

Nosema ceranae n. sp. (Microspora, Nosematidae), Morphological and Molecular Characterization of a Microsporidian Parasite of the Asian Honey bee *Apis cerana* (Hymenoptera, Apidae)

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SUMMARY

Based on light microscopic and ultrastructural characteristics as well as on the nucleotide sequence of the small subunit ribosomal RNA coding region, the microsporidium *Nosema ceranae* n. sp., a parasite of the Asian honey bee *Apis cerana* is described. Merogonial stages and sporonts are diplokaryotic. Merozoites are mostly formed by cytoplasmic fission in quadrinucleate meronts and the number of merogonial cycles may vary. The sporogony is disporoblastic. The living mature spore is ovocylindrical, straight to slightly curved and measures $4.7 \times 2.7 \mu\text{m}$ whereas fixed and stained spores measure $3.6 \times 1.7 \mu\text{m}$. The polar filament is isofilar with a diameter of 96–102 nm and is arranged in 20–23 coils in the posterior and mid-part of the spore. In the anterior part of the polaroplast there are closely packed approximately 11 nm thick lamellae. The lamellae of the posterior polaroplast are thicker and less regular. In the posterior part of the mature spore a well fixed posterior body interpreted as a posterosome was often observed. Phylogenetic analysis, based on the sequence of the small subunit ribosomal RNA, places *Nosema ceranae* in the *Nosema* clade, as defined by *Nosema bombycis*, the type species of the *Nosema* genus.

Introduction

The only identified microsporidian species reported from the genus *Apis* until the present time is *Nosema apis*, infecting the European honey bee *A. mellifera*. This parasite is spread world-wide wherever beekeeping occurs [27]. There are reports of other microsporidian infections in *A. mellifera* [8, 10], in *A. florea* [3, 4, 5] and of *N. apis* in *A. cerana* [24, 30, 37]. However, in all these reports, the identity of the parasite is

uncertain. The reports of *N. apis* in *A. cerana* are based simply on observations in the light microscope of spores resembling those of this parasite.

This paper reports on a new microsporidian infection in *A. cerana*. The life cycle is briefly described along with the cytology with emphasis on the ultrastructure. As classification of microsporidia to a particular genus based only on morphological features may be deceptive [29], the small subunit ribosomal RNA (SSUrRNA) coding region sequence for *N. ceranae* is

