Vairimorpha imperfecta n.sp., a microsporidian exhibiting an abortive octosporous sporogony in *Plutella xylostella* L. (Lepidoptera: Yponomeutidae)

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SUMMARY

The microsporidian genus *Nosema* is characterized by development in direct control with host cell cytoplasm, diplokaryotic nuclei throughout development and disporous sporogony. The genus *Vairimorpha* exhibits the same features plus an octoporous sporogony producing uninucleate spores in a sporophorous vesicle. A microsporidium from diamondback moth, *Plutella xylostella*, falls between *Nosema* and *Vairimorpha* in that it initiates but fails to complete the octosporous sequence in this host. The name *Vairimorpha imperfecta* n.sp. is proposed. Merogony is mainly by formation of buds from multinucleate meronts, the buds remaining attached in chains. Diplokaryotic spores measure $4{\cdot}3 \times 2{\cdot}0$ μm (fresh) and have 15·5 coils of the polar tube in 1 rank. The octosporous sporogony is aborted owing to irregular formation of nuclear spindles, incomplete cytoplasmic fission and bizarre deposition of electron-dense episporontal secretions. Phylogenetic analyses of the sequences of the small subunit rRNA genes of *V. imperfecta* and of several *Nosema* and *Vairimorpha* spp. place *V. imperfecta* in a clade with *Nosema* spp. from Lepidoptera rather than in the clade containing the more typical species of *Vairimorpha*. It is suggested that the ancestors of the *Vairimorpha*/*Nosema* complex of species exhibited both disporous and octosporous sporogonies, as does the type species of *Vairimorpha*, *Vairimorpha necatrix*. It would follow that true *Nosema* spp. have lost the ability to express an octosporous sequence and that *V. imperfecta* is in the process of losing it. It is proposed that the genera *Nosema* and *Vairimorpha* be placed in the same family Nosematidae Labbé 1899, rather than in separate families and orders as at present.

Key words: *Vairimorpha imperfecta* n.sp., *Plutella xylostella*, ultrastructure, rDNA sequence, genetic analysis.

INTRODUCTION

The genus *Vairimorpha* was established for a microsporidian species isolated from *Trichoplusia ni* (Pilley, 1976). It had previously been noted that microsporidia from this host and other lepidopterans had sometimes shown a mixture of disporoblastic, diplokaryotic stages characteristic of the genus *Nosema* and octosporoblastic, monokaryotic stages characteristic of the genus *Thelohania* (Tanada & Chang, 1962; Tanada, 1962). Kramer (1965) assumed that these stages in *Pseudaletia unipuncta* represented 2 species and named them *Nosema necatrix* and *Thelohania diazoma*. It was later shown that the different developmental sequences were part of a single dimorphic life-cycle (Maddox, 1966; Fowler & Reeves, 1974), thus laying the foundation for Pilley (1976) to establish the new genus using the specific name *Vairimorpha necatrix* (Kramer, 1965). *Thelohania diazoma* (Kramer, 1965) thus became a synonym. Several other microsporidia exhibiting the same type of dimorphism have since been recognized and placed in the genus *Vairimorpha*.

The occurrence of a dimorphic microsporidium of the genus *Vairimorpha* in the diamondback moth *Plutella xylostella* was first reported by Linde, Norten & Madel (1988) and Norten *et al*. (1988). Some data on its development have been given by Linde & Norten (1992). The diplokaryotic free spores measuring $4{\cdot}15 \times 2{\cdot}3$ μm were predominant, while the small monokaryotic spores packed in groups of 8 in sporophorous vesicles were rare and found only in adults. The unnamed species produced a chronic infection with no significant effects on the mortality of its host. The same isolate was studied further by Jungen (1995) who found that there was reduced fecundity in the *Vairimorpha*-infected female moths but no other effects on the life-cycle. The microsporidia were transmitted vertically, with 90% of the progeny of infected females becoming infected. However, Jungen (1995) did not succeed in transmitting the infection horizontally. Recently,

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Nahif & Jungen (1998) published a description of the life-cycle of this *Vairimorpha* sp., including disporous, diplokaryotic sporogony and octosporous, monokaryotic sporogony in larvae as well as adults of *P. xylostella* reared at 20–23 °C.

Haque, Canning & Wright (1998) reported on the pathogenicity of a *Vairimorpha* sp. which had been imported into England in *P. xylostella* collected from Sundai Palas, Cameron Highlands, Malaysia. Its biological interaction with its host was quite different from that of the *Vairimorpha* sp. studied by Norten *et al.* (1988) and Jungen (1995). At all doses from $1{\cdot}5 \times 10^6$ to $1{\cdot}5 \times 10^3$ spores per larva there was 100 % mortality and the time of death was dose dependent, ranging from 3·7 days to 11 days for the highest and lowest doses. The pathogen reduced food consumption by the host by as much as 95 % with the highest dose. It was transmitted horizontally, and transovarially via females which survived doses of 10^2 spores per larva and, in both cases, had a marked influence on progeny performance. In this paper we describe the Malaysian isolate of *Vairimorpha* at light- and electron-microscopical level recording that the monokaryotic, octosporoblastic sequence of sporogony is commonly initiated but always abortive. SSU rDNA sequence data show paraphyly for the genera *Nosema* and *Vairimorpha*. The 2 *Vairimorpha* spp. which infect *P. xylostella* cluster in a clade with *Nosema* spp. from Lepidoptera rather than with the other *Vairimorpha* sp. analysed. Nevertheless, we consider that the initiation of the octosporoblastic sequence indicates affinity with *Vairimorpha* spp. and we propose to name it *Vairimorpha imperfecta* n.sp.

MATERIALS AND METHODS

Infection of P. xylostella *with* V. imperfecta

Spores of *V. imperfecta* were harvested from cadavers of *P. xylostella* larvae reared from infected eggs sent from Malaysia. The larvae were surface treated by immersion in phosphate-buffered saline (PBS) containing 10000 i.u./ml penicillin and 10000 μg/ml streptomycin, then homogenized in the same antibiotic solution and filtered through cheese cloth. The filtrate containing the spores was centrifuged at 1500 ***g*** for 10 min. The pellet was resuspended in PBS and the spores purified by centrifugation in 50 % Percoll at 1000 ***g*** for 15 min. The purified spores were resuspended in distilled water and quantified by haemacytometer. *P. xylostella* from an uninfected colony (ROTH strain) kept separately from the Malaysian insects were infected by feeding on 5 mm cabbage leaf discs on to which doses of $1{\cdot}5 \times 10^2$ spores had been dispensed. The larvae were allowed to pupate and emerging adults laid eggs. Second- and early third-instar larvae transovarially infected in this way and reared at 20 °C were used in life-cycle studies of the parasite.

Microscopy

Larvae were fixed in alcoholic Bouin's fluid, dehydrated in a graded series of ethanol solutions, cleared in xylene, embedded in Paraplast wax and 5–10 μm sections were stained by Giemsa, with acetone differentiation. Smears made from fat body, midgut, salivary glands and Malpighian tubules were dried, fixed in methanol and stained with Giemsa. For electron microscopy small pieces of tissue were fixed in Karnovsky's fixative in 0·1 M cacodylate buffer, pH 6·5, post-fixed in 1 % (w/v) OsO_4 in cacodylate buffer, washed in buffer, dehydrated in a series of ethanol dilutions and embedded in Spurr's resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an AEI EM801 electron microscope.

