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Aquatic tetrasporoblastic microsporidia from caddis flies (Insecta, Trichoptera): Characterisation, phylogeny and taxonomic reevaluation of the genera *Episeptum* Larsson, 1986, *Pyrotheca* Hesse, 1935 and *Cougourdella* Hesse, 1935 ☆

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Abstract

Seven microsporidian species infecting caddis fly larvae, corresponding to conventional genera *Episeptum, Pyrotheca* and *Cougourdella* were studied using light and electron microscopy. Parts of their small subunit, ITS and large subunit ribosomal RNA genes were sequenced and compared with sequences of rDNA obtained from syntype slides of *Cougourdella polycentropi* Weiser 1965 and *Pyrotheca* sp. from *Hydropsyche pellucidula*. All studied caddis fly microsporidia form a closely related group. Their developmental stages in trichopteran hosts are restricted to fat body cells and oenocytes and have isolated nuclei. In late merogony, uninucleate meronts and binucleate plasmodia are formed. In sporogony a sporogonial plasmodium with four nuclei gives rise by rosette-like budding to four sporoblasts within a non-persistent sporophorous vesicle. Sporoblasts mature into pyriform to lageniform spores. The shape and size of spores, the number of polar filament coils, the structure of the polaroplast and of the exospore, together with morphometric characters present a set of markers unique for respective species. Four new species are established. The new genus *Paraepiseptum* is proposed to replace the tetrasporoblastic *Pyrotheca* and *Cougourdella* species from caddis flies. The genus *Episeptum* is redefined. Field and laboratory examinations as well as the phylogenetic position within the aquatic clade of microsporidia suggest that the life cycle of trichopteran microsporidia probably involves an alternate (copepod?) host and (or) transovarial transmission.

Keywords: Microsporidia; rDNA phylogeny; *Paraepiseptum* gen.n.; *Episeptum*; *Pyrotheca*; *Cougourdella*

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Introduction

Trichoptera are frequent hosts of microsporidia as 11 microsporidian genera have been reported from caddis flies. This generic heterogeneity becomes evident when the spectrum of microsporidian genera in Trichoptera is compared with that of other insect orders, particularly with terrestrial Lepidoptera, which are phylogenetically the closest group of insects to Trichoptera. Eight microsporidian genera have been described from Lepidoptera, but only two of them (*Gurleya* and *Vavraia*) exist also in Trichoptera.

A hypothesis that the aquatic way of life of trichopteran larvae is responsible for this phenomenon is of interest, as there is an increasing suspicion that many microsporidia from aquatic animals have complicated life cycles involving more than one host. So far a host exchange is known only in some mosquito microsporidia, the life cycle of which obligatorily includes a crustacean (copepod) phase (Becnel and Andreadis 1999). We selected a group of tetrasporoblastic microsporidia from caddis flies for study because of their close phylogenetic relationship to the tetrasporoblastic (rarely octosporoblastic) microsporidian *Marssoniella elegans* infecting crustacean copepods (Vávra et al. 2005).

From 11 described microsporidian genera infecting Trichoptera, four are tetrasporoblastic: *Gurleya* Doflein, 1898, *Cougourdella* Hesse, 1935, *Pyrotheca* Hesse, 1935 and *Episeptum* Larsson, 1986. Representatives of all these genera have been studied by electron microscopy (Larsson 1986, 1989, 1996; Xie and Canning 1986), but their generic affiliation and phylogeny was only cursorily examined using molecular biology techniques (Vávra et al. 2005). Except the genus *Episeptum,* the diagnosis of the other three genera has been based on old and incomplete descriptions of their type species parasitising hosts other than caddis flies (Doflein 1898; Hesse 1903, 1935).

During the years 2000–2002, the trichopteran fauna from 26 localities in the Czech Republic and Slovakia was screened. Tetrasporoblastic microsporidia were found at seven of them and were studied by light and electron microscopy, techniques of molecular taxonomy and using infection experiments mimicking field conditions.

Materials and methods

Origin of isolates; comparative isolates/species

The collected microsporidia were assigned to seven types, corresponding to conventionally defined microsporidian genera as listed in Canning and Vávra (2002): *Cougourdella*-like type (C1), *Pyrotheca*-like type (P2), *Episeptum*-like types (E3–E7), which will be referred to hereafter by these code names. Each type was recorded in a single host species and its fine structure and molecular biology was further studied using a single host larva. The origin of the respective types and the host data are given in Table 1. The occurrence of microsporidia infections was examined during 2 successive years at the collection sites, the prevalence values given below are the average values over 2 years.

The seven types were compared with syntype and paratype slides of *Cougourdella polycentropi* Weiser, 1965, *Episeptum inversum* Larsson, 1986, *Episeptum invadens* Larsson, 1996 and *Episeptum circumscriptum* Larsson, 1996. Slides for comparison were borrowed from the slide collection of Dr. Jaroslav Weiser, partly deposited in the Natural History Museum in Vienna, Austria and partly were provided directly by J. Weiser.

The GenBank accession numbers of sequenced microsporidia used for phylogenetic analysis are given in the legend to Fig. 52.

Examination of infected host tissues; transmission electron microscopy (TEM)

Fresh tissues and tissue smears stained with Giemsa (Sigma® Diagnostic Accustain) were examined for the presence of developmental stages and spores using light microscopy. Spores were immobilised using the agar method (Vávra 1964a), were measured ($n = 25$) with the Image Splitting Eyepiece (Vickers Instruments Ltd.) (Vávra and Maddox 1976) and using an Olympus BX 51 microscope equipped with 100 × oil immersion objective.

For TEM, pieces of infected adipose tissue were fixed for 24 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4 in the same buffer. Fixed tissue was dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite. Thin sections were cut on a Reichert-Jung Ultracut E ultramicrotome and stained using uranyl acetate and lead citrate. Sections were examined and photographed using Jeol JEM-1010 and JEM-1011 electron microscopes. Fine structure measurements were performed using a Megaview III camera and analySIS 3.2 software (Soft Imaging System®).

rDNA sequences; phylogenetic analysis

DNA was isolated from fresh purified spores of microsporidia C1, P2, E3–E7 (Table 1) and from the Giemsa-stained syntype slides of *Cougourdella polycentropi* Weiser, 1965 and *Pyrotheca* sp. from *Hydropsyche pellucidula,* according to the protocol of Hyliš et al. (2005). The sets of primers ss530f: ls580r for microsporidia C1, P2, E3, E4, E6 and ls26f: ls580r for microsporidia E5, E7, *Cougourdella polycentropi* Weiser, 1965 and *Pyrotheca* sp. from *Hydropsyche pellucidula*

Table 1. Reported *Cougourdella*, *Pyrotheca* and *Episeptum* spp. from Trichoptera

Species/isolate	Host	Locality—date
Cougourdella polycentropi Weiser 1965* (isolate no. 27#) = *Paraepiseptum polycentropi* (Weiser 1965)	*Polycentropus flavomaculatus* (Pictet 1834) (Polycentropodidae)	Bílek, Czech Republic; small river
Cougourdella rhyacophilae Baudoin 1969	*Rhyacophila obliterata* McLachlan, 1865 (Rhyacophilidae)	Besse, France; stream—July
Cougourdella polycentropi Larsson 1989	*Hydropsyche saxonica* McLachlan, 1884 (Hydropsychidae)	Bjellerup, Sweden; small stream—August 5.
Cougourdella sp.	*Glossosoma nigrior* Banks, 1911 (*Glossosomatidae*)	Michigan, USA; trout streams
Microsporidium sp.	*Brachycentrus americanus* (Banks, 1899) (Brachycentridae)	Michigan, USA; trout streams
Microsporidium C1 (isolate no. 9#) = *Paraepiseptum polycentropi* n. g.	*Hydropsyche fulvipes* (Curtis, 1834) (Hydropsychidae)	Mukařov-Želeč, Czech Republic; small stream—July 15.
Pyrotheca hydropsycheae Xie and Canning 1986	*Hydropsyche siltalai* Doehler, 1963 (Hydropsychidae)	Tilford, UK; river—autumn, spring
Pyrotheca sp. (isolate no. 43#)* = *Paraepiseptum* sp.	*Hydropsyche pellucidula* (Curtis, 1834) (Hydropsychidae)	Kokořín, Czech republic; small river (stream)—October 29.
Microsporidium P2 (isolate no. 10#) = *Paraepiseptum plectrocnemiae* n. g. et n. sp.	*Plectrocnemia conspersa* (Curtis, 1834) (Polycentropodidae)	Mukařov, Czech Republic; small stream—May 21.
Episeptum inversum Larsson 1986	*Holocentropus picicornis* (Stephens, 1836) (Polycentropodidae)	Vikhög, Sweden; small pond—summer
Episeptum invadens Larsson 1996	*Limnephilus fuscicornis* Rambur, 1842 (Limnephilidae)	Veberöd, Sweden; small river (stream)—July
Episeptum circumscriptum Larsson 1996	*Hydropsyche siltalai* Doehler, 1963 (Hydropsychidae)	Veberöd, Sweden; small river (stream)—July 8.
Microsporidium E3 (isolate no. 12#) = *Episeptum trichoinvadens* n. sp.	*Potamophylax cingulatus* (Stephens, 1837) (Limnephilidae)	Sněhov, Czech Republic; small stream—May 30.
Microsporidium E4 (isolate no. 17#) = *Episeptum circumscriptum* Larsson 1996	*Hydropsyche incognita* Pitsch, 1993 (Hydropsychidae)	Praha-Radotín, Czech Republic; small river—Juni 17.
Microsporidium E5 (isolate no. 15a#) = *Episeptum pseudoinversum* n. sp.	*Sericostoma* sp. (Sericostomatidae)	Moštenica, Slowakia; small river—March 10.
Microsporidium E6 (isolate no. 15b#) = *Episeptum pseudoinversum* n. sp.	*Sericostoma personatum* (Spence, 1826) (Sericostomatidae)	Mukařov-Končiny, Czech Republic; stream—April 15.
Microsporidium E7 (isolate no. 16#) = *Episeptum anaboliae* n. sp.	*Anabolia furcata* Brauer, 1857 (Limnephilidae)	Praha-Komořany, Czech Republic; pond—May 10.

*From slide collection of Dr. Jaroslav Weiser.
#Isolate designation used in Vávra et al. (2005).

were used to amplify conserved and variable regions of the small and large subunit rDNA. These primers, with the sequences ss530f GTG CCA GCA GCC GCG G; ls26f GCA TAT CAA TAA GCG GAG GAA AAG and ls580r GGT CCG TGT TTC AAG ACG G are slightly modified from primers of Weiss and Vossbrinck (1999). The PCR reaction (95 °C for 2 min; 30 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min and finally 72 °C for 10 min) was conducted in a total volume of 25 µl with 50–100 ng of DNA, 25 pmol of each primer, 1 unit Taq polymerase (TAKARA BIO INC. Otsu, Shiga, Japan) and buffer/dNTP (TaKaRa) according to manufacturers instructions. PCR products were separated using 1% agarose gel electrophoresis, extracted from the gel, purified using the DNeasy Tissue Kit® (QIAGEN, Germantown, MD, USA), cloned (TOPO TA Cloning Kits®, Invitrogen, Carlsbad, CA, USA) and sequenced on an automatic sequencer (Beckman CEQ 2000 XL). The sequences were aligned using the ClustalX program (Thompson et al. 1997), gaps and ambiguously aligned regions were omitted from further analyses. Maximum parsimony (MP) trees were constructed using PAUP 4b10 (Swofford 2000) with TBR as a branch-swapping method and 1000 bootstrap replicates. Maximum likelihood (ML) trees were constructed by PHYML program (Guindon and Gascuel 2003), using the GTR model for nucleotide substitutions with discrete gamma distribution in 8 + 1 categories; all

parameters (gamma shape, proportion of invariants) were estimated from the data set. Multiple data sets for ML bootstrap analyses were prepared using SeqBoot (PHYLIP 3.6.3; Felsenstein 2001). ML bootstrap support was computed in 300 replicates using PHYML program with the HKY85 model for nucleotide substitutions and one category of sites with a TI/TV ratio estimated from the data set.

