

Morphological and Molecular Investigations of *Tubulinosema ratisbonensis* gen. nov., sp. nov. (Microsporidia: Tubulinosematidae fam. nov.), a Parasite Infecting a Laboratory Colony of *Drosophila melanogaster* (Diptera: Drosophilidae)

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ABSTRACT. A new species of microsporidia from *Drosophila melanogaster* was investigated by light and electron microscopy and by ribosomal RNA (rRNA) sequencing. This microsporidium and the previously described *Nosema kingi* and *Nosema acridophagus* have been transferred to the new genus *Tubulinosema* gen. nov. with the following characters: nuclei are in diplokaryotic arrangement during the life cycle. All stages are in direct contact with the host cell cytoplasm, slightly anisofilar polar tube with the last coils being smaller in diameter arranged in one or two rows on both sides of the diplokaryon and small tubuli on the surface of late meronts. Spores are oval or slightly pyriform. Thick endospore wall, thinner over anchoring disc. This new genus and the genus *Brachiola* have been placed in a new family Tubulinosematidae fam. nov. Phylogenetic analysis of small subunit rRNA sequences by different methods placed *Tubulinosema* spp. in one clade with the genus *Brachiola* forming its sister clade, which is distant from the clade containing the true *Nosema* spp. including *Nosema bombycis*.

Key Words. *Nosema*, parasite, rRNA, taxonomy.

MICROSPORIDIA are a large group of obligatory intracellular parasites frequently using insects as the primary host. There is a wide variation in the types of pathologies that can occur in insects as a result of infection with microsporidia. Infections are usually chronic, slow-acting, and rarely acute. The various signs and symptoms associated with microsporidiosis in insects range from obvious tissue manifestations to abnormal developmental and behavioural changes (Becnel and Andreadis 1999).

The genus *Nosema* has more than 150 described species. Microsporidia of this genus usually occur in invertebrate hosts and have been described from at least 12 orders of insects. *Nosema* spp. are commonly found in Lepidoptera and Hymenoptera, although other taxa are parasitized, including Diptera (Sprague 1978). Well-known diseases are pebrine disease in silkworms caused by *Nosema bombycis* and a dysentery in honeybees caused by *Nosema apis*. Infections of fruit flies (*Drosophila*) by microsporidia have been reported rarely (Armstrong et al. 1986; Bell 1952; Burnett and King 1962; Kramer 1964; Stalker and Carson 1963; Wolfson, Stalker, and Carson 1957), and the responsible species appeared to be *Nosema kingi* in all reported cases (Armstrong et al. 1986).

The description for the type species of *Nosema*, *N. bombycis* (Naegeli 1857), was based on morphological features of spores and developmental stages. Meronts are binucleate with fused nuclei (diplokarya) and spores are also binucleate. All life cycle stages are in direct contact with the host cell cytoplasm. For *N. bombycis*, two types of spores are described: primary spores that develop early in the course of the infection and that serve to transmit the parasite between cells and tissues, and secondary spores that develop later in the infection and that are spread between hosts. Other members of the genus *Nosema* only produce one kind of spore, which spreads both between cells and tissues and between different hosts.

Using molecular data for phylogenetic studies, some *Nosema* and *Vairimorpha* spp. are consistently grouped together with the type species *N. bombycis*, whereas other *Nosema* spp. form clades of their own containing, for example, *Brachiola* (*Nosema*) *algerae*, *Nosema locustae*, *N. kingi*, and *Nosema acridophagus* (Baker et al. 1994; Müller et al. 2000). It has been concluded that some characteristics of the genus *Nosema* (e.g. being diplokaryotic throughout the life cycle) have evolved more than once and that the genus

Nosema is a polyphyletic collection of microsporidia (Baker et al. 1994; Sprague 1978). Subsequently, several of these unrelated *Nosema* spp., including *N. algerae* (= *B. algerae*), *Nosema conori* (= *B. connori*) (Cali et al. 1998), *Nosema corneum* (= *Vittafirma corneae*) (Silveira and Canning 1995), *N. locustae* (= *Paranosema locustae*), *Nosema grylli* (= *P. grylli*) (Sokolova et al. 2003), and *Nosema cristatellae* (= *Pseudonosema cristatellae*) (Canning et al. 2002), have been given new generic designations and other new genera (*Trichonosema*, *Bryonosema*, *Fibrillanosema*) (Canning et al. 2002; Slothouber Galbreath et al. 2004), and even a new family, Pseudonosematidae, has been created (Canning et al. 2002).

In this report, we describe an infection of *Drosophila melanogaster* by a new microsporidium, *Tubulinosema ratisbonensis* gen. nov., sp. nov., which occurred spontaneously in the *Drosophila* colony at the Department of Zoology, University of Regensburg, Germany. We examine the ultrastructure and life cycle of this parasite and undertake phylogenetic analysis of ribosomal RNA (rRNA) sequences to determine its relationship with *N. kingi*, *N. acridophagus*, with the *Brachiola* spp., with the “true” *Nosema/Vairimorpha* group, and the recently erected genera *Pseudonosema*, *Bryonosema*, *Trichonosema*, and *Paranosema*. Here, we propose the designation of *Tubulinosema kingi* comb. nov. and *Tubulinosema acridophagus* comb. nov., respectively, for *N. kingi* and *N. acridophagus*, which share similar morphological characteristics with *T. ratisbonensis* and are closely related to this microsporidium and are distantly related to the “true” *Nosema/Vairimorpha* clade and the other new genera in DNA-based phylogenies.

MATERIALS AND METHODS

Rearing conditions of the host. The laboratory stocks of *D. melanogaster* have been maintained for many years at the Department of Zoology, Lehrstuhl für Entwicklungsbiologie, University of Regensburg, Regensburg, Germany. Fly stocks were kept in standard condition at 25 °C and 65% humidity. Larvae and adult flies were fed on a standard medium containing yeast, maize, agar, and 3 g/liter Nipagin (Merck, Darmstadt, Germany).

Laboratory sampling. The microsporidian infection described here was found in several laboratory stocks of *D. melanogaster*, especially in those stocks reared at 25 °C. It is not clear from where the primary infection originated. Studies were conducted with larvae and adults of *D. melanogaster* from two microsporidian-infected laboratory stocks. To evaluate the infection

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rate, about 40 larvae and 40 adults were examined for microsporidian infection.

Light microscopy. Wet-mount preparations of dissected flies were examined by light microscopy and fresh spores were measured. Methanol-fixed Giemsa smears (Merck) were prepared for assessment of vegetative stages and measurements. Additional smears were stained with 1% (w/v) Uvitex 2B in PBS (Fungiqual A, Dr. D. Reinehr Spezialchemikalien für die medizinische Diagnostik, Kandern, Germany), Gram, 0.1% (w/v) toluidine blue O (Sigma, Taufkirchen, Germany), and a modified chromotrope 2R (Sigma) technique. In addition, adults and larvae were fixed with 4% (v/v) formalin in water and embedded in paraffin. Serial sections at 3–5 µm were stained with haematoxylin and eosin, Giemsa, toluidine blue O, a modified chromotrope 2R technique, Uvitex 2B, and tissue Gram stains (Sigma). A Leica DMRB fluorescence microscope (Leica, Bensheim, Germany) equipped with a mercury high-pressure lamp, an excitation filter with a transmission rate of 355–425 nm, and a suppression filter of 470 nm (filter cube D after Ploem) were used for all light microscopic studies and for preparing micrographs. Slides were photographed using a spot camera (Spot RT color, Diagnostic Instruments, Burroughs). Measurements were obtained using a computerized image analysis program [MetaVue, Version 5.0r1 (Visitron Systems, Munich, Germany)] and further imaging was carried out using Photoshop, Version 7.0 (Adobe Systems). Sizes are given as mean ± standard deviation and standard error.