Spore and nucleic acid preparation for sequencing

Two separate gDNA preparations, each derived from approximately 5×10^7 Percoll-purified *V. imperfecta* spores, were prepared on separate occasions from the same batch of purified spores. Another gDNA preparation was prepared directly from a new batch of ground up *V. imperfecta*-infected larvae without purification of spores. In each case the pellets of spores were resuspended 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) on low speed for 30 s. A 15 μl aliquot of a 10 mg/ml proteinase K stock was added and the samples were incubated at 55 °C for 2 h. The samples were beaten with beads as before for a further 30 s. The suspension was extracted twice with phenol : chloroform (1 : 1) and the DNA precipitated with sodium acetate and ice-cold ethanol (Sambrook, Fritsch & Maniatis, 1989). The DNA was resuspended in TE buffer, pH 8·0, and stored at −20 °C until required.

PCR amplification and sequencing

The small subunit (SSU) rRNA gene was amplified from each of the 3 separate gDNA preparations using the 18f (5′-CACCAGGTTGATTCTGCC-3′) and 1537r (5′-TTATGATCCTGCTAATGG-TTC-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 comprised of 92 °C denaturation for 2 min, followed by 30–40 cycles of 92 °C denaturation for 30 s, 48 °C annealing for 30 s

Table 1. Species of microsporidia for which the SSU rDNA sequences were aligned, then analysed by maximum likelihood and parsimony methods

Species	Locality or isolate name	Host	Accession number
Nosema ceranae	China	*Apis cerana*	U26533
Nosema vespula	No information given	No information given	U11047
Nosema sp.*	NISM11	*Bombyx mori*	D85501
Nosema oulemae	'Hostounsky'	*Oulema melanopus*	U27359
Vairimorpha sp.	NISM12	*Bombyx mori*	D85502
Nosema necatrix†	ATCC 30460	*Pseudaletia unipuncta*	U11051
Nosema apis	UMSP	*Apis mellifera*	U26534
Nosema bombycis	SES-NU	*Bombyx mori*	D85503
Nosema tyriae	Beale (UK)	*Tyria jacobaeae*	AJ012606
Vairimorpha imperfecta	Malaysia	*Plutella xylostella*	AJ131645
Vairimorpha sp.	Germany	*Plutella xylostella*	AF124331
Nosema sp.	Sd NU IW 8401	*Spodoptera depravata*	D85504
Nosema furnacalis	'Ankeny'	*Ostrinia furnacalis*	U26532
Encephalitozoon cuniculi	'Donovan'	*Homo sapiens*	X98470
Nosema trichoplusiae	ATCC 30702	*Trichoplusia ni*	U09282

* Now considered to be *Vairimorpha* sp. (Iguchi *et al*. 1997).
† Now named *Vairimorpha necatrix* (Pilley, 1976).

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 the first 2 gDNA preparations were cloned into the pGem-T Easy Vector system (Promega, UK). One clone was selected from each gDNA amplification. These were further amplified with SP6 and T7 vector system primers, and cleaned up 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′-TCTAAGGA/CT/TGCAGCAGG/AG/G-3′, 4F = 5′-CACCACCAGGAGTGGAGTGTG-3′, 5R2 = 5′-TTAAGCCGCACAATCCACTCC-3′ and 6R = 5′-GCCTGCTGCTGTCCTTGGAC-3′. A partial sequence was obtained from the third gDNA preparation by direct sequencing of the amplification product using primers 5R2, 3F* and 1R = 5′-TTATGATCCTGCTAATGGTTG-3′.

Phylogenetic analysis

The species analysed, together with the Accession numbers of the SSU rDNA sequences are given in Table 1. Sequences were pre-aligned using the CLUSTAL W program (Thompson, Higgins & Gibson, 1994) and then re-aligned with MALIGN (Wheeler & Galdstein, 1994), using *Encephalitozoon cuniculi* (Accession no. X98470) as an outgroup. This second alignment with MALIGN gave a much tighter alignment of the sequences. When sequence data were not available, owing to incomplete sequencing at the 3′ end of the SSU rRNA gene for several of the aligned species, the final 31 nucleotides at the 3′ end were removed from the analysis, so that phylogenetic analysis was restricted to the regions of the aligned rRNA genes where sequence data was available for all the species under consideration (1317 bases). The aligned sequences were analysed by parsimony and a subset of the data (the clade containing *V. imperfecta*, plus *N. apis* and *N. ceranae* from the other clade and *E. cuniculi* as outgroup) was analysed also by maximum likelihood, using PAUP* version 4.0b1 (Swofford, 1998). The branch and bound option of PAUP was used to generate the most parsimonious tree and bootstrap analysis was performed to give a measure of the confidence which can be placed in the resulting gene tree.

RESULTS

Light microscopy

Sections of larvae revealed that infection was generalized, with the fat body and midgut epithelium very heavily infected. Many of the cells were grossly hypertrophied and the tissue virtually destroyed.

Meronts were diplokaryotic. Stages with 1, 2 or 4 diplokarya (Fig. 1A–D) were the most common but stages with up to 12 diplokarya (Fig. 1E, H) were seen, with shapes ranging from simple rounded forms (usually the smallest) to highly irregular forms. Measurements of stained parasites, with means and standard errors, were $4{\cdot}3 \pm 0{\cdot}31 \times 3{\cdot}6 \pm 0{\cdot}34$ μm ($n = 10$) for meronts with a single diplokaryon, $5{\cdot}3 \pm 0{\cdot}41 \times 4{\cdot}7 \pm 0{\cdot}43$ μm ($n = 10$) for meronts with 2 diplokarya, $8{\cdot}2 \pm 0{\cdot}43 \times 6{\cdot}8 \pm 0{\cdot}42$ μm ($n = 7$) for meronts with 4 diplokarya and $13{\cdot}8 \pm 1{\cdot}16 \times 11{\cdot}7 \pm 1{\cdot}51$ μm ($n = 5$) for meronts with more than 4 diplokarya.

Division of meronts was by binary fission of tetranucleate forms (Fig. 1B, C) and by budding of

