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Nosema tyriae n.sp. and *Nosema* sp., Microsporidian Parasites of Cinnabar Moth *Tyria jacobaeae*

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Nosema tyriae **n.sp. was found in 63% of a population of Cinnabar moth larvae (*Tyria jacobaeae*). The infection was found in the gut wall, silk glands, and fat body and was probably generalized but appeared to be of low pathogenicity. Merogony and sporogony were by binary fission of diplokaryotic stages. Fresh spores were elongate, slightly pointed at the anterior end, and measured 4.7 × 2.0 μm. Ultrastructural features of special interest were 20-nm tubules connecting the surface of sporonts with host cell cytoplasm and, in the spores, a deeply domed polar sac, polaroplast consisting of closely packed longitudinally arranged membranes and loosely packed horizontally arranged membranes, and 10.5–14 coils of the polar tube in a single rank. The 16S rRNA genes of *N. tyriae* and *Nosema bombycis* from silkworms, *Bombyx mori,* differed by only six nucleotides and *N. tyriae* spores gave a moderately positive reaction with a monoclonal antibody raised to *N. bombycis. N. tyriae* was infective to *B. mori* but was less virulent than *N. bombycis.* However, no amplification product was obtained by PCR using *N. tyriae* DNA and primers considered to be specific for *N. bombycis.* Also, the spores of the two species are of entirely different shapes. A second diplokaryotic microsporidium, *Nosema* sp., found as a light infection in only one of the larvae had much smaller developmental stages and spores measuring 3.8 × 2.0 μm (fixed). Ultrastructurally it was distinguished by an abundance of dense membranes in cytoplasmic vesicles in both meronts and sporonts. Spores with up to 15 coils of the polar tube in irregular clusters or with about 12 coils in a single rank were observed in the tissues fixed from the one larva infected with this parasite. As this larva had been kept with *N. tyriae*-infected larvae for a few days before examination, it is possible that the two types of spores resulted from a double infection.**

Key Words: *Nosema tyriae* n.sp.; *Nosema* sp.; Cinnabar moth, *Tyria jacobaeae;* ultrastructure; ribosomal DNA sequence; comparison with *Nosema bombycis*.

INTRODUCTION

During a visit to Beale Bird Park, Berkshire, England (National Grid Reference SU619781) in August 1997 an infestation of Cinnabar moth caterpillars, *Tyria jacobaeae*, on ragwort, *Senecio jacobaeae*, was noticed causing extensive defoliation of the plants. A collection was made of various instars of the larvae and examination showed that a high percentage were infected with microsporidia. One larva showed spores of a size and shape which differed from those in the majority of larvae. In the one previous report of microsporidia in *T. jacobaeae* (Philogène and Massalski, 1977) infection had been identified in diapausing pupae from a laboratory colony in Canada. The parasites were considered to be "Nosématidée" but were not assigned to a genus or species. Ultrastructural data were given for the spores, which were the only stages observed. Here we present details, at light- and electron-microscopic levels, of the recently found microsporidia and compare these with the species examined by Philogène and Massalski. We have also sequenced the 16S rRNA gene of the larger species and compared this sequence with sequences in the GenBank of other *Nosema* species. As there were only six bases which differed from the sequence of *Nosema bombycis* isolate SES-NU (GenBank Accession No. D85503) derived from silkworms (*Bombyx mori*), we further examined its relationship with *N. bombycis* by (a) testing its reactivity with a monoclonal antibody (Mab) raised against *N. bombycis* isolate SES-NU; (b) attempting to obtain an amplifica-

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tion product using primers specific for *N. bombycis* (Kawakami *et al.*, 1995), and (c) attempting to infect silkworms with purified spores from Cinnabar moth. Analysis of the total data leads us to conclude that the larger species from Cinnabar moth is not *N. bombycis* and we propose to name it *Nosema tyriae* n.sp. The smaller species exhibits ultrastructural features of the genus *Nosema* which are new but because there was a risk of double infection with *N. tyriae* in this larva and it was found only once, we have not named the species.

MATERIALS AND METHODS

Light and Electron Microscopy

The collection of 54 larvae of *T. jacobaeae* were fed on ragwort leaves until examined. The head and last abdominal segments were removed and the gut was drawn out together with the silk glands. After this, the fat body and other organs were squeezed out. Small pieces of tissue were examined fresh and in Giemsa-stained smears. Fresh spores of *N. tyriae* were measured after immobilization at a water/paraffin oil interface and those of *Nosema* sp. were measured in Giemsa-stained smears. For electron microscopy, silk glands and pieces of gut wall and fat body were fixed at room temperature in Karnovsky's fixative in 0.1 M cacodylate buffer, pH 6.5, postfixed in 1% (w/v) Os O_4 in cacodylate buffer, washed in buffer, dehydrated in a series of ethanol dilutions, and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an AE1 EM 801 electron microscope.

Purification of Spores of N. tyriae and N. bombycis

Tissues of *T. jacobaeae* larvae infected with *N. tyriae* were crushed in phosphate-buffered saline (PBS) and 2 ml of the suspension were layered onto a gradient made up of 2 ml each of 25, 50, and 75% Percoll in PBS and 100% Percoll. The gradient was spun at 2000*g* for 30 min. Spores were collected from the bottom of the tube and were washed by repeated centrifugation in PBS.

The strain of *N. bombycis* was SES-NU maintained in silkworms, *B. mori*. Infected larvae were crushed in sterile distilled water; the homogenate was filtered through absorbent cotton, sandwiched between two layers of cheese cloth. The filtrate was centrifuged at 900*g* for 5 min and washed by repeated centrifugation in distilled water.

Reactivity of Spores with Monoclonal Antibodies

Latex beads coated with a monoclonal antibody (Mab) raised against *N. bombycis* SES-NU or against *Vairimorpha* spp., M11 and M12 (Mike *et al.*, 1988), were purchased from Yakult Co. Tokyo. Although M11 was considered to be a *Nosema* sp. by Mike *et al.* (1988), it was later shown on morphological grounds and on sequence of the ssu rDNA to be a typical *Vairimorpha* sp. (Iguchi *et al.*, 1997; Hatakeyama *et al.*, 1997). Drops containing 8 µl of the sensitized beads and 8 µl of spore suspension were introduced into wells on glass slides. The test slide contained spores of *N. tyriae* from Cinnabar moth mixed, in three separate wells, with beads coated with anti-SES-NU, anti-M11, or anti-M12 Mabs. Three control slides contained spores of SES-NU, M11, or M12 with their respectively coated beads. Reactions between spores and monoclonal antibody-coated beads was considered positive when more than 50% of spores adsorbed beads onto their surface or agglutinated to form a clump, negative when 90% of spores were free of beads, and intermediate with reactions between these figures.

