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Development of a new microsporidian parasite, *Intrapredatorus barri* n.g., n.sp. (Microsporida: Amblyosporidae) from the predacious mosquito *Culex fuscanus* Wiedemann (Diptera: Culicidae)

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Abstract

A microsporidium infecting the predacious mosquito *Culex fuscanus* Wiedemann, collected from Liu-Chiu Islet of Taiwan, was shown to be heterosporous. Two different types of haploid spores, one oval and the other lanceolate, were concurrently produced in the infected larvae. Merogony preceding the sequence leading to oval meiospores ended with the formation of a binucleate sporont with similar ultrastructural features to meronts. Synaptonemal complexes, suggesting that meiosis was involved during this sporogony, appeared in both nuclei of the sporont. The polar filament in the mature meiospore was anisofilar. Nine coiled turns of the polar filament were shown turning about the posterior portion of the spore. The polaroplast was composed of an anterior lamellate part and a posterior vesicular part. The anchoring disc was at the top of the straight portion of the polar filament. The meront of the lanceolate spore cannot be confirmed in this study. Two uninucleate lanceolate spores were eventually formed via nuclear dissociation. The polaroplast of the lanceolate spore was divided into an anterior multi-chambered part and a posterior of reticulate part. The polar filament was of the isofilar type, consisting of at least 5–6 coils. This parasite was extremely similar to the species *Amblyospora trinus*; in spore shape and development; but different in spore size and their hosts. Although it was similar to species of *Amblyospora* in host/parasite relations, we would rather assign this parasite to a newly established genus, i.e. *Intrapredatorus* and the name *Intrapredatorus barri* n.g., n.sp. was given for this microsporidium. The characteristics of the new genus was discussed in this article. However, its transmission routes remained uncertain thus far.

Keywords: *Intrapredatorus barri*; *Culex fuscanus*; Microsporidium; Ultrastructure

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1. Introduction

Microsporidia are intracellular eukaryotic parasites with a broad host spectrum, from protozoa to man. They are common parasites of mosquitoes, as more than 120 species have been described from these hosts [1–4]. The pathogenicity of microsporidia to mosquitoes is variable, from benign to lethal. Often, the life cycle of species parasitising mosquitoes is complex [5–7]. Of microsporidia, species belonging to the genus *Amblyospora* Hazard and Oldacre, 1975 [8] are the most common and widespread parasites with mosquito populations in nature [9,10]. It has been reported from 71 different mosquitoes [9]. Species of *Amblyospora* usually need an intermediate host to complete their life cycles [6,7,9,11–13]. In addition to the sporulation occurring in the intermediate host, there are usually two additional sporulations in which two mosquito generations are involved to form different types of spores in larvae and adult females, respectively [4,9,14].

In 1990, the microsporidian parasite *Amblyospora trinus*, Becnel and Sweeney, 1990, was reported from an Australian predacious mosquito *Culex halifaxi* Theobald, 1903 [15]. This was the first microsporidian parasite reported from the predacious *Culex* mosquitoes. During this study, specimens of another predacious mosquito *C. fuscanus* with microsporidian infections were collected from Liu-Chiu Islet in southern Taiwan [16]. The microsporidia from these two mosquitoes posses some similarities in morphology and developmental sequence. We here describe the ultrastructure and morphogenesis of this microsporidium, leading to the differentiation between these two similar microsporidia. Its taxonomy, especially the reclassification at the genus level, was also elucidated.

2. Materials and methods

2.1. Mosquitoes

Larvae of *C. fuscanus* used in this study were collected in March 1991 from a large metal container at Liu-Chiu Islet in southern Taiwan. All 15 fourth-instar larvae and the one pupa collected had patent fat-body microsporidial infections. Fresh tissues from some of the infected mosquitoes were smeared with a drop of insect saline on a glass slide to prepare for light microscopy. Spores were then observed and measured using an ocular micrometer under the light microscope. Spores which used for Giemsa staining were smeared on the glass slide, air-dried, fixed with methyl ethanol for 10 min. The smears were then immersed in Giemsa's stain solution (BDH Chemical, England) for 20 min. The slides were washed with running water and then observed under the light microscope.

2.2. Electron microscopy

Infected tissues for electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C for 2 h. They were then post-fixed in 1% OsO_4 for 3–4 h. The OsO_4 was replaced with 0.5% aqueous uranyl acetate for 1 h and after further washing, dehydration was run through an ascending ethanol series. Finally the tissues were embedded in Spurr's resin. Gold or silver thin sections were post-stained with 2% methanolic uranyl acetate for 5 min and followed by 0.08% lead citrate for 5–10 min. The prepared specimens were observed and photographed under a JOEL JEM-2000 electron microscope at 100 kV.

