

Ultrastructure and Molecular Phylogeny of *Mrazekia macrocyclopis* sp. n. (Microsporidia, Mrazekiidae), a Microsporidian Parasite of *Macrocyclops albidus* (Jur.) (Crustacea, Copepoda)

Irma V. ISSI¹, Yuri S. TOKAREV¹, Vladimir N. VORONIN², Elena V. SELIVERSTOVA³, Olga A. PAVLOVA¹, Vyacheslav V. DOLGIKH¹

¹All-Russian Institute of Plant Protection, Russian Academy of Agricultural Sciences, St. Petersburg-Pushkin, Russia; ²State Research Institute of Lake and River Fisheries, St. Petersburg, Russia; ³I.M. Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St. Petersburg, Russia

Summary. The ultrastructure and molecular phylogeny of a new microsporidium *Mrazekia macrocyclopis* sp.n., a parasite of the copepod *Macrocyclops albidus* (Jur.) in North-West of Russia are described. All stages of its life cycle are diplokaryotic. Fresh spores are rod-shaped and $7.3\text{--}10.5 \times 1.6\text{--}2.3 \mu\text{m}$ in size. Spore ultrastructure is typical of *Mrazekia*. The polar tube consists of the anterior clavate manubrium followed by a thin filament arranged in 3.5–4.5 nearly vertical coils. Spores are enclosed in individual sporophorous vesicles. SSU rDNA sequence analysis showed attribution of the new species to a cluster of microsporidia infecting insects (*Cystosporogenes*, *Endoreticulatus*), microstrustaceans (*Glugoides*), vertebrates (*Vittaforma*) and ciliates (*Euplotespora*) nested within the clade IV sensu Vossbrinck, Debrunner-Vossbrinck (2005). *Mrazekia macrocyclopis* is not therefore closely related to *Bacillidium vesiculiformis*, another microsporidium with rod-shaped spores, and the polyphyletic nature of the family of Mrazekiidae is obvious.

Key words: Microsporidia, *Mrazekia macrocyclopis* sp.n., Copepoda, *Macrocyclops albidus*, ultrastructure, molecular phylogeny.

INTRODUCTION

Fresh-water crustaceans of the order Copepoda are infected in North West Russia with microsporidia of the genera *Unikaryon* (6 species), *Gurleya* (3), *Cougourdella* (1), *Pyrotheca* (2), *Flabelliforma* (1), *Holobis-*

pora (1), *Lanatospora* (1), *Thelohania* (2) and *Microsporidium* sp. (6) (Voronin 1986, 1999). All these microsporidia produce oval or pyriform spores. It was not before fall of 2008 that cyclops were found infected with microsporidia possessing rod-shaped diplokaryotic spores.

To date, five microsporidian genera, namely *Bacillidium*, *Hrabyeia*, *Jirovecia*, *Mrazekia* and *Rectispora*, are known to produce diplokaryotic rod-shaped spores as a result of disporoblastic sporogony. Distinctions between these genera are based mainly on the ultrastruc-

Address for correspondence: Irma V. Issi, All-Russian Institute of Plant Protection, Russian Academy of Agricultural Sciences, Podbelskogo sh. 3, St. Petersburg-Pushkin 196608, Russia; Phone: +7(812)4704384; Fax: +7(812)4705110; E-mail: irma_issi@mail.ru

ture of the spore wall and the polar filament. The latter consists of a diversely structured manubrium and a thin filament with coils of varying number.

The first description of the genus *Mrazekia* from *Asellus aquaticus* dates back to 1916 (Leger and Hesse, 1916) and does not satisfy the demands of modern morphological taxonomy, based on ultrastructural criteria. Later, a survey of the ultrastructure of sporogonial stages of *Mrazekia* (= *Bacillidium*) *cyclopis* was published (Larsson *et al.* 1993, Canning and Vávra 2000), allowing an improved diagnosis of the genus of *Mrazekia*. According to this diagnosis, the structure of the extrusion apparatus of representatives of the genus *Mrazekia* is unique as compared to other microsporidia with rod-shaped spores. The manubrium widens from the anterior pole of the spore to the posterior one and constricts abruptly to form a thin filament with several coils.

The newly found microsporidium from *Macrocyclops albidus* (Jur.) possesses rod-shaped spores with a polar filament that is characteristic of the genus *Mrazekia*. A set of characters distinguishes this form from other known representatives of the genus, which allows us to establish it as a new species. The present paper describes the ultrastructure and the rDNA-based molecular phylogeny of this new species.

MATERIALS AND METHODS

Copepods of the species *Macrocyclops albidus* (Jur.) (Crustacea, Copepoda) were collected from a pond in the Victory Park in St. Petersburg in October 2008 (59°51'63"N, 30°24'99"E). For light microscopy (LM), smears of infected tissues were fixed with methanol for 5 min. and stained with Giemsa. Digital images were

acquired using Carl Zeiss Axio 10 Imager M1 with an attached digital camera. Measurements of spores were performed with Carl Zeiss Axiovision software version 4.4.6.

For transmission electron microscopy (TEM), samples were fixed with 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer with 4% sucrose for 1–2 hrs and postfixed with 1% cacodylate-buffered osmium tetroxide for 1 h. Tissues were dehydrated in an ascending ethanol series and absolute acetone and embedded into epon-araldite resin. Ultrathin sections were cut using Ultratome-III (LKB) and stained with 2% uranylacetate in 50% ethanol and lead citrate for 10–20 min. The material was examined using electron microscope JEM-100 CX II at an accelerating voltage of 80 kV.