Transmission electron microscopy. For ultrastructural studies, infected tissues from adult flies and larvae were fixed in 0.1 M cacodylate-buffered Karnovsky's solution (2.5% (w/v) glutaraldehyde and 1% (w/v) paraformaldehyde; overnight, room temperature) and postfixed in 1% (w/v) osmium tetroxide (2 h) at pH 7.3, dehydrated in graded ethanol, and embedded in the EmBed-812 epoxy resin (all reagents from Science Services, Munich, Germany; automated LYNX-tissue processor Leica, Bensheim, Germany). After 48 h heat polymerization at 60 °C, semithin (0.8 µm) sections were cut, stained with toluidine blue and basic fuchsin, and after light microscopic selection of representative tissue areas, the Epon block was trimmed for ultrathin sectioning. Ultrathin (80 nm) sections were cut with a diamond knife on a Reichert Ultracut-S ultramicrotome (Leica) and double contrasted with aqueous 2% (w/v) uranyl acetate and lead citrate solutions for 10 min each. The sections were examined in a LEO912AB electron microscope (Zeiss, Oberkochen, Germany) operating at 80 kV, equipped with a bottom-mounted CCD camera (Proscan, Lagerlechfeld, Germany) capable of recording pictures with 1,024 × 1,024 pixel. Imaging and measuring were carried out with the AnalySIS-software, Ver. 3.2 (Soft Imaging System, Münster, Germany).

Isolation and purification of microsporidia from infected insects. Microsporidian spores were isolated and purified from infected adult flies. Flies were macerated in 0.5 ml PBS using a manual tissue grinder (Micropistill, Eppendorf, Hamburg, Germany) and the suspension was filtered through 150-µm nylon mesh, 80-µm nylon mesh, and Kimwipes (Kimberly-Clark, Mühlheim, Germany).

DNA isolation, PCR amplification, and DNA sequencing. For amplification of rRNA sequences, microsporidian DNA was isolated from purified spores and infected flies by using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The small subunit (SSU) rRNA, the intergenic spacer, and the 5'-end of the large subunit (LSU) rRNA were amplified with the primers V1 (5'-CAC CAG GTT GAT TCT GCC TGA C-3'), 1492 (5'-GGT TAC CTT GTT ACG ACT T-3'), 530f (5'-GTG CCA GC(C/A) GCC GCG G-3'), 580r (5'-GGT CCG TGT TTC AAG ACG G-3') (Vossbrinck et al. 1993), and NOSr (5'-AAT ATA AGC TTC GCC TAC CTC-3'). Amplifications were carried out in 50-µl reaction

mixtures under the following conditions: 25 pmol of each primer, 200 µM concentration of each deoxynucleoside triphosphate, 10 mM TRIS-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, and 2.5 U of Taq DNA polymerase (Roche, Basel, Switzerland). Reactions were run in a Biometra thermocycler (Biometra, Göttingen, Germany) using a step-cycle programme. After initial denaturation of DNA at 94 °C for 3 min, 35 cycles were run at 94 °C for 1 min, at 42–50 °C for 2 min, and at 72 °C for 3 min, with a 10-min extension at 72 °C being carried out after the 35 cycles.

PCR products were separated on ethidium bromide-stained 1.5% agarose gels and the desired bands were extracted from 1.5% agarose gels by using the QIAquick Gel Extraction Kit (Qiagen). Isolated DNA fragments were directly sequenced on an automated DNA sequencer (ABI PRISM, Applied Biosystems, Foster City, CA) using the primers V1, 530f, 580r, and NOSr. Sequencing of both strands of each PCR fragment was carried out twice and two PCR products were sequenced for each DNA fragment. The resulting DNA sequences were assembled using GeneTool Lite, version 1.0, and the consensus sequence was submitted to the GenBank database under Accession no. AY695845.

Phylogenetic analysis. Sequences similar to those of our isolate were obtained from GenBank using a BLAST search. The sequences were automatically aligned on a personal computer using Clustal X, version 1.81 (Thompson et al. 1997) using the default parameter settings and edited visually using BioEdit 7.0.0 (Hall 1999). Phylogenetic analyses using the resultant alignment as the data set were based on the comparison of ~1,100 sites that were judged to be in alignment.

Phylogenetic analysis of the data sets was carried out by different algorithms (neighbour-joining distance criteria, maximum parsimony criteria, and maximum likelihood criteria) using PAUP*, version 4.0 (Swofford 2002) and the PHYLIP package, version 3.6b (Felsenstein 1993). The branch-and-bound option of PAUP* was used to find the most parsimonious trees. Maximum-likelihood models were chosen by likelihood ratio tests and AIC criteria in Modeltest 3.6 (Posada and Crandall 1998). The selected models were TrN+I+G and GTR+I+G, respectively. Several independent calculations were carried out using different random orders of addition of sequences to increase the probability of finding the shortest or most likely tree. Bootstrap values for all tree-building methods were obtained from 100 resamplings. Consensus trees were calculated by a family of consensus-tree methods, including strict consensus and majority-rule consensus available in CONSENSE (PHYLIP). *Basidiobolus ranarum* (GenBank Accession number D29946) was used as the outgroup. Handling, manipulation, and plotting of trees were carried out with TreeView, version 1.6.6.

The contribution generally follows the terminology and conceptual basis proposed for the microsporidia by Sprague, Becnel, and Hazard (1992).

RESULTS

Infected flies did not exhibit external or gross signs of infection. All examined flies ($n = 40$) and larvae ($n = 40$) from the two stocks were infected with *T. ratisbonensis* gen. nov., sp. nov.

Light microscopy. Development of this microsporidium occurred in larvae and adults of *D. melanogaster*. Sections of larvae revealed that infection was mainly localized in the fat body and the midgut (Fig. 1). Sections of adult flies showed generalized infection with microsporidian spores in the midgut, ovaries, fat body, malpighian tubules, muscles, neural tissue, and salivary glands (Fig. 2–4).

Studies of the microsporidian life cycle in Giemsa-stained preparations showed round rarely elongated, mononucleated,

