

## Fine Structure and Sporogonic Development of a *Vavraia* sp. (Microsporida: Pleistophoridae) in the Biting Midge *Culicoides edeni* (Diptera: Ceratopogonidae)

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A microsporidium with ultrastructural characteristics of the genus *Vavraia* was found in the fat body of an adult specimen of *Culicoides edeni* (Diptera: Ceratopogonidae) collected in northern Florida. The sporogonial stages developed within sporophorous vesicles, which contained variable numbers of oval spores at maturity. The wall of the sporophorous vesicle was composed of two electron-dense outer layers and an electron-lucent intermediate layer. Sporonts contained haplokaryotic nuclei and divided by rosette formation. Mature spores had anisofilar polar filaments and measured  $3.8 \pm 0.28 \mu\text{m}$  in length and  $2.2 \pm 0.16 \mu\text{m}$  in width in thick sections of resin-embedded material. This is the first report of a *Vavraia* sp. from a species of *Culicoides*. © 1990 Academic Press, Inc.

**KEY WORDS:** *Vavraia*; Pleistophoridae; *Culicoides edeni*; Ceratopogonidae; microsporidia; biting midge; ultrastructure; electron microscopy; morphology.

### INTRODUCTION

Ceratopogonid flies are important vectors of viral, protozoan, and helminth parasites (Linley et al., 1983; Linley, 1985), yet little is known about their pathogens or parasites (Wirth, 1977). Only a few reports have documented the occurrence of microsporidia in species of *Culicoides* (Chapman et al., 1968, 1969; Levchenko and Issi, 1973; Dubitskii et al., 1979; Kline et al., 1985). Ultrastructural observations are limited to a single study of a *Nosema*-like species from salt marsh habitats in Florida (Kline et al., 1985).

During an ultrastructural study of the development of *Haemoproteus meleagridis* (Haemosporina: Haemoproteidae) from wild turkeys in its ceratopogonid vector (Atkinson, 1989), the sporulating stages of a microsporidium were found in an adult specimen of *Culicoides edeni*. This report describes the fine structure of the sporogonial stages of the parasite.

### MATERIALS AND METHODS

Specimens of *Culicoides* were captured in a turkey-baited Bennett trap (Bennett, 1960) at Paynes Prairie State Preserve, Alachua County, Florida, as described by Atkinson (1988). Midges were aspirated from the trap and kept in screened cardboard cartons with a pad of cotton which had been moistened with a solution of 10% (w/v) sucrose. Within 24 hr after capture, specimens of *Culicoides* spp. were aspirated from the cardboard cartons, blown into a Petri dish containing RPMI 1640 tissue culture medium with a small drop of Triton X-100, and identified by wing pattern under a dissecting microscope. Midguts from specimens of *C. edeni* were dissected into a drop of 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, with 4% (w/v) sucrose and transferred to fresh fixative for 1 hr. The midguts were washed with 0.1 M cacodylate buffer that contained 4% (w/v) sucrose and embedded in a thin layer of warm 2% (w/v) agar made with the same solution. After the agar solidified, small blocks containing individual midguts were cut from the agar to facilitate handling of

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the tissue and post-fixed for 1 hr with 1% (w/v) osmium tetroxide in 0.1 M cacodylate buffer with 4% sucrose. The agar blocks were washed with 0.1 M buffer, dehydrated, and embedded in Spurr's epoxy resin. One-micrometer sections and ultrathin sections were cut with a glass or diamond knife. Thick sections were stained with methylene blue borax. Thin sections were stained with 2% (w/v) uranyl acetate in 50% methanol, followed by Reynold's lead citrate, carbon-coated, and examined with a JEOL 100CX electron microscope.

## RESULTS

Sporogonial stages of a microsporidium were found in a piece of fat body that remained attached to the midgut of an unengorged specimen of *C. edeni* during dissection and processing for electron microscopy. The fat body was completely filled with sporogonial plasmodia and sporophorous vesicles (SV) containing both sporoblasts and mature spores (Fig. 1, upper inset). The SVs were oval to spherical and contained a variable number of mature spores. Serial thick sections of the tissue indicated that each SV contained as few as 8 to more than 32 spores. Prior to dissection, the midge had a darkly pigmented abdomen, as is characteristic of parous specimens of *Culicoides* (Dyce, 1969). No sign of an overt microsporidiosis was evident.

