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Vavraia lutzomyiae n. sp. (Phylum Microspora) infecting the sandfly *Lutzomyia longipalpis* (Psychodidae, Phlebotominae), a vector of human visceral leishmaniasis

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Abstract

Vavraia lutzomyiae (Microsporida; Pleistophoridae) is a new species parasitic in the tropical phlebotomine sandfly, *Lutzomyia longipalpis* (Diptera, Psychodidae, Phlebotominae), a major vector of *Leishmania chagasi* in Latin America where human visceral leishmaniasis is endemic. Infected larvae and pupae were parasitized in the abdomen, and some adults were parasitized in Malpighian tubules and midgut. The sporogonial plasmodium divided by multiple divisions into up to 64 uninucleate sporoblasts. These stages were surrounded outside the plasmalemma by a thick, amorphous dense coat and transformed into a merontogenetic sporophorous vesicle within which the sporonts developed into sporoblasts. The mature microsporidian spores were broadly ellipsoidal and measured $6.1 \pm 0.43 \times 3.1 \pm 0.15$ μm. The spore wall consisted of a transparent endospore (~100 nm) and a thin electron dense exospore (~30 nm) with the outer limit slightly undulated. Spores contained a polar filament arranged peripherally in a single layer of eight to nine wide anterior coils (~125 nm diameter), and three to four narrow posterior coils (~70 nm diameter). Transverse sections revealed a concentric layer organization with the internal layer surrounded by numerous (up to 25) longitudinal microfibrils. The angle of tilt of the polar filament was about 65–68°.

Keywords: Life cycle; *Lutzomyia longipalpis*; Microsporidia; Phlebotominae; *Vavraia lutzomyiae* n. sp.; Ultrastructure

Introduction

In several countries numerous genera of microsporidia infect mosquitoes and other Diptera (Becnel and Andreadis 1999; Becnel and Fukuda 1991; Becnel and Sweeney 1990; Garcia and Becnel 1994; Kettle and Piper 1988; Lainson et al. 1977; Larsson 1999; Pell and Canning 1992, 1993; Sprague et al. 1992). Some

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phlebotomine species are major vectors of visceral leishmaniasis, particularly in Latin America (Costa et al. 1990; Gontijo et al. 1995; Lawyer 1984; Soares and Turco 2003; Vexenat et al. 1994; Ward and Killick-Kendrick 1974). Currently, there are about 70 genera of microsporidia that have insects as their type host, and among them, about 40 genera have been described as infecting dipteran hosts (Becnel and Andreadis 1999; Garcia and Becnel 1994; Hazard and Savage 1970; Larsson 1994). Of these, only a few have been reported from phlebotomine sandflies (Becnel and Andreadis 1999; Lainson et al. 1976, 1977; Lawyer 1984; Sprague et al. 1992).

Microsporidian infections in sandflies have mainly been reported from Brazil (Canning 1977). Descriptions without species identification, figures or drawings have included an unidentified microsporidian in the midgut of *Psychodopygus lainsoni* (Psychodidae) (Ward and Killick-Kendrick 1974), some developmental stages and spores of a microsporidian in the Malpighian tubules or midgut of *P. complexus* (Lainson et al. 1977) and a microsporidiosis in a species of *Lutzomyia* (Lainson et al. 1976). From phlebotomine sandflies, some life cycle stages of a *Thelohania*-like parasite were found in *Psychodopygus maripaensis* (Canning 1977) and massive infections by an unidentified microsporidian were found in female *Lutzomyia diabolica* from Texas (Lawyer 1984). Only one identified microsporidian species has been described from a sandfly, this is *Flabelliforma montana*, infecting *Phlebotomus ariasi* (Psychodidae) in France (Canning et al. 1991). No other references to microsporidians infecting sandflies were found.

