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Molecular Characterization and Taxonomy of a New Species of Caudosporidae (Microsporidia) from Black Flies (Diptera: Simuliidae), with Host-Derived Relationships of the North American Caudosporids

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A new species of microsporidium, *Caudospora palustris* (Microsporidia: Caudosporidae), is described from 3 species of black flies (*Cnephia ornithophilia* and diploid and triploid cytospecies of *Stegopterna mutata*), bringing to 7 the total species of caudosporids recorded from North America. This new species of caudosporid is recorded from swamp streams of the Coastal Plain from New Jersey to Georgia, with single records from the New Jersey mountains and the Upper Peninsula of Michigan. Densities of patently infected larvae (up to 10,600/m^2) and spore production (nearly 8×10^{11}/m^2) are the greatest recorded for any microsporidium of black flies. The ultrastructure of this new species is presented, along with the first molecular characterization for a microsporidium of black flies. The phylogenetic position of black fly microsporidia within the phylum Microsporidia is presented; however, the analysis does not support the inclusion of *C. palustris* in any clade. Key features of all North American caudosporids are provided, and possible evolutionary trajectories are proposed based on optimization of caudosporid species on the phylogeny of their 22 known host species, including 16 that represent new host species records.

***Key Words:* *Caudospora palustris; Caudospora; Cnephia ornithophilia; Stegopterna mutata;* microsporidia; black flies; new species; phylogeny; molecular sequence; ribosomal RNA; ultrastructure.**

INTRODUCTION

The family Caudosporidae Weiser includes three genera (*Caudospora* Weiser, *Ringueletium* Garcia, and *Weiseria* Doby and Saguez) and 9 species worldwide that attack black flies. Six of these species, in the genera *Caudospora* and *Weiseria*, occur in North America (Jamnback, 1970; Issi *et al.*, 1990). The monotypic genus *Ringueletium* parasitizes black flies in South America (Garcia, 1990). All three genera are characterized by sporonts that produce 8 or 16 binucleate spores, usually with ornamented exospores bearing cauda, alae, ridges, or rugosity (Sprague *et al.*, 1992). All species are specific to black flies (Crosskey, 1990), although they eventually might be found in alternate hosts once the complete life cycle of microsporidia attacking black flies is known (Lacey and Undeen, 1987). Black flies are common hosts of microsporidia, with patent infections in larvae typically destroying adipose and other tissues and appearing as large, lobate cysts (Weiser and Undeen, 1981). About 260 species of black flies are known from North America north of Mexico (Adler and McCreadie, 1997). Their immature stages are found in virtually all flowing-water habitats from the coastal plains to the barren alpine regions (Crosskey, 1990).

Weiser and Undeen (1981) suggested that most microsporidian species that attack black flies have been discovered. However, we present an ultrastructural and molecular description, plus bionomics, for a new species of caudosporid from black flies in eastern North America, representing the seventh species of caudosporid recorded from North American black flies. We also provide a comparison of the spores of the new species with those of other North American caudosporids of black flies. To place this new caudosporid in a phylogenetic context, we present a molecular phylogeny of 26 microsporidia from other host groups, plus a phylogeny of the 22 known hosts of North American caudosporids on which we optimize the parasites.

MATERIALS AND METHODS

Patently infected larvae of the black fly *Cnephia ornithophilia* Davies, Peterson, and Wood were collected in the Coastal Plain of South Carolina and Georgia in March of 1991, 1997, and 1998. Patently infected larvae of *Stegopterna mutata* (Malloch) (diploid and triploid cytospecies) collected in New Jersey in March 1998 were discovered in unidentified collections

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of black flies shipped to us in a 1:3 solution of acetic ethanol. A single, heavily infected larva from Michigan was discovered in an unrelated examination of alcohol-preserved black flies in the National Museum of Natural History, Washington, DC.

The new microsporidium was described using standard techniques for microsporidia (Undeen and Vávra, 1997). Air-dried smears from infected larvae were fixed in methanol and stained for 10 min with 10% Giemsa stain buffered at pH 7.4. Fresh spores from *C. ornithophilia* were measured using a Vickers A. E. I. Image Splitting Micrometer. Spores from host larvae of the *Stegopterna mutata* species complex that had been preserved in acetic ethanol were examined by macerating infected tissue in a drop of 50% acetic acid and flattening it under a coverslip. Conspecificity of the new microsporidium from different hosts was established by comparing spore morphology with bright field and phase contrast at 1250× magnification.

Infected tissues (from topotypical larvae of *Cnephia ornithophilia*) were fixed for 2.5 h at room temperature in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 0.1% $CaCl_2$ and postfixed in 1% aqueous OsO_4 (w/v). These tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite. Thin sections were poststained with methanolic uranyl acetate followed by lead citrate and were examined and photographed with a Hitachi H-600 electron microscope at 75 kV.

For analysis of the 16S ribosomal gene of the new species, a spore preparation was obtained by decapitating four heavily infected topotypical larvae collected on 16 March 1998, removing the food plugs, and grinding the remaining bodies in approximately 400 µl of deionized water in 1.5-ml microfuge tubes. The spores were pelleted and washed once in deionized water. Approximately 1×10^7 spores and 200 µl of 0.1-mm-diameter siliconized glass beads were resuspended in 200 µl of STE (sodium chloride/Tris/EDTA) buffer in a 0.5-ml microfuge tube. Spores were broken mechanically in a mini beadbeater (Biospec) to release the DNA. The homogenate was heated immediately at 95°C for 5 min to inactivate DNAses and then centrifuged at 10,000*g* for 5 min. The resulting supernatant containing the DNA served as a template for PCR amplification of the 16S ribosomal gene. The following temperature cycling profile was used: 94°C (1 min), followed by 35 cycles of 94°C (1 min), 54°C (1 min), 72°C (1 min), and a final extension step at 72°C (15 min). Gene products from three separate PCRs were pooled and sequenced directly. The sequence was completed by redundant sequencing of both strands and compared to 16S ribosomal gene sequences of select microsporidia from different host groups (Diptera, Hymenoptera, Lepidoptera, fish, and mammals) to get a first indication of the phylogenetic position of the new species relative to other dipteran microsporidia (Table 1). PCR and sequencing primers are listed in Table 2. Detailed methods for 16S rRNA gene amplification by PCR, sequencing of the pooled PCR product, and sequence comparisons are given by Moser *et al.* (1998). The sequence Accession No. is AF132544, reposited with GenBank, Los Alamos, New Mexico.

