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Nosema portugal, N. SP., Isolated from Gypsy Moths (*Lymantria dispar* L.) Collected in Portugal

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A microsporidium *Nosema portugal* n. sp. was isolated from gypsy moths, *Lymantria dispar* L, collected near Lisbon, Portugal, in 1985. The life cycle includes two sequential developmental cycles, a primary and a secondary cycle. The primary cycle occurs in midgut epithelial cells, where primary spores are produced within 48 h. The primary spores immediately extrude their polar filaments, presumably to infect other cells. In the target tissues (salivary glands and fat body) the secondary development cycle is followed by the formation of environmental spores. Primary spores were also sometimes present in target tissues. Fresh unfixed and unstained primary spores have a large posterior vacuole and measured 4.8 × 2.7 μm. Ultrastructurally, they have 5–8 polar filament coils, a large posterior vacuole, abundant endoplasmic reticulum, and were binucleate. Mature unfixed and unstained environmental spores were highly refractive and the posterior vacuole and nuclei could not be seen through the spore coat. Fresh environmental spores measured 4.5 × 1.9 μm. Ultrastructurally, environmental spores were binucleate, with a typical polaroplast, 10–11 isofilar polar filament coils, and a series of 4–6 thin polar filament-like tubules situated at the posterior end of the row of typical polar filament coils. The ssu rRNA sequences strongly suggest that this species is more closely related to the *Vairimorpha* subgroup within the *Nosema/Vairimorpha* clade than to the *Nosema* subgroup.

Key Words: **life cycle; biological control; taxonomy; ultrastructure; ribosomal RNA sequence.**

INTRODUCTION

The gypsy moth, *Lymantria dispar* L., a native of Eurasia, is an episodic pest of hardwood trees throughout much of its Eurasian distribution. Unintentionally introduced into the United States in the late 1860s (McManus and McIntyre, 1981), the gypsy moth has spread from the original release site in Massachusetts and is now distributed over much of northeastern North America, where it is often a severe pest of urban and forest trees. There are numerous reports of European gypsy moths infected with microsporidia and six species of microsporidia have been described from European gypsy moth populations (Table 1). In contrast, microsporidia have never been recovered from gypsy moths collected in North America (McManus *et al.,* 1989). Our initial interest in locating gypsy moth microsporidia in European populations focused on evaluating their potential as classical biological control agents against North American gypsy moth populations. Microsporidia have been reported from gypsy moths collected in Spain and Portugal (Romanyk, 1966; Cabral, 1977) but the biological characteristics of these microsporidia have not been previously determined nor have they been examined ultrastructurally. This paper describes the microsporidium reported previously from Portugal and isolated by us from gypsy moth larval populations near Lisbon, Portugal.

MATERIALS AND METHODS

The microsporidium we describe herein was isolated from gypsy moth larvae collected in 1985 by M. Cabral (Station of Forest Biology, Lisbon, Portugal), J. Maddox, and M. Jeffords from cork oaks (*Quercus suber* L.) in the vicinity of Lisbon, Portugal. The larvae were dissected and individual tissues were examined for the presence of spores in squash preparations in saline. Infected organs were then ground in a glass homogenizer in distilled water and the homogenate was cleaned by several cycles of water washes and centrifugation. Spores were stored in distilled water containing 3% Pen/Strep Fungizone and returned to the Center for Economic Entomology, Illinois Natural History Survey, Champaign, Illinois, where all subsequent studies on

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TABLE 1
Microsporidia Described or Reported from European Gypsy Moths

Microsporidian species	Location	Reference
Nosema lymantriae	Czechoslovakia	Weiser (1957)[a]
Nosema muscularis	Czechoslovakia	Weiser (1957)[a]
Nosema muscularis	Spain	Romanyk (1966)
Nosema muscularis	USSR, Ukraine	Zelinskaya (1980)
Nosema serbica	Yugoslavia	Weiser (1964)[a]
Nosema serbica	USSR, Ukraine	Zelinskaya (1981)
Nosema serbica	Bulgaria	Pilarska and Vavra (1991)
Thelohania disparis	Russia	Timofejeva (1956)[a]
Thelohania similis	Czechoslovakia	Weiser (1957)[a]
Vavraia schubergi	Czechoslovakia	Weiser (1964)
Vavraia schubergi	USSR, Ukraine	Zelinskaya (1980)
Nosema sp.	Portugal	Cabral (1977)
Nosema sp.	Yugoslavia	Sidor (1979)
Nosema sp.	Romania	Saftoiu *et al.* (1978)

[a] Species descriptions.

this microsporidium have been conducted under quarantine. For longer storage, spores obtained from laboratory-infected gypsy moth larvae were kept in liquid nitrogen (Maddox and Solter, 1996).

Infecting gypsy moth larvae. Spores were fed either by spreading the spore suspension directly on the surface of artificial gypsy moth diet (Bell *et al.*, 1981) or, when the absolute number of spores fed to a larva was important, a suspension of spores was fed to individual larvae with a 1-µl inoculating loop. Spore dosages ranged from 10^2 to 10^6 per larva. Third or fourth instar gypsy moth larvae, starved for 6–12 h, readily consumed the suspension when the loop was placed against their mouth parts. Only larvae that consumed the entire suspension were used in experiments. Infected larvae were then placed on artificial diet and held at 22°C.

Examining infected larval tissues. Tissues from infected larvae were examined at predetermined intervals for the presence of developmental forms and spores using phase-contrast and Nomarski interference phase-contrast microscopy as well as Giemsa (Sigma Diagnostics Accustain) staining. To determine the number of nuclei in spores, hydrochloric acid (1 N) at 60°C, followed by Giemsa staining (Vávra and Maddox, 1976), was used. Spore size and shape were recorded for spores immobilized by the agar method of Vávra (1964). The addition of a few drops of 8% glutaraldehyde to 5 ml of warm 1.5% agar prevented primary spore polar filament discharge. Immobilized spores were measured with an Image Splitting Eyepiece (Vickers Instruments Ltd.) and photographed with a Zeiss photomicroscope. For transmission electron microscopy (TEM), infected tissues were fixed for 24 h in 2.5% (v/v) glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% (w/v) aqueous OsO_4. Fixed tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon–Araldite. Thin sections were cut on a Reichert OMU2 ultramicrotome and stained using uranyl acetate and lead citrate. Sections were observed and photographed using a Zeiss EM9S electron microscope.

rDNA sequences. Total nucleic acids were isolated from purified spores of both this isolate and from *Vairimorpha lymantriae* by the method of bead beating (Baker *et al.*, 1995). PCR products were purified using QIAquick PCR purification kit manufactured by Qiagen Corp. following the manufacturer's instructions. The small subunit rRNA was amplified using the primer pair ss 18f (CACCAGGTTGATTCTGCC) and 1492r (GGTTACCTTGTTACGACTT) and the ITS spacer region was amplified using the primer pair ss530f (GTGCCAGC(C/A)GCCGCGG) and ls212r (GTT(G/A)GTTTCTTTTCCTC). Both species were sequenced with sufficient overlapping sequences to resolve any discrepancies between the two isolates. Sequencing primers were as follows: (Small subunit rDNA) ss18f CACCAGGTTGATTCTGCC; ss350f CCAAGGA(T/C)GGCAGCAGGCGCGAAA; ss350r TTTCGCGCCTGCTGCC(G/A)TCCTTGG; ss530f GTGCCAGC(C/A)GCCGCGG; ss530r CCGCGGC(T/G)GCTGGCAC; ss1047r AACGGCCATGCACCAC; ss1061f GGTGGTGCATGGCCG; ss1492r GGTTACCTTGTTACGACTT and (ITS region) ss1061f GGTGGTGCATGGCCG; ss1492f AAGTCGTAACA(T/A)GG(T/C)(T/A)GC; ls212r GTT(T/A)GTTTCTTTTCCTC. Sequencing was accomplished by direct dideoxy sequencing of PCR products using *Taq* FS DNA polymerase and fluorescently labeled dideoxynucleotides in a thermal cycling protocol. To reduce compressions, the reactions include dITP instead of dGTP.

Sequences were compared to sequences of other microsporidia available from the insect pathology laboratory at University of Illinois and from GenBank, National Center for Biotechnology Information, Bethesda, Maryland.

RESULTS

Prevalence of infection in nature. Although Cabral (1977; personal communication) had observed higher prevalences of this microsporidium in previous collections, only 5 of 300 larvae that we examined in 1985 were infected. As the spores obtained from individual larvae were identical, they were pooled and further used as single population.