Light and transmission electron microscopy (TEM) have been used to examine the development and the spores of a new microsporidian infecting the phlebotomine sandfly *Lutzomyia longipalpis*. This is the first ultrastructural record of microsporidiosis in this phlebotomine species from an endemic leishmaniasis area of South America.

Material and methods

Phlebotomine larvae, pupae and adults of a wild population of the sandfly, *L. longipalpis* Lutz and Neiva, 1912 (Diptera, Psychodidae, Phlebotominae), vector of visceral leishmaniasis due to *Leishmania chagasi*, were collected, during a survey project for leishmaniasis, between the Paty and Parnaíba rivers, near Teresina city (05°05′13″S, 42°48′41″W), Piauí State, Brazil. The phlebotomine sandflies were collected from a natural population, some of which were identified as infected sandflies by the presence of small, externally visible, whitish spots. The infection was primarily observed in the subcuticular region of the abdominal segments of some larvae and pupae and, in few cases, in the Malpighian tubules of adults. In a few cases, in more advanced infection stages, the microsporidia were found in the midgut and other surrounding organs. In continuous laboratory cultures similar whitish spots were observed in only a few larvae and pupae.

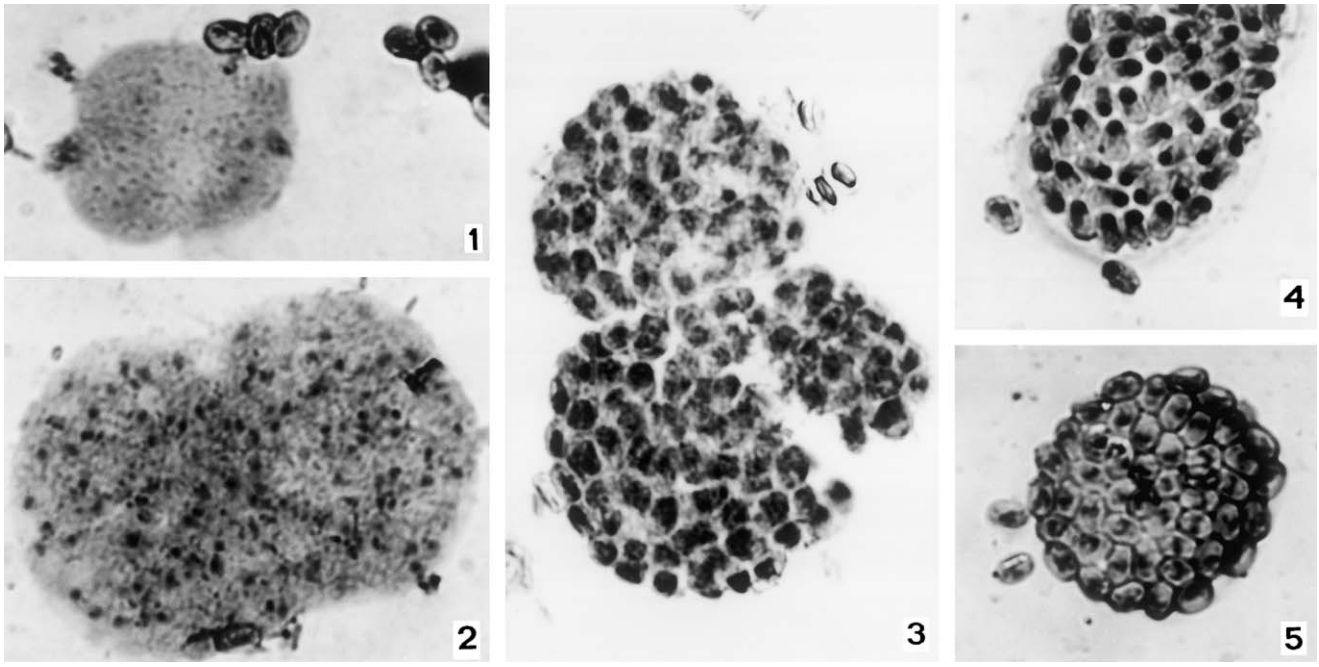
The whitish spots observed by light microscopy (LM) were found using differential interference contrast (DIC) optics to contain spores; so smears of relevant tissues were fixed in methyl alcohol and stained with Giemsa. For TEM the infected organs were fixed in 3% glutaraldehyde buffered with 0.2 M sodium cacodylate buffer (pH 7.2) at 4 °C for 24 h, held overnight at 4 °C in the same buffer, postfixed in buffered 2% OsO_4 at 4 °C for 3 h, dehydrated in an ascending series of ethanol, propylene oxide (three changes each for 2 h) and embedded in Epon. Semithin sections for LM were stained with methylene blue-Azur II, and the ultrathin sections were double-stained with uranyl acetate and lead citrate, observed and photographed with a JEOL 100CXII TEM, operated at 60 kV.

