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Ultrastructural Characterisation and Molecular Taxonomic Identification of *Nosema granulosis* n. sp., a Transovarially Transmitted Feminising (TTF) Microsporidium

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ABSTRACT. A novel microsporidian parasite is described, which infects the crustacean host *Gammarus duebeni*. The parasite was transovarially transmitted and feminised host offspring. The life cycle was monomorphic with three stages. Meronts were found in host embryos, juveniles, and in the gonadal tissue of adults. Sporoblasts and spores were restricted to the gonad. Sporogony was disporoblastic giving rise to paired sporoblasts, which then differentiated to form spores. Spores were not found in regular groupings and there was no interfacial envelope. Spores were approximately 3.78 × 1.22 μm and had a thin exospore wall, a short polar filament, and an unusual granular polaroplast. All life cycle stages were diplokaryotic. A region from the parasite small subunit ribosomal RNA gene was amplified and sequenced. Phylogenetic analysis based on these data places the parasite within the genus *Nosema*. We have named the species *Nosema granulosis* based on the structure of the polaroplast.

Key Words. Comparative sequence analysis, *Gammarus duebeni*, microsporidia, SSU rRNA, transovarial transmission.

MICROSPORIDIA are a large and diverse group of intracellular parasites, which have been recorded in many invertebrate hosts (Canning 1990). The majority of microsporidia are horizontally transmitted (Canning and Lom 1986). However, vertical (transovarial) transmission also occurs in the life cycle of a number of species, for example, *Amblyospora* spp. (Andreadis and Hall 1979; Dickson and Barr 1990), *Nosema* spp. (Han and Watanabe 1988; Raina et al. 1995) and *Edhazardia aedis* (Becnel et al. 1989). A range of microsporidia have been recorded in the crustacean genus *Gammarus* and these can be divided into two types, those found predominantly in muscle tissue and those found in gonadal tissue. The species of microsporidia which infect the musculature, such as *Glugea mulleri* and *Thelohania mulleri* (Van Ryckeghem 1930; Friedrich et al. 1995), result in massive focal infection, which is visible as white markings through the amphipod cuticle. These parasites are present in high burdens in the host and cause severe pathology, which is related to the release of spores for horizontal transmission.

The second group contains microsporidia that rely on vertical transmission. This group includes two species described as *Octosporea effeminans* and *Thelohania hereditaria,* both recorded in *Gammarus duebeni* from the Elbe estuary, Germany (Bulnheim and Vavra 1968; Bulnheim 1971) and a further unidentified microsporidium, observed in *G. duebeni* from Budle Bay, Northumberland, UK (Dunn et al. 1993). These parasites are found predominantly in the gonadal tissue of adult female *G. duebeni* (Bulnheim and Vavra 1968; Bulnheim 1971; Dunn et al. 1993) and exhibit some very unusual traits. The most unusual characteristics, which unite this group, are that they appear to rely solely on transovarial transmission and they have been shown to have a feminising influence on host offspring. These microsporidia also appear to be apathogenic, having no effect on host reproduction, growth or longevity (Bulnheim and Vavra 1968; Bulnheim 1971; Dunn et al. 1993; Dunn et al. 1995). We refer to parasites that use this strategy as transovarially transmitted feminisers (TTFs).

It is unclear how many species of TTF microsporidia exist but current morphological descriptions suggest that there are at least two distinct parasite species in *G. duebeni*. *Octosporea effeminans* was described by light microscopy (LM) as a parasite, which was binucleate throughout its life cycle with spores found in irregular loose groupings in gonadal tissue (Bulnheim and Vavra 1968). *Thelohania hereditaria,* again described by LM, is a parasite that primarily infects muscle tissue, although low numbers were found in the gonad of the female host (Bulnheim 1971). The spores of this parasite were mononucleate and were described as being found in groups of eight within a pansporoblast membrane (interfacial envelope). Electron microscopy (EM) of a third, unidentified microsporidium from Budle Bay revealed irregular groups of 3–5 spores in gonadal tissue, similar to *O. effeminans*. However, these spores were contained within a pansporoblast membrane and the number of nuclei could not be confirmed (Dunn et al. 1993).

These earlier studies suggest that a number of microsporidia have evolved to become TTFs. However, the phylogenetic status of these parasites is currently unclear due to the paucity of ultrastructural and comparative molecular data. We have recently discovered a novel TTF microsporidium infecting a population of *G. duebeni* from Millport on the Isle of Cumbrae, UK (Terry et al. 1997). This parasite shows the two definitive characteristics of a TTF, transovarial transmission (Terry et al. 1997) and feminisation (Terry et al. 1998; Dunn and Rigaud 1998), but differs from previously described TTFs in terms of its burden and host pathogenicity (Terry et al. 1998; Dunn et al. 1998). We present a full description of the parasite life cycle and ultrastructure, which together with analysis of SSU rRNA, demonstrate that the parasite is a novel species belonging to the genus *Nosema*. We propose that the parasite described here represents a new species, *Nosema granulosis* n. sp.

MATERIALS AND METHODS

Gammarus duebeni were collected from White Bay, Isle of Cumbrae, maintained in the laboratory in brackish water at 12° C, and were fed with *Enteromorpha* (marine algae) and sycamore leaves. To determine the infection status of female hosts, embryos were screened for the presence of microsporidian parasites and the females allocated to control or infected groups (Terry et al. 1998). *Gammarus duebeni* females lay their eggs into the ventral brood pouch where they are fertilised. Females were examined daily for the presence of embryos, which were flushed from the brood pouch, permeated with 5 M HCl, rinsed in distilled water, and fixed in acetone at −20° C. Fixed embryos were squashed on a microscope slide and nuclei stained with DAPI (4,6-diamidino-2-phenyl-indole: 1 μg DAPI in 400 μl of 10% glycerol and 100 μl of saturated NaH_2PO_4 solution). Slides were screened for the presence of parasites using a Zeiss Axioplan fluorescence microscope.