Infectivity and Virulence of N. tyriae to Silkworms, B. mori

B. mori larvae were exposed to the microsporidian spores painted onto the surface of diet used for rearing silkworms, obtained from Nihon Nousan Kohgyo, Yokohama, Japan. For this purpose a 5-cm-diameter roll of diet was cut into 0.5-cm-thick discs which were then cut in half to produce semicircular slices. Suspensions of spores of *N. tyriae* or *N. bombycis* (SES-NU) in 0.15 ml PBS were applied to the half pieces of diet, providing final dosages of 10^3 or 10^4 spores per larva. Fifty newly moulted second instar *B. mori* larvae were allowed to feed on the discs painted with *N. tyriae*, using three replicates for each spore dose. Thirty of the same batch of larvae were fed on discs painted with the same concentrations of *N. bombycis* spores but no replicates were used because the virulence of this strain to *B. mori* is well understood. Control larvae were fed on diet to which 0.15 ml distilled water had been added. The diet was replaced by fresh (uninfective) diet when consumed. Dead larvae and pupae were individually ground with a pestel and mortar in physiological saline and examined for the presence of spores. The survivors were examined as adults. Prior to this test of infectivity to *B. mori*, the viability of the *N. tyriae* spores was examined. Ten Cinnabar moth larvae, collected from a site at Silwood Park where a low prevalence of microsporidia had been found, were examined and found negative for *N. tyriae*. The remaining 35 from the collection were allowed to feed on ragwort leaves painted with the spore suspension that was used in the infectivity trial with *B. mori*. Dead larvae and pupae were removed but not examined (due to field work elsewhere) and the survivors were examined 18 days post infection.

Germination of Spores

Purified spores of *N. tyriae* were suspended in a solution of 3% H_2O_2 in 0.1 M KCl and were examined by light microscopy for extrusion of polar tubes.

Preparation of DNA from Purified Spores of N. bombycis and N. tyriae

A suspension of about 5×10^6 spores of *N. bombycis* was centrifuged at 900*g* for 2 min. The pellet was resuspended in 5 µl distilled water and 200 µl of STE medium (100 mM NaCl, 100 mM Tris–HCl, pH 8.0, 1 mM EDTA), shaken in a Vortex mixer, and centrifuged at 800*g* for 1 min. The pellet was disrupted with 150 mg of glass beads (Sigma G-8772, 425–600 µm diameter) and 150 µl STE in a Vortex mixer for 30 s and placed immediately at 95°C for 5 min. The tube was centrifuged at 17,500*g* for 2 min and 10 volumes of supernatant were added to 1 volume of 3 M sodium acetate. The DNA was precipitated with ethanol, rinsed in ethanol, and redissolved in TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA).

For *N. tyriae* 3×10^7 spores were suspended in 300 µl TE buffer. An equal volume of 0.5-mm Zirconium beads was added and the spores disrupted in a Minibead Beater (Biospec Products) for 30 s. The disrupted spores were incubated with 2 µl of a 10 mg/ml stock solution of Proteinase K for 2 h at 55°C. The DNA was extracted in phenol/chloroform and precipitated with ethanol.

Comparison of PCR Amplification Products of N. tyriae and N. bombycis (SES-NU)

Two sets of primers were used:

(1) KAI-01 = 5′-GAATTCAAGCTTGTAGTAGAGACCCAAATATC-3′ and
KAI-02 = 5′-GAGCTCGCATGCACTGTTCAGATATGGTCCTTATCG-3′; and
(2) VN001F = 5′ CTGCAGGTACCACCAGGTTGATTCTGCCTGAC-3′ and
VN001R = 5′GAGCTCGCATGCGGTTTACCTTGTTACGACTT-3′.

KAI-01 and KAI-02 were designed from a sequence of a putative pseudogene of the small subunit rRNA (SSU rRNA) of *N. bombycis* strain SES-NU (Kawakami *et al.*, 1995). VN001F and VN001R were based on highly conserved regions of the SSU rRNA (Vossbrinck *et al.*, 1987).

The PCR mixture (100 µl) contained 5 pm of each primer, 0.2 mM each dNTP, 2.5 units Taq DNA polymerase, and 10 ng of appropriately diluted template DNA in the PCR buffer (10 mM Tris–HCl buffer, pH 8.4, 50 mM KCl, 1.5 mM Mg Cl_2). The mixture was overlain with 20 µl mineral oil and the reaction was run in a thermocycler (Astec, Program Temp Control System PC-700) for 40 cycles each of 94°C for 1 min, 55°C for 2 min, and 72°C for 30 s.

A 3-µl aliquot of the PCR product was mixed with 5 µl TE buffer and 2 µl of 40% sucrose containing 0.5% bromophenol blue and subjected to agarose gel electrophoresis using 0.7% agarose (Sigma type II medium EEO). pHY DNA markers (Takara Shuzo Co.) were also applied. The gels were stained with ethidium bromide.

PCR Amplification and Sequencing of SSU rDNA of N. tyriae

The following primers, designed by Baker *et al.* (1995) were used:

18f = 5′-CACCAGGTTGATTCTGCC-3′ and
1537r = 5′-TTATGATCCTGCTAATGGTTC-3′.

