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Nosema scripta N. Sp. (Microsporida: Nosematidae), a Microsporidian Parasite of the Cottonwood Leaf Beetle, *Chrysomela scripta* (Coleoptera: Chrysomelidae)[1]

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ABSTRACT. *Nosema scripta* (Microsporida: Nosematidae), a new species of microsporidian parasite, is described from the cottonwood leaf beetle, *Chrysomela scripta* F. (Coleoptera: Chrysomelidae), in North America. Studies using light and electron microscopy reveal that this species completes its life cycle in direct contact with the cytoplasm of cells within the fat body, midgut, hindgut, muscles, central nerve cord, Malpighian tubules, tracheal end cells, and ovaries. The microsporidium is monomorphic, all life stages are diplokaryotic, and spores develop from disporous sporonts. The mature spores are broadly oval and measure 4.2 ± 0.10 × 3.4 ± 0.04 μm. Spores contain a tubular polar filament that is arranged peripherally in a single layer of 13–15 coils. Both horizontal transmission and vertical transmission have been demonstrated for this microsporidium in *C. scripta* in the laboratory. The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), was susceptible to infection with this pathogen in cross-infectivity studies.

Supplementary key words. Chronic disease, cross-infectivity, *Leptinotarsa decemlineata*, microsporidian ultrastructure, *Populus*, transovarial transmission.

THE cottonwood leaf beetle (CLB), *Chrysomela scripta* F. (Coleoptera: Chrysomelidae), is a native defoliator of cottonwood and poplar (*Populus* spp.) throughout North America. The cottonwood leaf beetle, a multivoltine species with as many as seven generations per year in the southern United States, is considered a major pest in plantations and nurseries. No pathogens are known from the CLB, although CLB is susceptible to *Bacillus thuringiensis* [2]. Population regulation is believed to result primarily from predators and parasitoids [3, 5, 6].

Adult CLB were shipped to our laboratory from Dr. E. R. Hart's laboratory and field plots at Iowa State University, Ames, Iowa over the last few years. These beetles were noticeably smaller than our laboratory-colony adults, and displayed symptoms of chronic disease that included reduced fecundity and adult longevity. Microscopic examination revealed the presence of massive infections of adults and larvae with microsporidian spores.

This paper describes the pathology and taxonomic status of a previously unknown microsporidium of CLB, using light and electron microscopy. The results of cross-infectivity studies with two other chrysomelid species, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and the imported willow leaf beetle, *Plagiodera versicolora* (Laicharting), are also reported.

MATERIALS AND METHODS

Colonies of CLB are maintained in our laboratory on poplar foliage according to the methods described by Bauer [2]. A naturally infected colony of beetles was maintained for several generations for transmission electron microscopy (TEM) and for studies on tissue specificity and pathogen transmission.

Tissue specificity was determined after dissecting and washing individual tissues, followed by observations of wet mounts for the presence of spores using the compound microscope (Leitz, ×400). In addition, observations were also made of semi-thick sections cut with a glass knife from the TEM blocks. Mode of vertical transmission was determined by observing the presence of spores in neonate larvae, eggs, and embryos at various stages of maturation with a compound microscope (×400).

Infected larvae were obtained for Giemsa-stained smears by inoculating individual second-instar larvae by placing spores on small leaf discs. Fresh spores were purified from infected adults by homogenization, filtration, and density gradient centrifugation. Spores remained viable for at least two years when refrigerated (6 ± 2° C) or when frozen at −20° C or in liquid nitrogen.

For electron microscopy, infected tissue was dissected directly into cold 2.5% (v/v) glutaraldehyde in 0.1 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.2) under a dissecting microscope. The tissue segments (ca. 2 mm long), were placed in individual vials of the cold glutaraldehyde solution and fixed overnight. Following a buffer wash, the segments were postfixed for 1 h at room temperature in 1% OsO_4 (w/v) in the same buffer as above. The segments were then dehydrated through an ethanol series, treated with propylene oxide, and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin sections were cut with a diamond knife mounted on an Ultratome III (LKB Instruments, Inc., Rockville, MD), stained with uranyl acetate and lead citrate, and examined with an electron microscope (CM-10; Philips Electronic Instruments, Co., Mahwah, NJ).