Infection experiments

Twenty-five *Potamophylax cingulatus* (Stephens, 1837) larvae and 18 *Hydropsyche fulvipes* (Curtis, 1834) larvae collected from a site where no microsporidian infection was found (side branch of the stream in the village Mukařov, Czech Republic, 50° 40′ 1.83″ North; 15° 11′ 21.03″ East) were infected individually with spore suspensions of microsporidia obtained from infected larvae of the same host species: E3 (in *Potamophylax cingulatus*) and C1 (in *Hydropsyche fulvipes*). The larvae were held individually and starved for 24 h prior to the treatment and the spore suspension (1–2 µl of 10^{3-4} spores/ larva) was introduced directly into the oral cavity of the larva using a bacteriological loop. Five larvae of each group were examined 5 h after inoculation to find out the number of ingested/extruded spores. The remaining larvae were divided into groups of five (*Potamophylax cingulatus*) or three (*Hydropsyche fulvipes*) and were immersed in the stream in a nylon net weighted with a stone covered by algae and moss. Uninfected controls using the same number of larvae were similarly manipulated and held in the same area of the stream. Fourteen days after inoculation, the larvae were dissected and their tissues examined in fresh and Giemsa-stained smears.

Results

Light microscope observations

Infection was restricted to adipose tissue and oenocytes in all examined hosts. The average and range of measurements from 25 fresh spores of each species studied here are given in Table 2, together with some data from the literature on other species.

The life cycle stages of microsporidia C1, P2 and E3–E7 showed a high degree of similarity and the same developmental pattern. Merogonial stages were relatively rare. The earliest stages found were small rounded uninucleate meronts (2–4 µm) and binucleate (4–5 µm) (rarely tetranucleate, 4–5 µm) merogonial plasmodia with intensely stained cytoplasm. In C1, P2 and E3 we occasionally observed irregular merogonial plasmodia (5–7 µm) with eight nuclei and dark-stained cytoplasm.

The sporogonial stages were more numerous and had less intensely stained cytoplasm in comparison with meronts. Uninucleate sporonts (4–5 µm) followed by binucleate plasmodia (5–8 µm) and four-lobed sporogonial plasmodia (6–12 µm), arising by rosette-like budding, were observed in all examined microsporidia. Bilobed (5–8 µm) and eight-lobed (6–12 µm) sporogonial plasmodia were rarely observed in P2 and E7. The sporogonial plasmodia gave rise to four sporoblasts. All isolates formed spore groups consisting of four spores. These groups were fragile and easy to disperse.

The spores were the sole stage of the life cycle that enabled the different species to be distinguished. Spores of C1 were lageniform (Figs 1 and 28) and indistinguishable in shape and size on Giemsa-stained smears from spores of the species *Cougourdella polycentropi* Weiser, 1965 (Table 2). Spores of P2 were short lageniform to long pyriform in shape (Figs 5 and 29) and on Giemsa-stained smears they resembled the spores of *Pyrotheca* sp. (“isolate no. 43”) from *Hydropsyche pellucidula* (Table 2). Spores of E3 (Figs 9 and 30) had a raindrop, regularly pyriform shape. Spores of E4 (Figs 12 and 31) were slightly longer and narrower than those of E3 and had a raindrop to pyriform shape. Spores of E5 and E6 were short and pyriform (Figs 15, 32 and 18, 33). Spores of E7 were also pyriform (Figs 21 and 34).

The spores of E3–E7 were similar to the spores of the three *Episeptum* species described by Larsson (1986, 1996) when compared on the respective paratype slides. The spores of C1 were arranged in groups with spores pointing in one direction (Fig. 1), in P2 and E3–E7 the spores in groups were randomly oriented (Figs 5, 9, 15 and 21). Spores of all isolates had a large distinct posterior vacuole and showed slight variation in shape (Figs 28–34).

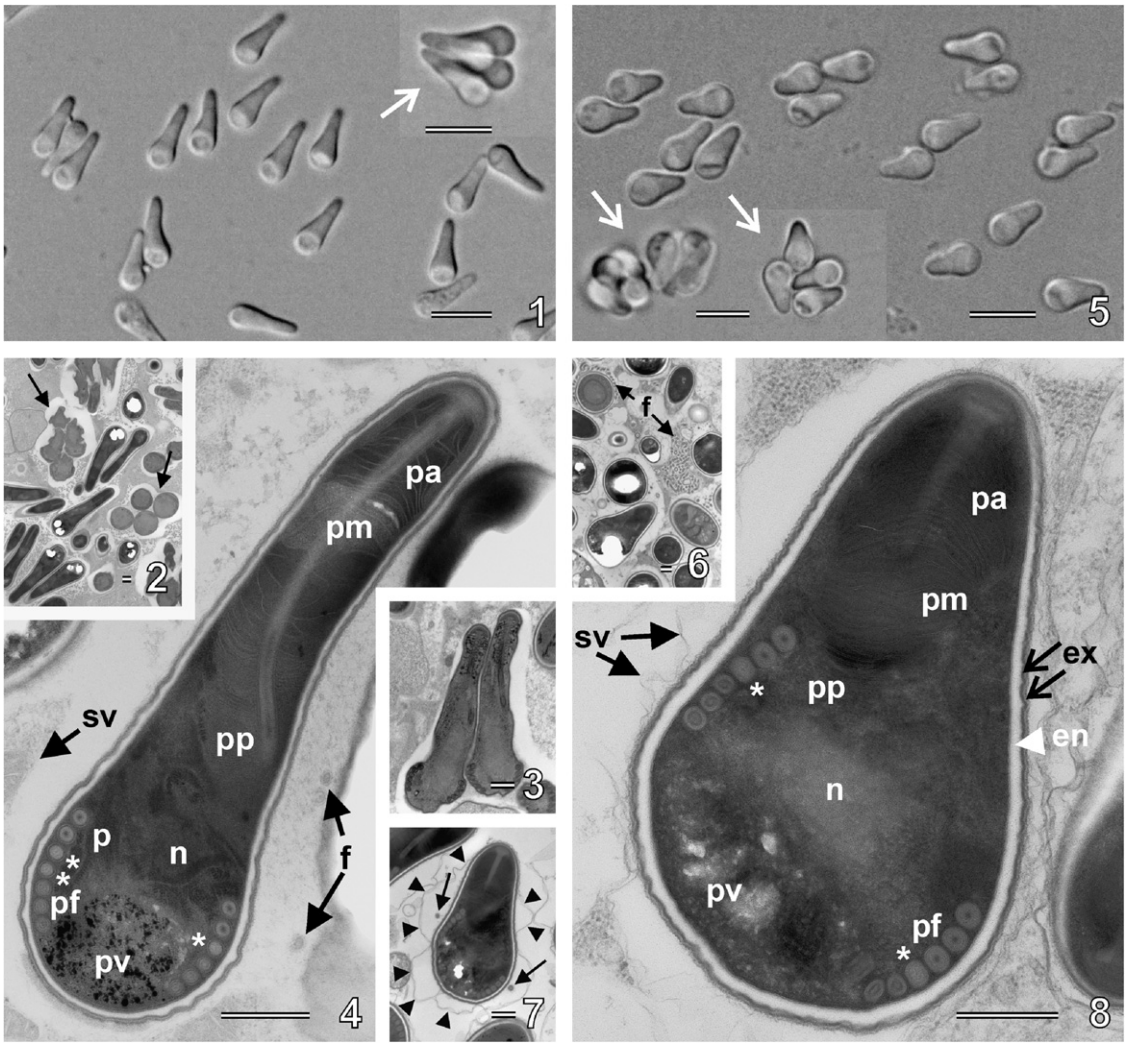
Ultrastructural observations

Merogonial stages were irregular in shape in all isolates. In their electron-dense cytoplasm abundant ribosomes, nuclei with distinct nucleoli and sporadic traces of endoplasmic reticulum were seen. Sporogonial stages had less electron-dense cytoplasm and a distinct ultrastructure. Even before the thickening of the plasma membrane by an external deposit and the division of the initial sporont into a binucleate plasmodium, the formation of an abundant endoplasmic reticulum occurred. The first nuclear division of the sporont occurred simultaneously with the thickening of the plasma membrane and the detachment of the sporophorous vesicle (SPOV) wall from the plasma membrane coat. During the second nuclear division of the sporont, the formation of the SPOV in all isolates was completed. The sporont division proceeded as rosette-like budding.

Table 2. Distinctive characters of reported *Cougourdella*, *Pyrotheca* and *Episeptum* spp. from Trichoptera