Early sporogonial plasmodia contained nuclei with one or more centriolar plaques, scattered endoplasmic reticulum, and abundant ribosomes (Fig. 1). A thickened, electron-dense surface coat was external to the

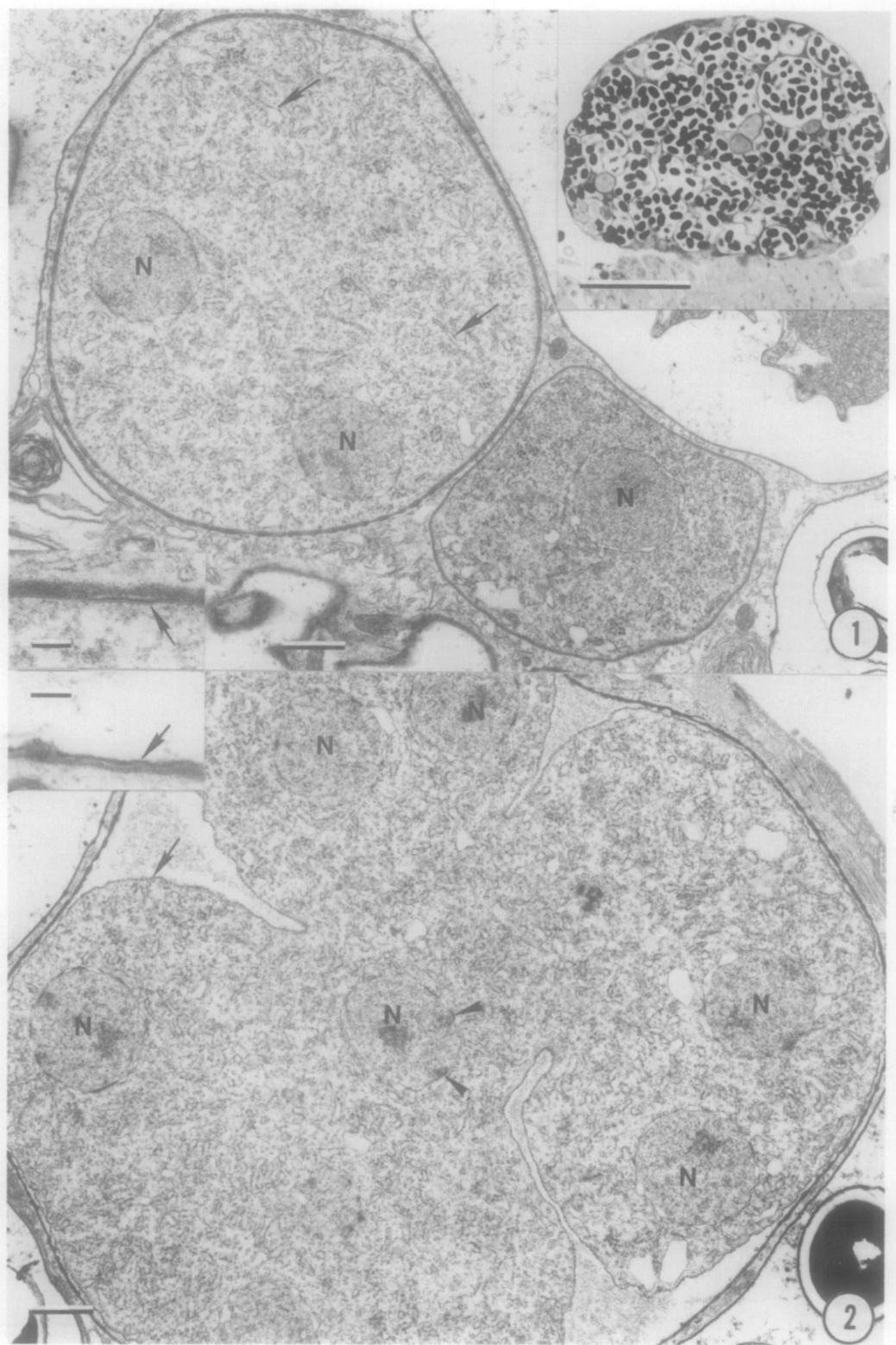
sporont plasmalemma (Fig. 1, lower inset). Plasmodia eventually contracted to form a space between the electron-dense surface coat and the plasmalemma of the parasites (Fig. 2). The surface coat was crenated in outline on its inner surface where the plasmalemma had retracted. The electron-dense coat became modified into a wall that was composed of two electron-dense layers separated by a thin, electron-lucent intermediate layer (Fig. 2, inset). Sporoblasts developed by rosette formation within the wall of the SV (Fig. 2). Each lobe of a rosette often contained more than one nucleus. No evidence of meiosis was observed.

