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Flabelliforma montana (Phylum Microsporidia) from *Phlebotomus ariasi* (Diptera, Psychodidae): ultrastructural observations and phylogenetic relationships

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Ultrastructural observations are presented on *Flabelliforma montana* (type species) (Phylum Microsporidia) from sandflies *Phlebotomus ariasi* (Diptera, Psychodidae). All stages have isolated nuclei. The sporophorous vesicle (SV) arises by separation of a 5 nm layer, via small blisters, from the 15 nm surface membrane of sporonts. Sporoblasts form by deep invagination of the sporont surface, the SV at first folding inwards around the invaginations but later unfolding to form a rounded vesicle enclosing spores. The vesicle contains tubules with bulbous terminal expansions. Spores have a deeply domed anchoring disc within a polar sac which covers the anterior polaroplast, consisting of membranes acutely angled to the longitudinal axis around the straight section of the polar tube. Posterior polaroplast membranes, in the region where the polar tube changes course towards the periphery, appear almost transverse in sagittal section. The 130 nm thick endospore is overlain by several membrane-like layers, which together with amorphous material constitute the exospore. There are 3.5–4.0 coils of the polar tube. *Flabelliforma ostracodae*, *Flabelliforma diaptomi* and *Flabelliforma magnivora* conform well in morphology with the type species. However, when *F. magnivora* was included in parsimony and maximum likelihood analyses, using 16S rDNA sequences of 20 microsporidia, it emerged as unrelated to *F. montana*. Disparities were found between morphological characters and molecular groupings of several of the microsporidia investigated. Within clades, nuclei could be isolated or diplokaryotic, sporophorous vesicles present or absent and life cycles simple or complex.

Key words: *Flabelliforma* spp.; Sandflies; Microcrustaceans; Ultrastructure; Phylogeny; 16S rDNA.

Introduction

The polysporous microsporidium *Flabelliforma montana* was described from midgut epithelial cells of sandflies, *Phlebotomus ariasi*, and was transmitted orally to larvae of *P. ariasi*, including those hatching from eggs laid by an uninfected female (Canning et al. 1991). A dichotomous key was presented to distinguish *Flabelliforma* from twelve other polysporous microsporidia which had been described previously. *F. montana* was shown to have isolated nuclei throughout its development and to undergo sporogony by division of a sporogonial plasmodium via deep lobes within a sporophorous vesicle (SV), which at first followed the contours of the lobes but finally became rounded at the time of sporoblast separation. The

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SV was a fragile structure, containing about 30 sporoblasts. Spore preservation was poor but a membranous polaroplast and 3.5 coils of the polar tube (= polar filament) were observed.

Three further species have since been ascribed to the genus: *Flabelliforma ostracodae* from *Candona* sp. (Crustacea, Ostracoda) (Bronnvall and Larsson 1994), *Flabelliforma diaptomi*, a new combination proposed by Voronin (1996) for *Stempellia diaptomi* Voronin, 1977 from *Diaptomus gracilis* (Crustacea, Copepoda) (Voronin 1977) and *Flabelliforma magnivora* from *Daphnia magna* (Crustacea, Cladocera) (Larsson et al. 1998). *F. magnivora* had previously been referred to as *Tuzetia* sp. (Mangin et al. 1995). The 3 species were distinguished from the type species by features such as number of sporoblasts produced by a sporont, spore size and number of coils of the polar tube. Larsson et al. (1998) placed *F. magnivora* in the family Duboscqiidae adopting the systematic position used by Bronnvall and Larsson (1994) for *F. ostracodae*. The pathogenicity and epidemiology of infections of *F. magnivora* in *D. magna* have been described by Mangin et al. (1995) and Ebert et al. (2000).

In the summer of 1998 we collected adult *P. ariasi* at a site in Laumède in the Cevennes mountains, France and obtained several new infections of *F. montana*. We have used this material and samples of *D. magna* infected with *F. magnivora* to obtain 16S rDNA sequences. We present new ultrastructural data on *F. montana* to add to the description of the type species and assess the systematic positions of the other species currently attributed to *Flabelliforma*, using the ultrastructural data and a molecular phylogeny based on 22 sequences of the 16S rDNA from 20 species of microsporidia, derived mainly from invertebrate hosts. We also obtained sequences of *Cystosporogenes operophterae* and *Orthosomella operophterae* from winter moth *Operophtera brumata* and included these sequences in the phylogenetic analysis.

Materials and methods

Sources of *Flabelliforma* spp., *O. operophterae* and *C. operophterae*

Adult male and female *P. ariasi* were caught in light traps set in the cellar, stable and gardens of a house in Laumède, Cevennes mountains, France between 16/08/98 and 20/08/98. The gut of each fly was dissected into phosphate buffered saline (PBS) within 24 h of trapping and examined by microscopy. Guts showing signs of infection with *F. montana* were either fixed in 96% ethanol for DNA extraction or were stored in PBS before being fed to larvae of *P. ariasi*. Spores from one heavily infected fly were placed in a pot with 126 *P. ariasi* eggs laid by two sandflies and spores from one heavily infected and one lightly infected sandfly were placed in a pot with 258 eggs laid by five sandflies. Some emergent larvae were examined at the fourth instar. The remainder entered diapause and were examined as adults eight months after exposure to spores. Infected guts from the experimental infections were preserved in 96% ethanol for DNA extraction. A few remaining spores were added to a new batch of *P. ariasi* larvae and, from these, two heavily infected guts were fixed in Karnovsky's fixative for electron microscopy.

Daphnia magna infected with *F. magnivora* were collected from ponds, one in Oxfordshire, UK and the other near Moscow, Russia in 1992 (Larsson et al. 1998; Ebert et al. 2000). Females were kept singly in filtered pond water and their parthenogenetic offspring were transferred to fresh pond water within 10 hours of birth. Clones were raised from these offspring, all of which were infected and infected hosts have been maintained in the laboratory by transovarial transmission since 1993. After maintenance for 3 years infected *D. magna* were preserved in 96% ethanol for extraction of DNA.

Winter moth larvae were collected from Wytham Wood, Oxford, UK and Wistman's Wood, Dartmoor, UK in 1997. Salivary glands were dissected and those infected with *O. operophterae* or *C. operophterae* were fixed whole in 96% ethanol or suspended in PBS for DNA extraction.

Electron microscopy of *F. montana*

The previously fixed guts were transferred to 0.1 M cacodylate buffer pH 7.2, post fixed in 1% Os O_4 in cacodylate buffer and dehydrated in an ethanol series. After transfer to propylene oxide, then mixtures of 70:30 and 30:70 propylene oxide : Agar 100 resin (Agar Scientific, Stansted, UK), the material was embedded in Agar 100 resin. Sections were stained with uranyl acetate and lead citrate and examined under an AE1 EM801 electron microscope.