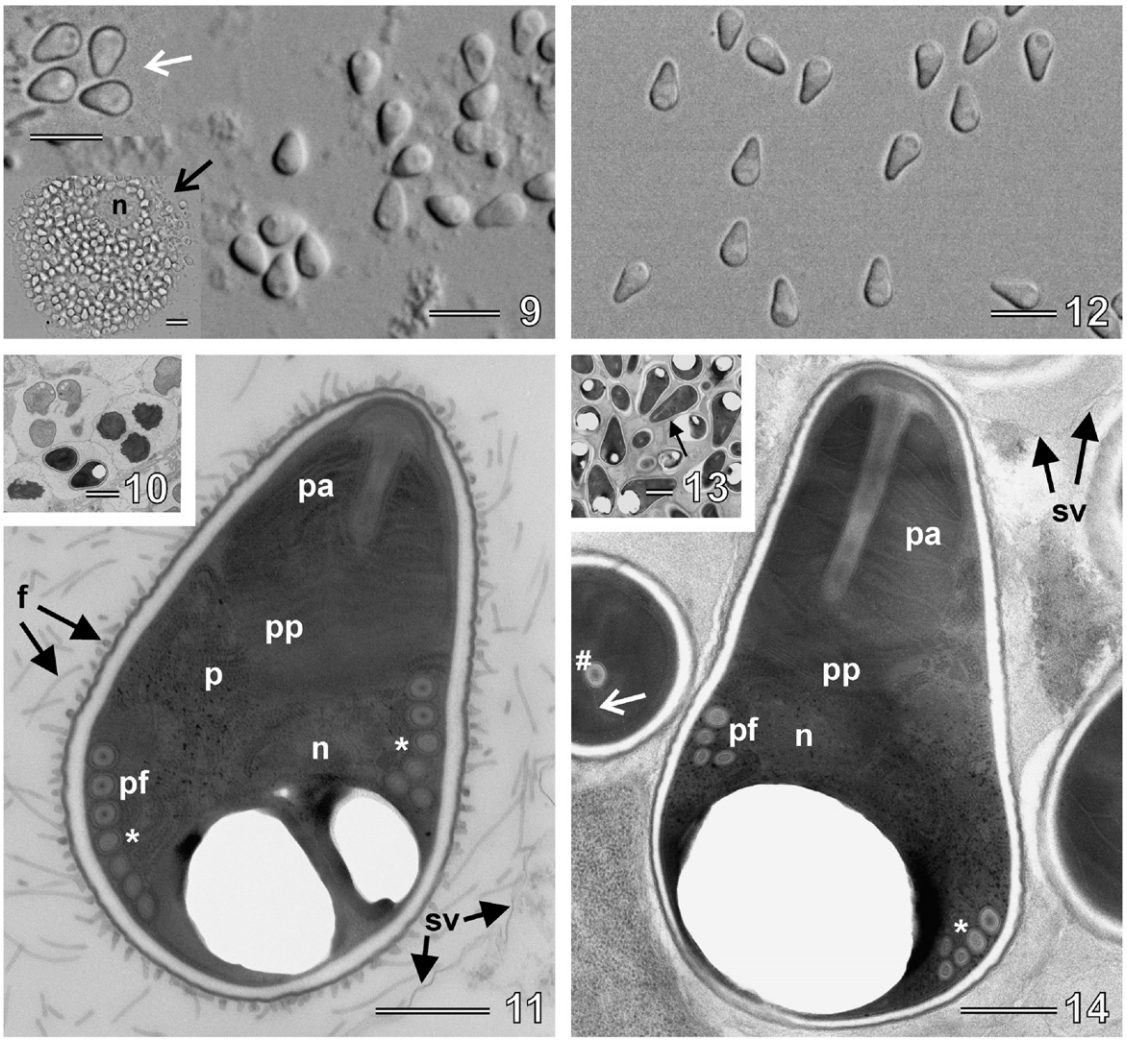
Species/isolate	Spore size (μm)	Polar filament coils: number, diameter wide, narrow (nm)	Anterior polaroplast	Exospore without episporal layer (nm)	Episporal layer
Cougourdella polycentropi Weiser 1965a (isolate no. 27[a]) = *Paraepiseptum polycentropi* (Weiser 1965)	6 × 1.5 (5.2–7 × 1.4–1.8) ?				
Cougourdella rhyacophilae Baudoin 1969	6.3 × 2.7 ?				
Cougourdella polycentropi Larsson 1989	7.0–7.7 × 1.4–2.1 uf	6–7 c, 3 (125–142), 3–4 (82–106)	Wide irregular chambers	16	Thick granular
Cougourdella sp.	5.25 × 3[b] uf				
Microsporidium sp.	6 × 4.5[b] uf				
Microsporidium C1 (isolate no. 9[a]) = *Paraepiseptum polycentropi* n. g.	6.1 × 2.3 (5.7–6.7 × 2.1–2.4) uf	6/7 c, 3–4 (128–150), 3–4 (96–112)	Loosely arranged lamellae (irregular chambers)	16–19	Moderately thick, thick cap at the bottom
Pyrotheca hydropsycheae Xie and Canning 1986	8.2–9.2 × 1.7–2.3 f	6–7 c	Loosely packed membranes arranged as partitions at angles		Fuzzy coat
Pyrotheca sp. (isolate no. 43[a])[c] = *Paraepiseptum* sp.	6.4 × 1.7 (6.1–6.7 × 1.7–1.8) f	6/6 c[d]	Loosely arranged lamellae (irregular chambers)[d]		Moderately thick, thick cap at the bottom[d]
Microsporidium P2 (isolate no. 10[a]) = *Paraepiseptum plectrocnemiae* n. g. et n. sp.	5.0 × 3.0 (4.1–6.4 × 2.8–3.7) uf	6/7–7/7 c, 3–4 (125–136), 2–3 (94–107)	Loosely arranged lamellae (irregular chambers)	21–25	Moderately thick
Episeptum inversum Larsson 1986	2.5–3.2 × 2.1 uf	5–6 c, 3 (130–140), 2–3 (108–113)	Wide lamellae	24–27	Wide, chambered
Episeptum invadens Larsson 1996	3.2–3.6 × 2.2–2.5 uf	5–7 c, 2–3 (100–128), 3–5 (95–96)	Wide lamellae (chambers)	25–26	Thin irregular
Episeptum circumscriptum Larsson 1996	2.5–3,7 × 2.1 uf	4–5 c, 1–2 (94–102), 2–3 (77–90)	Chambers	19–21	Thin irregular
Microsporidium E3 (isolate no. 12[a]) = *Episeptum trichoinvadens* n. sp.	3.5 × 2.4 (3.4–3.7 × 2.3–2.5) uf	6/6–6/7 c, 3–4 (124–134), 3–4 (95–110)	Moderatelly wide lamellae	21–24	Thin irregular, persistent fibrils
Microsporidium E4 (isolate no. 17[a]) = *Episeptum circumscriptum* Larsson 1996	4.0 × 2.4 (3.9–4.2 × 2.3–2.5) uf	4/5–5/5 c, 1–2 (125–130), 2–4 (93–98)	Wide chambers	20–23	Thin irregular
Microsporidium E5 (isolate no. 15a[a]) = *Episeptum pseudoinversum* n. sp.	3.5 × 2.3 (3.3–3.7 × 2.3–2.4) uf	5/5–5–6 c, 3 (128–144), 2–3 (107–123)	Wide lamellae (small chambers)	29–36	Moderately thick, wavy surface
Microsporidium E6 (isolate no. 15b[a]) = *Episeptum pseudoinversum* n. sp.	3.6 × 2.4 (3.6–3.9 × 2.3–2.5) uf	5/5–5/6 c, 3 (127–139), 2–3 (106–121)	Wide lamellae (small chambers)	27–35	Moderately thick, wavy surface
Microsporidium E7 (isolate no. 16[a]) = *Episeptum anaboliae* n. sp.	4.2 × 2.6 (4.1–4.3 × 2.5–2.7) uf	7/7–7/8 c, 4–5 (134–147), 3–4 (102–117)	Wide lamellae	26–33	Moderately thick

f, uf, ? = spores fixed, unfixed, not given.
[a]Isolate designation used in Vávra et al. (2005).
[b]Personal communication Dr. Steve Kohler.
[c]From slide collection of Dr. Jaroslav Weiser.
[d]Obtained from old photographic material of Dr. Jaroslav Weiser.



Figs. 1–8. Light and transmission electron micrographs of spores of C1 (*Paraepiseptum polycentropi*) (1–4) and P2 (*Paraepiseptum plectrocnemiae*) (5–8). **1.** Fresh lageniform spores with a large posterior vacuole in Nomarski interference contrast. Four spores typically pointing in one direction and originally enveloped by a fragile sporophorous vesicle (SPOV) are in the inset at arrow (simple light micrograph). **2.** Infected fat body tissue with stages of sporogony; at arrows are four sporoblasts in SPOV – note the parallel orientation of spores. **3.** Detail of cross-sectioned sporoblasts in SPOV. **4.** Longitudinal section of the spore with 7/6 coils of the polar filament. **5.** Fresh spores in Nomarski interference contrast showing shape variation from short to somewhat longer lageniform. Spores in the SPOV are randomly oriented (at arrows in the inset) (simple light micrograph). **6.** Section of infected fat body tissue with different stages of sporogony. Arrows point to thick tubules occurring temporarily around immature spores in the episporontal space. **7.** SPOV (arrowheads) with cross-sectioned spore and fibrils in the episporontal space (arrows) showing balloon-like blisters. **8.** Section of a mature spore. Abbreviations: en – endospore, ex – exospore, f – fibrous material in SPOV, n – nucleus, p – polyribosome aggregate, pa – anterior polaroplast, pf – polar filament, pm – middle polaroplast, pp – posterior polaroplast, pv – posterior vacuole, sv – sporphorous vesicle envelope. Asterisks mark transitional coils between the wide and narrow coils in which the electron-dense core is missing. Bars for Figs 1 and 5 = 5 μm; Figs 2–4, 6–8 = 0.5 μm.

Sporont daughter cells revealed all ultrastructural characters typical of sporogony (Figs 24 and 25): abundant endoplasmic reticulum with adhering ribosomes, primitive Golgi complex producing numerous vesicles, nuclei moving to the distal pole of the daughter cells, occurrence of mitosomes (Fig. 25) and continued thickening of the plasma membrane. The occurrence of a fibrillar deposit within the episporontal space of the SPOV was also noted. In E3 the fibrillar deposits probably formed connections between the exospore and the envelope of the SPOV (Fig. 25). The presence of some material forming net-like tubular structures within the SPOV was observed in all isolates (C1, P2 and E3–E7) but its quantity varied among isolates. The structure of this material changed during sporogenesis from a fine granular form (Figs 24 and 25) to thicker fibrils (Figs 6 and 27) with a multilayered structure in cross-section (Fig. 26). The material nearly disappeared

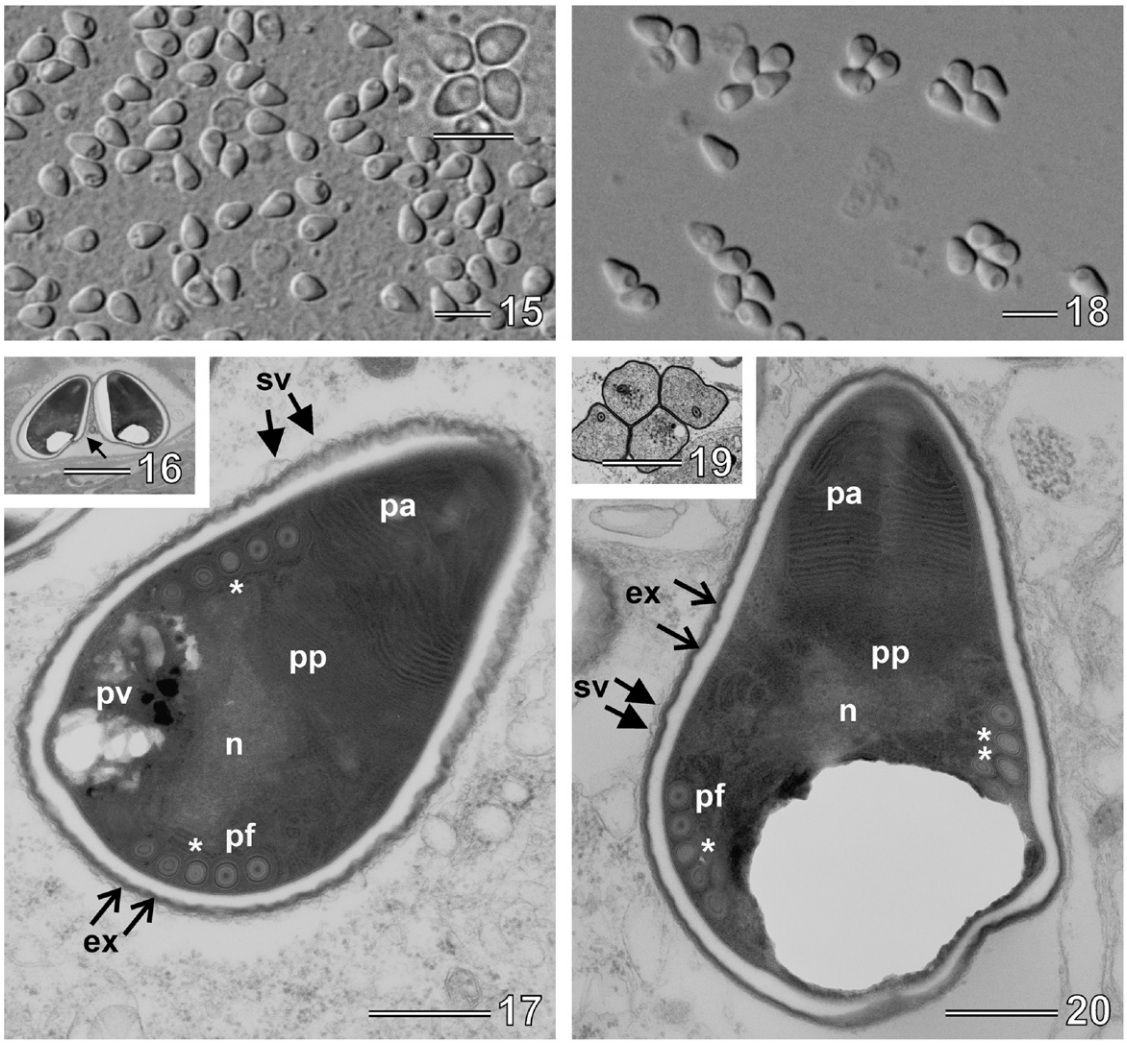


Figs. 9–14. Light and transmission electron micrographs of spores of E3 (*Episeptum trichoinvadens*) (9–11) and E4 (*Episeptum circumscriptum*) (12–14). **9.** Fresh spores in Nomarski interference contrast. Insets (both simple light micrographs) show four spores liberated from a disintegrated sporophorous vesicle (SPOV) (white arrow) and fat body cell (*n* = nucleus) filled with spores (black arrow). **10.** Section of infected fat body tissue with three SPOVs and different stages of sporogony. **11.** Longitudinally sectioned spore. **12.** Fresh spores in Nomarski interference contrast. **13.** Fine structure of infected fat body tissue with mature spores; at arrow are two spores within an SPOV. **14.** Section of a mature spore; note characteristic wide chambers of anterior polaroplast and tightly arranged lamellae of posterior polaroplast. White arrow marks a petal-like compartment of the polaroplast at transversely sectioned apex of the spore; polar filament is at #. Abbreviations as for Figs 1–8. Bars in Figs 9 and 12 = 5 μm; Figs 10 and 13 = 2 μm; Figs 11 and 14 = 0.5 μm.

when spores reached maturity (Figs 4, 7 and 23), except in E3, where the fibrillar net persisted until full maturity of spores (Figs 11, 25, 39 and 47).