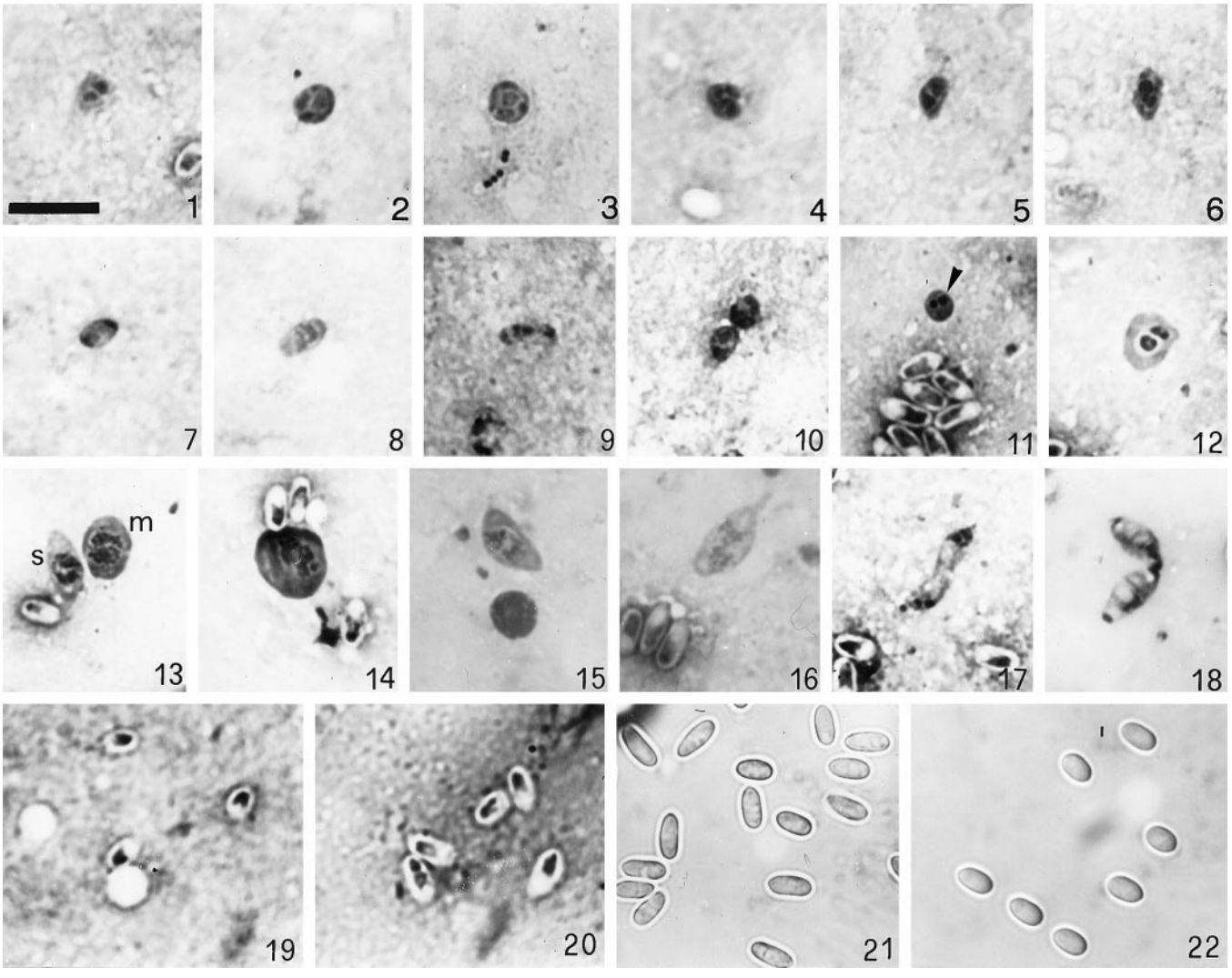
Reactions were carried out in 40-µl volumes containing 10 pmol each primer, 0.2 mM each dNTP, 1 unit of Taq polymerase and 10 ng of *N. tyriae* DNA in PCR buffer (10 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$). The reactions were run in a thermocycler (Techne, GeneE) at 94°C for 2 min, then 45 cycles of 92°C for 20 s, 45°C for 15 s, and 72°C for 45 s, with a final extension of 72°C for 5 min. PCR products were cleaned using Wizard PCR preps (Promega, UK) and cloned into the pGem-T Easy Vector system (Promega, UK). One clone was amplified with SP6 and T7 (the pGem-T Easy Vector primers) for sequencing with the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham) and run on an Applied Biosystems 373 automated sequencer. Six primers were used to obtain the whole sequence in both directions: SP6, T7, and 3F = 5′-GTCCAAGGA/TC/GGCAGCAGGC-3′, 4F = 5′-CACCACCAGGAGTGGAGTGTG-3′, 5R = 5′-CACA/AC/TCCACTCCTTGTGG-3′, and 6R = 5′-GCCRGCTGCTGTCCTTGGAC-3′.

RESULTS

Thirty four out of 54 (63%) *T. jacobaeae* larvae collected from Beale Park were infected with microsporidia, 33 being *N. tyriae* and 1 being a *Nosema* sp. smaller than *N. tyriae*. The infection with *Nosema* sp. was light. Most of the infections with *N. tyriae* were heavy and generalized, probably infecting all tissues, although only silk glands, gut wall, and adipose tissue were studied in detail. The larvae appeared unaffected and continued to move actively and eat voraciously until killed.

Nosema sp.

All stages of this species were noticeably small even for microsporidia and were difficult to photograph by light microscopy. The single infection was very light and it was not possible to obtain sufficient fresh spores for photography at a paraffin/oil interface. All developmental stages were diplokaryotic (Figs. 1–10). Rounded stages with one or two diplokarya and nuclei consisting of chromatic material within a pale area (Figs. 1–4) were probably meronts. These measured 2.0–4.0 µm in



FIGS. 1–10. Light micrographs of developmental stages of *Nosema* sp. Bar on Fig. 1, 10 µm and refers to all micrographs. Figs. 1–3. Binucleate meronts. Fig. 4. Tetranucleate meront. Figs. 5–9. Elongate stages, probably sporonts. Fig. 10. Dividing meront.

FIGS. 11–18. Light micrographs of developmental stages of *Nosema tyriae.* Fig. 11. Putative sporoplasm (arrowhead). Fig. 12. Binucleate meront with compact nuclei. Fig. 13. Binucleate meront (m) and sporont (s) with thread-like nuclear material. Fig. 14. Meront with dividing diplokaryon. Figs. 15 and 16. Sporonts with thread-like nuclear material. Fig. 17. Tetranucleate sporont with compact diplokarya at the poles. Fig. 18. Dividing sporont; note banded cytoplasm.

FIG. 19. *Nosema* sp. stained spores.