Nucleic acid preparation

DNA extraction was carried out on guts of infected *P. ariasi*, whole infected *D. magna* and salivary glands of *O. brumata*. Samples were suspended in 300 µl of TE buffer, pH 7.2, and shaken with 300 mg of 0.5 mm Zirconium beads in a Mini-Bead beater (Biospec Products Ltd.) at low speed for 30 s. A 15 µl aliquot of a 10

mg/ml^{-1} Proteinase K stock solution in H_2O was added and the samples were incubated at 55 °C for 2 h. After further bead-beating for 30 s, the suspension was extracted twice with phenol:chloroform (1:1) and the DNA was precipitated with sodium acetate and ice cold ethanol (Sambrook et al. 1989). The DNA was suspended in TE buffer, pH 8.0 and stored at –20 °C.

PCR amplification and sequencing

The 16S rDNA was amplified using the 18f (5′-CACCAGGTTGATTCTGCC-3′) and 1537r (5′-TTATGATCCTGCTAATGGTTC-3′) primers designed by Baker et al. (1995). PCR amplification was carried out in 20 µl volumes, using about 10 ng DNA, 5 pmol of each primer, 0.2 mM of each dNTP, 2 mM $MgCl_2$ and 1 unit of Taq polymerase (Life Technologies Ltd., Inchinnan, Paisley). The reactions were comprised of 92 °C denaturation for 2 min, followed by 30–40 cycles of 92 °C denaturation for 30 s, 48–58 °C annealing for 30 s and 72 °C extension for 45 s, with a final extension of 5 min. PCR products were cleaned using the Wizard PCR preps (Promega, UK) and cloned into the pGem-T Easy Vector system (Promega, UK). One to three clones were selected, amplified with SP6 and T7 vector system primers, and cleaned, as before, for sequencing with the Thermo Sequenase dye terminator cycle sequencing pre-mix kit (Amersham, UK). Six primers were used to obtain the whole sequence in both directions: SP6, T7, 3F (= 5′-GTCCAAGGA/TC/GG CAGCAGGC-3′), 4F (= 5′-CACCACCAGGAGTGG AGTGTG-3′), 5R (= 5′-CACA/AC/TCCACTCCTT GTG G-3′) and 6R (= 5′-GCCTGCTGCTGTCCTTG-GAC-3′).

Table 1. Species and isolates of microsporidia used in the phylogenetic analyses, their hosts, the accession numbers and sequence lengths of the 16S rDNA.

Microsporidia	Hosts (Systematic Group[1])	Accession Number	Sequence Length (bp)
Amblyospora stimuli	*Aedes stimulans* (DC)	AF027685	1361
Ameson sp.[2]	*Litopenaeus setiferus* (CD)	AJ252959	1305
Bacillidium sp.	*Lumbriculus* sp. (O)	AF104087	1386
Caudospora palustris	*Cnephia ornithophilia* (DS)	AF132544	1374
Culicosporella lunata	*Culex pilosus* (DC)	AF027683	1343
Cystosporogenes operophterae	*Operophtera brumata* (LG)	AJ302320	1258
Edhazardia aedis	*Aedes aegypti* (DC)	AF027684	1448
Endoreticulatus schubergi	*Lymantria dispar* (LL)	L39109	1252
Flabelliforma magnivora (C1, Russia)	*Daphnia magna* (CC)	AJ302318	1197
Flabelliforma magnivora (E-11-18, Oxford)	*Daphnia magna* (CC)	AJ302319	1304
Flabelliforma montana	*Phlebotomus ariasi* (DP)	AJ2052962	1115(p)[3]
Janacekia debaisieuxi	*Simulium* sp. (*S. ornatum* or *S. equinum*) (DS)	AJ252950	1417
Nosema algerae	*Anopheles stephensi* (DC)	AF069063	1365
Nosema tyriae	*Tyria jacobaeae* (LA)	AJ012606	1233(p)
Orthosomella operophterae (Dartmoor)	*Operophtera brumata* (LG)	AJ302316	1197(p)
Orthosomella operophterae (Oxford)	*Operophtera brumata* (LG)	AJ302317	1280
Polydispyrenia simulii	*Simulium* sp. (*S. ornatum* or *S. equinum*) (DS)	AJ252960	1291(p)
Thelohania solenopsis	*Solenopsis invicta* (H)	AF031538	1382
Trachipleistophora hominis	*Homo sapiens* (PH)	AJ002605	1364
Vavraia culicis	*Aedes albopictus* (DC)	AJ252961	1364
Vairimorpha imperfecta	*Plutella xylostella* (LY)	AJ131645	1231
Vittaforma corneae	*Homo sapiens* (PH)	U11046	1237
Giardia lamblia (outgroup)	(Portland 1 strain)	M54878	1453

[1]CC = Crustacea, Cladocera; CD = Crustacea, Decapoda; DC = Diptera, Culicidae; DP = Diptera, Psychodidae; DS = Diptera, Simuliidae; H = Hymenoptera; LA = Lepidoptera, Arctiidae; LG = Lepidoptera, Geometridae; LL = Lepidoptera, Lymantriidae; LY = Lepidoptera, Yponomeutidae; O = Oligochaeta, Lumbriculidae; PH = Primates, Hominidae. [2]Referred to as *Pleistophora* sp. (LS) in Cheney et al. (2000). [3]p = partial

Phylogenetic analysis

For phylogenetic analysis, the 16S rDNA sequences of *F. montana* and *F. magnivora* were aligned with those of 18 other species of microsporidia (two sequences of *O. operophterae*), which are listed with their hosts in Table 1. The corresponding sequence of *Giardia lamblia* (Portland 1 strain) was used as outgroup.

Sequences were aligned using the CLUSTAL W programme (Thompson et al. 1994), which gave an alignment of 1559 characters. Only those sites which could be unambiguously aligned among all the microsporidia and the outgroup were used to construct phylogenetic trees (1168 bp). The aligned sequences were analyzed by both parsimony and maximum likelihood methods using PAUP version 4.0b2a (Swofford 1998). For the parsimony tree, an initial heuristic search was carried out on the 23 CLUSTAL aligned sequences, using the tree bisection and reconnection swapping algorithm and 1000 bootstrap resamplings with 10 subreplicates and random addition of the sequences. The resulting bootstrap tree was used to construct a 90% majority rule (majrule) consensus tree. The nexus file from the aligned sequences was then imported into MacClade version 3.07 (Maddison and Maddison 1997), together with the 90% majrule tree, and the rescaled consistency (RC) index used to weight the data, treating the polytomies as soft. The weighted data set was then imported back into PAUP and the branch and bound option of PAUP was used to generate the most parsimonious tree, using the 90% majrule consensus tree as a constraint and the MacClade calculated RC character weights. Bootstrap analysis (100 replicates) was performed to give a measure of the confidence that can be placed in the resulting gene tree. Maximum Likelihood analysis was carried out on a subset of 10 microsporidian species and *G. lamblia*, using either a heuristic search or a branch and bound search, both with 100 bootstrap resampling, but using a 90% majrule consensus tree generated from an initial parsimony analysis as a constraint for the latter. A topological constraint tree, which constrained the *Flabelliforma* spp. together, was constructed using the same search strategy as that used to find the minimum (unconstrained) trees (a heuristic search with 100 bootstrap resamplings and 10 subreplicates on unweighted data) and the winning-sites of Kishino-Hasegawa test options in PAUP.