Giemsa-stained smears were prepared from second- and third-instar larvae at various intervals after inoculation. These smears were prepared by dissecting infected tissues, gently drying the tissue before smearing on a slide, allowing the slides to air dry, and fixing in methanol for 5 min. After drying, the slides were stained for 10 min in a 10% (v/v) solution of Modified Romanowsky's Giemsa stain (Fisher Chemical Co., Chicago, IL) diluted in pH 7.4 buffer, rinsed in tap water, and allowed to air dry. The slides were later observed under the compound microscope (×900, oil), and selected life stages were measured and photographed with a Zeiss Laser Scanning Confocal Microscope (Carl Zeiss, Inc., Thornwood, NY).

Fresh spores were measured using a micrometer on the compound microscope (n = 20), and confirmed using the image area measurement capabilities of a Zeiss Laser Scanning Confocal Microscope (n = 10).

Purified spores were used for cross-infectivity studies. Second-instar larvae of Colorado potato and imported willow leaf beetles were inoculated on their respective host plant foods (potato and willow) as described for CLB inoculations. Infection was confirmed by the presence of spores observed with the compound microscope.

RESULTS

Symptomology, pathology, and transmission. No overt behavioral or morphological signs or symptoms were observed in newly infected CLB. However, after several generations in the

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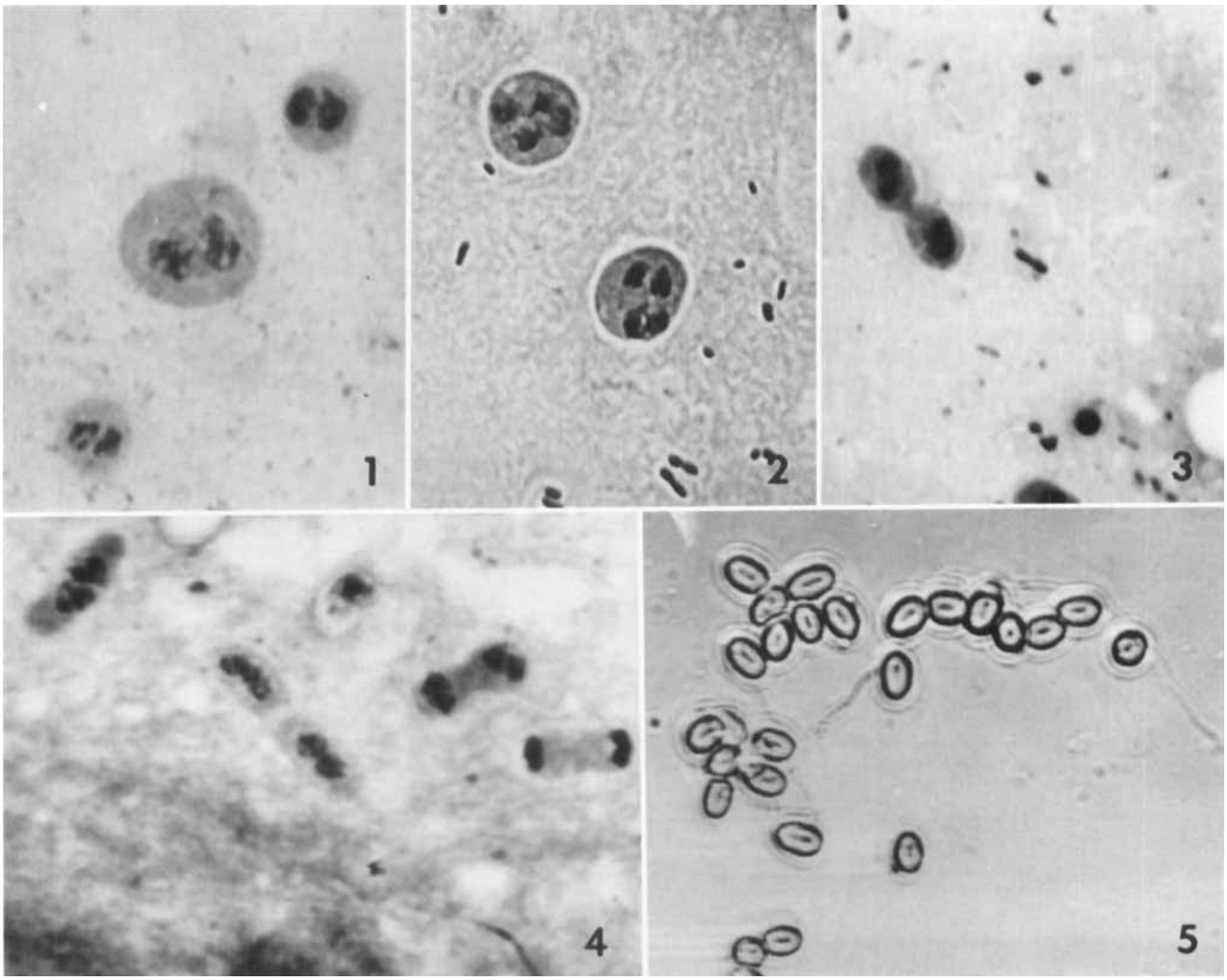