Electron microscopy. In order to observe the complete par-

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asite life cycle within its host, parasite structure and distribution were studied at three different times in the host life cycle. EM examination was carried out on gonadal tissue of adult males and females, on whole juveniles at the time of sex determination (moult 4), and on embryos during early embryogenesis (1–100 cells).

Adult and juvenile specimens were fixed overnight in 3% (w/v) glutaraldehyde in Sorensen's phosphate buffer, pH 7.4 (SPB), rinsed in SPB, post-fixed in 1% osmium tetroxide in SPB overnight, rinsed in SPB, dehydrated through a graded ethanol series and embedded in araldite (Luft, 1961). Transverse sections were taken throughout the animal tissues. Ultra-thin sections were taken every 10 μm, stained with uranyl acetate and lead citrate, and examined on a JEOL 1200EX electron microscope. Embryos were fixed in 2% paraformaldehyde and 0.05% glutaraldehyde in SPB, and then processed as above.

Nucleic acid preparation and PCR amplification. DNA was extracted from infected and uninfected *G. duebeni* gonadal tissue and from eggs using a proteinase K digestion followed by phenol/chloroform extraction and ethanol precipitation (Kocher et al. 1989). PCR amplification of SSU rRNA was carried out with two microsporidian specific primers, 18f (5′ CACCAGGTTGATTCTGCC 3′) and 1537r (5′ TTATGATCCTGCTAATGGTTC 3′) (Baker et al. 1995). A negative control (uninfected *G. duebeni* eggs) was included in all PCRs. Amplifications were performed in 25 μl volumes, overlaid with mineral oil. Each PCR included 1 μM of each primer, 200 μM of each dNTP and 0.625 Units of DNA polymerase (Promega, Madison, Wisconsin, USA) in buffer containing 1.5 mM $MgCl_2$. The amplification cycle consisted of 95° C for 5 min, followed by 35 cycles of 95° C for 1 min, 47° C for 1 min and 72° C for 1 min. The final elongation step was extended to 5 min. PCR products were electrophoresed through agarose gels and visualised by ethidium bromide staining.

Sequencing. PCR products were cleaned using QIAQuick PCR Purification kits (Qiagen, Inc. Crawley, Sussex, UK). PCR fragments amplified from infected embryos were sequenced using dye terminators (Applied Biosystems Inc. Warrington, UK) and analysed on an ABI 373 automatic sequencer. PCR products from infected ovaries were manually sequenced with the T7 sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Both strands of the fragments were sequenced using the amplification primers 18f and 1537r and internal primers 702r (5′ TCCTCTAGCTTACGTCCTT 3′), 530f (5′ GTGCCAGCAGCCGCGG 3′) (Lane 1991), 1047nr (5′ AACGGACCTGTTTTAA 3′) and 350nf (5′ CTAAGGATTGCAGCAGGGGCGAAA 3′) (modified from 1047r and 350f of Baker et al. 1995). The sequence has been deposited in GENBANK, Accession No. AJ011833.

Sequence analysis. Sequence homology searches were done in the EMBL and GenBank libraries using the FASTA program (Pearson and Lipman 1988). Aligned SSU rRNA sequences of 28 microsporidia were selected from the rRNA database (Van de Peer et al. 1998) as follows: *Amblyospora californica* (U68473), *Amblyospora stimuli* (AF027685), *Antonospora scoticae* (AF024655), *Culicosporella lunata* (AF027683), *Edhazardia aedis* (AF027684), *Encephalitozoon cuniculi* (L39107), *Encephalitozoon hellem* (L39108), *Endoreticulatus schubergi* (L39109), *Enterocytozoon bieneusi* (AF024657), *Enterocytozoon salmonis* (U10883), *Glugea atherinae* (U15987), *Ichthyosporidium* sp. (L39110), *Nosema apis* (U26534), *Nosema bombycis* (D85503), *Nosema ceranae* (U26533), *Nosema furnacalis* (U26532), *Nosema oulemea* (U27359), *Nosema trichoplusiae* (U09282), *Nosema vespula* (U11047), *Parathelohania anophelis* (AF027682), *Pleistophora* sp. (D85500), *Septata intestinalis* (L39113), *Spraguea lophii* (AF033197), *Trachipleistophora hominis* (AJ002605), *Vairimorpha* sp. (D85502), *Vairimorpha necatrix* (U11051), *Vavraia oncoperae* (X74112), and *Vittaforma cornae* (U11046). The SSU rDNA sequences were manually aligned on the basis of conserved regions and secondary structures. Only those portions of the sequences which could be unambiguously aligned (1050 bp, insertions and deletions excluded) were used in the phylogenetic analysis. This data matrix was analysed using parsimony, maximum likelihood, and distances methods in the PHYLIP suite of programs (Felsenstein 1993). Genetic distances were calculated as described by Kimura (1980) with nucleotide transversions to transitions weighted 2:1. For distance based methods, the trees were constructed using the neighbour-joining algorithm (Saitou and Nei 1987). Additionally, bootstrap analyses (1000 replicates) were generated using the SEQBOOT and CONSENSE programs.