Results

Prevalence of *F. montana*

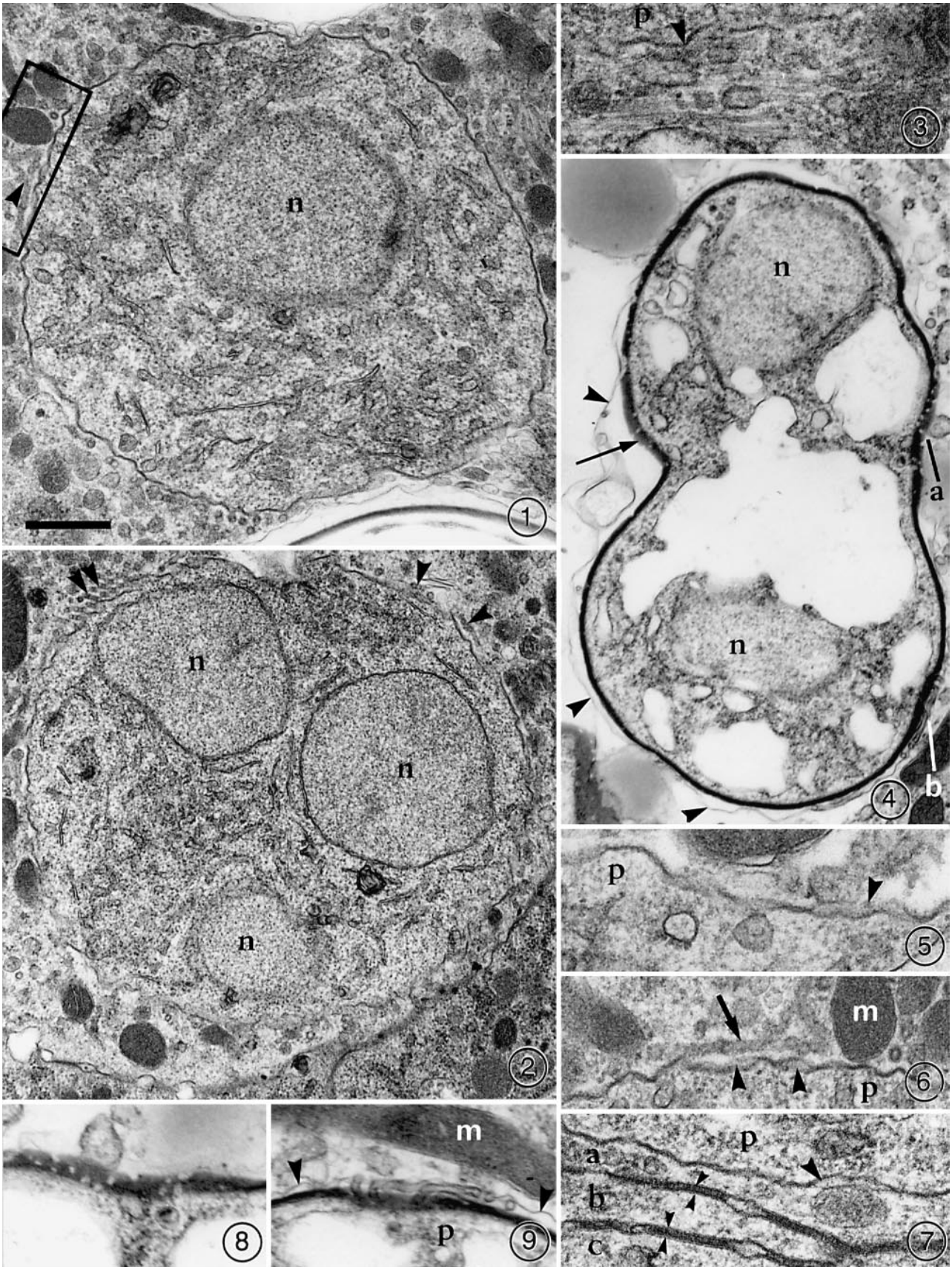
F. montana was found in 6 wild caught sandflies, 3 males and 3 females out of 331 dissected. On a scale of 1+ to 5+ (low to high intensities of infection): 2 were 2+, 2 were 4+ and 2 were 5+.

Ultrastructure of *F. montana*

Undivided meronts (Figs. 1, 2) and meronts dividing by plasmotomy were observed with up to four nuclei in one plane. Structures, appearing as rows of 45 nm diameter beads lying in host cell cytoplasm close to meront surfaces (Figs. 1, 2), were sometimes seen emanating from the meront surface (Fig. 2). Microtubules, 25 nm diameter, were common in the host cell cytoplasm surrounding all stages of the parasite (Fig. 3).

Sporogony was initiated by deposition of a thick surface coat on the sporont (Fig. 4) at the time of separation of a fine membrane from the plasma membrane to form a sporophorous vesicle (SV) (Fig. 4), within which spores were eventually held. The mode of formation of the vesicle envelope was not determined with certainty but there was strong evidence that it was parasite derived as follows: the surface structure of the meront measured 15 nm and was thick enough to have incorporated two tightly apposed membranes. These appeared to separate (Figs. 5–7) in preparation for deposition

Figs. 1–9. *Flabelliforma montana*: merogony and early sporogony. p = parasite, n = nuclei, m = host cell mitochondria. Bar on Fig. 1 applies to all figures. 1, 2. Meronts with one and three nuclei in the plane of section, are in direct contact with host cell cytoplasm. Beaded structures indicated by arrowheads appear to emanate from the parasite's surface in Fig. 2. The boxed area in Fig. 1 is enlarged in Fig. 6. Bar = 400 nm (Fig. 1), 625 nm (Fig. 2). 3. Microtubules in host cell cytoplasm near surface (arrowhead) of parasite. Bar = 200 nm. 4. Binucleate sporont with electron dense, pitted surface coat (arrow) surrounded by a very fine vesicle envelope (arrowheads). Enlargements of (a) and (b) are shown in Figs. 8 and 9. Bar = 625 nm. 5–7. Formation of sporophorous vesicle envelope. The electron dense surface layer of the parasite appears to split into two leaflets (large arrowheads). Junctions of three host cells (small arrowheads) are included in Fig. 7 to show the appearance of unit membrane for comparison with the parasite's surface. Fig. 6 is an enlargement of the boxed area in Fig. 1 and shows the beaded structures (arrow) close to the parasite's surface. Bar = 275 nm (Fig. 5) and 220 (Figs. 6, 7). 8. Enlargement of (a) from Fig. 4 showing pitted surface coat of sporont. Bar = 333 nm. 9. Enlargement of (b) from Fig. 4, showing tubular structures emanating from surface of parasite and lying within the fine sporophorous vesicle envelope arrowheads. Bar = 333 nm.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of the sporont surface coat which was secreted, at first in patches (Fig. 12), then as a continuous coat (Fig. 4). In patches lacking the surface coat (Figs. 10, 12) the SV envelope remained attached to the plasma membrane, indicating that it is the secretion of the surface coat that finally forces the separation of the SV envelope from the parasite's surface. Tangential sections revealed the surface coat to be a pitted layer (Figs. 4, 8, 10) even up to the stage when sporoblasts were being separated (Fig. 16). Tubules, measuring about 40 nm in diameter running between the SV envelope and the sporont surface coat appeared to connect with the sporont plasma membrane possibly through the pits in the surface coat (Fig. 9). In its final form on the sporont, the surface coat was a layered structure (Fig. 11) consisting of a 10 nm amorphous centre separating less dense 5 nm outer and inner layers, giving a total thickness of 20 nm. The coat was separated from the 7.5 nm plasma membrane by a 5–10 nm gap. The SV envelope became separated in places as two leaflets (Figs. 10, 12).

