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ORIGINAL PAPER

Life cycle, ultrastructure, and molecular phylogeny of *Crispospora chironomi* g.n. sp.n. (Microsporidia: Terresporidia), a parasite of *Chironomus plumosus* L. (Diptera: Chironomidae)

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Abstract The life cycle, ultrastructure, and molecular phylogeny of a new microsporidium *Crispospora chironomi* g.n. sp.n., a parasite of the midge *Chironomus plumosus*, are described. The parasite infects the gut epithelium of the host larvae and possesses sporogonies of two types, polysporoblastic and disporoblastic, respectively, proceeding within the same host cell. In the sporogonial sequence of the first type, dozens of spherical monokaryotic spores within a thick-walled capsule are formed. The spores are 1.5–2.0 μm in diameter; the exospore possesses two to three bundles of tubular protrusions. In the sporogonial sequence of the second type, diplokaryotic oval spores, 2.5×1.5 μm in size, are formed within a compartment, partially surrounded with multilayered membranes. Spores of both types are similar in respect to inner structure, possessing a well-developed extrusion apparatus with (a) the anterior vesicular part of the polaroplast covering the lamellar posterior one and (b) isofilar polar filament with several coils in one row. Small subunit ribosomal DNA phylogeny showed position of the new microsporidium in a cluster uniting microsporidia of terrestrial origin infecting diverse hosts, nested within Clade IV, corresponding to Class Terresporidia sensu Vossbrinck and Debrunner-Vossbrinck (Folia Parasitol 52:131–142, 2005).

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Introduction

The midge larvae are among the most preferred insect hosts for the microsporidian parasites. This fact can be explained by long-term development (e.g., 2–3 years in Northwestern Russia) and mass propagation of these hosts in the silt layer of the lake bottom, both features being favorable for prolonged acquisition of microsporidian spores from various sources. Over 50 species of microsporidia are known to parasitize chironomids (Voronin 1999) though ribosomal DNA sequence data, inevitable for phylogenetic studies of microsporidia, have been acquired previously only for one species, *Anisofilariata chironomi* (Tokarev et al. 2010). The majority of the species known infect fat body, and only four species are found in the gut epithelium of the host, namely *Nosema chironomi* (Kudo 1924), *Thelohania hessei* (=*Cocconema octospora*; Weiser 1961), *Thelohania chironomi* (Debaisieux 1928), and *Stempellia halliellae* (Codreanu-Bălcescu and Codreanu 1980). Of these, ultrastructural analysis has been performed only for *S. halliellae*, while taxonomic position of other species mentioned was not validated.

In 2008, a microsporidian infection of the epithelial gut cells of the *Chironomus plumosus* larvae was revealed, caused by a parasite producing spores of two types with ultrastructure differing from that of known microsporidian species. The present paper describes the

life cycle, ultrastructure, and molecular phylogeny of this new microsporidium *Crispospora chironomi*, being a type species of a new genus *Crispospora*.

Materials and methods

The microsporidium being the subject of the present study was found for the first time in *C. plumosus* larvae sampled in Leningrad Region in 1998. It was studied using light (LM) and electron microscopy (EM), but was not described. The infected midge larvae of *C. plumosus* L. (Diptera: Chironomidae) were collected in the Pobednoe Lake, Vyborg District of Leningrad Region (60°21′64″N, 29°25′86″E), in July–October 2008, alongside with larvae of the same host, infected with *A. chironomi* (Tokarev et al. 2010).

For LM, smears of the infected insect tissues were fixed with methanol 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 EM, samples were fixed with 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer with 4% sucrose for 1 h and postfixed with 1% cacodylate-buffered osmium tetroxide for 1 h. The tissues were dehydrated in ascending ethanol series and absolute acetone, subsequently embedded into epon–araldite resin. Ultrathin sections were cut using Reichert Ultracut and stained with 2% uranylacetate in 50% ethanol and lead citrate for 10–20 min. The ultrathin sections were examined with JEM-100CX II electron microscope at an accelerating voltage of 80 kV.

For rDNA extraction, an infected midge larva was 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 mgml^{-1}) were added to the samples and incubated for 3 h at 65°C. DNA was extracted routinely with phenol–chloroform (Sambrook et al. 1989) and resuspended in 50 μl UHQ water. To amplify the small subunit (SSU) rRNA gene of microsporidia, universal microsporidia primers V1f (Weiss et al. 1994) and 1492r (Weiss and Vossbrinck 1999) were used.

PCR was run using a Bio-Rad iCycler in 20 μl volume containing 5 μl DNA template, 2 μl of 10× PCR buffer, 1 μl of 10 μM dNTPs mixture, 1 μl of each forward and reverse 10 μM primers (Evrogen, Russia), and 1 U of Taq-polymerase (Sileks, Russia). A first cycle of denaturation was carried out at 92°C for 3 min, and a last cycle of extension was carried out at 72°C for 10 min. Samples were amplified for 30 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s, and elongation at 72°C for 60 s. The PCR product, about 1,200 bp in size, was gel purified and cloned into pAL-TA vector (Evrogen, Russia). Three resulting plasmids, purified with phenol–chloroform, were sequenced in both directions using M13F and M13R primers with identical results.