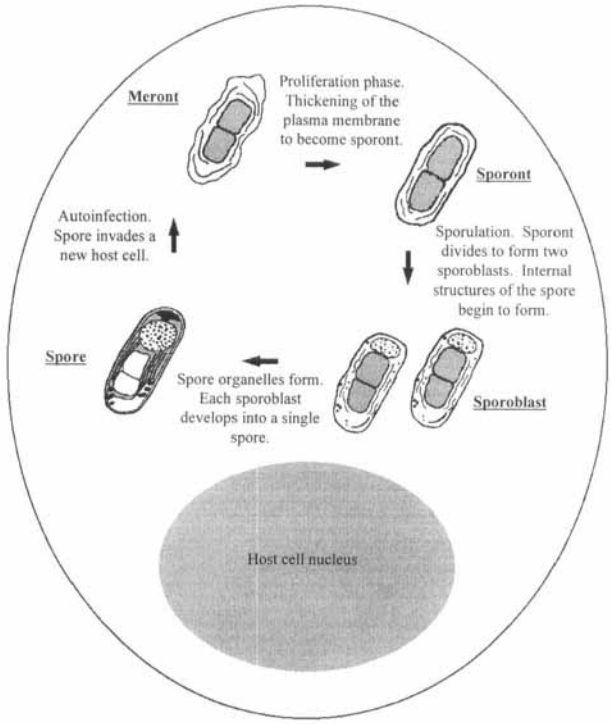
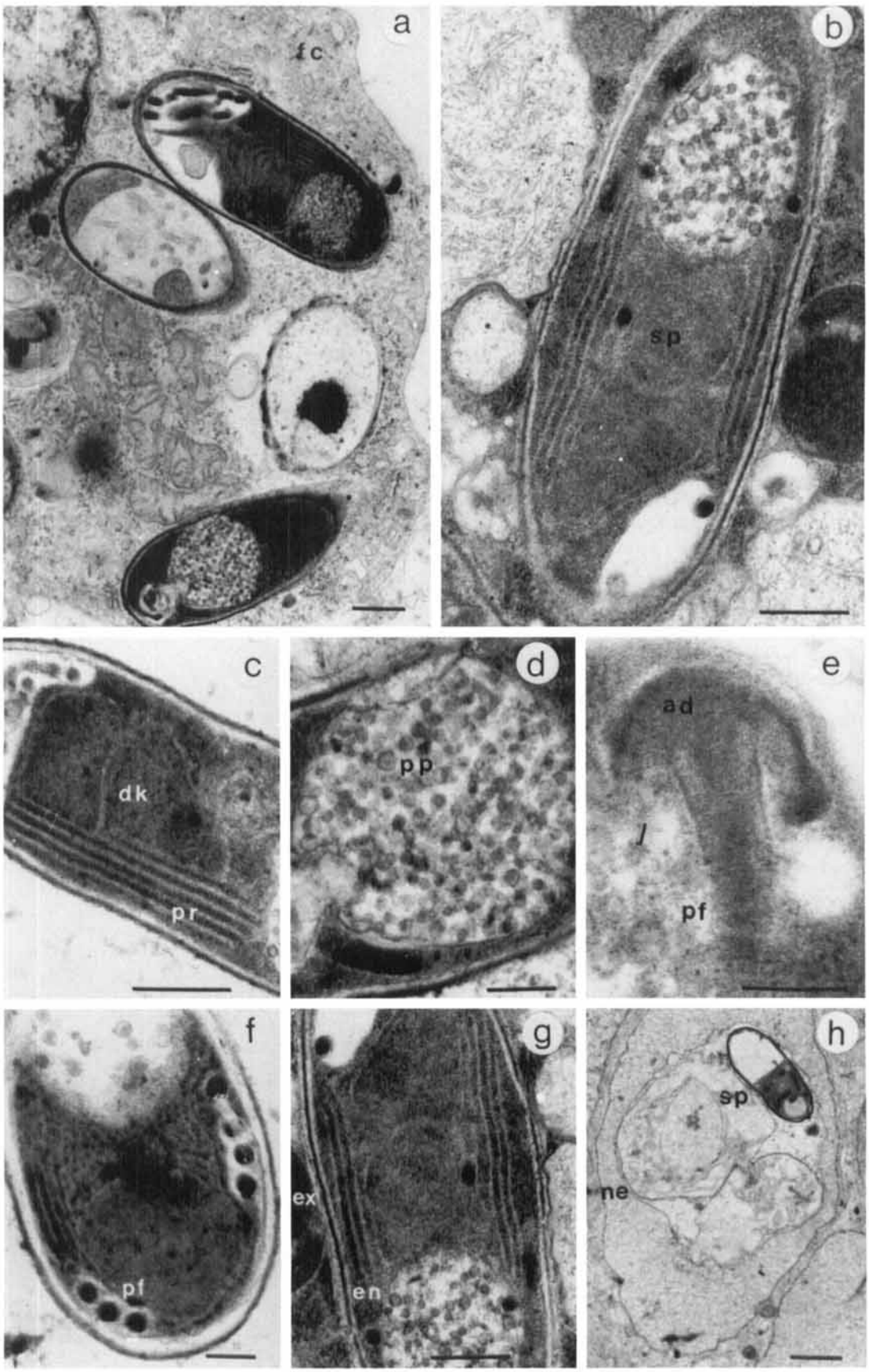


Fig. 1. Diagrammatic representation of the life cycle of *Nosema granulosis* n.sp. within the follicle cell of the *Gammarus duebeni* host ovarian tissue.

RESULTS

Morphological data. The parasite infecting *G. duebeni* from White Bay exhibited morphological characteristics typical of the phylum Microsporidia. The parasite was intracellular throughout its life cycle and three distinct stages: meronts, sporonts, and spores were observed in the gammarid host. A diagrammatic representation of the parasite life cycle is shown in Fig. 1. Meronts were seen to undergo replication in host embryos and in female gonadal tissue. However, sporulation occurred only in the female gonad. Sporogony was disporoblastic, generating two sporoblasts, which then underwent spore morphogenesis. Spore morphology was identical in successive host generations indicating that the parasite was monomorphic.

Spores were observed in the gonadal tissue of adult males and females but were not seen in embryonic or juvenile hosts. They were never found in regular groupings and there was no interfacial envelope (Fig. 2a). Spores were ovoid in shape and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measured 3.78 μm ± SE 0.15 in length and 1.22 μm ± SE 0.05 in width, n = 12 (Fig. 2.2). Spores contained two main components, the sporoplasm and the extrusion apparatus. The sporoplasm comprised a diplokaryotic nucleus (Fig. 2b) and cytoplasm, which contained abundant rows of polyribosomes (Fig. 2b, c). The extrusion apparatus consisted of the polaroplast, anchoring disc, and polar filament. The polaroplast was normally positioned towards the anterior end of the spore and was 1.05 μm ± SE 0.08 diam. It had an unusual structure with numerous discrete granules measuring between 0.02 and 0.07 μm in diameter, contained within a thick envelope (Fig. 2d, 4d). The polar filament was attached to the anterior of the spore by an anchoring disc (Fig. 2e). It then passed through the polaroplast (not shown) and coiled 3× in the region of the posterior vacuole (Fig. 2f). The polar filament appeared to be isofilar with a diameter of 0.09 μm ± SE 0.01, n = 15. The spore wall consisted of two layers, a dense exospore wall approximately 0.03 μm in width and a thin translucent endospore wall of 0.03 μm (Fig. 2g). Spores were observed in very low numbers in male gonadal tissue and were similar in shape and basic structure to those seen in females. However, spores in males frequently appeared vacuolar and degenerate and were often enclosed within a pocket formed by the host-cell nuclear envelope (Fig. 2h).

