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New microsporidia parasitizing bark lice (Insecta: Psocoptera)

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ABSTRACT

Two species of bark lice, *Xanthocaecilius sommermanae* Mockford and *Polypsocus corruptus* Hagen, collected in a canopy Malaise trap placed in Great Smoky Mountains National Park as part of a survey of the park's fauna, were found to be infected with microsporidia. Diagnosis was originally based on light microscopy, and was confirmed by PCR amplification and electron microscopy. This is the first record of microsporidia infection in the insect order Psocoptera. Four morphological spore types corresponded to four original SSUrDNA sequences (Genbank accession no. FJ865221-24), suggesting infection with four microsporidia species. Two of those species were examined by electron microscopy. We describe here one new genus and two new species based on morphological and sequence data: *Antonospora psocopterae* sp. n. with elongated diplokaryotic spores, 4.4 ± 0.05 × 1.9 ± 0.03 μm and *Mockfordia xanthocaeciliae* gen. n. sp. n. with ovocylindrical monokaryotic spores, 2.5 ± 0.10 × 1.4 ± 0.02 μm. *A. psocopterae* displayed high sequence (95%) and structural similarity with *Antonospora scoticae*, fell within a well supported dichotomy with *A. scoticae* inside the *Antonospora–Paranosema* clade in phylogenetic analyses by NJ, PS and ML. *M. xanthocaeciliae* did not exhibit much sequence or structural similarity with any of known microsporidia species, except *Encephalitozoon* spp. *M. xanthocaeciliae* fell within one clade with *Encephalitozoon* spp. in phylogenies and shared with encephalitozoons structural resemblance and about 80% of SSUrDNA sequence identity. The other two species were not described and provisionally were placed to the collective genus *Microsporidium* as *Microsporidium* sp. 1 and *Microsporidium* sp. 4 from bark lice because of insufficient morphological data. The finding that samples fixed and stored for months in propylene glycol ("antifreeze") are good enough for DNA sequence analysis and can be used for morphological analyses (if no better fixation alternatives are available), is promising for future surveys for microsporidia.

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1. Introduction

Microsporidia may be the most widely distributed and abundant group of eukaryotic entomopathogens. The evolutionary origin and speciation of Microsporidia as a phylum of parasitic fungi-related protists is believed to be linked with diversification of arthropods during the Paleozoic and particularly with the radiation of insects during the Carboniferous (Issi, 1986; Sokolova, 2009). The majority of contemporary insect microsporidia species have been described either from aquatic or economically important species. In the former group, primarily insect larvae, the transparent integuments make infections conspicuous and easily observed by an unaided eye as whitish conglomerates of spores. Economically important species, beneficial insects or agricultural and forest pests, display reproductive outbursts and high population densities favorable for epizootics (Becnel and Andreadis, 1999). Studies on epizootics caused by microsporidia in populations of several harmful and beneficial insects has revealed the potential of microsporidia as long-term regulators of population dynamics and inspired interest in their value as biological control agents (Becnel and Johnson, 2000; Ebert et al., 2000; Henry and Onsager, 1982; Kohler and Hoiland, 2001; Lewis et al., 2009; Odindo, 1990; Oi and Williams, 2002; Sweeney and Becnel, 1991; Weiser, 2005). At the same time in spite of the ubiquitous distribution of microsporidia in insects[1], incidence, diversity, and role of these parasites in insect populations from natural terrestrial ecosystems remain practically unknown. Low prevalence (1–4% maximum, except during epizootics (Sokolova and Issi, 2001), small size of spores, and a lack of gross pathology render individual collecting ineffective as a method of screening for microsporidia. Habitats such as forest canopies, well known for diversity and density of insect populations are relatively inaccessible without special collecting methods. Use of

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[1] The number of microsporidia parasitizing contemporary insects may be comparable to the total number of insect species (Issi, 1986).

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mass collecting traps has potential to significantly improve productivity of sampling for microsporidia in insect populations. However, no records exist of the use of any traps for studies of microsporidia distribution in terrestrial, non agricultural systems. Meanwhile, many mass-collecting methods are used in biodiversity studies of terrestrial arthropods, including Malaise traps, mercury vapor/ultraviolet light traps, Lindgren funnels, pitfall traps, etc. Could the samples from such traps be used for screening of microsporidia? To address this question we took an advantage of an ongoing research project to survey the insect fauna in Great Smoky Mountains National park (GSMNP) as a part of a larger all taxa biodiversity inventory (ATBI) (Sharkey, 2001). The most abundant insects captured in some canopy Malaise traps were adults of bark lice (Psocoptera), some of which were infected with microsporidia. To our knowledge, psocopterans have never been recorded as hosts for microsporidia. This fact and the systematic position of Psocoptera as a part of the Hemipteroid assemblage of insect orders and sister group to ectoparasitic lice (Phthiraptera) enhance the importance of the specific identification of bark lice microsporidia. Another important aspect of this research was confirming the suitability of biological material fixed and stored in propylene glycol (a routine fixative for bulk samples of insects) for molecular and ultrastructural analyses, mandatory for microsporidia identification.

2. Materials and methods

2.1. Sampling of hosts

Malaise Canopy traps (Sante Traps, Lexington, KY), are tent-like structures that funnel flying insects into collecting jars containing commercial automotive coolant (low-toxicity antifreeze, Prestone Inc., Danbury, CT), diluted 1:1 with tap water. The main component of this coolant is propylene glycol; supplemented with ethylene and diethylene glycols. Identical or similar mixtures are routinely used for fixation of arthropods captured in varies traps in ecological studies (Rubink et al., 2003). On June 30, 2007 the trap that produced all insects in this study was installed in a beech tree 15 m from the ground in mountain deciduous forest, GSMNP, White Oak Sink, 35. 63533°N, 83 74967°W, elevation 650 m (Blount Co., TN). On August 12, 2007 insects from this trap were collected, transferred into 100% ethanol, and stored thereafter at room temperature.

Two Psocoptera species from two families dominated the sample. They were identified as *Xanthocaecilius sommermanae* Mockford (Psocoptera; Psocomorpha; Caeciliusidae) and *Polypsocus corruptus* Hagen (Psocoptera; Psocomorpha; Amphipsocidae) using Mockford's North American guide to the order (Mockford, 1993). Most specimens were translucent due to prolonged immersion in glycol fixative, and digestive tracts and egg masses were clearly visible. Some individuals exhibited whitish opaque and slightly enlarged abdomens, which drew our attention. The slightest touch of a needle against such abdomens caused a release of whitish spore-filled suspension, which we suspected to be evidence of infection with microsporidia.