Fig. 1–5. Life stages of *Nosema scripta* as seen in Giemsa-stained smears (1–4) and wet mount (5). **1**. Diplokaryotic meronts. ×2,300. **2**. Meronts undergoing karyokinesis. ×1,800. **3**. Ovoid, diplokaryotic sporonts undergoing binary fission. ×2,300. **4**. Sporonts producing sporoblasts, maturing spores. ×2,300. **5**. Fresh spores. ×1,452.

laboratory, individuals surviving to the adult stage were smaller, produced fewer eggs, and lived shorter lives compared to healthy cohorts (LSB, unpubl. observ.). There was a dose-dependent increase in egg, larval, and pupal mortality in populations infected with this pathogen.

Observations using light and electron microscopy showed that in naturally infected late-stage larvae and adults most tissues were infected, including body fat, midgut, hindgut, muscles, central nerve cord, Malpighian tubules, tracheal end cells, and ovaries. The microsporidium was transmitted transovarially, as confirmed by observations of spores within late-stage embryos. In addition, eggs with no visible embryonic development and many neonates also contained large numbers of spores. Our chronically infected colony of CLB, maintained in the laboratory for 7 mo, experienced ca. 90% transovarial transmission efficiency.

Light microscopy. Giemsa-stained meronts were spherical, diplokaryotic, and ranged in size from 6 to 12.5 μm (Fig. 1). Many merogonic forms (ca. 6.5 μm) were observed producing two daughter cells by karyokinesis and contained two diplokarya (Fig. 2). Nuclei were centrally located within the cells, stained unevenly, and were spherical or oppressed laterally in shape.

Sporonts were ovocylindrical and diplokaryotic, and many were observed undergoing binary fission (Fig. 3). Observations of Giemsa-stained smears suggest that spores were produced from disporous sporonts. Sporoblasts were diplokaryotic, ovoid, with lightly stained cytoplasms (Fig. 4).