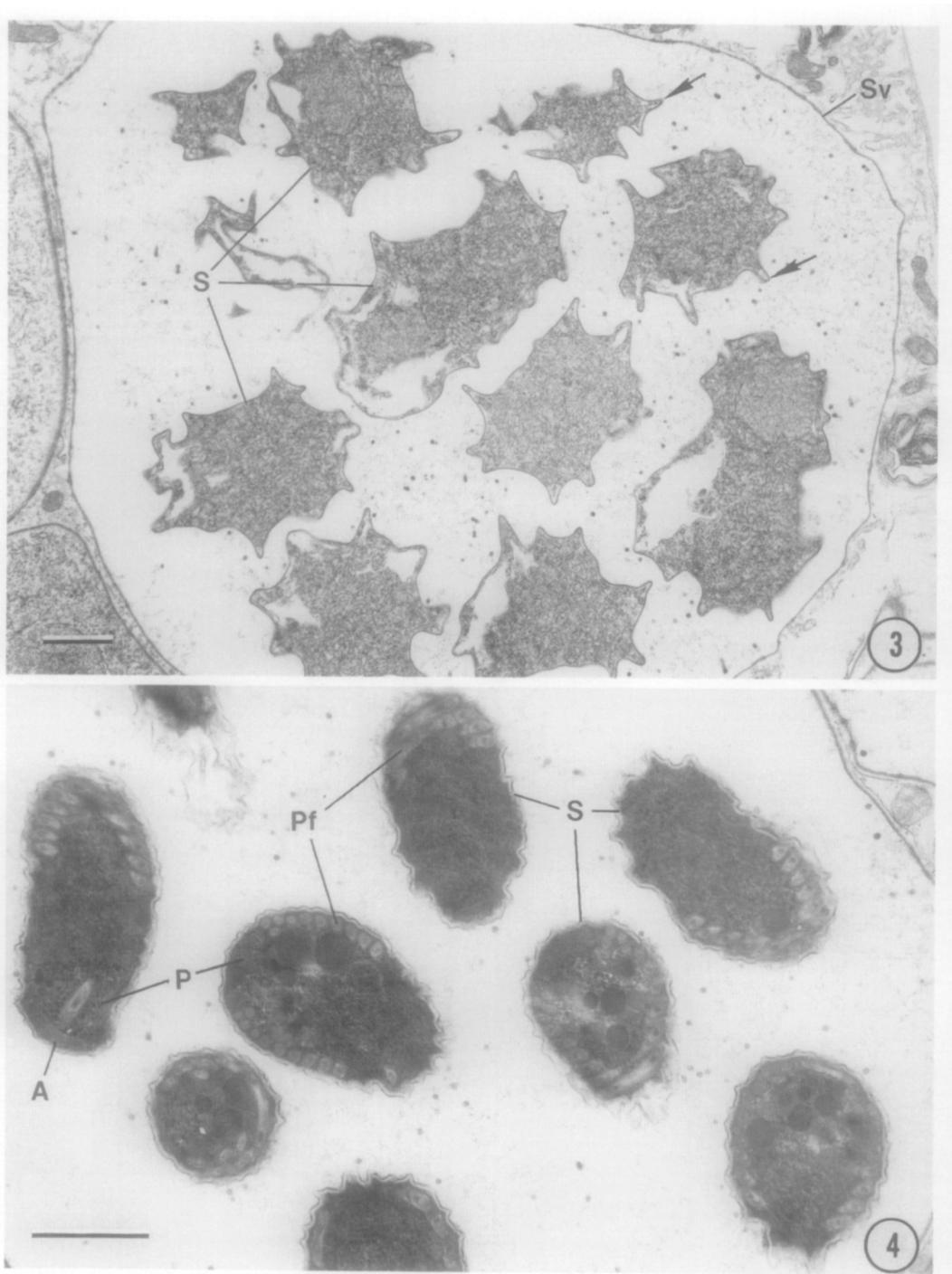
Immature sporoblasts had numerous projections on their surface which formed finger-like extensions in the sporogonial space of the SV (Fig. 3). During final stages of sporulation, a thick wall developed around each sporoblast and internal organelles differentiated, including the polar filament, polaroplast, and anchoring disk (Fig. 4). Mature spores were oval, uninucleate, and uniform in shape and dimension, measuring  $3.8 \pm 0.28 \mu\text{m}$  in length and  $2.2 \pm 0.16 \mu\text{m}$  in width in thick sections of fixed, resin-embedded material ( $n = 25$ ) (Fig. 1, inset). Mature spores had a crenated, electron-dense exospore measuring 20–40 nm in thickness which was lined on its inner surface by fine, granular material (Fig. 5). The electron-lucent endospore was thinner at the anterior pole of the spore in the region of the anchoring disc. A single nucleus was located in the center of the spore. A posterior vacuole that contained amorphous