Newly obtained rDNA sequence, submitted to GenBank under accession number GU130407, 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 (Hall 1999). Regions containing gaps and ambiguous sites were removed, leaving an alignment of 1,093 bp length. The number of parsimony informative characters was 348. Phylogenetic reconstructions were carried out with Bayesian Inference (BI) using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and Maximum Likelihood (ML) using PAUP* v4.0 β 10 (Swofford 2003), following the approach of Refardt et al. (2008). ML and BI settings were selected using jModelTest v0.1.1 (Posada 2008) by AIC and BIC, respectively.

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, which estimates the precision of the clade probabilities, reached 0.009 after 100,000 generations. Tree search in PAUP* was done heuristically with random stepwise addition (ten replicates) and TBR branch swapping. A majority rule consensus tree was calculated from 100 bootstrap replicates.

Results

Smears and ultrathin sections of the infected midge gut demonstrate merogonial and sporogonial stages, including spores of two types: the monokaryotic ones within the thick-walled formations referred to as “capsules” and the diplokaryotic ones within the host cell cytoplasm. Sporogonies of both types occur within the same host cell.

Light microscopy

Smears of the host gut contain prespore stages being few in number, multiple spore-containing capsules and rare single spores. Developmental merogonial stages are presented by round diplokaryotic cells and plasmodia with 2–8 diplokarya (Figs. 1, 2, 3). The sporogonial stages of the predominant type are characterized by formation of multinuclear oval plasmodia, limited by a

Table 1 List of microsporidian species used in the molecular phylogeny study of *Crispospora chironomi* (in bold letters)

Microsporidia species	Host	Accession no.
Anisofilariata chironomi	*Chironomus plumosus* (Diptera: Chironomidae)	GU126383
Crispospora chironomi	*Chironomus plumosus* (Diptera: Chironomidae)	GU130407
Cystosporogenes operophterae	*Operophtera brumata* (Lepidoptera: Geometridae)	AJ302320
Enterocytozoon bieneusi	*Macaca mulatta* (Primates: Cercopithecidae)	AF023245
Endoreticulatus bombycis	*Bombyx mori* (Lepidoptera: Bombycidae)	AY009115
Euplotespora binucleata	*Euplotes woodruffi* (Spirotrichea: Euplotidae)	DQ675604
Glugoides intestinalis	*Daphnia magna* (Crustacea: Branchiopoda)	AF394525
Liebermannia dichroplusiae	*Dichroplus elongatus* (Orthoptera: Acrididae)	EF016249
Mrazekia macrocyclopis	*Macrocyclops albidus* (Copepoda: Cyclopidae)	FJ914315
Nucleospora salmonis	*Oncorhynchus tshawytscha* (Pisces: Salmonidae)	AF185992
Orthosomella operophterae	*Operophtera brumata* (Lepidoptera: Noctuidae)	AJ302317
Vittaforma corneae	*Homo sapiens* (Primates: Hominidae)	U11046

thin wall which becomes thicker on certain regions of the capsule wall (Figs. 4, 5, 6). At the beginning of the spore maturation, the capsule wall becomes thick over its entire surface (Fig. 7). The large spherical capsules, 30–50 μm in diameter, are prevailing in the smears. They contain tens to hundreds of small spherical spores, 1.5–2.0 μm in size (fixed), surrounded by a narrow translucent rim (Fig. 8). Liberated from the capsules, monokaryotic spores on the background of the stained host tissue smear demonstrate wide asymmetric halo (Fig. 9). In the case of a heavy infection, solid masses of spore-bearing capsules fill up the entire host gut epithelium (Fig. 12). The capsule wall is 2–4 μm thick and is retained even after the spores are released from the capsule (Fig. 9).

Sporonts and sporoblasts of the sporogony of the second type are oval cells with one or two diplokarya and intensively stained cytoplasm developing in direct contact with the host cell cytoplasm (Fig. 10). This sporogony is disporoblastic, producing diplokaryotic spores. Spores are oval wide, 2.5×1.5 μm in size (fixed), with a clearly seen posterior vacuole. Narrow translucent rim surrounds thin spore wall of the stained spores on the smears (Fig. 11).

Electron microscopy

The earliest developmental stages found on the ultrathin sections are the diplokaryotic meronts of the second merogony. The meront is a round cell about 1.5 μm in diameter with one diplokaryon *ca.* 1.0 μm in diameter (Fig. 13). The diplokaryon divides repeatedly and a rosette-like merogonial plasmodium with 5–8 nuclei, being 0.5–0.75 μm in diameter, is formed (Fig. 14). Further development of microsporidia proceeds in one of two developmental sequences, each leading to formation of spores of one type.