Meronts were found in adults, juveniles, and embryos, and their structure was consistent throughout. Meront size was variable and ranged from 2.76–10.01 μm in length and 1.43–5.11 μm in width (Fig. 3a) with a thin, undulating plasma membrane surrounding the nucleus and cytoplasm (Fig. 3b). The nucleus was approximately 3.37 μm ± SE 0.11, n = 55, in length and was diplokaryotic with the two nuclei flattened together at a broad junction (Fig. 3c). A double nuclear membrane enclosed both individual nuclei, each having a darkened area rich in chromatin. The cytoplasm contained abundant endoplasmic reticulum (ER) and ribosomes (Fig. 3d). Meronts were always in direct contact with the host-cell cytoplasm and were often seen in close association with the host-cell mitochondria (Fig. 3e). Meront replication was most often seen in host embryos and dividing meronts were occasionally observed (Fig. 3f).

Sporonts (Fig 4a) were observed in both the ovary and testis of the adult host but were not found in host embryos or juveniles. Sporogony appeared to be disporoblastic as sporonts were frequently paired (Fig 4b). Sporonts could be distinguished from meronts by the presence of a thickened plasma membrane and were 4.31 μm ± SE 0.28 in length and 3.03 μm ± SE 0.16, n = 8, in width and were irregular in shape with a diplokaryon nucleus, ER and ribosomes. These matured to form paired sporoblasts approximately 3.41 μm × 1.73 μm. Formation of the spore organelles, such as the polar filament (Fig. 4c) and the polaroplast (Fig. 4d), was also observed and differentiation of the plasma membrane to form endospore and exospore walls was clear (Fig. 4e).

Molecular data. A region of 1168 bp of the SSU rRNA gene was successfully amplified by PCR using generic microsporidian primers. Identical sequence information was generated from infected adult female ovary and from batches of eggs. No amplification was obtained from the negative controls. A FASTA program search revealed that this sequence had 98.9% identity with the SSU rRNA gene of *Nosema furnacalis*. Further analyses showed that the *G. duebeni* parasite sequence had more than 80% identity with the SSU rDNA sequences of the genera *Nosema* and *Vairimorpha*. The SSU rRNA gene of this parasite had a GC content of 33.2%, which was comparable to that of other *Nosema* species.

The trees obtained using parsimony, maximum likelihood, and distance methods were nearly identical with the exception of the branching pattern among members of the *Ichthyosporidium* group, which was chosen as the outgroup. The inferred tree (Fig. 5) gave the same overall topology as the tree obtained by Baker et al. (1995). The phylum Microsporidia was divided into four distinct groups supported by high bootstrap scores. According to the sequence of SSU rDNA, the microsporidium from *G. duebeni* belongs to the *Nosema/Vairimorpha* group and is closely related to *N. bombycis,* the type species of the genus *Nosema.* Other close relatives are *N. furnacalis* and *N. trichoplusiae* recorded from lepidopteran hosts. These data clearly support the inclusion of the parasite within the genus *Nosema.*

DISCUSSION

The microsporidium described here is morphologically distinct from those previously described within gammarid hosts. Based on observations of the life cycle and ultrastructure, in particular the granular polaroplast and on rDNA sequence data, we propose that it should be named as a new species, *Nosema granulosis,* n. sp.

The diplokaryotic nucleus, absence of an interfacial envelope or of regular spore groupings, and the disporoblastic sporogony are all characteristic of the genus *Nosema.* However, the life cycle of *N. granulosis* is unusual in that it is monomorphic. Some of the structural features of the *N. granulosis* spore, the short three-coil polar filament, and thin endospore and exospore walls, may be adaptations to the transovarial route of transmission. Polar filament length varies considerably among microsporidia, but spores with short filaments, termed FC (few coils) spores have previously been noted and have been associated with autoinfection (Iwano and Ishihara 1991; Johnson et al. 1997) and/or transovarial transmission (Ni et al. 1995). It has been postulated that the short filament would penetrate nearby or adjacent host cells (Iwano and Ishihara 1991; Iwano and Kurtti 1995). In the case of *N. granulosis,* spores in follicle cells appeared to inject sporoplasms into adjacent oocytes (Terry et al. 1997). Thin spore walls are also a feature of other FC spores. As these spores are not released into the environment, a protective thick wall may not be necessary (Iwano and Ishihara 1991). These characteristics are seen in the spores of several microsporidian species from at least five genera and it interesting to speculate whether it is possible to define a transovarial transmission (TT) spore type.

Many parasites within the genus *Nosema* have a morphologically similar spore type involved in autoinfection and/or transovarial transmission (Iwano and Ishihara, 1991; Iwano and Kurtti 1995). However, these species are dimorphic and possess a second spore-type associated with horizontal transmission (Han and Watanbe 1988; Iwano et al. 1994). More widely, di-

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Fig. 2. Electron micrographs showing details of *Nosema granulosis* spores. Sporulation occurs in the follicle cells (fc) of the female gonad of *Gammarus duebeni* (Fig. 2a). Spores (sp) have a diplokaryotic nucleus (dk) and abundant polyribosomes (pr) (Fig. 2b, 2c). The polaroplast (pp) has an unusual granular appearance (Fig. 2d) and the polar filament (pf) has an anchoring disc (ad) (Fig. 2e) and is short with only three coils (Fig. 2f). The spore has thin exospore (ex) and endospore (en) walls (Fig. 2g). Spores were also seen in the male gonad (Fig. 2h). These were often enclosed in pocket formed by the nuclear envelope (ne) and were frequently empty or degenerate in appearance. Figure 2d, 2e, 2f bars = 200 nm. Figure 2a, 2b, 2g bars = 500 nm. Figure 2c bar = 1 μm. Figure 2h bar = 2 μm.