Mature spores were broadly oval, measuring 4.2 ± 0.10 ×

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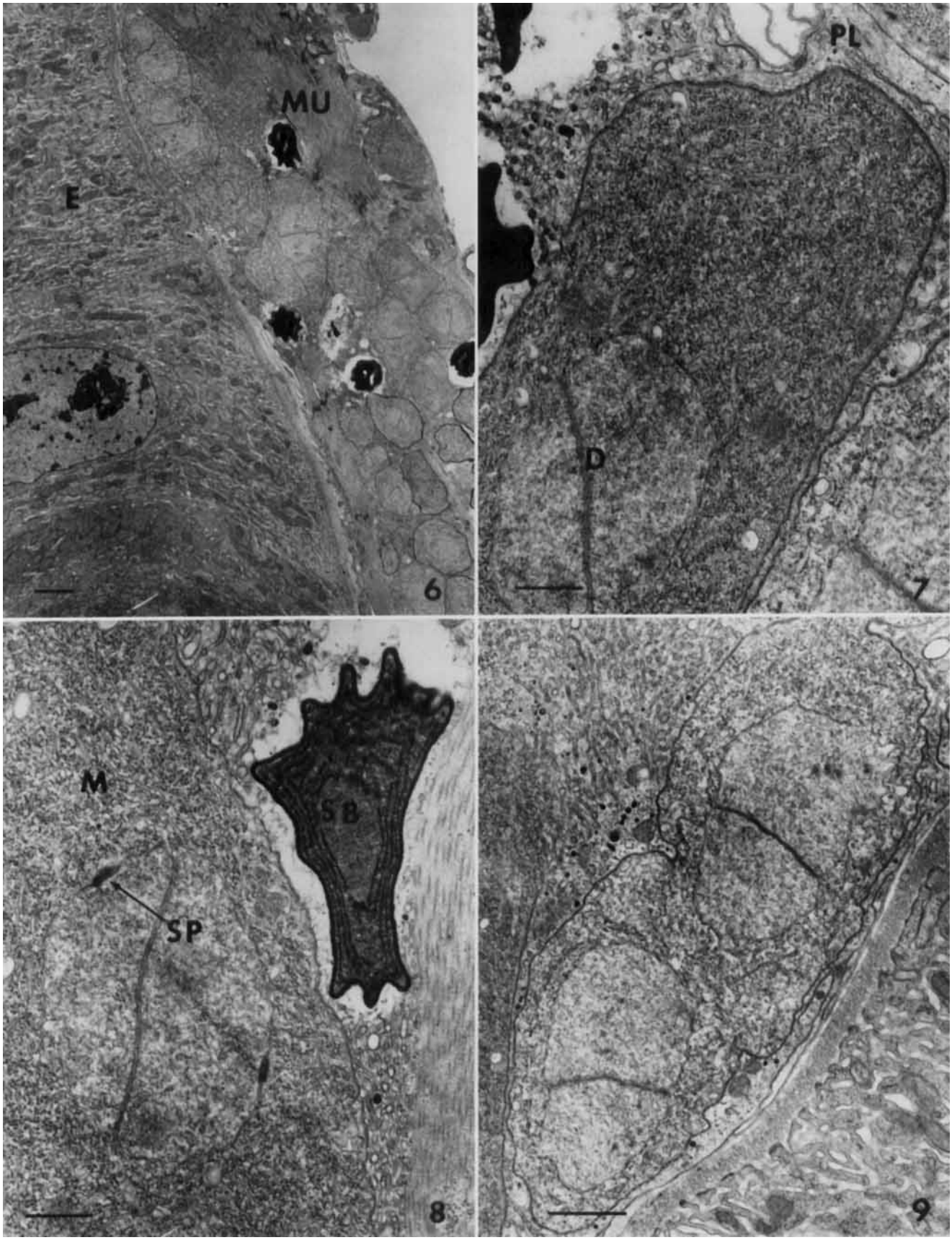
Fig. 6–9. Electron micrographs of the developmental stages of *Nosema scripta*. **6**. Overview of life stages in muscle (MU) surrounding midgut epithelium (E). Bar = 2 μm. **7**. Meront with diplokaryon (D), simple plasmalemma (PL), rough endoplasmic reticulum, ribosomes, and small vesicles. Bar = 0.5 