3. Results

The microsporidium from *C. fuscanus* was heterosporous with two concurrent sporulations in the larvae. Each sporulation ended with the different types of uninucleate spores. One was oval (meiospores), produced in a sporophorous vesicle in groups of eight (Fig. 1a) and the other was lanceolate (Fig. 1b). Infected larvae showed signs of infection in the form of externally visible white masses. Heavily infected larvae moved slowly. Ten of the 15 infected larvae eventually died before pupation.

3.1. Meronts and merogony

Meronts were irregular in shape and had diplokaryotic nuclei (Fig. 2a, Fig. 3). Electron

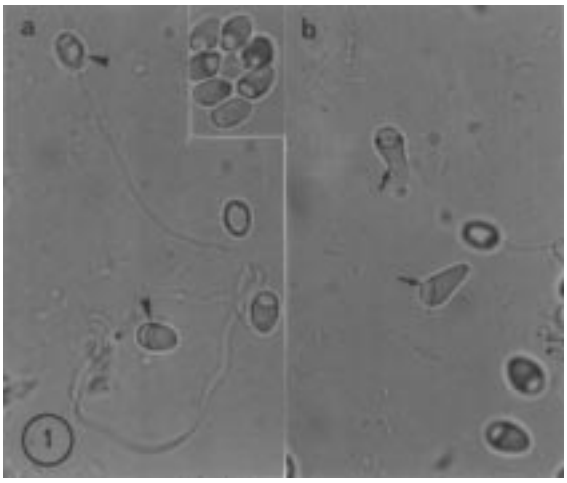

Fig. 1. Fresh spores of *Intrapredatorus barri* n.g., n.sp. (1000 ×). (a) Ungerminated and germinating (arrow heads) meiospores. Inset: Mature spores grouped in 8 within the sporophorous vesicle. (b) Lanceolate spores (arrows).

micrographs showed the closely apposed nuclear envelopes of two nuclei (Fig. 3). Meronts with two diplokarya, after nuclear division, were also observed (Fig. 2b, Fig. 4). A cell (Fig. 2c, Fig. 5) formed by fusion of the diplokaryotic nuclei, i.e. karyogamy, may be interpreted as the transitional stage between merogony and sporogony. The nuclei then separated and synaptonemal complexes appeared in each nucleus (Fig. 6). It resulted in the formation of a binucleate sporont (Fig. 2d, Fig. 7).

3.2. Sporulation sequence involving the formation of meiospores

The newly-formed binucleate sporont was enveloped by a thicker amorphous, electron-dense surface coat. This surface coat eventually formed the sporophorous vesicle (Fig. 7). Granular metabolites were frequently seen in the space between the sporont plasmalemma and the sporophorous vesicle (Fig. 7). The individual sporont underwent karyokinesis within the sporophorous vesicle to produce a binucleate, tetranucleate and, ultimately, octonucleate sporo-