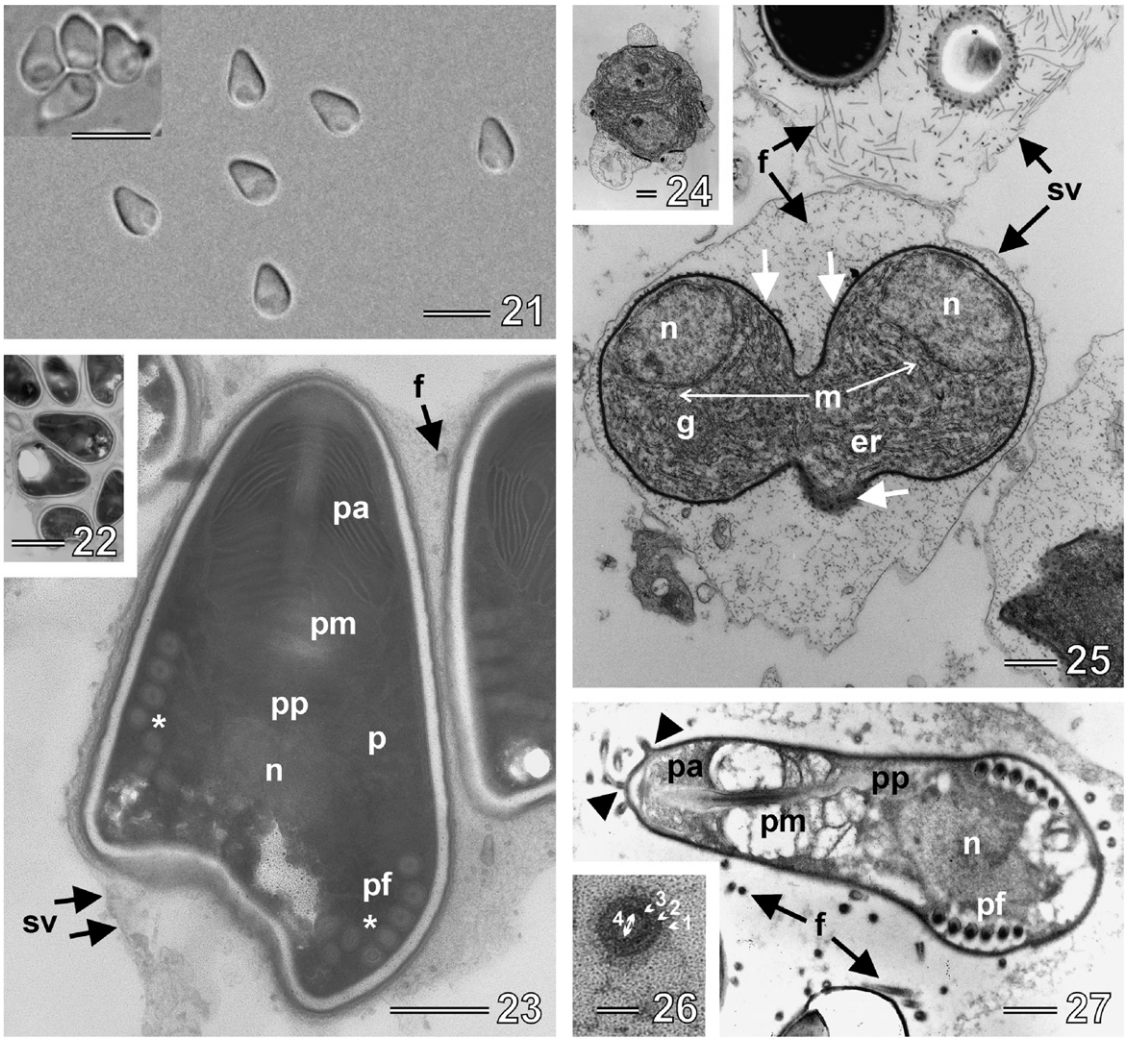
The SPOV was very thin and rather inconspicuous in all examined microsporidia. In E3 the SPOV was a balloon-like sac with spores inside (Figs 10 and 25), in E5 and E6 the SPOV membrane adhered closely to the spores (Figs 16, 17 and 20). In the remaining microsporidia, the SPOV membrane adhered to the spore surface at some places, at other sites it formed balloon-like blisters (Fig. 7).

The spores formed inside the SPOV offered some ultrastructural characters specific to respective examined microsporidia. In all types the spore wall was relatively thin and consisted of a plasma membrane limiting the cytoplasm of the spore, electron transparent endospore and electron-dense exospore. (Figs 45–51). The full development of the endospore occurred only at the end of the sporogenesis, it was absent in sporoblasts and young spores (Figs 3 and 27). The thickness of the endospore layer (80–120 nm) was similar in mature spores of all examined microsporidia and did not allow the distinction of individual isolates. In all types the exospore consisted of two distinct layers: an internal electron-dense layer and an external layer of medium electron density (Figs 45–51). The thickness of the



Figs. 15–20. Light and transmission electron micrographs of spores of E5 (*Episeptum pseudoinversum*, isolate 15a) (15–17) and E6 (*Episeptum pseudoinversum*, isolate 15b) (18–20). **15.** Fresh spores in Nomarski interference contrast. In inset is a group of four spores from a sporophorous vesicle (SPOV) (in phase contrast). **16.** Longitudinally sectioned spores with SPOV wall tightly adhering to spores (arrow). **17.** Section of a mature spore; note the characteristic wavy surface of spores caused by SPOV adhering to the exospore. **18.** Fresh spores in Nomarski interference contrast. **19.** Four early sporoblasts within an SPOV. **20.** Ultrastructural details of a mature spore. Abbreviations as for Figs 1–8. Bars for Figs 15 and 18 = 5 μm; Figs 16 and 19 = 2 μm; Figs 17 and 20 = 0.5 μm.

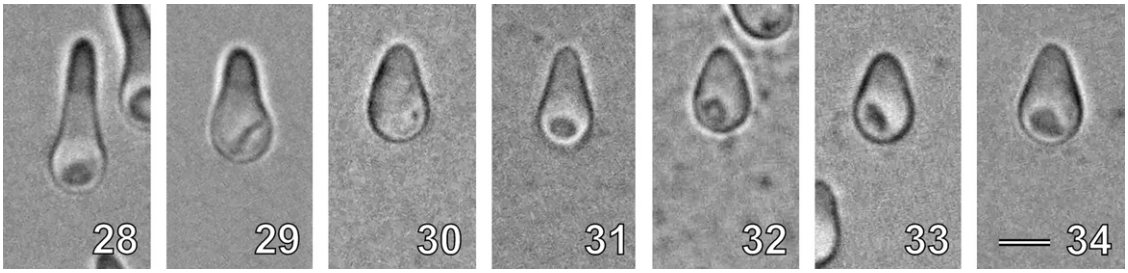
exospore enabled the respective microsporidia to be distinguished as it was relatively thin in C1 and P2 and nearly twice as thick in E3–E7 (Table 2). In all microsporidia except E3, there was an additional external layer on the exospore as defined above. This layer corresponded to the episporal layer of Larsson (1996) and showed some variability among C1, P2 and E4–E7. As the deposition of the material of the episporal layer in each isolate was not uniform around the exospore circumference and depended on the maturity of the spore, it was impossible to record its metric values. Microsporidia C1 (Figs 36 and 45) and P2 (Figs 38 and 46) possessed a relatively thick layer of episporal material, nearly as thick as the exospore. In C1 there was frequently (ca. 90% of spores) an additional, thicker (as thick as the whole spore wall) cap-like layer of material, covering the posterior pole of the spore (Figs 4 and 45). Similar cap-like material was identified in Weiser's unpublished photomicrograph of *Pyrotheca* sp. ("isolate no. 43") from *Hydropsyche pellucidula* (Fig. 27) (Table 2). Microsporidium E3 had a simple, single-layered electron-dense exospore covered by a layer forming persistent filamentous outgrows with periodic structure at the limit of resolution (Figs 11, 39 and 47). Microsporidium E4 had a thin, irregular layer of the episporal material (Figs 14 and 48). Spores of E5 and E6 had a wavy episporal layer of medium and varying thickness (Figs 17, 41, 49 and 20, 42, 50), due to the adhering SPOV (a more or less wavy surface of the epispore was present in all isolates but only in E5 and E6 was it prominent). Microsporidium E7 had a medium thick episporal layer when compared with other isolates



Figs. 21–27. Light and transmission electron micrographs of the spores of E7 (*Episeptum anaboliae*) (21–23) and of the developmental stages and structures of other microsporidia (24–27). **21.** Fresh spores in Nomarski interference contrast. Inset shows a ruptured sporophorous vesicle (SPOV) with four spores (phase contrast). **22.** Fat body tissue with mature spores. **23.** Longitudinally sectioned spore. **24.** Young sporogonial plasmodium of E3 (*Episeptum trichoinvadens*) **25.** Sporogonial plasmodium of E3 (*Episeptum trichoinvadens*) budding in rosette-like fashion and enclosed in SPOV. The fibrils in the episporontal space (f) appear first in the form of granular outgrowths on the plasma membrane of sporogonial stages (thick white arrows). Later they seem to connect the surface of spores with the envelope of the SPOV. The cytoplasm of the sporogonial plasmodium shows rich endoplasmic reticulum (er), vesicles of the Golgi complex (g) and mitosomes (m). **26.** Detail of a cross sectioned fibril in the episporontal space of P2 (*Paraepiseptum plectrocnemiae*) consisting of four concentric layers. **27.** Longitudinal section of a young spore of *Pyrotheca* sp. from *Hydropsyche pellucidula* (isolate 43) (Table 1) within SPOV (unpublished picture by courtesy of Dr. Jaroslav Weiser). The episporontal space exhibits tubules (f) communicating with the spore wall (arowheads). Three regions (fixation artefacts) of the polaroplast can be recognised in the ''neck'' of the spore, the middle part being bubble-like. Abbreviations as for Figs 1–8. Bars in Fig. 21 = 5 μm; Fig. 22 = 2 μm; Figs 23–25, 27 = 0.5 μm and Fig. 26 = 50 nm.

(Figs 23, 44 and 51). The structure of the episporal layer appeared as an appropriate character for distinquishing C1, P2, E3–E7, and its characters are summarised in Table 2.

The polar sac-anchoring disk complex showed similar structure in spores of all examined microsporidia. The disk had layers of different electron density and formed the apical end of the polar filament. It was enveloped by the umbrella-like polar sac extending posteriorly to surround the anterior part of the polaroplast (Figs 36–44). The polaroplast occupied one third to one half of the presumed spore volume and one half to two thirds of the spore length (Figs 4, 8, 11, 14, 17, 20 and 23). The polaroplast of all isolates possessed generally the unique character – some variant of the inverted polaroplast: anterior part with wide lamellae or (and) chambers and posterior part with compressed lamellae (Figs 35–44). A longitudinal section through



Figs. 28–34. Comparative views of fresh spores in the light microscope. **28.** C1 (*Paraepiseptum polycentropi*). **29.** P2 (*Paraepiseptum plectrocnemiae*). **30.** E3 (*Episeptum trichoinvadens*). **31.** E4 (*Episeptum circumscriptum*). **32.** E5 (*Episeptum pseudoinversum*, isolate 15a). **33.** E6 (*Episeptum pseudoinversum*, isolate 15b). **34.** E7 (*Episeptum anaboliae*). Bar = 2 μm.

the spore revealed 2–3 regions of the polaroplast with variations characteristic of each isolate. The posterior region was composed of closely packed parallel lamellae and was similar in all isolates. The middle region had the form of wide, irregular chambers and was recognised only in C1 (Figs 4, 35 and 36), P2 (Figs 8, 37 and 38) and E7 (Figs 23, 43 and 44). It was recognised also in *Pyrotheca* sp. (''isolate no. 43'') from *Hydropsyche pellucidula* (Fig. 27). The anterior region of the polaroplast was slightly variable within each isolate depending on the maturity of the spore but revealed structural variations usable to distinguish between C1, P2 and E3–E7 (Table 2). This region had the form of loosely arranged lamellae forming irregular chambers in C1 and P2 (Figs 4, 36 and 8, 37, 38). The anterior polaroplast in E3 had moderately wide lamellae (Figs 11 and 39). The anterior region of the polaroplast of E4 had an exceptional structure, formed from extremely wide chambers (Figs 14 and 40) resembling rather the middle part of the polaroplast of C1 (Figs 4, 35 and 36), P2 (Figs 8, 37 and 38) and E7 (Figs 23, 43 and 44). In E5 and E6 the anterior region of the polaroplast was composed of rather wide lamellae forming often small chambers which became progressively thinner posteriad (Figs 17, 41 and 20, 42). The anterior polaroplast of E7 had wide, uniform lamellae (Figs 23 and 44). In cross-sections of the anterior part of the polaroplast, a petal-like arrangement of the polaroplast lamellae was observed in all isolates (Fig. 14).

