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Establishment of the new genus *Paranosema* based on the ultrastructure and molecular phylogeny of the type species *Paranosema grylli* Gen. Nov., Comb. Nov. (Sokolova, Selezniov, Dolgikh, Issi 1994), from the cricket *Gryllus bimaculatus* Deg

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Abstract

The ultrastructure of the microsporidian parasite *Nosema grylli*, which parasitizes primarily fat body cells and haemocytes of the cricket *Gryllus bimaculatus* (Orthoptera, Gryllidae) is described. All observed stages (meront, meront/sporont transitional stage ("second meront"), sporont, sporoblast, and spore) are found in direct contact with the host cell cytoplasm. Nuclei are diplokaryotic during almost all stages of the life cycle, but a brief stage with one nucleus containing an abundance of electron-dense material is observed during a "second merogony." Sporogony is disporous. Mature spores are ovocylindrical in shape and measure $4.5 \pm 0.16 \,\mu\text{m} \times 2.2 \pm 0.07 \,\mu\text{m}$ (n = 10) on fresh smears and $3.3 \pm 0.06 \,\mu\text{m} \times 1.4 \pm 0.07 \,\mu\text{m}$ (n = 10) on ultrathin sections. Spores contain 15–18 coils of an isofilar polar filament arranged in one or two layers. Comparative phylogenetic analysis using rDNA shows *N. grylli* to be closely related to another orthopteran microsporidian, *Nosema locustae*, and to *Nosema whitei* from the confused flour beetle, *Tribolium confusum*. *Antonospora scoticae*, a parasite of the communal bee *Andrena scotica*, is a sister taxon to these three *Nosema* species. The sequence divergence and morphological traits clearly separate this group of "*Nosema*" parasites from the "true" *Nosema* clade containing *Nosema bombycis*. We therefore propose to change the generic name of *N. grylli* and its close relative *N. locustae* to *Paranosema* n. comb. We leave *N. whitei* in former status until more data on fine morphology of the species are obtained.

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1. Introduction

The genus *Nosema* has been defined as those microsporidia which are apansporoblastic, monomorphic, disporous, and diplokaryotic throughout their life cycle. It is the most speciose genus of Microsporidia (Cali, 1971; Issi, 1986; Sprague, 1978). More than 150 Nosema species have been described from at least 12 orders of insects (Sprague et al., 1992). Phylogenetic analysis based on large subunit rRNA sequence has shown that, in addition to the "true" Nosemal Vairimorpha group (a group of insect microsporidia related to Nosema bombycis), there is a group of unrelated Nosema species which includes N. locustae and N. kingi (Baker et al., 1994). It was therefore concluded that the character of being diplokaryotic throughout the life-cycle, the defining character for Nosema, has evolved more than once and that the genus

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Nosema is a polyphyletic collection of microsporidia (Baker et al., 1994). Subsequent small subunit rDNA (SSU rDNA) analyses (Adler et al., 2000; Cheney et al., 2001; Terry et al., 1999; Weiss and Vossbrinck, 1999) showed similar phylogenies. As a result of these studies, several of the unrelated *Nosema* species, including *N. algerae* (= *Brachiola algerae*), (Lowman et al., 2000) and *N. corneum* (= *Vittaforma corneae*, Silveira and Canning, 1995) have been given new generic designations.

Presenting a phylogenetic analysis together with an ultrastructural or morphological description allows for the direct evolutionary comparison of these characters among microsporidia. Such an analysis helps to validate the designation of a new genus on evolutionary grounds (Andreadis and Vossbrinck, 2002; Maddox et al., 1999). A sequence-based phylogenetic analysis may yield surprising results, as in the case of *Nosema granulosis*, which parasitizes amphipods and has an aberrant internal spore ultrastructure, but which shows a high degree of sequence similarity to *N. bombycis* (a parasite of the lepidopteran *Bombyx mori*) based on SSU rDNA sequence (Terry et al., 1999).

Nosema grylli was first described in a brief morphological and ultrastructural characterization (Sokolova et al., 1994), from a laboratory population of the cricket Gryllus bimaculatus. Since 1994 a laboratory culture of the microsporidian has been established. We present here a comprehensive study of the N. grylli-G. bimaculatus parasite-host system. We examine the ultrastructure and life cycle of the parasite with respect to that of its host. In addition we undertake phylogenetic analysis of SSU rDNA sequence data to determine the relationships between N. grylli, N. locustae, and N. whitei as well as to the Nosema (bombycis)/Vairimorpha (*necatrix*) group. We here propose the designation of Paranosema grylli n.comb. and Paranosema locustae n.comb, respectively for N. grylli and N. locustae which are distantly related to "true" Nosema clade.

2. Materials and methods

2.1. Host culture

A population of the cricket *G. bimaculatus* Deg. was maintained in the insectary at the Laboratory of Microbiological Control of the All-Russia Institute for Plant Protection, St. Petersburg under the following controlled conditions: $28 \,^{\circ}$ C, 12L:12D photoperiod, 40-45% RH; fed with wheat seedlings, seasonal grass, and an artificial diet for rats.