μm. **8**. Meront (M) with spindle plaques (SP) and sporoblast (SB) with peripherally aligned rough endoplasmic reticulum. Bar = 0.5 μm. **9**. Meront undergoing mitosis and cytokinesis with extracellular vesicles shown at higher magnification in Fig. 10. Bar = 1.0 μm.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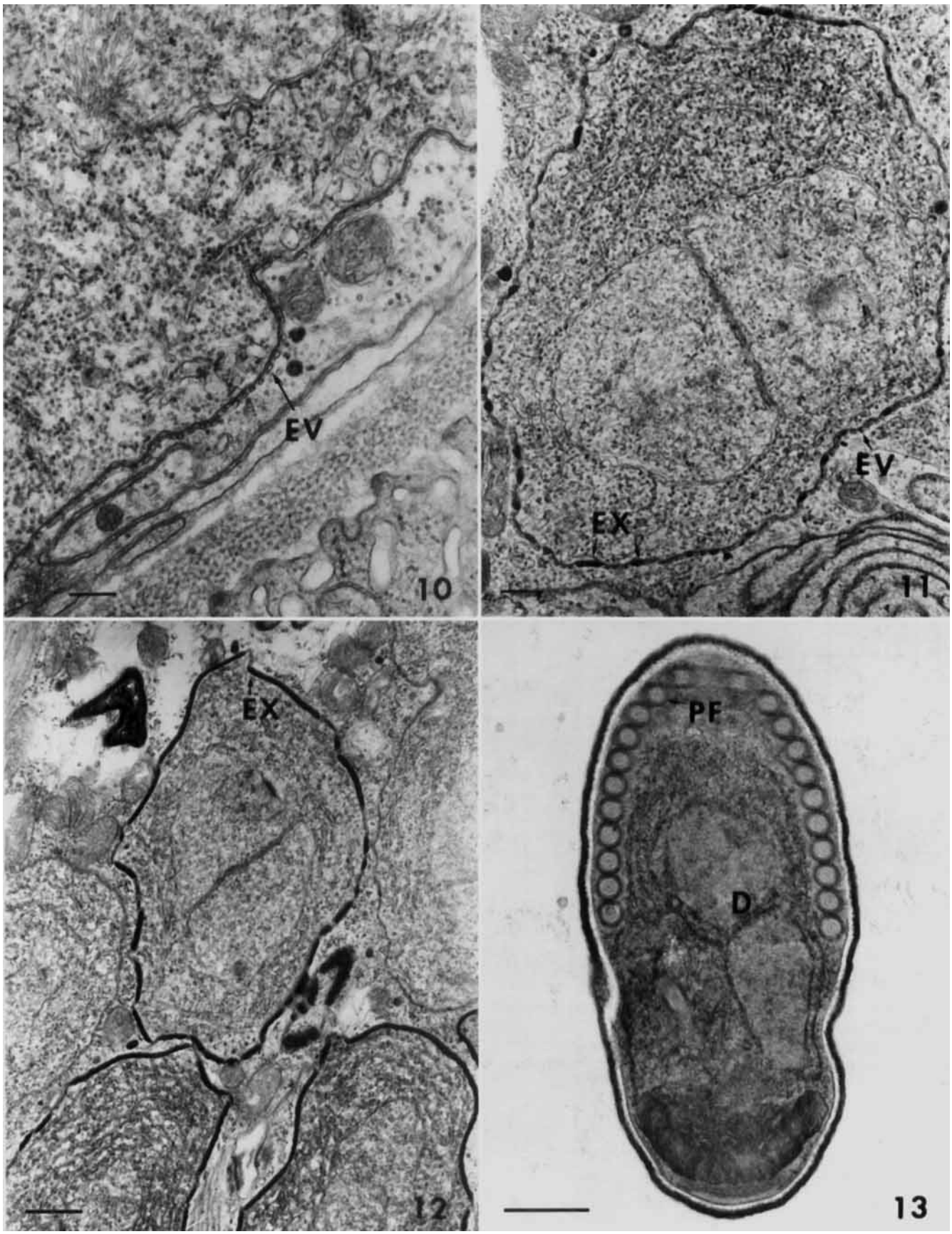
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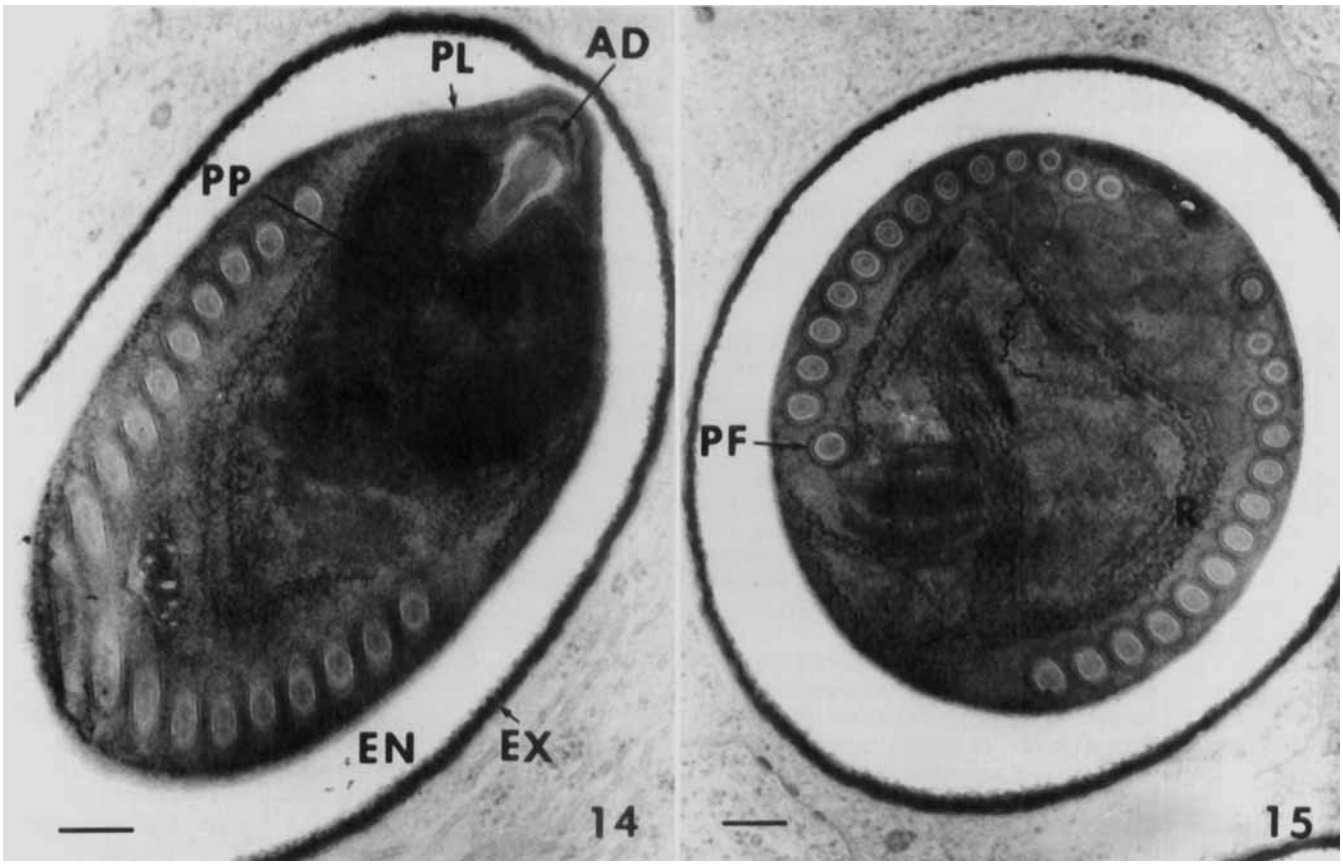