All known black fly hosts of North American caudosporids (Jamnback, 1970; Maurand, 1975), including 16 new host records reported here for the first time, were incorporated into a phylogeny that is based on the framework provided by morphological (Currie, 1988), cytological (Ottonen and Nambiar, 1969; Rothfels, 1979; Henderson, 1986; Adler, unpublished), and molecular (Moulton, 1997) evidence. The classification system for black flies follows that of Currie (1997). Caudosporids were associated with their hosts on the cladogram, and a hypothesis of their relationships was based on the earliest appearance of each caudosporid species in the host phylogeny.

RESULTS

Caudospora palustris New Species

Type host: *Cnephia ornithophilia* Davies, Peterson, and Wood (Diptera: Simuliidae).

Additional hosts: *Stegopterna mutata* (Malloch) diploid cytospecies, *Stegopterna mutata* (Malloch) triploid cytospecies (Diptera: Simuliidae).

Site of infection: Adipose tissue of larva.

Interface: Presporulation stages apparently in direct contact with the host cell hyaloplasm. Sporulation stages more or less covered with an electron-dense surface coat at the onset of sporogony. Coat apparently incorporated into formation of the exospore. No sporophorous vesicle.

Other parasite–host cell relations: The larval fat body was destroyed by the parasite and replaced by masses of spores, forming white lobate cysts.

Development: All stages were diplokaryotic. The earliest stages observed were small diplokaryotic stages (late meronts?) (Fig. 1) limited by a simple plasmalemma (Fig. 10). Divisions resulted in the formation of paucinucleate plasmodia (Figs. 2 and 11). In Giemsa-stained smears, stages in sporulation were identifiable by the larger size of the plasmodia and the nuclei of the diplokarya (Figs. 3–5). Sporogonial plasmodia were identified in EM by formation of an incomplete electron-dense surface coat on the plasmalemma, as demonstrated on the lower portion of the plasmodium in Fig. 12. Sporulation apparently involved formation of multinucleate plasmodia (Fig. 3) that sometimes divided by plasmotomy (Fig. 4) to produce sporogonial plasmodia (Figs. 5 and 13). Repeated nuclear divisions resulted in formation of multinucleate sporogonial plasmodia, typically with four diplokarya, that underwent multiple

TABLE 1
Microsporidian Species Used in the Phylogenetic Analysis (*Escherichia coli* as Outgroup)

Species name	Type host	GenBank Accession No.
Amblyospora californica	*Culex tarsalis* (Insecta: Diptera)	ACU68473
A. salinarius	*Culex salinarius* (Insecta: Diptera)	ASU68474
A. stimuli	*Aedes stimulans* (Insecta: Diptera)	AF027685
Antonospora scotiae	*Andrena scotica* (Insecta: Hymenoptera)	AF024655
Culicosporella lunata	*Culex pilosus* (Insecta: Diptera)	AF027683
Edhazardia aedis	*Aedes aegypti* (Insecta: Diptera)	AF027684
Encephalitozoon cuniculi	*Oryctolagus cuniculus* (Mammalia: Lagomorpha)	Z19563
E. hellem	*Homo sapiens* (Mammalia: Primates)	L19070
Septata intestinalis	*Homo sapiens* (Mammalia: Primates)	U09929
Endoreticulates schubergi	*Lymantria dispar* (Insecta: Lepidoptera)	L39109
Enterocytozoon bineusi	*Homo sapiens* (Mammalia: Primates)	AF024657
Glugea anomala	*Gasterosteus aculeatus* (Osteichthyes: Gasterosteidae)	AF044391
Ichthyosporidium sp.	*Leiostomus xanthurus* (Osteichthyes: Gasterosteidae?)	L39110
Loma salmonae	*Oncorhynchus tshawytscha* (Osteichthyes: Gasterosteidae?)	U78736
Nosema algerae	*Anopheles stephensi* (Insecta: Diptera)	AF069063
N. apis	*Apis mellifera* (Insecta: Hymenoptera)	U97150
N. bombycis	*Bombyx mori* (Insecta: Lepidoptera)	L39111
Nosema sp.	Species unknown (Insecta: Hymenoptera)	U11047
Nucleospora salmonis	*Oncorhynchus tshawytscha* (Osteichthyes: Gasterosteidae?)	U78176
Parathelohania anophelis	*Anopheles quadrimaculatus* (Insecta: Diptera)	AF027682
Thelohania solenopsae	*Solenopsis invicta* (Insecta: Hymenoptera)	AF031538
Vairimorpha necatrix	*Malacosoma americanum* (Insecta: Lepidoptera)	Y00266
Vairimorpha sp.	*Solenopsis richteri* (Insecta: Hymenoptera)	AF031539
Vavraia oncoperae	*Wiseana* sp. (Insecta: Lepidoptera)	X74112
Vittaforma corneae	*Homo sapiens* (Mammalia: Primates)	L39112
Caudospora palustris	*Cnephia ornithophilia* (Insecta: Diptera)	AF132544
Escherichia coli	*Homo sapiens* (Mammalia: Primates)	X80724

fission by budding (Fig. 6) or were in the form of a rosette (Fig. 7). Occasionally, multiple fission of plasmodia with up to eight diplokarya was observed (Fig. 8). Products of these divisions transformed into diplokaryotic sporoblasts (Figs. 14 and 15).

Spore: Spores binucleate and oblong-ovate with thick walls (Figs. 9a, 9b, and 16). Endospore approximately three times as thick as the rugose, unlayered exospore (Fig. 17). Polaroplast lamellar, occupying the anterior third of the spore. Polar filament with about nine turns, tapered slightly toward the tip (Figs. 16 and 17). Size (mean ± standard error, $n = 32$) of fresh spores: 5.58 ± 0.07 µm × 3.01 ± 0.04 µm.