FIGS. 1 AND 2. Light and electron micrographs of *Vavraia* sp. from an abdominal fat body of *Culicoides edeni*.

FIG. 1. Early sporonts contain nuclei (N), scattered endoplasmic reticulum (arrows), and ribosomes. Bar = 1  $\mu\text{m}$ . Lower inset: An outer wall composed of amorphous electron-dense material coats the sporont plasmalemma (arrow). Bar = 100 nm. Upper inset: Thick section of abdominal fat body attached to outer midgut. The fat body is packed with sporophorous vesicles containing mature and immature spores. Bar = 50  $\mu\text{m}$ .

FIG. 2. Sporont beginning to divide by rosette formation within the wall of the sporophorous vesicle. Each lobe of the sporont contains several nuclei (N) with centriolar plaques (arrowheads). Note striations on the plasmalemma of the sporont (arrow). Bar = 1  $\mu\text{m}$ . Inset: Higher magnification of the wall of the sporophorous vesicle. Wall (arrow) is composed of two outer electron-dense layers and an inner electron-lucent layer. Bar = 100 nm.





FIGS. 3 AND 4. Electron micrographs of sporoblasts of the *Vavraia* sp. from *Culicoides edeni*.

FIG. 3. Immature sporoblasts are present within a sporophorous vesicle (Sv). Sporoblasts (S) are deeply contoured in outline and have fingerlike projections (arrows) that extend into the sporophorous vesicle. Bar = 1  $\mu$ m.

FIG. 4. Maturing sporoblasts. Sporoblasts (S) are bounded by a thickened wall and contain developing anchoring disks (A), polar filaments (Pf), and polaroplasts (P). Bar = 1  $\mu$ m.

electron-dense material was immediately posterior to the nucleus. The polaroplast occupied the anterior half of mature spores and was composed of stacked lamellae that measured from 15 to 20 nm in thickness at the anterior end of spores and 30 to 40 nm in thickness at the posterior end, next to the centrally located nucleus (Fig. 6). An anchoring disk which measured approximately 400 nm in diameter was at the anterior end of the spores. The polar sac was approximately the same width as the anterior lamellae of the polaroplast (Fig. 6). The polar filament was anisofilar with wide anterior and narrower posterior coils (Fig. 7). Spores had 5 or 6 wide anterior coils and 5 or 6 narrower posterior coils. Anterior coils were usually arranged in a single layer adjacent to the external plasma membrane of the spore. Posterior coils were occasionally observed in two layers. In cross sections, the polar filament contained a central core that was surrounded by concentric layers of fine, granular material. Adjacent to these layers was a ring of 21 or 22 electron-lucent fibers that appeared to be spirally wound around the long axis of the filament in tangential sections (Fig. 7). Exterior to these were two concentric layers that differed slightly in electron density. The polar filament was bound at its periphery by a unit membrane.