General life cycle. Our interpretation of the general life cycle (Fig. 1) is presented under Discussion.

Primary merogonic cycle. The first merogonic stages, observed in the cytoplasm of midgut epithelial cells

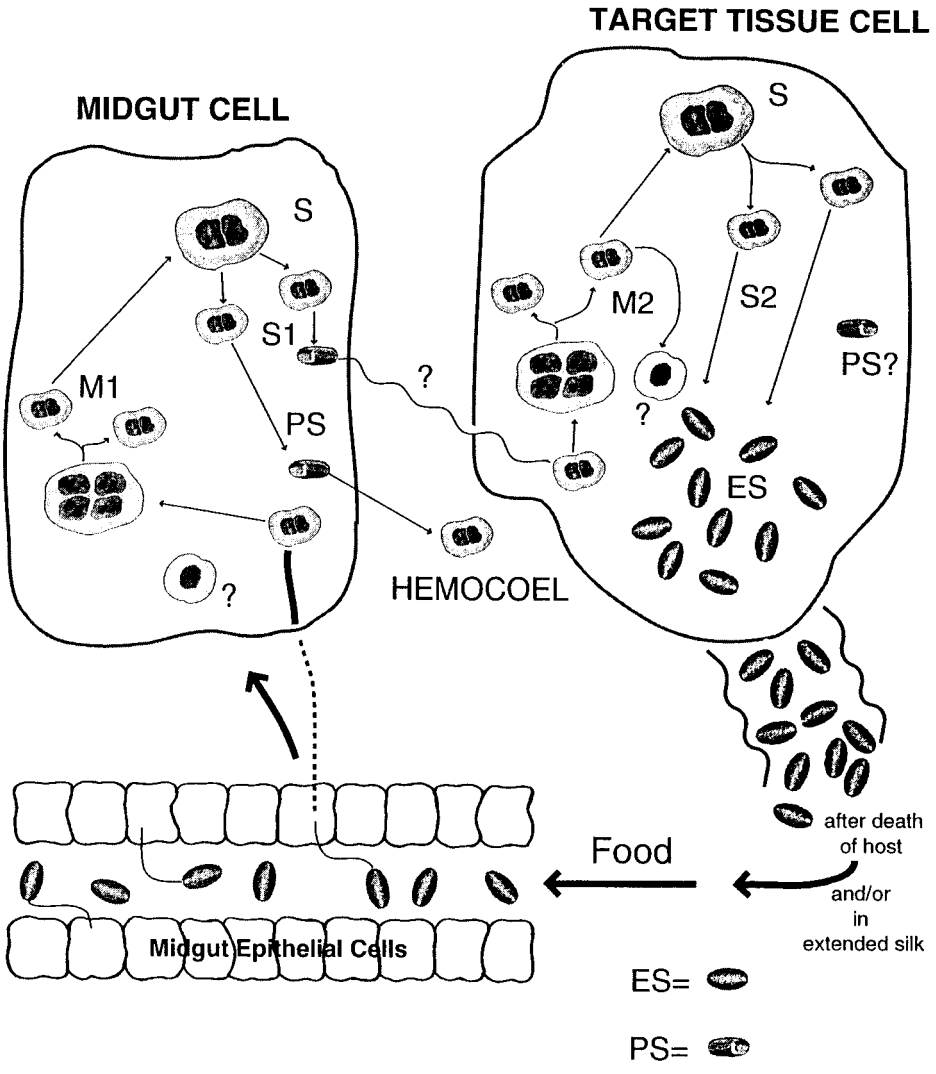
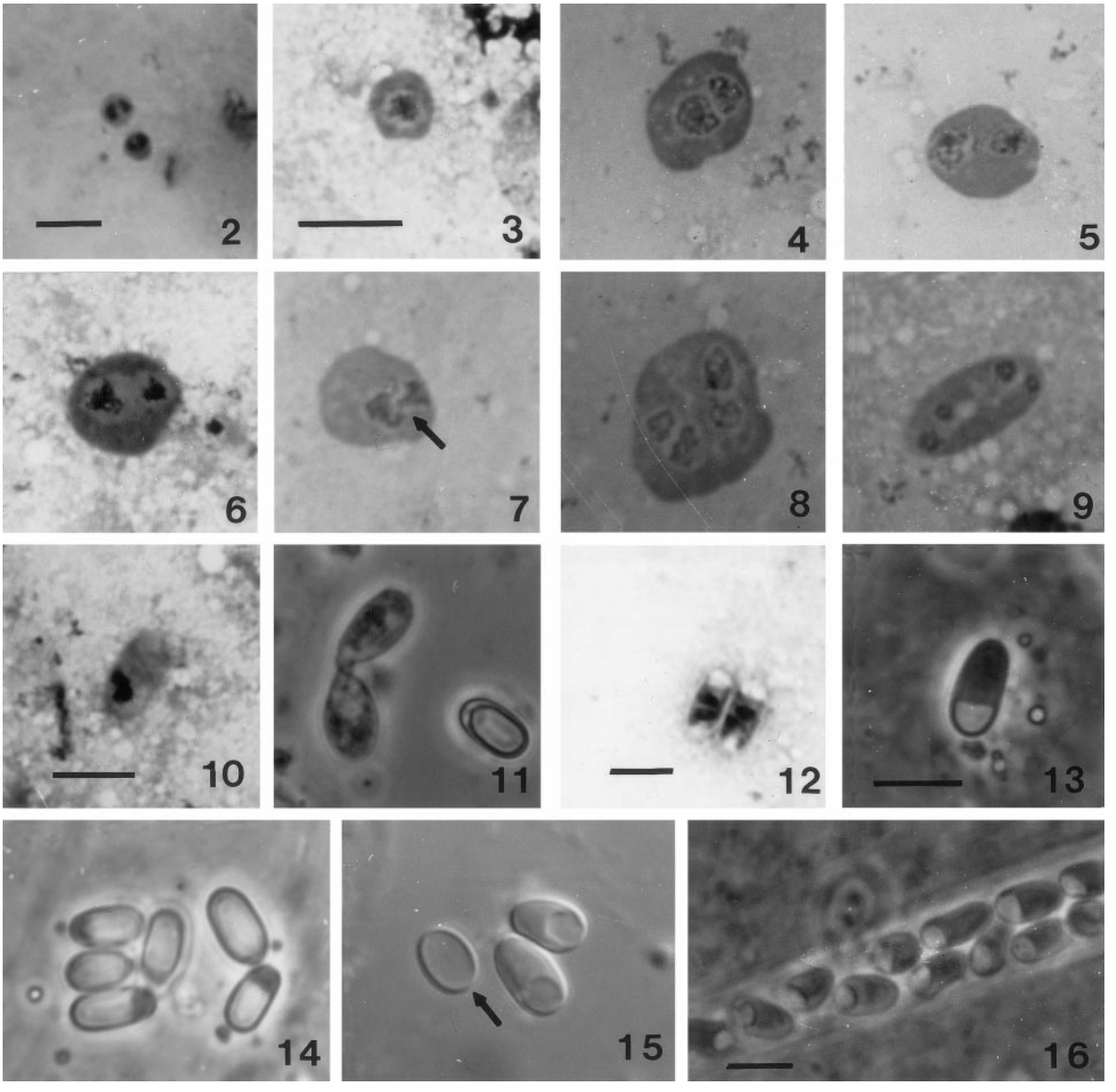


FIG. 1. The primary cycle in midgut cells is initiated when the host larva ingests environmental spores (ES). ES germinate in the midgut lumen and inject sporoplasms directly into midgut cells. A merogonial multiplication "primary merogony" (M1) follows. M1 is probably a single division followed by sporogony, "primary sporogony" (S1), which results in the formation of "primary spores" (PS). The PS extrude their polar filaments and inject sporoplasms either into target tissue cells and/or into the hemocoel, where secondary merogony (M2) occurs. M2 is a cycle; many merogonal divisions cause an enormous increase in the population of M2 meronts. M2 is followed by "secondary sporogony" (S2), which results in the formation of environmental spores (ES). Small numbers of uninucleate meronts occur in both M1 and M2 cycles. We do not know the significance of these forms. PS are also observed in some of the cells of the target tissues, indicating that M1 and S1 occur to some extent in these tissues. We do not know how this fits into the life cycle we present in Fig. 1.