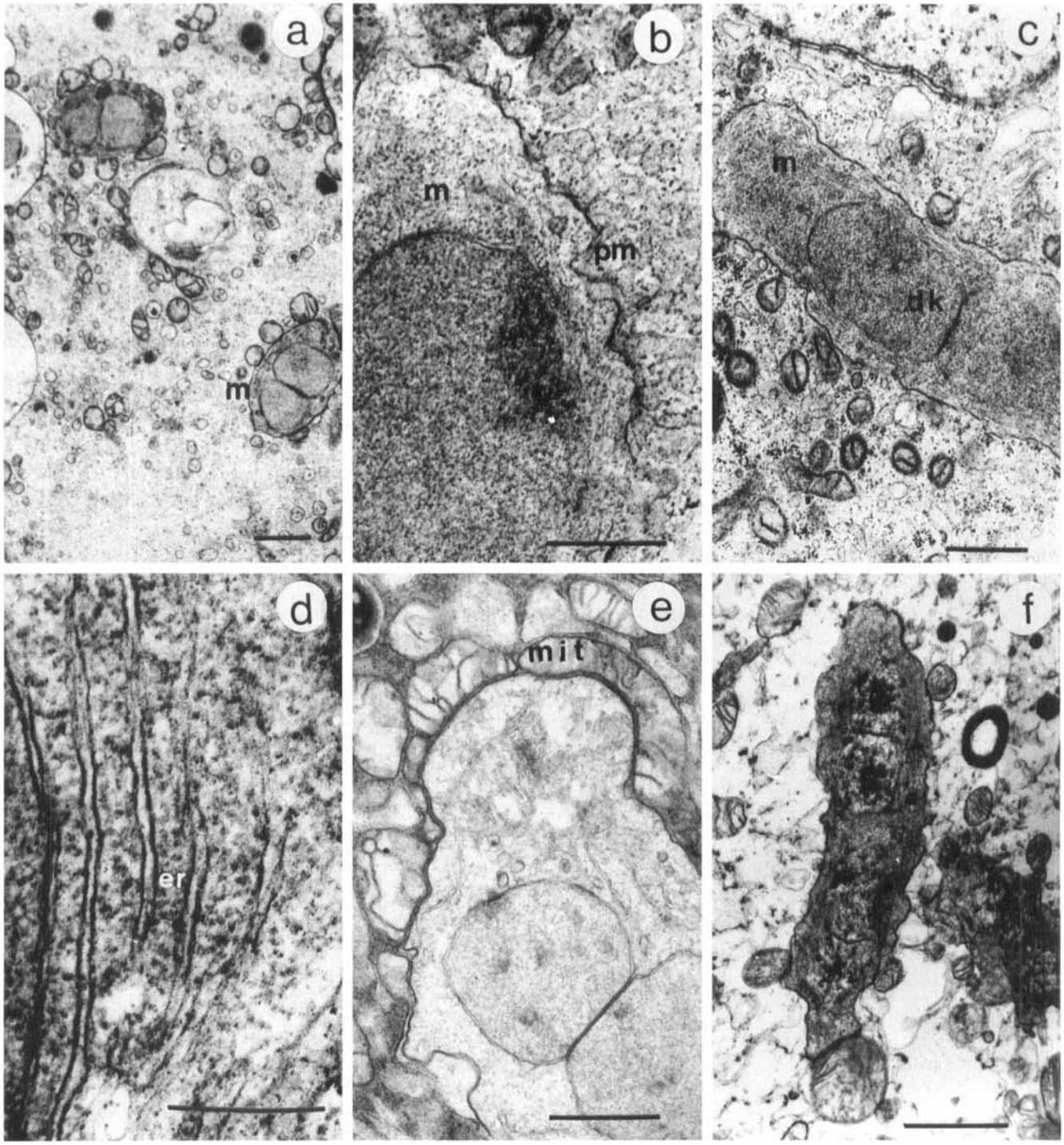

Fig. 3. Electron micrographs of *Nosema granulosis* meronts (m) in the cytoplasm of an early *Gammarus duebeni* embryo (Fig. 3a). Meronts had an undulating plasma membrane (pm) (Fig. 3b), a diplokaryotic nucleus (dk) (Fig. 3c) and abundant endoplasmic reticulum (er) (Fig. 3d). Meronts were closely associated with host cell mitochondria (mit) (Fig. 3e). Occasionally dividing meronts (pl) were seen (Fig. 3f). Figure 3d bar = 200 nm. Figure 3b, 3c bars = 500 nm. Figure 3e, 3f bars = 1 μm. Figure 3a bar = 2 μm.

morphic life cycles are associated with alternate transovarial and horizontal transmission in a variety of microsporidian parasites including *Thelohania* (Hazard and Weiser 1968), *Amblyospora* (Andreadis and Hall 1979) and *Culicospora* (Becnel et al. 1987). Based on the observation that *N. granulosis* has efficient vertical transmission (Terry et al. 1997), that the parasite is present in low burdens throughout the host life cycle (Terry et al.1998), and that there is no evidence for a second spore type, we propose that the parasite has lost its capacity for active horizontal transmission.