Results

Light microscopy

During routine examination of larvae, pupae and adults of the phlebotomine sandfly, *L. longipalpis* by DIC, several vesicles with microsporidian spores and early developmental stages of microsporidians were seen at sites visible externally on the hosts as small whitish spots. In the larvae and pupae the whitish spots were located internally in the abdominal segments (Fig. 1), while in the adults these spots were located in the Malpighian tubules and midgut. In the sample taken from the wild population, three out of 20 larvae, five out of 25 pupae and two out of 30 adult hosts were parasitized, giving a prevalence of 13.3%. It was observed that, after the appearance of the whitish spots, the parasitized specimens became less mobile and died after 1–2 days. The sex of the parasitized larvae and pupae was not determined.

The number of nuclei in the plasmodia was variable in Giemsa-stained preparations (Figs. 1 and 2). Multiple divisions of plasmodial stages produced a variable number of up to 64 uninucleate spores within subpersistent parasitophorous vesicles that had diameters up to 25 μm (Figs. 3–6). Early life cycle stages were observed in some vesicles. As the vesicles were easily ruptured, many free spores were observed (Figs. 1, 4 and 5). Isolated fresh spores, observed after rupture of the vesicles, were ellipsoid, 6.1 ± 0.43 μm long, and 3.1 ± 0.15 μm wide ($n = 50$) (Figs. 7 and 8). Macrospores were never observed.