For rDNA amplification and sequencing, two heavily infected cyclops were homogenized with a plastic pestle in 100 µl lysis buffer, containing 2% CTAB, 1.4 M NaCl, 100 mM EDTA, 100 mM Tris-Cl (pH 8.0). After homogenization, 500 µl lysis buffer with 0.2% β-mercaptoethanol and 10 µl proteinase K (20 mg mL⁻¹) were added to the samples and incubated for 3 hrs at 65°C. DNA was extracted with phenol-chloroform (Sambrook *et al.* 1989) and resuspended in 50 µl UHQ water. To amplify the small subunit (SSU) rRNA gene, universal microsporidia primers V1f 5'-CACCAGGTTGATTCTGCCTGAC-3' (Weiss *et al.* 1994) and 1492r 5'-GGTTACCTTGTTACGACTT-3' (Weiss, Vossbrinck 1999) were used.

PCR was run using a Bio-Rad MyCycler in 20 µl volume containing 5 µl DNA template, 2 µl 10 × PCR buffer 0.25 mM dNTPs, 0.25 mM; 1 U Taq-polymerase (Sileks, Russia), and 1 pMol each of forward and reverse primers (Evrogen, Russia). The PCR profile consisted of an initial denaturation step (92°C for 3 min.), 30 amplification cycles (denaturation at 92°C for 30 s; annealing at 54°C for 30 s; elongation at 72°C for 30 s) and a final extension step (72°C for 10 min.). The PCR product with an expected size of about 1200 bp. was gel purified and cloned into pAL-TA vector (Evrogen, Russia). The plasmid was purified with phenol-chorophorm and sequenced in both directions using primers M13F and M13R.

Newly obtained rDNA sequence, submitted to Genbank under accession number FJ914315, was compared to those available in NCBI using the built-in BLAST utility (www.ncbi.nlm.nih.gov/Blast.cgi). The alignment of the newly obtained sequence with those showing significant homology (Table 1) was done automatically using CLUSTAL W algorithm and edited by eye in BioEdit v7.0.8.0

Table 1. List of microsporidian species used in the molecular phylogeny study of *Mrazekia macrocyclopis* sp.n.

Microsporidia species	Host	Accession #
<i>Cystosporogenes operophterae</i>	<i>Operophtera brumata</i> (Lepidoptera: Geometridae)	AJ302320
<i>Cystosporogenes legeri</i>	<i>Lobesia botrana</i> (Lepidoptera: Tortricidae)	AY233131
<i>Endoreticulatus bombycis</i>	<i>Bombyx mori</i> (Lepidoptera: Bombycidae)	AY009115
<i>Endoreticulatus schubergi</i>	<i>Choristoneura fumiferana</i> (Lepidoptera: Tortricidae)	L39109
<i>Euplotespora binucleata</i>	<i>Euplotes woodruffi</i> (Spirotrichea: Euplotidae)	DQ675604
<i>Glugoides intestinalis</i>	<i>Daphnia magna</i> (Cladocera: Daphniidae)	AF394525
<i>Liebermannia dichroplusiae</i>	<i>Dichroplus elongatus</i> (Orthoptera: Acrididae)	EF016249
<i>Mrazekia macrocyclopis</i>	<i>Macrocyclops albidus</i> (Cyclopoida: Cyclopidae)	FJ914315
<i>Orthosomella operophterae</i>	<i>Operophtera brumata</i> (Lepidoptera: Noctuidae)	AJ302317
<i>Vittaforma corneae</i>	<i>Homo sapiens</i> (Primates: Hominidae)	U11046

(Hall 1999). Regions containing gaps and ambiguous sites were removed, leaving an alignment of 1087 bp length. Phylogenetic reconstruction was carried out with Bayesian inference in MrBayes v3.1.2 (Ronquist, Huelsenbeck 2003) and maximum likelihood in PAUP* v4.0 β 10 (Swofford 2003).

Maximum Likelihood (ML) and Bayesian Inference (BI) settings (TrN + I + G) were selected using jModelTest v0.1.1 (Posada 2008) by AIC and BIC, respectively. Tree search in PAUP* was done heuristically with random stepwise addition (10 replicates) and TBR branch swapping. A majority-rule consensus tree was calculated from 100 bootstrap replicates. MrBayes was run for 100,000 generations and every 100th generation was sampled. The first 25% of samples was discarded as burn-in, parameter values were summarized, and a consensus tree was constructed. Standard deviation of split frequencies reached 0.007 after 100,000 generations.

RESULTS

Description of *Mrazekia macrocyclopi* sp.n.

Light microscopy

Copepods were heavily infected and their bodies were white being completely filled with spores. Smears prepared from infected cyclops (Fig. 1) contained all developmental stages of the parasite (Figs 2–3). Merogonial and sporogonial stages possess a diplokaryotic nuclear apparatus and develop in direct contact with the host cell cytoplasm.

Meronts are round cells 6–10 μ m in size with large nuclei of typical diplokaryotic arrangement (Fig. 4). Sporonts with one diplokaryon are round cells 4–6 μ m in size while sporonts with two diplokaryons are lanceolate cells with nuclei located near each end of the cell. Sporoblasts are round diplokaryotic cells 6–8 μ m in size surrounded by granules, intensively stained red.

Living mature spores are rod-shaped, straight, refractive, 7.3–10.5 \times 1.6–2.3 μ m in size (average 8.5 \times 2.0 μ m) (Fig. 5). Stained spores usually lay parallel to each other in packs by two or by four (Fig. 8). Rarely spores are found in groups by eight, shorter and oriented randomly towards each other (Fig. 7). Extruded manubrium lies at an angle to the spore axis and terminates with a thin polar tube (Fig. 8). Some spores are embedded in a densely stained material attached to the spore wall (Fig. 6), though it is not clear whether this feature corresponds to the exospore structure or is a staining artifact.