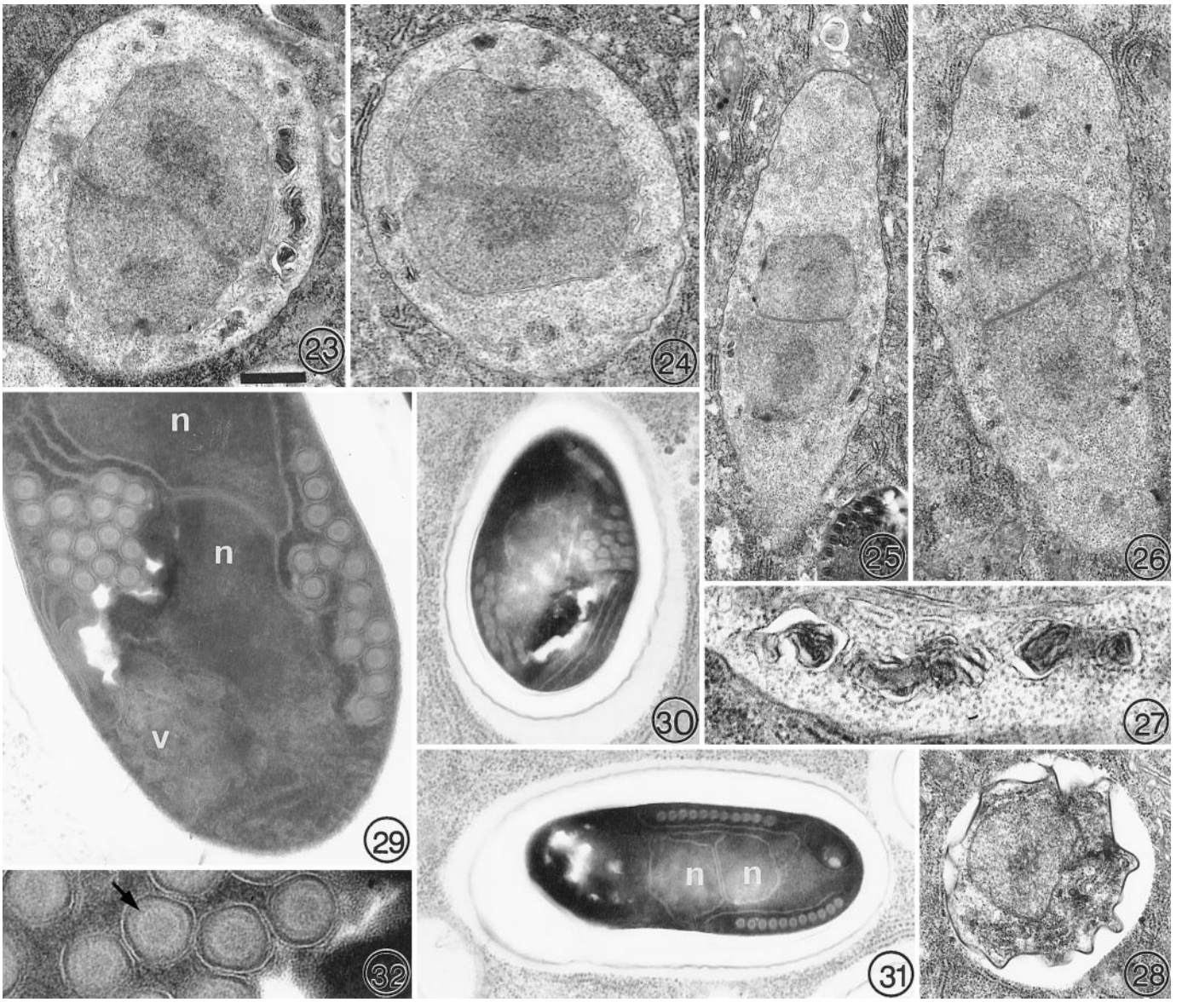
FIG. 20. *N. tyriae* stained spores.

FIG. 21. *N. tyriae* fresh spores.

FIG. 22. *Nosema bombycis* (isolate SES-NU) fresh spores.

diameter (mean = 3.1 × 2.9 µm, $n = 14$) when binucleate and 3.5 µm in diameter ($n = 3$) when tetranucleate. Elongate stages with more compact nuclei placed at central or polar positions, some with banded cytoplasm (Figs. 5–9) were probably sporonts. These measured 4.0 × 2.0 to 7.0 × 3.0 µm (mean = 5.1 × 2.6 µm, $n = 7$). A single dividing stage observed (Fig. 10) was probably merogonic. Spores in stained smears (Fig. 19) were ovoid and measured 3.8 ± 0.14 × <2.0 ± 0.08 µm ($n = 9$).

At the ultrastructural level *Nosema* sp. exhibited prominent vesicles in the cytoplasm containing electron-dense material and folded membranes. The cytoplasmic organization was well preserved, showing that these vesicles were not artifacts. They were present in meronts with simple plasma membranes (Figs. 23, 24, and 27) and early sporonts with surface coats (Figs. 25 and 26). Nuclei often contained a single dense mass of chromatin even when division was indicated by the presence of electron-dense spindle plaques (Fig. 24). Sporoblasts were crenated and had abundant cisternae of endoplasmic reticulum (Fig. 28). Spores with polar



FIGS. 23–32. Transmission electron micrographs of *Nosema* sp. Scale bar on Fig. 23 applies to all figures. Figs. 23 and 24. Diplokaryotic meronts, showing prominent membrane aggregates in cytoplasm. Bar, 0.57 µm. Figs. 25 and 26. Diplokaryotic sporonts also with membrane aggregates in cytoplasm. Bars, 0.8 µm and 0.6 µm, respectively. Fig. 27. Enlargement of part of Fig. 23, showing detail of membrane aggregates in cytoplasm. Bar, 0.24 µm. Fig. 28. Crenated sporoblast. Bar, 0.6 µm. Fig. 29. Posterior region of spore showing diplokaryon (n), polar tube coiled in an irregular cluster, and posterior vacuole (v). Bar, 0.2 µm. Fig. 30. Spore showing irregular cluster of polar tube coils. Bar, 0.4 µm. Fig. 31. Spore showing diplokaryon (n) and polar tube in a single rank. Bar, 0.4 µm. Fig. 32. Transverse sections of polar tube coil showing concentric layers including a ring of about 20 longitudinally running fibrils (arrow). Bar, 65 nm.

tubes in two arrangements were observed in this material. Some showed up to 15 isofilar coils in irregular clusters (Figs. 29 and 30) with about 20 longitudinally running "fibrils" in cross section (Fig. 32). Others had about 12 isofilar coils in a single rank (Fig. 31). Other details of the spores were not seen.

Nosema tyriae n.sp.

As with the unnamed *Nosema* sp. merogony was limited to binary fission of stages with two diplokarya but all stages were appreciably larger. Tetranucleate stages (Fig. 14) were rare, as were dividing stages, so that binucleate stages (Figs. 12 and 13) were massively predominant. This suggests that cytoplasmic fission follows rapidly after nuclear division. Meronts were rounded when extracellular but were more elongate when packed intracellularly, indicating plasticity of the plasma membrane at this stage. The cytoplasm was uniformly stained, sometimes dark, sometimes light according to the degree of spread in the smear. Nuclei

appeared either as compact red spots within a clear area in the cytoplasm (Fig. 12) or as a labyrinth of threads resembling chromosomes (Fig. 13). Measurements varied according to the area of the smear with large, more spread out stages in the thinner areas. They ranged in size from 2.0 μm in diameter to 7.0 × 6.5 μm, with some binucleate stages attaining the same size as the largest tetranucleate stages. Means and standard errors were 4.4 ± 0.31 × 4.0 ± 0.30 μm ($n = 19$) for binucleate meronts and 4.8 ± 0.27 × 4.4 ± 0.28 μm ($n = 13$) for tetranuclate meronts.