Nuclear division continued during sporogony. A centriolar plaque was surmounted by two or more double-membrane vesicles (Figs. 12, 13) and was the point from which spindle microtubules radiated into the nucleoplasm and terminated in chromosomes (Fig. 10). Division of the sporont occurred by constriction of the cytoplasm around uninucleate segments. In one sporont prominent paramural bodies were present at the bases of the invaginations, at points where the surface coat was absent (Figs. 14, 15). In the early stages of sporoblast separation some host cell cytoplasm, in contact with the SV envelope, was carried in with the constricting sporont surface (Figs. 16–18) but finally the contours of the vesicle smoothed out (Figs. 19, 21) so that the sporoblasts and spores lay in a rounded vesicle. The vesicle envelope remained in direct contact with host cell cytoplasm at the end of sporogony. Some fragments of the sporont contained two nuclei (Fig. 16) before final division into uninucleate sporoblasts. A complex of 20 nm diameter tubules was seen in tangential section within or beneath the surface coat of one sporoblast (Fig. 21).

Vesicles containing spores almost invariably also contained tubules consisting of an outer 60–100 nm cylinder and inner 30–50 nm tube (Fig. 23). The tubules terminated in bulbous expansions, involving an increase in diameter of the inner tube to 70 nm and outer tube up to 140 nm. The maximum length of a tubule observed in section was 1.2 µm (Fig. 22). Spores were only seen fresh in gut squashes and were not measured. They were flattened on one side, convex on the other with both ends tapering. The exospore derived from the sporont surface coat was a layered structure (Fig. 25), consisting of an outer 8 nm amorphous coat, three or four parallel membranes together measuring about 16 nm and an inner 11 nm amorphous layer, measuring about 35 nm in total. The endospore measured about 130 nm, except over the anchoring disc where it