Fig. **14, 15**. Electron micrographs of mature spores. Mature spore with thin plasmalemma (PL), thick electron-transparent endospore (EN), electron-dense exospore (EX), anchoring disc (AD), and polarplast region (PP). Bar = 0.2 μm. **15**. Mature spore with tapering, isofilar polar filament (PF), and organized ribosome region (R). (13–15 coils.) Bar = 0.2 μm.

3.4 ± 0.04 μm in wet-mount preparations (Fig. 5). Polar filaments extruded readily from mature spores under the cover slip as wet mounts began to dry down.

Transmission electron microscopy. All developmental stages observed were diplokaryotic and developed simultaneously within the cytoplasm of individual cells (Fig. 6). Meronts were spherical to ovoid, bounded by a plasmalemma which forms elongate membraneous protrusions, forming a high-surface area interface with the host cytoplasm (Fig. 7, 8). The granular cytoplasm contained rough endoplasmic reticulum (RER), small endoplasmic vesicles and vacuoles, and abundant free ribosomes. All nuclei were paired as diplokarya. The nucleoplasm also appeared granular, although slightly less electron dense than the cytoplasm. Spindle plaques formed adjacent to the nuclear envelope (Fig. 8), and remained closely associated with the nuclear membrane as spindle microtubules formed during mitosis. The rapid proliferation of vegetative stages was demonstrated by the abundance of multinucleate meronts observed undergoing mitosis and cytokinesis simultaneously (Fig. 9). Regularly-spaced extracellular vesicles were observed in intimate association with the plasmalemma of meronts during cytokinesis (Fig. 10).

Small extracellular vesicles were also observed surrounding sporonts, with associated primordial exospore material appearing as intermittent electron-dense regions, (Fig. 11). In maturing sporonts these regions expanded and coalesced into thickened zones, forming the exospore that surrounded the cell (Fig. 12). The RER in late-stage sporonts was more highly organized into a linear pattern around the single diplokaryon. Sporoblasts, crenate in outline, were thick-walled and highly electron dense, and they contained peripherally aligned RER (Fig. 8).

Spores were broadly oval and diplokaryotic (Fig. 13). The mature spore had the typical trilaminar spore wall that included a thin plasmalemma, thick electron-transparent endospore, and electron-dense exospore (Fig. 14). The spore cytoplasm contained what appeared to be membrane-associated ribosomes arranged peripherally around the diplokaryon and a lamellar polarplast region (Fig. 14, 15). The slightly tapering polar filament was isofilar, with 13–15 turns coiled in a single layer close to the plasmalemma (Fig. 15).