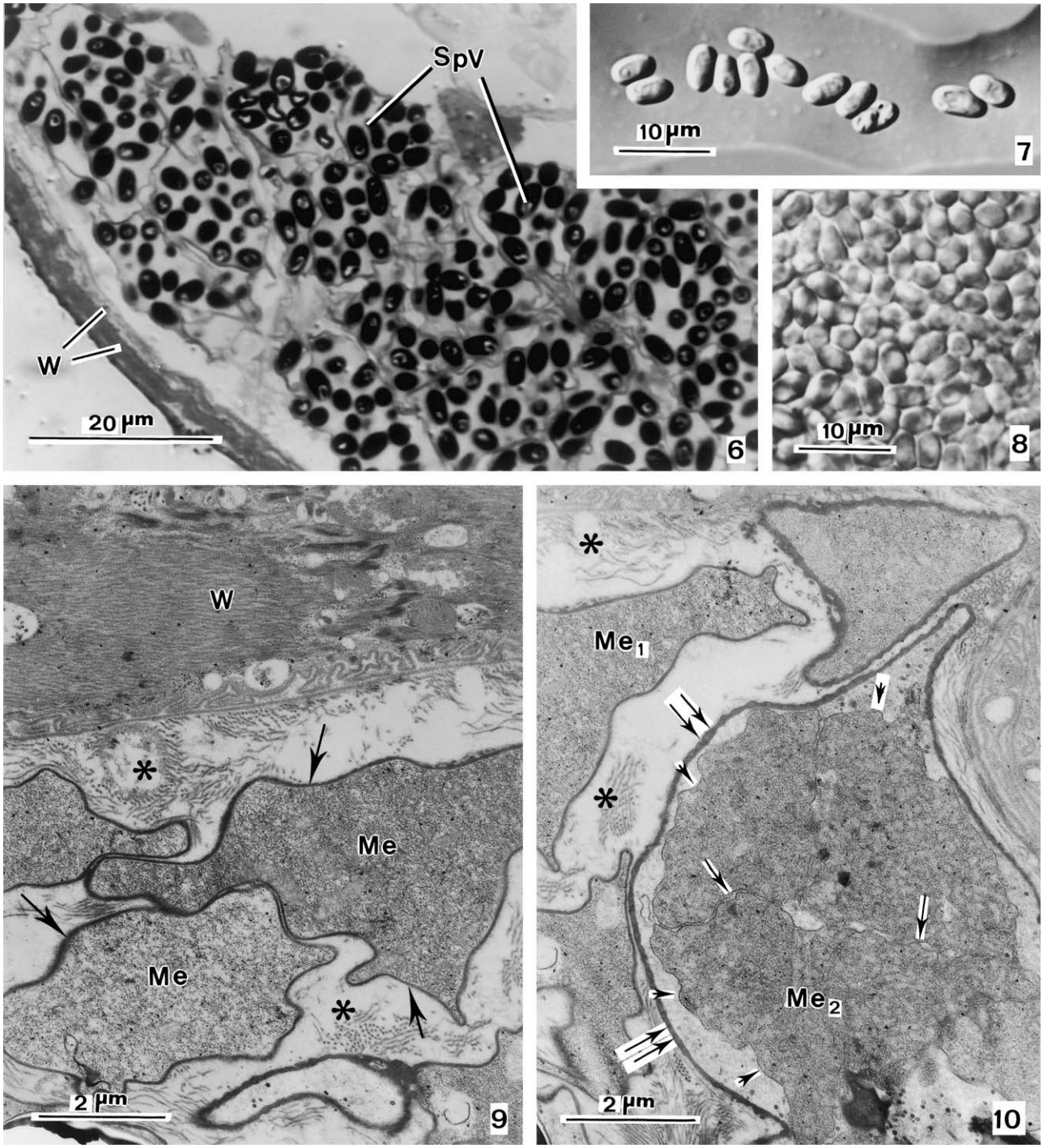
Figs. 1–5. Developmental stages of *Vavraia lutzomyiae* n. sp. as seen in Giemsa-stained smears observed in DIC. All magnifications; × 1525. **1, 2**: Sporogonic plasmodia with numerous nuclei. **3, 4**: Two sequential phases of the uninucleated spore maturation. **5**: A group of mature spores, possibly surrounded by the parasitophorous vesicle.