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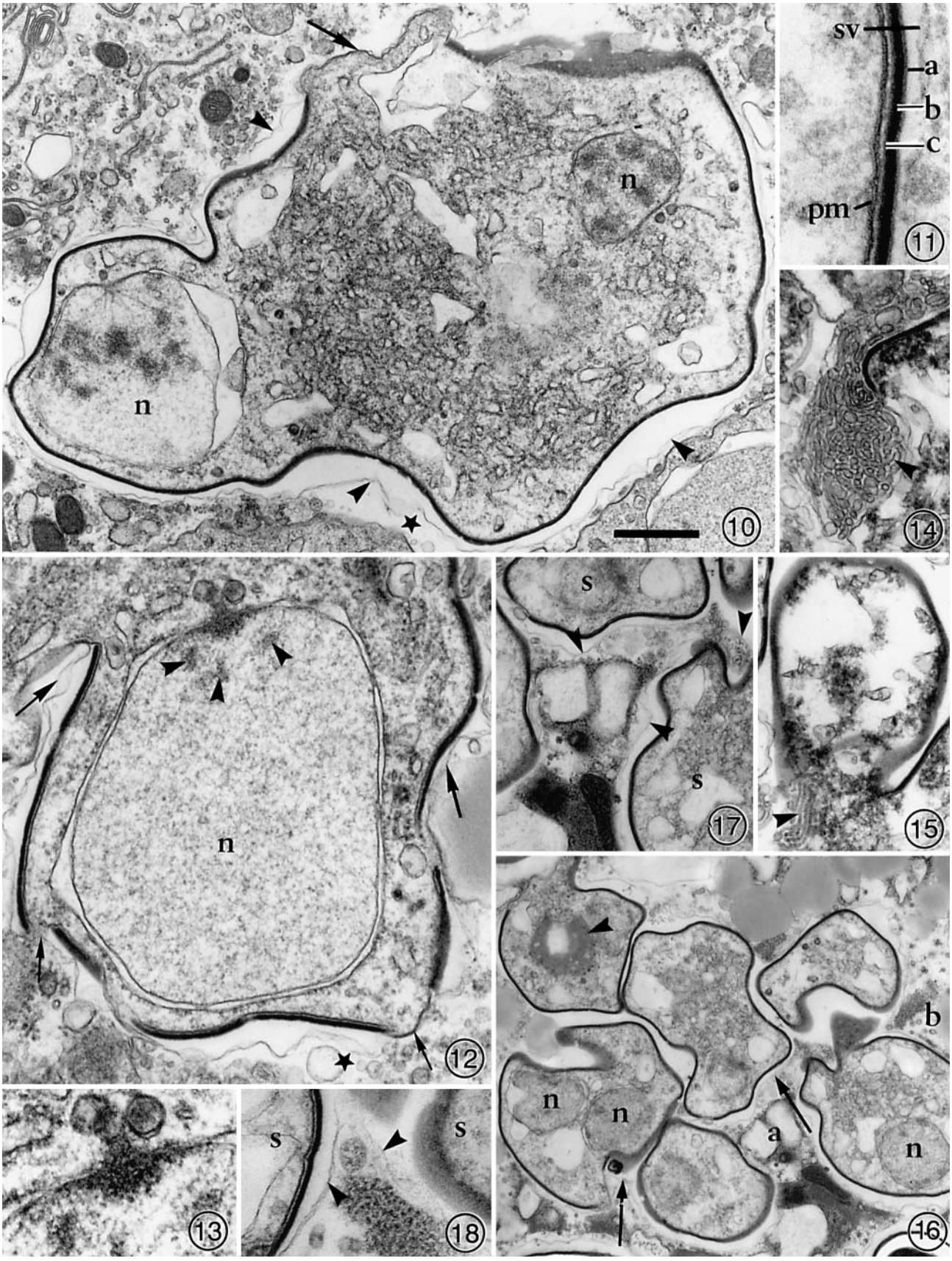
Figs. 10–18. *Flabelliforma montana* : stages of sporogony. n = nuclei. Bar on Fig. 10 applies to all figures. 10. Early sporont, with pitted surface coat, enclosed by a fine sporophorous vesicle envelope (arrowheads) which has separated in places into 2 leaflets (star). In a patch lacking the surface coat, the sporophorous vesicle envelope is still closely apposed to the plasma membrane (arrow). Nuclei show chromosomes and spindle of mitosis. Bar = 680 nm. 11. Surface of a mature sporont showing plasma membrane (pm) surface coat of three layers (a, b, c) and sporophorous vesicle envelope (sv). Bar = 110 nm. 12. Formation of a cytoplasmic bud in a sporont with incomplete surface coat. Chromosomes (arrowheads) close to a spindle pole indicate end of karyokinesis. Note that the sporophorous vesicle (large arrows) follows the contours of the bud and is attached to the plasma membrane in patches where the surface coat is lacking (small arrows). In other places (star) it has separated into 2 leaflets. Bar = 375 nm. 13. Enlargement of spindle plaque from Fig. 12 showing electron dense plaque in nuclear envelope, surmounted by double-walled vesicles. Bar = 200 nm. 14, 15. Complex tubular paramural bodies (arrowheads) at points on the surface of sporonts where buds emerge to form sporoblasts. 16. Almost complete division of sporont into sporoblasts (the buds are connected in another plane of section). At this stage the sporophorous vesicle, accompanied by host cell cytoplasm, follows the contours of the sporoblast buds (arrows). Note that one branch still has two nuclei, implying further division will take place. The surface coat is now almost devoid of pits but some pits remain (arrowhead). Areas a and b are enlarged in Figs. 17, 18. Bar = 1.1 µm. 17. Enlargement of (a) from Fig. 16. Sporophorous vesicle envelope (arrowheads) and host cell cytoplasm occupying the space between sporoblast buds (s). Bar = 680 nm. 18. Enlargement of (b) from Fig. 16 showing host cell cytoplasm and sporophorous vesicle envelope (arrowheads) between sporoblast buds (s). Bar = 280 nm.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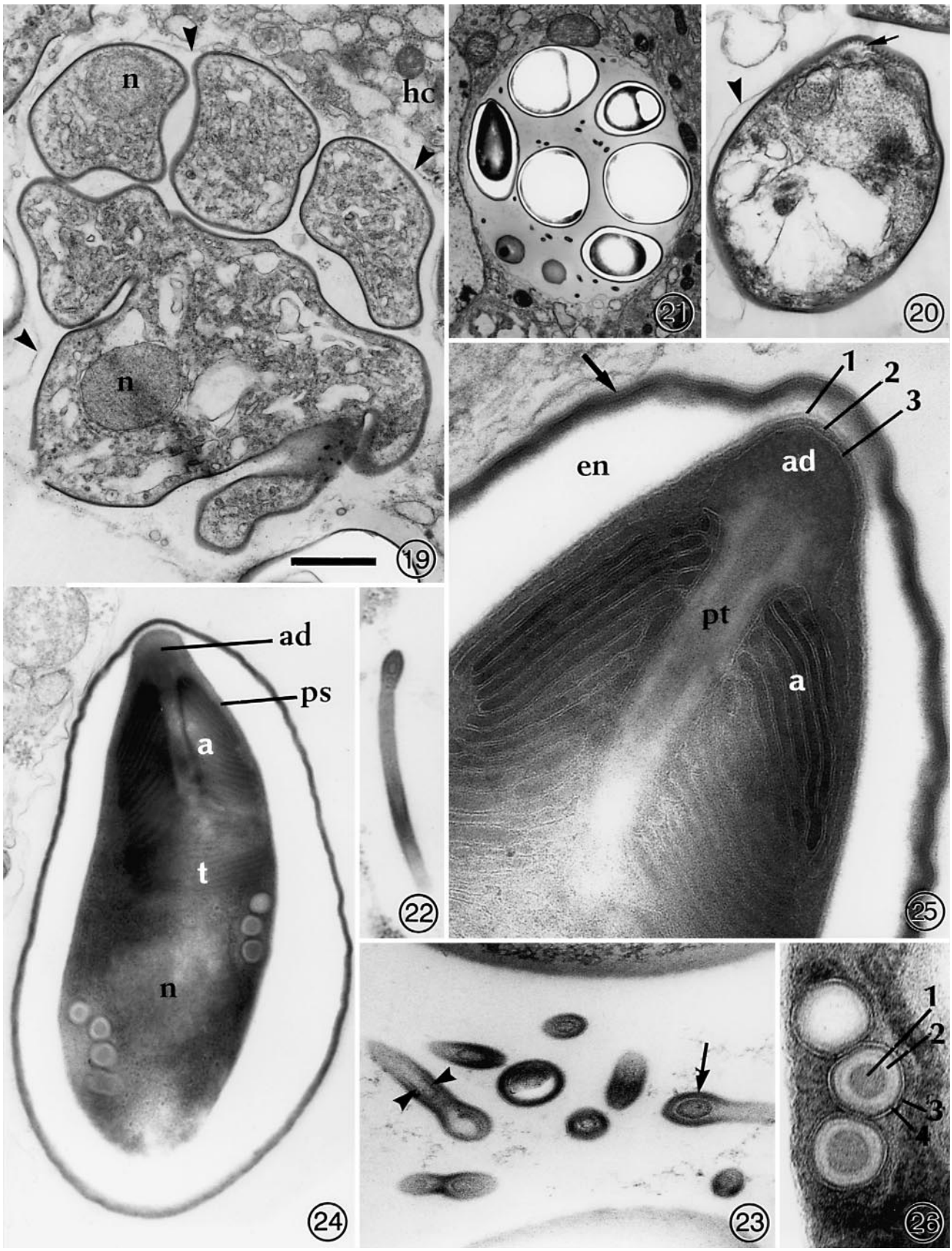
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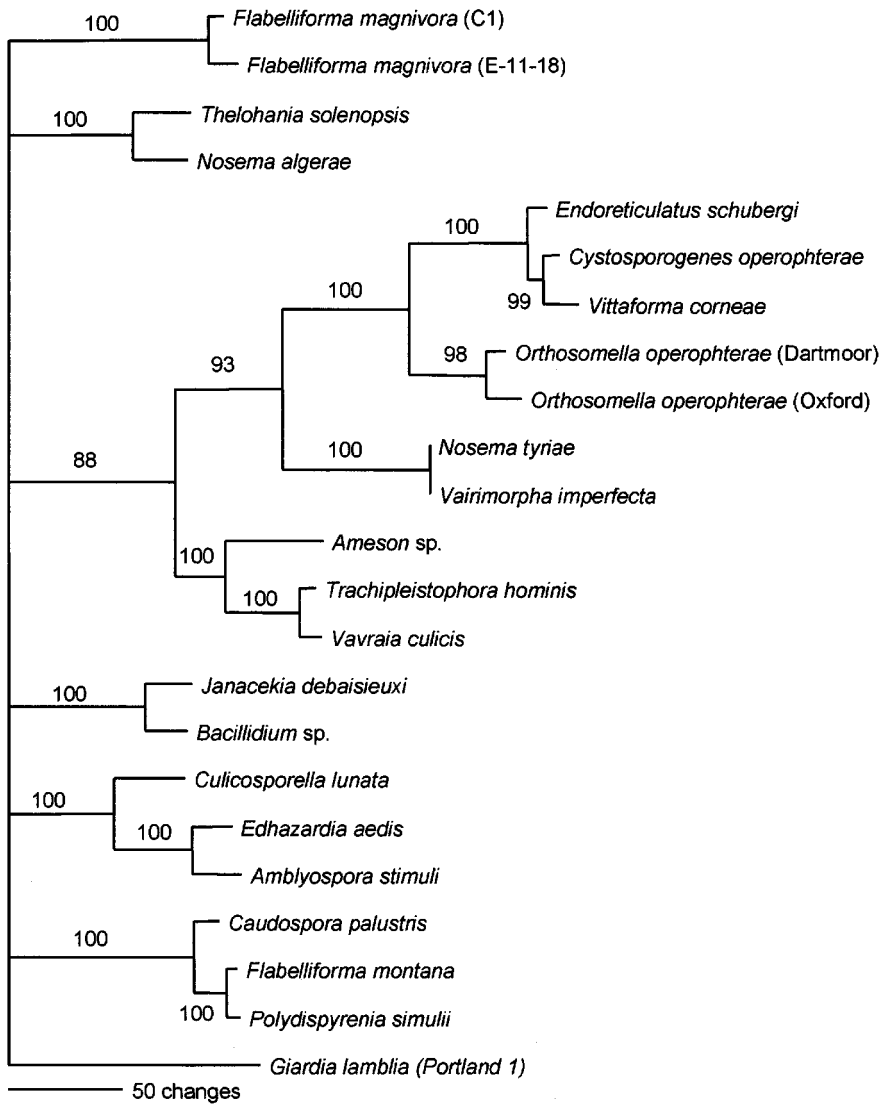