Electron microscopy

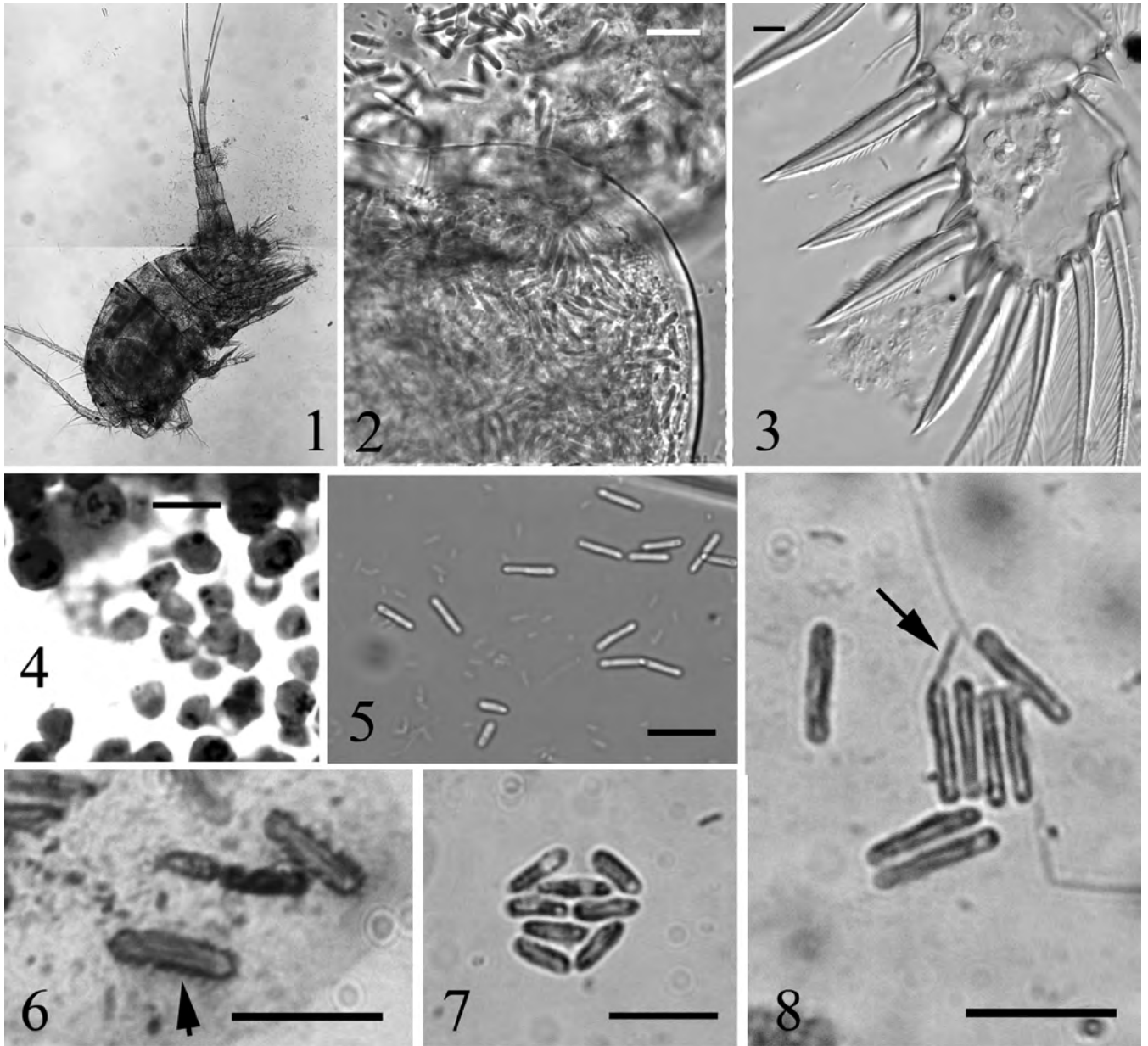
Spores are the prevailing stages observed on ultrathin sections of the infected tissue. Occasionally later meronts, sporonts and sporoblasts are found as well.

The meront is an oval cell, 3.0–3.3 \times 2.6–2.8 μ m in size, with a large diplokaryon 2.6 \times 1.3–1.4 μ m in size, located in its center. The cytoplasm is of moderate electron density with a feebly developed endoplasmatic reticulum (Fig. 9).

The sporont is either a round cell, 3.7–4.2 μ m in size, with one diplokaryon in its center, or an elongated cell, 5.5–5.6 \times 2.0 μ m in size, with two diplokarya at opposite ends. The boundary between the nuclei of the diplokarya, both about 1.8 μ m in diameter, is clearly visible. The cytoplasm is of moderate electron density (Fig. 10).

Sporoblasts are either round, 1.7–2.8 μ m in diameter, oval, 1.6–1.8 \times 3.0–4.8 μ m in length (Fig. 12), or strongly deformed (Fig. 14). They differ from the preceding stages by higher electron density of the cytoplasm, well-developed endoplasmatic reticulum and presence of primordia of the extrusion apparatus in the later sporoblasts (Fig. 14). At this stage, formation of the sporophorous vesicle (SPV) wall begins, with the detachment of a region of the sporoblast plasma membrane from the parasite cell. Sometimes the lumen of the future SPV contains tubular structures and fine granules (Figs 10–13).