2.2. Light microscopy

Insects were dissected and their abdomens removed, re-hydrated in water, smeared on microscopic slides, and observed under phase contrast optics at 400–1000X. Smears presumably positive for microsporidia (those, containing spore-like structures) were dried, fixed with methanol, and stained with Trichrome Blue, or Calcofluor (Remel, Lenexa, KS) , or Giemsa (Sigma, St. Louis, MO). Spores were photographed by Nikon Eclipse E-600 digital camera. Spore measurements were taken using imaging software (MetaView. 1998, Meta Imaging Series 4.5. Universal Imaging Corporation, West Chester, PA).

2.3. DNA isolation and sequencing

Abdomens containing microscopically confirmed infections were washed in five changes of distilled water to eliminate ethanol. They were transferred into 150 μl of guanidine buffer (4.2 M guanidine thyocyanate, 50 mM TrisHCl, pH 7.6, 10 mM EDTA, 25% lauryl sarcosinate), and stored at +4 °C for at least 24 h. Before DNA extraction individual abdomens were transferred to 150 μl of lysis guanidine buffer (4.2 M guanidine thyocyanate, 50 mM TrisHCl, pH 7.6, 10 mM EDT, 25% lauryl sarcosinate + 10% mercaptoethanol), bead-beaten in a Mini-Beadbeater (Biospec Products, Bartlesville, OK) at maximum speed for 30 s, and heated in a thermoblock for 10 min at 95 °C. Afterwards the samples were spun down and supernatants were subjected to phenol–chloroform-isoamyl alcohol DNA extraction followed by alcohol precipitation. Alternatively they were re-suspended in 150 μl of TAE buffer (0.04 M Tris acetate, 0.01 M EDTA) and used directly as a DNA template for PCR amplification (Vossbrinck et al., 2004). The primers for PCR amplification were V1 (5′-CAC CAG GTT GAT TCT GCC TGA C-3′) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′). Bands of about 1300 base pairs were excised from 2% agarose gel; DNA was extracted from gels with a Zymoclean DNA recovery kit (Zymo Research, CA). The sequencing reactions were performed with Applied BioSystems BigDie Terminator (version 3.1) and run on the Beckman Coulter Seq 8000 DNA sequencer in the GenLab, School of Veterinary Medicine, Louisiana State University. The primers for sequencing were V1, 530r (5′-CCG CGG C(T/G)G CTG GCA C-3′), 530f (5′-GTG CCA GC (G/A) GCC GCG G), 1061f (5′-GGT GGT GCA TGG CCG-3′), and 1492r (Vossbrinck et al., 2004; Weiss and Vossbrinck, 1999). These primers produced overlapping sequences that were assembled with Chromas. Pro. 1.34 software (http://www.technelysium.com.au/ChromasPro.html). Direct PCR amplification and sequencing were performed at least twice for each DNA sample.

2.4. Phylogenetic analysis

Twenty-five microsporidia sequences were selected for phylogenetic analyses. Among those, four novel sequences were recovered from this study (designated as *Microsporidium* sp. 1, 2, 3 and 4), and 21 sequences were obtained from the NCBI Genbank database (for accession numbers and host species see Table 1). Those 21 sequences either displayed the highest identity with the novel sequences in the BLAST search or belonged to representatives of the major groups of terrestrial microsporidia. The microsporidium *Systenostrema alba*, a parasite of *Aeshna* spp. larvae, only distantly related to other microsporidia used for the analyses, based on preliminary analyses, was selected as an outgroup.

All sequences were trimmed from the 5′-end beginning with the last nucleotide of the V1 universal primer, and at the 3′-end to a final length of 1300 characters including gaps. They were aligned with the CLUSTAL X program (Thompson et al., 1997) without additional changes. The resultant alignment was analysed by neighbor joining (NJ), maximum parsimony (PS), and maximum likelihood (ML) algorithms with PAUP*, version 4.0 (Swofford, 2002). A TrN + I + G model of nucleotide substitution was suggested as a best-fit by Hierarchical Likelihood Ratio tests and a GTR + I + G model by Akaike Information Criteria in Modeltest 3.6 (Posada and Crandall, 1998). Settings of the latter model were applied to the ML analyses presented in this paper; application of TrN + I + G model settings did not change the ML tree topology. Bootstrap values for all tree-building methods were obtained from 100 re-samplings. Trees were manipulated with Tree-View, version

Table 1
Hosts and GenBank accession numbers of the SSUrDNA sequences of 25 microsporidia species used in the phylogenetic analyses.

#	Microsporidia	Host	Acc #
1	*Anncaliia meligethi*	*Meligethes aeneus* (Insecta, Coleoptera)	AY894423
2	*Antonospora scoticae*	*Andrena scotica* (Insecta, Hymenoptera)	AF024655
3	*Encephalitozoon cuniculi*	*Homo sapiens* (Mammalia, Primates)	X98470
4	*Encephalitozoon hellem*	*Homo sapiens* (Mammalia, Primates)	AF338366
5	*Encephalitozoon* (=*Septata*) *intestinalis*	*Homo sapiens* (Mammalia, Primates)	U09929
6	*Encephalitozoon romaleae*	*Romalea microptera* (Insecta, Orthoptera)	EU502838
7	*Endoreticulatus schubergi*	*Choristoneura fumiferana* (Insecta, Lepidoptera)	L39109
8	*Hazardia* sp.	*Anopheles crucians* (Insecta, Diptera)	AY090066
9	*Heterovesicula cowani*	*Anabrus simplex* (Insecta, Orthoptera)	EU275200
10	*Kneallhazia* (=*Thelohania*) *solenopsae*	Solenopsis invicta (Insecta, Hymenoptera)	AF134205
11	*Liebermannia dichroplusae*	*Dichroplus elongatus* (Insecta, Orthoptera)	EF016249
12	*Liebermannia patagonica*	*Tristira magellanica* (Insecta, Orthoptera)	DQ239917
13	*Microsporidium* sp. 1	*Xanthocaecilius sommermanae* (Insecta, Psocoptera	FJ865221
14	*Antonospora psocopterae* (*Microsporidium* sp. 2)	*Xanthocaecilius sommermanae* (Insecta, Psocoptera)	FJ865222
15	*Mockfordia xanthocaeciliae* (*Microsporidium* sp. 3)	*Xanthocaecilius sommermanae* (Insecta, Psocoptera)	FJ865223
16	*Microsporidium* sp. 4	*Polypsocus corruptus* (Insecta, Psocoptera)	FJ865224
17	*Nosema bombycis*	*Bombyx mori* (Insecta, Lepidoptera)	L39111
18	*Orthosomella operophterae*	*Operophtera brumata* (Insecta, Lepidoptera)	AJ302316
19	*Ovavesicula popilliae*	*Popillia japonica* (Insecta, Coleoptera)	EF564602
20	*Paranosema grylli*	*Gryllus bimaculatus* (Insecta, Orthoptera)	AY305325
21	*Paranosema locustae*	*Locusta migratoria* (Insecta, Orthoptera)	AY305324
22	*Paranosema whitei*	*Tribolium castaneum* Insecta, Coleoptera	AY305323
23	*Systenostrema alba*	*Aeshna* sp. (Insecta, Odonata)	AY953292
24	*Tubulinosema ratisbonensis*	*Drosophila melanogaster* (Insecta, Diptera)	AY695845
25	*Vairimorpha necatrix*	*Trichoplusia ni* (Insecta, Lepidoptera)	Y00266

1.6.6. A pairwise sequence comparison in the form of a data matrix was calculated by the Kimura-2-Parameter analysis built in PAUP*, version 4.0 (Swofford, 2002).