Fig. 27. The most parsimonious tree (tree length = 1192.72), found using the branch and bound option of PAUP 4.0b2a. Bootstrap analysis (100 replicates) was performed to give a measure of the confidence that can be placed in the resulting tree, with bootstrap values being located on the tree branches.

Figs. 19–26. *Flabelliforma montana* : sporogony and spores. n = nuclei. Bar on Fig. 19 applies to all figures. 19. Late sporogonic division. The sporophorous vesicle envelope (arrowheads) and host cell cytoplasm (hc) has now retracted, leaving the dividing sporont in a rounded cavity. The surface coat is almost complete but the layered structure over the plasma membrane is not yet clear. Bar = 880 nm. 20. Sporoblast showing tubular structures (arrow) within or beneath the surface coat. Arrowhead indicates sporophorous vesicle envelope. Bar = 670 nm. 21. Sporophorous vesicle containing mature spores and tubules. Bar = 1.7 µm. 22, 23. Detail of tubules in the sporophorous vesicle cavity showing inner tubule (arrowheads) and termination in a bulb (arrow). Bar = 400 nm (Fig. 22) and 240 nm (Fig. 23). 24. Mature spore showing deeply domed anchoring disc (ad), polar sac (ps) polaroplast of membranes angled (a) and appearing transverse (t) to the long axis, nucleus and 3.5 coils of the polar tube. Bar = 333 nm. 25. Anterior end of mature spore showing: exospore (arrow) composed of three or four membranous layers bounded externally and internally by amorphous coats; lucent endospore (en), plasma membrane (1), cytoplasmic layer (2), membrane of polar sac/anchoring disc complex (3), polar tube (pt) with denser core terminating in the anchoring disc (ad). a = acutely angled polaroplast membranes. Bar = 110 nm. 26. Transverse sections of polar tube. The middle section shows the full range of layers consisting of a central core (1), lucent ring (2) containing longitudinal fibrils and outer two membranes (3, 4). Bar = 125 nm.

was thinned to 30 nm or less (Figs. 24, 25). The anterior end of the polar tube terminated in a 200 nm diameter deeply domed central region (anchoring disc) of the polar sac, which was uniformly dense except for a slightly denser 11 nm layer immediately beneath its anterior border (Fig. 25). The sac itself was separated from the endospore by a narrow band of cytoplasm 11 nm thick and the plasma membrane. Laterally the polar sac extended back over the polaroplast, which was entirely of lamellar construction with membranes 16–20 nm apart separated by amorphous material. The anterior region was arranged as a system of folds at an acute angle to the straight part of the polar tube (Fig. 25). The polaroplast membranes probably maintained this angled relationship with the polar tube when the tube changed direction towards the periphery, so that the membranes appeared transverse to the longitudinal axis in some planes of section (Fig. 24). The single nucleus lay immediately posterior to the polaroplast, surrounded by cytoplasm

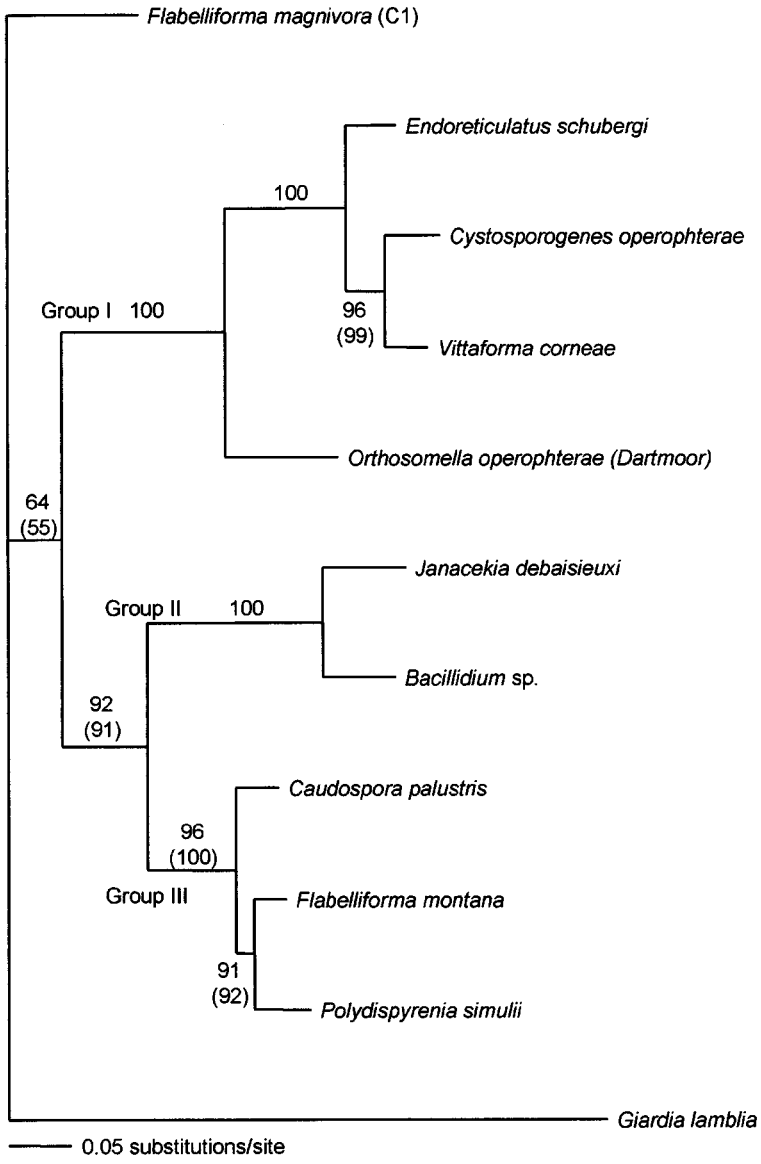

Fig. 28. A maximum likelihood tree (-ln Likelihood 7467.27) of a subset of 11 species. The same tree topology was generated from both heuristic and branch and bound searches, both carried out with 100 bootstrap resamplings of the data. Bootstrap values indicated on the tree branches are taken from the heuristic search, with those in parentheses representing the values obtained from the branch and bound search where these differed from heuristic analysis.

packed with ribosomes and the 3.5–4.0 coils of the polar tube (Figs. 24). A membrane-bound posterior vacuole with amorphous contents was observed only rarely. Cross sections of the polar tube revealed several concentric layers, including a wide electron lucent layer in which a circlet of fibres ran longitudinally (Fig. 26).