2.2. Culture and isolation of Nosema grylli

Spores of *N. grylli* were first discovered in 1993 in crickets brought from Middle Asia (Turkmenistan,

Kopet-Dag Mtn, vicinity of Ashhabad). A culture of N. grylli has been maintained in laboratory populations of G. bimaculatus since 1994. Peroral infections of third to fourth instar larvae were performed by adding a suspension of spores to the crickets' drinking water (approximately 10⁵ spores per ml). It took an average of 7-9 weeks for the microsporidia to produce the next generation of spores. Crickets were dissected and the fat bodies were homogenized in water with a teflon pestle. Homogenates were then centrifuged at 200g for 10 min and the white layer at the bottom of the tube, containing mature spores, collected and washed 2-4 times with water. Each wash was followed by centrifugation to pellet the spores, until no contaminating particles were visible among the spores in the microscope field under phase contrast optics at $400\times$. The spores could be stored in an aqueous suspension at 4°C without noticeable loss of infectivity for up to 6 months.

Nosema whitei spores were obtained from a culture of *Tribolium confusum* maintained in Connecticut Agricultural Experimental station, New Haven, Connecticut, *N. locustae* spores were obtained from commercial source (M&R Durango, Bayfield, CO).

2.3. Stimulation of N. grylli sporoplasm extrusion

Spores were stimulated to extrude according to the protocol of Kurtti et al. (1994) with the following modifications. Purified spores were resuspended in solution 1 (1 mM Tris, 10 mM EDTA), kept at room temperature for 30 min and then centrifuged at 600g for 10 min. The pellet was resuspended in solution 2 (10 mM KOH, 170 mM KCl). After 30 min spores were pelleted again and resuspended in solution 3 (25 mM Tris, 10 mM EDTA, 170 mM KCl, and 30 mM glucose). Extrusion of the sporoplasm began within 1–15 min of the addition of solution 3.

2.4. Light microscopy

Smears prepared from either a suspension of spores or from infected fat body were air dried, fixed with absolute methanol for 5 min, and stained with Giemsa. DAPI (a 0.01% w/v solution in phosphate-buffered saline, pH 7.2), a specific nuclear fluorescent stain, was applied in some cases to visualize parasite nuclei in fresh or methanol-fixed smears.

2.5. Transmission electron microscopy

Infected fat body, spore pellets, and sporoplasms obtained after extrusion were fixed using the following protocols:

 Classical fixation for the preservation of cytoplasmic organelles in host cells and in the majority of parasite stages: 2.5% glutaraldehyde (v/w) or a mixture containing 1% paraformaldehyde and 2% glutaraldehyde in a 0.1 M phosphate buffer, pH 7.2, with 0.3 M sucrose and 0.005 M CaCl₂, for 3–12 h. After rinsing with 2 volumes of cacodylate buffer (pH 7.2) samples were postfixed in 0.1 M cacodylate buffer containing 1% OsO₄ for 1 h.

- (2) Fixation with "reduced osmium" was performed as described elsewhere (Polishchuk et al., 1999). This protocol was effective for demonstrating membrane structures of the secretory pathway in microsporidian cells. In brief, the tissue is fixed with 1% glutaraldehyde in 0.2 M Hepes buffer for 5–30 min, then postfixed for 1 h in an aqueous solution containing 2% OsO₄ and 3% potassium ferrocyanide.
- (3) Osmium impregnation techniques were used for visualization of early Golgi vesicles (Jezernik and Pipan, 1991); samples were incubated in 2% aqueous OsO₄ for 2 × 24 h at 40 °C.

After incubation in osmium all samples were rinsed with water, dehydrated in an ethanol and propylene oxide series, and embedded in an epon–araldite resin mixture. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Hitachi H-300 electron microscope.

2.6. Isolation and purification of spores

DNA isolation, gene amplification, and DNA sequencing were performed as described elsewhere (Vossbrinck et al., 1998). Spores from *N. grylli*, *N. locustae*, and *N. whitei* were purified using a 50% Ludox cushion in a 1.5 ml eppendorf microcentrifuge tube. DNA was isolated from spores using standard bead beating techniques and amplified using the Taq Kit (Qiagen), following the manufacturer's instructions. Amplified DNA was purified on QIAquick PCR purification spin columns (Qiagen) and submitted for sequencing using previously published primers (Vossbrinck et al., 1998; Weiss and Vossbrinck, 1998, 1999).

2.7. Phylogenetic analysis

The three novel sequences, *N. grylli* (AY305325), *N. locustae* (AY305324), and *N. whitei* (AY305323) were aligned with 8 other microsporidian species from Gen-Bank as follows: *Amblyospora californica* (U68473), *Antonospora scoticae* (AF024655), *Brachiola algerae* (=*Nosema algerae*) (AF069063), *Encephalitozoon cuniculi* (L39107), *Endoreticulatus schubergi* (L39109), *Nosema bombycis* (D85504), *Nucleospora salmonis* (U78176), and *Vairimorpha necatrix* (Y00266). The sequences were aligned by eye using Bioedit (Hall, 2001) and were rooted against the obligate anaerobic flagellate *Tritrichomonas foetus* (M81842). Only those portions that could be unambiguously aligned were used in the phylogenetic analysis. Phylogenetic analyses were per-

formed using PAUP*^{4.0b10} (Swofford, 2002) on a power Macintosh computer. Sequence comparisons, in the form of a data matrix, were made with percentage GC content, percentage identities and a distance matrix calculated using Kimura-2 parameter analysis. Neighbor Joining, Parsimony, and Maximum Likelihood (ML) analyses were performed. A branch and bound search was performed to find the most parsimonious tree. A neighbor joining tree was used as the start point for ML analysis. The base frequencies, transition/ transversion ratio, γ -shape parameter, proportion of invariable sites and R matrix were estimated from the data set and used in the analysis. A reduction of the general time reversible substitution model (4 γ classes) was used with a heuristic search and random stepwise addition (100 replicates). Nodal support was assessed by bootstrap analysis (100 replicates) for both parsimony and ML analyses.