2.5. Transmission electron microscopy

Previously re-hydrated infected abdomens were immersed in fixative (2.5% v/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) and further dissected under a dissecting microscope. Tissues were removed from chitin integuments and fixed for 1 h in a fresh portion of fixative, postfixed in 1% aqueous OsO_4, and en block stained with uranyl acetate. Dehydration was through an ascending alcohol series and acetone. Before the final incubation in 100% acetone, the samples were exposed to a saturated solution of lead acetate in 1:1 alcohol/acetone for 1 h (Elliott, 2007). Samples were embedded in Spurr's resin. Thin sections were examined and digitally photographed under a JEOL-JEM-1011 electron microscope without additional staining. Electron microscopy was performed only for *Microsporidium* 2 and 3, because we failed to retrieve intact *Microsporidium* 1-infected Malpighian tubules for EM analyses, and obtain sufficient material from the only one insect infected with *Microsporidium* 4.

3. Results

3.1. Symptoms of infection

Among several psocopteran species captured in the Malaise canopy traps, two species of barklice, *X. sommermanae* and *P. corruptus*, were infected with microsporidia. All *X. sommermanae* males that were examined ($n = 9$) were free from infection. *P. corruptus* was represented exclusively by females. Enlarged milky-white abdomens containing spore-filled fat bodies were the salient features of microsporidiosis in *X. sommermanae* specimens. In *P. corruptus* females, eggs that were clearly visible through the integument in uninfected individuals were masked by whitish masses of spores in infected insects. Some infected insects did not display visible signs of microsporidiosis prior to microscopic examination of tissues.

3.2. Light microscopy

Overall 215 specimens of *X. sommermanae* and 198 of *P. corruptus* were tested. Observations under phase contrast optics confirmed by staining with Blue Trichrome, or Giemsa, or Calcofluor stains (not shown) revealed four types of microsporidia spores with distinct morphology and tissue tropism (Fig. 1, Table 2). The first, referred to as "*Microsporidium* sp. 1" displayed broad, oval and highly refractive spores measuring $5.67 \pm 0.058 \times 3.50 \pm 0.049$ μm ($x \pm$ SE).[2] It was recovered from both species at prevalences of 26% in *P. corruptus* and 28% *X. sommermanae*, and was found in Malpighian tubules only. The "*Microsporidium* sp. 2" spore type was even more common (33% and 34% in *P. corruptus* and *X. sommermanae*, respectively), and was characterized by elongated diplokaryotic spores measuring $4.39 \pm 0.045 \times 1.90 \pm 0.033$ μm. It was recovered from fat body. The other two morphotypes occurred at much lower frequencies in the sampled material. "*Microsporidium* sp. 3" with ovacylindrical small spores ($2.48 \pm 0.097 \times 1.41 \pm 0.018$ μm) infected muscles, and was found only in four specimens of *P. corruptus* and two of *X. sommermanae*. "*Microsporidium* sp. 4" ($3.6 \pm 0.04 \times 2.2 \pm 0.03$ μm) was discovered in one specimen of *P. corruptus*, and its tissue tropism was not determined. Approximately 20% of infected insects contained both *Microsporidium* sp. 1 and 2 spore types. One specimen of *P. corruptus* contained three spore types, *Microsporidium* sp. 1, 2 and 3. We were unable to unequivocally determine the number of nuclei in *Microsporidium* sp. 1, 3, and 4 spore types by Giemsa (or DAPI) staining.

3.3. Sequence analysis and phylogenetic relationships with other microsporidia

PCR amplification of SSUrDNA and consequent sequencing were successful in spite of long storage of material in automotive coolant at relatively high environmental and room temperatures. The first question we addressed by sequence analysis was whether these spore types belonged to separate species, or to one or two polymorphic species. Each spore type corresponded to a specific

[2] Spore measurement were performed on material fixed and stored as described above in Section 2, and thus can be compared only with measurements of fixed spores of other microsporidia.

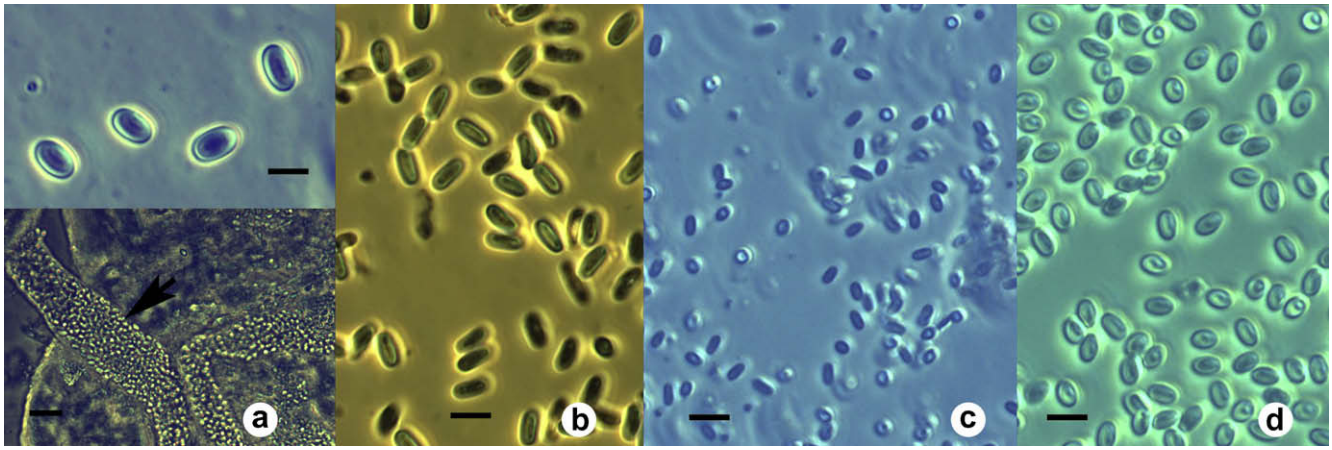

Fig. 1. Spores of microsporidia from bark lice. (a) *Microsporidium* sp. 1. Upper image – spores; lower image – infected Malpighian tubules. (b) *Antonospora psocopterae* n. sp. (c) *Mockfordia xanthocaeciliae* n. sp. (d) *Microsporidium* sp. 4. scale bars: a, upper image, b, c, d – 5 μm; a, lower image – 20 μm.