Transmission electron microscopy

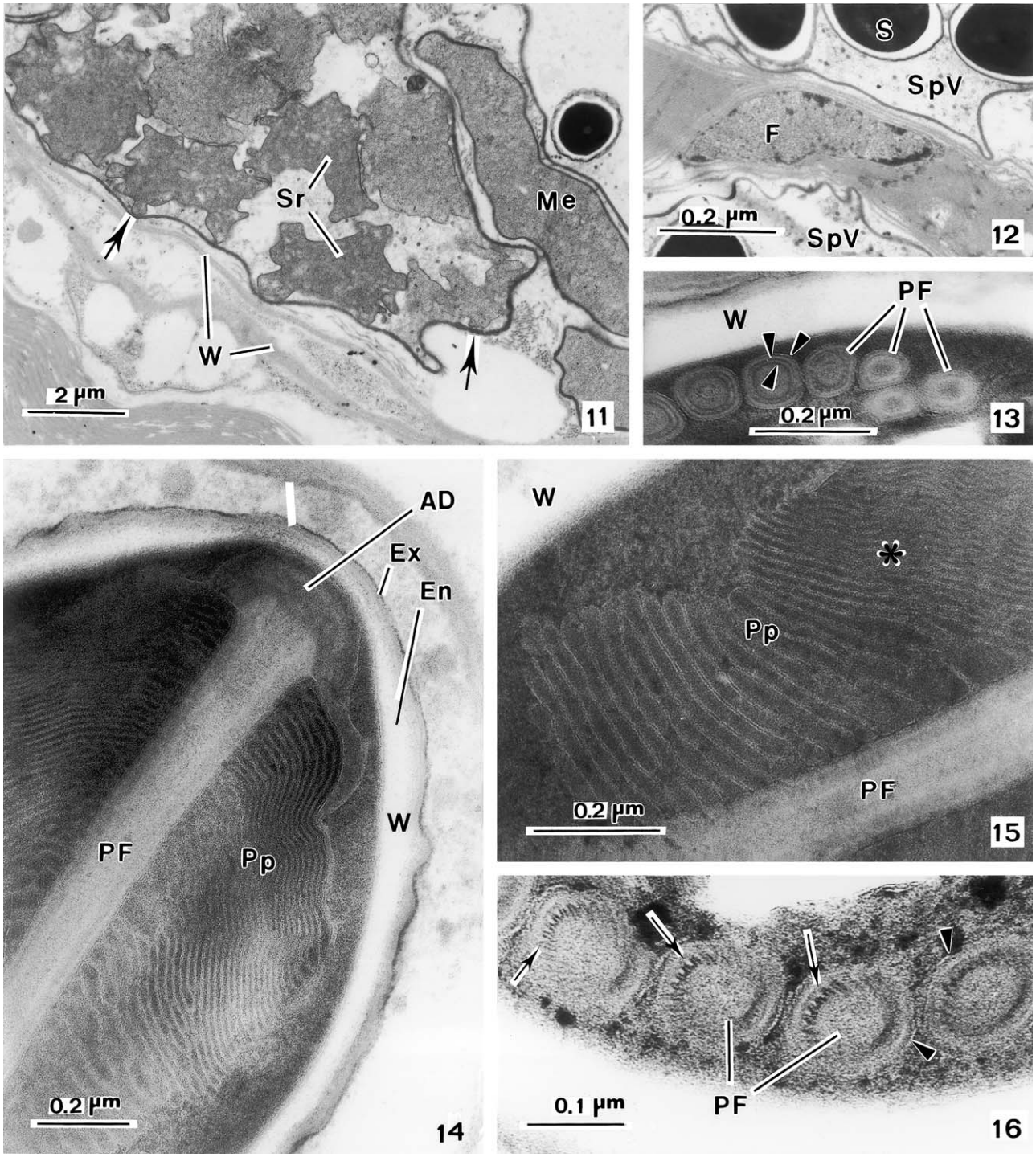
Among adult phlebotomine sandflies infection with microsporidia was observed only in females, and *L. chagasi* was never observed in hosts parasitized with microsporidia. Some groups of cells representing different life cycle stages, meronts (Fig. 9), dividing meronts (sporonts) (Fig. 10), sporoblasts (Fig. 11), and numerous vesicles containing spores (Figs. 6 and 12) were observed by TEM. The meronts and dividing meronts (sporonts) with irregular contours had uniform cytoplasm but their nuclear organization was hardly visible (Figs. 10 and 11). All these stages were surrounded by thick and amorphous dense coats external to the plasmalemmas (Figs. 9 and 10). These structures gradually gave rise to the merontogenetic sporophorous vesicles (MSV) by detachment of the dense external coat from the plasmalemma of the parasite (Fig. 10). During this dividing process the multinucleate cells became sporonts (Fig. 10), which gave rise to a variable number of sporoblasts (Fig. 11). The nucleus had a similar electron density to the surrounding cytoplasm. This process gave rise to the MSV containing numerous spores (Fig. 11). Some fibroblasts (Fig. 12) were present among the MSV.

