Russian" isolate was performed using homogenization of 100 μl of spore suspension with a plastic pestle in a 1.5 ml and incubation in 500 μl lysis buffer (2% CTAB, 1.4 M NaCl, 100 mM EDTA, 100 mM Tris-Cl; pH = 8.0) followed by phenol-chloroform separation, isopropanol sedimentation, and 70% ethanol washing (Sambrook et al. 1989). The dried pellet after ethanol evaporation was resuspended in 50 μl of molecular grade deionized water.

To amplify 16S rRNA gene portion, primers 18f:1492r were used (Weiss and Vossbrinck 1999). The initial primers for RPB1 amplification were RPB1-F1:RPB1-R1 (Cheney et al. 2001) but produced mixed PCR products that could not be read unambiguously after first 240 peaks. In an attempt to overcome this problem, specific primers RPB1_AF and RPB1_AR were designed, basing upon start regions of the obtained forward and reverse reads, respectively. This primer pair, however, also generated mixed products which were finally cloned using a TOPO TA Cloning Kit (Invitrogen) and a PAL-TA vector (Evrogen) for UK and "Russian" isolates, respectively. PCR was run in 25 μl reaction mixtures using either GoTaq (Promega) or Taq (Invitrogen) polymerase, with 1 mM of dNTPs, 0.4 μM of each primer, and 1 μl of DNA sample of varied dilution. DNA was amplified in a Primus thermocycler (MWG Biotech) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, the appropriate annealing temperature gradient for 1 min and 72 °C for 2 min. This was followed by a final elongation step of 72 °C for 7 min. For the "Russian" isolate, PCR was run under similar conditions using Colored Taq polymerase (Sileks) and Tertsik termocycler (DNK-Teknologiya). The amplicons were separated by 1% agarose gel electrophoresis and eluted using QIAquick Gel Extraction Kit, following the manufacturer's instructions for gel extraction with a microcentrifuge. Alternatively, the amplicons were purified from the excised gel pieces by melting with 3 M GITC buffer at 60 °C, mixing with an aliquot of Glass Milk, precipitating by centrifugation, washing with a buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 25 mM EDTA and 50% ethanol) and eluting by molecular grade deionized water (Vogelstein and Gillespie 1979). Sequencing was performed by standard Sanger dideoxy method using ABI Prism 3500 Genetic Analyzer.

The obtained sequences were edited manually and aligned using Clustal W multiple alignment in BioEdit 7.2.5 (Hall 1999). The pairwise genetic distances between taxa were calculated in MEGA 7 (Kumar et al. 2015) using Kimura two-parameter model for nucleotide sequences (Kimura 1980) and Jones-Taylor-Thornton (JTT) model for amino acid sequences (Jones et al. 1992). Phylogenetic reconstructions were performed by Maximum Likelihood approach in raxmlGUI v. 1.5 (Silvestro and Michalak 2012) with 100 bootstrap replicates. For the SSU rRNA gene sequence alignment, 1558 bp long, GTR model with gamma distributed rates among sites (+G) and a proportion of invariable sites (+I) was applied. For the RPB1 gene sequence alignment, 1995 aa long, JTT+G+I model was applied.

Results

Light and electron microscopy

Out of 30 examined crickets originating from the UK pet food supplier, 5 were infected with microsporidia, corresponding to ~ 17% prevalence rate. The prevalence rate in crickets from the Moscow Zoo was not estimated. The spore masses of the microsporidium were found in thoracic muscles and fat body. The infected tissues were either unaltered (when infection was of low intensity) or hypertrophied; the infected adipose tissue changed its color from yellow to white (Fig. 1a). Examination of infected tissue smears of crickets both from the UK and Russia revealed small clumps of sporophorous vesicles (SPVs) in thoracic muscles and fat body. The number of spores per SPV varied (Fig. 1b). In particular, SPVs containing 16 and 8 spores constituted about 70–80% and 10–20%, respectively, while proportion of SPVs with 20–32 spores was below 5–10% in both "British" ($N = 37$) and "Russian" ($N = 52$) samples. In one heavily infected cricket from the UK, the fat body released a dense mass of individual spores, possibly due to disintegration of the fragile SPV sheath during prolonged persistence of mature spore masses within the host body. Similarly, after storage of infected tissue samples for several days, as well as after spore centrifugation, the spores were no longer retained by the SPV sheath.