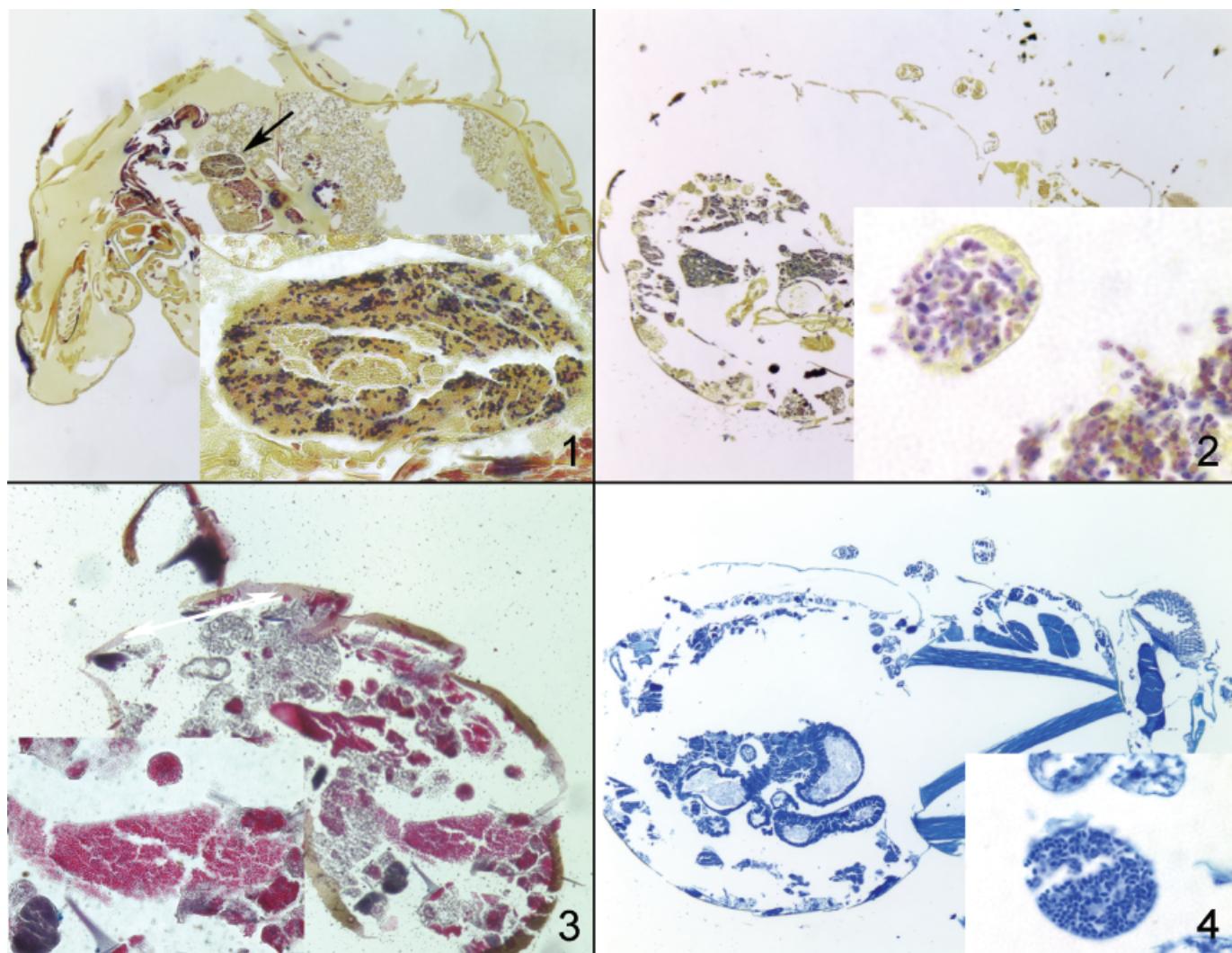


Fig. 1–4. Light microscopic sections of *Drosophila melanogaster* with patent infection with microsporidian spores. 1. Longitudinal section of larvae showing infection with microsporidia (see arrow); Gram stain. 2–4. Longitudinal section of adult fly showing disseminated infection with microsporidia; 2. Gram stain, 3. Modified chromotrope stain, 4. Giemsa stain.

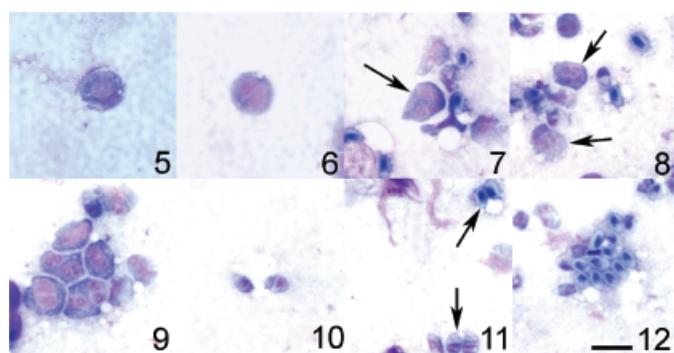


Fig. 5–12. Developmental stages of *Tubulinosema ratisbonensis* gen. nov., sp. nov. as seen in Giemsa-stained smears of infected flies. 5. Monokaryotic meront; 6. Diplokaryotic meront; 7. Meront with two diplokaryotic nuclei (arrows); 8. Two sporonts each with two diplokaryotic nuclei (arrows); 9. Several diplokaryotic developmental stages with one or two pairs of nuclei; 10. Two sporoblast with two band-like nuclei; 11. Two sporoblasts with two nuclei and two spores (arrows); 12. Group of spores. Scale bar = 5 μm.

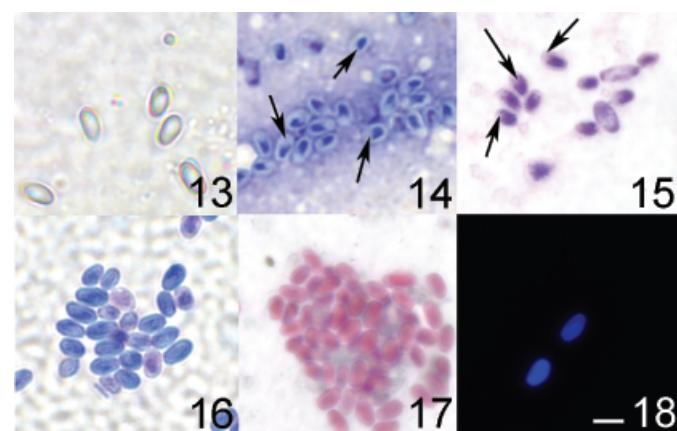


Fig. 13–18. Light microscopy of spores of *Tubulinosema ratisbonensis* gen. nov., sp. nov. 13. Wet mount without staining; 14. Giemsa stain; 15. Gram stain; 16. Toluidine blue O stain; 17. Modified chromotrope stain; 18. Uvitex 2B stain. The unstained posterior vacuole (arrows) can be seen in Giemsa (14) and Gram (15) stains. Scale bar = 3 μm.

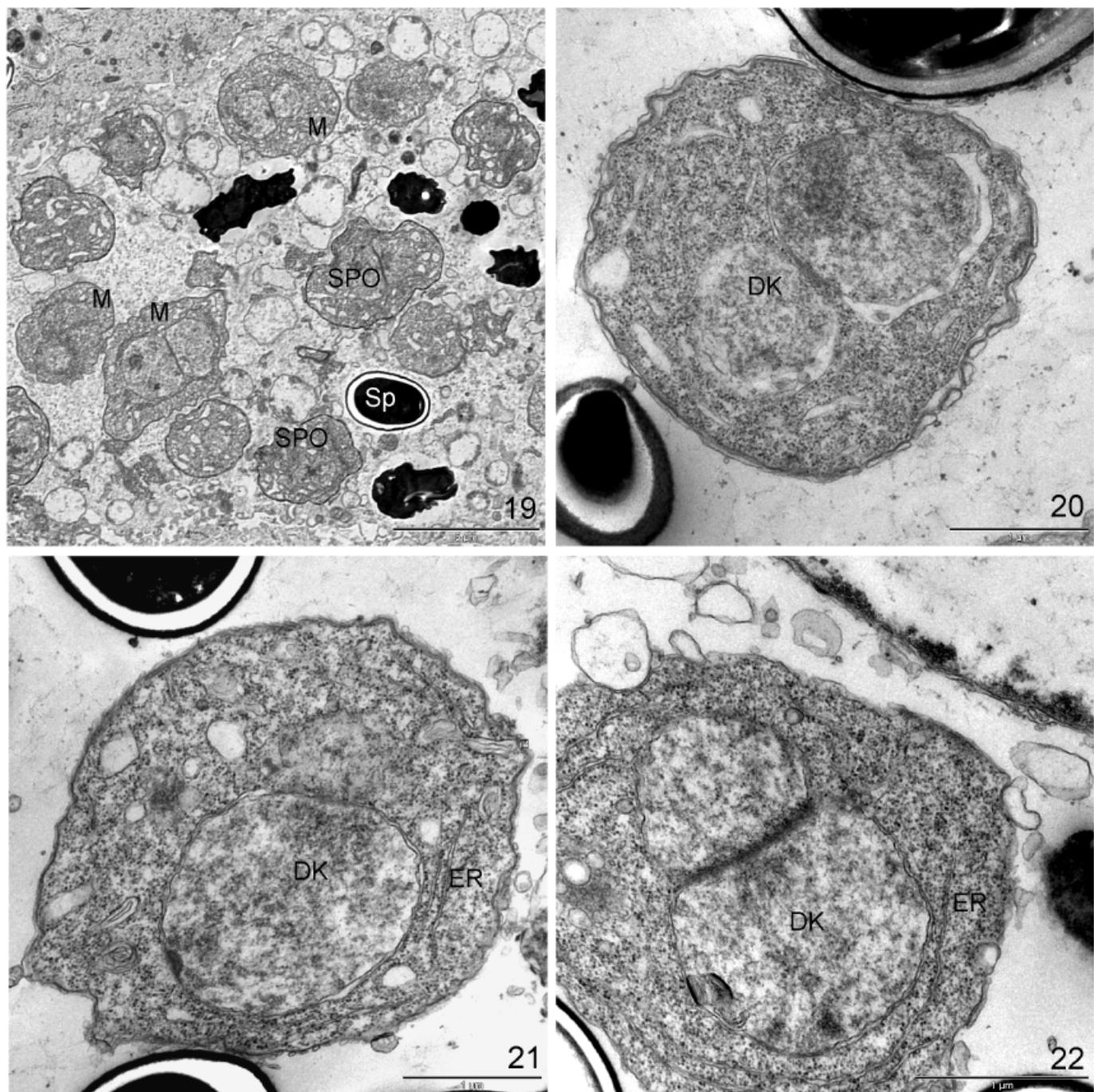


Fig. 19–22. Electron micrographs of proliferative cells of *Tubulinosema ratisbonensis* gen. nov., sp. nov. **19.** Several meronts (M) with diplokaryotic nuclei, some sporonts (SPO) and sporoblasts, and one spore (SP). Scale bar = 5 μ m. **20.** Cross-section through a typical meront with numerous free ribosomes and nuclei in diplokaryotic arrangement. Scale bar = 1 μ m. **21.** Meront with numerous free ribosomes and cisternae of endoplasmatic reticulum. Scale bar = 1 μ m. **22.** Meront with nuclei in diplokaryotic arrangement. Scale bar = 1 μ m. DK, diplokaryon; ER, endoplasmatic reticulum; M, meront; SP, spore; SPO, sporont.