In the developmental sequence of the first type, the next stage observed on the ultrathin sections is the meront/sporont transitional stage in which electron-dense matter is being deposited in form of loops and rings (Fig. 15). The consequent sporogonial plasmodium develops within a membrane-bound compartment (the spore-bearing capsule to be) and several rosette-like plasmodia are formed within a capsule (Figs. 16, 17). Nuclear division goes on unless the lumen of the capsule is packed with the parasite cells. Then the rosette buds separate into sporoblasts 1.5×1.2 μm in size (Fig. 18). Number of sporoblasts and consequently spores varies from several dozens to hundred and more. Young spores possess five coils of the polar filament, cytoplasm of varying electron density and the spore wall presented by the exospore in the form of a thick membrane with adjacent layer of tubuli (Fig. 19).

The mature spores of the first type are spherical, possessing a complicated structure and a developed extrusion apparatus (Figs. 24, 25, 26, 27, 28). The diameter of the spores on the ultrathin sections is 1.6–2.0 μm (average 1.7 μm) without taking into account the tubular protrusions of the exospore. The anchoring disk, 100 nm in diameter, lays eccentrically to the long axis of the spore. Edges of the polar sac cover the anterior part of the polaroplast (Fig. 25). The polaroplast is bipartite and also lays eccentrically to the long axis of the spore (Fig. 24). On the transverse sections, the anterior part of the polaroplast is horseshoe-shaped, consists of small vesicles limited by a thin membrane, and covers the posterior part (Figs. 24, 27). In young spores, the posterior part of the polaroplast consists of short loose lamellae, forming a narrow zone around the anterior region of the polar filament. In mature spores, it is made up by tightly packed thin lamellae, deposited obliquely to the polar filament. The polar filament is isofilar, 40 nm in diameter; its anterior region is bent turning round the nucleus and 5–6 coils form one layer (Figs. 25, 29). Single nucleus, found in the center of the spore, is spherical, 0.5–0.6 μm in diameter (Fig. 29), or is elongated, 0.4×0.8 μm in size (Fig. 28). The spore wall