Rod-shaped spores are 7.3–10.5 \times 1.6–2.3 μ m in size and remain straight after fixation (Fig. 18). The extrusion apparatus has a complicated structure. The mushroom-like anchoring disc, 300 nm in diameter, lies in the center of the anterior pole of the spore (Fig. 19). The polar sac covers the anterior part of the bipartite polaroplast and protrudes towards the posterior pole by 0.5 μ m (Figs 18–19). Each part of the polaroplast is bounded by a thin membrane. The anterior part of the polaroplast is composed of tightly packed lamellae while the posterior part is composed of spherical or tubular structures 10 nm in diameter with a dark core (Fig. 18). The clavate manubrium is located obliquely as in *Mrazekia cyclopi*. It widens more than twofold towards the posterior pole from 130 to 350 nm in diameter (Figs 18, 24). The manubrium wall is composed of an amorphous material of moderate electron density, bounded by an electron-dense sheath. A thin channel, about 70 nm in diameter, penetrates the center of the manubrium. The thickened distal portion of the manubrium sharply changes into the thin filament, 80 nm in diameter (Fig. 21). The filament possesses 3.5–4.5 coils, positioned at an angle of 10–30 (mainly 15–20) degrees to the long axis of the spore, laterally to the manubrium (Figs 18, 21–23). Such a position of the polar filament coils can be explained by the fact that in many spores



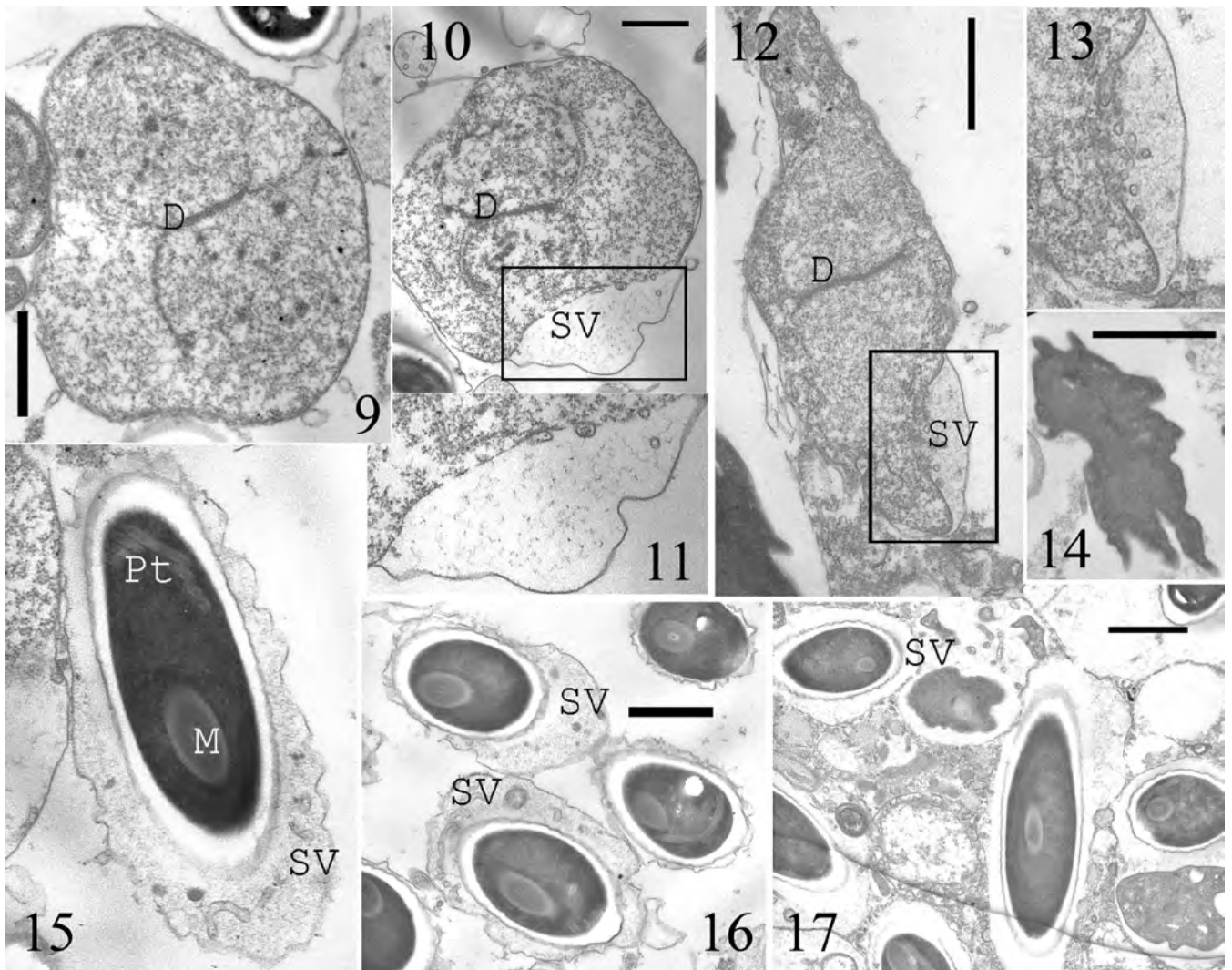
Figs 1–8. Light microscopy of the infected copepod *Macrocyclus albidus* and its microsporidian parasite *Mrazekia macrocyclus*. Nomarski contrast (1–3, 5), Giemsa staining (4, 6–8). **1** – microsporidia-infected copepod; **2, 3** – copepod tissues, loaded with developmental stages of the microsporidium; **4** – prespore developmental stages of the microsporidium; **5** – living spores; **6** – stained spores within a Giemsa-stained coat; **7** – group of 8 spores; **8** – a spore with extruded manubrium (arrow). Scale bar: 10 μm .

the manubrium reaches the posterior pole of the spore, leaving no space for the coils at the spore end. The polar filament coils surround the posterosome, an oval membrane-bound structure of moderate electron density with appearance of elements of the Golgi complex.

The diplokaryon is presented by two beanlike nuclei, $0.8 \times 0.3 \mu\text{m}$ in size, in the middle part of the spore. The

nuclear membrane is covered by aggregations of ribosomes (Figs 18, 20) in a manner resembling *Octosporea* (Canning and Vávra 2000) The nuclei are round on the transverse sections and nearly adjoin but do not enclose the manubrium (Fig. 16).