Phylogenetic analysis demonstrates that this parasite belongs within the 'true *Nosema*', a subset of the current genus *Nosema* that shows the highest similarity to the type species. This is the first record of a true *Nosema* that has not been recorded in a

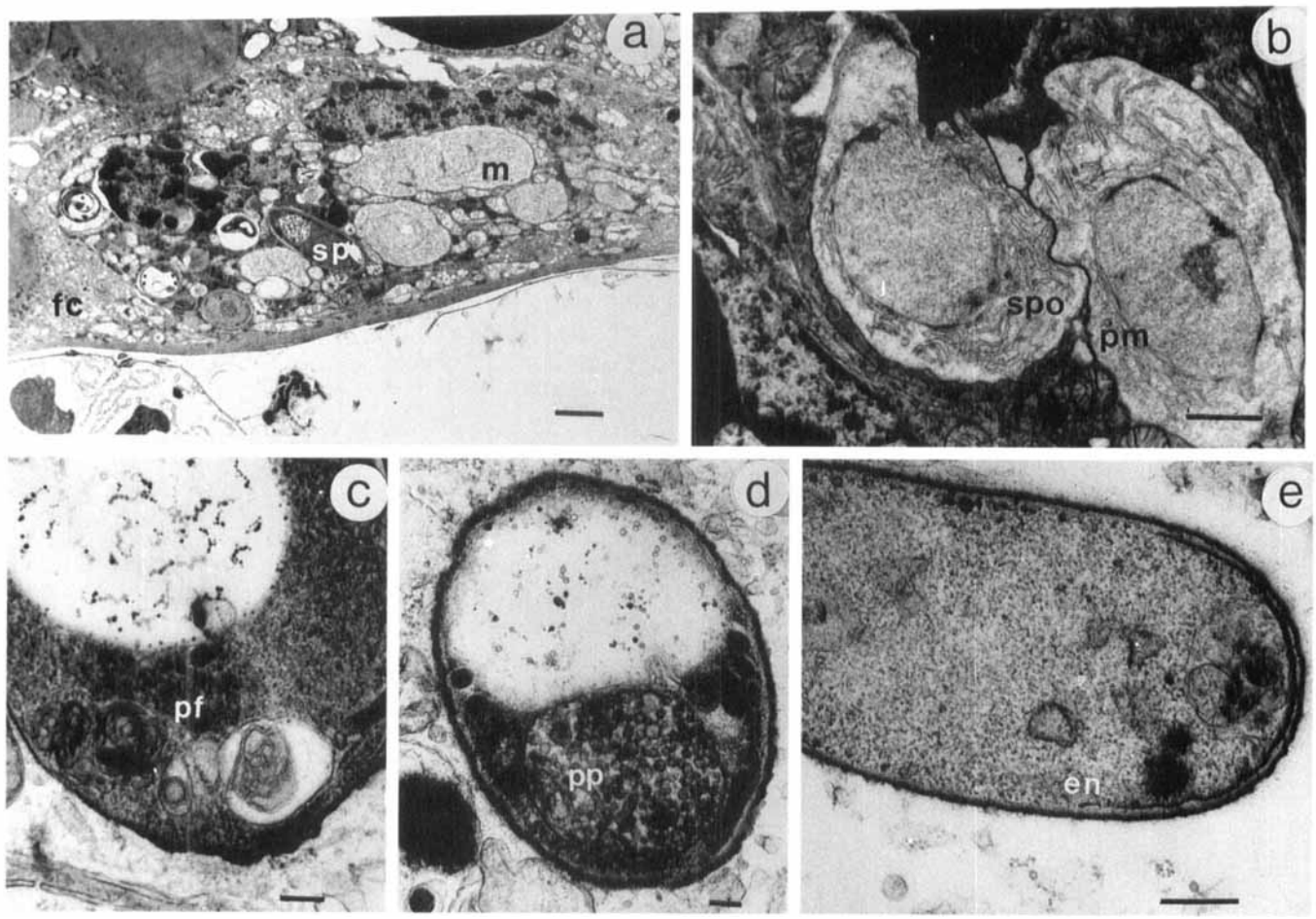

Fig. 4. Electron micrographs showing the process of sporulation in *Nosema granulosis*. Sporulation occurs in the follicle cells (fc) of the adult female gonad, which contain all stages from meronts (m) to spores (sp) (Fig. 4a). Sporulation appears to be disporoblastic, giving rise to paired sporonts (spo) which can be recognised by thickening of the plasma membrane (pm) (Fig. 4b). Developing organelles, such as the polar filament (pf), polaroplast (pp), and endospore wall (en), can be seen within maturing spores (Fig. 4c–4e). Figure 4c, 4d bars = 200 nm. Figure 4e bar = 500 nm. Figure 4b bar = 1 μm. Figure 4a bar = 2 μm.

lepidopteran host. The peculiarity of the ultrastructure and life cycle of this parasite emphasises the need for molecular phylogenetic studies in addition to microscopy when examining the taxonomic classification of such a complex phylum.

This parasite is clearly distinct from other feminising microsporidian species discovered in *G. duebeni*. It is present at relatively high burdens and possesses binucleate spores that are not seen in regular groupings or within an interfacial envelope (Terry et al. 1997). It is also the only TTF recorded to date that has a pathogenic effect on its host (Terry et al. 1998). It is possible that TTF microsporidia may have arisen several times resulting in parasites from different genera all utilising the same distinct method of transmission enhancement. Molecular phylogenetic techniques represent the only methods for examining the true evolutionary history of this unusual group of microsporidian parasites. The discovery of this fourth, ultrastructurally unique parasite from a single host species raises questions on the true abundance and diversity of TTF microsporidia.

Taxonomic summary—*Nosema granulosis* n. sp.

Type host. The brackish water amphipod, *Gammarus duebeni*.

Transmission. Transovarial. Spores present in the follicle cells of host ovary are thought to germinate and inject their sporoplasms into developing host oocytes.

Site of infection. Gonadal tissue of adult hosts. Subcuticular cells of juveniles at sex determination. The majority of cells in the early host embryos (1–100 cell stages).

Interface. All stages of parasite are found directly in the host cell cytoplasm.

Other parasite-host cell relations. Parasites are observed within the host cell cytoplasm but are never found within organised or regular groupings. Parasites have an unusual feminising effect on host offspring.

Haplophase. No haplophase was observed within the parasite life cycle.

Merogony. Diplokaryotic meronts undergo binary division.

Transition to sporogony. Sporogony is first identified by a thickening of the parasite plasma membrane.

Sporogony. The sporont is an irregular shaped diplokaryotic cell. Disporoblastic sporogony.