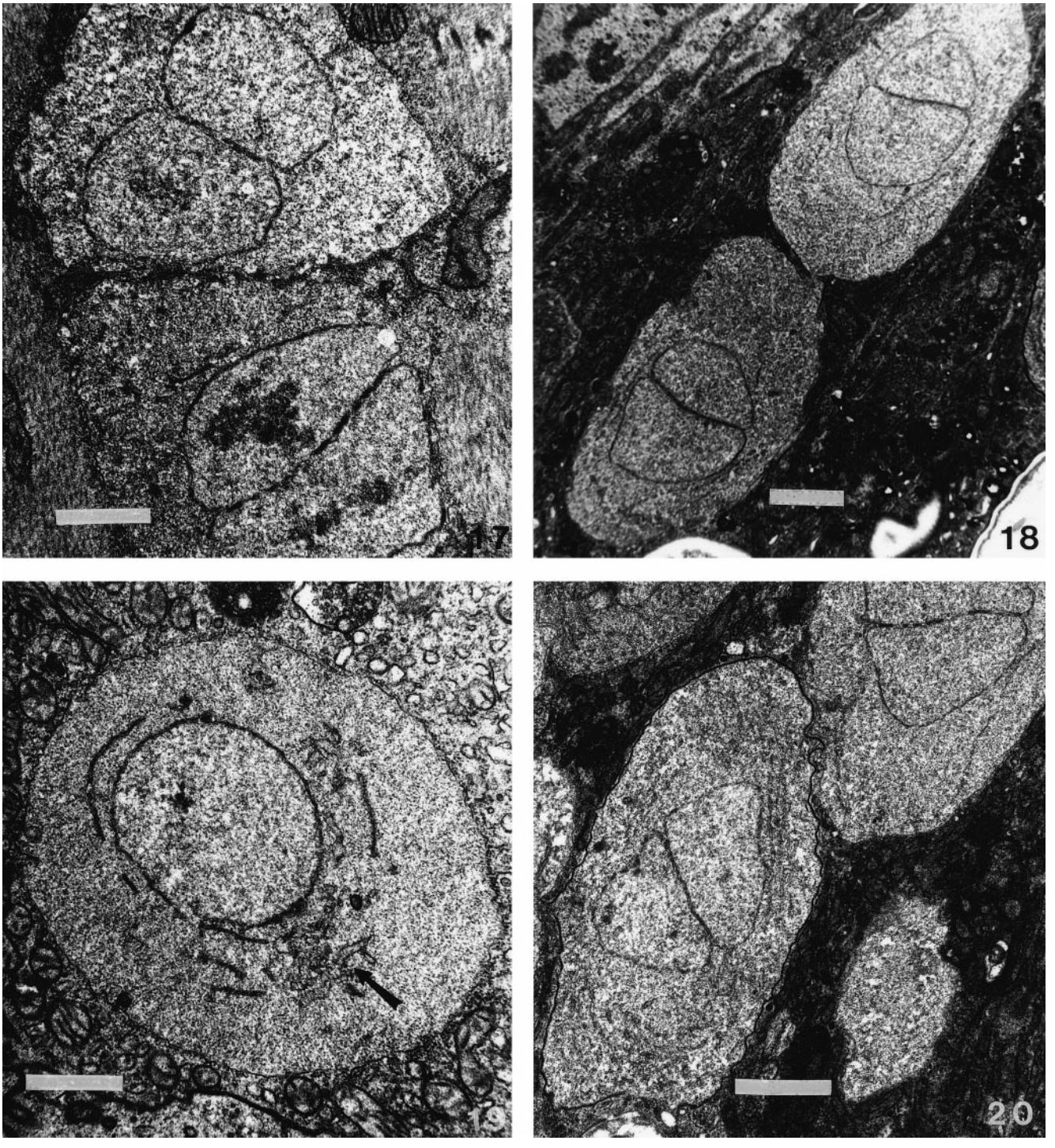
24 h postinfection (Fig. 2), were small (1.5 × 1.5 μm) cells, with compact diplokaryotic nuclei. They were probably the sporoplasms injected into the midgut epithelial cells when the polar tubes were extruded from the environmental spores (ES) fed to the larvae. These forms never appeared later in either the M1 or M2 cycles. We were unable to obtain TEM material for these forms. Meronts were observed in midgut epithelial cells at 48 h postinfection (pi) and were larger than the sporoplasms mentioned above (Figs. 3–8). Forms were observed in the TEM (Figs. 17 and 18) that suggested that meronts divide by binary fission, but chains of more than two meronts were never observed. At the TEM level meronts were enclosed in a single unit membrane, and their cytoplasm contained numerous free ribosomes and only traces of endoplasmic reticulum (ER) (Figs. 17 and 18). Nuclei often contained one or two distinct nucleoli (Fig. 17). Most of the meronts contained either two or four nuclei (Figs. 4, 5, 7, and 8) but small numbers of uninucleate meronts were also observed (Fig. 3). Some Giemsa-stained binucleate meronts contained clearly delineated diplokaryotic nuclei (Fig. 4) but others contained nuclei with an array of chromatin arrangements (Figs. 5–7). We do not know how many merogonic divisions occurred before the onset of sporogony, but because spores appeared within



FIGS. 2–16. Light micrographs of the primary developmental cycle of *Nosema portugal* in midgut epithelial cells and longitudinal midgut muscle (Fig. 16) of *Lymantria dispar*. M1, primary merogony; PS, primary spores. **FIG. 2.** Giemsa-stained diplokaryotic sporoblasts/meronts 24 h postinfection. Bar, 3 μm. **FIG. 3.** Giemsa-stained uninucleate meront 48 h postinfection. Bar, 4 μm. **FIGS. 4–7.** Giemsa-stained binucleate meronts with different nuclear configurations 48 h postinfection, under the same magnification as Fig. 3. Note the invaginated nucleus in Fig. 7. **FIG. 8.** Giemsa-stained tetranucleate meront under the same magnification as Fig. 3. **FIG. 9.** Presumed S1 sporont in the process of dividing under the same magnification as Fig. 3. **FIG. 10.** Giemsa-stained sporoblast of M1 48 h postinfection. Bar, 5 μm. **FIG. 11.** Two sporoblasts/young spores resulting from a single division of a sporont (disporoblastic sporogony). Note the germinated PS. Unfixed, unstained, phase contrast. Same magnification as Fig. 10. **FIG. 12.** Giemsa-stained PS showing large posterior vacuole and two nuclei in each spore. Bar, 5 μm. **FIG. 13.** PS with large vacuole. Loss of refringence suggests that the spore is germinating. Unfixed, unstained, phase contrast. Bar, 5 μm. **FIG. 14.** A group of germinated PS. Unfixed, unstained, phase contrast. Same magnification as Fig. 13. **FIG. 15.** Nomarski interference-contrast micrograph of primary spores. One spore (arrow) is extruded and empty. The two unextruded have large vacuoles in the posterior end. Same magnification as Fig. 13. **FIG. 16.** PS in fiber of longitudinal midgut muscle. Unfixed, unstained, phase contrast. Bar, 5 μm.

2 days pi and meronts were not abundant, it is unlikely that merogonic reproduction was extensive.