binucleated, or tetranucleated meronts with large nuclei that occupied about half of the area of the meronts and sporonts (Fig. 5–9). Diplokaryons consist of two nuclei often in a coffee bean-like association. Meronts measured $5.35 (\pm 0.80/0.18)$ μ m in diam. ($n = 20$), with nuclei $2.42 \times 1.66 (\pm 0.69/0.15 \times 0.44/0.06)$ μ m in diam. ($n = 20$). No multinucleated plasmodia were seen so that division of meronts was probably by binary fission of tetranucleated forms. Bi- or tetranucleated, round sporonts measured 3.84

($\pm 0.75/0.23$) μ m in diam. ($n = 10$) and had more condensed nuclei as compared with meronts [$1.54 \times 1.13 (\pm 0.41/0.13 \times 0.30/0.09)$ μ m ($n = 10$)]. Division of sporonts gave two oval sporoblasts, each measuring $4.50 \times 2.85 (\pm 0.48/0.11 \times 0.33/0.07)$ μ m ($n = 20$) with two small, band-like nuclei. The sporogonic sequence was diplokaryotic and disporoblastic (Fig. 5–12).

The unfixed mature spores were bean-shaped or slightly pyriform, measuring $4.18 \times 2.48 (\pm 0.26/0.05 \times 0.17/0.03)$ μ m

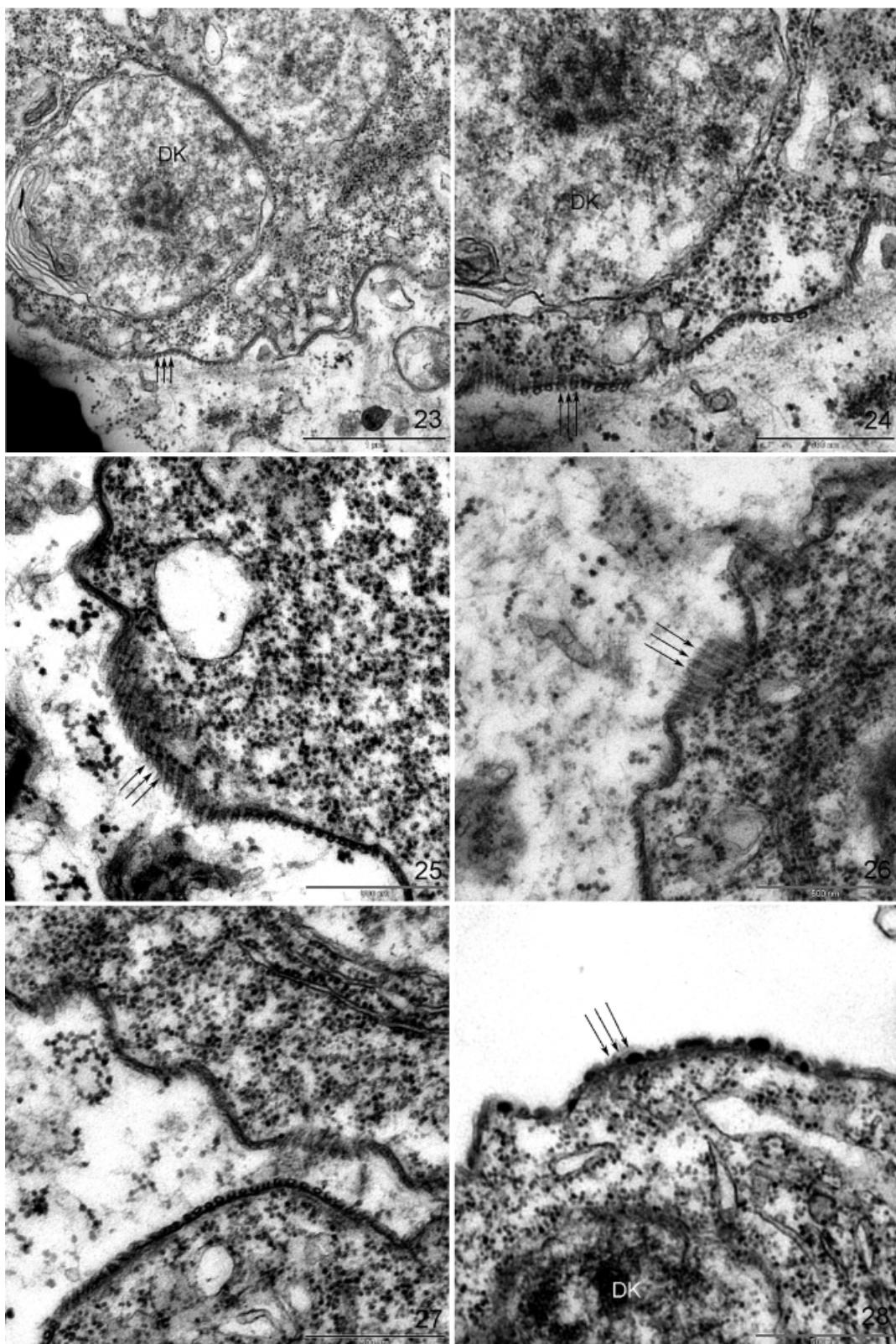
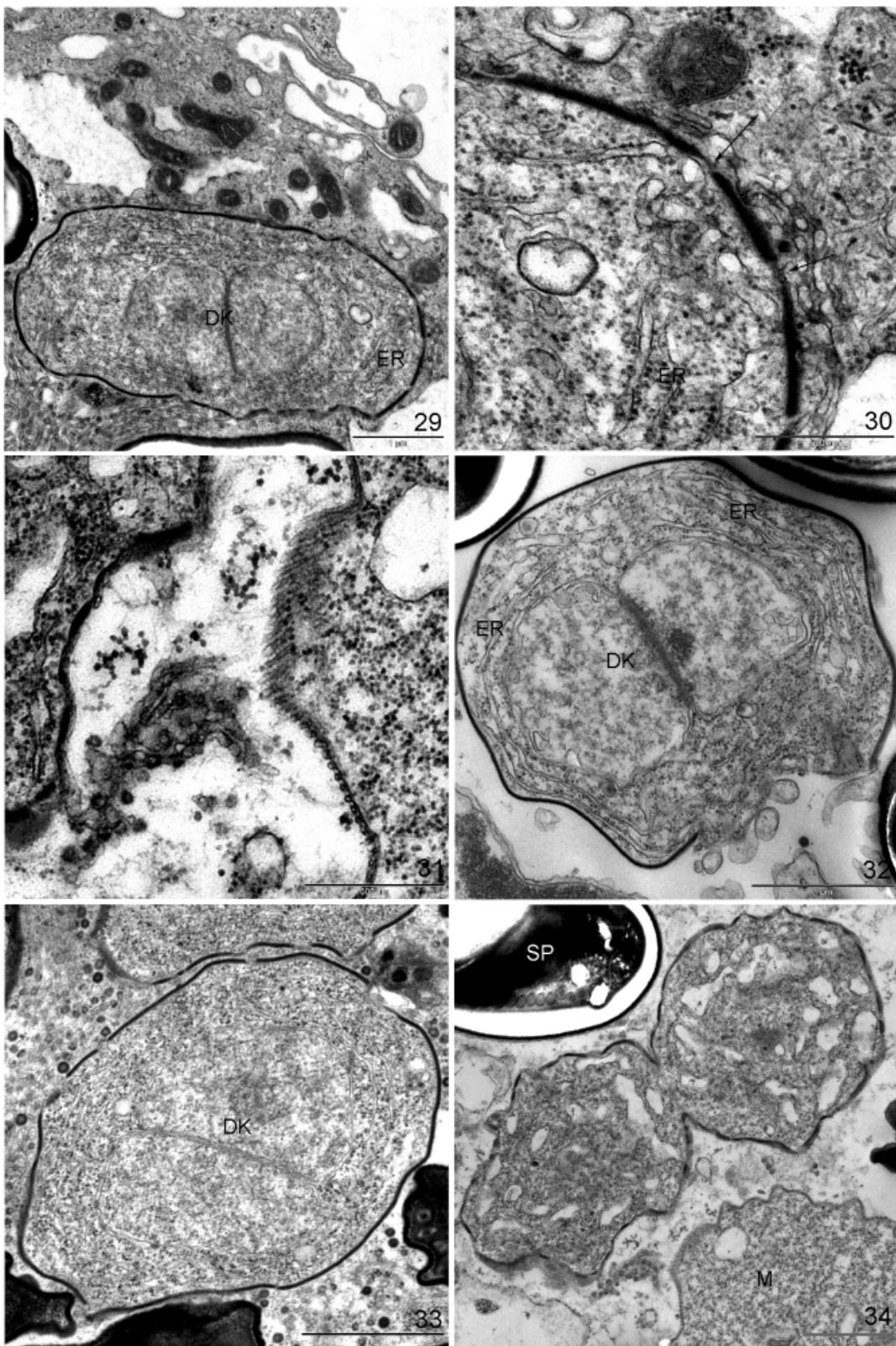