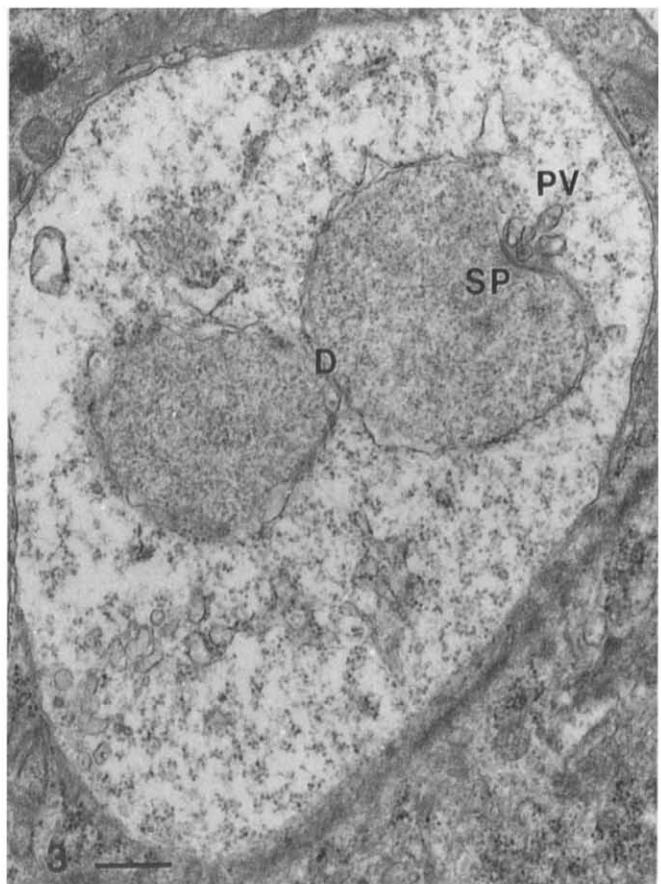
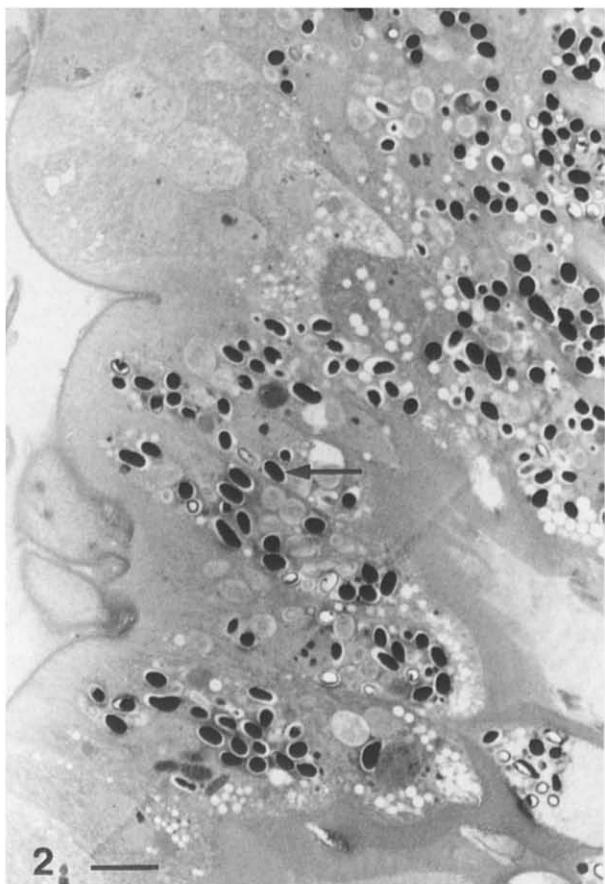
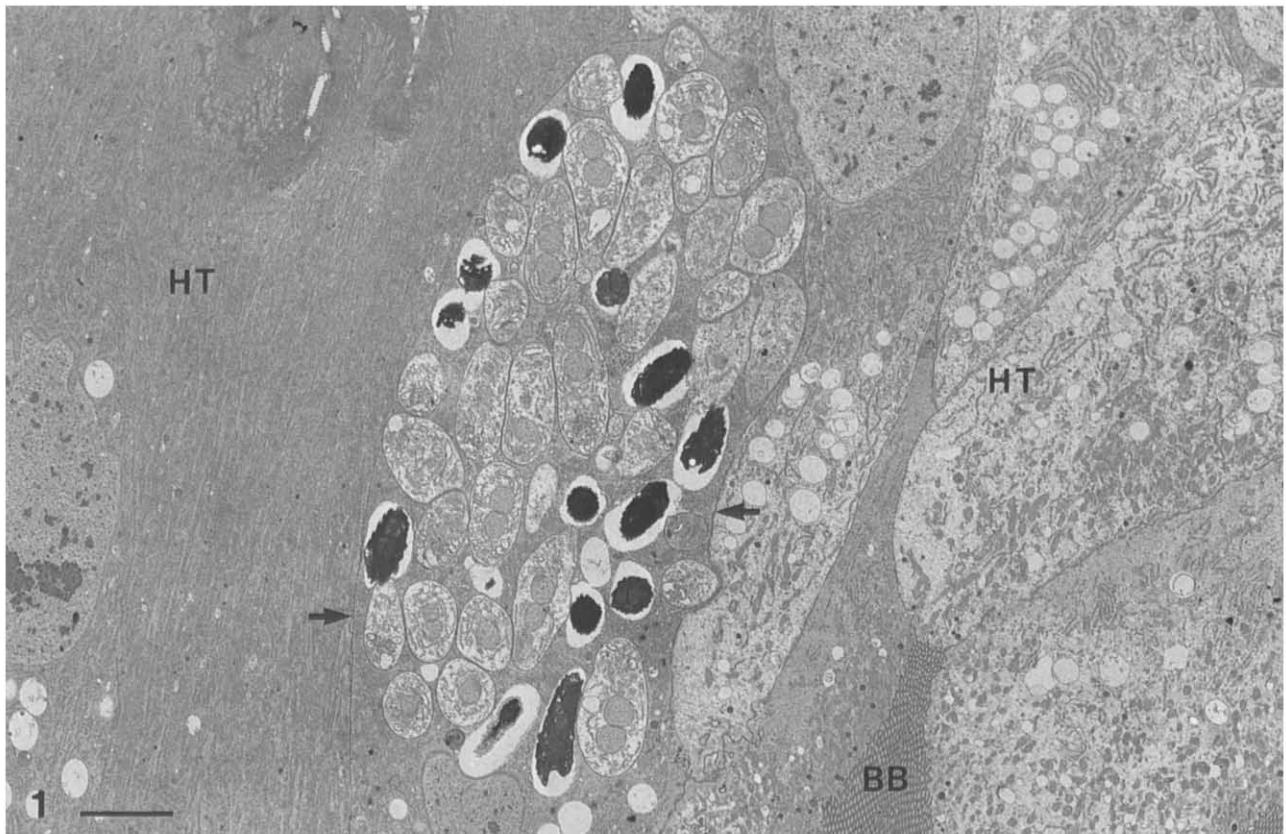


Fig. 1. A ventricular cell of *Apis cerana* infected by *Nosema ceranae* n. sp. surrounded by seemingly healthy tissue. Arrows indicate cell membrane of infected cell. BB = brush border, HT = healthy tissue. Scale bar = 10 μm . – Fig. 2. Light micrograph of heavily infected tissue with tightly packed parasites. Arrow indicates localization of the holotype (slide No. 950515-1 IF). Scale bar = 10 μm . – Fig. 3. Meront preparing for nuclear division. Note invagination of the nuclear membrane at the site of the spindle plaque. D = diplokaryon, SP = spindle plaque, PV = polar vesicles. Scale bar = 0.4 μm .

described. Based on the SSUrRNA sequence data, the relation of this species to some other species of microsporidia is discussed.

Material and Methods

One hundred worker bees of *Apis cerana* Fabricius, 1793 were collected in each of three managed colonies located in the mountain region 120 km south of Beijing, P.R. of China. Each bee was individually dissected and a small part of each ventriculus examined for the presence of microsporidian spores. Each infected specimen was cut in two halves. One half was used to make a spore solution in tap water which was kept frozen at -28°C . The other half was used for microscopy. On a separate occasion more spores were harvested from infected bees and frozen.

Preparations for Microscopy

Tissue specimens were fixed for transmission electron microscopy and light microscopy using 4% glutaraldehyde (v/v) in 0.067 M cacodylate buffer, pH 7.4, for three weeks. The material was kept refrigerated ($+7^{\circ}\text{C}$) during pre-fixation. After washing in cacodylate buffer, the specimens were post fixed for 2 h in 2% OsO₄ (w/v) in 0.1 M S-colloidine buffer. After dehydration in an ascending concentration series of ethanol solutions, followed by a propylene oxide solution, the tissue pieces were embedded in Epoxy resin (Agar 100) by routine procedures for electron microscopy.

Sections of the embedded material were mounted for light microscopy after contrast coloring with toluidine blue.