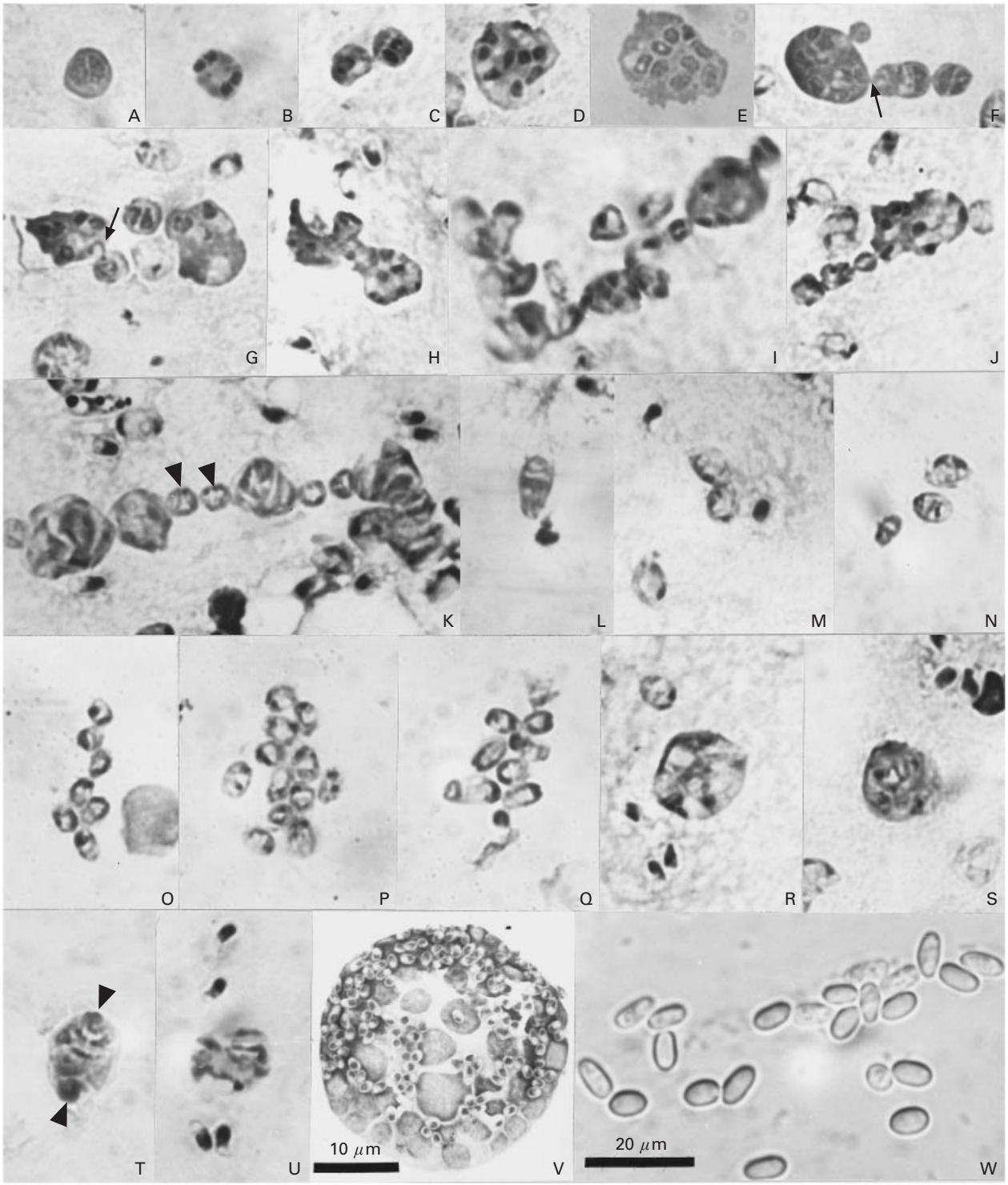

Fig. 1. Light micrographs of merogonic and sporogonic stages of *Vairimorpha imperfecta* in fat body of *Plutella xylostella*. A–V, Giemsa stained, W, fresh preparation. Scale bar on W applies also to A–U. (A–E) Meronts with 1 to several diplokarya, C is undergoing binary fission. (F, G) Early chain formation of buds: one nucleus of a diplokaryon is squeezing through the narrow cytoplasmic isthmus connecting parent meront and bud (arrows). (H) Multinucleate meront initiating bud formation. (I–K) Chain formation of meront buds, some buds undergoing further nuclear division and some possibly converted to sporonts (arrowheads on K). (L–Q) Sporogony of the diplokaryotic, disporous sequence. (L) Sporont with central nuclei. (M) Adjacent (probably attached) sporonts with lateral nuclei. (N) Two sporonts and a sporoblast. (O) Chain of sporoblasts. (P, Q) Groups of attached sporonts and sporoblasts. (R–U) Stages of the abortive octosporous sporogony: in T large clumps of episporontal secretion (arrowheads) are seen at the poles. (V) Malpighian tubule cell packed with meronts and spores. (W) Fresh spores.

the larger forms (Fig. 1 F–K). The budding process consisted of formation of a cytoplasmic bud into which passed a diplokaryon, followed by constriction of the bud and formation of a new nucleated bud between the parent body and the first bud. Sometimes a nucleus of the diplokaryon was visible squeezing through the constriction between parent meront and bud (Fig. 1 F, G). The buds remained attached to one another, so that chains of up to 12 buds were formed, depleting the parent body of nuclei. Nuclear division then occurred in the buds so that groups of multinucleate meronts were formed, which could continue division. Nuclei were large and irregular and occupied about half of the area of the meront, irrespective of the number of diplokarya.

Two types of sporogonic sequences were initiated. The predominant sequence was diplokaryotic and disporoblastic (Fig. 1 L–N). Sporonts were barrel-shaped with 1 or 2 diplokarya according to stage of development and measured $4{\cdot}8 \pm 0{\cdot}13 \times 2{\cdot}7 \pm 0{\cdot}14$ μm ($n = 9$). The nuclei were very compact, almost pinpoint, and occupied a central position or, when 2 diplokarya were present, often central and polar positions. Division gave 2 sporoblasts each measuring $3{\cdot}0 \pm 0{\cdot}8 \times 2{\cdot}1 \pm 0{\cdot}4$ μm ($n = 25$). The compact nuclei of sporoblasts were located in median, polar or lateral positions. Sporoblasts were frequently found in chains or groups, suggesting that binucleate meronts derived from chains of buds, remained attached during transition to sporogony (Fig. 1 O–Q). Spores were ovoid, measuring $3{\cdot}5 \pm 0{\cdot}13 \times 2{\cdot}1 \pm 0{\cdot}06$ μm ($n = 10$) when stained and $4{\cdot}3 \pm 0{\cdot}1 \times 2{\cdot}0 \pm 0{\cdot}1$ μm ($n = 25$) when fresh (Fig. 1 W). Spherical or slightly elongate bodies (Fig. 1 R–U) measuring $8{\cdot}6 \pm 0{\cdot}5 \times 7{\cdot}2 \pm 0{\cdot}5$ μm ($n = 15$) represented an abortive octosporoblastic sporogony within sporophorous vesicles. In Giemsa-stained smears, these exhibited strands of cytoplasm, pink-stained clumps interpreted as episporontal secretions (Fig. 1 T) and deep red-stained nuclei, which were compact or elongate. No groups of spores were observed within these vesicles. Infected cells showed diplokaryotic spores formed alongside dividing merogonic stages (Fig. 1 V).

Electron microscopy

The fine structure was observed in heavily infected fat body, the cells of which were in complete disarray (Fig. 2 A). Meronts were observed with 2 to several diplokarya in one plane (Fig. 2 B, C). These were of highly irregular shapes with cytoplasmic processes insinuating between organelles and secretions in the fat body. The nuclei were joined as diplokarya by apposition of the two envelopes along a short section of their surface. Elsewhere the 2 nuclear membranes, the outer of which was prominently studded with ribosomes, were often widely separated except at occasional points which were probably sites of nuclear pores. Often the space within the nuclear envelope was occupied by irregular membranes and sometimes apparently by cytoplasm, as if cytoplasm had invaginated into the space from a point outside the plane of section (Fig. 2 D, H). Spindle termini, consisting of electron-dense plaques in shallow invaginations (Fig. 2 E) of the nuclear envelope or at the surface (Fig. 2 G) were common, as were intranuclear microtubules terminating in electron-dense structures which could be chromatin or kinetochores. The spindle plaques consisted of electron-dense material within and external to the nuclear envelope, surmounted by double membrane-bound vesicles. The cytoplasm was largely free of organelles but there were some small vesicles with a single membrane, long stretches of cytoplasmic microtubules (Fig. 2 D, F) and very rare vesicles with 2 membranes (Fig. 2 B insert). Some of the cytoplasmic microtubules appeared to originate in the region of the spindle plaque but others, stretching between nuclear pairs or running near the periphery of the cells, had no obvious nuclear association. The budding mechanism, by which the diplokarya were squeezed through a cytoplasmic isthmus into a bud was easily seen (Fig. 2 I), as was the tenuous, persisting isthmus between buds (Fig. 2 J).