Fig. 23–28. Electron micrographs of the cell surface of meronts of *Tubulinosema ratisbonensis* gen. nov., sp. nov. showing the microtubuli (arrows), diagnostic for the genus. **23.** Meront with diplokaryon and numerous small tubular elements on the surface of the plasma membrane. Scale bar = 1 μ m. **24.** Higher magnification of the parasite plasmalemma showing the tubular elements in cross-section. Scale bar = 500 nm. **25, 26.** Higher magnifications of the parasite plasmalemma in oblique section showing the tubular elements on the surface of the plasma membrane of the parasite. Scale bar = 500 nm. **27.** Plasma membrane of two meronts with microtubuli on the surface. Scale bar = 500 nm. **28.** Development of dense material (arrows) in association with microtubuli on the plasma membrane of the parasite. Scale bar = 500 nm. DK diplokaryon.



($n = 30$) (Fig. 13). Spore size measured on methanol-fixed, Giemsa-stained smears was 3.85×2.36 ($\pm 0.23/0.05 \times 0.21/0.05$) μm ($n = 20$) (Fig. 14). Spores stained pinkish red with the modified chromotrope 2R stain (Fig. 17), light blue with toluidine blue O (Fig. 16), and intense blue after staining with Uvitex 2B (Fig. 18). A fine, bicorn line, representing the polar tube inside the spores, could be seen in some spores at $1,000 \times$ after staining with Uvitex 2B. Spores were Gram positive and the posterior vacuole, which was clearly seen in Giemsa and Gram stains, was located at the wider end of the spores (Fig. 14, 15). Spores were packed tightly inside cells but no sporophorous vesicles could be observed. In squash and smear preparations from infected flies, several spores were released from ruptured cells.

Electron microscopy. Meronts were the earliest stages in the life cycle of *T. ratisbonensis* gen. nov., sp. nov., which we recognized. All meronts were in direct contact with the host cell cytoplasm and there was no evidence of grouping or enclosure within a membrane. They were round or rarely oval in shape measuring $3.25\text{--}3.75 \mu\text{m}$ in diam. as measured in ultrathin sections (Fig. 19–22). Meronts usually had two nuclei closely apposed to form a diplokaryotic arrangement. Both nuclei are limited by double unit membranes and separated by a perinuclear space. Nuclei ranged in diam. from 1.25 to $2.0 \mu\text{m}$ and sometimes contained dense intranuclear particles (Fig. 20–22). Merogony was not observed. However, the absence of large meiotic plasmodia suggests that division was likely to occur by binary fission. More advanced meronts were surrounded by a plasma membrane with a surface coat of dense material deposited in the form of membrane-bound small tubular elements (microtubuli) that encircled the parasite and that were closely packed in one single layer (Fig. 23–28). The meront cytoplasm had numerous ribosomes that were dispersed freely in the cytoplasm of the cells and a few profiles of rough endoplasmatic reticulum.

The appearance of consolidated dense material that developed from the membrane-bound tubular elements on the surface of the plasma membrane leads to sporont formation. Strands of dense material without any ornamentation were deposited on the plasmalemma surface of the parasite (Fig. 29–34). Sporonts were round or slightly oval in shape measuring $3.0\text{--}4.6 \mu\text{m}$ in diam. as measured in ultrathin sections. The cytoplasm of sporonts contained a diplokaryon, ribosomes, and endoplasmatic reticulum as in meronts. Sporogony was by binary fission, with the sporont dividing to produce two diplokaryotic sporoblasts (disporoblastic) (Fig. 34).

Most observed spores were slightly pyriform in shape rather than oval. Spores contained two nuclei in diplokaryotic arrangement (Fig. 35–37). Spores showed a dense exospore layer and a thick lucent endospore layer, which was thinner over the anchoring disc (Fig. 39). The sporoplasm of the spores was enclosed inside a plasma membrane. The polar filament coiled nine to 14 times (mean 12, $n = 20$), lateral to both nuclei and the posterior vacuole and was arranged in a single row. The polar tube was slightly anisofilar with a small difference in polar filament layers and diameter, with the posterior three or four coils being smaller in diameter. The average diam. of the polar filament in the anterior coils was 157 nm and in the posterior coils was 125 nm (Fig. 37, 38). Inside the spores, ribosomes were arranged in a crystalline

pattern as polyribosomes (Fig. 40). No spore dimorphism was observed.

rRNA sequence. An ~ 1.5 kb DNA band was readily amplified from genomic DNA preparations of infected flies and isolated spores by PCR using primers 530f and 580r (data not shown). Sequencing of this DNA fragment utilizing primers 530f and 580r provided a 1,459-bp DNA sequence. The primer pair V1 and 1492 failed to amplify a proper DNA fragment of the SSU rRNA gene. So we designed a reverse primer (NOSr) that binds inside the previously sequenced 530f region of the SSU rRNA gene. A 570-bp DNA band was amplified from the genomic DNA preparations using this primer and the primer V1 (data not shown). These DNA sequences were assembled and the resulting DNA sequence of the SSU rRNA gene, the intergenic spacer region, and the 5'-end of the LSU rRNA gene of *T. ratisbonensis* were submitted to the GenBank database under Accession no. AY695845.