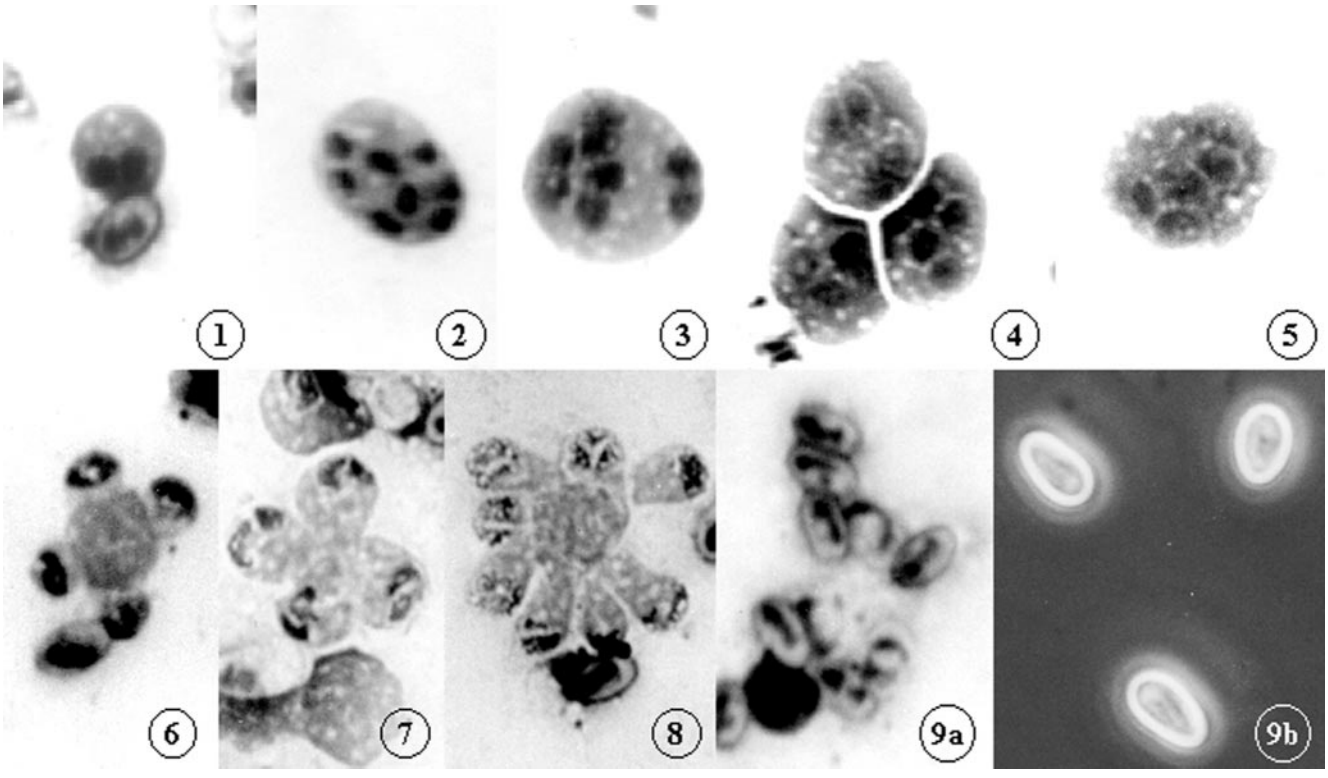
Type locality: South Carolina, Sumter County, Woods Bay State Park, swamp outflow, 33°57′N 80°00′W; types collected 14 March 1997 (P. H. Adler).

Additional collections: Georgia, Calhoun Co., Keel Creek, Route 37, 31°26.36′N 84°29.00′W, 3 March 1991 (P. H. Adler), ex *Cnephia ornithophilia;* Michigan, Schoolcraft Co., Seney Wildlife Refuge, Marsh Creek, 26 May 1967 (I. B. Tarshis), ex *Cnephia ornithophilia* or *Cnephia dacotensis* Dyar and Shannon; New Jersey, Atlantic Co., tributary of Great Egg Harbor River, Estell Manor County Park, 39.4028°N 74.7317°W, 17 March 1998 (D. S. Bidlack), ex *Stegopterna mutata* (triploid cytospecies); New Jersey, Atlantic Co., tributary of Great Egg Harbor River, Weymouth County Park, 17 March 1998 (D. S. Bidlack), ex *Stegopterna mutata* (diploid cytospecies); New Jersey, Sussex Co., Delaware Water Gap National Recreation Area, 41.1090°N 74.9125°W, 25 March 1998 (D. S. Bidlack), ex *Stegopterna mutata* triploid cytospecies; South Carolina, Sumter Co., Woods Bay State Park, 16 March 1998 (P. H. Adler and C. E. Beard) and 18 March 1999 (C. E. Beard and D. Werner), ex *Cnephia ornithophilia*.

Deposition of type specimens: Type slides (USNM Nos. 51473, 51474) have been deposited in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC. Additional slides, host specimens in ethanol, and specimens embedded in plastic resin are in the Clemson University Arthropod

TABLE 2
List of PCR and Sequencing Primers

	Nucleotide sequence of primer (5′–3′)
Forward primers	
JM27/18f	TTT GAA TTC CAC CAG GTT GAT TCT GCC
BAM1	CTG TCC TGT GGG TAA ATG TG
RP7/530f	GTG CCA GC(AC) GCC GCG G
Reverse primers	
RP4/1492r	TTT GGA TCC GGT TAC CTT GTT ACG ACT T
RP8/1047r	AAC GGC CAT GCA CCA C
RP10/530r	CCG CGG C(GT)G CTG GCA C



FIGS. 1–9. Developmental stages of *Caudospora palustris* n. sp. in adipose tissue of *Cnephia ornithophilia*. Giemsa stained (except 9b), ×2000. **FIG. 1.** Small diplokaryotic stage (meront?). **FIG. 2.** Multinucleate merogonial plasmodium. **FIG. 3.** Sporogonial plasmodium. **FIG. 4.** Plasmotomy of sporogonial plasmodium. **FIG. 5.** Late-stage sporogonial plasmodium. **FIG. 6.** Division of a quadrinucleate plasmodium by budding. **FIG. 7.** Division of a quadrinucleate plasmodium in the shape of a rosette. **FIG. 8.** Division of an octonucleate plasmodium in the shape of a rosette. **FIG. 9.** Mature binucleate spores; (a) Geimsa stained; (b) unstained, unfixed.

Collection, Clemson, South Carolina, and the Center for Medical, Agricultural and Veterinary Entomology, USDA, Gainesville, Florida.