Early diplokaryotic sporonts of irregular shape, recognized by partial deposition of the surface coat on the plasma membrane, showed both nuclei of the diplokarya, still in close apposition, undergoing division, the spindles of both nuclei running parallel to the line of apposition (Fig. 3 A). In these sporonts were the first signs of flattened cisternae of endoplasmic reticulum. Division followed the pattern seen by light microscopy in that elongate sporonts, derived from the chains of meront buds, remained in partial continuity while undergoing disporoblastic division (Fig. 3 B). One such connection between sporonts showed microtubules running in 1 potential sporoblast up to the last remnants of its connection with the next sporont in the chain (Fig. 3 B, C).

Sporoblasts and spores lay free in host tissue but sporoblasts were not well fixed. A prominent nucleolus was present in sporoblast nuclei, which also showed the developing polar tube and rugose exospore. Mature spores (Fig. 3 D) had a 15 nm rugose exospore and 75 nm endospore, thinned to 25 nm over the anterior end. The 2 nuclei lay along the anterior–posterior axis within the polar tube coil and were slightly separated. The polar tube coils were organized in a single row and the maximum number of coils was $15\frac{1}{2}$. The anterior end of the polar tube was inserted into an anchoring disc of which the anterior layer beneath the enveloping polar sac was denser than the rest (Fig. 3 E). The polar sac extended over about two-thirds of the polaroplast and appeared as a prominent electron-lucent cover over the anchoring disc. The polaroplast

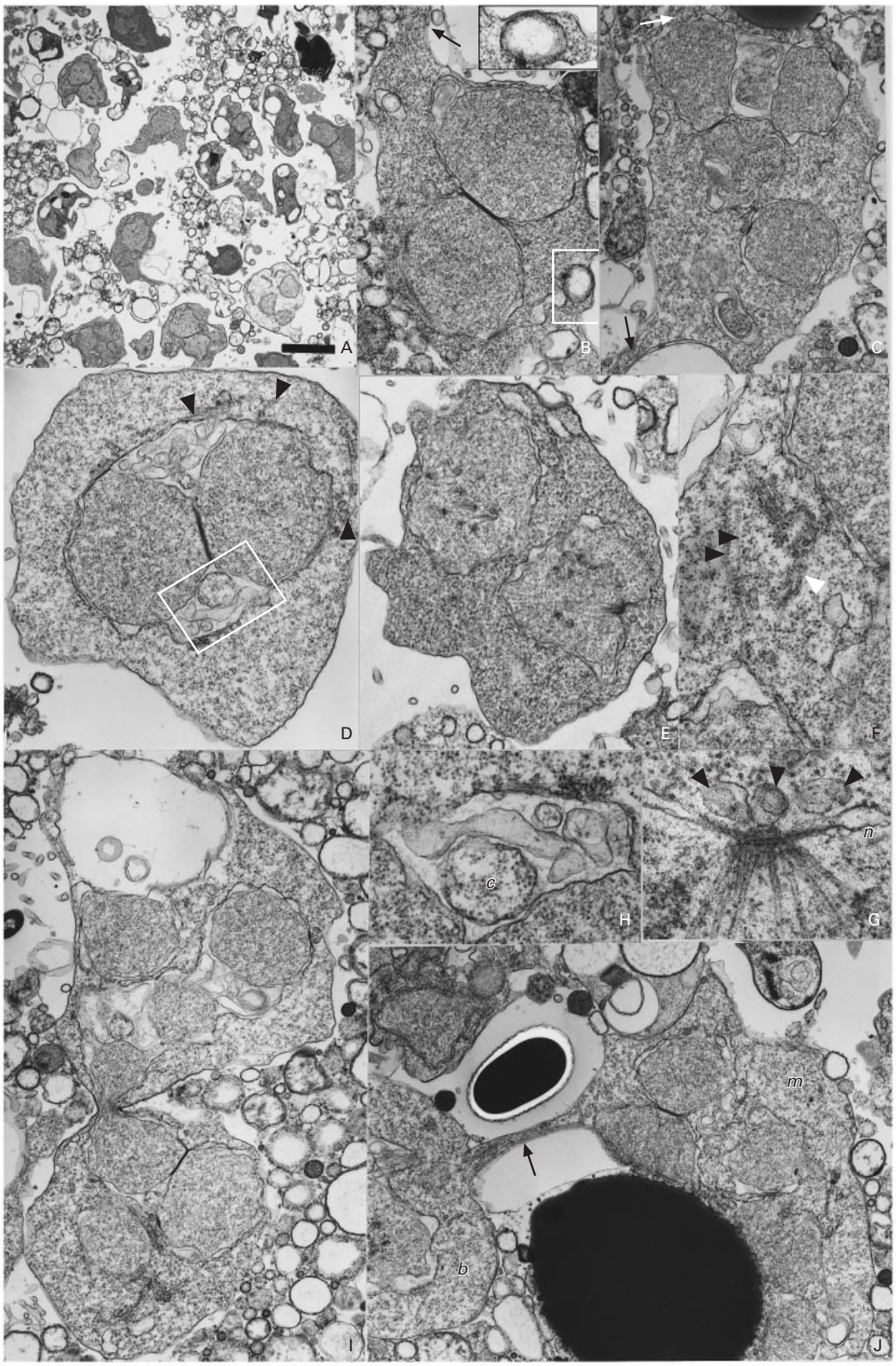

Fig. 2. For legend see opposite.

consisted of membranes which became more widely separated in a posterior direction (Fig. 3E). Cross-sections of the polar tube coils (Fig. 3F) showed an outer sleeve of 2 dense bands separated by a light ring. Within the sleeve were about 5 alternating light and dark rings around a central dense core. An organelle consisting of membranes around amorphous material represented the posterior vacuole (Fig. 3D).

The octosporoblastic sporogony was common (Fig. 3G) but consistently abortive. The first signs were separation of the 2 nuclei of the diplokaryon (Fig. 3H) and separation of a fine sporophorous vesicle envelope (SPV) from the plasma membrane (Fig. 3I), then deposition of coarse electron-dense granules from cytoplasmic vesicles into the space between the plasma membrane and the SPV (Fig. 3J). As the amount of secreted granules increased to surround the sporont, the granules fused to form rows (Fig. 4A). Cytoplasmic microtubules were occasionally observed. Nuclear division was initiated in 1 or both of the separate nuclei with typical intranuclear spindles arising from plaques, surmounted by vesicles, and microtubule termini associated with kinetochores (Fig. 4B, D). Subsequent development was anomalous with spindle microtubules running in all directions in the nucleoplasm (Fig. 4C). Signs of nuclear division were still visible even in the most degenerate cells (Fig. 4H).