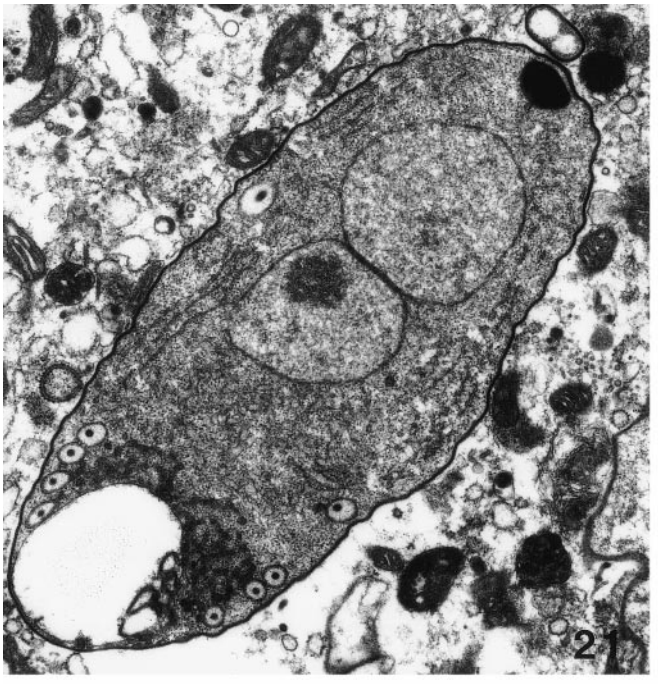
Primary sporulation cycle. Until they began to acquire polarity, sporonts and sporoblasts were not easily identified with light microscopy (Fig. 10). The onset of the sporulation cycle was easily distinguished ultrastructurally by thickening of the plasmalemma, the presence of Golgi-like structures, and the appearance of multiple lamellae of the ER (Figs. 19 and 20). Two sporoblasts appeared to develop from a single sporont (Figs. 11 and 20). Sporoblasts have four to five polar fila-

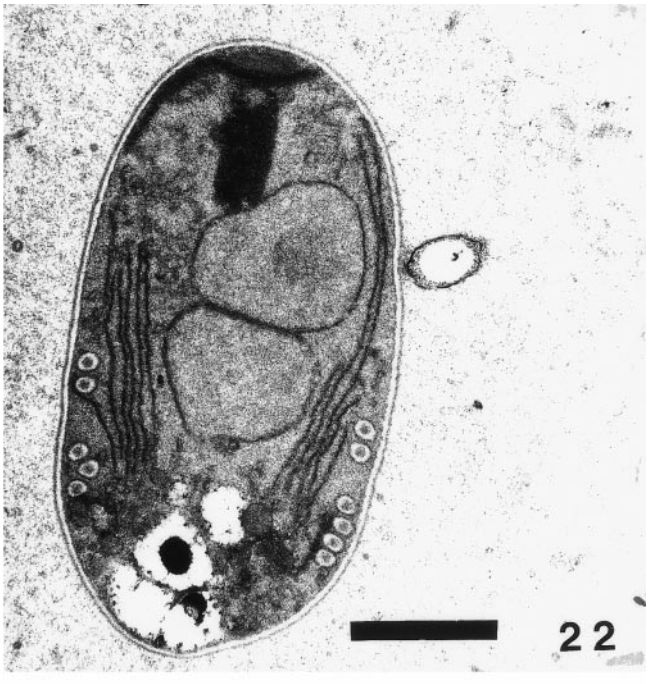


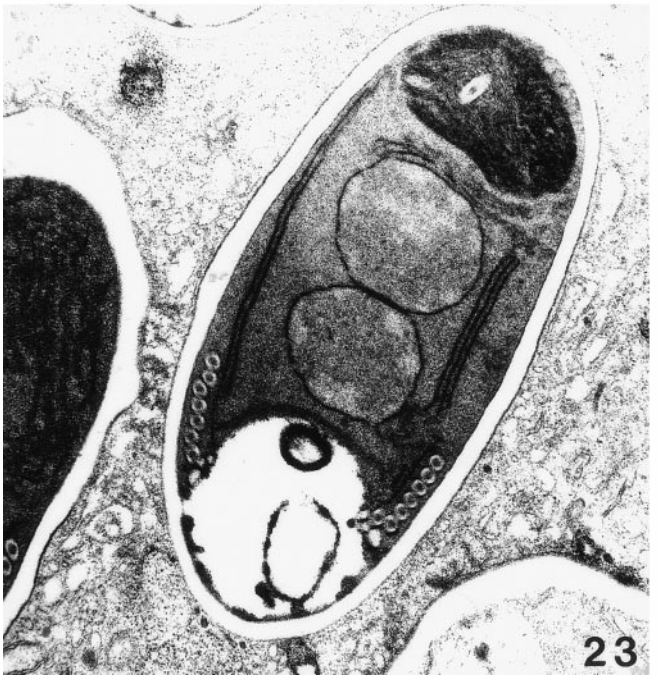
FIGS. 17–20. Electron micrographs of the primary developmental cycle of *N. portugal* in the midgut epithelial cells of *L. dispar*. M1, primary merogony; S1, primary sporogony. **FIG. 17.** Two closely adjacent meronts of M1, probably resulting from division of a tetranucleate stage. The cytoplasm is rich in ribosomes but ER is nearly nonexistent. Bar, 1 µm. **FIG. 18.** Two meronts of M1 immediately after division of a tetranucleate stage. Bar, 1 µm. **FIG. 19.** Early sporont from S1, 72 h postinfection. Identified by Golgi-like structure, increasing number of endoplasmic reticulum lamellae and the thickening of the plasmalemma. Bar, 1 µm. **FIG. 20.** Two very early sporoblasts immediately after the division of the sporont. Bar, 1 µm.

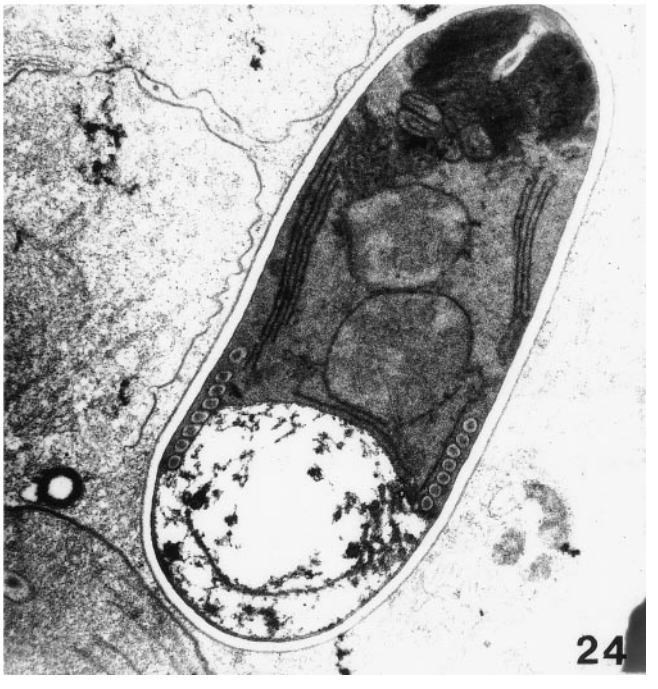
ment coils as they approach maturity (Fig. 21). Otherwise, they are similar to sporoblasts of ES (Fig. 41).

Primary spores (PS). Fresh unfixed and unstained PS have a large posterior vacuole and appear less refractive than the ES when viewed under the light microscope using phase (Figs. 13, 14, and 16) or Nomarski interference contrast (Fig. 15). Giemsa-stained PS also have a large posterior vacuole and their twin nuclei can often be seen (Fig. 12). When squash preparations are made from infected midgut tissue, PS often extrude their polar tubes, leaving the midgut cells filled with empty spore cases (Figs. 14 and 15). Fresh PS measured 4–4.8–5.7 (±0.45) × 2–2.7–3.2 (±0.26) μm









FIGS. 21–24. Electron micrographs showing variability in the structure of the primary spores of *N. portugal* in midgut epithelial cells of *L. dispar* 72 h postinfection. Fig. 21 is an immature spore in the process of sporegenisis. Note the very thin endospore and incomplete number of polar filament coils when compared with mature spores in Figs. 22–23. Bar in Fig. 22, 1 μm. Figs. 21–24 are all the same magnification.

($n = 50$). Ultrastructurally, PS have five to eight polar filament coils, a large posterior vacuole, abundant ER, and two nuclei (Figs. 22 and 24). The polaroplast is of the more common type with the tight lamellae anterior and irregular lamellae or sacs posterior (Fig. 24). The exospore forms a single layer of electron-dense material that is relatively thinner than the ES exospore. The surface of the exospore is smooth and, generally, the PS endospore is thinner (0.1 µm) than the endospore of ES (0.25 µm) (Figs. 42–44). We found a great deal of variability in the ultrastructure of PS (Figs. 21–23). This is probably because some spores were immature.