The spores were ovoid and slightly bent, with a slightly pointed anterior end. A pale spot occupied the posterior end of the spore (~ 1.5 μm across), corresponding to the large refractive posterior vacuole (PV). The anterior end of each spore was also particularly refractive. The unfixed spores were 5.5–7.2 (mean 6.3) × 3.3–4.3 (mean 3.7) μm in size. The spores stained with DAPI were monokaryotic, presenting one bright fluorescent spot (~ 1 μm in diameter) near the center of the spore (Fig. 1c). Calcofluor white intensively stained the chitinaceous layer of the spore wall (Fig. 1d). During prolonged examination of fresh smears, when the water dried and receded across the slide, spores extruded their polar filaments (PF) onto the dry slide surface. During extrusion, PFs looked like cracks appearing in the glass, with growth only at the terminus. After extrusion, the sporoplasm was occasionally visible at the end of the PF (not shown). The length of individual PFs was variable, ranging from ~ 100 to ~ 200 μm. Moreover, the spores were easily discharged when the coverslip was slightly pressed against the glass slide so that empty spores and everted PFs were observed (Fig. 1e).

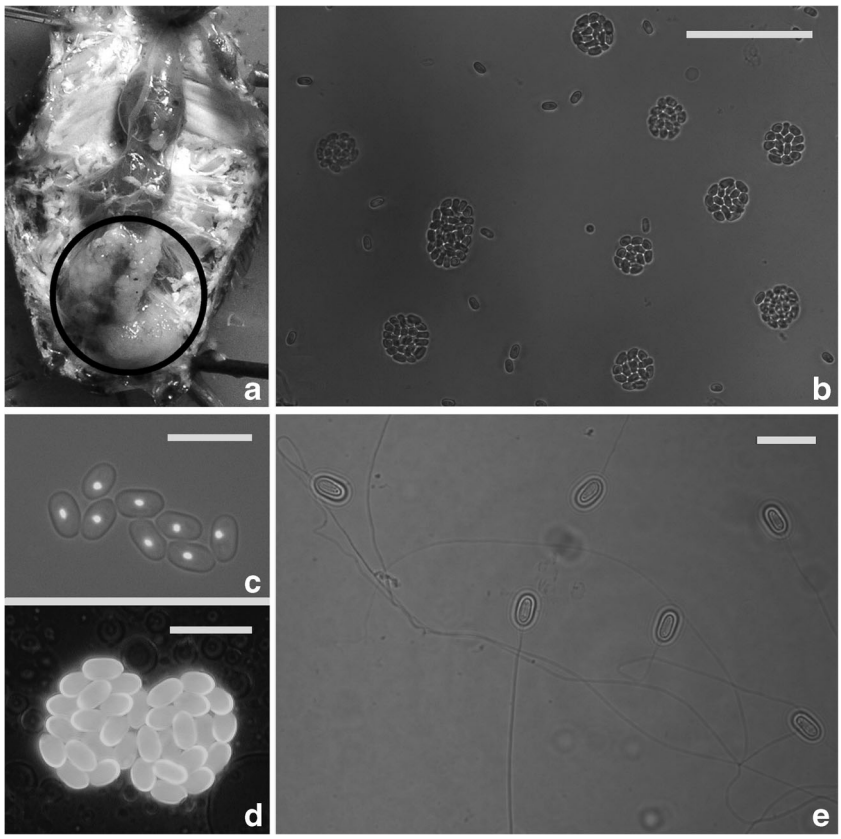

Fig. 1 *Microsporidium grylli* sp. nov. in infected tissues of cricket *Gryllus bimaculatus*. **a** Hypertrophied adipose tissue lobes (encircled) around midgut of a dissected adult cricket. **b** Sporophorous vesicles with variable number of spores on a fresh tissue smear (bright field). **c** DAPI staining of nuclear apparatus. **d** Calcofluor White staining of chitinaceous layer of the spore wall (epifluorescence combined with bright field). **e** An extruded spore with full-length everted polar tube and a sporoplasm on its tip (Nomarski contrast). Scale bar = 50 μm (B), 1 μm (**c**–**e**)