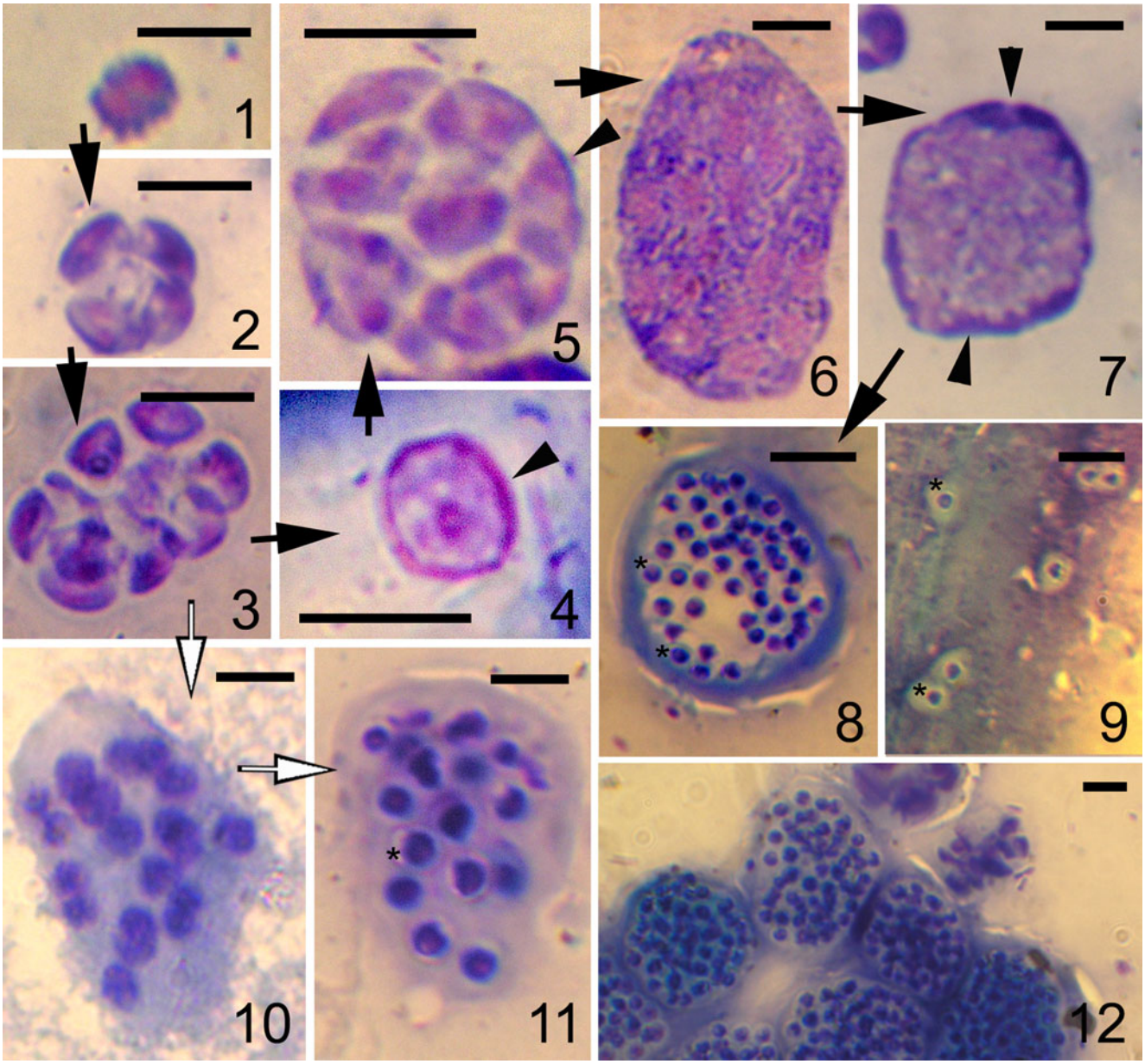

Fig. 1 *Crispospora chironomi* sp. n. Light microscopy of the Giemsa-stained slides. **1–3** Consequent diplokaryotic stages of the first merogony. **4–7** Formation of the capsule wall (*arrowheads*) in developmental stages of the second merogony (*4*, *5*) and the sporogony of the first type (*6*, *7*). **8** Capsule with thick walls (*arrowhead*) containing the mature spores surrounded by a narrow translucent rim (*asterisk*). **9** Monokaryotic spores on a smear of the host tissues with an asymmetric halo seen around the spores (*asterisk*). **10**, **11** Sporogony of the second type with disporoblastic division of sporonts (*10*) and spores (*11*) surrounded by a translucent rim (*asterisk*). **12** Several spore-bearing capsules on a smear from heavily infected host tissue. *Black* and *white arrows* indicate direction of developmental sequences of the first and the second type, respectively. *Scale bar*—2 μm (*1–7*), 5 μm (*10*, *11*), and 10 μm (*8*, *9*, *12*)

consists of the endospore, 80 nm thick, and the trilaminar exospore which forms protrusions of long tubules 20 nm in diameter. In capsules tightly packed with the spores, these tubules form a continuous layer around the spore (Fig. 20). In spores which lie freely in the capsule lumen or are liberated from the capsule, the exospore protrusions are organized in 2-3 bundles. One of the bundles is located on the anterior pole of the spore and the other two (or one) are on the lateral sides of the spore (Figs. 24, 26, 27, 28), corresponding to the asymmetric halo observed on light micrographs (Fig. 9).

The lumen of the capsule containing the mature spores is filled with fine hairy filaments forming a radial network around the spores with rare granular inclusion of moderate electron density (Figs. 24, 25, 26).

The sporogony of the second type also proceeds with the plasmatomy of the merogonial plasmodium. The consequent developmental stages (all diplokaryotic) observed on