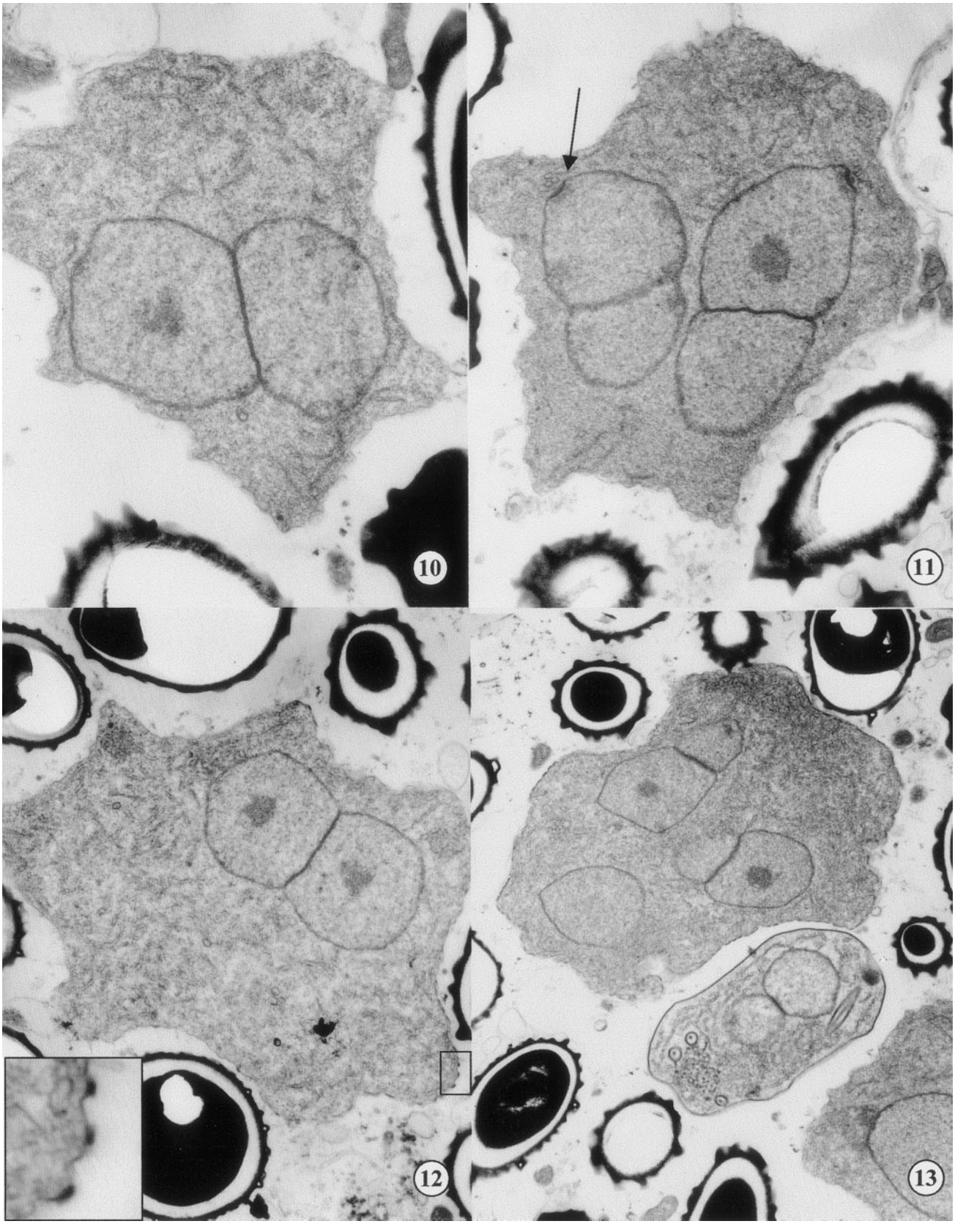
Etymology: The species name comes from the Latin *palustris,* meaning swamp or marsh dweller, in reference to the swampy habitat typically associated with streams of the larval simuliid hosts.

Remarks: Few stages during presporulation could be positively identified in either electron micrographs or Giemsa-stained smears. Most stages observed were in the sporulation process.

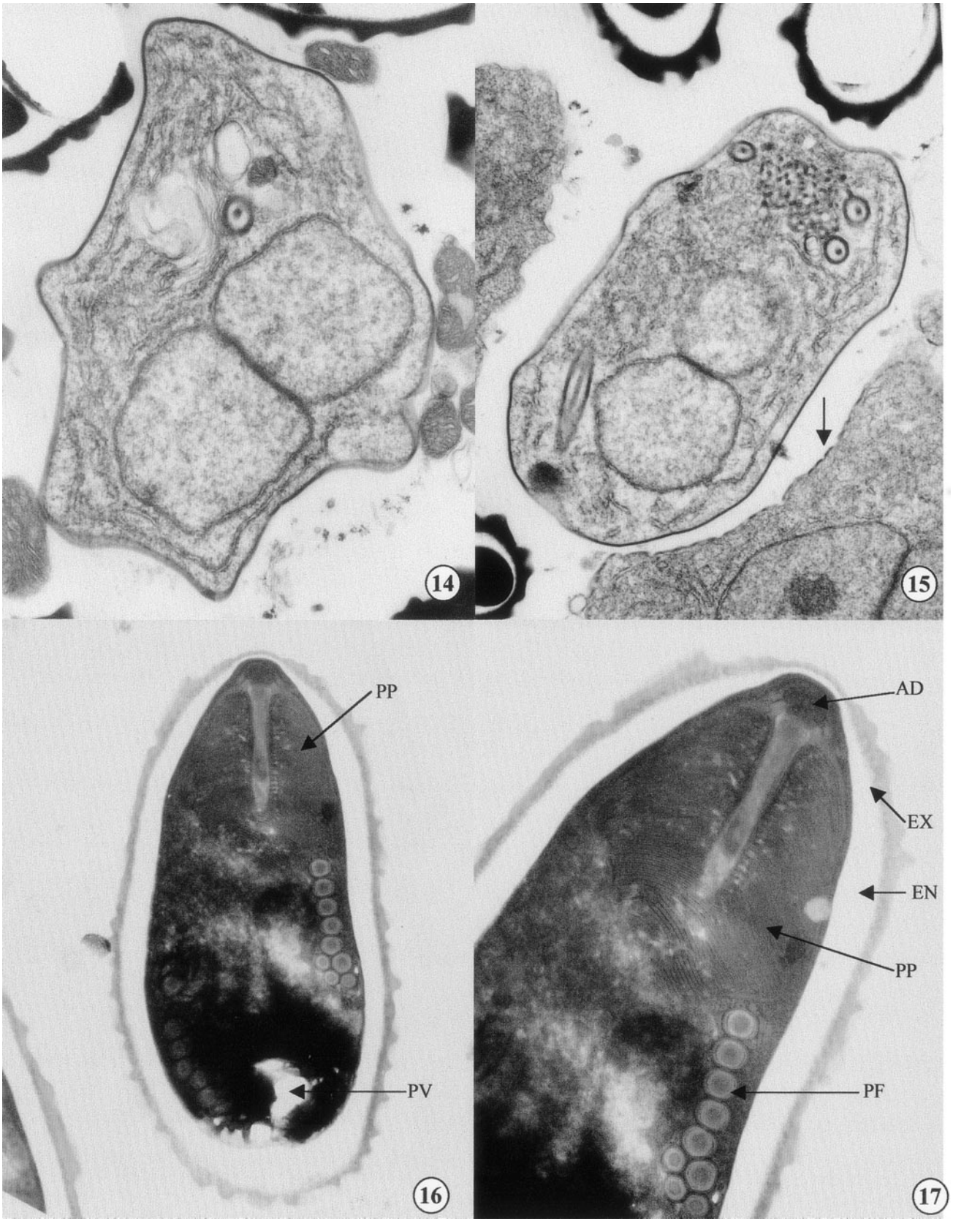
Molecular characterization: The complete 16S ribosomal gene sequence was 1386 bases long with a GC content of 50.5%. Based on the branching pattern of the most-parsimonious tree, *Caudospora palustris* did not form a clade with the other dipteran microsporidia included in the analysis (Fig. 18). Only minor branching-pattern differences were found when generating trees based on the distance data (data not shown). The phylogenetic position of *Caudospora palustris* was unresolved in the most parsimonious 50% majority rule consensus tree generated by 1000 bootstrap replications.