The endospore is thin, up to 150 nm, being 30–50 nm thick above the anchoring disc. The exospore of the

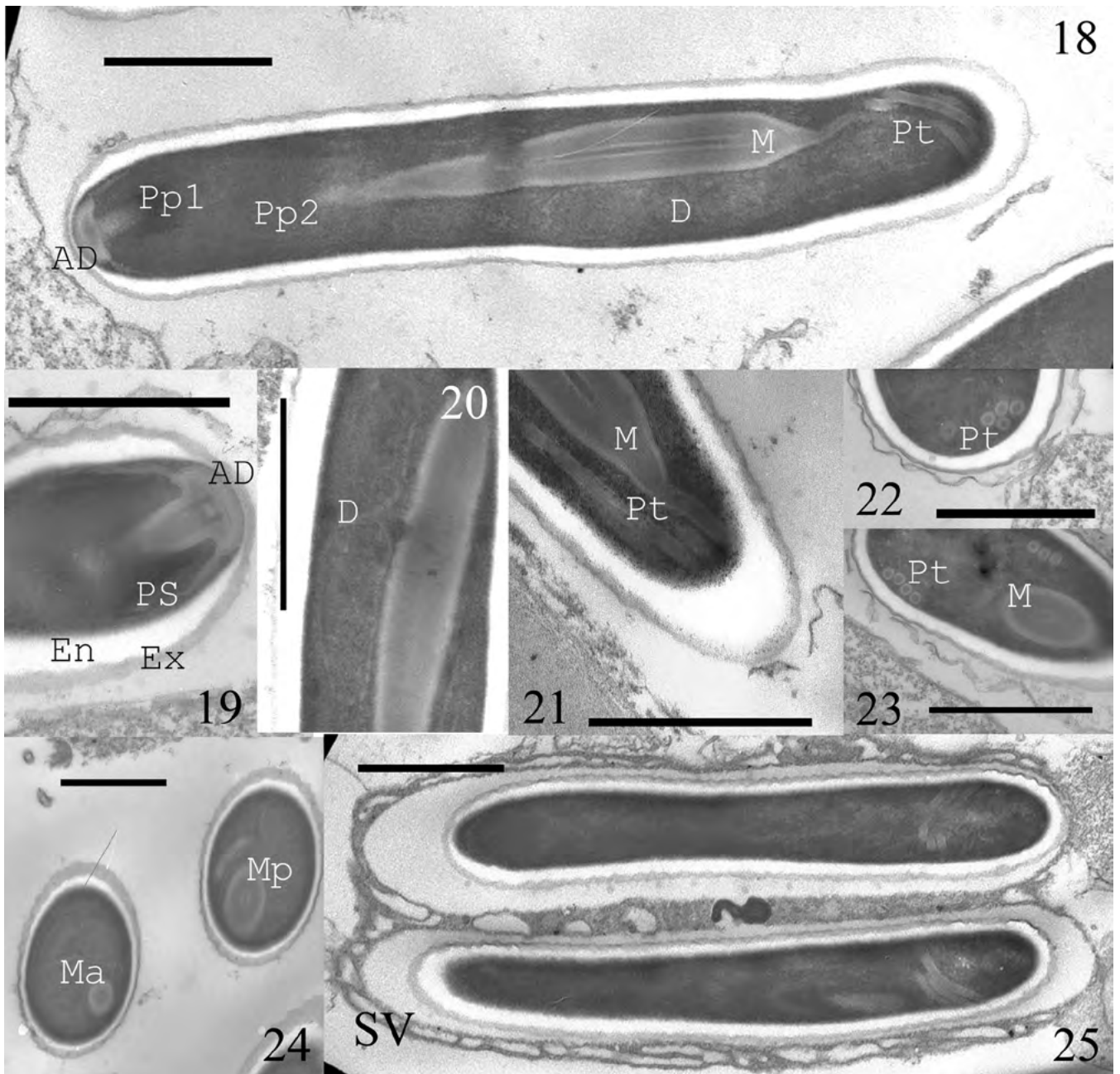


Figs 9–17. Ultrastructure of *Mrazekia macrocyclopis*. TEM. Sporogonial stages. **9** – late meront/early sporont transitional stage; **10** – late sporont at the beginning of SPV formation; **11** – an enlarged detail of Fig. 10. Tubular structures and fine granules are seen in the SPV cavity; **12** – a sporoblast in a partially formed SPV; **13** – an enlarged detail of Fig. 12. More tubular structures and fine granules are present, **14** – late sporoblast; **15–16** – young spores in SPVs; **17** – end of sporogony, SPV content disappears and only its wall remains. D – diplokaryon, M – manubrium, Pt – polar filament, SV – sporophorous vesicle. Scale bars: 0.75 μm (9, 10), 1 μm (12, 15, 16), 1.2 μm (14), 1.5 μm (17).

young spores is undulating, two-layered. Its outer layer is thin, being of high electron density. The inner layer is of moderate electron density and has varied thickness, making the spore surface undulating (Figs 19, 21, 24).