Table 2
Major characters of four novel microsporidia species from bark lice, *Xanthocaecilius sommermanae* (*X. s.*, *n* = 215) and *Polypsocus corruptus* (*P. c.*, *n* = 198).

Microsporidia	*Microsporidium* sp. 1	*Microsporidium* sp. 2 (=*Antonospora psocopterae*)	*Microsporidium* sp. 3 (=*Mockfordia xanthocaeciliae*)	*Microsporidium* sp. 4
Host	*X. sommermanae* *P. corruptus*	*X. sommermannae* *P. corruptus*	*X. sommermanae* *P. corruptus*	*P. corruptus*
Prevalence, % (*n*)	in *X. s.* 27.9 (60) in *P. c.* 26.2 (52)	in *X. s.* 34.0 (73) in *P. c.* 33.0 (65)	in *X. s.* 0.9 (2) in *P. c.* 2.0 (4)	0.5 (1)
Tissue tropism	Malpighian tubules	Fat body	Muscles	Not known
Spore size, μm (*n*)	5.67 ± 0.058*x* 3.50 ± 0.049 (31)	4.39 ± 0.045*x* 1.90 ± 0.033 (49)	2.48 ± 0.097*x* 1.41 ± 0.018 (21)	3.6 ± 0.04*x* 2.2 ± 0.03 (31)
Spore Shape, l/*w*	Oval 1.62	Elongated 2.3	Ovacylindrical 1.8	Oval 1.64

sequence, thus indicating the presence of four genetically and morphologically distinctive species. The novel sequences were deposited in Genbank as: FJ865221 (*Microsporidium* sp. 1), FJ865222 (*Microsporidium* sp. 2), FJ865223 (*Microsporidium* sp. 3), and FJ865224 (*Microsporidium* sp. 4). Relationships to other microsporidia and phylogenetic positions of these sequences were consistent in Neighbor Joining (NJ), Maximum Parsimony (MP), and Maximum Likelihood (ML) analyses (3), though general topology of the trees differed slightly in ML (Fig. 3a) versus MP and NJ (Fig. 3b) analyses.[3]

Microsporidium sp. 1 and *Microsporidium* sp. 2 belong to the *Paranosema–Antonospora* clade, within which *Microsporidium* 2 clustered with *Antonospora scoticae*. *Microsporidium* sp. 1 formed a dichotomy with the *Microsporidium* sp. 2–*Antonospora* clade. *Microsporidium* sp. 3 clustered with the *Encephalitozoon* branch, and *Microsporidium* sp. 4 clustered with *Heterovesicula cowani* sequence within the *Nosema–Vairimorpha–Encephalitozoon* clade. The *Heterovesicula–Microsporidium* sp. 4 branch formed a sister group to the *Vairimorpha–Nosema* dichotomy.

Evolutionary distances estimated by Kimura-2-parameter distance matrix analysis (Table 3), suggested that the closest sequences to those of *Microsporidium* sp. 1 are the *Microsporidium* 2 (sequence divergence score 0.061), and *Paranosema* spp. and *A. scoticae* sequences (about 0.08). The closest to *Microsporidium* sp. 2 are *A. scoticae* (0.054), *Microsporidium* sp. 1 and *Paranosema* spp. (0.06). Other sequences are more distant from *Microsporidium* 1 and *Microsporidium* 2 (0.30–0.625). The *Microsporidium* sp. 3 sequence displayed minimal divergence scores (0.187–0.211) against three *Encepalitozoon* spp., 0.314 – against *Microsporidium* sp. 4 sequence, and 0.348–0.566 – against other sequences analysed. The closest sequence to *Microsporidium* sp. 4 was that of *Heterovesicula cowani* (0.214).

3.4. Electron microscopy

Luckily, some key structures of spores and sporoblasts of *Microsporidium* sp. 2 and *Microsporidium* sp. 3 could be identified by electron microscopy, though, as expected, long-term storage in propylene glycol and subsequent transfer to ethanol destroyed most of intracellular membrane structures and negatively effected preservation of microsporidia spores and, especially, intracellular stages.

3.5. Microsporidium sp. 2.

Only the late sporogony stages were seen on the examined sections of infected *P. corruptus* and *X. sommermanae* fat bodies heavily loaded with spores. Diplokaryotic sporonts (Fig. 3a) resided in direct contact with host cell cytoplasm. Their membranes were ornamented with patches of electron-dense material, which eventually formed a continuous layer (Fig. 3a and b). Sporonts produced binucleate sporoblasts presumably by binary fission. Within the host cytoplasm networks of tubular structures were often observed in the vicinity of sporonts (Fig. 3a). These tubules eventually disappeared during spore maturation. Spores (3a, c) in sections measured 3.1–5.3 × 1.1–1.9 μm. Their exospores were 30–35 nm thick and exhibited a multilayered structure and undulating outline, the latter though might be an effect of unsuitable fixation. Translucent endospores were 150–200 nm thick, except for the anterior region. Spores contained diplokarya and isofilar polar

[3] Alignment is available from the first author upon request.