Ultrathin sections contained sporoblasts and mature spores. The preservation of sporoblasts was not satisfactory and the obtained ultrastructural data were not included in the analysis. The polaroplast, composed of thin lamellae, occupied about one third of the spore volume. The anchoring disc and polar sac were of moderate electron density, looking light as compared to surrounding polaroplast and cytoplasm. The distal part of polar sac was thin and comparatively long, reaching the center of the lateral sides of the polaroplast. There was a prominent layer of cytoplasm between the polaroplast and the endospore. The endospore was thick, up to 200 nm, thinning over the anchoring disc. Thin exospore was uniform, devoid of any ornamentation on its surface (Fig. 2a). The PF was isofilar, composed of 15–16 coils organized into a single row surrounding the PV and stacking into 2–3 rows towards the spore anterior. Coiled PF cross sections were composed of a 60–75-nm filament surrounded by a 10-nm thick wall (Fig. 2b).

Molecular phylogenetics

SSU rRNA gene sequencing resulted in a sequence (Genbank accession # MG663123), 1327 bp long, 100% identical in the UK and "Russian" isolates, indicating their attribution to the same species. In Genbank, the closest relatives were *Trachipleistophora hominis* (# AJ278945) and *Vavraia culicis* (# AJ278956) with 86–87% sequence similarity and 0.146–0.151 genetic distance (Table 1). Sequencing molecular clones of RPB1 gene portion resulted in two sequences for the UK isolate (clones UK1–2) and five sequences for "Russian" isolates (clones RU1–5). Interestingly, clones UK1 (## MG664544-MG664545) and RU1 (## MG664546-MG664548), both 844 bp long, possessed an identical insert at positions 182–189 which was absent in other clones (Fig. 3), so that overall sequence similarity

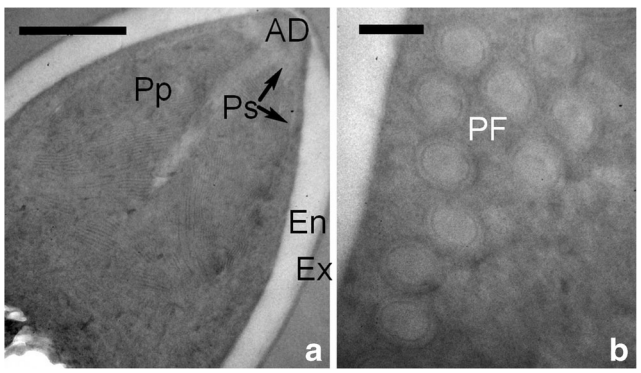

Fig. 2 Transmission electron microscopy of spores of *Microsporidium grylli* sp. nov. showing the lamellar polaroplast (**a**) and the polar filament coils (**b**). AD anchoring disc, En endospore, Ex exospore, PF polar filament, Pp polaroplast, Ps polar sac. Scale bar = 1 μm (**a**), 0.2 μm (**b**)

Table 1 Pairwise genetic distances estimated for *Microsporidium grylli* sp. nov. (in bold) and related taxa

#	Taxon name	Genbank accession #		Pairwise genetic distances for SSU rRNA nucleotide (upper-right) and RPB1 amino acid sequences (lower-left)											
		SSU rRNA	RPB1	1	2	**3**	4	5	6	7	8	9	10	11	12
1	*Trachipleistophora hominis*	AJ002605	CAC33868	ID	0.040	**0.146**	0.085	0.083	0.084	0.109	0.091	0.125	0.128	0.191	0.211
2	*Vavraia culicis*	AJ252961	CAC33869	0.124	ID	**0.151**	0.092	0.090	0.091	0.106	0.091	0.128	0.123	0.194	0.221
3	***Microsporidium grylli* sp. nov.**	**MG663123**	**MG664544**	**0.551**	**0.546**	**ID**	**0.172**	**0.170**	**0.172**	**0.186**	**0.167**	**0.196**	**0.181**	**0.232**	**0.267**
4	*Pleistophora typicalis*	AJ252956	CAC33864	0.382	0.375	**0.605**	ID	0.002	0.002	0.087	0.073	0.126	0.132	0.178	0.200
5	*Pleistophora mulleri*	EF119339	ABO63964	0.382	0.375	**0.605**	0.000	ID	0.001	0.084	0.071	0.123	0.129	0.175	0.197
6	*Pleistophora hippoglossoideos*	AJ252953	CAC33861	0.382	0.375	**0.605**	0.000	0.000	ID	0.086	0.072	0.125	0.131	0.177	0.200
7	*Ovipleistophora ovariae*	AJ252955	CAC33862	0.369	0.378	**0.627**	0.423	0.423	0.423	ID	0.053	0.119	0.131	0.184	0.203
8	*Heterosporis anguillarum*	AF387331	CAC33860	0.473	0.431	**0.657**	0.469	0.469	0.469	0.341	ID	0.117	0.126	0.175	0.200
9	*Glugea anomala*	AF044391	CAC33856	0.679	0.657	**0.842**	0.664	0.664	0.664	0.628	0.522	ID	0.099	0.161	0.213
10	*Loma acerinae*	AJ252951	CAC33858	0.635	0.641	**0.750**	0.637	0.637	0.637	0.616	0.589	0.291	ID	0.170	0.228
11	*Pseudoloma neurophilia*	AF322654	KRH94948	0.942	0.922	**1.056**	0.916	0.916	0.916	0.882	0.766	0.623	0.700	ID	0.260
12	*Spraguea lophii*	AF033197	EPR80047	0.897	0.915	**1.172**	0.847	0.847	0.847	1.007	0.915	0.997	0.945	1.156	ID