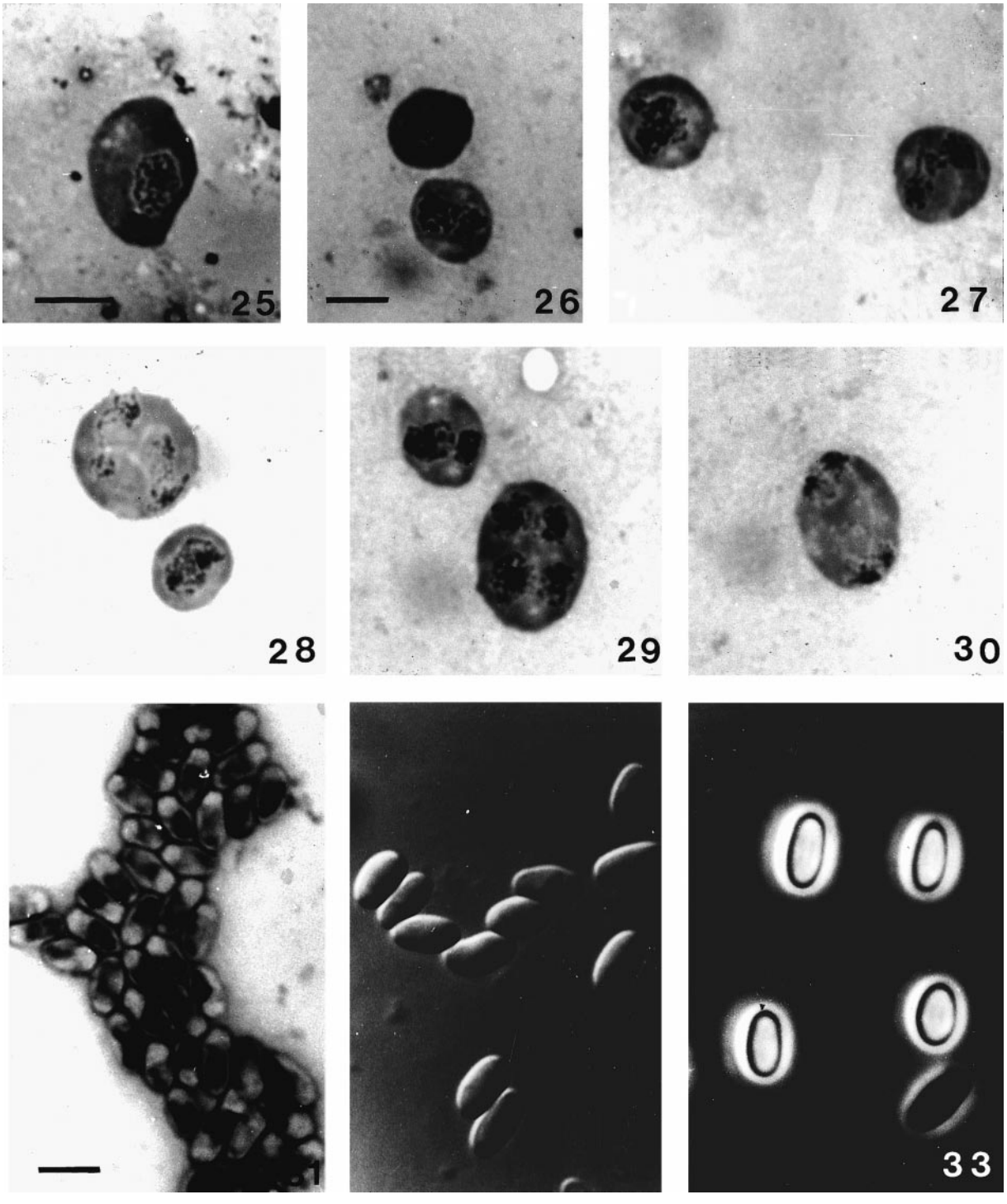
Secondary merogonic cycle. The second merogonic cycle was presumably initiated by the emergence of sporoplasms from extruded polar tubes of PS. We were unable to unequivocally identify this form. The investigation of the secondary merogonic cycle is further complicated because it is impossible to be absolutely certain that a primary-type merogonic cycle is not repeated later during the life cycle. We have observed that even in the target tissues, PS may occur in low numbers. The nuclear configuration in the secondary merogonic cycle is similar to the nuclear configuration in the primary merogonic cycle. As in the first merogonic cycle, most of the meronts contained two or four nuclei (Figs. 26–29), but small numbers of uninucleate meronts were also present (Fig. 25). In some nuclei, the chromatin was distributed in such way that it was impossible to decide if there was a diplokaryon present or if both the two nuclei have fused (Figs. 26–28). Some uninucleate meronts contained a distinct nucleolus (Fig. 25). Multiplication of meronts was evidently by binary fission of tetranucleate forms (Figs. 28, 29, and 34). Meronts of M2 appeared to contain more abundant fragments of ER when compared with those of M1 (Figs. 34 and 35). M2 meronts were often observed in nucleoplasm of the host cells, especially in nuclei of the salivary gland cells. They were observed inside the nucleoplasm, not invaginated into the nucleus (Fig. 40). Nuclei of some M2 meronts were not in contact in a straight plane, typical of most diplokaryotic associations, but in an irregular, intricate manner (Fig. 38).

Secondary sporogonic cycle. In ultrathin sections, the beginning of the sporulation cycle was indicated by thickened plasmalemma, development of a more completely organized ER, and the appearance of a Golgi zone consisting of a reticulum of small vesicles (Figs. 36 and 37). All stages in the sporulation sequence were diplokaryotic and some contained distinct nucleoli (Fig. 37). The nuclei of some forms in the S2 cycle were in contact in an irregular manner, as was observed in some M2 meronts (Fig. 39). Two sporoblasts developed from each sporont. Each mature sporoblast had 11 polar filament coils; immature sporoblasts had fewer coils (Fig. 41).

Environmental spores. When viewed under phase-contrast microscopy, mature unfixed and unstained ES were highly refractive and the posterior vacuole and nuclei could not be seen through the spore coat (Figs. 32 and 33). Unlike PS, polar tubes were rarely extruded in squash preparations. Fresh ES measured 3.4–4.5–5.5 ($\pm$0.41) $\times$ 1.5–1.9–2.3 ($\pm$0.17) µm. Ultrastructurally, ES were binucleate, with 10–11 typical polar filament coils (Figs. 42 and 43). The polaroplast was typical of the polaroplast reported from most species of *Nosema,* with the tightly packed lamellae anterior and the wider lamellae posterior (Larsson, 1986). The polar sac covered only the anterior portion of the polaroplast. The most unusual and distinctive feature of the mature ES was a series of four to six thin polar filament-like tubules situated at the posterior end of the row of typical polar filament coils (Figs. 42–44). This feature was observed in every ES sectioned in a plane that exposed the ultrastructural features of this part of the spore. The ES exospore was relatively smooth and the endospore was thicker (0.25 µm) than the endospore of PS (0.1 µm) (Figs. 42–44).

Tissues infected. Midgut epithelial cells and muscles surrounding midgut contained meronts within 25 h after the hosts ingested infectious ES. The M1 and S1 cycles were completed in these tissues. PS appeared in the midgut epithelial cells and surrounding muscles as early as 55 h pi, and meronts of M2 appeared in the cells of the fat body and silk glands within 100 h pi. The timing of these events was greatly influenced by spore dose and the temperature at which the host was maintained. Both the silk glands and the fat body became very heavily infected at 10–12 days pi. Occasionally, light infections were observed in gonads and nervous tissue. In advanced infections (10 or more days pi), PS were no longer observed in midgut epithelial cells. Apparently the PS have all germinated at this time and either the empty spores have been digested by the host cells or the infected tissues were replaced by newly regenerated epithelial cells.

Phylogenetic position of N. portugal. The sequence containing the small subunit rDNA and the internal transcribed spacer region for *N. portugal* are deposited in GenBank (Accession No. AF033316). In addition, a more recently sequenced small subunit rDNA sequence including the internal transcribed spacer region has been deposited for the *Vairimorpha* sp. which we believe was originally described as *Nosema lymantriae* GenBank (Accession No. AF033315). *N. portugal* and this *Vairimorpha* species differ by only a single nucleotide in the small subunit rDNA (T in *N. portugal* vs. C in the *Vairimorpha* sp. sequence at position 829) and by two nucleotides in the internal transcribed spacer region positions, T vs C at position 1258 and T vs A at position 1475 for *N. portugal* and *Vairimorpha* sp.,



FIGS. 25–33. Light micrographs of the secondary developmental cycle (S2) of *N. portugal* in salivary gland and fat body tissues. Figs. 25–31 are photographed from Giemsa stains. ES, environmental spores. **FIG. 25.** Uninucleate meront. Bar, 1.5 μm. **FIG. 26.** Binucleate meronts. Bar, 2 μm. **FIGS. 27 and 28.** Meronts undergoing nuclear transformations; nuclear division and/or the possibility of a fusion of diplokarya. Magnification is the same as Fig. 26. **FIG. 29.** Binucleate and tetranucleate meronts. Magnification is the same as Fig. 26. **FIG. 30.** Presumed S2 sporont of the second sporogony. Magnification is the same as Fig. 26. **FIG. 31.** Giemsa-stained ES. Bar, 5 μm. **FIG. 32.** Unfixed, unstained ES. Nomarski interference contrast. Same magnification as Fig. 31. **FIG. 33.** ES. Phase contrast. Same magnification as Fig. 31.

respectively. Therefore, *N. portugal* and the *Vairimorpha* sp. we believe was described as *N. lymantriae* are sister species on the small subunit rDNA phylogenetic tree (Baker *et al.*, 1994, 1995).