The posterior region of the spore contained a nucleus of irregular elongate shape embedded in electron-dense cytoplasm with abundant polyribosomal agregates and a large posterior vacuole (Figs 4, 8, 11, 14, 17, 20 and 23).

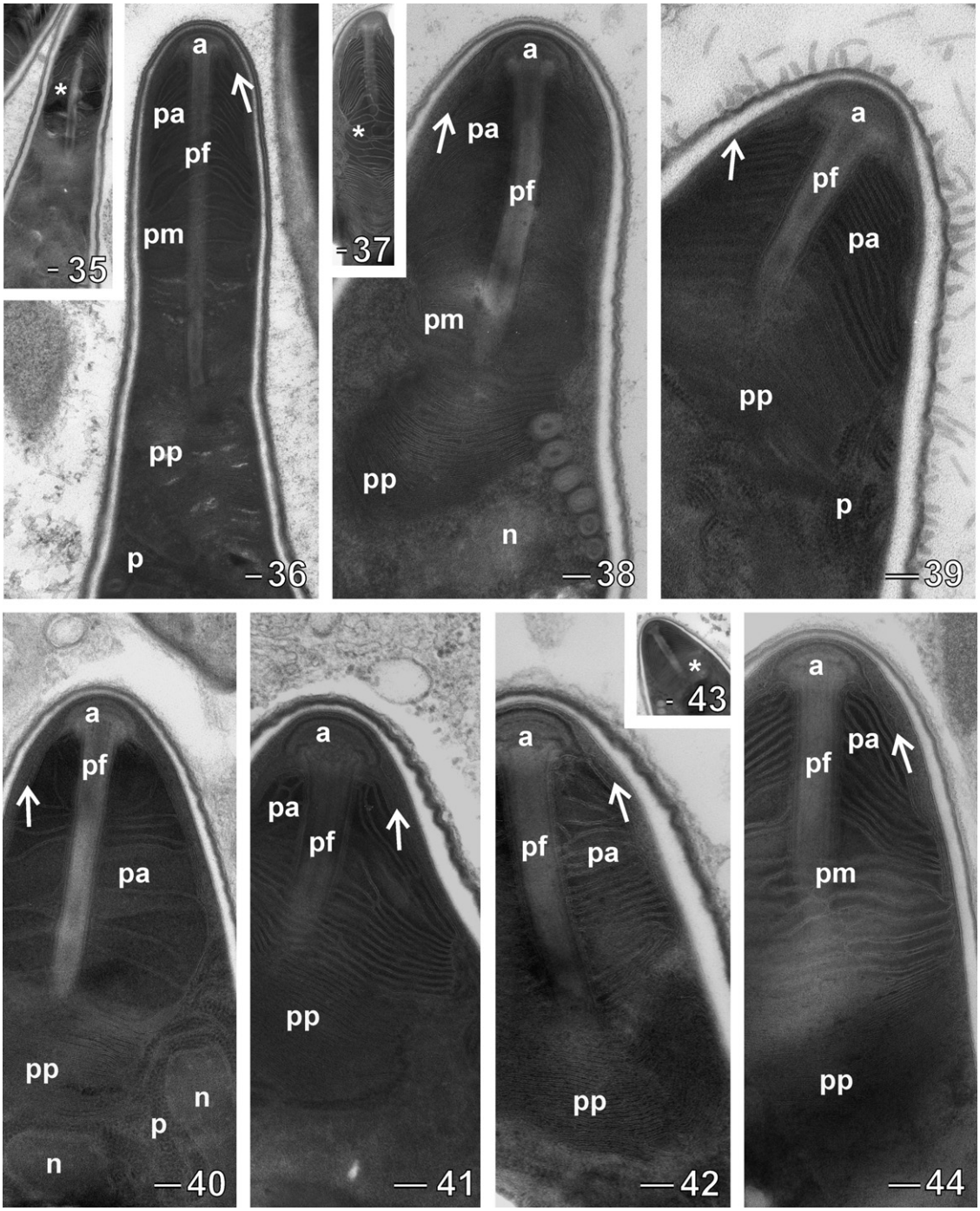
The polar filament of C1, P2 and E3–E7 was always slightly anisofilar and was similarly built from six layers of different electron density and thickness, but slightly variable among isolates (Figs 45–51): unit membrane-like layer (1), electron-dense layer (2), moderately dense layer (3), second electron-dense layer (4), second moderately dense layer containing fibrous material (5) and most lucent and thick layer (6) with electron-dense central part that is absent in one coil forming the transition between wide and narrow coils (transitional coil). The number of polar filament coils, the proportion of the wide and narrow coils and their respective diameters are most important characters for distinguishing C1, P2 and E3–E7 (see data in Table 2). In transverse sections, the most posterior coil in E3, E5, E6 and E7 had a wider diameter than the more anterior coils and its thickness was comparable to the apical thickest coils (Figs 47 and 49–51).

Molecular taxonomy evidence

All trees constructed using MP and ML analysis showed that the seven types of examined microsporidia represent a distinct clade of sister entities indicating the existence of several species distributed in two sister subclades probably representing different genera (Figs 52 and 53). One such tentative genus was represented by the isolates C1 and P2, the other one by the isolates E3–E7. The taxonomic designations of respective genera and species and phylogenetic relationships are presented and discussed below.

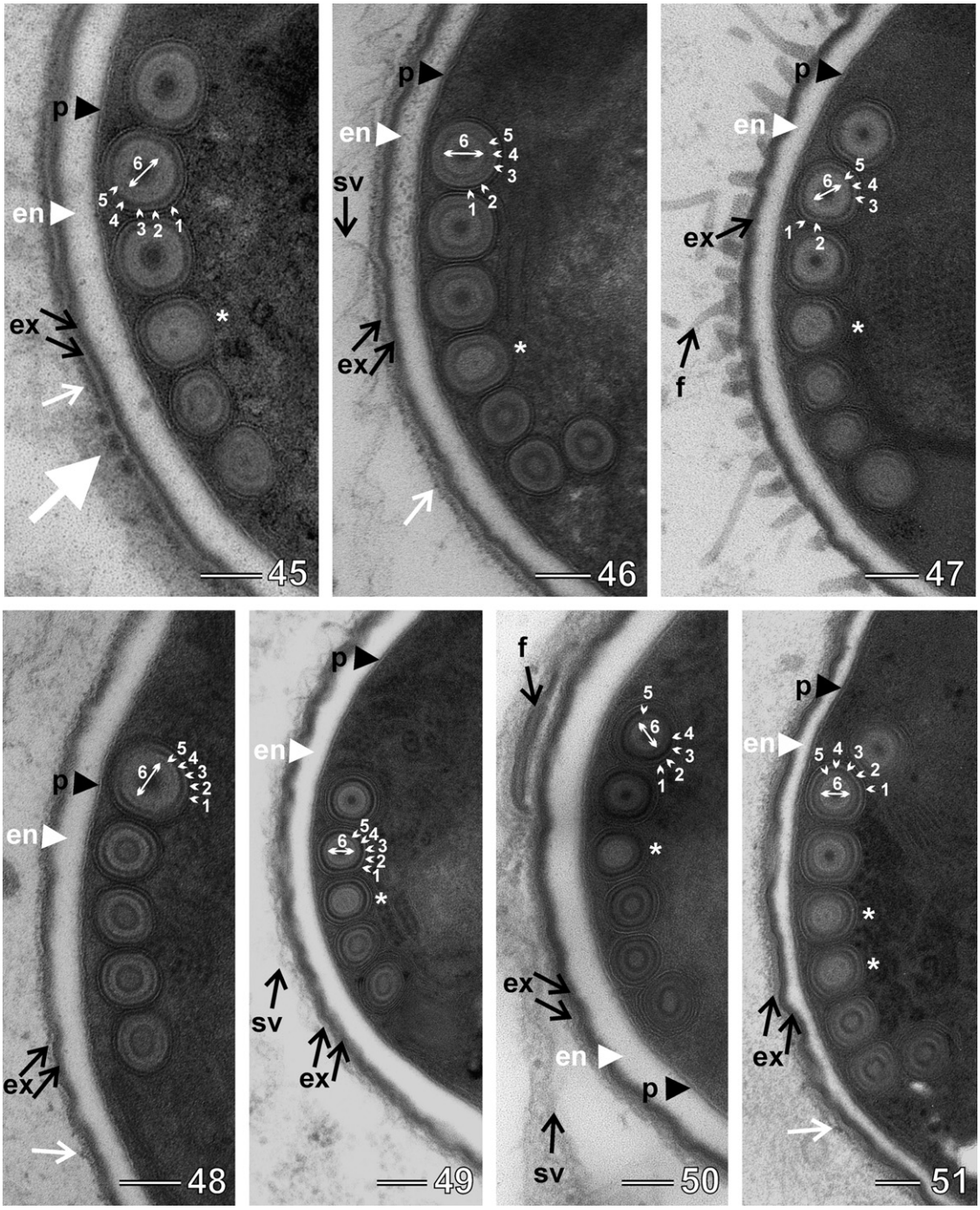
Host specificity; field prevalence of infection and transmission experiments

Microsporidia C1, P2 and E3–E7 appeared to be specific to their hosts as one host species was infected only by one microsporidian type (Table 1). There were several other trichopteran species present at the collection sites but these were uninfected. Types C1, P2, E3 and E6 were isolated from one small trout stream; E3 from its upper temporary branch, E6 and P2 from its middle part and C1 from the bottom part. A seasonal factor and specific prevalence of respective microsporidian types was observed: C1 was found in 62.5% ($n = 16$) of larvae of *Hydropsyche fulvipes* (Curtis, 1834); the host was of low density at the site and the infection occurred during July and August. P2 was found in 33.3% ($n = 12$) of larvae of *Plectrocnemia*



Figs. 35–44. Comparative sections of the polaroplast region of spores representing the respective spore types. The polaroplast differs in its construction in different microsporidia: the anterior region (pa) can be more or less wide and with more or less regularly arranged lamellae or chambers, its middle region (pm and asterisks) may consist of irregular chambers, while its posterior region (pp) is always represented by closely arranged lamellae. The polar sac (arrows) surrounds the proximal part of the polaroplast, the anchoring disc (a) and the proximal part of the polar filament (pf) in the spore apex. The nucleus (n) has lobular shape and the cytoplasm is rich in polyribosomal agregates (p). **35, 36.** C1 (*Paraepiseptum polycentropi*.). **37, 38.** P2 (*Paraepiseptum plectrocnemiae*). **39.** E3 (*Episeptum trichoinvadens*). **40.** E4 (*Episeptum circumscriptum*). **41.** E5 (*Episeptum pseudoinversum,* isolate 15a). **42.** E6 (*Episeptum pseudoinversum,* isolate 15b). **43, 44.** E7 (*Episeptum anaboliae*). All bars = 100 nm.

conspersa (Curtis, 1834); the host species was in low density and infection occurred during May and June. E3 was found in 2.4% ($n = 125$) of larvae of *Potamophylax cingulatus* (Stephens, 1837); the host was of very high abundance at the site and infection occurred from April to May. E4 was found in 4.1% ($n = 96$) of larvae of



Figs. 45–51. Comparative sections of the posterior parts of the different spore types. Details of construction of the polar filament and spore wall are shown. The polar filament is lightly anisofilar and differs in the total number of coils and in the ratio of the wide vs. narrow coils as well as in the thickness of individual coil layers (1–6). Asterisks mark the transitional coil with slightly different structure. The spore walls consist of an endospore (en) of different thickness and of more complex exospore (ex), composed usually of two distinctive layers (black arrows) and external (episporontal) layers of different thickness and structure. Sv – sporophorous vesicle, p – plasma membrane, f – fibrils in the episporontal space, white thin arrow – episporal layer, white thick arrow – deposition of the episporontal material at posterior pole of the spore. **45.** C1 (*Paraepiseptum polycentropi*). **46.** P2 (*Paraepiseptum plectrocnemiae*). **47.** E3 (*Episeptum trichoinvadens*). **48.** E4 (*Episeptum circumscriptum*). **49.** E5 (*Episeptum pseudoinversum*, isolate 15a). **50.** E6 (*Episeptum pseudoinversum*, isolate 15b). **51.** E7 (*Episeptum anaboliae*). All bars = 100 nm.