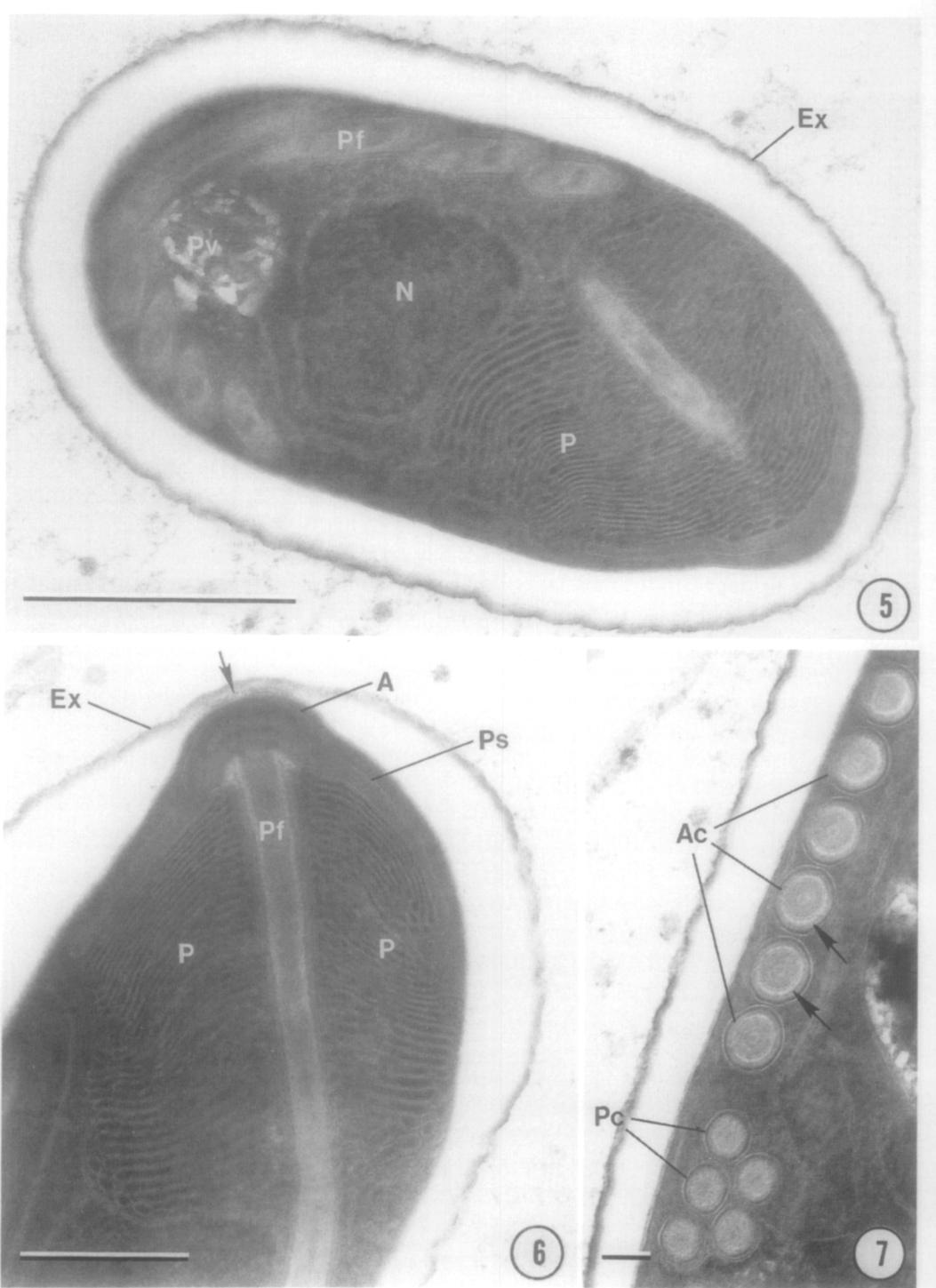
## DISCUSSION

The only prior records of microsporidia in species of *Culicoides* are limited to brief descriptions of *Pleistophora* and *Nosema* spp. in larvae that were collected in southern Louisiana, Florida, and southeastern Kazakhstan in the Soviet Union (Chapman et al., 1968, 1969; Chapman, 1973; Levchenko and Issi, 1973; Kline et al., 1985). Limited surveys of field-collected *Culicoides* larvae have shown that microsporidia have prevalences ranging from less than 1 to 4.5%, depending on season and geographic area (Chapman, 1973; Dubitskii et al., 1979; Kline et al., 1985). Little is known about their effectiveness as biological control agents, although several reports have described the discoloration and death of both naturally and experimentally infected *Culicoides* larvae (Chapman et al., 1968, 1969; Dubitskii et al., 1979).

In recent years, taxonomy of the pansporoblastic microsporidia has undergone extensive revision. The genus *Pleistophora* appears to be limited primarily to species which are parasitic in vertebrates (Canning and Hazard, 1982; Milner and Briese, 1986). Ultrastructural characteristics of sporogonial stages, nuclear organization, and origin and nature of the SV have been used to erect new genera for the microsporidia of insects which exhibit multi-sporous sporogony within a vesicle or vacuole, namely *Vavraia*, *Polydispyrenia*, *Cystosporogenes*, *Ovavesicula*, and *Endoreticulatus* (Canning and Hazard, 1982; Canning et al., 1985; Andreadis and Hanula, 1987; Brooks et al., 1988). Correct generic identification depends on many morphological details that can be resolved only by electron microscopy (Larsson, 1988).

The parasites from *C. edeni* have a number of ultrastructural features that are characteristic of the genus *Vavraia*, including the thickened wall of the SV with an electron-lucent inner space, haplokaryotic nuclei, and oval spores with anisofilar polar filaments (Larsson, 1988). They lack the electron-dense envelope that surrounds merogonic and sporogonic stages of *Cystosporogenes*, a parasitophorous vacuole derived from the host cell endoplasmic reticulum as is characteristic of *Endoreticulatus*, and the diplokaryotic nuclei that are characteristic of *Polydispyrenia* and *Ovavesicula*.