DISCUSSION

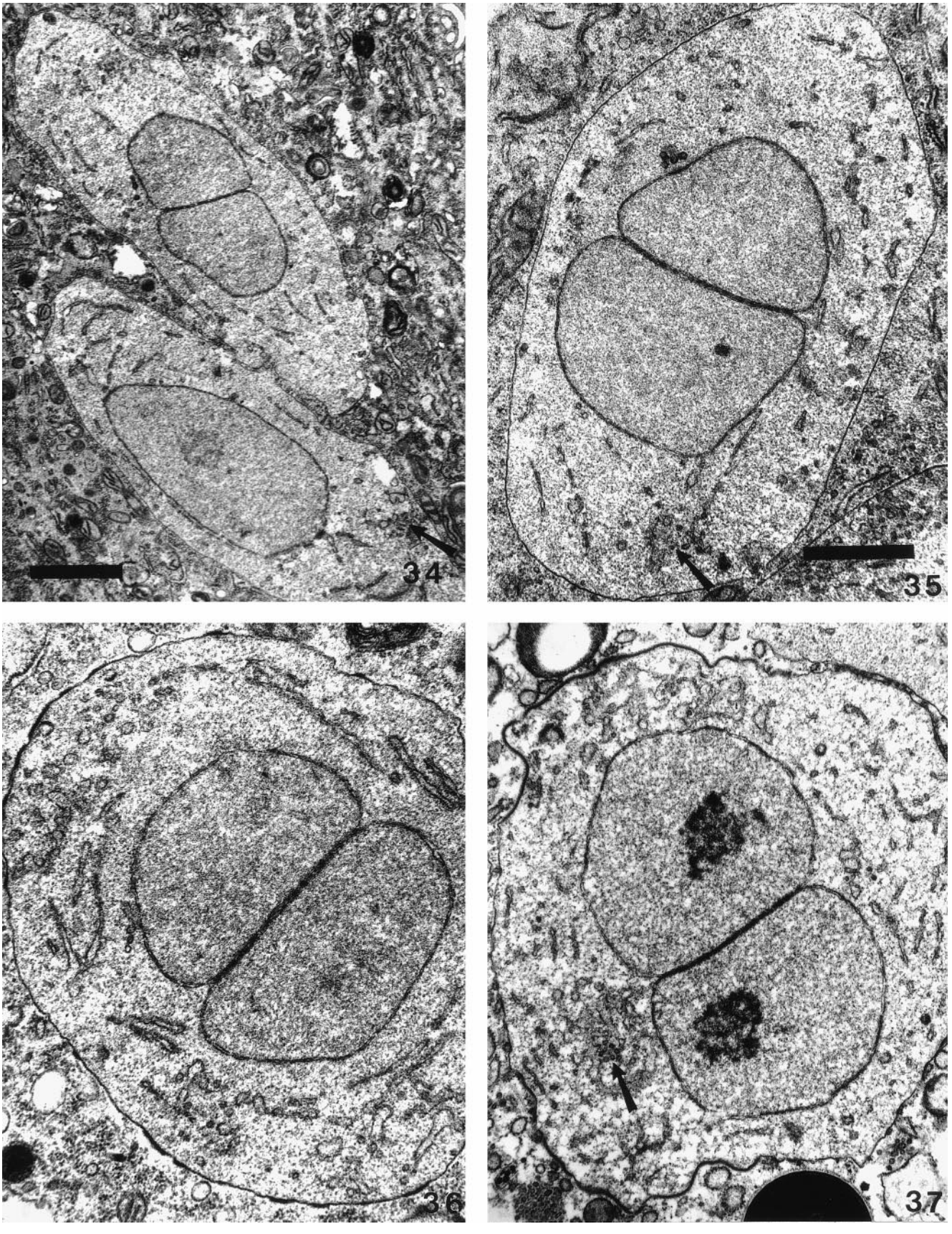
General life cycle (Fig. 1). The life cycle includes two sequential developmental cycles, a primary and a secondary cycle. The primary cycle begins when environmentally resistant spores, "environmental spores," ingested with food by a larva germinate in the gut lumen. The sporoplasms are injected directly into the gut epithelial cells and possibly into muscle cells surrounding the gut (not shown in Fig. 1). A merogonal multiplication "primary merogony" (M1) takes place in gut epithelial cells and in the muscle cells surrounding the gut. This is followed by sporogony, "primary sporogony" (S1), which results in the formation of "primary spores." The PS immediately extrude their polar filaments and inject sporoplasms either into target cells and/or into the hemocoel, where secondary merogony occurs. In target tissues "secondary merogony" (M2) takes place followed by "secondary sporogony" (S2), which results in the formation of ES. PS were also sometimes present in target tissues, indicating that M1 may occur at the onset of infections in target tissues. We do not know how this fits into the overall life cycle.

Primary spores. Iwano and Ishihara (1991) have used the terms "few coils" (FC) for spores that appear early after infection and "many coils" (MC) to spores that appear later. Although the spores of the first sporulation sequence of the microsporidium treated here also have fewer coils (5–8) than those appearing later in the infection (10–11), we prefer the terms primary spores and environmental spores (Solter *et al.*, 1997) because these terms are more appropriate to when they appear in the life cycle and the functional properties of these two spore types. The function of PS is probably to transmit the infection from the gut to other tissues of the host where a massive build up of ES numbers takes place. This explains why PS are very reactive and extrude their filaments soon after they are formed. The ES, on the contrary, are adapted to transmiting the infection from host to host, are environmentally resistant, and do not extrude the filaments until they encounter proper conditions within the host gut. The presence of PS is apparently not unique to the microsporidium described here; it also occurs in some other microsporidia both under *in vitro* and *in vivo* conditions (Kurtti *et al.*, 1983; Iwano and Ishihara, 1991; Fries *et al.*, 1992; deGraff *et al.*, 1994; Johnson *et al.*, 1997). PS are easily overlooked unless the initial infectious spore dose is very high. In the gypsy moth, PS are abundant in midgut cells and in muscles surrounding the midgut only when the initial spore dose exceeds 10^6 spores per larva. There is a generally accepted hypothesis that high spore dosages damage midgut epithelial cells to such an extent that the host dies, not from the development of the microsporidium in host tissues, but rather from the physical damage to the midgut caused by the polar filaments of the germinating spores (Maddox *et al.*, 1981). We believe that for the microsporidium we are describing, the formation of large numbers of PS and/or the extrusion of their polar filaments is responsible for this phenomenon. The timing is correct. Larvae infected with a very high spore dose begin to die at about 48 h pi, just when many PS germinate. Therefore, the midgut epithelial cells are damaged by the germination of intracellular PS, not by germinating ES, located extracellularly in the gut.

Uninucleate meronts. Our light microscope observations indicate that uninucleate meronts occur occasionally in the life cycle of this microsporidium. The occurrence of uninucleate stages in the life cycle of a *Nosema*-like microsporidium (which is supposed to have diplokarya throughout its life cycle) is of considerable interest. Several authors reported uninucleate stages in otherwise typical diplokaryotic microsporidia and have speculated about their significance. Oshima (1973) reported the presence of binucleate meronts and uninucleate meronts with small compact nuclei in *Nosema bombycis*. He suggested that the uninucleate meronts were formed by karyogomy. Sprague *et al.* (1992) believed that "the invading sporoplasm (or one of its early descendants) divides into 2 uninucleate cells, thereby initiating a proliferative sequence of uninucleate cells that ends with plasmogamy and nuclear association to form the first diplokaryotic cell in a new life cycle." We believe this is unlikely in the species we are describing because we found no evidence that uninucleate meronts divided into further uninucleate meronts; furthermore uninucleate meronts were relatively uncommon. If the scenario suggested by Sprague *et al.* (1992) did occur, we would expect to find larger numbers of uninucleate meronts in the early part of the first merogonic cycle. We did not observe this phenomenon; on the contrary, we observed uninucleate stages during advanced merogony in both the M1 and M2 cycles. As these stages were rare we cannot speculate about their significance or function.