Some of the secreted granular material was deposited patchily or in variable thickness on the undivided sporont or its products. The rest of the granules were organized as labyrinths (Fig. 4A, B, E) or tubules (Fig. 4E, F). Other signs of irregularity were large spaces created in the sporont (Fig. 4E, F) and the presence of concentric membrane formations (Fig. 4A, G).

Division of the sporont within the SPV was observed but each product, surrounded by an electron-dense coat, was abortive (Fig. 4F). The nearest to normal development was seen in 2 adjacent SPVs where there was rosette formation of sporoblasts but even in these vesicles there were areas which were clearly abnormal (Fig. 4G). In a few SPVs structures resembling sporoblasts were formed but their organization was equally chaotic, either with no formation of typical spore organelles or with aberrant formation (Fig. 4I). No SPVs containing spores were seen by light or electron microscopy

Small subunit rRNA

Two preparations of genomic DNA were made on separate occasions from the same batch of purified spores of *V. imperfecta*. Sequencing was conducted on 1 clone from each amplification product (designated isolates 1 and 2). The complete sequences of 1231 bases, which differed from one another by 4 bases, have been deposited in the GenBank under Accession Numbers AJ131645 (isolate 1) and AJ131646 (isolate 2). After the phylogenetic analyses, using sequence data from isolate 1, had indicated that *V. imperfecta* was closely related to several *Nosema* spp. from Lepidoptera, genomic DNA was prepared from a new batch of infected *P. xylostella* larvae. A partial sequence (nucleotides 84–1150) was obtained from direct sequencing of the amplification product. This confirmed the sequences obtained from the clones and, thus, the relationship of *V. imperfecta* to *Nosema* spp. from Lepidoptera.

The SSU rDNA sequence of *V. imperfecta* and those of the species recorded in Table 1 were aligned by CLUSTAL W then MALIGN and analysed by parsimony using *E. cuniculi* as outgroup. The parsimony analysis separated the species into 2 major clades with 100% bootstrap support (Fig. 5). *V. imperfecta* and the *Vairimorpha* sp. from *P. xylostella* in Germany were in the clade otherwise consisting of *Nosema* spp. from Lepidoptera. However, there were insufficient parsimony informative sites to resolve the relationships of species within this clade apart from *N. furnacalis*. The alignments had shown that there were only 33 variable sites among species within the clade and only 13 if *N. furnacalis* was omitted. Even fewer of these sites were phylogenetically informative in parsimony

Fig. 2. Electron micrographs of merogonic stages of *Vairimorpha imperfecta* in fat body of *Plutella xylostella*. Scale bar line on (A) applies to all figures. (A) Low-power view showing diplokaryotic meronts and early sporogonic stages in disorganized fat tissue. Bar = 3·8 μm. (B) Diplokaryotic meront with cytoplasmic process (arrow): the insert is an enlargement of the boxed area, showing a vesicle bounded by 2 membranes. Bar = 0·6 μm; insert = 0·35 μm. (C) Meront showing cytoplasmic processes (arrows) extending around vesicles in fat body. Bar = 1·0 μm. (D) Diplokaryotic meront showing inflation of the space between the two membranes of the nuclear envelope. Cytoplasmic microtubules (arrowheads) run a curved course apparently unrelated to the nuclei. Bar = 0·6 μm. The boxed area is enlarged in H. (E) Nuclear division showing kinetochore-like structures, attached to spindle fibres arising from centriolar plaques in the nuclear envelope. Bar = 0·8 μm. (F) Microtubules (arrowheads) in the cytoplasm. Bar = 0·3 μm. (G) Centriolar plaque in the nuclear envelope associated with microtubules in the nucleoplasm (*n*) and vesicles (arrowheads) in the cytoplasm. Bar = 0·25 μm. (H) Enlargement of the boxed area in D: the piece of cytoplasm (*c*) within the nuclear envelope almost certainly represents an invagination from a point out of the plane of section. Bar = 0·15 μm. (I) Division of a multinucleate meront: note that 1 nucleus of a diplokaryon is being squeezed as it passes through the isthmus where the cytoplasm is constricted. Bar = 1·0 μm. (J) Bud formation: note the narrow tenuous connection (arrow) of a bud (*b*) with a multinucleate meront (*m*). Bar = 1·0 μm.

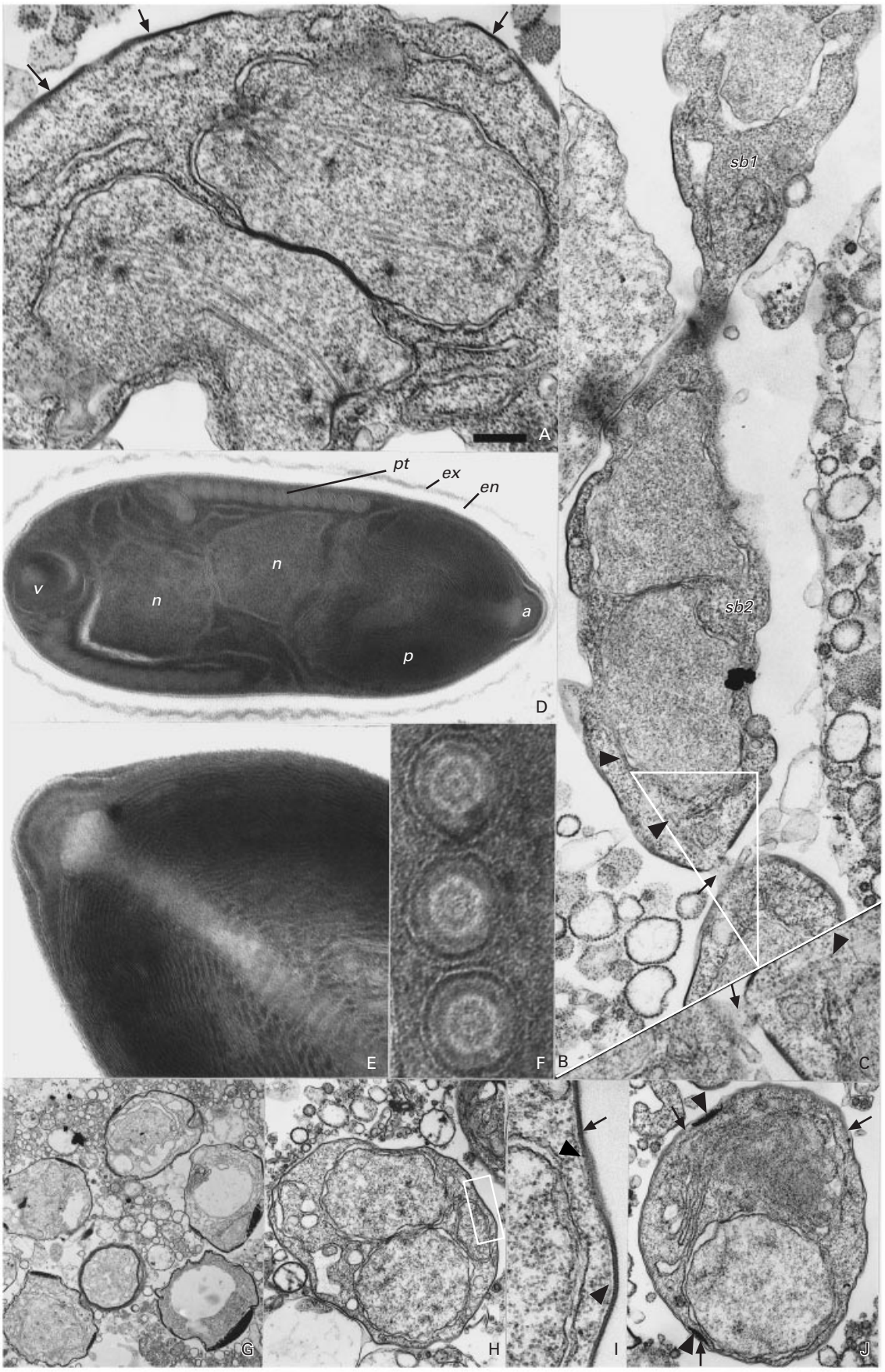

Fig. 3. For legend see opposite.