Phylogenetic Relationships

The accession numbers of the 22 16S rDNA sequences of the microsporidia used in the phylogenetic analyses and their sequence lengths are given in Table 1. The parsimony tree (Fig. 27) gave good resolution of the terminal branches but poor resolution of the clades with respect to one another, resulting in a polytomy. The 2 sequences of *F. magnivora* were not close to the clade containing *F. montana*, *Polydispyrenia simulii* and *Caudospora palustris*. Whereas there was about 0.5% sequence difference between *F. montana* and *P. simulii* and 0.7% between *F. montana* and *C. palustris*, the distance between *F. montana* and *F. magnivora* was 20%. The sequence difference between the two isolates of *F. magnivora* was 1.8% and between the two isolates of *Orthosomella operophterae* was 0.9%. When *F. magnivora* was constrained to cluster with *F. montana* in the parsimony tree, 19 more steps were required than in the original tree. This was significant (p = <0.039) but not highly significant. The ML tree (Fig. 28) constructed on a subset of the data, placed all but one of the species examined in one clade, albeit with low bootstrap support. The exception was *F. magnivora*.

As found previously (Canning et al. 1999), *Nosema tyriae* paired with *Vairimorpha imperfecta* with strong bootstrap support but these species were distant from *Nosema algerae* which paired with *Thelohania solenopsis* with equally strong support. *N. algerae* has been transferred to the genus *Brachiola* (Lowman et al. 2000). Of the new sequences obtained in this study, that of *C. operophterae* was close to those of *Vittaforma corneae* and *Endoreticulatus schubergi* and a sister to this clade was *O. operophterae*. A surprisingly close relationship, with 100% bootstrap support, was found between *Janacekia debaisreuxi* and *Bacillidium* sp. Finally the clades containing *Amblyospora stimuli*, *Edhazardia aedis* and *Culicosporella lunata* on the one hand and *Trachipleistophora hominis* and *Vavraia culicis* on the other were as found respectively by Alder et al. (2000) and Cheney et al. (2000). The species previously designated as *Pleistophora* sp. LS and found to be close to *Ameson michaelis* (see Cheney et al. 2000) has since been identified on ultrastructural data as a species of *Ameson* (unpublished data).

Table 2. Summary of topological constraint analyses.

Topological Constraint	Number of steps required [1]	p value [2]	
		Kishino-Hasegawa	Winning-sites
No constraint (best tree)	3088	–	–
Monophyly of all *Flabelliforma* spp.	3107	<0.0393	<0.0509

[1] Tree length from a heuristic search with 100 bootstrap resamplings and 10 subreplicates on unweighted data
[2] $p < 0.001$ highly significant; $p > 0.1$ not significant

Discussion

The new material of *F. montana* has permitted us to elucidate some of the features which were incompletely described in the original description and to compare it, as far as data permits, with the more recently described species, *F. ostracodae* Bronnvall and Larsson, 1994 from *Candona* sp. (Crustacea, Ostracoda), *F. diaptomi* (Voronin 1977) from *Diaptomus gracilis* (Crustacea, Calanoidea) and *F. magnivora* Larsson, Ebert, Mangin and Vávra, 1998 from *Daphnia magna* (Crustacea, Cladocera).

Important generic characters of *Flabelliforma* were confirmed, especially the unpaired nuclei throughout development, merogony in direct contact with host cell cytoplasm and multisporous sporogony in a sporophorous vesicle (SV) by formation and final separation of deep lobes in the sporogonial plasmodium. It was also confirmed that the SV envelope separates from the sporogonial plasmodium at the time of deposition of the surface coat, at first following the contours of the lobes and later rounding off to enclose the groups of sporoblasts and spores. New data of generic/specific importance were provided on mode of SV envelope formation by separation from the meront plasma membrane and the occurrence of tubules with a terminal bulbous expansion

in the SV cavity, similar to the type 1 tubules described by Takvorian and Cali (1983). Also on spore structure with details of the anchoring disc, the polaroplast composed entirely of lamellae and the exospore made up of 3 or 4 membrane-like layers bounded on either side by amorphous material. The spindle plaques and paramural bodies are of more widespread microsporidian occurrence, while the significance of the beaded structures emanating from the meront surface is not clear.

F. ostracodae conforms closely with the generic characters, notably in the unpaired nuclei, the separation of the very fine (8 nm) SV envelope as tiny blisters from the surface of the sporogonial plasmodium and sporoblast formation from a distinctly lobed sporogonial plasmodium. Its polaroplast is also of lamellar type only but in the posterior region the membranes are distinctly more widely separated than in *F. montana*. Other features which differ are the exospore structure of two membrane-like layers surmounted by an amorphous layer, the 13–16 coils of the polar tube and the large amount of "debris" in the SV.

Stempellia diaptomi Voronin, 1977 was transferred to the genus *Flabelliforma* by Voronin (1996) on the basis of unpaired nuclei, meronts in direct contact with host cell cytoplasm and division of the sporogonial plasmodium into sporoblasts via deep lobes. It is also likely that the SV envelope is formed as in *F. montana* but no evidence is available that it accompanies the invaginations of the plasmodium during early sporogony. At an early stage the SV cavity contains prominent, inclusions which appear as double-walled tubules as in *F. montana*. Although spore preservation was not good there is some evidence that the polaroplast is lamellar only but, in contrast to *F. montana*, has anterior and posterior close-packed lamellae and a middle region of widely separated lamellae. The exospore differs from that of *F. montana*, as it is composed of two prominent dense layers separated by a more lucent layer of the same thickness, with amorphous material disposed externally and internally.

F. magnivora conforms with the genus *Flabelliforma* in having isolated nuclei and in the general modes of division of meronts and sporogonial plasmodia and probably in the mode of formation of the SV envelope. However, the surface coat (future exospore) of the sporogonial plasmodium is constructed of a continuous inner layer and a compactly-folded outer layer, like a frill. Excess of this material, extruded into the SV cavity, is organised as thick frilled tubules or balls. As well as these, the cavity becomes packed with thin-walled tubules. The exospore eventually resolves as 5 layers at the surface of spores in an arrangement quite unlike that on spores of *F. montana*. The polaroplast is similar in being entirely lamellar but in the anterior region the membranes are tightly-packed, giving way to more widely spaced membranes posteriorly.