Bionomics: We found *Caudospora palustris* in streams 1–3 m wide, with water temperatures less than 15°C that issued from swamps and beaver ponds in Sumter Co., South Carolina (type locality); Calhoun Co., Georgia; and Atlantic and Sussex Cos., New Jersey. In the southeastern United States, hosts were larvae of *Cnephia ornithophilia* that usually hatch in October and pupate between January and the end of March, depending on the particular population. Larvae of *Stegopterna mutata* (diploid and triploid cytospecies) were hosts in New Jersey, where they are prevalent from late winter to midspring. The triploid cytospecies is an all-female, parthenogenetic black fly, whereas the diploid cytospecies reproduces sexually.

An enormous population of infected larvae was observed at the type locality on 16 March 1998. The host population at this site was pure for *Cnephia ornithophilia;* no other black flies were found at the site from 1997 to 1999. In some sections of the stream, the density of infected larvae was approximately 10,600/m^2, so great that from the stream bank larvae could be seen through the brown water at a distance of 6 m. Of 1721 larvae collected on 16 March, 57.4% were patently infected. These infected larvae reached 14 mm in length, whereas the greatest length for an apparently uninfected larva was 11 mm. We estimated 75 million spores per individual, based on 10 homogenized larvae.



FIGS. 10–13. Developmental stages of *Caudospora palustris* n. sp. in adipose tissue of *Cnephia ornithophilia*. **FIG. 10.** Meront (?) ×19,200. **FIG. 11.** Merogonial plasmodium, ×16,000. Note the spindle plaques on the nuclei of each diplokaryon in preparation for division. **FIG. 12.** Sporont identified by the initial modifications of the plasmalemma (see inset) to form an electron-dense surface coat, ×12,800. **FIG. 13.** Multinucleate sporogonial plasmodium with an extensive electron-dense surface coat, ×9600.



FIGS. 14–17. Sporogenesis of *Caudospora palustris* n. sp. in *Cnephia ornithophilia*. **FIG. 14.** Diplokaryotic sporoblast with primordium of the polar filament, ×27,200. **FIG. 15.** Elongated diplokaryotic sporoblast with the primordium of the anchoring disk at the anterior pole, ×24,000. Spore-wall formation indicated by a thickened plasmalemma. Arrow indicates the formation of the surface coat on the sporogonial