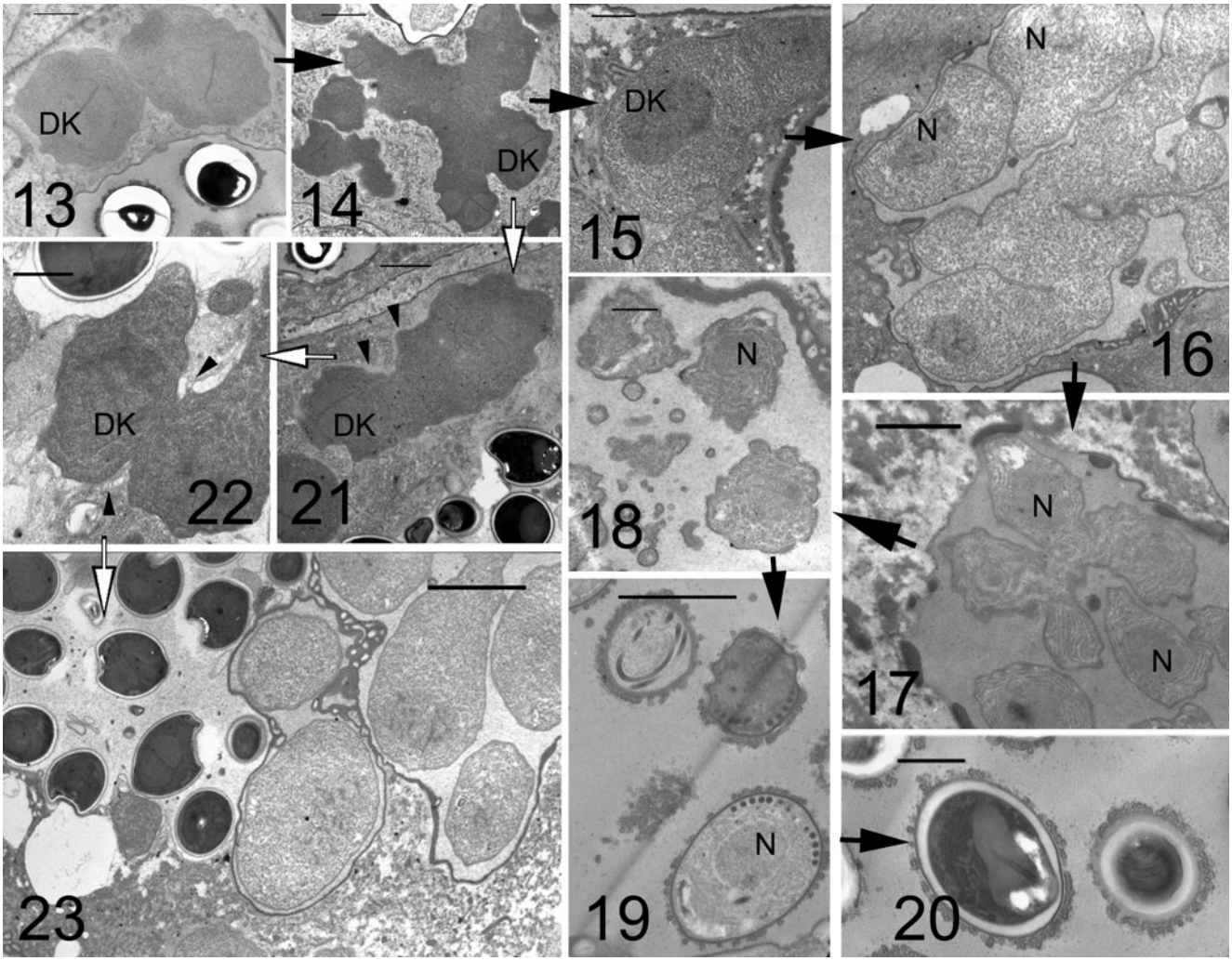

Fig. 2 Ultrastructure of the life cycle stages of *Crispospora chironomi*, presenting the developmental cycles of the first (*13–20*, *black arrows*) and the second type (*14*, *21–23*, *white arrows*). **13** Meronts of the second merogony developing in direct contact with the host cell cytoplasm. **14** Rosette-like merogonial plasmodium of the second merogony. **15** Meront/sporont transitional stage with electron-dense material accumulating between the membranous layers. **16** Rosette-like sporogonial plasmodia in the early phase of formation. **17** Rosette-like sporogonial plasmodia giving rise to the sporoblasts. **18** Sporoblasts at the phase of spore organelle formation. Cavities are seen within the lace-like capsule wall. **19** Young spore with eccentric extrusion apparatus and tubular protrusions of the exospore. **20** Mature monokaryotic spores. **22** Diplokaryotic sporont with multilayer membranes adjacent to its surface (*arrowheads*). **21** A pair of diplokaryotic sporoblasts surrounded with multilayer membranes (*arrowheads*). **23** Diplokaryotic spores occupying the same cell as the vacuole containing a rosette-like sporogonial plasmodium of the first developmental sequence. *DK* diplokaryon, *N* single nucleus. *Scale bars*—0.76 μm (*18*, *20*), 0.97 μm (*15*, *17*, *22*), 1.21 μm (*13*), 1.51 μm (*21*), 1.92 μm (*17*, *19*), 2.42 μm (*14*), and 3.84 μm (23)

the ultrathin sections are (a) the meront/sporont transitional stage with an additional sheath of comparatively lesser electron density on the plasma membrane (Fig. 21), (b) pairs of sporoblasts with cytoplasm containing well-developed endoplasmic reticulum (Fig. 22), (c) the spores (Fig. 23). The diplokaryotic spores are short oval, 0.9–2.0 (average 1.3)×0.6–1.4 (1.0) μm in size (fixed). The anchoring disk is 100 nm in diameter. The polaroplast is composed of two parts. The anterior part shows like a short bell on transverse sections and its cavity is filled with small vacuoles with electron-dense content (Fig. 30). The posterior part is lamellar with appearance of a large-scaled onion (Figs. 29, 33), adjacent to one of the lateral sides of the spore. Two nuclei, each 0.5×0.6 μm in size, are located vertically on the opposite side of the spore. The polar filament forms three to four coils in one layer (Figs. 32, 34). The posterior vacuole is limited by a double membrane. Due to the vacuole shrinking during fixation, the posterior pole becomes either flattened or invaginated (Fig. 32). The spore wall is thin as the endospore is up to 40 nm thick and the exospore is about 20 nm thick (Figs. 33, 34, 35). At higher magnifications, fragile thin filaments or semitransparent villi are seen on the outer surface of the exospore (Fig. 34). Stacks of short or long