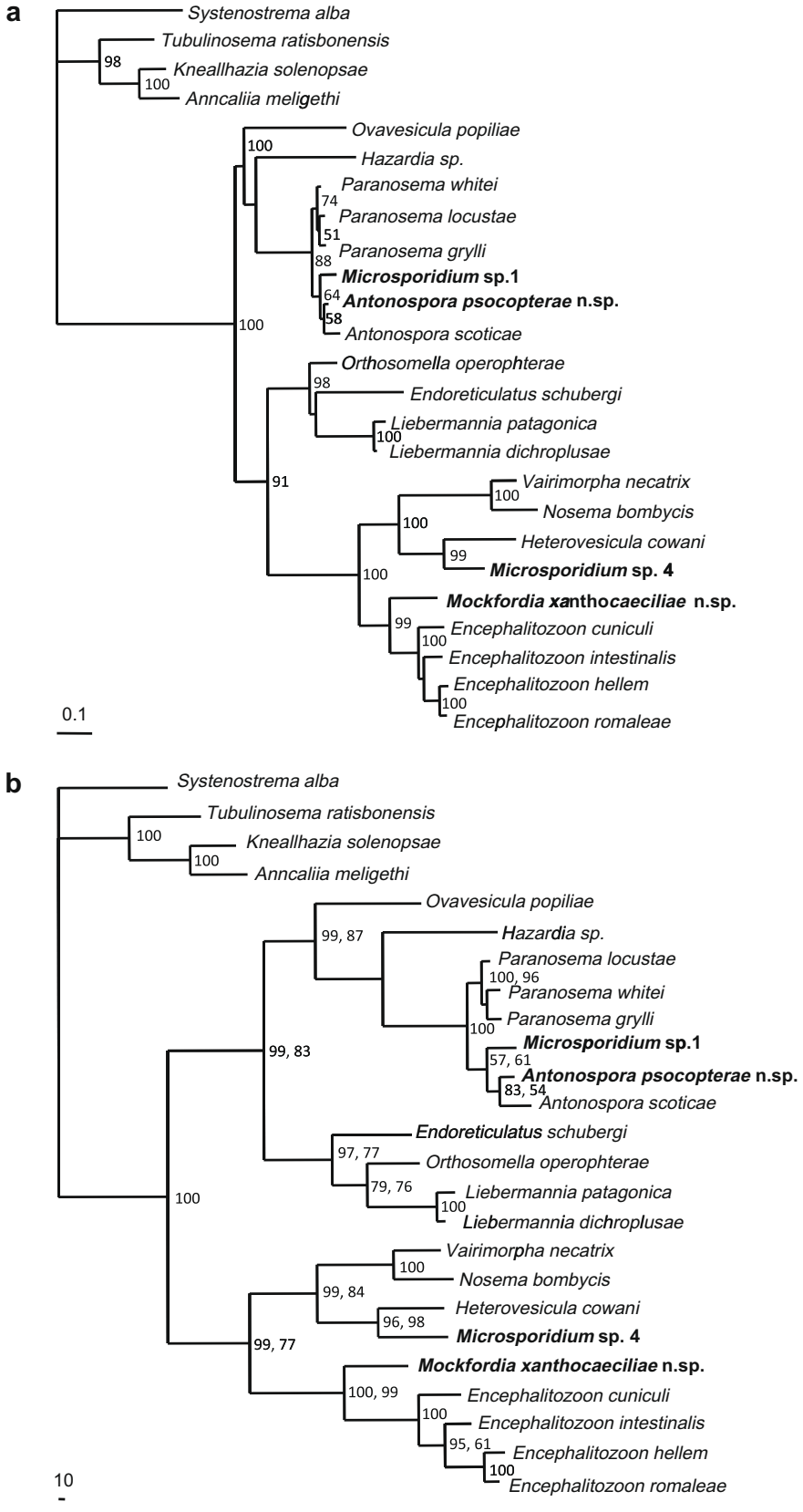

Fig. 2. SSUrDNA-based phylogenetic relationships of four novel microsporidia from Psocoptera (*Microsporidium* sp. 1, *Antonospora psocopterae* n.sp, *Mockfordia xanthocaeciliae* n.sp, and *Microsporidium* sp. 4, with 21 other microsporidia species. Branch lengths represent evolutionary distance. The scale bar represents one substitution per 10 nucleotides. Bootstrap support is indicated only if more than 50; number of replicates for all analyses equaled 100. (a) Maximum likelihood tree. (b) Parsimony tree. The first number at each node indicates bootstrap values from neighbor joining analysis, which displayed identical tree topology, the second – from parsimony analysis. One number at a node means that both values equaled 100.

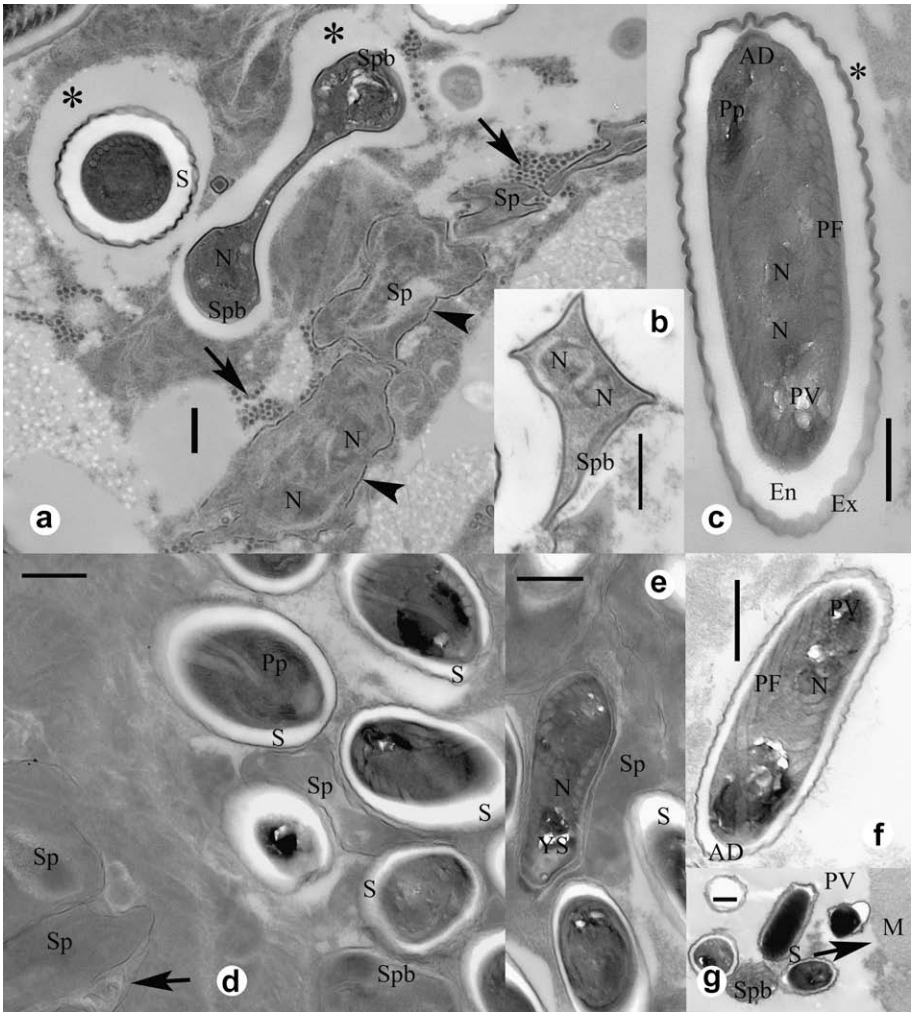

Fig. 3. Electron microscopy. (a–c) *Antonospora psocopterae* n. sp. (d–g) *Mockfordia xanthocaeciliae* n. sp. (a) Sporonts reside in direct contact with host cell cytoplasm and their membranes are ornamented with patches of electron-dense material (arrowheads). Sporont undergoes binary fission to produce two binucleate sporoblasts. Network of transversally cut tubular structures are observed in vicinity of sporonts (arrows). (b) Star-like sporoblast with a clearly seen diplokaryon. (c) Longitudinal section through the spore. (d) Spores and sporogonic stages. Arrow indicates the membrane, presumably the membrane of a parasitophorous vacuole. (e) Spores and an elongated sporont inside the infected cell. (f) Longitudinal section through the spore. (g) Section through a muscle cells; transparent zone free from myofibrils is presumably occupied by parasitophorous vacuole, with boundaries indicated by an arrow. AD – anchoring disk; En – endospore; Ex – exospore; M – myofibrils; N – nucleus, a counterpart of a diplokaryon; PF– polar filament; Pp – polaroplast; PV – posterior vacuole; S – spore; Sp – sporont; Spb – sporoblast. Asterisks mark transparent 250–300 μm zones around spores and sporoblasts. Scale bars: 500 nm.