Thin sections of the embedded specimens were mounted on copper grids and stained with uranyl acetate followed by lead citrate. The preparations were examined in a Philips 201 or Philips 420 electron microscope at an accelerating voltage of 60–80 kV.

DNA Isolation, PCR Amplification, Cloning, Sequencing of the SSUrRNA Coding Region, and Phylogenetic Analysis

DNA extraction, PCR amplification, cloning, and sequencing of the SSUrRNA sequence was done as described previously [35]. Briefly, spores were disrupted with glass beads (Cat. No. G-9139 Sigma, St. Louis, MO) in a buffer containing proteinase K and 1% lauryl alcohol polyether (Laureth 12, PPG Industries Inc., Gurnee, IL). After an overnight incubation at 55°C , proteinase K was inactivated by heating the sample at 95°C for 10 min. This DNA preparation was stored at 4°C until used. For amplification of the entire *N. ceranae* SSUrRNA coding region, PCR primers MICRO-F and MICRO-R were used [35].

For phylogenetic analysis, the following microsporidial SSUrRNA sequences were retrieved from the GenBank database: *N. apis* (U26534), *N. furnacalis* (U26532), *N. necatrix* (U11051), and *N. bombycis* (U09282). The archaeabacterium, *Halobacterium halobium* (M11583), was included in the analysis as an outgroup to root the phylogenetic tree. The *N. necatrix* SSUrRNA sequence was used instead of a sequence submitted earlier as *Vairimorpha necatrix* [36]. The latter sequence shows 8 differences with the U11051 sequence. These differences may result from sequencing errors in the *V. necatrix* sequence (Pieniazek, unpublished). Sequences were aligned with the program CLUSTAL W [33]

and the phylogenetic analysis was accomplished with the computer program package PHYLIP [11].

Results

Prevalence, Pathology and Life Cycle

The prevalence of infected bees was low. Of 100 bees investigated in each of three colonies spores were found in 1, 3 and 5 bees, respectively. Spores were only found in the intestinal tract where the epithelial cells of the ventriculus were infected. Since no spores were found in other parts of infected insects, the infection may be tissue specific.

Infected cells or cell regions, even in heavily infected specimens, often occurred isolated surrounded by seemingly healthy tissue (Fig. 1). Infected cells became filled with tightly packed parasites in all developmental stages (Fig. 2) and infected cells were shed into or burst open into in the gut lumen where mature spores were released. These spores may germinate and reinfect the epithelial cells or leave the insect with the feces.

There may be more than one merogonial cycle. Only meronts with two diplokarya dividing by simple binary fission were observed. Each sporont appeared to divide at least once yielding stages that developed into sporoblasts. All stages throughout the life cycle are probably diplokaryotic.

There were no outward clinical signs of disease in infected individuals. Heavily infected ventriculi appeared whitish and swollen compared with uninfected ventriculi but it was necessary to confirm the presence of infection by microscopic examination.

Presporal Stages

The cytoplasm of merogonial stages observed contained a large number of free ribosomes and weakly developed rough endoplasmic reticulum (RER). The plasma membrane was approximately 10 nm thick and in direct contact with the host cytoplasm (Fig. 3). Apparently newly released merozoites were 3.2–3.7 μm wide with the diplokaryon measuring 3.1–3.3 μm . The largest merozoite nuclei observed measured 1.6 μm across the line of apposition. The nuclear envelope had two unit membranes with pores and a perinuclear cisterna of traditional type. Cytoplasmic fission was observed in meronts containing two diplokarya only. The number of merogonial cycles could not be established and may be variable. Spindle plaques were sometimes observed in invaginations of the nuclear membrane (Fig. 3). As the spindle tubules converging from the chromosomes towards the plaque became visible, the nuclei often appeared pointed towards the plaque (Fig. 4).

At the onset of sporogony, the electron dense primordium of the exospore measuring about 16 nm was secreted in patches on the plasma membrane of the developing sporont (Fig. 5). The sporonts, and their diplokarya, were of similar size as in merozoites. Spo-