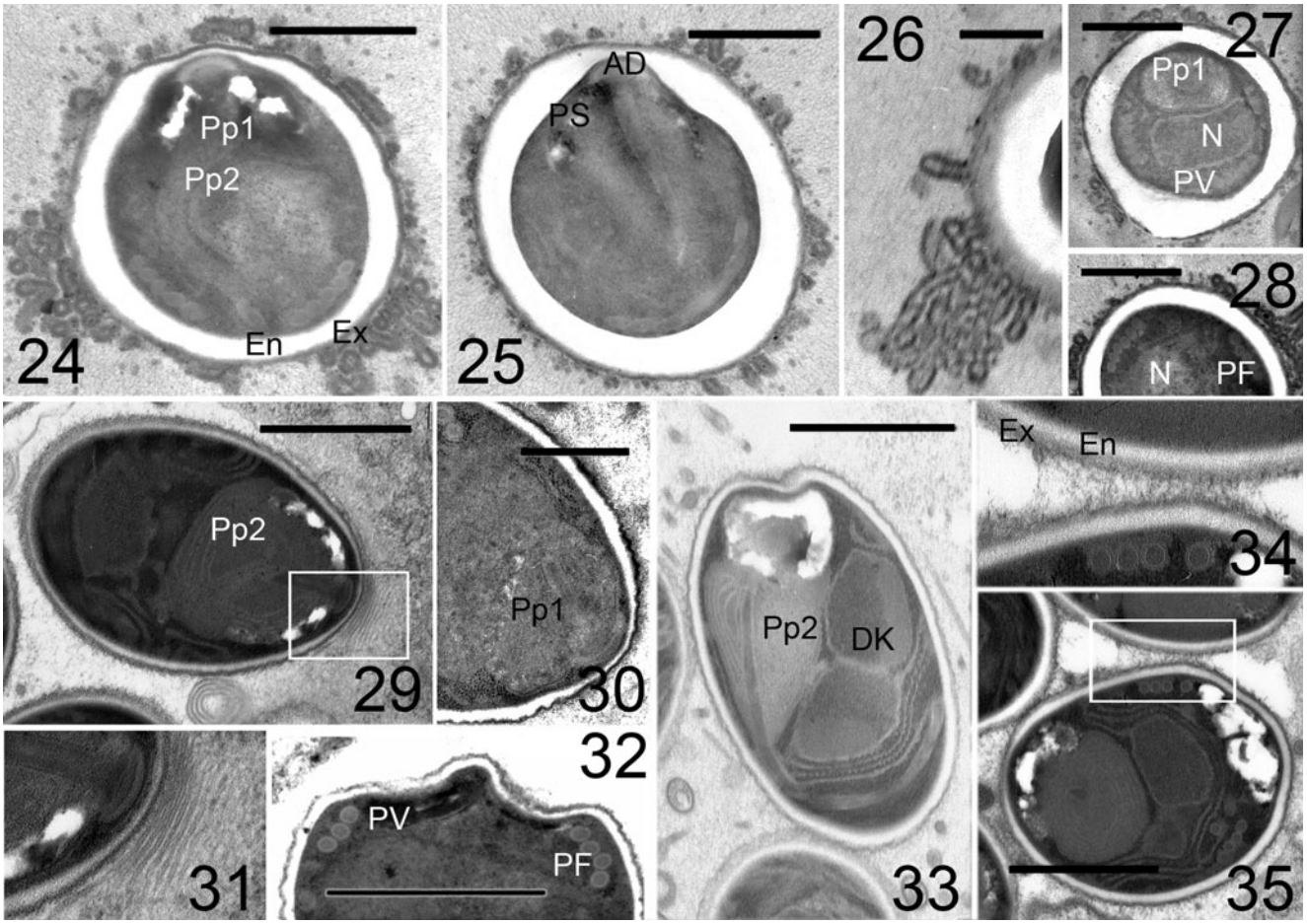

Fig. 3 Details of ultrastructure of monokaryotic (*24–28*) and diplokaryotic spores (*29–35*) of *Crispospora chironomi*. **31**, **34** Enlarged fragments of **29** and **35**, respectively. *AD* anchoring disk, *En* endospore, *Ex* exospore, *Pp1* anterior part of the polaroplast, *Pp2* posterior part of the polaroplast, *PS* polar sac, *PF* polar filament coils, *PV* polar vacuole; other abbreviations as in Figs. 13–23. *Scale bars*—0.38 μm (*26*, *30*), 0.76 μm (*24*, *25*, *27*, *28*, *32*), and 0.97 μm (*29*, *33*, *35*)

membranes are found around the spores in the host cell cytoplasm (Fig. 31).

As a rule, mature spores of the second type are found within the host cells alongside with the sporogonial plasmodia of the first developmental sequence (Fig. 23), the latter taking seemingly more time to proceed due to repeated division of merogonial and sporogonial plasmodia with formation of dozens of spores within each capsule.

DNA sequence analysis

Sequencing of the SSU rDNA fragment from the newly found microsporidium produced a sequence of 1,230 bp, deposited in GenBank under accession number of GU130407. A BLAST search against GenBank found the maximal sequence similarity of 91.1% between this parasite and *Vittaforma corneae* (GenBank Accession # U11046). Sequence similarity between *A. chironomi* (GenBank Accession # GU126383), another microsporidian parasite of *C. plumosus*, and the one studied in the present work is only 73.2%. SSU rDNA sequences of ten closely related species of microsporidia were used for phylogenetic reconstruction, including *Liebermannia dichroplusiae* and *Orthosomella operophterae* chosen as an outgroup (Table 1).