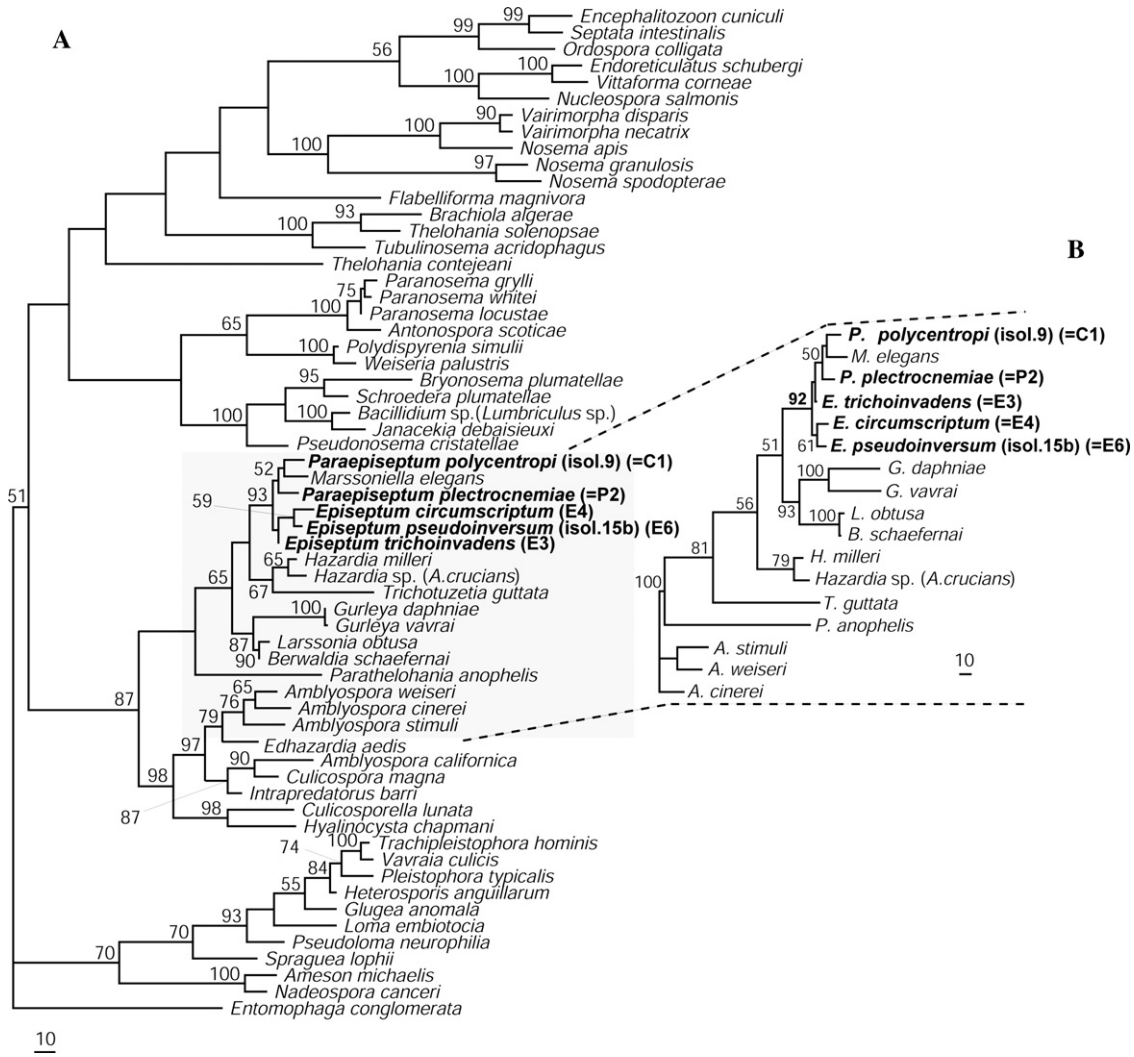


Fig. 52. Maximum parsimony (MP) trees as inferred from partial SSU rRNA gene sequences. Numbers above branches indicate MP bootstrap support (1000 replicates). (**A**) One of 12 equally parsimonious trees; alignment used contained 503 characters, of which 320 were parsimony informative. TL = 2555; CI = 0.323; HI = 0.699; RI = 0.684; RC = 0.221. (**B**) One of two equally parsimonious trees; alignment contained 575 characters, of which 191 were parsimony informative. TL = 588; CI = 0.670; RI = 0.689; RC = 0.462. The GenBank accession numbers of sequences used were as follows (with names of new sequences printed in bold type): *Amblyospora californica* (U68473), *Amblyospora cinerei* (AY090057), *Amblyospora* sp. (*A. salinarius*) (L28960), *Amblyospora stimuli* (AF027685), *Amblyospora weiseri* (AY090048), *Ameson michaelis* (L15741), *Antonospora scoticae* (AF024655), *Bacillidium* sp. (*Lumbriculus* sp.) (AF104087), *Berwaldia schaefernai* (AY090042), *Brachiola algerae* (AF069063), *Bryonosema plumatellae* (AF484690), *Culicospora magna* (AY326269), *Culicosporella lunata* (AF027683), *Edhazardia aedis* (AF027684), *Encephalitozoon cuniculi* (AJ005581), *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (AF023245), ***Episeptum anaboliae*** (DQ864443), ***Episeptum circumscriptum*** (AY880953), (DQ864440), ***Episeptum pseudoinversum*** **(isol. 15a)** (DQ864442), ***Episeptum pseudoinversum*** **(isol. 15b)** (AY880952), (DQ864441), ***Episeptum trichoinvadens*** (AY880954), (DQ864439), *Flabelliforma magnivora* (AY649786), *Glugea anomala* (AF044391), *Gurleya daphniae* (AF439320), *Gurleya vavrai* (AF394526), *Hazardia milleri* (AY090067), *Hazardia* sp. (*A. crucians*) (AY090066), *Heterosporis anguillarum* (AF387331), *Hyalinocysta chapmani* (AF483837), *Intrapredatorus barri* (AY013359), *Janacekia debaisieuxi* (AJ252950), *Larssonia obtusa* (AF394527), *Loma embiotocia* (AF320310), *Marssoniella elegans* (AY090041), *Nadelspora canceri* (AF305708), *Nosema apis* (U97150), *Nosema granulosis* (AJ011833), *Nosema spodopterae* (AY211390), *Nucleospora salmonis* (U78176), *Ordospora colligata* (AF394529), ***Paraepiseptum plectrocnemiae*** (AY880955), (DQ864438), ***Paraepiseptum polycentropi*** **(isol. 9)** (AY880951), (DQ864437), ***Paraepiseptum polycentropi*** **(isol. 27)** (DQ864444), ***Paraepiseptum*** **sp. (isol. 43)** (DQ864445), *Paranosema grylli* (AY305325), *Paranosema locustae* (AY305324), *Paranosema whitei* (AY305323), *Parathelohania anophelis* (AF027682), *Parathelohania anophelis* (L28969), *Pleistophora typicalis* (AF044387), *Polydispyrenia simulii* (AJ252960), *Pseudoloma neurophilia* (AF322654), *Pseudonosema cristatellae* (AF484694), *Schroedera plumatellae* (AY135024), *Septata intestinalis* (U09929), *Spraguea lophii* (AF033197), *Thelohania contejeani* (AF492593), *Thelohania solenopsae* (AF031538), *Trachipleistophora hominis* (AJ002605), *Trichotuzetia guttata* (AY326268), *Tubulinosema acridophagus* (AF024658), *Vairimorpha disparis* (AF033315), *Vairimorpha necatrix* (Y00266), *Vavraia culicis* (AJ252961), *Vittaforma corneae* (U11046), *Weiseria palustris* (AF132544). *Entomophaga conglomerata* was used as the outgroup (GenBank accession number AF368509).

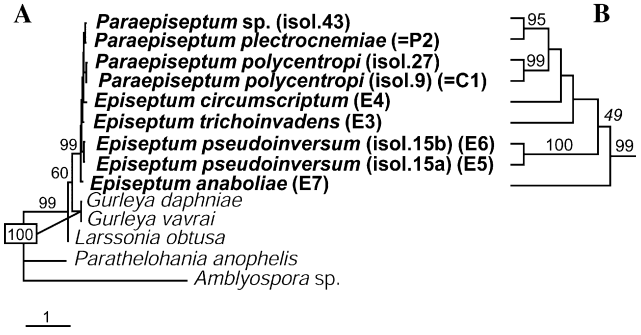


Fig. 53. (**A**) Maximum likelihood (ML) phylogenetic tree ($\log lk = -2042.81095$) as inferred from partial LSU rRNA gene sequences. Tree was constructed using a HKY 85 model for nucleotide substitutions with discrete gamma distribution in 8 + 1 categories. All parameters (gamma shape = 0.335; TV/TS ratio = 4.834; proportion of invariants = 0.000) were estimated from the data set. Numbers above branches indicate ML bootstrap support (HKY model, TS/TV ratio and proportion of invariants estimated from the data set, 300 replicates). Tree was rooted using *Amblyospora* sp. sequence as an outgroup. (**B**). Detailed topologies of particular clusters with ML-bootstrap support computed as mentioned.

Hydropsyche incognita Pitsch, 1993; the host species was present in abundance and the infection occurred from June to August. E5 was found in 9.5% ($n = 42$) collected larvae of *Sericostoma* sp. in March; the host was present in very high numbers, but the locality was not repeatedly examined. E6 was found in 5.3% ($n = 56$) larvae of *Sericostoma personatum* (Spence, 1826); the host was in high density and the infection occurred from March to April. E7 was found in 7.1% ($n = 28$) larvae of *Anabolia furcata* Brauer, 1857; the host population was of middle density at the habitat and the infection occurred from April to May.

None of the *Potamophylax cingulatus* and *Hydropsyche fulvipes* larvae infected experimentally was found infected when examined 14 days after inoculation. The midgut lumen of all larvae examined 5 h after inoculation contained a number of ingested spores. Less than 10% of spores of E3 and more than 50% spores of C1 were found extruded.