Table 3
Sequence divergence (lower diagonal) and percentage of identity (upper diagonal) among seventeen SSUrDNA sequences estimated by Kimura-2-parameter analysis[a].

	Eh[b]	Er	Ei	Ec	Mx	Vn	Nb	Hc	M4	Pw	Pl	Pg	Ap	M1	As	Lp	Ld
Eh	0	96.1	90.6	88.0	80.9	64.6	61.3	64.8	67.5	53.2	53.0	52.6	54.8	54.4	56.9	57.9	58.8
Er	0.039	0	90.6	88.2	81.3	65.4	63.4	65.5	68.3	52.8	53.6	53.1	55.4	55.0	57.4	56.9	57.5
Ei	0.094	0.094	0	89.6	79.8	65.3	62.5	64.4	68.3	56.2	56.8	56.3	58.1	58.1	60.0	61.0	61.4
Ec	0.120	0.118	0.104	0	78.9	62.9	59.6	63.4	66.6	56.8	57.4	56.9	59.1	58.8	59.8	59.6	60.7
Mx	0.191	0.187	0.202	0.211	0	65.2	63.0	64.3	68.6	53.6	54.8	54.0	56.1	56.7	57.2	60.1	61.0
Vn	0.354	0.346	0.347	0.371	0.348	0	85.2	67.7	66.9	44.7	45.1	44.2	47.4	45.7	47.6	49.3	51.0
Nb	0.387	0.366	0.375	0.404	0.370	0.148	0	67.7	62.5	36.3	37.1	37.0	40.0	37.5	39.7	46.7	48.2
Hc	0.352	0.345	0.356	0.366	0.357	0.323	0.323	0	78.6	48.0	48.0	48.5	50.3	50.1	50.3	53.6	55.2
M4	0.325	0.317	0.317	0.334	0.314	0.331	0.375	0.214	0	56.7	56.6	57.2	58.5	58.8	58.5	58.9	60.4
Pw	0.468	0.472	0.438	0.432	0.464	0.553	0.637	0.520	0.433	0	97.0	96.9	93.9	92.1	91.9	65.3	67.8
Pl	0.470	0.464	0.432	0.426	0.452	0.549	0.629	0.520	0.434	0.030	0	96.8	93.7	92.0	92.1	65.3	67.6
Pg	0.474	0.469	0.437	0.431	0.460	0.558	0.630	0.515	0.428	0.031	0.032	0	93.6	91.8	91.3	65.3	67.5
Ap	0.452	0.446	0.419	0.409	0.439	0.526	0.600	0.497	0.415	0.061	0.063	0.064	0	93.9	94.6	65.2	67.7
M1	0.456	0.450	0.419	0.412	0.433	0.543	0.625	0.499	0.412	0.079	0.080	0.082	0.061	0	91.7	64.5	66.8
As	0.431	0.426	0.400	0.402	0.428	0.524	0.603	0.497	0.415	0.081	0.079	0.087	0.054	0.083	0	65.4	67.0
Lp	0.421	0.431	0.390	0.404	0.399	0.507	0.533	0.464	0.411	0.347	0.347	0.347	0.348	0.355	0.346	0	96.3
Ld	0.412	0.425	0.386	0.393	0.390	0.490	0.518	0.448	0.396	0.322	0.324	0.325	0.323	0.332	0.330	0.037	0

[a] The whole matrix for 25 sequences is available from the senior author upon request.

[b] Abbreviations: As – *Antonospora scoticae*; Ap – *A. psocopterae* n. sp.; Ec – *Encephalitozoon cuniculi*; Eh – *E. hellem*; Er – *E. romaleae*; Ei – *E. intestinalis*; Hc – *Heterovesicula cowani*; Ld – *L. dichroplusae*; Lp – *Liebermannia patagonica*; M1, M4 – *Microsporidium* sp. 1, *Microsporidium* sp. 4 from bark lice; Mx – *Mockfordia xanthocaecilli*ae n. sp., Nb – *Nosema bombycis*; Pg – *Paranosema grylli*; Pl – *P. locustae*; Pw – *P. whitei*; Vn – *Vairimorpha necatrix*.

filaments of 65–75 nm in diameter arranged in 13–17 coils. Polar sacs and polaroplasts were not well preserved but their remnants suggested presence of typical umbrella-like polar discs and lamellar polaroplasts. Posterior vacuoles were seen in some spores as ruptures at the posterior ends (Fig. 3c). Sporoblasts and spores were surrounded by 250–500 nm-wide transparent zones with no limiting membranes (3a, c).

3.6. Microsporidium sp. 3.

Fragments of muscles of *X. sommermanae* infected with this microsporidium were heavily loaded with small-sized spores measuring 1.7–2.1 × 0.3–05 μm on ultrathin sections (Fig. 3d–g). Interfacial envelopes (IE) were hardly distinguishable among masses of spores (3d), though their membranes could be distinguished, especially in places where the content surrounded by IE was washed out (Fig. 3g). The spores and sporoblasts were intermingled with ribbons of elongated sporonts with slightly thickened plasma membranes (Fig. 3e), which eventually transformed into the exospore. Exospore and endospore in mature spores were 18–23 and 100–150 nm thick, respectively. Each spore contained 6–8 coils of isofilar polar filament 60–65 nm in diameter (Fig. 3d–f), one nucleus (Fig. 3e and f), a two-partite polaroplast (Fig. 3d and f) and a posterior vacuole (Fig. 3f).

4. Discussion

4.1. New host-parasite association

As far as we know, no sampling for microsporidia in Psocoptera has been undertaken until now (Becnel and Andreadis, 1999; Canning and Vavra, 2000; Sprague, 1977a). This study added Psocoptera to the list of insect orders containing species known as hosts for microsporidia. Practically no records exist of other natural pathogens of bark lice either. The three known fungal parasites naturally occurring in barklice (two ascomycetes and one zygomecete) were described only recently (Toledo et al., 2008). Those authors speculated that such scarcity of parasitic fungal flora is related to the evolution of specific cuticular compounds that work as antifungal defenses for bark lice (Lord and Howard, 2004), but inadequate sampling would probably be a more obvious explanation. Microsporidia, with their unique mode of transmission, would not be sensitive to such cuticular defenses anyway.