BI and ML methods resulted in phylograms of similar topology, placing the new microsporidium among microsporidia of terrestrial origin belonging to the Clade IV, Class Terresporidia sensu Vossbrinck and Debrunner-Vossbrinck (2005). The particular branch containing the new species includes microsporidia from diverse hosts: ciliates (*Euplotespora binucleata*), microcrustaceans (*Glugoides intestinalis*, *Mrazekia macrocyclopis*), lepidopteran insects (*Cystosporogenes* spp., *Endoreticulatus* spp.), and human (*V. corneae*) with *A. chironomi* in a basal position. BI method indicated the new species in a position basal to the cluster of *Mrazekia*, *Cystosporogenes*, and *Euplotespora*, while ML tree was not fully resolved as it contained the polytomous branch of *Mrazekia/Cystosporogenes*, *Endoreticulatus*, *Euplotespora*, *Vittaforma*, and the new species. This poly-

tomy could not be removed using different taxa combinations, search methods, and model parameters (Fig. 36).

Discussion

Differential diagnosis

Besides the newly described microsporidium, there is only one other species of a microsporidium, *Kinorhynchospora japonica* from gut epithelium of kinorhynches, forming spores of two types, uninucleate pyriform and binucleate horseshoe-shaped ones, markedly different from those described in this study (Adrianov and Rybakov 1991). However, no other microsporidia are known from gut epithelium of chironomids or any other animal hosts to form spores of two types, with spherical monokaryotic spores developing in thick-walled structures and short oval diplokaryotic ones—in direct contact with the host cell cytoplasm. Moreover, only in this newly found species exospore of monokaryotic spores bears bundles of tubular protrusions and spores of both types, in spite of rather minute sizes, possess well-developed extrusion apparatus. These features allow us to establish a new genus *Crispospora* with the type species *C. chironomi.*

Diagnosis of *Crispospora* g.n.

Microsporidium is dimorphic, diplokaryotic, and monokaryotic. Sporogony with two sequences, producing spores of two morphological types within the same host cell. Diplokaryotic merogonial plasmodium divides to produce cells with one diplokaryon. In the first developmental sequence, merogonial stages are surrounded by membranous structures, which give rise to the capsule wall during sporogenesis. Repeated rosette-like fission of merogonial and sporogonial plasmodia leads to formation of multiple spherical uninucleate spores within a thick-walled capsule. The exospore possesses protrusions in the form of tubular structures. The sporogony of the second type is disporoblastic, producing diplokaryotic spores which retain direct contact with the host cell cytoplasm. Spores of both types possess well-developed extrusion apparatus, isofilar polar filament, and bipartite polaroplast.

Type species: *C. chironomi*

Etymology: The genus name is given in respect to the exospore protrusions in the form of bundles of tubular structures, resembling curls (*crispus* in Latin) in the spores of the first type.

Diagnosis of *C. chironomi* sp.n.

Type host: *C. plumosus* L. (Diptera: Chironomidae)

Type localization: midgut epithelium of the host larvae

Type locality: the Pobednoe Lake of Vyborg District, Leningrad Region, Northwestern Russia (60°21′64″N, 29° 25′86″E)

NCBI GenBank nucleotide accession number: *C. chironomi* (*C. plumosus*)—GU130407

The species diagnosis corresponds to that of the genus. Spherical monokaryotic spores are 1.5–2.0 μm in diameter (fixed), isofilar polar filament possesses five to six coils, the polaroplast is bipartite with the anterior part made up by small vesicles, limited by a thin membrane, and covering the posterior part, made up by tightly packed thin lamellae, deposited obliquely to the polar filament. The exospore possesses protrusions in the form of tubules up to 0.5 μm long, organized in two to three bundles or continuous layer around the spore. One capsule contains dozens of spores. Diplokaryotic spores are short oval, 2.5×1.5 μm in size (fixed), possessing isofilar polar filament with three to four coils and bipartite polaroplast.

Etymology: The species name corresponds to the genus name of the type host.