Mature spores were ellipsoidal and possessed all the classic microsporidian structures. The spore wall consisted of a transparent endospore (100 nm thick), thinning to 45 nm over the anchoring disk, and a thin electron dense exospore (30 nm thick), the outer limit of which was slightly undulated (Fig. 14). The polaroplast, which nearly filled the anterior third of the spore, consisted of two membranous systems. The anterior region contained closely packed lamellae and the posterior region more widely spaced lamellae (Figs. 14 and 15). The polar filament, connected anteriorly to a biconvex anchoring disc with bands of differing density, formed eight to nine wide anterior coils, about 125 nm in diameter, and three to four narrow posterior coils, about 70 nm in diameter (Figs. 13 and 16). The anterior coils were arranged in a single layer and the posterior coils in a single or double layer. Transverse sections of the polar filament showed three concentric layers, the internal layers surrounded by numerous (up to 25) microfibrils positioned side by side and slightly obliquely to the polar filament axis (Fig. 16). The angle of tilt between the anterior filament coil and the longitudinal axis of the spore was 65–68°. The nucleus, which was barely visible, was located near the center of the spore, between the polaroplast and the posterior vacuole. The posterior vacuole was filled with dense granular material intermingled with some light areas. Ultrastructural details of the spore were schematically drawn using the information from serial ultrathin sections (Fig. 17).

Based on the morphological and ultrastructural aspects of the spores and host specificity, we propose to establish a new species according to the differences found in relation to the previously described species and



Figs. 6–10. Light and electron micrographs of *Vavraia lutzomyiae* n. sp., from the phlebotomine sandfly, *Lutzomyia longipalpis*. **6**: Semithin section of a larva at the abdominal region level showing several sporophorous vesicles (SpV) containing numerous spores near the subcuticular wall (W) observed in DIC. **7**: Twelve free fresh spores observed in DIC. **8**: A smear of numerous fresh spores observed in DIC. **9**: Ultrathin section of the periphery of the subcuticular wall (W) of a pupa formed by numerous dense fibers and, more internally, meronts (Me) with a thick wall (arrows). Among the meronts several collagen fibers (*) are present. **10**: Ultrathin section of meronts (Me_1), the initial phase of separation of dividing meront (sporont) (Me_2) membranes (arrows), and the separation of the sporont membranes (arrowheads) from the merontogenetic sporophorous vesicle (double arrows). Numerous collagen fibers (*) surround the parasites.



Figs. 11–16. Ultrastructural aspects of the sporophorous vesicles and spores of the microsporidian *Vavraia lutzomyiae* n. sp. from phlebotomine sandfly, *Lutzomyia longipalpis*. **11**: A merontogenetic sporophorous vesicle, located in the subcuticular region of the abdomen of a larva, containing several sporoblasts (Sr). A meront (Me) is nearby. **12**: Ultrastructural aspects of two adjacent sporophorous vesicles (SpV) containing spores (S). Between them a fibroblast (F) and numerous groups of collagen fibers are observed. **13**: Ultrathin section of the spore wall (W) and polar filament (PF) sectioned at different levels, showing two different diameters. The PF shows the three concentric membranous layers (arrowheads). **14**: Ultrathin section of the apical region of a spore showing the spore wall (W) (Ex – exospore; En – endospore), the anchoring disc (AD) and attachment of the polar filament (PF) surrounded by the polaroplast (Pp). **15**: Detail of the polaroplast (Pp) showing the two membranous systems composed anteriorly of closely packed lamellae (*) and posteriorly of more widely spaced lamellae. The transverse section of the spore wall (W) and longitudinal section of the anterior part of the polar filament (PF) are visible. **16**: Ultrathin transverse section of the polar filament (PF) showing the internal longitudinal translucent microfibril organization (arrows). The concentric membranous layers of the PF (arrowheads) are sectioned obliquely.