Sporonts were always spindle shaped, whether intra- or extracellular, presumably because the presence of the surface coat prevented adoption of a spherical form when released from cells. Sizes varied from 7.0 × 2.5 to 11 × 2.5 μm, with means of 8.1 ± 0.23 × 3.25 ± 0.13 μm for binucleate forms ($n = 20$) and 9.8 ± 0.4 × 3.3 ± 0.3 μm ($n = 5$) for tetranucleate forms. The shorter, stouter forms had uniformly stained cytoplasm similar to that of the meronts (Figs. 15 and 16) and the nuclei often appeared as labyrinths of threads. In mature sporonts, four compact nuclei were arranged as two pairs lying toward the extremities (Fig. 17). Division stages were rare, each sporont giving rise to two binucleate sporoblasts which were ellipsoid with banded cytoplasm and two compact nuclei (Fig. 18).

Spores were elongate ellipsoid, slightly pointed at the anterior end and usually without an obvious posterior vacuole. Fresh spores (Fig. 21) measured 4.7 ± 0.08 × 2.03 ± 0.02 μm ($n = 50$). In stained smears, spores of *N. tyriae* (Fig. 20) were noticeably longer than those of the smaller *Nosema* sp. (Fig. 19) but were of the same width.

One example only was found of a small rounded stage 2.5 μm in diameter with pale uniformly stained cytoplasm and two compact round nuclei (Fig. 11). This may have been a sporoplasm extruded from a spore.

Ultrastructural studies showed that all stages were in direct contact with host cell cytoplasm. Meronts were rounded or slightly elongate cells lying in close contact with host cell cytoplasm (Fig. 33). Some showed the complete diplokaryon, whereas others showed only one nucleus due to the plane of section. The cytoplasm was permeated by endoplasmic reticulum and occasional small vesicles. Division was rarely seen (Fig. 36). Sporogonic stages had universally contracted away from the host cell cytoplasm, presumably because of less rapid penetration of fixative through the surface coat (Figs. 37–39). Several sections were seen in which the surface of sporonts was connected to host cell cytoplasm by tubular structures 20 nm in diameter with prominent cross banding (Figs. 37 and 38). Sporonts were the least altered in shape (Figs. 37 and 39) but sporoblasts were markedly stellate (Fig. 40). In meronts and sporonts, there were signs of nuclear activity in the form of division spindles (Fig. 35) or centriolar plaques surmounted by 2–3 vesicles at the nuclear surface or at the base of pockets indented deeply into the nuclei (Fig. 34). There were also electron-dense intranuclear structures forming a single mass (Fig. 39) or separate smaller structures associated with spindle microtubules (Figs. 33 and 37). Division of sporonts was not observed but the regular occurrence of pairs of sporoblasts (Fig. 45) and spores (Fig. 46) provided evidence supporting the disporoblastic sporogony seen by light microscopy.

Spores had a 130-nm-thick endospore and a 20-nm exospore with a wavy outline (Fig. 41). The anchoring disc of the polar tube lay within the polar sac, which formed a deep dome at the anterior extremity and extended laterally over the compact membranes of the polaroplast (Figs. 41–43). Over the anchoring disc the endospore was thinned to about 35 nm. The straight section of the polar tube narrowed gradually from about 160 nm at its insertion into the anchoring disc to 90 nm where it began to coil (Fig. 43). The polaroplast was organized into compact and loose membranes (Figs. 41–43). The compact membranes of about 20 double layers were directed in an almost anterior to posterior direction around the straight section of the polar tube. The loose membranes were arranged almost at right angles to the anterior–posterior axis. The polar tube altered direction as it passed through the loose membranes from the center of the spore to the peripheral cytoplasm (Fig. 43). The coiled region of the polar tube was arranged as 10.5 to 14 isofilar turns in various spores, always in a single rank close to the spore wall. The diplokaryon nuclei were slightly separated, lying one behind the other within the polar tube coil. Transverse sections of the polar tube (Fig. 44) showed a lucent ring (a) around a dense ring, (b) a grey ring, (c) bounded internally by about 18 longitudinally running fibrils (f). Within these was another lucent ring (d) around a central core (e). At the posterior end of the spore was a membrane-bound vacuole (Fig. 41) with amorphous contents. The cytoplasm occupying the rest of the spore was packed with ribosomes around a few longitudinally running cisternae of endoplasmic reticulum.

A sequence of 1236 bp was obtained for the 16S rRNA gene of *N. tyriae* (GenBank Accession No. AJ012606), using primers 18f and 1537r. The sequence differs by 6 bases from *Nosema bombycis* (SES-NU, GenBank Accession No. D85503). There was one insertion, three transitions, and two transversions. Using primers KAI-01 and KAI-02, no amplification was achieved with *N. tyriae* but a product of approximately 860 bp was obtained with *N. bombycis* SES-NU. Primers VN001F and VN001R gave products of 1250 bp with *N. tyriae* and with SES-NU.

In the tests with monoclonal antibodies (Mabs), *N. tyriae* reacted positively, but at a moderate level, with

about 50% of spores adsorbing the Latex beads coated with anti-SES-NU Mab. *N. tyriae* reacted negatively with the anti-*Vairmorpha* M11 and M12 Mabs, with less than 10% of spores binding the Mab-coated beads. *N. bombycis* SES-NU and *Vairimorpha* spp. M11 and M12 were strongly positive with their respective Mabs.

Of 35 Cinnabar moth larvae which had been fed on ragwort leaves contaminated with *N. tyriae,* 16 were examined 18 days post infection and all were heavily infected. The same harvest of spores was used to test the infectivity and virulence of *N. tyriae* to silkworms. Up to 93.2% of these spores germinated in the H_2O_2 in KCl solution. It was found that most of the *B. mori* larvae which had been fed with *N. bombycis* spores died as 3rd instars, i.e., 23/30 fed with diet painted with 10^4 spores and 24/30 fed with diet painted with 10^3 spores. The remainder died as 4th instars. All larvae were examined at death and all were infected. In contrast, many *B. mori* fed with *N. tyriae* spores survived to adult: with discs seeded with 10^4 spores, the adult figures were 25/50, 11/50, and 9/50 for the three replicates and with discs seeded with 10^3 spores, the figures were 20/50, 7/50, and 11/50. Six larvae from each of two replicates fed with 10^3 spores of *N. tyriae* showed no infection when they died as 4th instars. All the other larvae were infected. The others died as 4th or 5th instars or pupae, none as 3rd instars. None of the control larvae became infected.