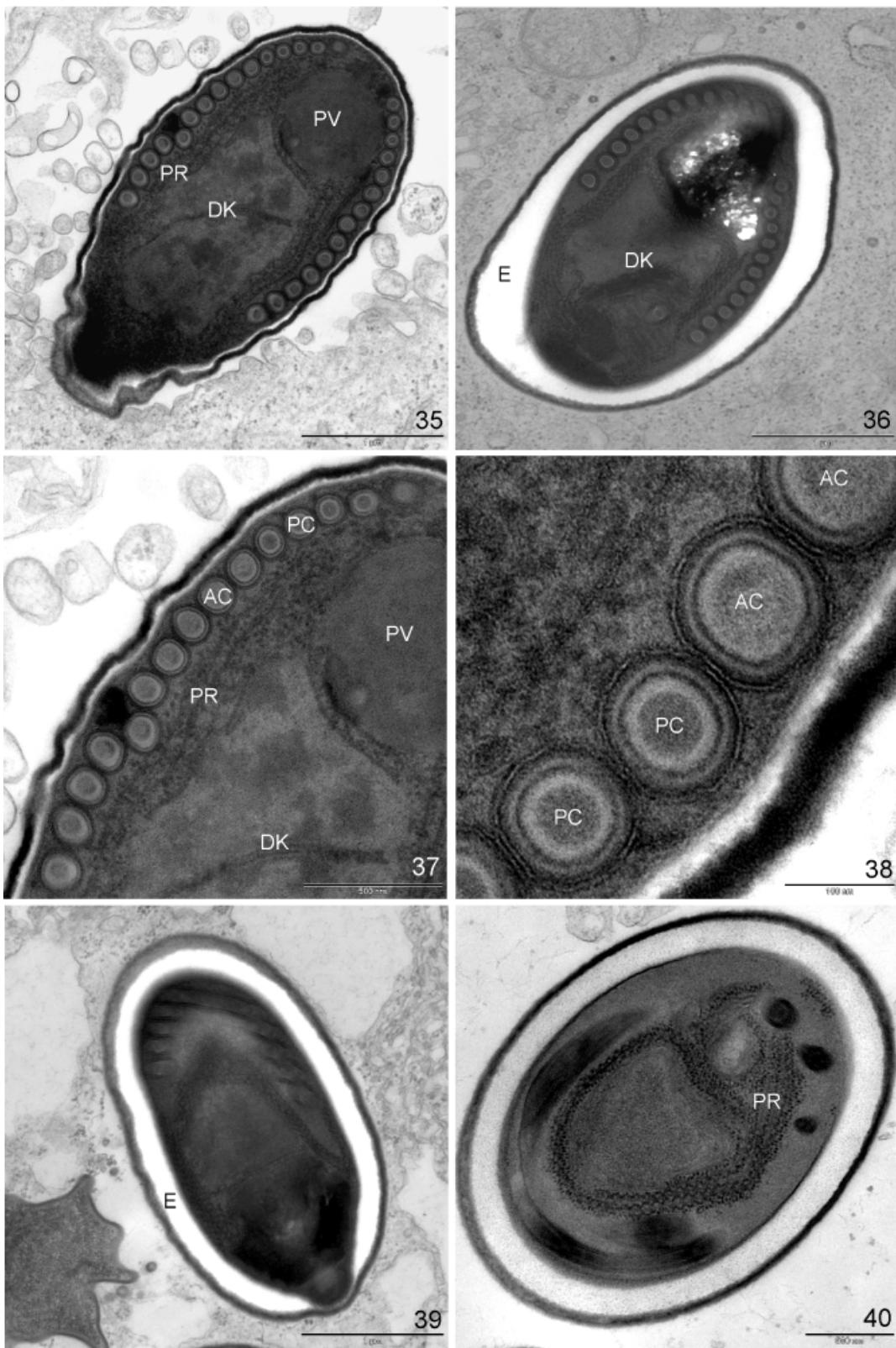
The rRNA sequence was 1,855 bp in length with a GC content of 42%. A BLAST search of the GenBank database with the sequence obtained from *T. ratisbonensis* detected close matches to other microsporidian rRNA sequences. The SSU rRNA sequence of *T. ratisbonensis* showed 99% sequence identity with a sequence submitted as *Visvesvaria acridophagus* (Accession no. AF024658) and 95% sequence identity with an unclassified *Nosema* sp. (AF240356). The LSU rRNA 580r region of *T. ratisbonensis* showed 97% sequence identity with that of *N. kingi* (330 bp fragment). For *N. kingi*, no SSU rRNA sequence or other sequences are currently available in the databases.

Phylogenetic analysis based on SSU rRNA sequence. Sixteen SSU rRNA sequences including those with high BLAST scores were aligned with our *T. ratisbonensis* rRNA sequence and analysed. All applied methods revealed the same general tree topology and in all trees, *T. ratisbonensis* clustered with the sequence submitted as *V. acridophagus* (AF024658) and with *B. algerae* (AF069063) and *Thelohania solenopsae* (AF031538), indicating that these four microsporidia belong to the same group (Fig. 41). Bootstrap values for the trees were above 60% for all the branches of the upper part of the trees and therefore support exists for these trees.

DISCUSSION

The genus *Nosema* is currently under extensive reconstruction and several new genera have been established, but the microsporidium described here does not fit in any of these genera. It does not belong to the family Pseudonosematidae with the genera *Pseudonosema*, *Trichonosema*, and *Bryonosema* because the members of these genera, which are all parasites of freshwater bryozoans, have large spores with numerous polar tube coils arranged as a single row anteriorly and posteriorly and as two or three rows in the middle (Canning et al. 2002). Other genera with high BLAST scores are also out of the question, irrespective of some morphological similarities. The genus *Fibrillanosema* has pockets that contain a fibrillar matrix on the surface of developing sporoblasts (Slotheruber Galbreath et al. 2004); spores of the genus *Paranosema* have 15–20 coils of the polar tube (Sokolova et al. 2003); the genera *Thelohania* and *Janacekia* are characterized by octosporoblastic sporogony and their uninucleated spores are contained within a sporophorous vesicle; the genus *Bacillidium* has

◀ Fig. 29–34. Electron micrographs of sporonts of *Tubulinosema ratisbonensis* gen. nov., sp. nov. 29. Elongated sporont with diplokaryon, free ribosomes, endoplasmatic reticulum, and thickened plasma membrane. Scale bar = $1 \mu\text{m}$. 30. Higher magnification of the thickened plasma membrane of the parasite. The dense material on the surface of the plasma membrane has been deposited in strands with some gaps inside the material (arrows). Scale bar = 500 nm . 31. One meront with tubular structures on the plasma membrane (right) and one sporont with a thickened plasma membrane (left). Scale bar = 500 nm . 32. More advanced sporont with multiple strands of endoplasmatic reticulum and thickened plasma membrane without gaps in the dense material. Scale bar = $1 \mu\text{m}$. 33. Typical sporont with diplokaryon and thickened plasma membrane. Scale bar = $1 \mu\text{m}$. 34. Sporont undergoing binary fission to produce two sporoblasts. Scale bar = $1 \mu\text{m}$. DK diplokaryon; ER, endoplasmatic reticulum; M, meront; SP, spore.



large (17–25 µm) rod-shaped spores; and the genus *Culicospora* shows two sporulation sequences. All these features are not applicable for the microsporidium described in this report. Moreover, the rRNA sequence of the microsporidium and the phylogenetic analysis supports the conclusion drawn from the morphological data that this microsporidium is a new species that should be placed in a new genus as *Tubulinosema ratisbonensis* gen. nov., sp. nov.

The most characteristic ultrastructural feature of *T. ratisbonensis* is the production of small tubular structures on the surface of the parasite. Similar structures could be observed in other microsporidia that also showed high similarities in BLAST searches using SSU and LSU rRNA sequences of *T. ratisbonensis* (Table 1) (Cali et al. 1998; Canning and Sinden 1973; Street and Henry 1993).

In *B. algerae*, a mosquito parasite, and *Brachiola vesicularum*, a human parasite, the cell membrane is covered with a thin layer of dense material interspersed with microtubuli similar to the microtubuli on the cell membrane of *T. ratisbonensis*. In addition to these tubuli, *B. vesicularum* shows numerous vesiculotubular structures on the parasite cell surface, which were not present on the surface of *T. ratisbonensis*. *Brachiola* also develops in direct contact with the host cell cytoplasm and has nuclei in diplokaryotic arrangement, but the number of the coils of the polar filament, which is also slightly anisofilar with the posterior coils smaller in diam. as in *T. ratisbonensis*, did not exceed 10 (*B. vesicularum*) and 12 (*B. algerae*). Moreover, the SSU rRNA data clearly show that *T. ratisbonensis* is distinct from *B. algerae* and *B. vesicularum*, which might be identical species.