3. Results

3.1. Light microscopy

Round diplokaryotic stages were especially abundant on fresh smears from the infected cricket larvae (Fig. 1A). Spores were ovocylindrical in shape and, with the exception of a few aberrantly large spores, were uniform both in shape and size (Figs. 1B and C). Fresh spores measured $4.5 \pm 0.16 \,\mu\text{m} \times 2.2 \pm 0.07 \,\mu\text{m}$ (average \pm SE, n = 10), whereas methanol fixed and Giemsa stained spores measured $4.3 \pm 0.20 \,\mu\text{m} \times 2.0 \pm 0.05 \,\mu\text{m}$ (n = 10). Spores readily discharged their polar filament as smears dried. The maximum length of the discharged tube was 200 µm. Firing of the polar filament could also be stimulated by 3% hydrogen peroxide or by a pH shift in the presence of EDTA. DAPI staining revealed diplokaryotic arrangement of nuclei in spores and sporoplasms (Fig. 1D). On Giemsa-stained smears (Figs. 1E-J) diplokaryotic stages were frequently seen. Undivided cells containing 4 paired nuclei were common (Fig. 1I). Occasionally cells with one large dense nucleus and stages with two or four unpaired nuclei were observed (Fig. 1G). Sporoblasts and spores were easily recognized by their smaller size and elongated shape (Fig. 1J).

3.2. Electron microscopy

Meronts of *N. grylli* life-cycle were round or oval cells which measured $4.4 \pm 0.18 \,\mu\text{m}$ (n = 13) in diameter (Figs. 2A–J). The cells at this stage were surrounded by a single plasma membrane (7–9 nm thick). The meronts, like all other stages of the *N. grylli* life-cycle, developed in direct contact with the host cell cytoplasm. The meront cytoplasm contained numerous free ribosomes and several cisternae of rough endoplasmic reticulum



Fig. 1. Light microscopy of *Nosema grylli*. (A) Diplokaryotic stages, DIC; (B) spores and sporoblasts, DIC; (C) spores, "empty" spores after sporoplasm extrusion look dark (non-refractive), phase contrast; (D) diplokaria in spores, DAPI, fluorescence; (E–I) stages of *N. grylli* life cycle on methanol-fixed smears after staining with Giemsa, bright field. (E) Sporoplasm or early meront with poorly resolved two nuclei; (F) diplokaryotic stages; (G) a stage presumably with one nucleus; (H) dividing stages with diplokaria. (I) a stage with four unpaired nuclei; (J) diplokaryotic sporoblasts and spores. (A–D) bar = $10 \,\mu$ m; (E–J) bar = $5 \,\mu$ m.

(RER), which seemed to extend from the expanded regions of perinuclear space (Figs. 2B-D). Interphase diplokarya had a homogenous nucleoplasm and the nuclear envelope was ornamented with nuclear pores (Figs. 2B and D). Merogony was the major proliferative stage in the N. grylli life-cycle. Prior to cellular division meronts elongated and microtubule organizing centers (spindle plaques) appeared in the nuclear envelope (Figs. 2C, E-H). During mitosis (presumably in the prophase/ anaphase stages) the chromosomes, attached to the spindle plaque microtubules, became visible (Figs. 2F and G). The chromosome number, counted on sample sections, did not exceed 8 for each member of the diplokaryon (Fig. 2D). Nuclear division occurred more rapidly than cytokinesis; as a result, elongated cells with two diplokarya were often observed (Fig. 2E).

3.2.1. Meront/sporont transitional stage ("second meronts")

Eventually meronts underwent a gradual transformation and became what we refer to here as "second meronts". This stage seems to be uninucleate (Figs. 3C– D) and may represent karyogamy prior to sporogenesis. However, we have not observed a spindle apparatus and are not sure if meiosis is taking place. We therefore, refer to this pre- or early sporogonic stage as second merogony. Second meronts (Figs. 3A–J) are approximately the same size as first meronts but can be distinguished from the previous stage by: (i) Electron-dense intranuclear particles, 50-100 nm in diameter, which became visible throughout the nucleoplasm (Figs. 3B-E). First the particles appeared in one member of the diplokaryon (Fig. 3B), and then in both followed by the appearance of uninucleate cells (Figs. 3C-D). (ii) Numerous smooth ER membrane cisternae, vacuoles, and stacks of lamellae appeared in the cytoplasm (Figs. 3C-J). (iii) Accumulations of vesicle-like membrane lamellae 30-60 nm in diameter, surrounded by coated membranes in the vicinity of the ER cisternae, similar to those described by Vavra as a "primitive Golgi" (Vavra and Larsson, 1999) (Figs. 3C-J). One or two large circular arrangements (0.5-2 µm in diameter) of tightly packed clusters of small tubules (CSTs; Sokolova et al., 2001) developed in close proximity to the nucleus (Figs. 3D-G). The CSTs were regularly observed to be surrounded by flattened cisternae of smooth ER (Fig. 3D). At this stage the parasites seemed to undergo cellular division. Incompletely divided cells and plasmodia containing 2-4 single nuclei which never displayed diplokaryotic arrangement, and up to four CSTs were observed (Figs. 3F-G). Nuclear envelopes were poorly resolved at this stage and no spindle plaques, microtubules or chromosomes, which might indicate the phase of the nuclear cycle, were seen. Occasionally, structures resembling ER cisternae with ribosomes located internally, and "rigid elongated frames" containing electron-dense material were observed (Figs. 3H and I), similar to strands of polysomes, described for sporonts of Becnelia sigarae