analysis. Conversely the 3 other *Vairimorpha* spp. from Lepidoptera (*V. necatrix*, *Vairimorpha* NIS MI2 and *Nosema* (= *Vairimorpha*) NIS MI1) were mixed with *Nosema* spp. from non-lepidopteran hosts in the other clade. The maximum likelihood analysis (heuristic search with 100 replicates) again showed 100% bootstrap support for the 2 major clades and gave no further resolution of the relationships within the clade containing *V. imperfecta* (data not shown).

DISCUSSION

The microsporidian species described herein as *V. imperfecta* was originally sent in pupae and eggs of *Plutella xylostella* collected in the Cameron Highlands of Malaysia for studies on its pathogenicity in *P. xylostella*, which is recognized as a serious pest of cruciferous crops in tropical and subtropical regions (Talekar, 1992). Laboratory studies have shown that it induced 100% mortality of its host even at doses of $1{\cdot}5 \times 10^3$ spores per larva and that it reduced feeding by as much as 95% using doses of $1{\cdot}5 \times 10^6$ spores per larva. It was considered to have potential as a biological control agent worthy of further investigation (Haque *et al*. 1999).

The unnamed species of *Vairimorpha* found in a laboratory colony of *P. xylostella* in Germany, reported in several publications (Linde *et al*. 1988; Norten *et al*. 1988; Linde & Norten, 1992; Jungen, 1995; Nahif & Jungen, 1998) differs substantially in its host–parasite relations from our isolate. It was non-pathogenic to *P. xylostella* except that it reduced fecundity, it could not be transmitted horizontally and it completed an octosporous sporogony in larvae and adults with production of uninucleate spores. Furthermore, merogony, described as binary fission of tetranucleate meronts, appeared to be more restricted. However, stages described as giant sporonts 'with four-, six- and octonucleate forms' actually exhibited those numbers of diplokarya and were certainly part of the merogonic cycle, rather than precursors of the octosporous sporogony. There was no indication that these giant meronts would undergo prolific budding as in *V. imperfecta* but would obviously have divided by some means. It could be that the differences between the German isolate and ours resulted from the prolonged laboratory culture of the former but, in the absence of ultrastructural data on the German isolate, it cannot be determined unequivocally whether or not they are identical. The sequences of the SSU rRNA genes of the 2 isolates have also failed to resolve this problem. There were 4 base differences between isolates 1 and 2 of *V. imperfecta*, indicating different copies of the gene in a natural population and there were 4 differences between our isolate 1 and 6 differences between our isolate 2 and the German isolate. As there are only 33 variable sites between all the species in the clade containing the *Nosema* spp. from Lepidoptera, it is clear that this highly conserved gene is not useful in distinguishing between very closely related species, even those which can be distinguished on morphological criteria.

The data presented here show that the predominant developmental cycle of *V. imperfecta* involves diplokaryotic stages. The proliferative stage is unusual in that division is mainly by formation of chains of buds from multinucleate meronts and that nuclear division occurs within these buds even while the parent meronts are still forming new buds. Some of the buds will separate and divide further, by binary fission or bud formation, but many buds remain attached to one another by tenuous bridges. All of these stages have simple plasma membranes indicating they belong to the merogonic cycle. At some stage in the repeated proliferations, the diplokaryotic buds lay down an electron-dense surface coat signalling the conversion of meronts to sporont at the onset of sporogony. One further nuclear division in the sporont is followed by binary fission to give 2 diplokaryotic sporoblasts. The disporoblastic sporogony may occur in isolated sporonts or within a chain of tenuously attached sporonts to give rise to large groups of attached sporoblasts. Although sporoblast formation may occur simultaneously in

Fig. 3. Electron micrographs of diplokaryotic sporogony (A–F) and initiation of the abortive octosporous sporogony (G–J) of *Vairimorpha imperfecta* in fat body of *Plutella xylostella*. Scale bar on A applies to all figures. (A) Early diplokaryotic sporont undergoing division of both nuclei: note partial deposition of surface coat (arrows). Bar = 0·4 μm. (B) Diplokaryotic sporont constricting to form 2 sporoblasts (*sb1*, *sb2*): microtubules (arrowheads) lead through the cytoplasm of 1 sporont towards the very tenuous connection (arrow) with the next sporont in the chain. Bar = 0·5 μm. (C) The boxed area of (B) enlarged to show the microtubules apparently running through the connection (small arrow) to the next sporont. Bar = 0·4 μm. (D) Diplokaryotic spore showing exospore (*ex*), endospore (*en*), anchoring disc (*a*), polaroplast (*p*), nuclei (*n*) about 13 coils of the polar tube (*pt*) and posterior vacuole (*v*). Bar = 0·24 μm. (E) Detail of the anterior end of spore showing layers in the anchoring disc and increasingly widely-spaced polaroplast membranes in a posterior direction. Bar = 0·1 μm. (F) Transverse sections of the polar tube showing concentric rings. Bar = 0·04 μm. (G) Several early octosporous sporonts. Bar = 3·0 μm. (H) Early octosporous sporont showing the beginning of separation of the diplokaryon. Bar = 0·9 μm. (I) Enlargement of the boxed area of H, showing dense sporophorous vesicle (arrow) overlying the delicate plasma membrane (arrowheads). Bar = 0·2 μm. (J) Early octosporous sporont showing small deposits of episporontal secretions (arrowheads) within the sporophorous vesicle envelope (arrows). Bar = 0·9 μm.