Discussion

Microsporidia in Trichoptera and the classification of the investigated microsporidia

About 20 species of microsporidia belonging to 11 genera (*Amblyospora*, *Cougourdella*, *Episeptum*, *Gurleya*, *Chytridiopsis*, *Issia*, *Pegmatheca*, *Pyrotheca*, *Tardivesicula*, *Thelohania* and *Vavraia*) have so far been recorded from Trichoptera. From this list seven genera are out of the scope of this discussion as they are not tetrasporoblastic. As far as the tetrasporoblastic microsporidia are concerned, only four genera are represented among trichopteran microsporidia: *Gurleya* Doflein, 1898, *Cougourdella* Hesse, 1935, *Pyrotheca* Hesse, 1935 and *Episeptum* Larsson, 1986. The conventional distinguishing characters of these tetrasporoblastic genera are structural with the shape of spores as the main criterion. The spore shape is described as pear-shaped in *Episeptum* and *Gurleya*, cylindric-bulbous (resembling the fruits of *Lagenaria vulgaris* Ser., var. *cougourda* Ser.) in *Cougourdella* and long, drop-shaped, slightly curved in *Pyrotheca*.

Except for the genus *Episeptum*, all these genera have type hosts other than Trichoptera. Presently, there are three species described in the genus *Episeptum* (all of them from caddis flies), six species of the genus *Cougourdella* (two from caddis flies, three from copepods and one from oligochaetes), eight species of the genus *Pyrotheca* (one from caddis flies, two from dipterans and five from copepods and cladocerans) and about 22 species of *Gurleya* (one from caddis flies, others from different crustaceans and insects but also from arachnids and ciliophorans).

Little modern information is available on trichopteran tetrasporoblastic microsporidia except the genus *Episeptum*, the development and fine structure of which has been described in detail (Larsson 1986, 1996). Partial developmental and ultrastructural data exist also on caddis fly microsporidian species attributed to genera *Pyrotheca* (in Xie and Canning 1986), *Cougourdella* (in Larsson 1989) and *Gurleya* (in Larsson 1995). Molecular data on tetrasporoblastic trichopteran microsporidia are limited to ssuRNA gene sequences of five undescribed isolates (Vávra et al. 2005).

Microsporidia C1, P2, E3–E7 in our study resembled in spore shape all four tetrasporoblastic genera known to occur in Trichoptera. C1 looked like the genus *Pyrotheca*/*Cougourdella* combination, P2 resembled the genus *Pyrotheca* and E3–E7 could be conventionally ranged into the genera *Gurleya* or *Episeptum* when spore shape was considered alone. We decided, however, to arrange the investigated microsporidia in a way different from the conventional classification scheme suggested by simple spore shape, which seems to be in fact only a variation of the basic pear-shaped type.

The main reason why we decided to arrange C1 and P2 into a newly proposed genus *Paraepiseptum* (see taxonomic definition below) and E3–E7 into the redefined genus *Episeptum* (see below) is that the three genera (*Gurleya, Cougourdella* and *Pyrotheca*), conventionally available for the generic assignment of the respective investigated microsporidia, have their type hosts among Crustacea and not among Trichoptera. Recent research data indicate that microsporidia are evolutionarily closely related to their hosts and have speciated with them (Baker et al. 1998; Agnew et al.

2003; Vossbrinck et al. 2004, Vossbrinck and Debrunner-Vossbrinck, 2005). Generally, molecular data show that many conventional genera (i.e. genera defined on structural characters) are in reality polyphyletic assemblages. Some of the members of these genera have already been reclassified (*Vairimorpha* Pilley, 1976; *Paranosema* Sokolova, Selezniov, Dolgikh, Issi, 1994; *Tubulinosema* Franzen, Fischer, Schroeder, Scholmerich, Schneuwly, 2005), *Dictyocoela* (provisionally designated by Terry et al., 2004). Other examples of "polyphyletic" genera (e.g. *Thelohania*, *Amblyospora*) are evidently awaiting reclassification (Cheney et al. 2001; Vossbrinck et al. 2004, Vossbrinck and Debrunner-Vossbrinck, 2005; Brown and Adamson 2006).

We believe that this fact should be taken into consideration when microsporidian genera are treated. Presently, most microsporidian genera are defined simply by structural characters which do not reflect the host component and the evolutionary signals revealed by molecular phylogeny. The proposed genus *Paraepiseptum* contains microsporidian species forming a clade in phylogeny trees which is supported by relatively high bootstrap values and is a sister clade to microsporidia of the genus *Episeptum*. Thus the way in which our investigated microsporidia are classified reflects their structural similarity as well as their molecular phylogeny. Both *Episeptum* and *Paraepiseptum* in our definition include only trichopteran microsporidia. It is further proposed that the existing trichopteran microsporidia in the genera *Pyrotheca* and *Cougourdella* are transferred to the new genus *Paraepiseptum*. Uniting *Pyrotheca* and *Cougourdella*-like microsporidia from Trichoptera in one genus also solves the problem of where to place microsporidia whose spore shape is very similar (see Larsson 1989; Xie and Canning 1986).

Together with the establishment of the new genus *Paraepiseptum*, we propose that the genera *Pyrotheca* and *Cougourdella* are retained for microsporidia from their original type hosts (copepods) and host groups related to them (crustaceans). Reported polysporoblastic *Cougourdella*-like and *Pyrotheca*-like microsporidia from cyclopoid copepods are not tetrasporoblastic, form multisporous fragile SPOVs (Voronin 1993; Ovcharenko and Wita 2001) and correspond by spore size to the original description of these two genera by Hesse (1935) more than to the tetrasporoblastic microsporidia from caddis flies.

Our establishment of the new genus *Paraepiseptum* and the redefinition of the genus *Episeptum* solves the problem of the generic assignment of the tetrasporoblastic microsporidia collected during our study, as well as the generic assignment of tetrasporoblastic microsporidia described by other authors and placed into the genera *Cougourdella* and *Pyrotheca*. However, there exists one tetrasporoblastic microsporidian described in the literature (Larsson, 1995), the phylogenetic position of which cannot be properly decided as no molecular data on this species are yet available. This microsporidian, *Gurleya dorisae,* is not identical at the generic or specific level with any microsporidium from our study. Its spores have a very thick endospore, 20–25 coils of the polar filament and are enclosed in a persistent SPOV, structural characters absent in our microsporidia.

The genus Episeptum Larsson, 1986

The type species is *Episeptum inversum* from the caddis fly *Holocentropus picicornis* (Stephens, 1836). Two further species were described in 1996 by the same author, together with a list of species-distinguishing characters. In accordance with Larsson (1986, 1996), we observed in E3–E7 a unique character of the polaroplast – the inverted polaroplast: anterior part with wide lamellae or chambers and posterior with tightly arranged lamellae. Our observations however differ from those of Larsson (1986) in that they show that the "plurilayered exospore with a thick chambered coat" should be removed from the genus characters. This character is the species marker of *Episeptum inversum* only. It is absent *de facto* in *Episeptum invadens* and *Episeptum circumscriptum* later described by Larsson (1996) as well as in our microsporidia E3–E7.

Of the species characters given by Larsson (1996), our observations confirmed that the following are indicative: spore size, number of polar filament coils and their distinction into wide and narrow coils, arrangement of membranous lamellae in the anterior part of the polaroplast, thickness and structure of the episporal material and the character of the material ocurring in the episporontal space during sporogenesis. As additional characters, the dimensions of the polar filament, of the exospore and the thickness of the "primordium of sporont wall" can be employed. Using these characters, we assigned our microsporidium E4 to *Episeptum circumscriptum* Larsson, 1996, and the microsporidia E3, E5/E6 and E7 are described as new species.

Taxonomic summary

Microsporidium E3: *Episeptum trichoinvadens* n. sp.

Type host: *Potamophylax cingulatus* (Stephens, 1837) (Trichoptera, Limnephilidae), larva.

Transmission: Unknown. Presumed per os. Spores found not to be autoinfective.

Site of infection: Adipose tissue and oenocytes.

Interface: Merogonial stages in direct contact with host cell cytoplasm, sporogonial stages enclosed in a non-persistent SPOV.

Development: Late merogonial stages: uninucleate and binucleate (no diplokarya) meronts. Irregular four–eight nucleate plasmodia rarely observed, probably represent other merogonial stages. Sporogony tetrasporoblastic: uninucleate sporont in an SPOV gives rise to tetranucleate plasmodium. Four uninucleate sporoblasts are formed by rosette-like division.

Spore: Pyriform uninucleate, 3.5 × 2.4 μm (3.4–3.7 × 2.3–2.5) when fresh. Polar filament slightly anisofilar, forming 6/6–6/7 coils in a single row, the anterior part in 3–4, 124–134 nm wide coils and posterior part in 3–4, 95–110 nm wide coils. Polaroplast with two lamellar parts: anterior with moderately wide lamellae and posterior with narrow lamellae. The exospore (without episporal layer), 21–24 nm thick. Episporal layer thin, the spore bears numerous persistent thick hairs.

Type locality: Upper part of a small trout stream in the vicinity of the village Sněhov, Northern Bohemia, Czech Republic (50° 39′ 43.22″ North; 15° 11′ 41.25″ East)

Remarks: Partial nucleotide sequences, SSU, ITS and LSU rDNA, deposited in the NCBI GenBank, Accession Nos. AY880954 and DQ864439. Syntype slides, Giemsa-stained smears of infected adipose tissue of *Potamophylax cingulatus*, are deposited at the Smithsonian Institute, Washington, DC, USA, Accession No. USNM 1099762, in the type slide collection of Dr. Jaroslav Weiser, Prague, Czech Republic and in the collection of the first author.

Etymology: Species name alludes to the persistent fibrillar hairs on the spore surface and to the similarity in size and shape to *Episeptum invadens* Larsson, 1996.

Microsporidium E5 and E6: *Episeptum pseudoinversum* n. sp. (type of the species is the microsporidium E6)

Type host: *Sericostoma personatum* (Spence, 1826) (Trichoptera, Sericostomatidae), larva.

Transmission: Unknown. Presumed per os.

Site of infection: Adipose tissue and oenocytes.

Interface: Merogonial stages in direct contact with host cell cytoplasm, sporogonial stages enclosed in non-persistent SPOV.

Development: Late merogonial stages: uninucleate and binucleate (no diplokarya) meronts. Irregular four nucleate plasmodia rarely observed, probably represent other merogonial stages. Sporogony tetrasporoblastic: uninucleate sporont in an SPOV gives rise to tetranucleate plasmodium. Four uninucleate sporoblasts are formed by rosette-like division.

Spore: Pyriform, uninucleate, 3.6 × 2.4 μm (3.6–3.9 × 2.3–2.5) when fresh. The slightly anisofilar polar filament is arranged in 5/5–5/6 coils in a single row, the anterior part in 3, 127–139 nm wide coils and posterior part in 2–3, 106–121 nm wide coils. Polaroplast with two parts: anterior composed of wide lamellae up to small chambers and posterior with narrow lamellae. The exospore (without episporal layer) is 27–35 nm thick. The spore surface is wavy, formed by fusion of the SPOV, episporal layer and multilayered exospore.

Type locality: Middle part of trout stream close to the village Mukařov-part Končiny, Northern Bohemia, Czech Republic (50° 40′ 10.76″ North; 15° 12′ 2.25″ East)

Remarks: Partial nucleotide sequences, SSU, ITS and LSU rDNA, deposited in the NCBI GenBank, Accession Nos. AY880952 and DQ864441. Syntype slides, Giemsa-stained smears of infected adipose tissue of *Sericostoma personatum*, are held at the Smithsonian Institute, Washington, DC, USA, Accession No. USNM 1099763, in the type slide collection of Dr. Jaroslav Weiser, Prague, Czech Republic and in the collection of first author.

Etymology: Species name alludes to the size and shape resembling to *Episeptum inversum* Larsson, 1986.

Microsporidium E7: *Episeptum anaboliae* n. sp.

Type host: *Anabolia furcata* Brauer, 1857 (Trichoptera, Limnephilidae), larva.

Transmission: Unknown. Presumed per os.

Site of infection: Adipose tissue and oenocytes.

Interface: Merogonial stages in direct contact with host cell cytoplasm, sporogonial stages enclosed in fragile SPOV.

Development: Late merogonial stages: uninucleate and binucleate (no diplokarya) meronts. Irregular four nucleate plasmodia rarely observed, probably represent other merogonial stages. Sporogony tetrasporoblastic: uninucleate sporont in an SPOV gives rise to tetranucleate plasmodium. Four uninucleate sporoblasts are formed by rosette-like division.