Fig. 2. Ultrastructure of Nosema grylli meronts (A) section through an adipocyte (N, adipocyte nucleus), heavily infected with meronts (M); (B) cross section through a typical meront with numerous free ribosomes, cisternae of endoplasmatic reticulum (ER), and expanded regions (arrow) of diplokaryon (DK) envelope; (C) section through meronts (M) displaying their elongate shape and connection of perinuclear space with ER (arrows); (D) nuclear envelope is ornamented with nuclear pores (arrows), seven chromosomes (Ch) are visible inside the nucleoplasm; (E) elongated meronts with two diplokarya (DK) are undergoing karyokinesis without destruction of the nuclear envelope (closed mitosis), saccules of ER are being distributed between the daughter cells along the long axis of the dividing mother cell, microtubules (arrows) are attached to spindle plaques; (F) part of the diplokaryon undergoing mitosis at larger magnification: spindle plaque (SP), the microtubule organizing center, located in the invagination of the nuclear envelope, gives rise to numerous microtubules (arrow), some of which are attached to kinetochores of chromosomes (Ch) by their distal ends. Others are still connected to the spindle plaque of the sister cell diplokaryon, small thick arrows indicate nuclear pores; (G) cross-section through a multilayered spindle plaque (SP) with the attached microtubules, arrow points to chromosomes; (H) tangential section through a nuclear envelope: two spindle plaques in "perpendicular arrangement" demonstrating structural similarity to typical centrioles of higher eukaryotes, arrows point to chromosomes; (I) crosssection through the adipocyte cytoplasm in close proximity to the meronts (M): numerous microtubules (arrows) are seen attached to the parasite plasma membrane; (J) section through a meront undergoing transition to the second meront stage. The meront cytoplasm shows more membrane structures, including small vacuoles (V), cisternae of ER, and a few conglomerates of vesicle-like membrane lamellae 30-60 nm in diameter, surrounded by coated membranes in the vicinity of the ER cisternae (arrows). Abbreviations: B, symbiotic bacteria; Ch, chromosomes; DK, diplokaryon; ER, endoplasmic reticulum; M, meront; Mit, host cell mitochondria; N, host cell nuclei; SP, spindle plaque; V, vacuole. Scale bars: (A) 10 µm; (B-F, J) 1 µm; (G-I) 0.5 µm.



Fig. 3. Ultrastructure of the sporont/meront intermediate stage, referred to in this paper as the "second meront". (A) Cross-section through the parasite cell showing an increase in the number of various membrane structures, including vacuoles (V), endoplasmic reticulum (ER), and conglomerates of vesicle-like lamellae (arrow): the nucleoplasm contains electron-dense particles distributed unevenly between two parts of the diplokaryon, the nucleolus (Nu) is visible in one part of the diplokaryon; (B) two meronts are visible: the upper one is a typical meront; the lower one is undergoing transition to the second meront stage, which is marked by the appearance of electron-dense granules in one nucleus of the diplokaryon. Host mitochondria (Mit) and a spore (S) are visible; (C) section through the 2nd meront cytoplasm: vacuoles (V) and membrane profiles fill the cytoplasm; electron-dense particles are abundant in the nucleus of the parasite cell (Np), arrows indicate tubular structures which appear in host cell cytoplasm at this particular stage of the parasite life cycle; (D) a circular arrangement of tightly packed clusters of small tubules (CST) surrounded by flattened cisternae of smooth ER, is located in close proximity to the nucleus (Np); (E) incompletely divided cell: two nuclei (Np) and 2 CSTs are visible; the nuclear envelopes and the membranes surrounding the CSTs are not visible; (F) section through a plasmodium containing four nuclei (Np): note the abundant vacuoles in the cytoplasm and the microtubules attached to the external surface of the parasite plasma membrane (arrows); (G) four CSTs are visible in this section through a 2nd meront; (H) strands of polysomes (arrow) are often observed in this stage in proximity to the parasite nucleus (Np); (I) rigid elongated frames containing electrondense material (arrow) and stacks of membrane material (asterisks) located near nucleus (Np), are typical of this stage; (J) section through the 2nd meront, showing stacks of membrane profiles in close proximity to the nucleus (long arrow) and an undulating profile of the parasite plasma membrane (PM) ornamented with patches of electron-dense material (exospore formation, short arrows). Abbreviations: CST, cluster of small tubules; N, host nucleus; Np, parasite nucleus; Nu, nucleolus; PM, plasma membrane; S, spore; Sp, sporont; other abbreviations are the same as on Fig. 2. Scale bars: (A–J) 1 µm.

(Tonka and Weiser, 2000). The plasma membrane of transitional stages was 10–12 nm thick and usually exhibited an undulating profile with numerous evaginations. Patches of electron-dense amorphous material were deposited randomly on the outer surface of the plasma membrane; over time these patches formed a continuous electron-dense envelope around the parasite cell suggestive of transition to sporogony (Fig. 3J).

Sporonts (Figs. 4A–F) measured $4.73 \pm 0.17 \times 3.56 \pm 0.19 \,\mu\text{m}$ (n = 12). These cells had an electron-dense 30 nm thick envelope (the primordial exospore) and were elongated in shape with stacks of rough ER oriented from one pole of the cell to the other. The ultrastructure of the diplokaryon at this stage was similar to that of the first meronts. Sporonts divided by binary fission, demonstrating "bisporous sporogony" (Vavra and Larsson, 1999).