As outlined above the assignments of the three new species to the genus *Flabelliforma* were based on reasonable morphological grounds, especially as there were deficiencies in the description of the type species, notably about its spore structure (Canning et al. 1991). However the present phylogenetic study, based on 16S rDNA sequences of 20 microsporidia, of which 18 including *F. montana* and *F. magnivora* were from invertebrate hosts, has indicated that these two species are not closely related. *F. montana* was found to be most closely related to *P. simulii* and *C. palustris* with 100% bootstrap support. These are both parasites of blackflies. Cheney et al. (2000) also found that *F. montana* paired with *P. simulii* with 100% bootstrap support, in a parsimony tree constructed with a different set of microsporidia of which 13/21 were from vertebrate hosts.

The parsimony tree (Fig. 27) failed to resolve the relationships of the various clades to one another. Resolution was marginally improved in the ML tree (Fig. 28) based on a subset of the species and it was again clear that *F. magnivora* was not close to *F. montana*. This was supported by the 20% difference in their sequences and the topological constraints analysis which required 19 more steps than the original tree when *F. montana* and *F. magnivora* were constrained to be monophyletic. On the basis of the present molecular data, as opposed to morphological data, *F. magnivora* should not be placed in the genus *Flabelliforma*. We were not able to include *F. diaptomi* or *F. ostracodae* in our molecular study. These, and *F. magnivora* are parasites of freshwater microcrustaceans and it would be interesting, if material becomes available for 16S rDNA sequencing of *F. diaptomi* and *F. ostracodae*, to determine whether these form a clade which evolved in the crustacean groups.

Groups I–III in the ML tree (Fig. 28), reflecting the same groups in the parsimony tree, contain genera of markedly different morphology. In group I *P. simulii* is diplokaryotic in merogony but undergoes meiosis in sporogony to give rise to nu-

merous haploid spores in SVs (Canning and Hazard 1982). *C. palustris* is diplokaryotic in merogony and remains diplokaryotic in sporogony (apparently without meiosis), dividing into 4–8 diplokaryotic sporoblasts, not enclosed in SVs (Alder et al. 2000). *F. montana* has unpaired nuclei throughout development, producing about 30 uninucleate spores in SVs. As parasites of haematophagous Diptera producing masses of spores in the aquatic larval stage, it is likely that *P. simulii*, and possibly also *C. palustris*, have complex life cycles involving alternation of hosts, as is known for several species of *Amblyospora* infecting mosquitoes and copepods (Andreadis 1985; Sweeney et al. 1985, 1988; Becnel 1992). *F. montana* can be transmitted directly by feeding spores to larvae (Canning et al. 1991) and, as its host, *P. ariasi*, does not have an aquatic stage, similar complexities in the life cycle are unlikely. In the parsimony tree generated by Alder et al. (2000), *C. palustris* branched independently and was not included in the clade containing microsporidian parasites of Culicidae (Diptera). In our analyses, *C. palustris* was again distinct from the group parasitizing Culicidae but, by including *P. simulii*, *C. palustris* was found to cluster with it, indicating that these two taxa from Simuliidae are related.

In view of the morphological characters shared by *F. montana* and *F. magnivora* and the differences between *F. montana* and *P. simulii*, it is remarkable that 1.8% nucleotide difference was found in the 16S rDNA sequences obtained for isolates of *F. magnivora* collected in Oxford, UK and Russia (confirmed by independent sequencing of *F. magnivora*, D. Rifardt personal communication), while only 0.5% nucleotide difference was found between species of different genera, i.e. *F. montana* and *P. simulii*. No ultrastructural differences to suggest even different species were found between the Oxford and Russian isolates of *F. magnivora* (Larsson et al. 1998) but geographical isolation could have been responsible for divergence in the 16S rDNA. It has not been possible to obtain new material of *F. montana* but its sequence was obtained four months after that of *P. simulii*, rendering it unlikely that there was cross contamination. As morphological differences at least as great as those exhibited by the Group III organisms (Fig. 28) are found in microsporidia in Groups I and II (discussed below), it is not entirely surprising that Group III organisms are related.

In Group II the pairing of *Janacekia* and *Bacillidium* cannot be reconciled on morphological grounds. The polysporous genus *Janacekia* exhibits diplokarya which undergo meiosis at the onset of sporogony so that each sporoblast and spore has one haploid nucleus (Larsson 1983). The SV enclosing the sporogonial plasmodium divides with the plasmodium so that each spore is enclosed in its own SV. In contrast, the disporous genus *Bacillidium* is diplokaryotic throughout the life cycle and produces *Bacillus*-like spores from which a double membrane-like exospore layer may separate as a sac around individual spores (Larsson 1994). The sacs resemble SVs but have a different origin. The morphological differences coupled with host differences – blackflies for *Janacekia*, oligochaetes for *Bacillidium* suggest an uneasy partnership.

In Group III *Cystosporogenes* and *Endoreticulatus* are morphologically similar. Both have unpaired nuclei in all stages and develop in parasitophorous vacuoles in host cell cytoplasm. The two membranes forming the boundary of the vacuole enclosing *Endoreticulatus* were unequivocally identified as host endoplasmic reticulum (ER), as the outer membrane was ribosome studded (Brooks et al. 1988). The origin of the single membrane enclosing stages of *Cystosporogenes* was not determined when the genus was established (Canning et al. 1985) but recent ultrastructural observations (unpublished) show that the vacuoles can harbour merogonic and sporogonic stages concurrently (as in *Endoreticulatus*) and cannot therefore be SVs. The presence of one vacuolar membrane in *Cystosporogenes* and two in *Endoreticulatus* defines the difference between the genera. In contrast to these two genera, *Vittaforma* is diplokaryotic throughout development (Shadduck et al. 1990). Each stage is encased in host ER but merogonic and sporogonic stages do not coexist in the same vacuole. The feature common to this clade is, therefore, the envelopment of parasites by host ER. *Orthosomella*, the other genus in this group, which emerged as a sister taxon, has unpaired nuclei and develops directly in host cell cytoplasm with no intervening SV or parasitophorous vacuole (Canning et al. 1985). Sequence difference of 0.9% between the isolates of *O. operophterae* from two different woodlands in England was also greater than the distance between *F. montana* and *P. simulii*. Ultrastructural data are only available for the Dartmoor isolate (Canning et al. 1985) but light microscopy has revealed no differences (un-

published observations) which might suggest generic or specific differences.

The disparity between morphological and molecular data for the organisms in Groups I-III implies either that SVs, diplokarya and complex life cycles have evolved and been lost several times in microsporidian evolution or that 16S rDNA, as a highly conserved gene, does not contain sufficient information to discriminate between generic taxa. However, in two species, *F. magnivora* and *O. operophterae*, the gene varied considerably between isolates. To date, 16S rDNA sequences have been obtained for only about 30 of the 140 or so known genera of microsporidia and data for more of these taxa may resolve the apparent anomalies. At present, there is no clear guidance as to the morphological characters which are useful in systematics.

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