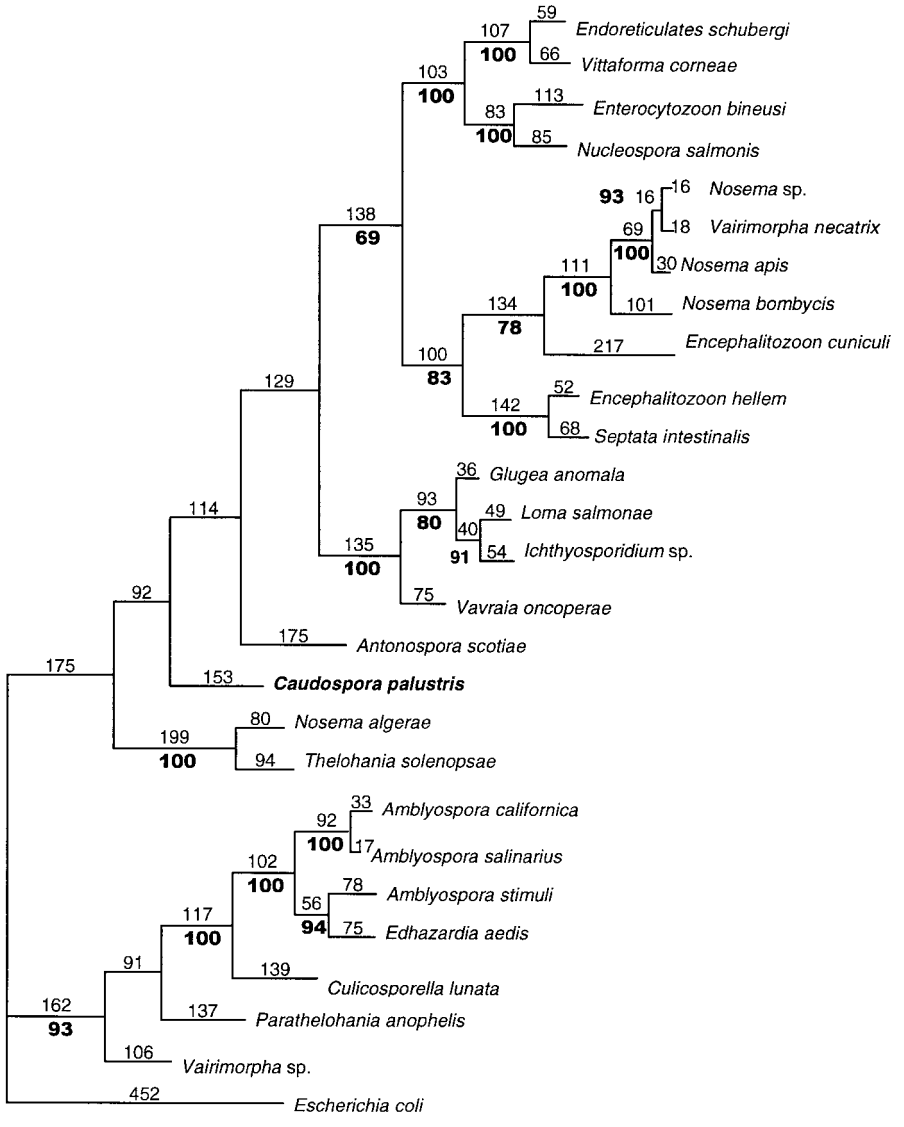


FIG. 18. Most-parsimonious tree based on the 16S rDNA sequences of 21 species of microsporidia. *Escherichia coli* was used as the outgroup. Boldface numbers on the tree indicate the percentage of bootstrap replicates which contained that topology.

Thus, spore production in some stream sections reached nearly 8×10^{11} spores/m^2. In other collections, prevalence of patent infection was 35% (n = 20 larvae; GA, Calhoun Co., 3 March 1991), 17% (n = 23; NJ, Sussex Co., 25 March 1998), 6% (n = 18; NJ, Atlantic Co., 17 March 1998), 5% (n = 84; SC, type locality, 18 March 1999), and 3% (n = 420; SC, type locality, 14 March 1998). In no other situation were the enormous densities of infected larvae observed.

One infected larva of *Stegopterna mutata* (triploid cytospecies) had a dual, patent infection of *Caudospora palustris* and the chytrid fungus *Coelomycidium simulii* Debaisieux. In southern Georgia, infections of *Caudospora palustris* and the microsporidium *Janacekia debaisieuxi* (Jírovec) Larsson were found in larvae of *Cnephia ornithophilia* in the same stream at the same time of year, but dual infections were not found. The unstained spore of *Janacekia debaisieuxi*, under the light microscope, superficially resembles that of *Caudospora palustris* but can be distinguished (at 1250× magnification under bright field) by the smooth, rather than slightly ragged, outline of the exospore.

Larvae of the following additional black fly species were present at sites where infections of *Caudospora*

plasmodium. **FIG. 16.** Mature, binucleate spore, ×25,500. **FIG. 17.** Mature, binucleate spore, demonstrating the lamellar polaroplast, the coils of the polar filament, and the rugose, unlayered exospore, ×39,000. AD, anchoring disk; EN, endospore; EX, exospore; PF, polar filament; PP, polaroplast; PV, posterior vacuole.

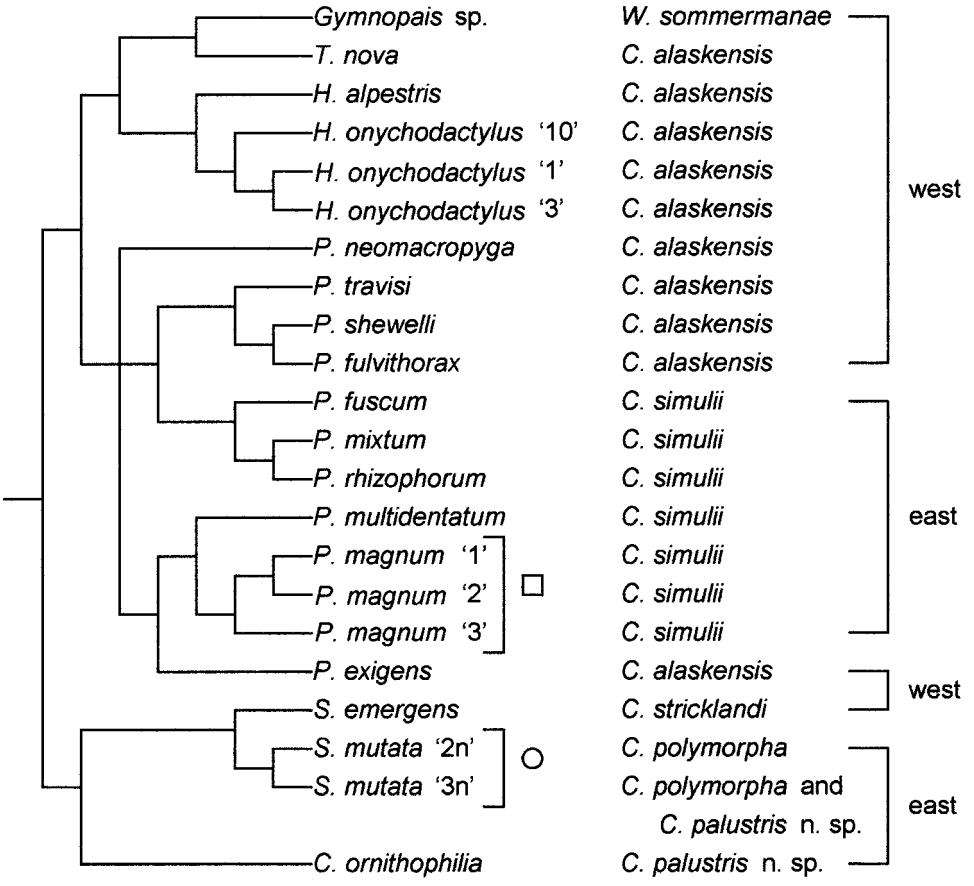


FIG. 19. Phylogeny of simuliid hosts of North American Caudosporidae, with optimization of caudosporid parasites. Only those simuliid taxa are shown in which caudosporids have been found. Brackets above cytospecies of *Prosimulium magnum* and *Stegopterna mutata* indicate that a caudosporid species (square = *Caudospora pennsylvanica,* circle = *Caudospora alaskensis*) was found in an unidentified host cytospecies of each of these species complexes. For hosts, *T = Twinnia, H = Helodon, P = Prosimulium, S = Stegopterna,* and *C = Cnephia.* For microsporidia (right side), *W = Weiseria* and *C = Caudospora.* New host records include *T. nova; H. onychodactylus* cytospecies '10', '1', and '3'; *P. neomacropyga; P. travisi; P. shewelli; P. fulvithorax; P. rhizophorum; P. magnum* cytospecies '1', '2', and '3'; *P. exigens; S. mutata* cytospecies '2n'; *S. mutata* cytospecies '3n' (for *C. palustris*); and *C. ornithophilia.*

palustris were found: *Simulium congareenarum* (Dyar & Shannon), *Simulium tuberosum* (Lundström) cytospecies F, *Simulium venustum* Say cytospecies CC, and *Simulium verecundum* Stone & Jamnback. None of these larvae had patent infections of *Caudospora palustris,* despite larval population levels as high or higher than those of the hosts. Purified spores fed to the mosquitoes *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say did not germinate in the guts or produce infections.