3.2.2. Sporoblasts/young spores

Daughter cells arising from the binary fission of sporonts gradually transformed into sporoblasts (Figs. 5A-F). The CSTs occupied a progressively larger area. Eventually they differentiated into a tubular network to form a typical microsporidian trans-Golgi organelle (Golgi-like complex; Cali and Takvorian, 1999) (Figs. 5B and C). Maturation of the polar filament proteins occurred in this trans-Golgi organelle during the resting phase sporogony. Sporoblasts, measuring of $5.74 \pm 0.14 \,\mu\text{m} \times 2.12 \pm 0.08 \,\mu\text{m}$ (n = 5), could be identified by their further elongation and polarization (Fig. 5A). Polarization was marked by the appearance of the apical portion of the polar filament anteriorly, and saccules containing electron-dense polar filament material in the posterior part of the cell (Figs. 5A and B). In addition to polar filament precursors, vacuoles,



Fig. 4. Transition to sporogony. Ultrastructure of sporonts. (A) Amorphous material deposited in patches covers most of the outer surface of the undulating plasma membrane (PM), the parasite nucleus (Np) still contains numerous electron-dense granules; (B) the elongated shape of the cell, presence of stacks of endoplasmic reticulum (ER) oriented from one pole of the cell to the other, and deposition of electron-dense material (arrow) indicate the transition of the parasite to the sporont stage, features of the previous, second merogony stage (structure of the nucleus (Np) and the presence of the CST) are also noticeable; (C) numerous tubular structures (arrows) fill the cytoplasm of the adipocyte (HC) containing 2nd meronts and sporonts (Sp); (D) division of a sporont nucleus (Np) by closed mitosis, the continuous electron-dense envelope encloses the sporont (arrows); (E) binary fission of sporonts (Sp); (F) section through two sporonts displaying diplokaria (DK), the elongated arrangement of the ER, and the 30 nm-thick electron dense envelope, tubular structures in the host cytoplasm are indicated by arrows. *Abbreviations:* HC, host cell (adipocyte) cytoplasm; LG, lipid granule; other abbreviations are the same as on Figs. 2, 3. *Scale bars:* (A–F) 1 µm.



Fig. 5. Ultrastructure of sporoblasts and young spores. (A) A view of a typical sporoblast showing the diplokaryon (DK), ER cisternae oriented along the long axis of the cell, developing polar disk (PD) with the adjacent manubrial part of the polar filament, the distal part of the polar filament precursors (PF), and an envelope with two layers corresponding to future exo- and endospores (arrow); (B) sections through a young spore (left) and a sporoblast (right), polaroplast primordium (PPP) and 14 coils of the polar filament (PF) are seen inside the young spore; the section through the sporoblast displays the diplokaryon (DK) with an envelope ornamented with nuclear pores (arrows) and a typical Golgi organelle, containing tubular networks (TN), and saccules with polar filament material (PF); (C) a section through a Golgi organelle of the sporoblast shows clusters of small tubules (CST); tubular networks (TN) and a saccule with polar filament material (PF); the sporoblast exospore consists of several layers (arrow); (D) sporoblasts with polar filament precursors (PF), arrows indicate tubular structures in the host cytoplasm; (E) section through a young spore fixed by the reduced osmium method. Note the two layered exospore (Ex) ornamented with filamentous matrix (FM) deposited on the outer layer of the exospore; electron-dense bodies often seen in the posterior portion of immature spores, were shown to contain undeveloped polar tube coils (arrows); (F) oblique section through a young spore; En, endospore; Fm, filamentous matrix; PF, polar filament; PP, polaroplast; PPP, polaroplast primordium; and TN, tubular network; other abbreviations are the same as on Figs. 2–4. *Scale bars*: (A–F) 1 µm.

tubular networks, microtubules, and extended ER cisternae surrounding the centrally located diplokaryon were abundant in the sporoblast cytoplasm (Fig. 5E). Initially, the sporoblast was enclosed by a plasma membrane and by an electron-dense envelope (the primordial exospore). The sporoblast exospore was a multi-layered structure at this stage, with a minimum of three layers (Fig. 5C).

An abrupt reduction in sporoblast size occurred, after which the sporoblast could be defined as an immature spore. An additional layer started to form between the plasma membrane and the exospore; oblique sections through this layer occasionally revealed numerous tubular structures (Fig. 5F). In mature spores this layer converted into an electron-transparent endospore. Electron-dense bodies were often seen in the posterior portion of immature spores. These electron-dense bodies were fixed with reduced osmium and were shown to contain undeveloped polar tube coils (Fig. 5E).

3.2.3. Spores and sporoplasms

Spores fixed for transmission electron microscopy on ultrathin sections measured $3.3 \pm 0.06 \,\mu\text{m} \times 1.43 \pm 0.07 \,\mu\text{m} (n = 10)$. The organelles seen in a mature *N. grylli* spore

are typical for microsporidia (Vavra and Larsson, 1999) (Figs. 6A-E). The electron-transparent endospore was $0.3-0.5 \,\mu\text{m}$ thick; at the apical end of the spore the thickness of the endospore was reduced to $0.05-0.2 \,\mu\text{m}$. The exospore was 30-40 nm thick and consisted of three layers with an electron-transparent layer between two electrondense layers (Fig. 6A). Occasionally in mature spores residing inside host cells, and always in immature spores, a fourth layer was visible as an electron-dense, filamentous matrix deposited on the external (host cell) side of the spore envelope. The plasma membrane was 10-12 nm thick and lay beneath the endospore. After the sporoplasm was discharged the plasma membrane remained inside the emptied spore envelope (Figs. 6F and H). The polar filament exhibited a multilayered structure in transverse sections. It was isofilar and arranged in 15-18 coils packed irregularly in one or two layers (Figs. 6A-B). The length of the manubrial filament was about 2/5 the length of the entire spore. The angle of tilt between the polar filament and the long axis of the spore was $60-70^{\circ}$. The polaroplast had two regions, the anterior lamellar portion, consisting of tightly arranged membranes, and the posterior vesicular portion, which contained more loosely arranged membranes (Fig. 6B). No posterior vacuole was observed. The discharged sporoplasms were rounded (Fig. 6G) and their diameter, measured on fresh smears, varied from 3 to 6 μ m. Several membrane structures and two separate nuclei were visible inside the sporoplasms (Fig. 6G). The cytoplasm was electron-transparent and contained numerous circular membranes.