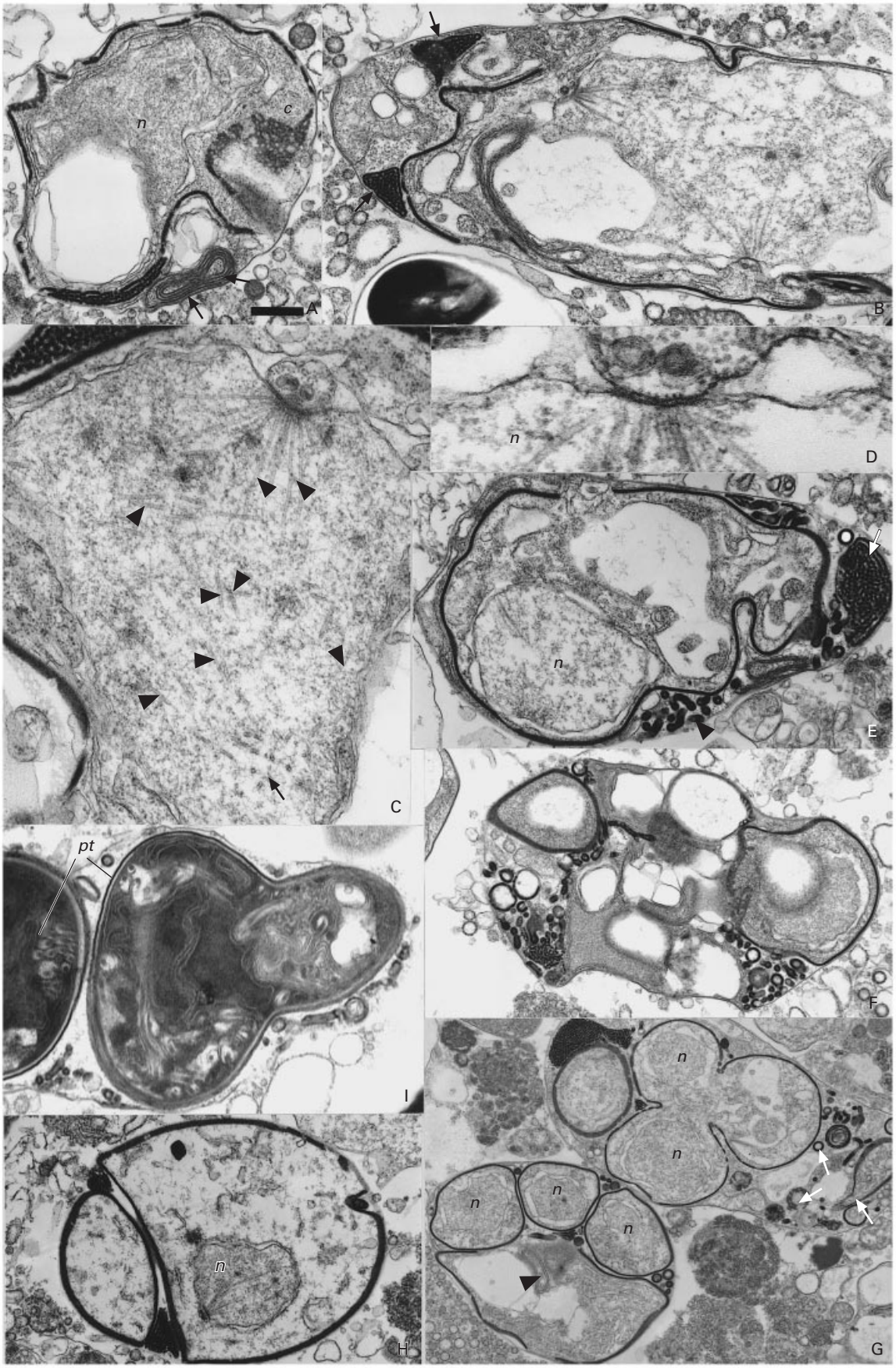

Fig. 4. For legend see opposite.

several sporonts in the chain, sporogony is considered to be disporoblastic because the initial division to produce the chain of buds belongs to the merogonic cycle. Mature spores break free from the chains.

Chain formation of buds would explain the high rate of proliferation responsible for massive and rapid destruction of the tissues involved. Cells of the adipose tissue and gut epithelium, in particular, are transformed into greatly hypertrophied bags of spores. It is likely that the destruction of midgut epithelium interferes with normal gut function, prevents absorption of food and accounts for the rapid cessation of feeding observed by Haque *et al*. (1999). Rather unusual features of the proliferative cells were the nuclear envelopes, the 2 membranes of which were often widely separated, the presence of cytoplasmic microtubules and rare vesicles with double membranes. It is likely that the irregular nuclear envelopes and their inclusions resulted from an osmotic imbalance during fixation, even though other cytoplasmic organelles appeared normal, or may have been an indication of degeneracy, as the host tissue was in a state of disarray. In either case it cannot be considered normal for the species. Cytoplasmic microtubules have rarely been reported in microsporidia. Recently Bigliardi *et al*. (1998) described cytoplasmic microtubules, originating outside the nuclear envelope in the electron-dense material of the spindle plaques, and proposed that both intranuclear and cytoplasmic microtubules were involved in nuclear division. In *V. imperfecta* the cytoplasmic microtubules were not specifically associated with nuclear spindle plaques and probably had a role in directing the formation of buds from multinucleate meronts and in assisting cytoplasmic constriction during merogony and sporogony. The revelation that microsporidia have symbiont-like HSP-70 genes (Germot, Philippe & LeGuader, 1997; Hirt *et al*. 1997) has led to speculation that microsporidia may once have had mitochondria. Double membrane vesicles such as those found in *V. imperfecta* may be relic mitochondria.

The systematic position of this parasite is enigmatic. It resembles the genus *Nosema* in having diplokaryotic stages in direct contact with host cell cytoplasm and disporoblastic sporogony and it has features in common with *Nosema apis*, a pathogen of honey bees, especially that meronts are multinucleate and produce division products as chains of buds. However, *V. imperfecta* has an important biological character in common with species of the genus *Vairimorpha*: in addition to the disporoblastic sporogony producing free spores, octosporoblastic sporogony is initiated within sporophorous vesicles. The first stage of this sporogonic sequence is nearly normal, with formation of a sporophorous vesicle envelope, separation of the nuclei of the diplokaryon and accumulation of electron-dense episporontal secretions. The secretions assume a number of forms as previously described (Moore & Brooks, 1992), ranging from rows of large granules to labyrinths and tubules. Some of the secretory products are deposited on the outside of the sporont and its irregular products. Thereafter, development within the sporophorous vesicle is abnormal in respect of nuclear and cytoplasmic divisions and overall chaotic organization. Thus, although the onset of octosporoblastic sporogony was common, it was never completed. Although we suspected that the fixation of our material was suboptimal as indicated by the inflated endoplasmic reticulum, it did not account for the abnormal appearance of the SPVs. The disporoblastic sporogony was completed with formation of normal free diplokaryotic spores but no octospores were formed in larvae, pupae or adult moths as observed by electron and light microscopy.

From the analyses of ribosomal DNA sequence data from a variety of species of *Nosema* and *Vairimorpha* (Baker *et al*. 1994), it was shown that

Fig. 4. Electron micrographs of the abortive octosporous sporogony in sporophorous vesicles (SPV) of *Vairimorpha imperfecta* in fat body of *Plutella xylostella*. Scale bar on (A) applies to all figures. (A) Dense episporontal secretions take the form of rows of granules and a labyrinth. Other anomalous features are the concentric membranes (arrow) and large spaces in the nucleus (*n*) and cytoplasm (*c*). Bar = 0·6 μm. (B) Part of SPV showing spindle microtubules arising from centriolar plaques and episporontal secretions deposited on the surface of the sporont and forming 2 labyrinths (arrows). Bar = 0·6 μm. (C) Abnormal nuclear division: spindle microtubules arising from centriolar plaques run in all directions in the nucleus, some seen in longitudinal section (arrowheads), others in cross-section (arrow). Bar = 0·3 μm. (D) Centriolar plaque in the nuclear envelope from which spindle microtubules radiate into the nucleus (*n*): 2 vesicles are seen above the plaque on the cytoplasmic side. Bar = 0·1 μm. (E, F) Two examples of chaotic SPVs, the undivided sporont in (E) surrounded by a dense surface coat formed from episporontal secretions, shows 1 nucleus (*n*) and residual episporontal secretions forming a labyrinth (arrow) and tubules (arrowhead). Bar = 0·7 μm. In (F) the sporont has divided but not all the products are enclosed in surface coat: episporontal secretions are mainly in the form of large tubules and vesicles. Bar = 0·8 μm. (G) Division of sporonts into uninucleate sporoblasts each with 1 nucleus (*n*): abnormal areas in the SPV contain concentric membranes (arrowhead) and spaces and a bizarre collection of tubules and other formations derived from the episporontal secretions (arrow). Bar = 1·2 μm. (H) Completely abortive sporont still showing centriolar plaque and spindle microtubules in the nucleus (*n*). Bar = 0·9 μm. (I) Sporoblasts within SPV showing some signs of spore wall and polar tube formation (*pt*) but are generally chaotic. Bar = 0·5 μm.