Spore. Binucleate, ovoid spore measuring 3.78 μm ± SE 0.15 in length and 1.22 μm ± SE 0.05 in width, n = 12. Exospore and endospore walls are both thin. Polaroplast has a distinctive granular structure, isofilar polar filament with three coils, small posterior vacuole and rows of polyribosomes.

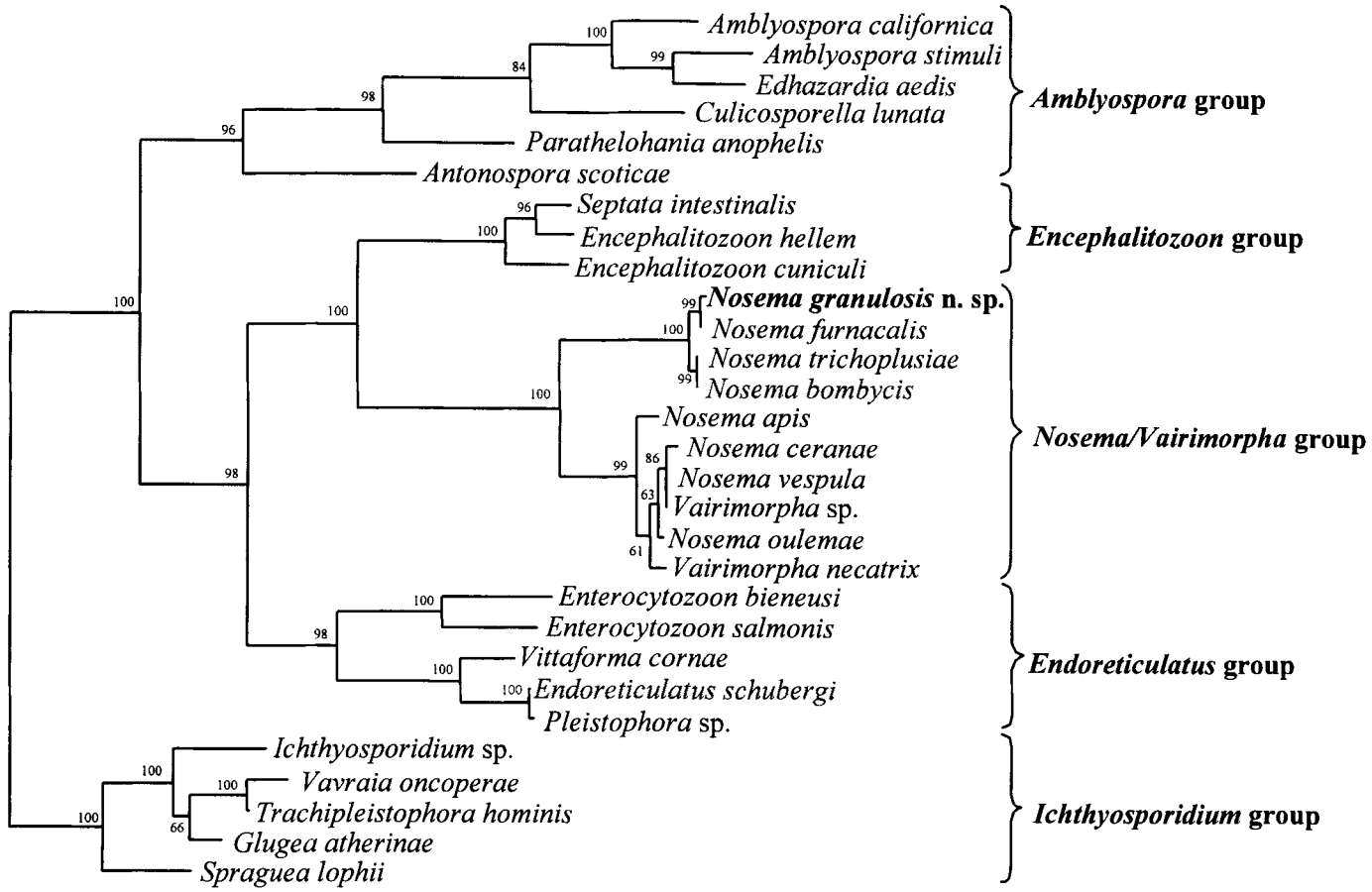

Fig. 5. Inferred phylogenetic tree of Microsporidia based on SSU rDNA, showing the position of *Nosema granulosis* n. sp., the TTF parasite of *Gammarus duebeni* (bold). The tree was generated by the neighbour-joining method using Kimura 2-parameter distances excluding insertions and deletions. The *Ichthyosporidium* group was used as an outgroup. Numbers next to the nodes refer to the bootstrap scores (%) in 1000 replicates.

Type locality. White Bay, Isle of Cumbrae, UK (OS grid reference—NS178591).

Remarks. Molecular phylogenetic studies indicate that this parasite is closely related to the true *Nosema* found in lepidopteran hosts. The unusual polaroplast and absence of a second spore type are unusual for a member of this group. A holotype slide, semi-thin section of infected female *G.duebeni* gonad stained with toluidine blue, has been placed in the Smithsonian culture collection, Washington, D.C. US Accession No. 51460. The nucleotide sequence of *N. granulosis* SSU rDNA has been deposited in the EMBL database under Accession No. AJ011833.

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LITERATURE CITED

Andreadis, T. G. & Hall, D. W. 1979. Development, ultrastructure, and mode of transmission of *Amblyospora* sp. (Microspora) in the mosquito. *J. Protozool.*, **26**:444–452.

Baker, M. D., Vossbrinck, C. R., Didier, E. S., Maddox, J. V. & Shadduck, J. A. 1995. Small subunit ribosomal DNA phylogeny of various microsporidia with emphasis on AIDS related forms. *J. Eukaryot. Microbiol.*, **42**:564–570.

Becnel, J. J., Hazard, E. I., Fukuda, T. & Sprague, V. 1987. Life cycle of *Culicospora magna* (Kudo, 1920) (Microsporida: Culicosporidae) in *Culex restuans* Theobald with special reference to sexuality. *J. Protozool.*, **34**:313–322.