Phylogeny of Caudosporidae

Optimizing caudosporid species on the host phylogeny (Fig. 19) produced two major groups (Fig. 20). One group consists of three species (*Caudospora stricklandi, Caudospora polymorpha,* and *Caudospora palustris*) that are found in the most derived of the infected host groups, *Stegopterna* and *Cnephia.* Host relationships indicate that these three caudosporids could be shown with *C. stricklandi* as the sister species of the other two species (as we arbitrarily have shown) or with *C. palustris* as the sister species of the other two. The other group consists of two pairs of species (*Weiseria sommermanae* and *Caudospora alaskensis*) and (*Caudospora simulii* and *Caudospora pennsylvanica*) that occur in the most basal of the infected host genera, *Gymnopais, Twinnia, Helodon,* and *Prosimulium.*

DISCUSSION

The description of *Caudospora palustris* brings to seven the number of nominal species of Caudosporidae known from North American black flies. Placement of this new species in the family Caudosporidae is based primarily on the ornamentation of the exospore, the binucleate spores, and the apparent specificity for primitive hosts of the family Simuliidae. Generic assignment is more problematic. Few developmental and

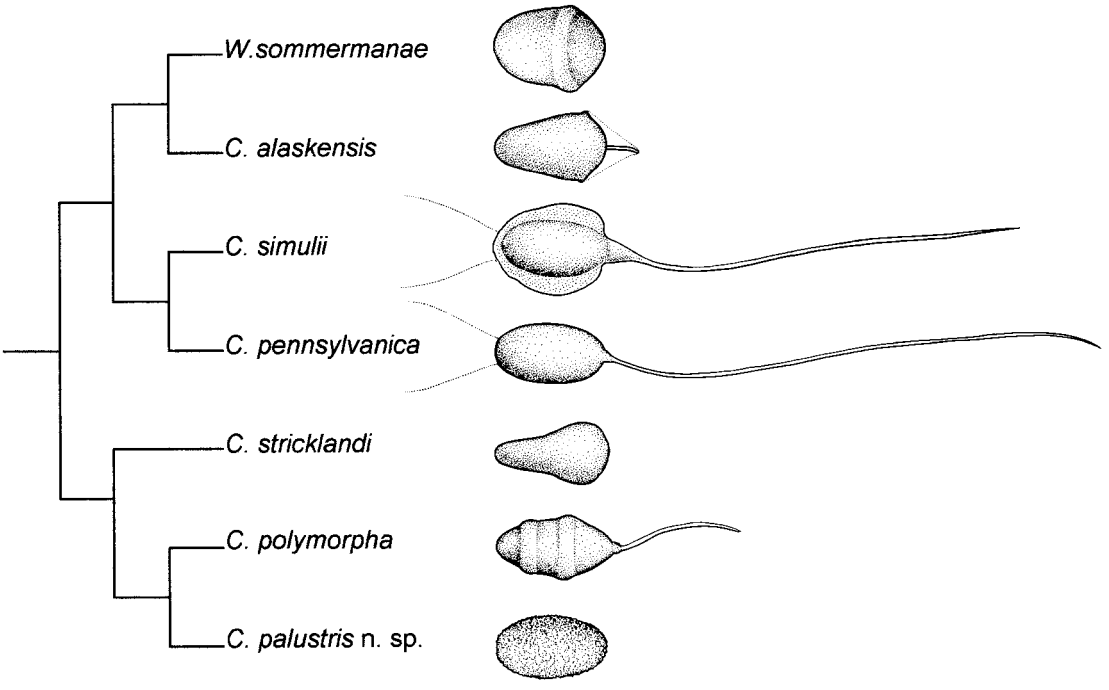


FIG. 20. Hypothesized relationships of microsporidia based on the earliest appearance of each caudosporid species in the host phylogeny (Fig. 19). Spores are illustrated beside each species name. Spore of *Caudospora stricklandi* was drawn from Maurand (1975). *C.* = *Caudospora; W.* = *Weiseria.*

morphological characters distinguish the three genera (*Caudospora, Ringueletium,* and *Weiseria*) in the family. The new species does not fit well in either *Ringueletium* or *Weiseria*. The Neotropical, monotypic *Ringueletium* is octosporoblastic and has an exospore with filamentous appendages (Garcia, 1990). The two species of *Weiseria* have thickened posterior ridges or crests and sporogonial plasmodia with up to 22 diplokarya (Doby and Saguez, 1964; Jamnback, 1970). Characterization of the genus *Weiseria* is complicated by the questionable distinction between *Weiseria laurenti,* the type species of the genus, and *Caudospora alaskensis* (unpublished data); further study is required to resolve their relation to one another. We prefer to avoid the establishment of a monotypic genus and by default conservatively place the new species in genus *Caudospora.* Placement of a species without a cauda in *Caudospora* is not without precedent. *Caudospora stricklandi,* which lacks a cauda, was transferred from the genus *Nosema* Naegeli (Vávra, 1981; Issi *et al.,* 1990). The combination of a rugose exospore and oval spore shape of *Caudospora palustris* distinguish this new species from other caudosporids (Table 3), as well as from all nominal microsporidia of black flies worldwide.

Gross spore morphology under a compound microscope and the size ranges for preserved spores of *Caudospora palustris* are the same in *Cnephia ornithophilia* and both cytospecies of *Stegopterna mutata,* suggesting that microsporidia from the three host species are conspecific. Molecular and ultrastructural confirmation of conspecificity, nonetheless, is needed. Similarity of spore sizes among hosts of *Caudospora palustris* contrasts markedly with significant differences in spore sizes among and within hosts of other caudosporids, as reflected in the large overall range of sizes in Table 3. Some of these other caudosporids, such as *Caudospora alaskensis* and *Caudospora simulii,* are possibly complexes of sibling species.