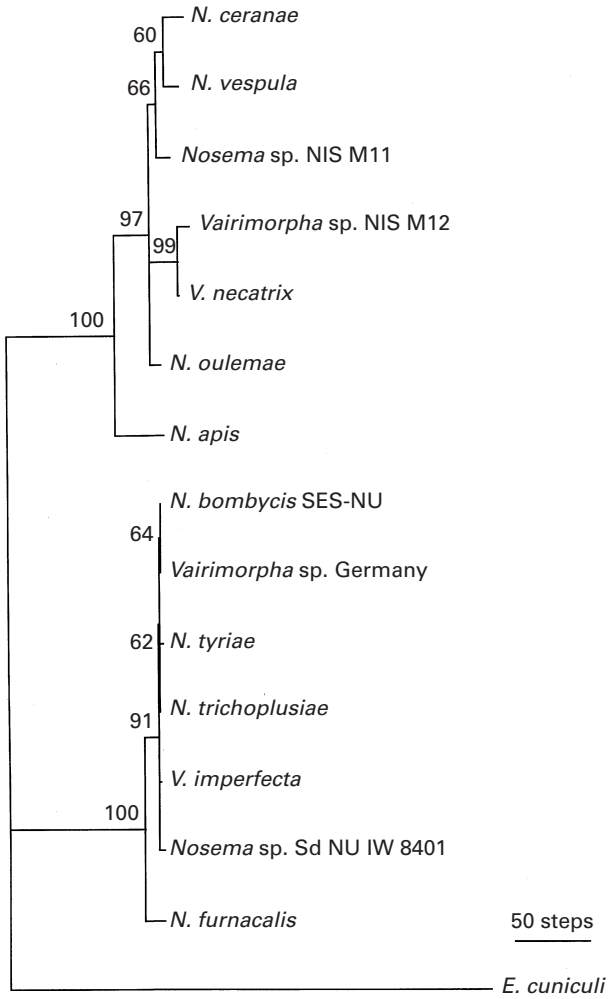

Fig. 5. Parsimony tree of SSU rDNA sequences to compare *Vairimorpha imperfecta* with selected sequences from other *Vairimorpha* and *Nosema* species. The numbers on the branches indicate bootstrap support from 1000 replicates using the branch and bound algorithm of PAUP* Version 4·0b1 (Swofford, 1998) and the furthest addition sequence. Branch lengths are proportional to number of steps as indicated by the bar. The tree is rooted on *E. cuniculi* (Accession number X98470). (Tree length = 647 with 206 parsimony informative characters).

the *Nosema* spp. from Lepidoptera formed a clade, as did *Vairimorpha* spp. from Lepidoptera and that these genera were more closely related to each other than either was to *Nosema* spp. from other hosts. Subsequently Fries *et al.* (1996) found that *N. apis* and *Nosema ceranae* from honey bees are more closely related to *Vairimorpha necatrix* (from lepidopteran hosts) than to *Nosema* spp. from Lepidoptera. The current work indicates that *V. imperfecta*, and the German isolate of *Vairimorpha*, both from the lepidopteran host *Plutella xylostella*, are more closely related to the *Nosema* spp. from Lepidoptera (including the type species *Nosema bombycis*), than to 3 *Vairimorpha* species included in the analysis. Conversely, a new species *Nosema portugal*, recently described from gypsy moths, *Lymantria dispar* (Maddox *et al.* 1999), was found, on the basis of the rDNA sequence (GenBank Accession No. AF033316) to be more closely related to *Vairimorpha* sp. from the same host (GenBank Accession No. AF033315). In fact there was only 1 nucleotide difference between their rDNA sequences yet the former had only disporoblastic sporogony, whereas the latter expressed both disporoblastic and octosporoblastic sporogonies. We conclude that the present classification of *Nosema* spp. in the family Nosematidae, and *Vairimorpha* spp. in the family Burenellidae is untenable, especially in a system where the two families are placed in different orders (Sprague, Becnel & Hazard, 1992). Baker *et al.* (1997) suggested that complex life-cycles in microsporidia are ancestral rather than more recently acquired. In the genus *Amblyospora*, which these authors found to have a basal position in the phylum, there are several sporogonic sequences. If the complex life-cycles are ancestral it is likely that, within the *Vairimorpha*/*Nosema* complex of species, the ability to express octosporoblastic sporogony has been lost completely by some (i.e. typical *Nosema* spp. including the type species, *N. bombycis*, and *N. portugal*), partially lost by others (i.e. *V. imperfecta*) and fully retained by others (i.e. typical *Vairimorpha* spp.). Probably, it would be most appropriate, at present, to classify all *Nosema* and *Vairimorpha* spp. in 1 family, Nosematidae Labbé 1899 and, when more sequence data become available, reconsider the systematic positions of the other genera currently classified with them.

In spite of the abortive nature of the octosporoblastic sequence, we have chosen to name the parasite as a species of *Vairimorpha* (*V. imperfecta*) because we consider that morphological characters are important for identification. It may be that the gene for octosporoblastic development could not be fully expressed in *P. xylostella* because this is not its natural host. An investigation of susceptibility of other insects in the type locality might reveal a host in which the octosporoblastic sequence is expressed.

Early studies of *V. necatrix*, the type species of *Vairimorpha*, indicated that the octosporoblastic sequence was temperature dependent and was only expressed at temperatures of 21 °C and below (Maddox, 1966; Pilley, 1976). However, Mitchell & Cali (1993) found that both sequences of *V. necatrix* were expressed at all temperatures (15–30 °C) used to rear the experimental host, *H. zea*. A microsporidium isolated from cotton leafworm, *Alabama argillacea* (Hamm, Nordlund & Mullinix, 1983) designated *Vairimorpha* sp. 696 was ascribed to the genus *Vairimorpha* on the basis of its protein profiles separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Streett & Briggs, 1982) but was not closely related to the other *Vairimorpha* spp. included in the study. Sedlacek *et al.* (1985) did not observe octosporous development of *Vairimorpha*

sp. 696 in an experimental host *Heliothis virescens* reared at 32 or 19 °C. Reasons for this could be that it is actually a *Nosema* sp. or, as in the case of *V. imperfecta*, it is a *Vairimorpha* sp. in which the octosporous sequence is often, if not always, incomplete or that this sequence can only be expressed in its natural host, *A. argillacea*. The studies on *Vairimorpha* sp. 696 further emphasize the close relationships of *Nosema* and *Vairimorpha* spp.

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