3.2.4. Tissue specificity and interactions with the host cells

The fat body was the primary site of infection. Infection also occurred in the female and male gonads,



Fig. 6. Ultrastructure of spores and sporoplasms. (A) Cross-section through a spore, fixed by the reduced osmium method: thick electron-lucid endospore (En), undulating multi-layered exospore (Ex), and filamentous structures scattered outside the parasite cell (arrows), are visible; the posterior end of the spore contains an electron-dense body (asterisk); (B) cross-section through a spore fixed by the osmium impregnation method, which resulted in negative contrast of the membranes: the polaroplast reveals two parts—the anterior portion (PP1) consisting of tightly arranged membranes, and the posterior portion (PP2) containing loosely arranged membranes, filament coils are arranged in two rows; (C) oblique section through the polar filament coils (arrow); (D,E) sections through the anterior portion of a mature spore showing a polar disk (PD) in the manubrial region of the polar filament, a polar cap (PC), and a polar sac (arrow); (F) section through a spore in the process of germinating, showing the diplokaryon (DK) and a small portion of the discharging sporoplasm, polar tube (PT) has been just fired; note that there is no membrane around the discharged tube, but the membranes of the cisternae in which the polar filament was packed (arrows), remain inside the spore after discharge; (G) extruded sporoplasm fixed 4–10 min after stimulation of polar tube extrusion; (H) an empty spore envelope after firing: arrows indicate the membranes that previously enclosed the polar filament. *Abbreviations:* PD, polar disk; PC, polar cap; other abbreviations are the same as on Figs. 2–5. *Scale bars:* (A–D) and (G–H) 1 µm; (E,F) 0.5 µm.

haemocytes, and in the pericardial cells (cricket haemopoetic organ; Nassonova et al., 2001; Sokolova et al., 2000). Throughout the N. grylli life cycle abundant morphological evidence of intimate interactions between host and parasite cells was observed. Small vesicles, host ER cisternae and mitochondria were seen in close proximity to the parasite cell from early merogony until spore formation. Groups of host microtubules were visible and attached to the parasite plasma membrane during the meront stage (Fig. 2I). The characteristic "tubular structure" described for various microsporidia infecting insect fat body (Darwish et al., 1989; Wang et al., 1991) appeared in the host cell cytoplasm when the parasite entered the second merogonial stage (Fig. 4C). Extensive replication of the parasite during merogony caused significant increase in the host cell volume and deformation of the host nucleus. Transition to sporogony and spore formation caused progressive destruction of adipocytes; eventually the fat body was replaced by a mass of spores.

3.3. Phylogenetic analysis

Sequencing of the SSU rDNA from N. grylli, N. locustae, and N. whitei generated sequence of between 1335 and 1337 bp with identities of 95–97% (Table 1). All three sequences had high GC contents (65%; Table 1) similar to that of Antonospora scoticae (62%) but considerably higher than those of the "true" Nosema (e.g., N. bombycis 33%). Phylogenetic analyses with 8 additional microsporidia and one outgroup were performed. Parsimony produced 2 trees of which the most parsimonious tree is shown in Fig. 7. The overall placement of N. grylli, N. locustae, and N. whitei within the tree was identical in all analyses performed. However, the exact relationship between these three sequences remains unclear. Parsimony indicated that N. grylli was most closely related to N. locustae. ML showed that N. whitei was most closely related to N. grylli (Fig. 7), this was identical to the results given

Table 1						
Comparison	of SSU	rDNA	gene	seg	uenco	es

by neighbor joining (data not shown). All analyses clearly demonstrated that *N. grylli*, *N. locustae*, and *N. whitei* formed a sister clade to *Antonospora scoticae* and that these sequences were clearly unrelated to the 'true' *Nosema* clade containing the type species *N. bombycis*.

4. Discussion

4.1. Morphology

Ultrastructurally, N. grylli shows the closest similarity to N. locustae. Both species possess a characteristic stage in their life cycle, here defined as a meront/sporont transitional stage or as a second meront (Sokolova et al., 1998; Sokolova and Lange, 2002). The most striking feature of this phase is that the nucleus is filled with numerous electron-dense bodies which were revealed by all three fixative methods, and did not react with antibodies against DNA (E.M. and Y.S., unpublished data). The question of whether the nucleus maintains its diplokaryotic arrangement at this stage or whether it becomes uninucleate is still unclear, since the analysis of random thin sections does not resolve the number of nuclei. The formation of electron-dense granules begins in one of the nuclei of the diplokaryon, but when these granules became abundant and are distributed throughout the entire diplokaryon, the boundary between the nuclei disappears. Cells with one nucleus or with two or four unpaired nuclei were occasionally observed both on Giemsa-stained smears and on ultrathin sections. The similar stages were observed by Canning in Nosema locustae (1953), and the presence of a uninucleate stage was included in the diagnosis of the species. Ultrustructural analysis of N. locustae could neither support nor neglect this finding (Sokolova and Lange, 2002). We reported previously (Sokolova et al., 1994) that all stages of the N. grylli life cycle were diplokaryotic. The results of the current light and electron microscopy study, however, suggest the presence of the