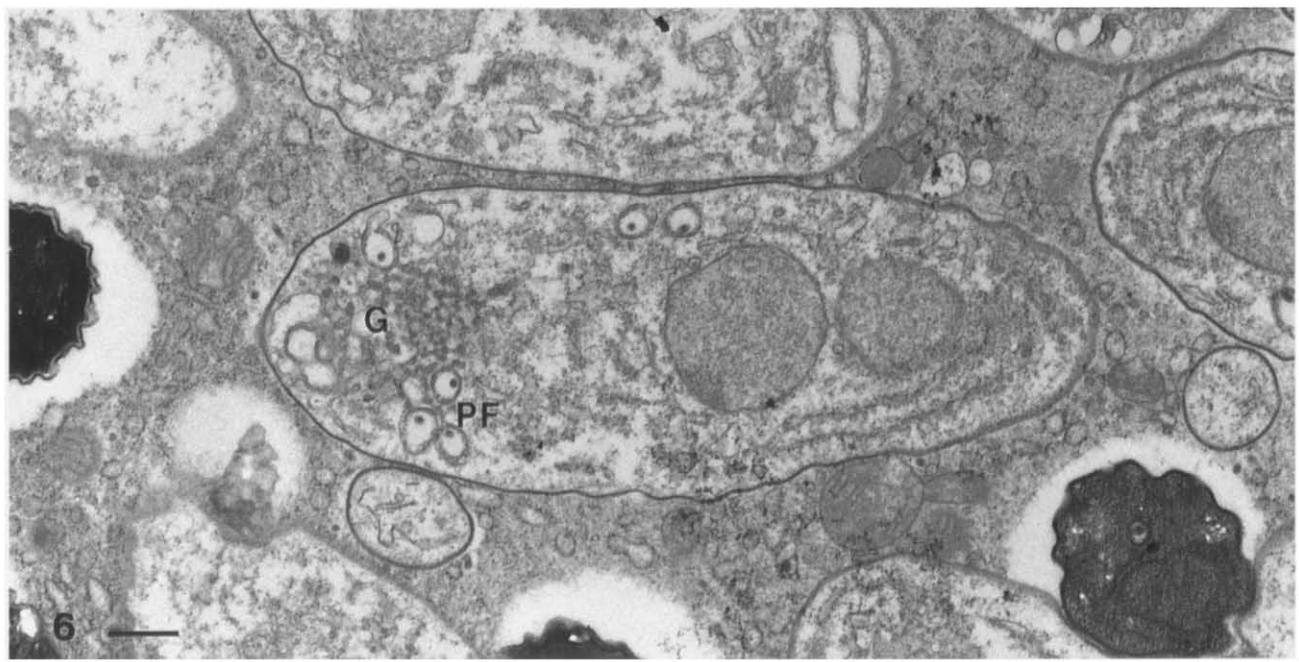
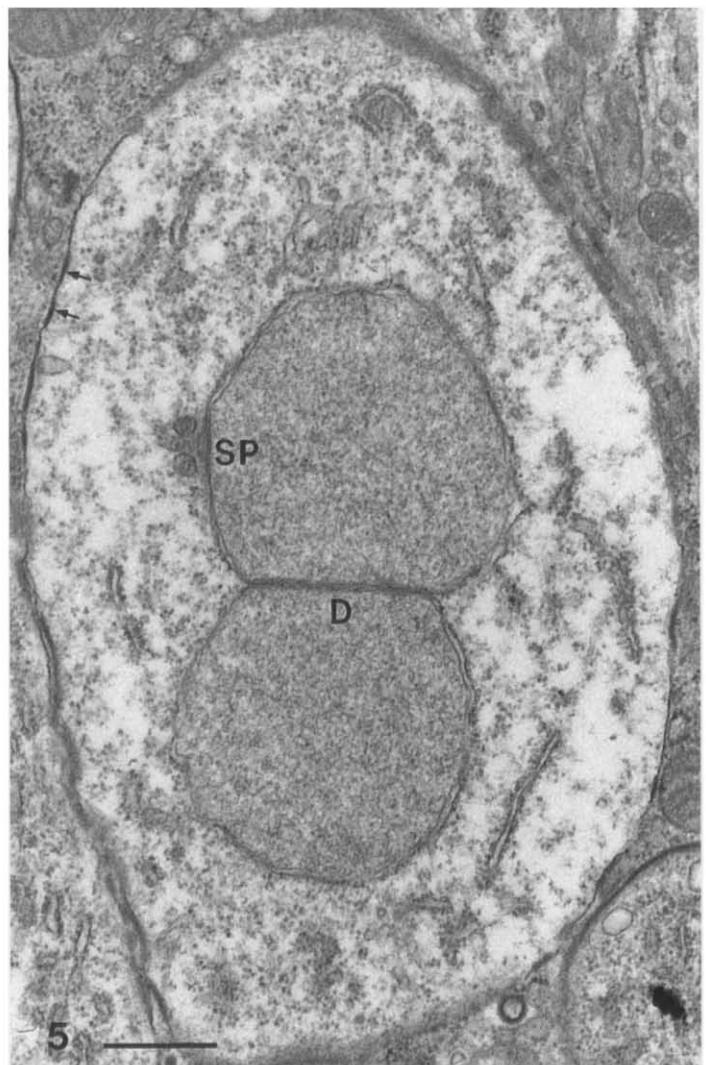
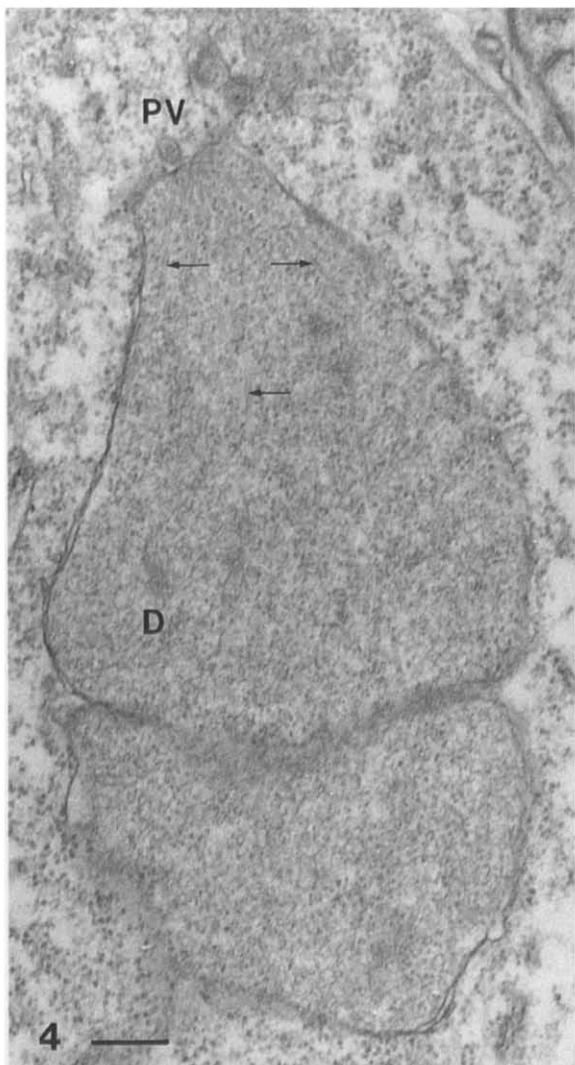


Fig. 4. Meront diplokaryon preparing for nuclear division. Note nucleus pointed toward the spindle plaque and spindle tubules (arrows) converging towards the plaque. D = diplokaryon, PV = polar vesicle. Scale bar = 0.25 μ m. - Fig. 5. Primordium of the exospore (arrows) secreted patchily on to the plasma membrane of a developing sporont. Note nuclear activity indicated by the spindle plaque. D = diplokaryon, SP = spindle plaque. Scale bar = 0.5 μ m. - Fig. 6. Sporoblast with developing polar filament. G = Golgi complex, PF = polar filament. Scale bar = 0.5 μ m.

ronts were distinguished from merozoites by the electron dense coat external to the plasma membrane. Preparations for nuclear division in the sporonts were seen already at the initial deposition of electron dense material external to the plasma membrane (Fig. 5).