DISCUSSION

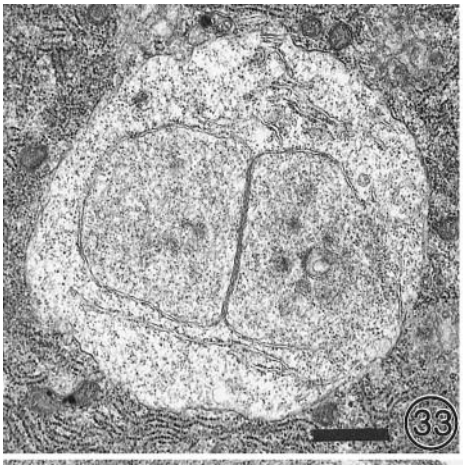
The parasite reported by Philogène and Massalski (1977) from *T. jacobaeae* was found in diapausing pupae. The authors were interested in the phenomenon of diapause and paid special attention to the cephalic neuroendocrine system, recording that there was a high concentration of microsporidia in the fat body surrounding the nerve centers but none in the nerve cells. There was no comment on the presence or absence of microsporidia in other tissues. They gave details of the spores, including measurements of 2.75×1.5 µm (presumably fixed and stained in resin-embedded sections) and presented electron micrographs showing up to 13 coils of the polar tube with three distinct concentric layers in cross section. Having classified the organism in "Nosématidée", they concluded that it was binucleate but this was not confirmed by observation. As far as we can determine, there have been no further studies on microsporidia in *T. jacobaeae.*

Our initial suspicion that our collection of *T. jacobaeae* larvae harbored two species of microsporidia was reinforced by studies which showed that all stages of the unnamed species of *Nosema* were smaller than those of *N. tyriae* and that only in the small *Nosema* sp. were there cytoplasmic vesicles enclosing electron-dense material and membranes. Spores of *N. tyriae* were more elongate than those of *Nosema* sp. Infection with both parasites was found in gut wall, fat body, and silk glands but other tissues were not processed and could not be excluded as sites of infection. On size alone it is not possible to identify either of our parasites as that found by the Canadian workers, as the measurements they gave, although appreciably smaller than those of our species, were probably taken from fixed and embedded sections. Our observations that infection did not cause mortality or inhibit feeding, is in accord with the Canadian work.

Light microscopic studies confirmed that all stages of both species are diplokaryotic and that, in *N. tyriae* at least, sporogony is disporoblastic. Electron microscopy showed that development is in direct contact with host cell cytoplasm. These are characters of the genus *Nosema* to which both species clearly belong.

Comparison of the 16S rRNA gene sequence of *N. tyriae* with the corresponding sequences of several species of *Nosema* showed that the parasite is closest to *N. bombycis* Naegeli, 1857 but differs from the sequence D85503 of the stock *N. bombycis* (SES-NU) from *Bombyx mori* held at the National Sericultural Experimental Station Tokyo. Several complete 16S rRNA sequences attributed to *N. bombycis* have been deposited in GenBank. However, not all of the isolates were derived from the type host, *B. mori.* The sequences D85504 and L39111 (actually sequences of the same isolate, designated Sd-NU-IW8401, obtained from *Spodoptera depravata*) differ from each other by six bases, indicating a sequencing error in one of these. Sequence D85504 differs by nine bases from D85503. We propose that the sequence D85503 should be used as the reference sequence of *N. bombycis* and that determination of the species status of other isolates should be made against this sequence. *N. tyriae* differs from D85503 by six bases.