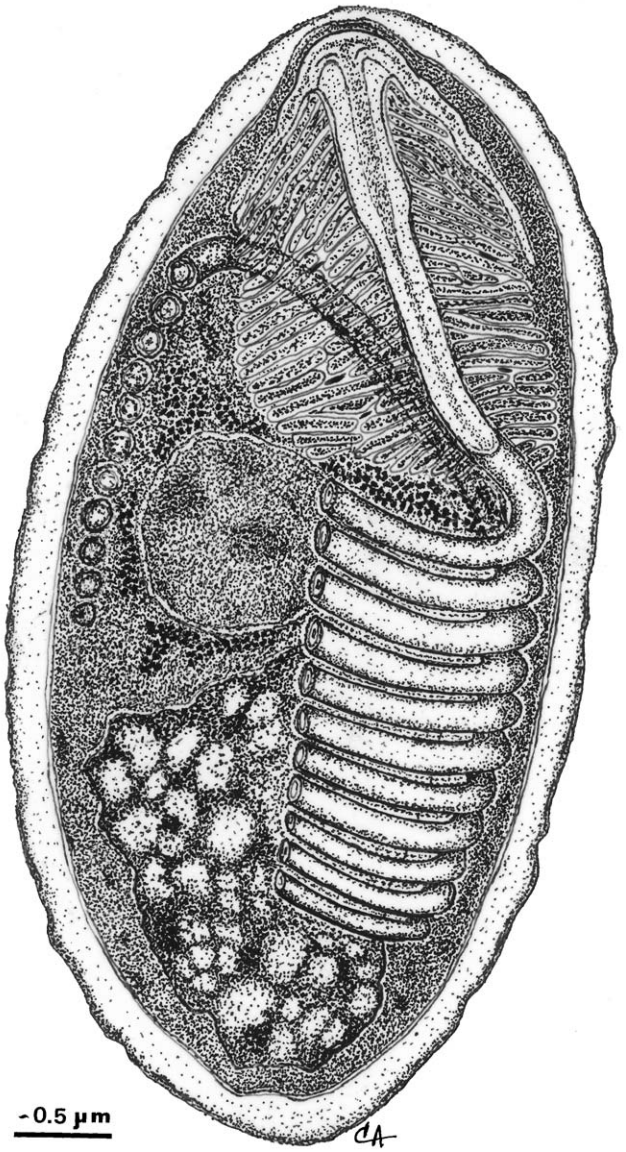


Fig. 17. Schematic drawing of the spore of the microsporidian *Vavraia lutzomyiae* n. sp., derived from micrographs of ultrathin serial sections as described in the text and illustrated in electron micrographs.

according to the classification proposed by Sprague, Becnel and Hazard (1992).

Diagnosis

Phylum Microspora Sprague, 1969
Class Haplophasea Sprague, Becnel & Hazard, 1992
Order Glugeida Issi, 1989
Family Pleistophoridae Doflein, 1901
Genus *Vavraia* Weiser, 1977.

Vavraia lutzomyiae n. sp. (Figs. 1–17)

Specific characters: Uninucleate ellipsoidal spores of only one spore type, $6.1 \pm 0.43 \times 3.1 \pm 0.15$ µm; spore wall with thin (30 nm), slightly undulated, external layer of moderate density, and thick (~100 nm) transparent endospore; polaroplast lamellate, bipartite, fills anterior third of spore, its apical part with packed lamellae and posterior lamellae more widely spaced; polar filament with eight to nine wide anterior coils in a single layer and three to four narrower posterior coils in a single or double layer, at all levels filament wall has three concentric layers of which the internal one is surrounded by numerous (up to 25) microfibrils positioned regularly side by side and slightly obliquely to the polar filament axis; angle of tilt of the anterior part of the polar filament 65–68° to spore axis.