Dividing sporonts were more commonly observed than dividing meronts. The sporonts divided at least once yielding two sporoblasts that each developed into the final spore. In the sporoblasts the RER content increased compared to earlier developmental stages. The polar filament originated from the Golgi apparatus in the posterior part of the sporoblast (Fig. 6). Golgi-like structures were not observed in sporonts or in earlier developmental stages.

The Mature Spore

The spores were formed free in the host cell cytoplasm without any sporophorous vesicle.

Mature spores were ovocylindrical, straight to slightly curved. Fresh spores measured $3.6-5.5 \times 2.3-3.0 \mu\text{m}$ (average $4.7 \times 2.7 \mu\text{m}$, $N=25$) (Fig. 7) whereas fixed and stained spores measured $3.2-3.6 \times 1.5-1.9 \mu\text{m}$ (average $3.4 \times 1.7 \mu\text{m}$, $N=14$) (Fig. 8). The spore wall measured 137–183 nm but was considerably thinner anteriorly. At the apical part, where the spore wall covered the anchoring disc of the polar filament, the spore wall was only about 36 nm thick due to a reduction of the endospore size (Fig. 8).

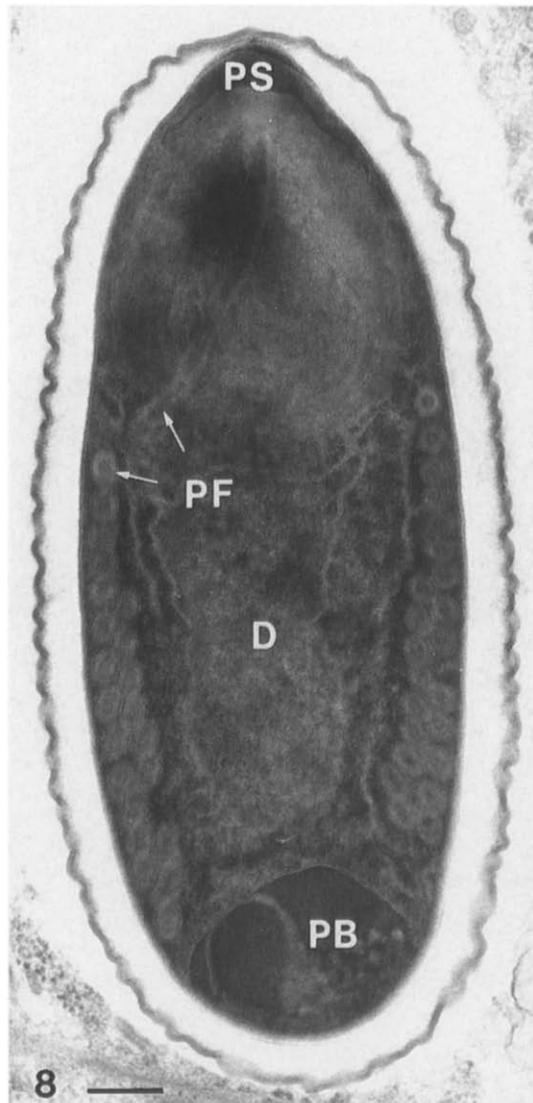
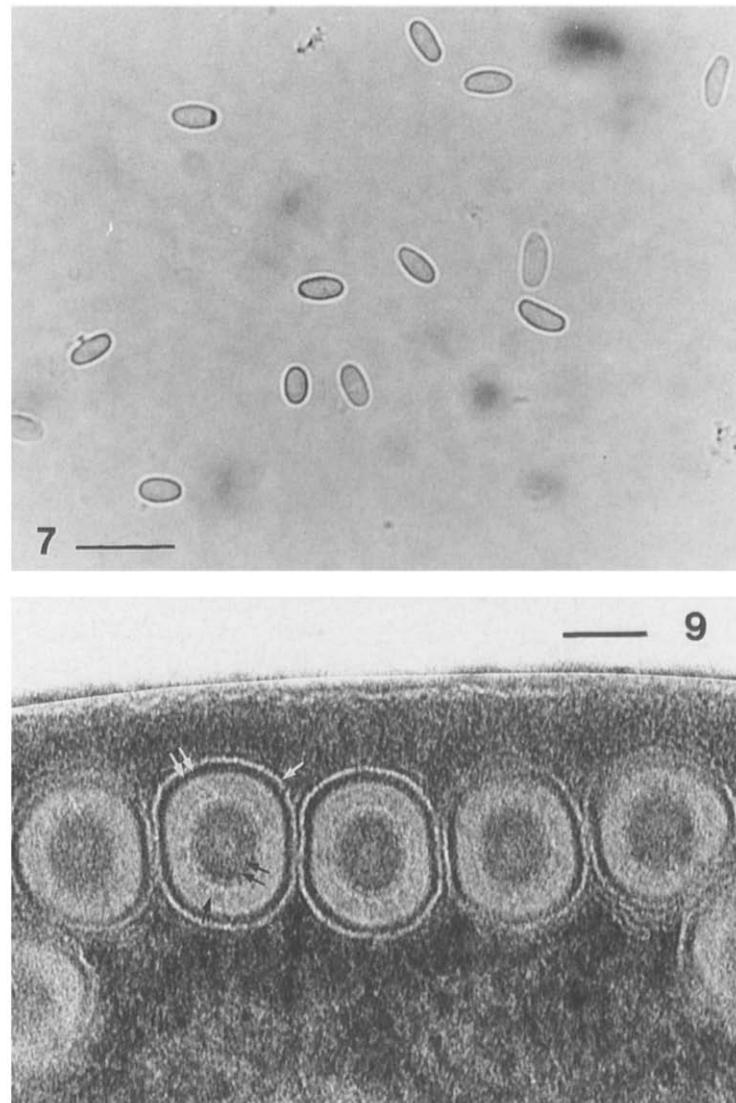