Because ultrastructural studies of black fly caudosporids are limited to *Caudospora simulii* (Vávra, 1968), a single spore of *Caudospora alaskensis* (Ledin, 1994), and a brief written description of *Caudospora stricklandi* (Vávra, 1981), comparative information is minimal. All four species have a lamellar polaroplast and no posterior vacuole. The polar filament in *Caudospora alaskensis* averages five turns, whereas in *Caudospora palustris* it makes about nine turns, in *Caudospora stricklandi* seven turns, and in *Caudospora simulii* about 12–13 turns. The taxonomic utility of the number of turns in the Caudosporidae is unknown.

The present study provides the first gene sequence for the small ribosomal subunit of a black fly microsporidium and the first approximation of the phylogenetic position of black fly microsporidia within the phylum Microsporidia. The analysis provides no support for inclusion of *Caudospora palustris* in any clade. If host groups are indicative of relationships, we would expect

TABLE 3
Morphological Features of Caudosporid Species that Attack Black Flies in North America

Species	Preserved spore size (μm)	Exposure ornamentation	Reference
Caudospora alaskensis Jamnback (=*Caudospora* sp. Frost and Nolan = undescribed species [Fig. 16 of Strickland])	3.7–6.1 × 1.7–3.5	Cauda (0.7–1.9 μm) subtended by membrane	Strickland (1911), Jamnback (1970), Frost and Nolan (1972), Ledin (1994)
Caudospora pennsylvanica Beaudoin and Wills	5.3 ± 0.06 × 3.2 ± 0.06	Cauda (23.5 ± 0.06 μm), dual anterior filaments	Beaudoin and Wills (1965)
Caudospora polymorpha (Strickland) Vávra (formerly *Caudospora brevicauda*)	3.1–6.8 × 1.6–4.4	Cauda (0.8–10.0 μm), pair of encircling ridges	Jamnback (1970), Frost and Nolan (1972), Vávra and Undeen (1981)
Caudospora simulii Weiser	3.0–7.0 × 1.9–6.0	Lateral alae[a], cauda (5.0–38.0 μm), dual anterior filaments	Jamnback (1970), Frost and Nolan (1972), Vávra and Undeen (1981)
Caudospora stricklandi (Jírovec) Vávra	5.0 × 2.5	Irregular ribs	Maurand (1975), Vávra (1976), Issi *et al.* (1990)
Caudospora palustris n. sp.	5.5–6.0 × 3.0–3.5[b]	Rugosity	Present study
Weiseria sommermanae Jamnback	4.8–6.0 × 3.4–4.8	Posterior thickened ridge	Jamnback (1970)

[a] Not all spores within a host have lateral alae.
[b] Same range for all hosts (*Cnephia ornithophilia, Stegopterna mutata* diploid and triploid cytospecies).

the inclusion of *Caudospora palustris* in the well-supported clade containing microsporidia from Diptera. To resolve the phylogenetic position of *Caudospora palustris* based on the 16S ribosomal gene, additional data on microsporidia from black flies are needed.

The North American caudosporids are restricted to the more basal lineages of the Simuliidae. *Caudospora nasiae* Jamnback, however, attacks an Afrotropical species of *Simulium* (Jamnback, 1970), the most derived host genus, and *Caudospora alaskensis, Caudospora simulii,* and *Caudospora stricklandi* each have been taken from a limited number of *Simulium* larvae in the Palearctic Region (Crosskey, 1990; Vávra, 1981; Adler *et al.*, 1999).

The phylogeny of caudosporid hosts is robust, representing a reconstruction from morphological, cytological, and molecular evidence. It provides a topology of caudosporid relationships that can be tested using molecular and other character sources. The phylogeny depicts the North American caudosporids as generally falling into eastern (*Caudospora pennsylvanica, Caudospora polymorpha, Caudospora simulii,* and *Caudospora palustris*) and western groups (*Caudospora alaskensis, Caudospora stricklandi,* and *Weiseria sommermanae*), reflecting the geographic distributions of their hosts. Only *Caudospora alaskensis* has been found in both eastern and western North America. Although fairly common in *Prosimulium* hosts in western North America, it is encountered infrequently in the eastern portion of the continent, where it has been found only in the *Stegopterna mutata* complex (as *Caudospora* sp. by Frost and Nolan [1972] and as *Glugea polymorpha* in part [Fig. 16] by Strickland [1911]). Eastern material might represent a new species, although it is depicted conservatively in Fig. 19 and Table 3 as *Caudospora alaskensis*. If host–parasite evolution has been congruent, *Weiseria sommermanae* would be one of the most ancestral species of the group and *Caudospora palustris* one of the most derived.

Like the other six species of North American caudosporids, *Caudospora palustris* attacks cold-water, univoltine hosts. Its occurrence in the southeastern Coastal Plain, however, is unique, deviating from the association with mountainous or hilly terrain typical of other caudosporid species. Its distribution might be determined more by the distribution of its hosts, which are found in lowland as well as mountainous areas.

Host specificity of *Caudospora palustris,* like that of other caudosporids, is limited despite the occurrence of at least four other species of black flies in the same streams as the hosts. We recorded only three host species for *Caudospora palustris* in over 10 years of prospecting for black flies and their microsporidia. During this same period of time, we found nine new host records for *Caudospora alaskensis* and four for *Caudospora simulii* (Fig. 19).

The high prevalence of patent infection and consequent levels of spore production at the type locality of *Caudospora palustris* in 1998 are a phenomenal exception to the low level (<1%) of patent infections typical of black flies (cf. Crosskey, 1990). These high levels were not observed in other collections of *Caudospora palustris,* including the type locality at the same time 1 year previous and subsequent and, therefore, might not be typical of this species. In certain copepod hosts, high prevalence of infection (up to 80%) with a tuzetiid microsporidium is seasonal and regular from year to year, producing about 2.7×10^9 spores per m^2 (Vávra *et al.*, 1996/97), far less than the nearly 8.0×10^{11} spores

per m^2 of *Caudospora palustris*. A seasonal component to prevalence of infection is unlikely in *Caudospora palustris* because the hosts are univoltine. Microsporidia of black flies, however, typically become more frequent and prominent as host populations age (Maurand, 1975).

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