There was 99% identity in the SSU rRNA sequence between *T. ratisbonensis* and an SSU rRNA sequence submitted to GenBank as *V. acridophagus* (Accession no. AF024658). However, *Visvesvaria* is not a valid taxonomic name, as the genus was suggested on the basis of two rRNA sequences submitted to GenBank only and has not been described in accordance with the relevant taxonomic code (GenBank entry under Accession no. AF024658). The valid name of this microsporidium is *N. acridophagus*, which is a microsporidian pathogen of grasshoppers (Henry 1967). This monomorphic microsporidian also develops in direct contact with the host cell cytoplasm, has diplokarya during most stages of the life cycle, and its cell membrane is covered with a thin layer of microtubuli similar to the microtubuli on the cell membrane of *T. ratisbonensis*. But there are clear differences in morphology as the meronts of *N. acridophagus* are predominantly elongated cells and the polar filament inside the spores coils only 10–12 times. Although the polar tube was reported to be isofilar, the authors stated that the diameters of the polar tube coil ranged from 75 to 105 nm (Street and Henry 1993). Quadrinucleate forms are common in *N. acridophagus* (Henry 1967), whereas we observed such forms only occasionally in *T. ratisbonensis*. The hosts of *N. acridophagus* are grasshoppers (Orthoptera) whereas the type host for *T. ratisbonensis* is a fruit fly of the genus *Drosophila* (Diptera), but transmission studies to determine host specificity were not carried out. Similar high degrees of rRNA sequence similarity between distinct microsporidian species have been observed earlier, as in the case of *Nosema granulosis*, a parasite of amphipods with an aberrant internal spore ultrastructure, but that shows a high degree of sequence similarity to *N. bombycis*, a parasite of

the lepidopteran *Bombyx mori*, based on SSU rRNA sequence, but transmission studies between hosts of these two species have not been carried out.

Tubulinosema ratisbonensis closely resembles *N. kingi* from *Drosophila willistoni* but there are also clear differences. Burnett and King (1962) first observed this microsporidium in *D. willistoni* and they described some ultrastructural data of the spores. In their line drawings of the microsporidian spores as seen in electron micrographs, the polar tube has 13 coils mostly in a single row at both sides of the spore and in a double row anteriorly. They reported that some photomicrographs showed what appears to be a double row of sectioned filaments. Such double rows of the polar filament were never observed in spores of *T. ratisbonensis*. Unfortunately, Burnett and King (1962) provided no transmission electron micrographs and the ultrastructural morphology of the spores is presented only as line drawings. Further ultrastructural data from other sites are not available for *N. kingi*. Kramer (1964) described the developmental and taxonomically important stages of this isolate (type species), but only light microscopy was used in this study. He observed binucleated meronts 4–5 µm in diam. and advanced stages that were 8–10 µm in breadth with two or four nuclei. Uninucleated cells were also observed and it was thought that the nuclei of the diplokaryon fuse, resulting in an uninucleated sporont. Furthermore, uninucleated sporoblasts were observed and sporogony was described as monosporoblastic (Kramer 1964). However, convincing evidence for fusion of nuclei to produce a sporont was not presented and the sporulation sequence could have been inaccurately interpreted (see Sprague 1977). Spores measured 4.31 × 2.57 µm when examined fresh and 3.67 × 2.06 µm after fixation with an extruded polar tube of 75–95 µm in length. Some of these morphological features resemble *T. ratisbonensis* but sporogony of *T. ratisbonensis* is disporoblastic and developmental stages greater than 6.5 µm in diam. were never observed for *T. ratisbonensis* by us, whereas meronts and sporonts of *N. kingi* are reported to be up to 10 µm in diam. (Kramer 1964). Armstrong (1986) examined microsporidian infections in *D. willistoni* and *D. melanogaster* and compared both isolates at the light microscopic level, but no ultrastructural or molecular data have been reported on these isolates and no material is remaining for comparative studies (Armstrong, E., pers. commun.). In addition to morphological similarities, the DNA sequence of the 580r region of the LSU rRNA gene of *T. ratisbonensis* clearly shows that our isolate is closely related to *N. kingi*. Only this small DNA fragment (338 bp) from the LSU rRNA sequence of *N. kingi* is available through the databases (Accession no. L28966). This LSU rRNA sequence was obtained from an *N. kingi* isolate provided by H. K. Kaya, University of California (Baker et al. 1994). No further information or ultrastructural data are available about this isolate and the original material has been lost (Kaya, H. K., pers. commun.). However, sequence data from the 580r region of the LSU rRNA gene are appropriate characters to use for examining relationships at or near the species level (Baker et al. 1994) and isolates that differ at one or more nucleotides in the 580r region have been found, in all cases, to be separate species (Preparata et al. 1989; Vossbrinck and Friedmann 1989).

Fig. 35–40. Electron micrographs of spores of *Tubulinosema ratisbonensis* gen. nov., sp. nov. 35. Section of an immature spore with diplokaryon, posterior vacuole, and ribosomes arranged in a crystalline pattern as polyribosomes, and 14 coils of the polar tube in cross-section. Scale bar = 1 µm. 36. Mature spore with dense exospore and fully developed thick endospore. Scale bar = 1 µm. 37. Higher magnification of the 14 coils of the polar tube. Scale bar = 500 nm. 38. Higher magnification of the slightly anisofilar polar tube with a small difference in polar filament layers and diameter in the anterior and posterior coils, with the last three or four coils being smaller in diameter. Scale bar = 100 nm. 39. Spore with a thick endospore that is thinner over the anchoring disc. Scale bar = 1 µm. 40. Oblique section of a spore showing ribosomes arranged in a crystalline pattern as polyribosomes. Scale bar = 500 nm. DK, diplokaryon; PR, polyribosomes; PV, posterior vacuole; E, endospore; PC, posterior coils of the polar filament; AC, anterior coils of the polar filament.

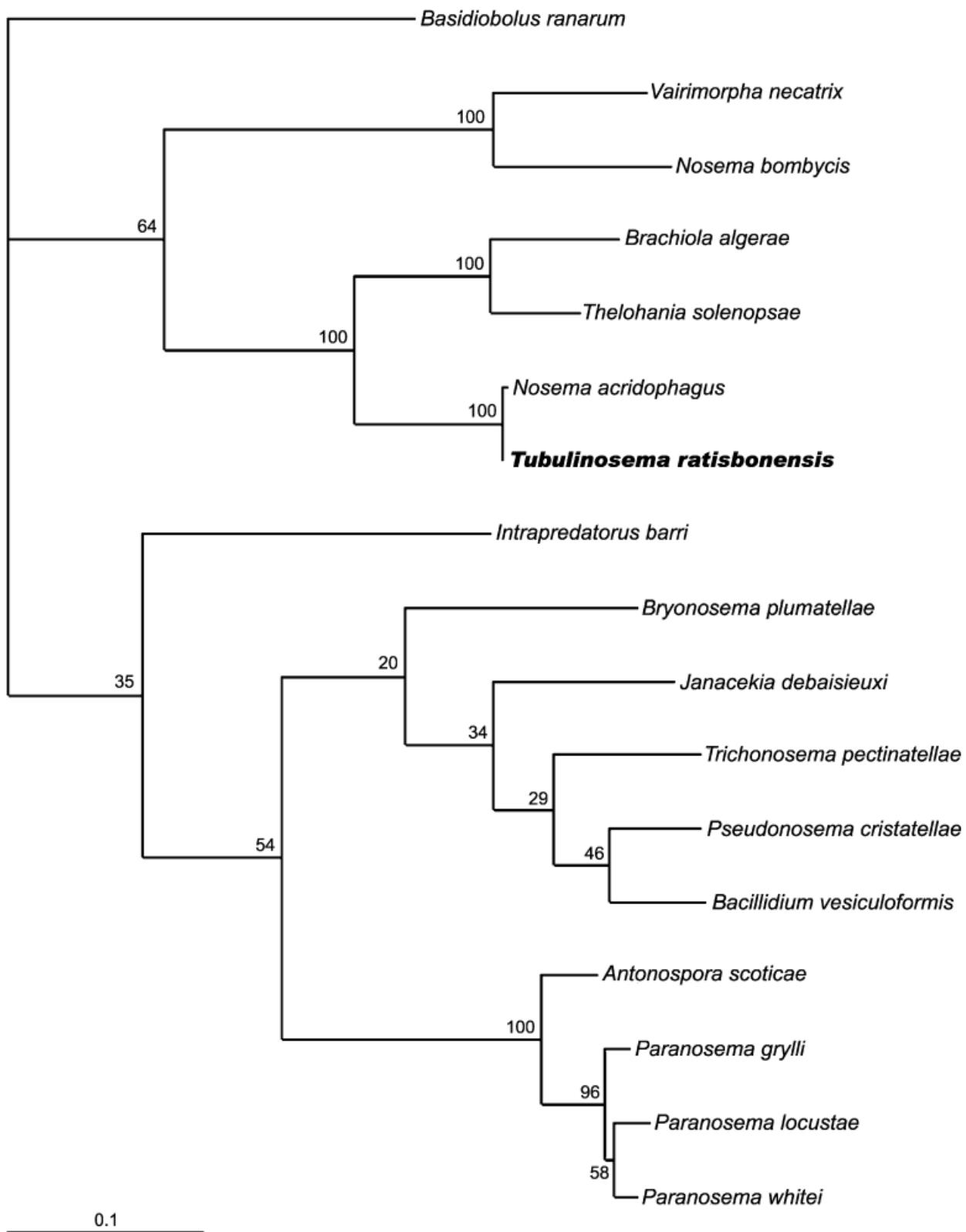


Fig. 41. Maximum likelihood analysis of small subunit rDNA showing the relationship of *Tubulinosema ratisbonensis* gen. nov., sp. nov. with other species of microsporidia. The analysis shows a close relationship of *T. ratisbonensis* with *Nosema acridophagus*. *Brachiola algerae* is a sister taxon to this group. The true *Nosema* spp. including *Nosema bombycis* do not fall into the same clade as *T. ratisbonensis*. Bootstrap values on the tree are from another analysis that provided an identical topology. Nearly the same topology resulted from the other analyses performed (data not shown).