The microsporidium described in this report is similar to *V. culicis* in morphology of the sporophorous vesicle, mature spores, mode of division, and host order (Canning and Hazard, 1982), but cross transmission studies are necessary to determine their exact taxonomic relationships. Attempts to transmit *Pleistophora* and *Nosema* spp.



FIGS. 5, 6, AND 7. Electron micrographs of mature spores of the *Vavraia* sp. from *Culicoides edeni*.

FIG. 5. This spore has a crenated, electron-dense exospore (Ex) and contains a nucleus (N), a posterior vacuole (Pv), a polaroplast (P) composed of stacked lamellae, and coils of the polar filament (Pf). Bar = 1  $\mu$ m.

FIG. 6. The anterior end of a mature spore contains an anchoring apparatus with an anchoring disk (A) and polar sac (Ps). Note granular material (arrow) between the anterior pole of the spore and the exospore (Ex) and polar filament (Pf) and stacked lamellae of the polaroplast (P). Bar = 0.5  $\mu$ m.

FIG. 7. Mature spores with an anisofilar polar filament are composed of 5 or 6 wide anterior coils (Ac) and 5 or 6 narrower posterior coils (Pc). In cross section, the polar filament contains a central core surrounded by concentric layers of granular material and a ring of 21 or 22 electron-lucent fibers (arrows). Bar = 100 nm.

that were isolated from larval *Culicoides* to mosquitoes have been unsuccessful (Chapman, 1973; Dubitskii et al., 1979). Levchenko and Issi (1973) described *Pleistophora culicoidi* from larval *Culicoides* in the Soviet Union, but provided few morphological details. The spores of *P. culicoidi* are the same shape as spores of the *Vavraia* sp. described here, but are much smaller (1.7–2.1  $\mu\text{m}$  long and 0.9–1.2  $\mu\text{m}$  wide). A precise identification will have to wait until more information is known about the distribution, life cycle, the host specificity of this species, and the range of morphological variations that might occur in different host species and families.

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