Careful examination of numerous ultrathin sections found no evidence for SPVs in sporonts and sporoblasts. However, small regions of additional outer membrane detach from the spore surface, and tubular structures extend from the parasite cell into the resulted electron-translucent cavity (Figs 15–17). In cross sections young

spores are enclosed within individual SPVs 1.7–3.0 \times 2.2–3.2 μm in size. The SPV cavity is bounded by a very thin membrane and contains tubular structures and numerous very small granules (Figs 15–16). Sometimes SPVs contain two spores of different maturity or even a spore and a sporoblast (Fig. 17). Later in development, the content of the SPV lumen disappears and only a thin fragile membranous layer is retained around mature spores, while the space between this layer and the spore becomes electron-translucent (Fig. 17). In some cases,



Figs 18–25. Ultrastructure of spores of *Mrazekia macrocyclopis*. TEM. **18** – general view of the spore; **19** – anterior pole of the spore; **20** – nuclei of the spore, adjoining the manubrium; **21** – transition of the manubrium into thin filament; **22–23** – polar filament coils; **24** – transverse section of spores at the level of anterior and posterior parts of the manubrium; **25** – spores with the SPVs lumen without inclusions. AD – anchoring disk, En – endospore, Ex – exospore, Ma – anterior with of which and posterior parts of the manubrium, respectively, Pp1 and Pp2 – anterior and posterior parts of the polaroplast, respectively, PS – polar sac, other abbreviations as in Figs 9–17. Scale bar: 0.75 μm (21), 1 μm (19, 22), 1.2 μm (18, 23), 1.5 μm (24–25).

the membranous layer tightly attaches to the exospore looking like an additional layer of the spore wall (Figs 22–23). This is typical of many microsporidia with rod-shaped spores (Canning and Vávra 2000, p. 41, Fig. 3).

Ultrathin sections demonstrate that the spores fill up the body of the host, including cells under the cuticle and the gut cells.

Phylogenetic analysis

Sequencing of the SSU rDNA fragment from the newly found microsporidium produced a sequence of 1233 bp, deposited in Genbank under accession number of FJ914315. A BLAST search against Genbank found that it belongs to the Clade IV sensu Vossbrinck, Debrunner-Vossbrinck (2005). *Bacillidium vesiculoformis*, the only other microsporidium with rod-shaped spores presented in Genbank (AJ581995), showed only 55.8% SSU rDNA similarity to *M. macrocyclopolis*, proving that these two species are not closely related forms. Closely related species of which sequence data are available are *Cystosporogenes* spp., *Euplotespora binucleata*, *Vittaforma corneae*, *Endoreticulatus* spp. and *Glugoides intestinalis*. Basing on a preliminary broad-scale phylogenetic analysis (not shown), *Orthosomella operophterae* and *Liebermannia dichroplusiae*, also belonging to Clade IV, were found to be an appropriate outgroup for molecular phylogeny.

BI and ML methods resulted in phylograms of identical topology, indicating *Mrazekia macrocyclopolis* as a sister taxon to the cluster of *Cystosporogenes* spp. Similarity of the SSU rRNA gene sequence of *M. macrocyclopolis* to *Cystosporogenes legeri* and *Cystosporogenes operophterae* was 91.1% and 90.7%, respectively.

DISCUSSION

Taxonomy of the species

The first microsporidium with rod-shaped spores was described as *Mrazekia argoisi* by Leger and Hesse

(1916). It infected fat body cells that surrounded the gut of *Asellus aquaticus*. Before microsporidia were studied using EM, species with rod-shaped spores were attributed to this genus. In his monograph, Kudo (1924) listed eight species of *Mrazekia*. Then, further examination of rod-shaped forms lead to the description of ten new species, based mainly on nuclear apparatus and spore wall structure (Canning and Vávra 2000). To date, there are five disporoblastic genera besides *Mrazekia* that possess diplokaryotic spores with manubrium (*Bacillidium*, *Jirovecia*, *Hrabyeia*, *Rectispora* and *Scipionospora*). However, only the manubrium of *Mrazekia* has a special structure.

A modern diagnosis of the genus *Mrazekia* is provided by Larsson *et al.* (1993) in an EM study of spores of *Mrazekia* (= *Bacillidium*) *cyclopolis* Vávra 1962 infecting *Acanthocyclops americanus*. The principal taxonomical character of the genus is the clavate manubrium. Its diameter increases more than twofold from anterior to posterior pole. *Mrazekia argoisi*, the type species of the genus, has a manubrium of similar shape in large living spores (17–23 × 3.5 µm in size), yet its ultrastructure has not been described.

Differential diagnosis

The new microsporidium we studied is most similar to *M. cyclopolis* (Table 2). Both species infect fat body cells of copepods and possess spores of similar shape. The species, described here, differs from *M. cyclopolis* by the following features: a) the mature spores are straight and not curved with spore index (length:width ratio) of 4.3 and not 5.2; b) the polar filament possesses 3.5–4.5 and not 2–3 coils; c) the manubrium widens gradually

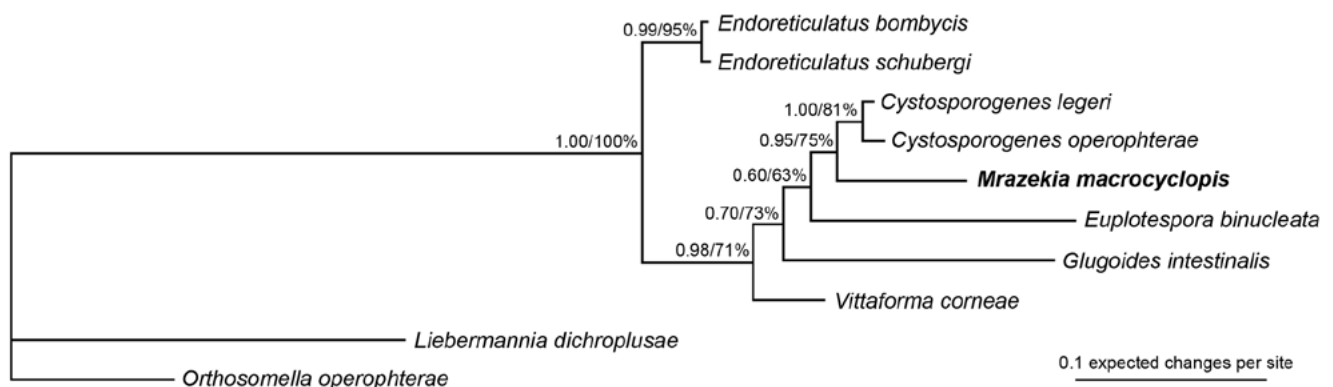


Fig. 26. Molecular phylogeny as obtained by Bayesian inference (BI) from an alignment of SSU rRNA genes of *Mrazekia macrocyclopolis* sp.n. (in bold letters) and nine other closely related microsporidian species. Maximum likelihood (ML) gave the identical topology. Branch support is given as probability (BI) and bootstrap value (ML).