Species	%GC	1	2	3	4	5	6	7	8	9	10	11
1. Nosema grylli	65.1	_	95.4	96.7	89.3	58.8	59.6	58.3	48.8	52.0	59.8	53.0
2. Nosema locustae	65.3	0.013	_	95.8	90.4	58.8	59.3	58.6	48.5	51.7	60.2	52.2
3. Nosema whitei	65.3	0.012	0.011	-	90.0	59.3	59.5	57.9	48.6	51.7	59.8	52.7
4. Antonospora scoticae	62.4	0.031	0.028	0.031	_	59.4	59.2	59.2	49.4	52.8	60.9	53.2
5. Nucleospora salmonis	48.5	0.253	0.250	0.254	0.243	-	70.0	61.8	58.8	59.6	55.3	52.8
6. Endoreticulatus schubergi	51.1	0.231	0.228	0.231	0.231	0.193	_	60.8	59.2	60.3	55.3	52.0
7. Encephalitozoon cuniculi	52.6	0.230	0.226	0.233	0.219	0.241	0.246	_	58.9	60.1	54.4	52.6
8. Nosema bombycis	33.5	0.357	0.359	0.362	0.353	0.288	0.294	0.261	_	81.4	51.8	48.7
9. Vairimorpha necatrix	36.9	0.311	0.315	0.319	0.306	0.276	0.275	0.234	0.085	_	52.1	50.1
10. Nosema algerae	46.7	0.240	0.241	0.244	0.232	0.288	0.290	0.311	0.320	0.314	-	52.1
11. Amblyospora californica	49.1	0.247	0.246	0.249	0.235	0.269	0.302	0.276	0.328	0.301	0.289	_

Percentage GC content (second column), percentage identity (top diagonal) and pairwise distances (bottom diagonal) obtained by Kimura-2 parameter analysis.

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Fig. 7. Maximum parsimony analysis of SSU rDNA showing the relationship of *Paranosema (Nosema) grylli* to other species of microsporidia. The analysis shows a close relationship among *N. grylli*, *N. locustae*, and *N. whitei. Antonospora scoticae* is a sister taxon to the *N. grylli* group. A more distant relationship is seen to *N. bombycis.* Inset shows alternate relationship based on Maximum Likelihood Analysis. Bootstrap analysis (100 replicates) was performed for both maximum parsimony and ML trees. The values on the tree represent the percentage of bootstrap replicates that gave that topology.

monokaryotic stage during the late merogony of the *N. grylli* life cycle.

4.2. Phylogeny

Analysis of sequence data has shown that *N. grylli*, *N. locustae*, and *N. whitei* cluster together alongside their sister taxon *Antonospora scoticae* to form a distinct clade in the microsporidian phylogenetic tree. This group of organisms is unrelated to the "true" *Nosema* of the *NosemalVairimorpha* group as previously observed (Baker et al., 1994). It is not surprising therefore that *N. locustae* and *N. grylli* show very similar ultrastructural and life cycle characteristics. They both are found in the adipose tissue of orthopteran hosts (Canning, 1953; Huger, 1960; Sokolova and Lange, 2002; ibid). *N. grylli* and *N. locustae* seem to have a uninucleate stage at the onset of sporogony and have a monomorphic spore with an ornamental exospore and thick endospore. The combination of ultrastructural and sequence data pre-

sented facilitate the reclassification of *N. grylli* and *N. locustae* to the novel genus *Paranosema*. The ultrastructure of *N. whitei* is poorly described, though such features as spore size and shape, parasitizing fat body cells, and organization of spore envelope (Milner, 1972) suggest similarity of this species to ortopteran microsporidia. Reclassification of this particular species demands additional ultrastructural information.

The genus Paranosema can be distinguished from the genus Nosema by ultrastructural characteristics, sequence divergence, host species, and tissue location. The life cycle of *Paranosema* is monomorphic, having only a single spore type. Whilst the *Nosema* life cycle includes two or more spore types (Becnel and Andreadis, 1999; Canning and Vavra, 2001; de Graaf et al., 1994a,b; Maddox et al., 1999; Solter and Maddox, 1998). Unlike Nosema the Paranosema life cycle includes the presence of a meront/ sporont transitional stage with electron-dense intranuclear particles, and a spore with an ornamented exospore and thick endospore. *Paranosema* parasitize the insects of the order Orthoptera. Nosema and Vairimorpha occur in insects belonging to the orders Lepidoptera, Hymenoptera, Coleoptera, and Diptera (Baker et al., 1994). Paranosema species do not parasitize tissues of epithelial origin, while Nosema species frequently parasitize epithelial cells (*Nosema bombycis*, Iwano and Ishihara, 1991; Nosema apis, de Graaf et al., 1994a,b; Nosema mesnili, Sokolova and Issi, 1997; Vairimorpha (= Nosema) necatrix, Solter and Maddox, 1998). Paranosema can also be differentiated from its sister group Antonospora (Fries et al., 1999). A. scoticae, a parasite of Andrena scotica (Hymenoptera), has larger and more elongated spores, a thinner exospore (24-32 nm) consisting of four distinct layers, and infects a hymenopteran host species.