Spore: Pyriform, uninucleate, unfixed measure 4.2 × 2.6 μm (4.1–4.3 × 2.5–2.7). The slightly anisofilar polar filament is arranged in 7/7–7/8 coils in a single row, the anterior part in 4–5, 134–147 nm wide coils and posterior part in 3–4, 102–117 nm wide coils. Polaroplast with three parts: anterior with wide lamellae, middle with small chambers and posterior with narrow lamellae. The exospore (without episporal layer) is 26–33 nm thick.

Type locality: Small pond communicating with a river, Prague-part Komořany, Czech Republic (49° 59′ 42.71″ North; 14° 24′ 8.06″ East)

Remarks: Partial nucleotide sequence of LSU rDNA, deposited in the NCBI GenBank, Accession No. DQ864443. Syntype slides, Giemsa-stained smears of infected adipose tissue of *Anabolia furcata*, are held at the Smithsonian Institute, Washington, DC, USA,

Accession No. USNM 1099764, in the type slide collection of Dr. Jaroslav Weiser, Prague, Czech Republic and in the collection of first author.

Etymology: Species name alludes to the genus name of the host.

The genus *Paraepiseptum* n.g.

The name was selected to express the fact that the genus is close to the genus *Episeptum* as far as the developmental cycle in trichopteran host, fine structure characters, host and tissue specificity are concerned. However, these microsporidia form a sister, yet distinct, clade to *Episeptum*. The genus is also defined with the aim to solve the *Pyrotheca*/*Cougourdella* conundrum, e.g. the one in which two, possibly conspecific species, *Cougourdella polycentropi* Weiser, 1965 and *Pyrotheca hydropsycheae* Xie & Canning, 1986, trichopteran microsporidia have been placed into two different genera: *Cougourdella* Hesse, 1935 and *Pyrotheca* Hesse, 1935. There exist three tetrasporoblastic trichopteran microsporidia as candidates to be transferred into the genus *Paraepiseptum*: *Cougourdella polycentropi* Weiser, 1965, *Cougourdella rhyacophilae* Baudoin, 1969 and *Pyrotheca hydropsycheae* Xie & Canning, 1986. Because of missing fine structure data about *Cougourdella polycentropi* Weiser, 1965, the existing distinctive characters of respective species are only spore size and host specificity (Tables 2 and 1). We carefully compared several slides of the type material of *Cougourdella polycentropi* Weiser, 1965 (unfixed, unstained and stained in different ways) with C1. The similarity in the spore size and shape, posterior vacuole size and staining properties suggest that C1 and *Cougourdella polycentropi* Weiser, 1965 are undoubtedly identical species as supported by sequence data (six base difference in the variable region of LSU rDNA). No modern data are available on *Cougourdella rhyacophilae* Baudoin, 1969. Its spore size, however, indicates that it might be conspecific with *Cougourdella polycentropi* Weiser, 1965.

An isolate of *Cougourdella polycentropi* studied by Larsson (1989) had larger spores and its host was different (Table 1). We belive that it represents a new species; however, the reclassification should be supported by molecular data. As far as *Pyrotheca hydropsycheae* Xie & Canning, 1986 is concerned, we believe that it represents a valid species.

We selected the former *Cougourdella polycentropi* Weiser, 1965 as the type species of the new genus *Paraepiseptum*. As far as our microsporidia are concerned, C1 is identical with the type species *Paraepiseptum polycentropi* (Weiser, 1965), P2 is proposed as a new species.

The genus *Paraepiseptum* gen. nov.

Diagnosis: Developmental cycle similar to that of *Episeptum*. Nuclei isolated at all stages of the developmental cycle. Eight-nucleate merogonial plasmodium produces uninucleate meronts. Sporogony tetrasporoblastic in a thin-walled subpersistent SPOV. Spores uninucleate long pyriform to lageniform – above 5 μm length. Polaroplast divided into three parts: anterior loosely lamellar, middle chambered and posterior tightly lamellar. Polar filament anisofilar. Parasites of adipose tissue and oenocytes of caddis flies. Spores not autoinfective. An alternate host probably involved in life cycle (copepod host and developmental cycle resembling *Marssoniella elegans* Lemmermann, 1900?).

Etymology: genus name alludes to the structural and phylogenetic relationship to the genus *Episeptum*.

Species:

1. *Paraepiseptum polycentropi* (Weiser, 1965) n. comb., type species

Synonym: *Cougourdella polycentropi* Weiser, 1965

2. *Paraepiseptum rhyacophilae* (Baudoin, 1969) n. comb.

Synonym: *Congourdella rhyacophilae* Baudoin, 1969

3. *Paraepiseptum hydropsycheae* (Xie & Canning, 1986) n. comb.

Synonym: *Pyrotheca hydropsycheae* Xie & Canning, 1986

Microsporidium P2: *Paraepiseptum plectrocnemiae* n. sp.

Type host: *Plectrocnemia conspersa* (Curtis, 1834) (Trichoptera, Polycentropodidae), larva.

Transmission: Unknown. Presumed per os.

Site of infection: Adipose tissue and oenocytes.

Interface: Merogonial stages in direct contact with host cell cytoplasm, sporogonial stages enclosed in a non-persistent SPOV.

Development: Late merogonial stages: uninucleate and binucleate (no diplokarya) meronts. Irregular four–eight nucleate plasmodia rarely observed, probably represent other merogonial stages. Sporogony tetrasporoblastic: uninucleate sporont in an SPOV gives rise to tetranucleate plasmodium. Four uninucleate sporoblasts are formed by rosette-like division.

Spore: Short lageniform to long pyriform, uninucleate, 5.0×3.0 μm (4.1–6.4 × 2.8–3.7) when fresh. Polar filament slightly anisofilar, forming 6/7–7/7 coils in a single row, the anterior part in 3–4, 125–136 nm wide coils and posterior part in 2–3, 94–107 nm wide coils. Polaroplast with three parts: anterior loosely lamellar, middle chambered and posterior tightly lamellar. The exospore (without episporal layer) is 21–24 nm thick. Episporal layer moderately thick, SPOV is irregularly

adhered and liberated from spore. Numerous subpersisting tubular structures occur within the episporontal space during sporogony.

Type locality: Middle part of a small trout stream in the village Mukařov, Northern Bohemia, Czech Republic (50° 40′ 0.2″ North; 15° 11′ 28.41″ East)

Remarks: Partial nucleotide sequences, SSU, ITS and LSU rDNA, deposited in the NCBI GenBank, Accession Nos. AY880955 and DQ864438. Syntype slides, Giemsa-stained smears of infected adipose tissue of *Plectrocnemia conspersa*, are deposited at the Smithsonian Institute, Washington, DC, USA, Accession No. USNM 1099765, in the type slide collection of Dr. Jaroslav Weiser, Prague, Czech Republic and in the collection of the first author.

Etymology. Species name alludes to the the genus name of the host.

Host–parasite relationships and classification

Our failure to infect two species of trichopteran larvae with their specific microsporidia indicates that these microsporidia belong to a large group of microsporidia infecting water dwelling hosts which do not form "autoinfective spores" (Vávra 1964b; Vávra et al. 2005) and cannot thus be simply infected by feeding spores of the same type (those harboured in tissues). These microsporidia are likely to have complex life cycles involving other hosts. So far such life cycles have been elucidated only in some mosquito microsporidia using copepods as intermediate hosts (Becnel and Andreadis 1999), but are probably more widespread as several other microsporidia infecting water dwelling hosts cannot be infected by spores that they carry (Vávra et al. 2005). In accordance with our results, Parsell et al. (1997), Parsell and Kohler (1998) and Heilveil et al. (2001) reported the impossibility of the direct (horizontal) transmission of *Cougourdella* sp. and *Microsporidium* sp., infecting the caddis flies *Glossosoma nigrior* and *Brachycentrus americanus,* respectively, into uninfected original hosts. In addition, no vertical (transovum) transmission was observed in these host–parasite systems by these authors. Despite the limited published data, we believe that both of these microsporidia represent tetrasporoblastic microsporidia which could be placed in the genus *Paraepiseptum* proposed in the present paper.

Our observations indicate that the trichopteran tetrasporoblastic microsporidia that we studied were host specific (as far as trichopteran hosts are concerned). Despite the presence of larvae of several other caddis fly species in the examined habitats, each species described in this paper infected only a single host species of trichopteran. Generally, the *Paraepiseptum* species were reported in the raptorial (carnivorous/omnivorous) trichoptera and the *Episeptum* species in the herbivorous/omnivorous ones. The question of host specificity should be studied further, however. The *Paraepiseptum polycentropi* selected here as type species of the new genus was found by Weiser (1965) in *Polycentropus flavomaculatus*, while C1, considered to be conspecific, was found by us in *Hydropsyche fulvipes*. There is a six-nucleotide difference in the sequenced 580R region of the LSU rDNA gene of these two microsporidians. In the situation when ultrastructure data and infectivity (for respective caddis flies or eventual alternate hosts) are lacking, we have opted for a conservative approach and we treat the two isolates as conspecific, but further research is needed to either confirm or negate our view. Judging from incomplete data, the *Cougourdella rhyacophilae* Baudoin, 1969 might also be conspecific with *Paraepiseptum polycentropi* as defined in this paper. Similarly, we decided to consider two isolates (E5, E6) as belonging to the same species, *Episeptum pseudoinversum*, n.sp., despite the fact that they infected different hosts (*Sericostoma personatum* vs *Sericostoma* sp.) and had a seven nucleotide difference in the sequenced portion of the LSU rRNA gene. This conservative approach is in agreement with the way that molecular data have been projected into classification of microsporidia in the past (Baker et al. 1994; Hyliš et al. 2006).

Phylogenetic relationships of tetrasporoblastic trichopteran microsporidia

In phylogeny trees constructed using both MP and ML analysis, the trichopteran tetrasporoblastic microsporidia form, with relatively high bootstrap support, a separate group included in a large clade (superclade) of microsporidia from aquatic hosts (copepod and cladoceran crustaceans) or hosts having aquatic larval stages (mosquitoes), in some cases having again Crustacea as second hosts (e.g. Fig. 52). In these trees, trichopteran tetrasporoblastic microsporidia group closely with a subclade of microsporidia from Cladocera (*Gurleya daphniae*, *Gurleya vavrai*, *Larssonia obtusa* and *Berwaldia schaefernai*), forming, with mosquito parasites *Hazardia milleri*, *Hazardia* sp. and a copepod parasite *Trichotuzetia guttata,* a bigger group, sister to *Parathelohania anophelis,* a mosquito parasite (see Fig. 52). Both trees based on the SSU rRNA gene sequence (Fig. 52A and B) show also the internal relationships within this group: splitting C1 (*Paraepiseptum polycentropi* isol. 9), P2 (*Paraepiseptum plectrocnemiae*) and *Marssoniella elegans* (Vávra et al. 2005) from E3 (*Episeptum trichoinvadens*), E4 (*Episeptum circumscriptum*) and E6 (*Episeptum pseudoinversum* isol. 15b). The ML trees (Fig. 53A and B) based on partial LSU rRNA gene sequences confirm the position of trichopteran tetrasporoblastic microsporidia as a separate group

closely related to *Gurleya daphniae, Gurleya vavrai* and *Larssonia obtusa,* all parasitising Cladocera, and separation of *Cougourdella*- and *Pyrotheca*-like microsporidia in the distinct *Paraepiseptum* subclade from the *Episeptum* species. Nucleotide differences within the respective genera *Episeptum* and *Paraepiseptum* and among their respective species are generally larger than those within the species of the genera *Nosema* and *Vairimorpha* parasitising lepidoptera (Baker et al. 1994; Hyliš et al. 2006). This supports our conclusions based on structural analysis that the genera *Paraepiseptum* and *Episeptum* are different and valid.

The above-mentioned phylogenetic relationships of trichopteran microsporidia with aquatic (mainly crustacean) microsporidia indicate that the eventual alternate host(s) of trichopteran microsporidia should be sought among Crustacea, and more specifically among copepods.

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