Infections of host cell nuclei. Development of microsporidia in host cell nuclei is uncommon for most species (Sprague *et al.*, 1992). In the microsporidium we are describing, we often observed infected host nuclei, but we never observed spores in the host nuclei, suggesting that the microsporidium did not complete its development in the nucleoplasm.

Anisofilar polar filament? The presence of the small polar filament-like tubes at the posterior end of the ES



FIGS. 34–37. Electron micrographs of the secondary developmental cycle of *N. portugal*. M2, secondary merogony; S2, secondary sporogony. **FIGS. 34 and 35.** M2 of *N. portugal* in the fat body, 200 h postinfection. Short pieces of the endoplasmic reticulum lamellae and Golgi zones (arrow) are starting to form in the cytoplasm. Bar in Fig. 34, 0.9 µm. Bar in Fig. 35, 0.74 µm. **FIGS. 36 and 37.** Early sporonts from

is apparently unique. Their proximity to the polar filament suggests that they are actually the terminal part of the filament. The ES of several groups of microsporidia are characterized by a tapered (anisofilar) polar filament (Larsson, 1986). Unlike previous descriptions of anisofilar polar filaments, which have a tapered end of the filament, the small polar filament-like coils observed in *N. portugal* are smaller in diameter (less than half the diameter of larger coils), do not have the concentric structure and electron of the larger portion of the polar filament, and, if they are an extension of the polar filament, represent an abrupt rather than a gradual (tapered) change in the diameter of the polar filament. Nevertheless, this structure was consistently present in ES and it is important to determine its nature and function. The numbers of coils on either side of the spore suggest that they are sections through coils of the polar filament.

Assignment to the genus Nosema. The ssu rRNA sequences strongly suggest that this species is more closely related to the *Vairimorpha* subgroup within the *Nosema/Vairimorpha* clade than to the "typical" *Nosema* subgroup as identified by Baker *et al.* (1994). This species, however, does not form uninucleate spores in an octosporous sporulation cycle, which is the defining characteristic of the genus *Vairimorpha* (Sprague *et al.*, 1992; Pilley, 1976). Although this species is *Nosema*-like it does possess a few characteristics that are atypical for this genus. Uninucleate meronts were observed in both merogonic cycles and the polar filament in ES is not a typical isofilar polar filament. All *Nosema* species that have been examined ultrastructurally have isofilar polar filaments. There is no consensus on the developmental cycle of *N. bombycis* Nageli, the type species of the genus (Sprague *et al.*, 1992), so the presence of uninucleate meronts is an acceptable character for a species placed in the genus *Nosema*. Because two spore types (PS and ES) have been reported for the type species *N. bombycis*, this feature of the life cycle presents no taxonomic problems. There are three logical taxonomic choices with regard to the generic placement of this species. They can (1) rely primarily on the molecular sequence data and assign this species to the genus *Vairimorpha*, (2) accept the close relationship between the sister groups in the *Nosema/Vairimorpha* group and assign the species to the genus *Nosema*, or (3) create a new genus for this species because it does not conform exactly with the generic characters of either *Nosema* or *Vairimorpha*. We have chosen to place this species in the genus *Nosema* because we believe this creates the least taxonomic confusion at this time. This decision was based on the fact that *N. portugal* does not form uninucleate spores in an octosporous sporulation cycle, the defining characteristic of the genus *Vairimorpha*. The rDNA sequences indicate that *N. portugal* is clearly more closely related to *Vairimorpha necatrix* (the type species for the genus *Vairimorpha*) than to *N. bombysis* (the type species for the genus *Nosema*) and its position is well within the *Vairimorpha* group as a sister species to the *Vairimorpha* sp. isolated from the gypsy moth. Until the life cycles and ultrastructural characteristics of more species from the *Nosema/Vairimorpha* group are thoroughly examined and their relationships evaluated using additional molecular information, the generic placement of species as well as the relative importance of morphological, developmental, and molecular characters is likely to remain an enigma.

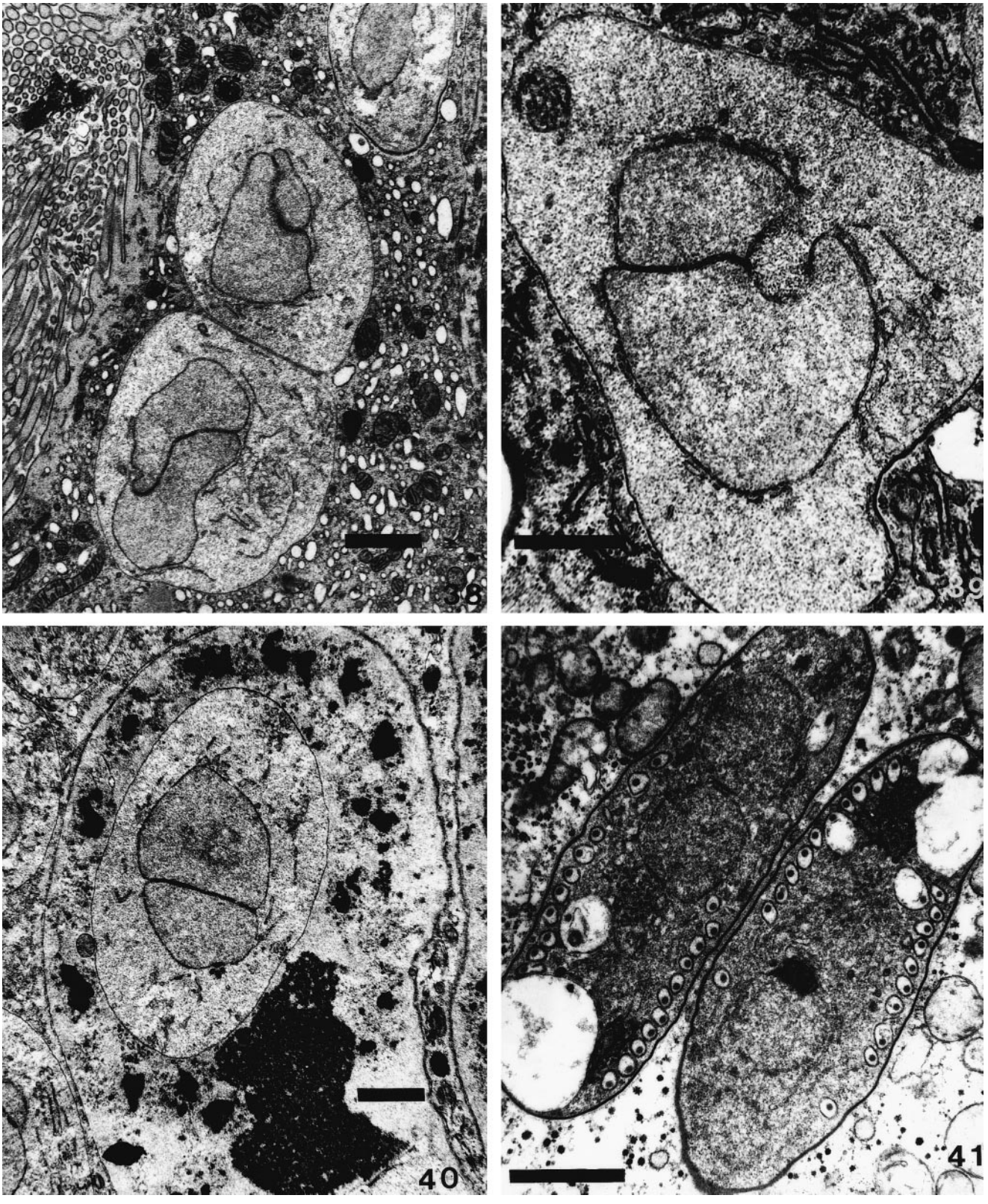
We have chosen the name *N. portugal*, suggesting the place of its origin. We dedicate the description to Maria Teresa E. C. Cabral, who observed this microsporidium in Portuguese gypsy moths for many years. Without her assistance we would never have obtained isolates of this species.