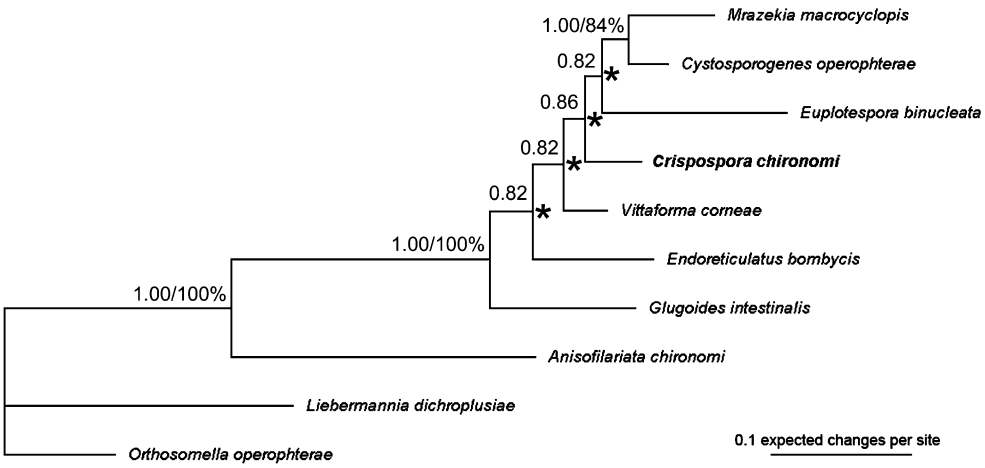

Fig. 4 Molecular phylogeny as obtained by Bayesian inference (BI) from an alignment of SSU rRNA genes of *Crispospora chironomi* (in *bold letters*) and nine other closely related microsporidian species. Maximum likelihood (ML) gave the similar topology. Branch support is given as probability (BI) and bootstrap value (ML) where applicable. *Asterisks* indicate nodes which are not resolved in ML, resulting in a polytomous branch with bootstrap value of 78%

Life cycle of *C. chironomi*

The genus of *Crispospora*, according to its diagnosis, possesses a complex life cycle with sporogonies of two types. A complex life cycle involving an obligate intermediate host is thought to be the ancestral condition in microsporidia (Baker et al. 1997). Microsporidia of genera like *Vairimorpha* switch to produce either diplokaryotic or monokaryotic spores within one host, and this is the case with *Crispospora* as well, which is not closely related to, but joins the same clade (i.e., presumably possesses the same ancestor) as *Vairimorpha*. The roles of spores of two types are not clear, but we may speculate the following. Formation of multiple monokaryotic spores retained within the capsules is likely to facilitate the success of infection of a new host acquiring dozens of spores in case of capsule ingestion, corresponding to the concept of "effective infection dose." Meanwhile, the sporogony producing the diplokaryotic spores is accomplished earlier and might serve for autoinfection so that more host cells become infected during the disease (Issi 1986).

Change of the nuclear phase is indicative of sexual process in microsporidian development and uninucleate spores are referred to as gametes (Vossbrinck and Debrunner-Vossbrinck 2005). This explains the presence of two developmental sequences as a requirement for sexual process. Moreover, the "effective infection dose" provides the intake of "more than one" uninucleate spore by one host being another requirement for sexual process.

Nuclear phase, type of sporogony, and other features of the microsporidian life cycle are shown to be extremely labile and presented oppositely in closely related forms. For example, two closely related taxa, *Binucleata daphniae* and *Senoma globulifera*, the parasites of a cladoceran and a mosquito, respectively, demonstrate SSU rDNA sequence similarity to each other *ca.* 96% and rather dissimilar spore ultrastructure (Simakova et al. 2005; Refardt et al. 2008). A possible explanation is the divergence of these forms from a common ancestor with two dissimilar developmental sequences proceeding within determinative and intermediate hosts, respectively. In another case, three species of the genus *Liebermannia*, all from orthopteran insects, are similar in ultrastructure but different in nuclear phase, so that the presence of diplokaryotic spores is removed from the revised genus diagnosis (Sokolova et al. 2009). Another example is the taxonomy of *Nosema*/*Vairimorpha* clade in which assignment to one of these two genera, formally based upon developmental sequence, is not congruent with phylogenetic relationships, substantiating the need for revision of these taxa (Baker et al. 1995; Kyei-Poku et al. 2008). It can be therefore expected that after a large-scale readjustment of microsporidian taxonomy, life cycle features will not be used in the diagnosis of microsporidian genera, including *Crispospora*.

Molecular phylogeny of *C. chironomi*

In the study of Vossbrinck and Debrunner-Vossbrinck (2005), microsporidia are grouped phylogenetically into five clades, forming the classes of Aquasporidia (paraphyletic, consisting of Clades I, II, and V), Marinosporidia (Clade III), and Terresporidia (Clade IV). These three classes correspond to the primary habitat (freshwater, marine, and terrestrial, respectively) of the hosts in which the microsporidia studied have been found. However, the Clade IV in the initial study incorporated few examples of microsporidia from freshwater (not terrestrial) hosts, for example, *G. intestinalis* and *Ordospora colligata* from *Daphnia* spp. Consequent works have found more freshwater microsporidia species branched within a particular cluster of Clade IV, including *E. binucleata* from a ciliate (Fokin et al. 2008), *M. macrocyclopis* from a cyclop (Issi et al. 2010), and *A. chironomi* from the midge *C. plumosus* (Tokarev et al. 2010), as well as *C. chironomi* from the same host, described in this study. Other members of this cluster are the parasites of orthopteran and lepidopteran insects and human. Phylogenetic branching of the freshwater microsporidia among Terresporidia is indicative of rather feasible evolutionary switches of microsporidia between hosts of variable habitats. Intermittent disposition of freshwater microsporidia among terrestrial ones in the phylogram might result from multiple independent events of adaptation of microsporidia originating from a terrestrial source to parasitism in freshwater hosts.

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