Several species of Microsporidia are known to infect representatives of other orders of the hemipteroid assemblage (Paraneoptera). One species was recorded from lice (Phthiraptera), one from aphids (Homoptera), and as many as 10 from true bugs (Heteroptera), most of the latter from aquatic species (Sprague, 1977a, 1977b; Tonka and Weiser, 2000). None of those species, most of which were described prior 1960 (i.e., lacking electron microscopy or molecular data), seem to be similar to the species described here, though the discrepancy of methods used for descriptions of new Microsporidia species then and now make the comparison problematic.

We report here discovery of four species of microsporidia from *X. sommermanae* and *P. corruptus*. These two species of barklice belong to closely related families, Caeciliusidae and Amphipsocidae of the suborder Psocomorpha (Johnson and Mockford, 2003; Mockford, 1993). Both species inhabit forests of North America, live on bark of trees, feed on lichens and fungi, and aggregate in "herds" during nymphal and adult stages (Mockford, 1993). The latter conspicuous feature of their behaviors is obviously favorable for peroral transmission and dispersal of the Microsporidia parasites. Given an overwhelming number of barklice in our probe, several such "herds" must have been captured in the single Malaise trap.

Phylogenetic analyses (Fig. 2) indicated that four novel microsporidia infecting two barklice fell into three clades belonging to two unrelated supertaxa of terrestrial microsporidia, namely the *Paranosema–Antonospora* and *Nosema–Encephalitozoon* lineages. Psocoptera is considered to be the most primitive order of hemipteroids, originating during the Permian Period 295–248 million years ago (Gillot, 1995). Interestingly that Psocoptera are closely related to Phthiraptera, sucking lice, which parasitize warm blooded animals including humans. These two orders are placed in the infraorder Psocodea and share a common ancestor, based on robust morphological and molecular evidence (Johnson et al., 2004). Though the majority of barklice are free living species, various species of Psocoptera inhabit plumage of birds and the pelage of mammals, as well as their nests. This short-term commensal-type relationship presumably gave rise to obligate parasitism characteristic to Phthiraptera (Johnson et al., 2004). Evidence of a close relationship of *Microsporidium* sp. 3 from Psocoptera to *Encephalitozoon* spp., ubiquitous parasites of birds and mammals, presented in this paper, supports the idea that the association of ancestral Psocodea with mammals and birds could be one of the avenues of transfer of Microsporidia from arthropods to warm blooded hosts. In fact, SSUrDNA-based phylogenetic analyses (Fig. 2, Table 3) suggest that *Microsporidium* sp. 2 is more closely related to, and shares more sequence similarity with *Encephalitozoon* spp., than insect Microsporidia. The *Microsporidium* sp. 3 sequence branches as sister group to the *Encephalitozoon* clade and occupies a basal position within the *Microsporidium* sp. 2–*Encephalitozoon* spp. superclade. Within the *Encephalitozoon* clade the position of recently discovered *Encephalitozoon romaleae* (Johny et al., 2009; Lange et al., 2009), which parasitizes the lubber grasshopper, but shares 96% with *Encephalitozoon hellem* (a microsporidium primarily infecting birds) certainly creates a problem. As an explanation of striking relatedness of *E. romalea* to *E. hellem* it may be speculated that this species has evolved as a result of reciprocal transfer of the *E. hellem*-related bird-infecting microsporidium back to insects, though our current knowledge is not sufficient to make definite conclusions.

Further surveys of Microsporidia in Psocoptera, Phthiraptera and related orders, as well as in other ectoparasitic or bloodsucking insects (fleas, bed-bugs, dipterans) and acarines (ticks, mites and chiggers) will shed light on evolutionary routes of host transfer of microsporidia as parasites of birds and mammals.

4.2. Propylene as a fixative for microsporidia surveys

This research established that insect material stored in propylene glycol ("antifreeze") for months at summer temperatures remains suitable for surveys for microsporidia. In this work microsporidia DNA extracted from barklice after 2-month storage in glycol was PCR amplified with nearly the same fidelity as from fresh insects. We believe that propylene glycol is more preferable for DNA fixation and long-term storage at high (>20 °C) temperatures, than 70–90% ethyl alcohol. Fixation and long-term storage of various arthropods in antifreeze at high temperatures adequately preserved both mitochondrial and nuclear genetic material for PCR and other analyses (Rubink et al., 2003; Vink et al., 2005). We now conclude that parasitic DNA is preserved in antifreeze as well as that of the host. More surprising, after fixation and storage in propylene glycol and subsequent transfer to absolute ethanol, samples were still suitable for limited analysis by electron microscopy. Preservation of many membranous internal structures of stages and spores was poor, but more robust morphological characters of spores and sporoblasts were evident. These characters are important for identification of microsporidia (general spore morphology, number of polar filament coils, type of polaroplast and some others) and could be resolved in propylene glycol-fixed

samples. Though glycol fixation cannot be recommended for general use due to its obvious flaws, it still may be used to characterize certain features of fine morphology of microsporidia, if not better alternative is available. Probably fixation and preservation in glycol was not as destructive for fine morphology of samples as their rapid transfer from glycol to 100% ethanol. The quality of EM after processing of glycol-fixed samples will be experimentally checked. We believe this work can guide future surveys for microsporidia using massive sampling of insects in various traps employed by ecologists and taxonomists. Such surveys would tremendously increase our knowledge on microsporidia diversity, speciation and parallel host-parasite evolution.

4.3. Phylogeny and taxonomy

Topologies of the obtained trees (Fig. 2) were in accord with the previously published phylogenies (Sokolova et al., 2005, 2006, 2009; Vossbrinck and Debrunner-Vossbrinck, 2005). Parsimony (PS) and neighbor joining (NJ) analyses produced trees of identical topologies. The only discrepancy between the PS–NJ and the maximum likelihood (ML) trees was the position of the *Orthosomella–Endoreticulatus–Liebermannia* clade, which clustered with the *Nosema–Encephalitozoon* clade in PS and NJ analyses, and with the *Paranosema–Antonospora* clade in ML analyses. More new sequences are required to determine the relations among these three groups. Position of the *Hazardia* sp. remains unresolved in all analyses. ML, PS and NJ as well as the distance matrix analyses suggested that *Microsporidium* sp. 1 and sp. 2 fit into either the genus *Paranosema* or *Antonospora* depending on morphological characters, while *Microsporidium* sp. 3 and sp. 4 belonged to yet undescribed genera. *Microsporidium* sp. 1 and *Microsporidium* sp. 4, cannot yet be assigned to any genus and await further morphological characterization. The corresponding sequences, Acc # FJ865221 and Acc # FJ865224, are deposited in the Genbank as *Microsporidium* sp. 1 from bark lice and *Microsporidium* sp. 4 from bark lice, respectively. We assign here *Microsporidium* sp. 2 to the genus *Antonospora* based on the high sequence and morphological similarity (Fries et al., 1999). We erect a new genus *Mockfordia* for *Microsporidium* sp. 3 given sequence dissimilarity with other microsporidia, morphological characters, and novel host range.