Type host: Phlebotomine sandfly, *L. longipalpis* Lutz and Neiva, 1912 (Diptera, Psychodidae, Phlebotominae).

Locality: Region between the Paty and Parnaíba rivers near the city of Teresina (5°5′13″S, 42°48′41″W), Piauí State, Brazil.

Pathogenic activity: All life cycle stages developed in sporophorous vesicles in the abdominal segments of the larvae and pupae and in the Malpighian tubules and midgut of the adult sandflies with macroscopic external signs, characterized by some small whitish spots; death of all parasitized hosts occurred 1–2 days after the appearance of the whitish spots.

Type specimens: One glass slide with syntype and one glass slide with semithin sections of the merontogenetic sporophorous vacuoles containing spores and earliest life cycle stages were deposited in the International Protozoan Type Slide Collection, National Museum of National History, Washington, DC 20560, USA (USNM no. 1076955).

Etymology: The specific name derives from the generic name of the host species.

Discussion

A wide variety of parasite organisms have been described in the dipteran phlebotomine sandfly *L. longipalpis*, ranging from viruses to helminths. While numerous microsporidia are associated with mosquitoes (Becnel and Andreadis 1999) and other dipterans (see Introduction), few phlebotomine sandflies are known to host microsporidians (Canning et al. 1991). Our data form the first and only ultrastructural report of a microsporidian species parasitizing *L. longipalpis*.

Based on the ultrastructural morphology of the spore and the developmental stages of the microsporidian described here, we consider that this parasite is a species of the family Pleistophoridae Doflein, 1901 (Canning et al. 1991; Sprague et al. 1992). Among the different genera of this family, only *Pleistophora, Flabelliforma, Trachipleistophora* and *Vavraia* develop the MSV from

the surface coat during merogony, as we observed in the present study. The genus *Pleistophora* comprises parasites in vertebrates and is equivalent to the genus *Vavraia*, whose species are parasites in invertebrates (Canning et al. 1991; Larsson 1999). *Flabelliforma* has fan-shaped plasmodia, crenated sporoblasts and laterally curved pyriform spores, having as host a phlebotomine sandfly of the same family (Psychodidae) (Canning et al. 1991) as that to which the parasite described in the present work belongs. *Trachipleistophora* and *Vavraia* exhibit several morphological differences. In sporogony of *Trachipleistophora* the MSV encloses an uninucleate sporont that undergoes repeated binary fission (Hollister et al. 1996), while *Vavraia* develops from a multinucleate merogonial plasmodium, which then becomes a sporogonial plasmodium by separation from the MSV and then undergoes multiple fission, as observed in the present study.

Although the development and ultrastructural organization of some species of the genus *Vavraia* which produce two spore types (macro and microspores), e.g. *V. anostraca* (Martinez et al. 1992), is similar to that of the present species, we have never observed more than one spore type in this study, and only one spore type has been reported in some other species of this genus, e.g. *V. mediterranica* (Azevedo 2001). On the other hand, *V. culicis* is described as producing, sometimes one type (Sprague et al. 1992) and sometimes two types of spores (Diarra and Toguebaye 1991).

Our results, especially those regarding the development of the MSV, are similar to the morphology and ultrastructural data previously described for different species of the genus *Vavraia* (Azevedo 2001; Becnel et al. 2005; Diarra and Toguebaye 1991; Langdon 1991). This seems a strong argument to place this parasite in the genus *Vavraia* Weiser, 1977, as re-defined by Canning and Hazard (1982). Our results compared with the previously described species of *Vavraia* present some ultrastructural differences. The polar filament with eight to nine wide anterior coils arranged in a single layer and three to four narrow posterior coils, as observed in the present study, is an organization never observed in other species of this genus. In addition, the spore size, internal organization, mainly of the polaroplast structure, and the host specificity, justify the establishment of the new species *V. lutzomyiae*. This study represents the first ultrastructural description of microsporidia from a phlebotomine sandfly in Brazil.

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