Fig. 7. Light micrograph of fresh spores in water. Scale bar = 10 μm . – Fig. 8. Longitudinally sectioned mature spore with internal structures partly visible. PS = polar sac, D = diplokaryon, PB = posterior body, PF = polar filament (arrows). Scale bar = 0.25 μm . – Fig. 9. Transversally sectioned polar filament showing the 4 layers. Single white arrow = layer 1, double white arrows = layer 2, single black arrow = layer 3, double black arrows = layer 4. Scale bar = 100 nm.



The spore wall consisted of a three-layered exospore, a lucent endospore and an internal plasma membrane that remained unchanged from earlier developmental stages. The exospore measured 14–17 nm and the endospore measured 134–158 nm.

The polar filament was isofilar with a width of 96–102 nm. The transversely sectioned filament was stratified in four distinct sections: 1) an external unit membrane 2) an electron dense layer of approximately the same width as the external membrane 3) a partly lucent layer with fibril-like structures towards the center measuring about 20 nm and 4) a granular center about 35 nm wide (Fig. 9). The filament was attached to the biconvex anchoring disc enclosed by the umbrella-like polar sac in the apical pole of the spore (Fig. 7, and Fig. 10). The polar filament was arranged in the

posterior and mid-part of the spore with 20–23 coils (Fig. 7). In the most posterior part of the spore, the filament was arranged in two layers. The angle of tilt of the most anterior coil of the polar filament to the long axis of the spore was about 55–60°. The polar sac overlapped a large part of the anterior polaroplast (Fig. 10).

The anterior polaroplast consisted of closely packed lamellae, approximately 11 nm thick, structurally connected to the posterior polaroplast (Fig. 10). The posterior polaroplast also consisted of packed lamellae but these were wider and less uniform than in the anterior polaroplast (Fig. 10) and extended down about 1/3 of the spore, beyond the most apical coil of the polar filament.