Becnel, J. J., Sprague, V., Fukuda, T. & Hazard, E. I. 1989. Development of *Edhazardia aedis* (Kudo, 1930) n. g., n. comb. (Microsporida: Amblyosporidae) in the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Protozool.*, **36**:119–130.

Bulnheim, H. P. 1971. Entwicklung, obertragung und parasit-wirtbeziehungen von *Thelohania herediteria* sp. n. (Protozoa, Microsporidia). *Zeitschrift für Parasitenkunde*, **35**:244–260.

Bulnheim, H. P. & Vavra, J. 1968. Infection by the microsporidian *Octosporea effeminans* sp. n., and its sex determining influence in the amphipod *Gammarus duebeni*. *J. Parasitol.*, **54**:241–248.

Canning, E. U. 1990. Phylum Microspora. *In:* Margulis, L., Corliss, J. O., Melkonian, M. & Chapman D. J. (ed.), Handbook of Protoctista. Jones & Bartlett, Boston. p. 53–72.

Canning, E. U. & Lom, J. 1986. The Microsporidia of Vertebrates. Academic Press, London. p. 289.

Dickson, D. L. & Barr, A. R. 1990. Development of *Amblyospora campbelli* (Microsporidia: Amblyosporidae) in the mosquito *Culiseta incidens* (Thomson). *J. Protozool.*, **37**:71–78.

Dunn, A. M., Adams, J. & Smith, J. E. 1993. Transovarial transmission and sex ratio distortion by a microsporidian parasite in a shrimp. *J. Invert. Path.*, **61**:248–252.

Dunn, A. M., Hatcher, M. J., Terry, R. S. & Tofts, C. 1995. Evolutionary ecology of vertically transmitted parasites: transovarial transmission

of a microsporidian sex ratio distorter in *Gammarus duebeni*. *Parasitology*, **111**:S91–S109.

Dunn, A. M. & Rigaud, T. 1998. Horizontal transfer of parasitic sex ratio distorters between crustacean hosts. *Parasitology*, **117**:15–19.

Dunn, A. M., Terry, R. S. & Taneyhill, D. E. 1998. Within-host transmission strategies of transovarial, feminizing parasites of *Gammarus duebeni*. *Parasitology*, **117**:21–30.

Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package), version 3.5c. Department of Genetics, University of Washington, Seattle.

Friedrich, C., Kepka, O. & Lorenz, E. 1995. Zu verbreitung, wirtsspektrum und ultrastruktur von *Thelohania muelleri*, Pfeiffer, 1895 (Microsporidia). *Archiv für Protistenkunde*, **146**:201–205.

Han, M.-S. & Watanabe, H. 1988. Transovarial transmission of two microsporidia in the silkworm, *Bombyx mori*, and disease occurrence in the progeny population. *J. Invert. Path.*, **51**:41–45.

Hazard, E. I. & Weiser, J. 1968. Spores of *Thelohania* in adult female *Anopheles*: Development and transovarial transmission, and redescriptions of *T. legeri* Hesse and *T. obesa* Kudo. *J. Protozool.*, **15**: 817–823.

Iwano, H. & Ishihara, R. 1991. Dimorphism of spores of *Nosema* spp. in cultured cell. *J. Invert. Path.*, **57**:211–219.

Iwano, H. & Kurtti, T. J. 1995. Identification and isolation of dimorphic spores from *Nosema furnacalis* (Microspora: Nosematidae). *J. Invert. Path.*, **65**:230–236.

Iwano, H., Shimizu, N., Kawakami, Y. & Ishihara, R. 1994. Spore dimorphism and some other biological features of a *Nosema* sp. isolated from the lawn grass cutworm, *Spodoptera depravata* Butler. *Appl. Entomol. Zool.*, **29**:219–227.

Johnson, M. A., Becnel, J. J. & Undeen, A. H. 1997. A new sporulation sequence in *Edhazardia aedis* (Microsporidia: Culicosporidae), a parasites of the mosquito *Aedes aegypti* (Diptera: Culicidae). *J. Invert. Path.*, **70**:69–75.

Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.*, **16**:111–120.

Kocher, T. D., Thomas, W. K., Meyer, A. Edwards, S. V., Pääbo, S., Villablanca, F. X. & Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl Acad. Sci. USA*, **86**:6196–6200.

Lane, D. J. 1991. 16S/23S rRNA sequencing. *In:* Stackebrandt, E. & Goodfellow, M. (ed.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Chichester, UK. Pp 115–175.

Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**:409–414.

Ni, X., Backus, E. A. & Maddox, J. V. 1995. A new microsporidium, *Nosema empoascae* n. sp., from *Empoasca fabae* (Harris) (Homoptera: Auchenorryncha: Cicadellidae). *J. Invert. Path.*, **66**:52–59.

Pearson, W. R. & Lipman, D. J. 1988. Improved Tools for Biological Sequence Analysis. *Proc. Natl Acad. Sci. USA*, **85**:2444–2448.

Raina, S. K., Das, S., Rai, M. M. & Khurad, A. M. 1995. Transovarial transmission of *Nosema locustae* (Microsporida: Nosematidae) in the migratory locust *Locusta migratoria migratorioides*. *Parasitol. Res.*, **81**:38–44.

Saitou, N. & Nei, M. 1987. The neighbor joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**:406–425.

Terry, R. S., Dunn, A. M. & Smith, J. E. 1997. Cellular distribution of a feminising microsporidian parasite: a strategy for transovarial transmission. *Parasitology*, **115**:157–163.

Terry, R. S., Smith, J. E. & Dunn, A. M. 1998. Impact of a novel, feminising microsporidium on its crustacean host. *J. Eukaryot. Microbiol.*, **45**:495–501.

Van de Peer, Y., Caers, A., De Rijk, P., De Wachter, R. 1998. Database on the structure of small ribosomal subunit RNA. *Nuc. Acids Res.*, **26**:179–182

Van Ryckeghem, J. 1930. Les Cnidosporidies et autres parasites du *Gammarus pulex*. *Cellule*, **39**:400–416.

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