Although it is difficult to be precise about the number of nucleotide differences, which represent differences between species, without knowing how much intraspecific variation there is within populations of the same species, the observation that no amplification was obtained from *N. tyriae* DNA using primers KAI-01 and KAI-02 (Kawakami *et al.*, 1995) support our conclusion that the Cinnabar moth isolate is a new species. It is thought that the primers of Kawakami *et al.* (1995) amplify a pseudogene closely related to ribosomal DNA. So far these primers have been effective in amplifying DNA *only* from *N. bombycis* derived from *B. mori* (the reference strain SES-NU and strains from Northern and Southern India and China). No DNA was amplified from *Nosema* sp. (originally identified as *N. bombycis*) from *Spodoptera exigua* (Yasunaga *et al.*, 1992). Amplification of DNA from *N. tyriae* with primers based on those designed by Baker *et al.* (1995) was successful under the same conditions, showing that the technique was not faulty.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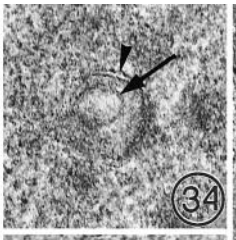
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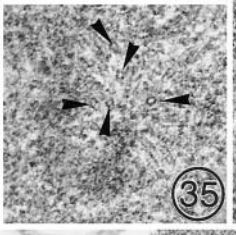
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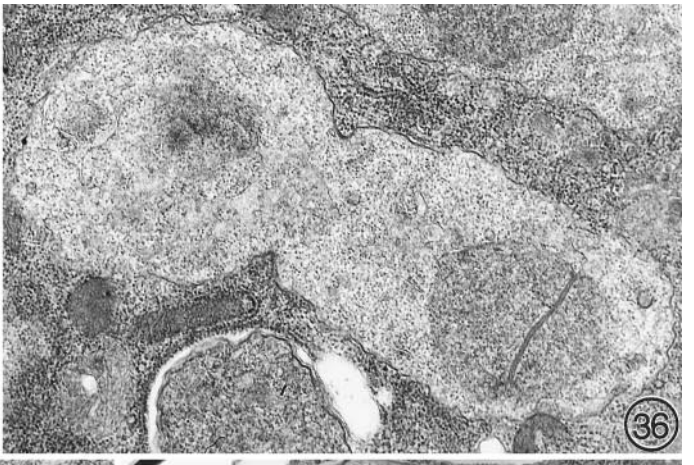
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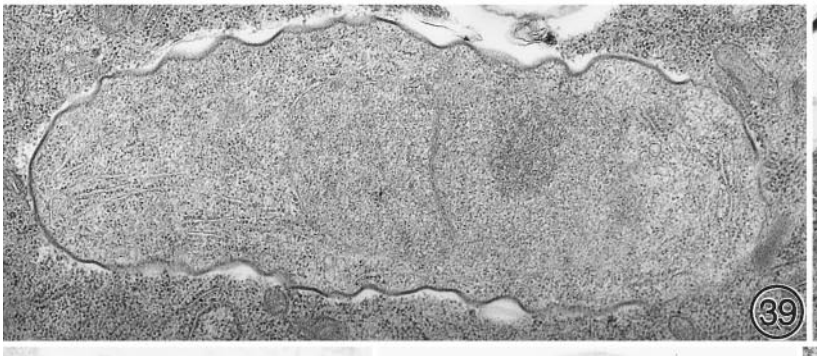
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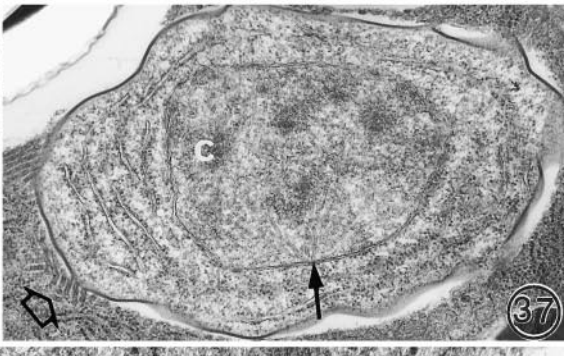
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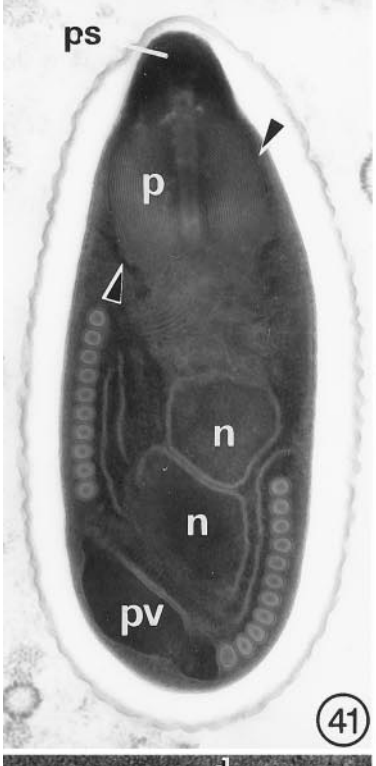
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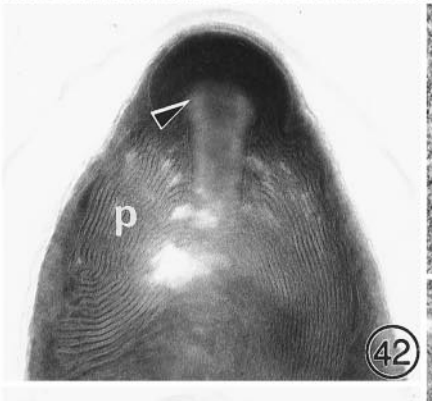
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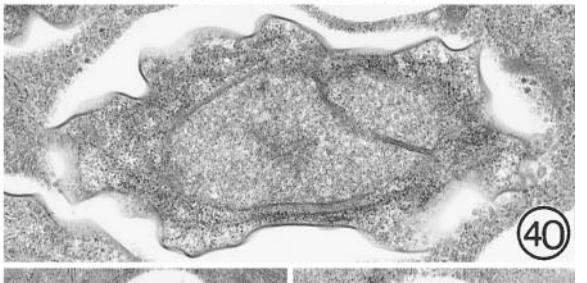
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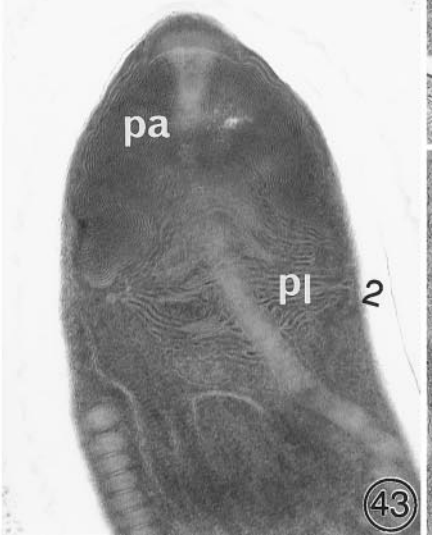
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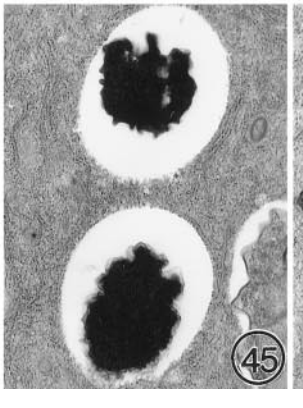
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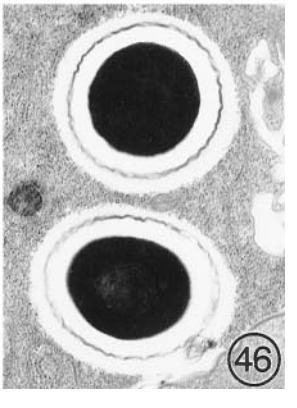
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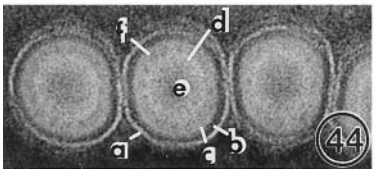
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A monoclonal antibody raised against *N. bombycis* strain SES NU (Yakult Co. Tokyo) reacted positively with *N. tyriae* but moderately in comparison with *N. bombycis* SES-NU. Reactivity of this Mab with two microsporidia from *S. depravata,* one with almost spherical spores measuring 3.1 × 2.4 μm and the other with elongate spores measuring 4.2 × 2.2 μm (Ishihara and Iwano 1991) led the authors to conclude, at that time, that both microsporidia were *N. bombycis.* Although it could be argued that *N. bombycis* might assume a different morphology in a different host, it is not reasonable to suppose that it would form two very different spore types in that host. As monoclonal antibodies are raised to a particular epitope, a more likely explanation of the reactivity observed with these microsporidia and with *N. tyriae* is that a similar epitope exists on closely related species so that cross reactivity occurs. One of these isolates from *S. depravata* is Sd NU IW8401 from which sequence D85504 is derived. This sequence differs by nine bases from the *N. bombycis* reference sequence D85503 and Sd NU IW8401 is no longer considered to be *N. bombycis.*

N. tyriae has elongate spores (Fig. 21), measuring 4.7 × 2.03 μm in comparison with the broadly ovoid spores of *N. bombycis* reference stock (Fig. 22), measuring 3.6 × 2.3 μm (Ishihara and Iwano, 1991). Few details are available on the ultrastructure of *N. bombycis* derived from silkworms which can be used to differentiate it from other *Nosema* spp.. Ishihara (1970) described prespore stages. Iwano and Ishihara (1991) showed that *N. bombycis* produces early spores responsible for within-host transmission with only 3–3.5 coils of the polar tube and that spores produced later in infection, which have 11 or more coils of the polar tube, are responsible for between-host transmission. It is not known whether *N. tyriae* produces early spores with a few coils of the polar tube, as all infections were well advanced. It is unlikely that the small *Nosema* sp. in *T. jacobaeae* represents the early stages of *N. tyriae,* producing spores responsible for autoinfection, as early spores of *Nosema* spp. typically have only a few coils (3–4) of the polar tube (Iwano and Ishihara, 1991; Sagers *et al.*, 1996; Solter and Maddox, 1998). The organization of organelles within the late spores of *N. bombycis* has not been described fully but available electron micrographs (Takizawa *et al.*, 1975; Iwano and Ishihara, 1991) suggest that the anchoring disc is flatter, less deeply domed than that of *N. tyriae.* The spore size and shape coupled with the six bases difference in the 16S rRNA gene sequence and nonamplification by primers KAI-01 and KAI0-2 led us to the conclusion that this species is not identical with *N. bombycis.* This view has been strengthened by the comparisons of *N. tyriae* and *N. bombycis* in their reactivity with a monoclonal antibody raised to *N. bombycis* and virulence in *B. mori.*