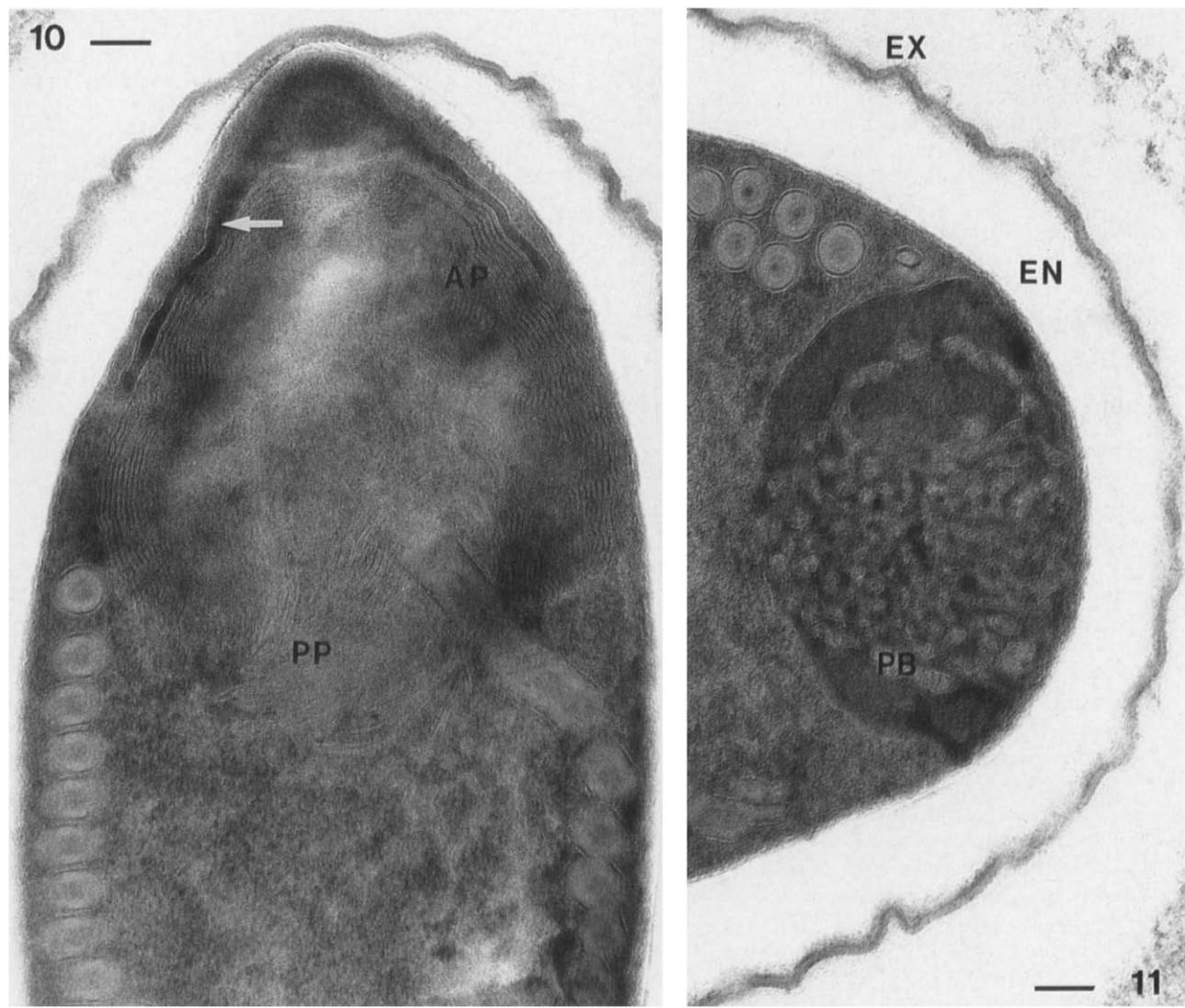


Fig. 10. Anterior part of the mature spore showing polar sac (arrow) and anterior polaroplast (AP) and posterior polaroplast (PP). Scale bar = 100 nm. – Fig. 11. Posterior body or posterosome (PB) of the mature spore. EN = endospore, EX = exospore. Scale bar = 100 nm.

The diplokarya of the spore was located in the mid-part of the spore and surrounded by the coiled polar filament. The diameter of the longest sectioned diplokarya in the mature spore was 0.9 µm and measured 0.4 µm across the line of apposition. A zone of cytoplasm separated the posterior nucleus from the posterior body.

In the posterior part of the mature spore, a unit membrane-lined posterior body interpreted as a posterosome was found in many spores (Fig. 11). The diameter of the posterosome was about 0.75 µm. The internal structures of the posterosome were cisternae-like and may be structurally connected to the polar filament.

Nosema ceranae Small Subunit Ribosomal RNA Coding Sequence

The amplified product of about 1,200 base pairs was cloned. Three independent clones were sequenced and a consensus sequence was inferred to compensate for Taq polymerase errors [32]. The small subunit ribosomal RNA (SSUrRNA) sequence for *Nosema ceranae* was deposited in the GenBank database (<http://www.ncbi.nlm.nih.gov>) under the Accession Number U26533. The complete *N. ceranae* SSUrRNA sequence is 1259 bases long. The base composition of this molecule is 36.3% GC, which is similar in range to the SSUrRNA base composition determined for other species from the genus *Nosema* (33.9 to 38.6%).

Phylogenetic Analysis

Five microsporidial SSUrRNA sequences and an archaeabacterial outgroup (*Halobacterium halobium*) were aligned using default options for the program CLUSTAL W and analyzed without editing using distance and maximum likelihood methods. The phylogenetic trees inferred with both methods were identical. Species from the genus *Nosema* and a *Vairimorpha* species represent a well-resolved, coherent phylogenetic group (Fig. 12), composed of two sister clades. *N. ceranae* branches together with *V. necatrix* and *N. apis*.

Discussion

From the presented material it must be concluded that the described microsporidium is a new species. It is placed in the genus *Nosema* based on the molecular phylogenetic analysis and because of the disporoblastic development, lack of sporophorous vesicle and the presence of diplokarya during all stages of the life cycle. In contrast to the type species, *N. bombycis*, where spores produced early in the infection (early spores) germinate inside the host cells [17], the development of the parasite described here does not seem to include intracellular germination of spores. Recently it has been reported that early spores that germinate intracellularly in both *in vitro* and *in vivo*

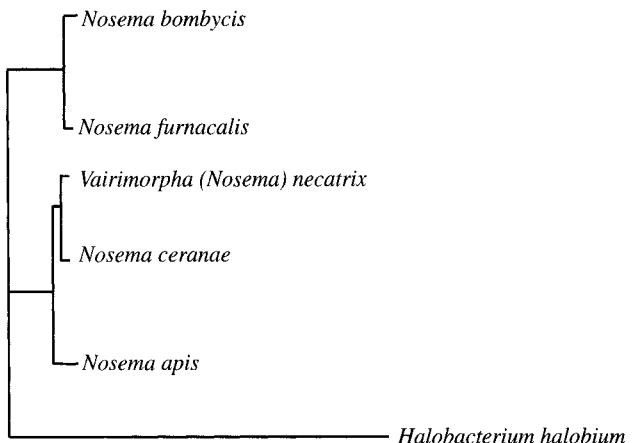


Fig. 12. Maximum likelihood small subunit ribosomal RNA phylogenetic tree for four microsporidian species in the genus *Nosema* and one in the genus *Vairimorpha*. The sequence of *Vairimorpha necatrix* is registered at GenBank under the name *N. necatrix*. The archaeabacterium, *Halobacterium halobium*, was used as the outgroup. The bar represents scaled evolutionary distance in expected number of substitutions. Vertical distances are for clarity only. The aligned data set is available upon request from the authors.

experiments develop in several species in the genus *Nosema* [14, 17, 19]. These spores may spread the infection within the host whereas spores that do not germinate within the host cytoplasm spread the infection between hosts. The main difference in these reported spore types consists of the number of filament coils and the thickness of the spore wall, where the young spores with few filament coils may eject the polar filament inside the host cytoplasm. In the present investigation there were no emptied spores or signs of intracellular germination. Thus, the recent hypothesis that microsporidia of the genus *Nosema* generally produce a spore which plays a role in transmitting parasites from cell to cell by *in situ* germination [18] may not include the species described here.