Table 2. Comparison of some morphological features for species of *Mrazekia* from Crustacea.

Microsporidian species, reference	Host, country	Spore size, μm ; spore index; shape	Polar tube coils number, inclination angle	Structures around spores
<i>M. argoisii</i> Leger et Hesse, 1916	<i>Asellus aquaticus</i> France	17–23 \times 3.5; 4.8* slightly curved	?	?
<i>M. cyclopiis</i> (Vávra 1962)	<i>Acanthocyclops americanus</i> Czech Republic	7.0–7.7 \times 1.4; 5.2* slightly curved	2–3 55–65	Membranous layer
<i>M. macrocyclopiis</i> Present paper	<i>Macrocyclus albidus</i> Russia	7.3–10.5 \times 1.6–2.3; 4.3 straight	3.5–4.5 15	Typical SPV Thin-membrane individual vesicle

* Estimated using images of living spores of respective species (Larsson *et al.* 1993, Figs 9, 13).

without a sharp change to the wider part; d) the posterior part of the polaroplast is vesicular and not lamellar; e) the young spores are contained in the typical sporophorous vesicles; f) the nuclei of the diplokaryon do not enclose the posterior end of the manubrium in a mantle-like fashion. Such differences in our opinion are sufficient to validate establishment of a new species, *Mrazekia macrocyclopiis*.

Membrane structures surrounding spores of *Mrazekia* spp. (excepting *M. argoisii* as no data on ultrastructure of this species are available) require special attention. The nature of the membranes that separate from the exospore during maturation of rod-shaped spores of *Bacillidium*, *Hrabyeia*, *Jirovecia*, *Mrazekia* and *Rectispora* is discussed by Larsson *et al.* (1993) and by Vávra and Larsson (1999), yet the final opinion is not given by the authors.

Examination of ultrastructure of the sporogonial stages of the new species allows us to consider these additional membranes as SPVs of a special type, formed at the final phase of sporogony. Any kind of membranes produced by the parasite that separate its cell from the host cell cytoplasm are recommended to refer to as a pansporoblast (=SPV) (Vávra 1976).

Merontogenetic and sporontogenetic SPV membranes have been described in microsporidia up to now. It seems that membranes in rod-shaped spores are formed during the transition phase from sporoblast to spore (Figs 12–13) and could be referred to as sporoblastogenetic. Separation of membranes with SPV cavity formation indicative of a sporoblastogenetic SPV was

reported in the cases of *Holobispora* and *Lanatospora* (Voronin 1990).

The modern diagnosis of the genus of *Mrazekia* (Larsson *et al.* 1993, p. 59) is based upon the study of *M. cyclopiis* (Vávra, 1962), which is not the type species for this genus. Both *M. cyclopiis* and the species we describe here are characterized by SPV formation during the final phases of sporogony, which has been confirmed for the former species upon revision of respective negatives (J. Vávra, pers. comm.). This character was not included in the genus diagnosis. Moreover, the absence of parasitophorous vesicles is stressed in the genus diagnosis. For these reasons, we find it necessary to emend the genus diagnosis with changes italicized.

***Mrazekia* Leger and Hesse, 1916, emended diagnosis**

Merogony and sporogony diplokaryotic. Disporoblastic. Spores diplokaryotic, cylindrical, without tail-like prolongations. *Sporophorous vesicles are produced during late sporogony and contain individual young spores.* Polar filament with a straight, wide anterior manubroid part, of which the posterior end is prominently enlarged, and a narrow, coiled posterior section. Polar filament bent anteriorly, forming an angle to the longitudinal axis of the spore; the filament is ejected at a more or less prominent angle to the spore. Only one sporogonial sequence observed.

Diagnosis of *Mrazekia macrocyclopiis* sp.n.

Type host: *Macrocyclus albidus* (Jur.) (Crustacea, Copepoda).

Localization: Adipose tissue; generalized infection at the final stage of the disease.

Type locality and collection date: The pond in the Victory Park, Saint Petersburg, Russia; October 2008 (59°51'63"N, 30°24'99"E).

Morphology of the life cycle stages: All stages of the life cycle are diplokaryotic. The rod-shaped spores are 7.3–10.5 × 1.6–2.3 µm in size (alive) with an ultrastructure typical of the genus. The polar filament consists of anterior clavate manubrium and a very thin distal part arranged in 3.5–4.5 coils. The spores are enclosed in the individual sporophorous vesicles.

Deposition of types: The slides with Giemsa-stained smears are deposited at the State Collection of Entomopathogenic and Phytopathogenic Microorganisms and their Metabolites affiliated to the All-Russian Institute of Plant Protection RAAS (Podbelsky sh. 3, 196608 St. Petersburg, Pushkin, Russian Federation). Deposition numbers 330–333.

Etymology: The species name alludes to the host genus.

Molecular phylogeny of *Mrazekia macrocyclopolis* sp.n.