As more sequence data is collected, the taxonomic significance of microsporidian characteristics such as SSU rDNA sequence, host, ultrastructure, and life cycle will become clearer. At present the single best character for indicating the relationship among microsporidia is the host, but there are exceptions. For example, the Nosemal Vairimorpha group does not always separate along host lines: Nosema portugal infects Lepidoptera while Nosema vespula, a closely related species, infects Hymenoptera (C.V., personal observation). Ultrastructural characteristics may vary greatly among closely related species. Nosema granulosis, which parasitizes amphipods (Terry et al., 1999), shows a very high SSU rDNA similarity to N. bombycis but has a strikingly aberrant internal spore ultrastructure. Perhaps some of the Nosema groups have developed strategies as generalist parasites and are able to change hosts relatively easily.

4.3. Systematics

Paranosema n.g. (diagnosis based on the characteristics of *Nosema locustae* and *N. grylli*). *Nuclear and life cycle.* Diplokaryotic during almost all stages of the life-cycle, brief uninucleate phase may take place at the end of merogony, meiosis has not been observed. Merogony and sporogony by binary fission of diplokaryotic stages; characteristic meront/sporont transitional stage (referred to in this paper as second merogony) with numerous electron-dense intranuclear particles; sporogony is disporous; one type of spores is produced (monomorphic).

Interfacial envelopes. Development of all stages in direct contact with host cell cytoplasm.

Spore ultrastructure. Spores are ovoid or ovocylindrical, thick endospore 200–300 nm, exospore 40–60 nm and possesses an undulating profile, associated with external electron-dense material, visible at least in immature spores; bipartite lamellae polaroplast (with tightly packed anterior and loose posterior lamellae); centrally or slightly posteriorly located diplokaryon, 15– 20 coils of isofilar polar tube are irregularly arranged in 1-2 rows.

SSU rDNA sequence analysis. There is 3–5% sequence divergence between N. grylli and N. locustae or N. whitei while the difference between N. grylli and Antonospora scoticae is about 10%. Therefore microsporidia which show from 10% difference or less from N. locustae could well be considered a Paranosema depending on structural and ecological considerations.

Tissuelorgan affected: adipocytes, haemocytes, and pericardial organ.

4.3.1. Differential diagnosis of the genus Paranosema

This genus can be distinguished from the genus Nosema by the following features. (1) Small subunit rDNA sequences difference greater than 10%; in the case of the Nosema/Vairimorpha group differences of approximately 50%. (2) Presence of a single spore type in the *Paranosema* life cycle (the *Nosema* life cycle includes an "early spore" (Becnel and Andreadis, 1999; Canning and Vavra, 2001; de Graaf et al., 1994a,b; Solter and Maddox, 1998)). (3) Morphological characteristics including: (i) presence of a meront/sporont transitional stage with electron-dense intranuclear particles in the *Paranosema*; (ii) number of polar filament coils in spores (15-20 coils in Paranosema versus 3-12) in Nosema (Canning and Vavra, 2001); (iii) posterior vacuole, absent or weak in Paranosema, prominent in Nosema species; and (iv) exospore, ornamented with electrondense material in Paranosema. (4) Host range: Paranosema species were found so far in insects of the order Orthoptera. Nosema and Vairimorpha occur in insects belonging to the orders Lepidoptera, Hymenoptera, Coleoptera, and Diptera (Baker et al., 1994). (5) Tissue specificity: Paranosema species do not parasitize epithelial cells, while Nosema species frequently parasitize epithelial cells (Nosema bombycis, Iwano and Ishihara, 1991; Nosema apis, de Graaf et al., 1994a,b; Nosema

mesnili, Sokolova and Issi, 1997; *Vairimorpha necatrix* (=Nosema necatrix), Solter and Maddox, 1998). While the ultrastructural description of *N. whitei* is not complete, the information presented (Milner, 1972; Weiser, 1953) is in agreement with the genus definition for *Paranosema*.

Antonospora (Fries et al., 1999), the sister group to *Paranosema*, can be differentiated from *Paranosema* by larger and more elongated spores, thinner exospore (24–32 nm) consisting of four distinct layers and host specificity (a parasite of *Andrena scotica*) (Hymenoptera).

Paranosema grylli (Sokolova et al., 1994) n. comb. (type species)

Type host. The cricket *Gryllus bimaculatus* Deg. (Or-thoptera, Gryllidae)

Tissue and cell specificity. Tissues of mesodermal origin: adipocytes, haemocytes, and pericardial organ. All stages develop in direct contact with the host cell cytoplasm.

Nuclear and life cycle: Nuclei are typical diplokarya during all stages of the life-cycle except the meront/ sporont intermediate stage ("second meront"). This stage exhibited a distinct fine structure and monokaryotic arrangement of the nucleus at least for a short period of time. Sporogony is disporous. Spores measure $4.5 \times 2.2 \,\mu$ m (fresh) or $4.3 \times 2.0 \,\mu$ m (methanol fixed), Mature spores contain 15–18 polar filament coils. No posterior vacuole was seen.

Small SSU rDNA sequence: We are including the small subunit rDNA sequence of *P. grylli* as part of the species definition (GenBank Accession No.: AY305325).

4.3.2. Synopsis of the genus Paranosema

Paranosema grylli (Sokolova, Seleznev, Dolgikh, Issi), new combination

Nosema grylli Sokolova et al. (1994, p. 493)

Paranosema locustae (Canning), new combination Nosema locustae Canning (1953, p. 290)

COL DNA 1 (CN 1 (N

SSU rDNA sequence data of *Nosema whitei* Weiser, 1953 (p. 205) (AY305323) and the few structural and life cycle characteristics available indicate this species also belongs to the genus *Paranosema*, though its inclusion into the genus is postponed until more structural data are obtained.

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