The Mab provided by Yakult Co. Tokyo was used by Pieniacek *et al.* (1996) to identify two strains of *N. bombycis,* one from *B. mori* and the other, designated Y9101, from *Spodoptera exigua.* In comparison with these reference strains, *Nosema trichoplusiae* (strain ATCC 30702) was synonymized with *N. bombycis,* as the 16S rRNA sequences were identical (GenBank Accession No. U09282). Although there may have been cross reactions using this Mab and the sequence was longer by one nucleotide than that for SES-NU obtained by Hatakeyama *et al.* (1997), it is reasonable to consider that this small difference falls within intraspecific variation. Nevertheless, it would be interesting to see whether DNA from any of the isolates examined by Pieniacek *et al.* (1996), including *N. trichoplusiae,* would be amplified by the primers of Kawakami *et al.* (1995).

The small *Nosema* sp. in *T. jacobaeae* was clearly differentiated from *N. tyriae* by size and by the presence of prominent vesicles containing folded membranes in the cytoplasm of prespore stages. Two types of spore were seen, one closely resembling those of *N. tyriae.* The two types of spore may represent variation within the small *Nosema* sp. but could also have arisen from an early double infection with *N. tyriae,* since the Cinnabar moth larvae had been kept together feeding

FIGS. 33–46. Transmission electron micrographs of *Nosema tyriae.* Scale bar on Fig. 33 applies to all figures. Meronts in close contact with host cell cytoplasm, sporogonic stages contracted away from host cell cytoplasm due to penetration difficulty of fixative. Fig. 33. Meront showing evidence of division in the diplokaryotic nuclei. Bar, 0.6 μm. Fig. 34. Enlargement of right-hand nucleus from Fig. 33 showing polar vesicle (arrow) within an invagination of the nuclear envelope (arrowhead) viewed from above. Bar, 0.2 μm. Fig. 35. Enlargement of left-hand nucleus from Fig. 33 showing spindle microtubules in cross section (arrowheads). Bar, 0.2 μm. Fig. 36. Dividing meront showing one of the two diplokarya in the plane of section. Bar, 0.5 μm. Fig. 37. Early sporont sectioned through one nucleus of the diplokaryon showing radiating spindle microtubules (arrow) and putative chromosomes (c): note electron-dense surface coat breached by tubular structures connecting parasite and host (open arrow). Bar, 0.48 μm. Fig. 38. Enlargement of part of Fig. 27 showing detail of tubular structures. Bar, 0.2 μm. Fig. 39. Elongate sporont showing one diplokaryon in the plane of section. Bar, 0.6 μm. Fig. 40. Sporoblast showing diplokaryon and crenated outline. Bar, 0.55 μm. Fig. 41. Longitudinal section of spore showing insertion of polar tube into polar sac (ps) with lateral extensions (arrowhead) over the polaroplast (p). The diplokaryon (n), polar tube coiled in a single rank, and posterior vacuole (pv) are also visible. Bar, 0.3 μm. Fig. 42. Anterior end of spore showing anterior insertion of polar tube into polar sac (arrowhead) and anterior lamellae of polaroplast (p). Bar, 0.15 μm. Fig. 43. Anterior and middle regions of spore showing compact anterior lamellae of polaroplast (pa), loosely arranged posterior lamellae (pl), and connection of straight part of polar tube with the coiled region. Bar, 0.26 μm. Fig. 44. Transverse sections of the polar tube showing concentric layers a–f including about 18 longitudinally running fibrils (f) around a central core (e). Bar, 0.05 μm. Fig. 45. Paired sporoblasts. Bar, 0.8 μm. Fig. 46. Paired spores. Bar, 1.0 μm.

on ragwort leaves in a confined space for several days before all had been examined. As the spore structure was confused, we have chosen not to name the species. Insufficient material was available for molecular studies on the *Nosema* sp.

Although molecular biology is useful in confirming identity of taxa based on similar morphology or, conversely, confirming nonidentity of taxa which differ in morphology, confusion is created when the two techniques give conflicting results. The problem of identification of species which are morphologically very similar is highlighted by the discovery of microsporidia in Cinnabar moth and our attempts to determine the defining characters of *N. bombycis*. We suggest that designation of one 16S rRNA sequence (D85503) for *N. bombycis* is a step toward eliminating or at least reducing confusion.

TAXONOMIC SUMMARY

Nosema tyriae n.sp.

Type host: *Tyria jacobaeae*, Cinnabar moth.

Type locality: Beale Bird Park, Berkshire, England (National Grid Reference SU 619781).

Sites of Infection: Gut wall, silk glands, adipose tissue, probably generalized.

Merogony: Binary fission of stages with two diplokarya.

Sporogony: Spindle-shaped sporonts become elongate with polar diplokarya and divide into two sporoblasts.

Spores: Elongate ovoid 4.7 × 2.03 μm (fresh). Anchoring disc deeply domed. Polaroplast consists of closely packed membranes arranged vertically around the straight part of polar tube and loose membranes arranged horizontally behind these; polar tube forms 10.5–14 isofilar coils in a single regularly arranged rank, cross sections showing about 18 longitudinally running fibrils and several rings of differing electron density. Posterior vacuole with amorphous dense contents, not generally visible in fresh spores.

16S rRNA gene sequence: GenBank Accession No. AJ012606.

Specific name derived from host generic name.

Nosema sp.

Type host, locality, and tissues infected: As for *N. tyriae*.

Merogony: Binary fission of diplokaryotic stages.

Sporogony: Probably binary fission of diplokaryotic sporonts.

Spores: Ovoid 3.8 × <2.0 μm (stained) with up to 15 isofilar coils of the polar tube in irregular clusters showing about 20 longitudinally running fibrils and several rings of differing electron density. Possibly some spores with polar tube coils as in *N. tyriae*.

Cytoplasm of developing stages contain prominent vesicles with electron-dense material and folded membranes.

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