A preliminary initial phylogenetic analysis based on the SSU rDNA sequence of *Bacillidium vesiculiformis* from an oligochaete, suggested that this species belongs to one of the earliest branches of the tree of the phylum Microsporidia. The unique morphology of this microsporidium (rod-shaped spores with manubrium), representative of the family Mrazekiidae, was suggested to be the result of a long isolation (Nilsen 1999). However, *M. macrocyclopolis* has a spore morphology similar to *B. vesiculiformis* yet is placed in the Clade IV, distant from the latter species in the phylogenetic system (Vossbrinck, Debrunner-Vossbrinck 2005). Thus, DNA sequence analysis does not confirm that these two species belong to different genera of the same family and the family Mrazekiidae appears to be polyphyletic. Special features found by Larsson *et al.* (1993) in manubrium ultrastructure of *Mrazekia* differentiate it from *Bacillidium* and this difference might be of greater importance than similarity in the spore shape. Noteworthy, a similar structure of the manubrium is observed in *Microfilum lutjani* (Faye *et al.* 1991), a gill parasite of fish which is strikingly different from *Mrazekia* in other morphological features. Unfortunately, SSU rDNA sequence data are not available for this species.

The cluster incorporating *M. macrocyclopolis* includes species of parasites that do not form rod-shaped spores and possess an isofilar polar filament and de-

velop mainly in the gut epithelium of the host (with the exceptions of *Vittaforma* and *Euplotespora*) or cause a generalized invasion. The host range of parasites of this clade is rather wide, including lepidopteran insects (*Cystosporogenes*, *Endoreticulatus*), microcrustaceans (*Glugoides*, *Mrazekia*), ciliates (*Euplotespora*) and humans (*Vittaforma*), although the latter is likely an opportunistic infection (Weiss and Vossbrinck 1999). Most of these genera, except for *Mrazekia* and *Vittaforma*, possess a monokaryotic nuclear apparatus. Placing of *M. macrocyclopolis* within this cluster is another confirmation of the conclusion that “the characters used to distinguish among the higher taxonomic levels of the Microsporidia change state very rapidly and taxonomies based on these characters result in unacceptable polyphyletic clades” (Vossbrinck and Debrunner-Vossbrinck 2005).

Acknowledgements. The authors are greatly indebted to Dr. Jiří Vávra (Charles University, Prague, Czech Republic) for critical reviewing of an early draft of the manuscript and to Igor V. Senderskiy (All-Russian Institute of Plant Protection, St. Petersburg, Russia) for technical assistance with sample preparation and light microscopy. The research is supported by the Russian Foundation for Basic Research, grant no. 10-04-00284, and by a grant of the President of the Russian Federation MK-3419.2009.4.

REFERENCES

- Canning E. U., Vávra J. (2000) Phylum Microsporidia Balbiani, 1882. In: An illustrated guide of the Protozoa, (Eds. J. J. Lee, G. F. Leedale, P. C. Bradbury). Society of Protozoologists, Lawrence, KS, **1**: 39–126
- Faye N., Toguebaye B. S., Bouix G. (1991) *Microfilum lutjani* n.g., n.sp. (Protozoa, Microsporidia), a gill parasite of the Golden African Snapper *Lutjanus Fulgens* (Valenciennes, 1830) (Teleost Lutjanidae): developmental cycle and ultrastructure. *J. Protozool.* **38**: 30–40
- Hall T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp.* **41**: 95–98
- Kudo R. (1924) A biologic and taxonomic study of the Microsporidia. *Illin. Biol. Monograph.* **9**: 1–268
- Larsson R., Vávra J., Schrevel J. O. (1993) *Bacillidium cyclopolis* Vávra, 1962 – Description of the ultrastructural cytology and transfer to the genus *Mrazekia* Leger and Hesse, 1916 (Microsporida, Mrazekiidae). *Eur. J. Protistol.* **29**: 49–60
- Leger L., Hesse E. (1916) *Mrazekia*, genre nouveau de Microsporidies a spores tubileuses. *C. R. Soc. Biol.* **79**: 345–348
- Nilsen F. (1999) Small subunit rDNA phylogeny of *Bacillidium* sp. (Microsporida, Mrazekiidae) infecting oligochaets. *Parasitol.* **118**: 553–558
- Posada D. (2008) jModeltest: Phylogenetic Model Averaging. *Mol. Biol. Evol.* **25**: 1253–1256
- Ronquist F., Huelsenbeck J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics.* **19**: 1572–1574

- Sambrook J., Fritsch E., Maniatis T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
- Swofford D. L. (2003) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4. Sinauer Associates, Sunderland, MA
- Vávra J. (1962) *Bacillidium cyclopis* n. sp. (Cnidospora, Microsporidia), a new parasite of copepods. *Vestn. Česk. Spol. Zool.* **26**: 295–299
- Vávra J. (1976) Structure of microsporidia. In: *Comparative pathobiology I*, (Eds. I. A. Bulla Jr., T. C. Cheng). Plenum Press, New York–London, 1–85
- Vávra J., Larsson R. (1999) Structure of the Microsporidia. In: *The Microsporidia and Microsporidiosis*, (Eds. M. Wittner, L. M. Weiss). ASM Press, Washington, 7–84
- Voronin V. N. (1986) The microsporidia of crustaceans. *Protozoology* (Leningrad). **10**: 137–165 (in Russian)
- Voronin V. N. (1990) The effect of methods of the host preparation on the Microsporidian extrasporal coat (Microsporidia). *Parazitologiya*. **3**: 242–244 (in Russian)
- Voronin V. N. (1999) The microsporidia of freshwater invertebrates and fish of Russia. The thesis of doctoral dissertation, Saint-Petersburg, 45 pp. (in Russian)
- Vossbrinck C. R., Debrunner-Vossbrinck B. A. (2005) Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitologica* **52**: 131–142
- Weiss L. H., Vossbrinck C. R. (1999) Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. In: *The Microsporidia and Microsporidiosis*, (Eds. M. Wittner, L. M. Weiss). ASM Press, Washington, 129–171
- Weiss L. M., Zhu X., Cali A., Tanowitz H. B., Wittner M. (1994) Utility of microsporidian rRNA in diagnosis and phylogeny: a review. *Folia Parasitol.* **41**: 81–90

Received on 27th July, 2009; revised version on 11th October, 2009; accepted on 23rd October, 2009