between clones with and without the insert was 97–98%. Besides this difference, the clones possessed minimal amount of SNPs providing 99.3–99.8% sequence similarity and, most essential, clones RU4 and RU5 were 100% identical to clone UK2 (#MG664545). It can be therefore concluded that there were no genetic borders between the isolates and this fairly corresponded to the fact that the "Russian" isolate originated from the London Zoo (see above), i.e., both isolates came from the UK. The amino acid sequence similarity to *V. culicis* and *T. hominis* was 60.8–61.3% and genetic distance reached 0.546–0.551 (Table 1). In phylogenetic reconstructions using either nucleotide sequences of SSU rRNA (Fig. 4a) or amino acid sequences of RPB1 (Fig. 4b), the new microsporidium occupied a position sister to the *Trachipleistophora/Vavraia* lineage in the crown of the Clade 5 (Vossbrinck et al. 2014), previously known as Clade III (Vossbrinck and Debrunner-Vossbrinck 2005).

Discussion

The majority of representatives of Clade 5 are the parasites of marine hosts from classes Pisces or Crustacea, while the *Trachipleistophora/Vavraia* lineage represents a group which evolved from these marine parasites and adapted to terrestrial insects and mammals. Infection of an orthopteran host provides support for the proposal that human *T. hominis* infections originate from natural insect hosts (Cheney et al. 2000) and this is further corroborated by the discovery of *Trachipleistophora extenrec* in an insectivorous host (Vavra et al. 2011). Another study of biological properties of its congener *T. hominis* revealed ability of its human isolate to develop in mosquitoes and retain infectivity to mammals, as concluded from an experimental infection of mice (Weidner et al. 1999). All these observations indicate ecological and evolutionary plasticity of *Pleistophora*-like microsporidia host ranges.

When the microsporidium was discovered both in the UK and Russia, only parasite spores were observed in infected tissues. Moreover, no attempts have been made to cultivate the parasite for tracking its life cycle, neither pieces of infected tissues were fixed for electron microscopy, which was performed using samples of semi-purified spores only. The data concerning thin morphology of the parasite and its interactions with the infected host cell are therefore not sufficient for a sound taxonomic description. It can be noted, however, that morphology

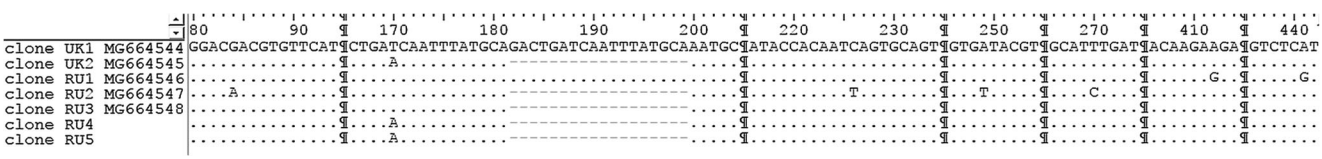

Fig. 3 An alignment of molecular clones of RNA polymerase II largest subunit gene portion sequenced for UK (UK1–2) and "Russian" (RU1–5) isolates of *Microsporidium grylli* sp. nov. Dots indicate identical positions as compared to the first sequence