Cross-infectivity. This microsporidium readily infected Colorado potato beetles (CPB) following inoculation as second-stage larvae. Viable spores were produced in similar tissues as reported for CLB, although infected midgut epithelial cells were

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Fig. **10–13**. Electron micrographs of *N. scripta*, developmental stages, cont'd. **10**. Extracellular vesicles (EV) characteristic of meronts and young sporonts. Bar = 0.2 μm. **11**. Maturing sporont with extracellular vesicles (EV) and small regions of primordial exospore (EX). Bar = 0.2 μm. **12**. Maturing sporont with expanding regions of exospore (EX). Bar = 0.5 μm. **13**. Immature spore with diplokaryon (D) and coiled polar filament (PF). Bar = 0.5 μm.

more common in CPB. Unlike CLB, individuals that survived to the adult stage had very low levels of infection, and these adults successfully transmitted this pathogen to progeny. Although vertical transmission was confirmed to progeny, most of the infected progeny died as neonates. We were not able to confirm that transovarially infected larvae survived to the adult stage.

No evidence was obtained to indicate that this microsporidium was infectious to the imported willow leaf beetle.

DISCUSSION

The ultrastructural characteristics and developmental cycle of this previously unknown microsporidium support its inclusion in the genus *Nosema* Naegeli, 1857. These generic criteria, as derived from the type species *Nosema bombycis,* are: diplokaryotic and monomorphic life stages that develop in direct contact with host cytoplasm, sporophorous vesicle lacking, merogony binary or multiple, and sporogony disporoblastic [8–10]. The distinctive early spores reported in the type species [7] were not observed in this study, perhaps since electron microscope observations were performed on chronically infected late-instar larvae. We name this microsporidium *Nosema scripta* n. sp. after the type host, *Chrysomela scripta.*

The most prevalent microsporidian genus described from coleopteran hosts is *Nosema,* a large heterogeneous group that primarily infects lepidopterans [9]. According to the most recent summary, 41 species of *Nosema* are known from Coleoptera [12], and ca. one quarter are pathogens of beetles within the family Chrysomelidae [12, 13]. *Nosema scripta* is the first microsporidium formally described from a chrysomelid in North America [12, 16], although an undescribed species was reported from the bean leaf beetle, *Ceratoma trifurcata* [4].

Ordinal specificity is still the primary method used to delineate species of Microsporida [3]. This character, as well as gross morphological characteristics such as spore size and shape, are summarized for previously described microsporidia infecting Coleoptera [3, 11], and more specifically, those infecting Chrysomelidae [16]. However, ultrastructural data are, by far, the most reliable way to distinguish species of microsporidia. Unfortunately, relatively few species have been described using TEM. These data are available for four Old World *Nosema* species described from chrysomelids—*N. galerucellae* [12], *N. couilloudi* [13], *N. birgii* [14], and *N. nisotrae* [15]. Similar data are also available for comparative purposes for the North American species *N. epilachnae* and *N. varivestis* described from the Mexican bean beetle, *Epilachna varivestis* (Coleoptera: Coccinellidae) [3]. After comparisons of these ultrastructural data, we determined one distinguishing character of *N. scripta* was the presence of small vesicles on the plasmalemma of meronts and early stage sporonts. Although somewhat similar structures were described on the sporont plasmalemma surface of *N. varivestis* [3] and *N. algerae* [1], they are more tubular in nature than those observed in *N. scripta.*

Biological control of the CLB has focused primarily on predators and parasites [6], and, more recently, on the use of *Bacillus thuringiensis* as a microbial control agent [2]. Before our study no indigenous pathogen had been described from this species, despite life table studies [6]. In a review of these life table data for four generations of CLB in Wisconsin, only 10 to 25% of the larval and pupal mortality could be explained by predators and parasitoids. The results from our studies on *N. scripta* suggest that this pathogen could be an important regulator of CLB. Additional studies are being done to determine both the geographical extent of this pathogen in North American CLB populations and the incidence in field populations in Iowa (E. Hart, pers. commun.).

The high infectivity and virulence levels of *N. scripta* in Colorado potato beetle suggest its potential as a microbial control agent. However, cost-effective methods needed for mass-rearing obligate intracellular pathogens, such as microsporodia, are not yet available. At present spores are produced in vivo, a method that is most efficient in a large insect host that can be reared on artificial diet (W. Brooks, pers. commun.). We have not explored this option for mass production of *N. scripta* spores. The utility of *N. scripta* as a biological control agent of Colorado potato beetle would be marginal because transovarially infected progeny do not survive to adulthood. Therefore, it is unlikely that an enzootic of *N. scripta* would be sustained in field populations of CPB.