4.4. Taxonomic summary

Antonospora psocopterae n. sp., Sokolova, Sokolov and Carlton
Type host: *X. sommermanae* Mockford, 1955 (Psocoptera; Caeciliusidae)
Other hosts: *P. corruptus* Hagen, 1861 (Psocoptera, Amphipsocidae)
Type locality: canopy of montane deciduous forest (White Oak Sink), 35. 63533°N, 83 74967°W, Great Smoky Mountains National Park, Blount Co., TN, USA
Infection site: fat body
Transmission: unknown
Merogony: unknown

Sporogony: sporonts are oval-to-elongated diplokaryotic cells surrounded by electron-dense envelope; they divide by binary fission to produce two sporoblasts.

Interfacial envelopes: development of all sporogony stages in direct contact with host cell cytoplasm. Sporonts are associated with the network of tubular structures inside the host cell.

Spores: elongated (length/wide ratio 2.3); when fixed measuring $4.39 \pm 0.045 \times 1.90 \pm 0.033$ μm (49); diplokaryotic; with 30–35 nm thick multilayered exospore, 150–200 nm thick endospore, and isofilar polar filament arranged in 13–17 coils.

Etymology: specific name after the host order.

We include SSUrRNA gene partial sequence under Genbank accession no. FJ865222 in the species characterization.

Differential diagnosis: Antonospora xanthocaeciliae can be differentiated from A. scoticae by SSUrDNA sequence divergence of 5.4%, less elongated (length/width ratio 2.3 vs. 2.5) spores, and host order (A. scoticae was described from a wild bee Andrena scotica (Hymenoptera, Andrenidae).

Synopsis of the genus Antonospora
A. scoticae Fries et al. (1999), type species.
A. psocopterae n. sp., Sokolova, Sokolov and Carlton.
Mockfordia n. gen., Sokolova, Sokolov and Carlton.

Uninucleate at least in the sporogonic phase of the life cycle. Small (about 2.5 μm in length) uniform ovocylindrical spores with extrusion apparatus of typical structure. One morphological spore type is produced. Sporoblasts formed from sequential division of elongate sporonts. Parasitophorous vacuole, if present, not conspicuous. Parasitizes muscles of terrestrial insects.

Type species: *Mockfordia xanthocaeciliae* n. sp., Sokolova, Sokolov and Carlton.

Differential diagnosis: Difference of SSUrDNA sequence between the type species and any other microsporidia sequence deposited in Genbank equals or exceeds 19%. There exist only six monomorphic monokaryotic genera which members produce ovacylindrical spores less than 3 μm in length and do not produce octospores. All those genera can be differentiated from *Mockfordia* by host species, morphology and tissue localization (Canning and Vavra, 2000). (1) *Canningia* Weiser, Wegensteiner and Zizka 1995 parasitize fat body and Malpighian tubules of scolytid beetles, and can be distinguished by the bulbous anchoring disk indenting into the spore, and strictly cylindrical spores. (2) Species of the genus *Cystosporogenes* Canning Barker Nicholas and Page, 1985 and (3) *Endoreticulatus* Brooks, Becnel and Kennedy, 1988 parasitize midgut epithelium of lepidopterans and chrysomelid beetles, and their sporonts are enclosed in conspicuous parasitoforous vacuoles containing 10–60 spores. (4) Spores of *Hessea* Ormieres and Sprague 1973, a parasite of midgut epithelium of fungus gnats, *Sciara* sp., reside inside thick-walled cysts, and have only 2–3 coils of polar filament. (5) Species of *Orthosomella* Canning, Wigley and Barker 1991 parasitizing lepidopterans, are extremely variable in length and on average are significantly larger. (6) Representatives of the genus *Liebermannia* Sokolova, Lange and Fuxa 2006 infect epithelium cells and also produce spores of variable length (Sokolova et al., 2006, 2007, 2009). In fact, *Microsporidium* sp. 3 from bark lice shares structural similarities, such as spore size, shape and ultrastructure, only with representatives of the genus *Encephalitozoon*, which may parasitize insects as well as vertebrates (Johny et al., 2009; Lange et al., 2009). *Mockfordia* can be differentiated from *Encephalitozoon* spp. from vertebrates by sequence divergence and host range, and from *E. romaleae* also by smaller size of spores, and by tissue specificity: *E. romaleae* infection is restricted to gut epithelium of lubber grasshoppers.

Synopsis of the genus Mockfordia: monotypic
M. xanthocaeciliae n. sp., Sokolova, Sokolov and Carlton
Type host: *X. sommermanae* Mockford, 1955 (Psocoptera; Caeciliusidae)
Other hosts: *P. corruptus* Hagen, 1861(Psocoptera, Amphipsocidae)
Type locality: canopy of montane deciduous forest (White Oak Sink), 35. 63533°N, 83 74967°W, Great Smoky Mountains National Park, Blount Co., TN, USA.
Infection site: muscles
Transmission: unknown

Merogony: unknown

Sporogony: sporonts or sporogonial plasmodia are elongated ribbon-like cells with yet undetermined arrangement of nuclei. They divide sequentially by binary or multiple fission to produce elongated sporoblasts.

Interfacial envelopes: sporogony inside inconspicuous parasitophorous vacuole.

Spores: ovocylindrical (length/wide ratio1.83); fixed spores measured $2.48 \pm 0.097 \times 1.41 \pm 0.018$ (21) μm; uniform, monokaryotic; with 18–23 nm thick multilayered exospore, 100–150 nm thick endospore, isofilar polar filament arranged in 6–8 coils, bipartite polaroplast, and moderately developed posterior vacuole.

Etymology: generic name is after Dr. Edward L. Mockford, Distinguished and Emeritus Professor of Entomology at Illinois State University, a world famous specialist in life history and taxonomy of bark lice. Specific name reflects the type host generic name.

We include SSUrRNA gene partial sequence under Genbank accession no. FJ865224 in the species characterization

Type material: Type slides "Microsporidia from Psocoptera, GSMNP-2008"; #1_Ps-sp 1-2, and #2_Ps-sp3 are deposited in the museum of Great Smoky Mountains National Park. Type slides #3_Ps-sp1-2, #4-Ps-sp2, #5_Ps-sp3, and #6_Ps_sp4 with Giemsa and Trichrome stained smears, EM blocks and frozen samples of isolated DNA of *A. psocopterae* and *M. xanthocaeciliae* have been deposited in the collection of Microsporidia at the Institute for Plant Protection, Russian Academy of Agricultural Scientises, St. Petersburg (Irma Issi's collection). Paratypes are kept at the private collection of the senior author.

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