In *N. bombycis* infecting *Bombyx mori* and *N. apis* infecting *A. mellifera*, emptied spores in the host cytoplasm occur as soon as mature spores can be found. This has been interpreted as the mechanism by which intercellular spread of the parasite is accomplished [12, 17]. The fact that infected epithelial cells often appear isolated, even when cells contain mature spores, indicates that intercellular spread of the parasite may be limited or absent. The route of transmission between cells would then probably be limited to autoinfection with germination of spores as infected cells burst into the gut lumen. Although indicated from some *in vitro* studies [16, 20, 22, among others], intercellular spread of vegetative stages of microsporidia has not been demonstrated *in vivo* and remains to be identified.

The formation of meronts in chains, common to *N. apis* in *A. mellifera* [15], does not occur in the microsporidian described in this paper. The formation of

merogonic chains has been claimed to be non-typical for the genus *Nosema* where meronts more commonly divide by binary fission and bud off merozoites before chains are formed [6].

Earlier reports of *N. apis* infections in *A. cerana* may be observations of the species described here. Since unique spore shapes are rare among microsporidia [23], ultrastructural characters and molecular phylogeny must be used to differentiate among many species of microsporidia. In the present case, the spores of the microsporidium described here are clearly smaller under the light microscope when compared with spores of *N. apis*. Fresh, unfixed spores of *N. apis* measure approximately $6 \times 3 \mu\text{m}$ [38] whereas the fresh spores described in this paper measured approximately $4.7 \times 2.7 \mu\text{m}$ (Fig. 5). In contrast to spores of *N. apis*, the spores described here are often slightly bent. Although the difference in the size of spores between these species is clear, it may still be difficult to detect the difference in routine diagnosis of infected bees using light microscopy.

The number of polar filament coils is one tool that helps to differentiate between species of *Nosema* [7]. In the studied specimens this number was 20–23 in mature spores, whereas the number of polar filament coils in spores of *N. apis* is always larger and often more than 30 [12, 25].

The presented molecular data show a close phylogenetic relationship between *Vairimorpha necatrix* and *N. ceranae* (Fig. 12). *N. necatrix* was first described by Kramer [21] who classified two different species of microsporidia from fat body cells of *Spodoptera exempta*. Later, Pilley [28] erected the new genus *Vairimorpha* when she found that the two species described by Kramer [21] in fact belonged to a single dimorphic species. In recent attempts to classify microsporidia [9, 31] *Vairimorpha necatrix* is placed in the family Burenellidae compiled of dimorphic species with both a “*Nosema*-like” and a “*Thelohania*-like” sporulation sequence. Intergeneric comparisons made among *Nosema* species suggest that this genus may be composed of several unrelated groups [1] and the data presented here also demonstrate that closely related species presently may be found in different families and suborders (Pansporoblastina/Apansporoblastina, [34]). This is unfortunate if the object of classification is to create groups of related organisms.

Baker et al. [1] analyzed partial sequences of the large subunit rRNA from several *Nosema* and *Vairimorpha* species and found that the lepidopteran *Nosema* species were more closely related to the *Vairimorpha* species compared to some *Nosema* species from hosts from other insect orders. Inclusion of more species to the sequences analysis showed that *N. apis* is more closely related to the *Vairimorpha* group than to the type species, *N. bombycis* and that, consequently, the octosporoblastic developmental cycle characteristic for *Vairimorpha* may not be an absolute character distinguishing between these two genera [2]. It should also be noted that

N. ceranae is more closely related to *V. necatrix* than to *N. apis* infecting *A. mellifera*. The relationship between the new *Nosema* sp. described in this paper, from another hymenopteran host, and *V. necatrix* demonstrates that a close phylogenetic relationship between *Nosema* and *Vairimorpha* species is not limited to parasites found in lepidopteran hosts only.

It has been suggested that the diagnostic characteristics for the heterogeneous genus *Nosema* should be reconsidered [26]. However, such classification probably should be based on molecular data as well as on morphological characters to avoid future confusions. Further molecular phylogenetic analysis of the genus *Nosema* and other microsporidian genera are essential to understand the phylogenetic relationship within and between these groups.

N. apis has a major negative impact on beekeeping with *A. mellifera* in temperate climates [13]. It is not known how the new parasite described in this paper will affect the production and pollination capacity of colonies of *A. cerana*. However, the similarities in physiology and general biology between *A. mellifera* and *A. cerana* and the location of the microsporidian infections in both honey bee species suggests that the infection in *A. cerana* may be a serious threat to profitable beekeeping with this honey bee.

Description

Nosema ceranae n. sp.

Merogony: Diplokaryotic merozoites are produced by binary fission of meronts. The number of cycles of merogony is unknown.

Sporogony: As for the genus. Two diplokaryotic sporoblasts are formed from each diplokaryotic spore.

Spores: Ovocylindrical, straight to slightly curved. Measurements of spores are $4.7 \times 2.7 \mu\text{m}$ when fresh and $3.4 \times 1.7 \mu\text{m}$ when fixed and stained. The spore wall is 137–183 nm thick with the endospore measuring 134–158 nm. The isofilar polar filament is 96–102 nm wide. It is arranged in 20–23 coils in the posterior part of the spore with the most posterior part in two layers. The angle of tilt is approximately 55–60°. Two nuclei are located in the mid-region of the spore. The polar cap overlies part of the anterior polaroplast which consists of closely packed lamellae. In the posterior part of the spore there is a Golgi-like structure in contact with the final part of the polar filament.

Host tissue involved: The epithelial cells of the ventriculus.

Type host: *Apis cerana* Fabricius, 1793 (Hymenoptera: Apidae).

Type locality: Managed honey bee colonies in the mountain region 120 km south of Beijing, China.

Type series: Holotype (Fig. 2) on slide No. 950515-1 IF, paratypes on slides 1–15.

Deposition of type: The slide with the holotype in the International Protozoan Type slide collection, Smithsonian Institution, Washington DC, USA. Paratypes are in the collection of the first author.

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