TAXONOMIC SUMMARY

Nosema scripta n. sp.

Type host. *Chrysomela scripta* F. (Coleoptera: Chrysomelidae).

Infection sites. Fat body, midgut, hindgut, muscles, central nerve cord, Malpighian tubules, tracheal end cells, and ovaries.

Development. Merogony and sporogony as for the genus. Life stages diplokaryotic, disporous sporont.

Spore. Spores free, broadly oval with smooth surface, $4.2 \pm 0.10 \times 3.4 \pm 0.04$ μm. Spore polar filaments isofilar, 13–15 coils.

Transmission. Transovarial and per os.

Type locality. Poplar plantations in Ames, Iowa.

Deposition of types. Syntypes were deposited at the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (slide no. A43115, B43116, C43117) and in the collection of LSB.

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Lateromyxa gallica N. G., N. Sp. (Vampyrellidae): A Filopodial Amoeboid Protist with a Novel Life Cycle and Conspicuous Ultrastructural Characters

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ABSTRACT. A large, uni- or multinucleate vampyrellid rhizopod, *Lateromyxa gallica* n. g., n. sp., has been isolated several times from two lakes in central France between September 1973 and August 1989 and cultivated for several months or years under laboratory conditions. No essential variations of feeding behaviour were found over the time; all isolated strains invade the trichomes of the green alga *Oedogonium* and move, divide and encyst inside the vanished plant cells. Penetration is performed by attacking the cross walls and only primary attacks are directed against the lateral cell walls of the algae. These findings contrast with the behaviour, life cycles and fine structure of all known species of the genera *Vampyrella, Hyalodiscus, Arachnula* and *Gobiella*. The establishment of a new genus, *Lateromyxa,* with the type species *Lateromyxa gallica* is therefore proposed.

Supplementary key words. Biological control, feeding behaviour, rhizopods, taxonomy.

ACCORDING to the most recent systems [8, 11], the naked filose amoebae are subsumed under a single taxon, the Aconchulinida De Saedeleer, 1934. This order is traditionally grouped within the class Filosea Leidy, 1879, which also comprises testate filose organisms. As in other rhizopodial assemblages, the relationships are based exclusively on the presence of the pseudopodial appendages and, as a consequence, on structures that are probably not homologous. However, the contrasting patterns of ultrastructural cell organization among representative genera, suggest that both of these high level assemblages are polyphyletic [13, 16]. Therefore, the use of the terms Filosea and Aconchulinida seems inappropriate for classification purposes.

Among these filopodial rhizopods or Aconchulinida, some genera exhibit a complex life cycle characterized by obligatory alternation between encysted stages and the freely moving filopodial amoebae or trophozoites. For more than a century such filopodial rhizopods have been considered members of the family Vampyrellidae Zopf, 1885 [18, 19]. This family includes genera with normal phagotrophy (like *Leptophrys* Hertwig & Lesser [1, 19]) as well as genera with the peculiar ability to attack hyphae or conidia of fungi (such as *Arachnula* [9, 10]) or filaments of algae [1, 4–7, 14, 15, 17–19] by dissolving holes in the affected cell walls.

Those Vampyrellidae which feed exclusively on penetrated algae currently comprise four genera, one from marine habitats (*Vampyrelloides* Schepotieff, 1912) and three from freshwater habitats (*Hyalodiscus* Hertwig & Lesser, 1874, *Vampyrella* Cienkowski, 1865 and *Gobiella* Cienkowski, 1881 [1, 4, 6, 15, 19]). In contrast to the more isodiametrical organized cells of *Vampyrella* and *Gobiella,* the members of the genus *Hyalodiscus* are characterized by granuloplasmic humps and filopodia-bearing lamellipodia [1, 2, 4, 5], a morphological criterion which seems to be sufficient for easy and correct determination. The remaining group of nearly 30 described *Vampyrella* and *Gobiella* species, however, is quite heterogeneous and comprises a number of mostly incompletely known species which do not correspond to the type species *Vampyrella lateritia* (Cienkowski, 1865) Leidy, 1879 and *Gobiella borealis* Cienkowski, 1881. In this species complex, only two species have been the subjects