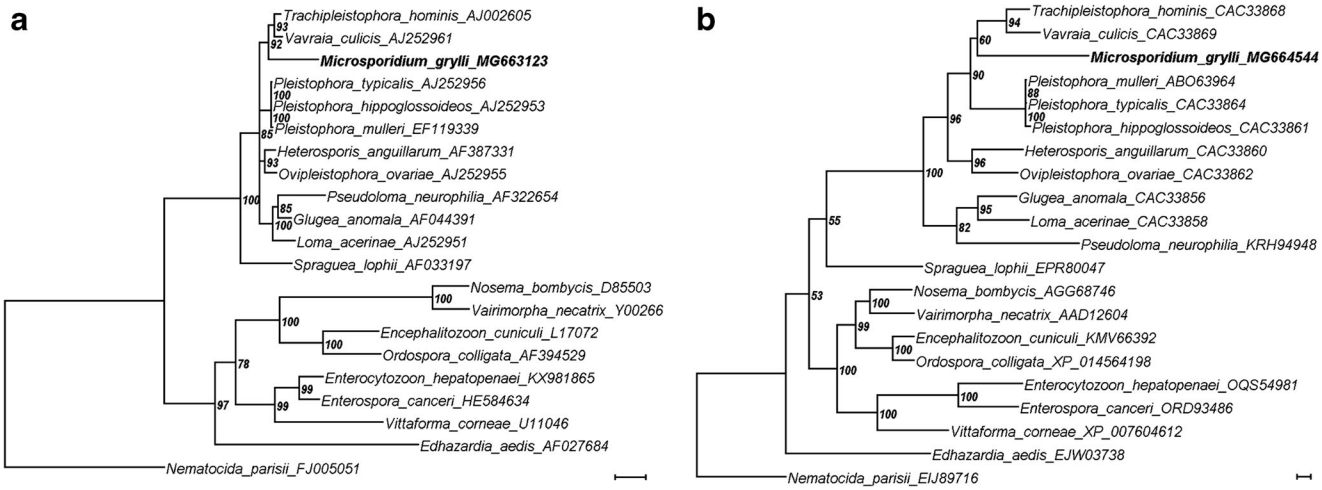

Fig. 4 Phylogenetic reconstruction inferred from nucleotide sequences of SSU rRNA (**a**) and RPB1 gene sequences (**b**) of *Microsporidium grylli* sp. nov. (in bold) and related taxa using Maximum Likelihood approach in raxmlGUI v. 1.5. Bootstrap values are given as branch support. The taxa are annotated with Genbank accession numbers. Scale bar = 0.1 expected changes per site

of *Trachipleistophora*, *Vavraia*, and the new microsporidium is quite similar and represents the typical *Pleistophora*-like appearance, including SPVs with variable number of spores divisible by 8, unpaired nuclei, lamellar polaroplast, large posterior vacuole, and a single row of polar tube coils (Sprague et al. 1992; Hollister et al. 1996; Weiser et al. 1997). Yet the newly found microsporidium is diverged genetically and might represent a separate genus if sufficient ultrastructural data of its developmental stages was available for a taxonomic description. We therefore provisionally place this parasite to the collective taxon *Microsporidium*.

Description of *Microsporidium grylli* sp. nov.

The spores ovoid, uninucleate, 6.3 × 3.7 μm in size (unfixed), discharge easily. Fragile sporophorous vesicle with variable spore number, divisible by 8. The polar tube isofilar with 15–16 coils, arranged in 1 row, anterior coils in 1–2 additional rows. The polaroplast composed of thin lamellae, occupying about one third of the spore volume. The endospore 200 nm thick, thinning over the anchoring disc. The exospore thin, uniform, devoid of ornamentation. The posterior vacuole filled with granular material.

Type host: *Gryllus bimaculatus* (Orthoptera, Gryllidae), a laboratory culture (UK).

Etymology: The species name alludes to the host genus.

Genbank accession numbers: MG663123 (SSU rRNA), MG664544-MG664548 (RPB1).

Taxonomic summary for *Microsporidium grylli* sp. nov.

Phylum: Microsporidia Balbiani 1882.

Family: *insertae sedis*.

Genus: *insertae sedis* (temporally assigned into a holding genus *Microsporidium* Balbiani 1884).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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