Comparison with other microsporidia described from the gypsy moth. Six species of microsporidia have been described from the gypsy moth. Weiser (1998) presented an historic perspective of these species. Based on ultrastructural observations we believe that only one of these species, *Nosema serbica* (Weiser, 1961b), has enough similarity to possibly be conspecific with the microsporidium we are describing as a new species. *N. serbica* was examined ultrastructurally by Pilarska and Vávra (1991). It lacks the small polar filament-like structures at the posterior end of the ES. Additionally, environmental spores of *N. serbica* have 8–9 polar filament coils, while *N. portugal* has 10–11 polar filament coils. All of the *N. lymantriae* (Weiser, 1957) isolates we have examined actually have the characteristics of a *Vairimorpha* sp. and as stated above we have deposited our sequence of this species as *V. lymantriae* (AF033315). *Nosema muscularis* Weiser only infects midgut epithelium and midgut muscle tissues and may be the PS form of another microsporidium (Weiser, personal communication).

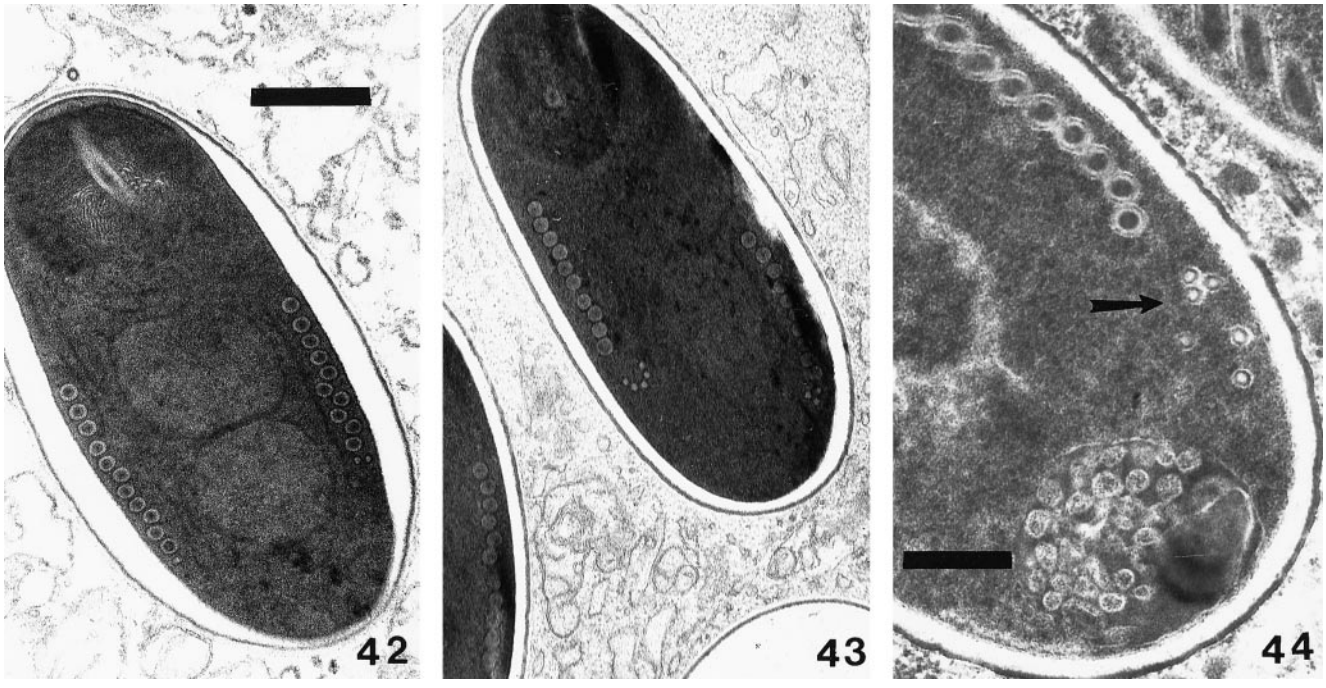
Systematics: Nosema portugal n. sp. J. V. Maddox and J. Vávra

Type host. Gypsy moth, *Lymantria dispar* L.

S2 in the fat body, 255 h postinfection. Note the thickening of the plasmalemma at certain points of the cell circumference, the increasing amount of lamella of the endoplasmic reticulum, and the development of Golgi-like structures (arrow). Magnifications in Figs. 36 and 37 are the same as in that in Fig. 35.



FIGS. 38–41. Electron micrographs of *N. portugal*. M2, secondary merogony: S2 secondary sporogony. **FIG. 38.** Meront from M2 with an unusual nuclear configuration in the silk gland, 200 h postinfection. Bar, 1.5 µm. **FIG. 39.** Sporont from S2 with unusual nuclear configuration in the silk gland, 200 h postinfection. Bar, 0.8 µm. **FIG. 40.** Meront of M2 in host fat body nucleus, 255 h postinfection. Bar, 0.9 µm. **FIG. 41.** Sporoblasts of S2 maturing into young spores, 255 h postinfection.



FIGS. 42–44. Electron micrographs of environmental spores of *N. portugal*. **FIG. 42.** Longitudinal section, 255 h postinfection, showing 10–11 polar filament coils in a single row. Note the small structures at the posterior end of the row of polar filament coils. These may represent cross sections of the smaller diameter of an anisofilar filament. Bar, 1 µm. **FIG. 43.** Spore showing 4–6 polar filament-like structures (anisofilar filament?) at the posterior end of the row of polar filament coils. Bar, 1 µm. **FIG. 44.** A detailed view of the polar filament-like structures (arrow) at the posterior end of the row of polar filament coils. Bar, 0.40 µm.

Transmission. *Per os*, demonstrated experimentally. Transovum (by inference) and L. Bauer (personal communication).

Site of infection. In gypsy moth larvae. First merogony and primary spores (PS) form in midgut epithelial cells and in the muscles surrounding the midgut. Second merogony and environmental spores (ES) form primarily in silk glands and fat body. In later stages of the infection, light infections may develop in the gonads and nervous system. Interface. Parasite plasmalemma is in direct contact with host cell cytoplasm. Meronts occasionally occur in nuclei of target tissues.

Development. There are two relatively simple merogonic and sporogonic cycles involving binucleate (one diplokaryon) and tetranucleate (two diplokarya) cells. Sporogony is disporoblastic in both sporogonic cycles. Rarely, uninucleate cells of unknown significance were observed. The first merogonic and sporogonic cycle occurs in the midgut epithelial cells and the muscle cells surrounding the midgut. It results in the formation of PS which germinate in the cytoplasm of midgut epithelial cells and the muscle cells surrounding the midgut. Low numbers of PS were occasionally observed in target tissues, indicating that these cycles occur also in target tissues. The second merogonic and sporogonic cycle does not occur in midgut epithelial cells but occurs in target host tissues, primarily the silk glands and fat body. The second sporogonic cycle produces ES.

Spore. Two spore types are produced: PS and ES. Both spore types are binucleate. Fresh, unfixed PS measure 4.8 × 2.7 µm. Under phase-contrast and bright-field light microscopy, PS are less refractive than environmental spores and have a large obvious posterior vacuole. Giemsa-stained PS also display the large posterior vacuole and the two nuclei are often visible through the spore coat. PS often germinate when infected tissues are squashed under a coverslip. Ultrastructurally, PS have 5–8 isofilar polar filament coils, a large posterior vacuole, abundant endoplasmic reticulum, and two nuclei. The polaroplast has a tighter lamellae anteriorly and irregular lamellae or sacs posteriorly. The surface of the exospore is smooth and the endospore generally thinner than the endospore of ES. Fresh, unfixed ES measure 4.5 × 1.9. Under phase-contrast and bright-field light microscopy ES are highly refractive. No internal features can be seen with the light microscope. Giemsa-stained spores have a dark blue center core and a lighter blue outer periphery typical of most microsporidia. Ultrastructurally, ES are binucleate, with 10–11 typical polar filament coils. ES also have 4–6 small polar filament-like structures at the posterior end of the row of typical polar filament

coils. This suggests that the polar filament of the ES is probably of a special anisofilar type. The polaroplast has tightly packed lamellae anterior and the wider lamellae posterior.

Type locality. Isolated from gypsy moth larvae collected from cork oak trees (*Quercus suber* L.) in the vicinity of Lisbon, Portugal, May, 1985.

Deposition of type specimens. At the Illinois Natural History Survey (slides in addition to viable spores in liquid nitrogen) and in the collection of Dr. J. Weiser, Prague, Czech Republic.

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