Table 1. Comparison of morphological and ultrastructural features of members of the genus *Tubulinosema*: *T. acridophagus*, *T. kingi*, and *T. ratisbonensis*.

Feature	<i>T. acridophagus</i>	<i>T. kingi</i>	<i>T. ratisbonensis</i>
Type host	<i>Schistocerca americana</i>	<i>Drosophila willistoni</i>	<i>Drosophila melanogaster</i>
Tissue/organ affected	Mg, G, P, N, A	Mg, G, Mt, A	Mg, G, N, A
Meronts shape	Elongate or round	Round to oval	Round to oval or irregular
size (μm)	6–8 (length)	4–5 (diameter)	4–5 (diameter)
nuclei	Two or four	One, two, or four	One, two, or four
Sporonts shape	Oval	Oval or irregular	Round to oval or irregular
size (μm)	4.2–6.4 (diameter)	8–10 (diameter)	4 (diameter)
nuclei	Two or four	Two or four	Two or four
Spore shape	Ovoid	Ovoid	Slightly pyriform
Size (μm)	4.1 × 2.6	4.3 × 2.6	4.18 × 2.48
No. of coils of polar tube	10–12	13	9–14
Arrangement	One row	One or two rows	One row
Polar filament diameter	Isofilar?	Isofilar?	Slightly anisofilar
Reference	Henry (1967) Street and Henry (1993)	Burnett and King (1962) Kramer 1964 Armstrong (1969)	Franzen et al., ibidem

A, adipose tissue; G, gonad; Mg, midgut; Mt, malpighian tubules; N, neural tissue; P, pericardium.

The morphological and molecular data support the placement of this microsporidium in a new genus. We therefore erect the genus *Tubulinosema*, taking the name from the tubuli on the cell surface together with ultrastructural homology of the observed life cycle to members of the genus *Nosema*. The species name *ratisbonensis* refers to the city of Regensburg in Germany where the parasite was isolated. The description includes diplokaryotic life cycle stages with disporoblastic sporogony, oval to slightly pyriform spores, and all stages lie in direct contact with the host cell cytoplasm. All these features are also characteristic for the *Nosema* spp. but there are several unusual ultrastructural features and the phylogenetic analysis places *T. ratisbonensis* alongside *N. acridophagus* and *B. algerae* and distant from the true *Nosema* spp. including *N. bombycis*.

It is probable that the *Nosema*-like microsporidia that produce tubuli on the surface of the meront during late merogony (*Tubulinosema*, *Brachiola*) are all members of one group. They share significant morphological features different from the true *Nosema* spp. and in addition to this uniform ultrastructural character, these microsporidia belong to a single clade in phylogenetic trees based on SSU rRNA analysis. Consequently, we propose to establish a new family *Tubulinosematidae* fam. nov. for them. We must exclude *Thelohania* from this new family on morphological grounds (the sporonts of *Thelohania* undergo octosporoblastic sporogony and the uninucleated spores are contained within a sporophorous vesicle), although this genus at present falls into the same molecular clade. We predict that when additional DNA data from other genes and from a greater number of microsporidian species are included, phylogenetic analyses will show that *Thelohania* is not closely related to the microsporidia that produce tubuli on the surface of their meronts.

Nosema acridophagus shares significant ultrastructural features with *T. ratisbonensis* and SSU-rRNA data clearly show that *N. acridophagus* is distinct from the true *Nosema* spp. and is closely related to *T. ratisbonensis*. At this time, we propose the removal of *N. acridophagus* from the genus *Nosema*, placing it as a second species in the new genus *Tubulinosema*. As rRNA data suggest that *N. kingi* does not belong to the true *Nosema* spp. but is closely related to *T. ratisbonensis*, we propose the removal of *N. kingi* from the genus *Nosema*, placing it as a third species in the new genus *Tubulinosema*.

TAXONOMIC SUMMARY

***Tubulinosema* gen. nov.** Nuclei are in diplokaryotic arrangement during the life cycle. All stages are in direct contact with the host cell cytoplasm. No parasitophorous vacuole or sporophorous vesicle present at any point in the developmental cycle. Merogonial division probably by binary fission of cells with four nuclei in diplokaryotic arrangement. No plasmodial stages. Slightly anisofilar polar tube, with the last coils being smaller in diameter arranged in one or two rows on both sides of the diplokaryon. Small tubuli on the surface of late meronts. Spores are oval or slightly pyriform. Thick endospore wall, thinner over anchoring disc. Generic name based on *Nosema* with the prefix *Tubuli* meaning “small tubes”, characteristic of the surface of late meronts. Type species: *T. ratisbonensis*.

***Tubulinosema ratisbonensis* sp. nov.** The features of the genus: parasites in cytoplasm are randomly dispersed and cells become packed with parasites. Merogony is probably by binary fission of tetranucleate cells, but was not observed. Small tubuli are present on the surface of late meronts. Thickening of the plasmalemma, starting from the small tubuli, leads to sporont formation. Sporonts divide into two sporoblasts by binary fission (disporoblastic). Spores are binucleate and slightly pyriform, $4.18 (\pm 0.26/0.05) \times 2.48 (\pm 0.17/0.03) \mu\text{m}$. The polar tube is slightly anisofilar, with nine to 14 coils in a single row. Moderate to large posterior vacuole. Species name based on the city of Regensburg where the parasite was isolated. Type host: the fruitfly, *D. melanogaster* (Drosophilidae). The infection was systemic in larvae and adults and spreads within the host by autoinfection. All life cycle stages were found in the cytoplasm of several cells. Horizontal transmission probably occurs by mouth, but was not determined. Vertical (transovarial) transmission likely, but not determined. Type locality: *Drosophila* colony at the Department of Zoology at the University of Regensburg, Regensburg, Germany.

Deposition of type specimens: Hapantotypes have been deposited in the US National Parasite Collection, USDA, ARS, ANRI, Bldg. 1180, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705-2350, USA (USNPC No. 094986.00). Additional slides with smears and specimens (embedded in paraffin or plastic resin) are held at the Department of Internal Medicine I,

University of Regensburg, Germany. rRNA: GenBank accession number AY695845.

Tubulinosematidae fam. nov. Nuclei are in diplokaryotic arrangement during most of the life cycle. All stages are in direct contact with the host cell cytoplasm. Merogonial division probably by binary fission of cells with four nuclei in diplokaryotic arrangement. No plasmodial stages. Slightly anisofilar polar tube, with the last coils being smaller in diameter arranged in one, two, or three rows on both sides of the diplokaryon. Small tubuli on the surface of late meronts. Type genus: *Tubulinosema*.

Synopsis of the genus *Tubulinosema*.

Tubulinosema ratisbonensis (Franzen, Fischer, Schroeder, Schölmerich & Schneuwly) gen. nov., sp. nov.

Tubulinosema acridophagus (Henry), comb. nov.

Synonymy. *N. acridophagus* (Henry 1967).

Tubulinosema kingi (Kramer), comb. nov.

Synonymy. *N. kingi* (Kramer 1964).

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