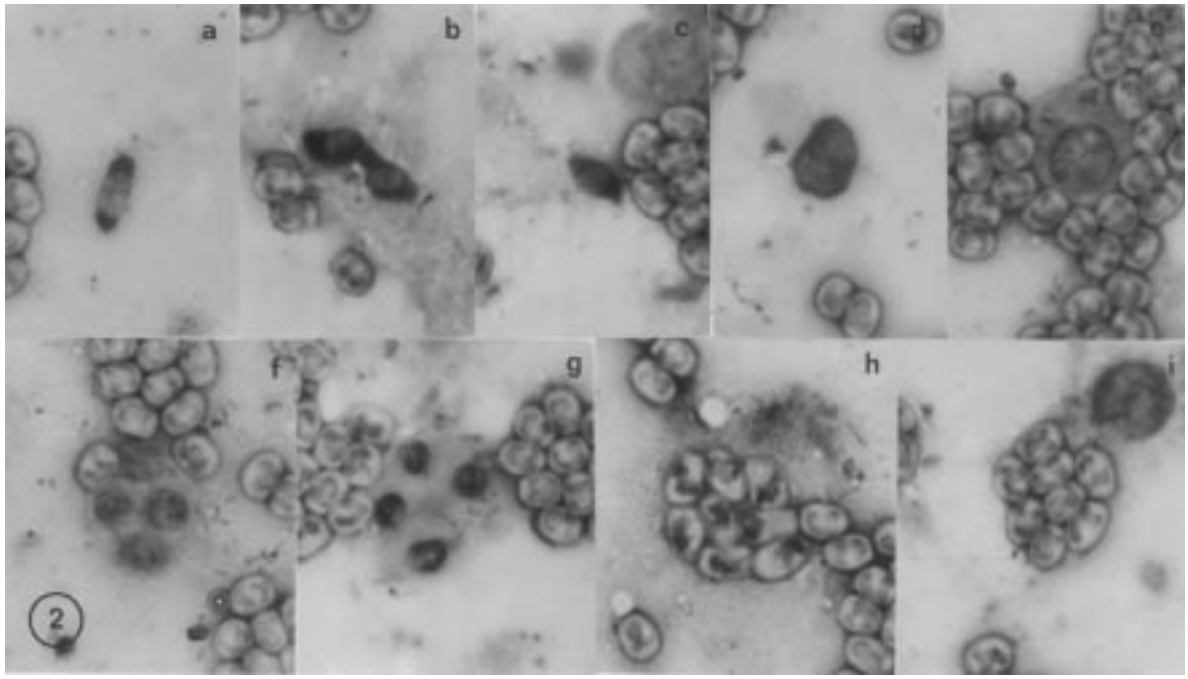

Fig. 2. Sporulation involving the formation of meiospores (1000 ×). (a,b) Merogony. (c–h) Meiosis. (i) Mature meiospores.

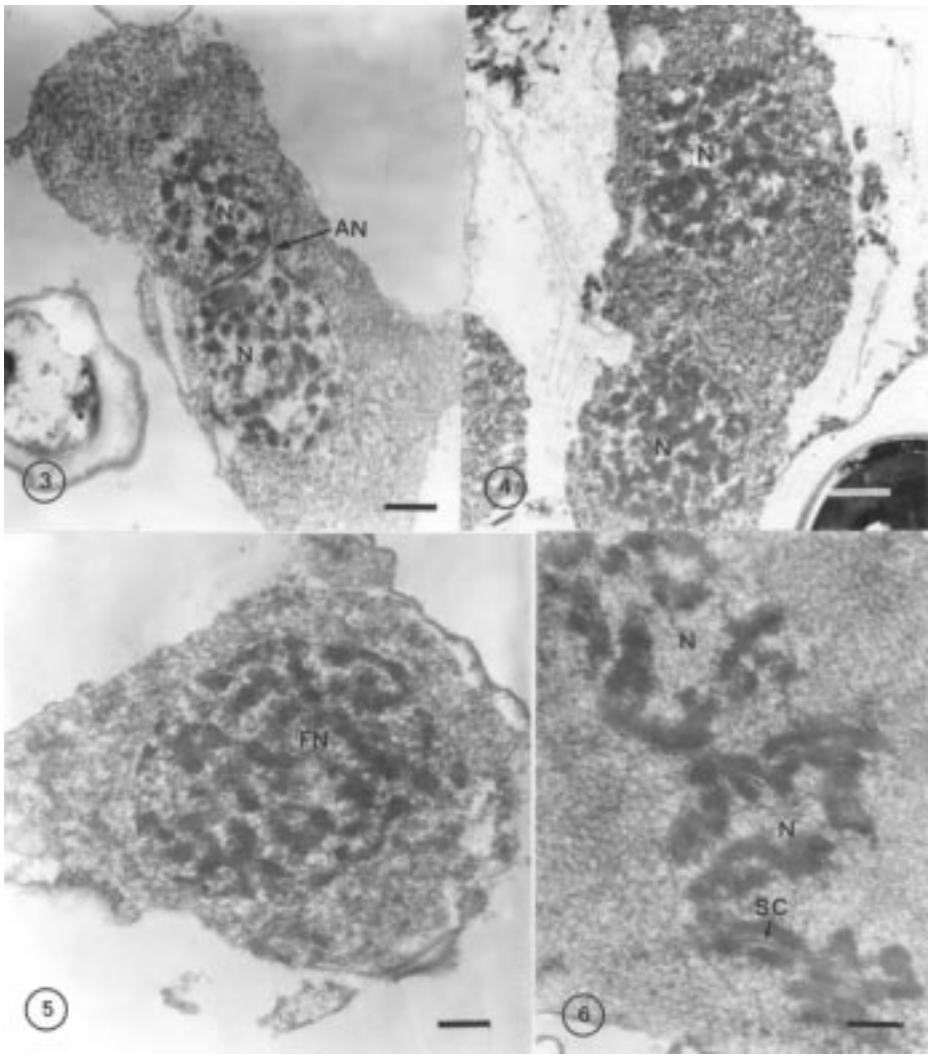

Fig. 3. Diplokaryotic cell or meront preceding both sporulation sequences. Note apposition of two nuclei (AN). N, nucleus. (Bar = 1 μm).

Fig. 4. Meront with two diplokarya before cytokinesis. N, nucleus. (Bar = 1 μm).

Fig. 5. A cell after karyogamy via fusion of the diplokaryotic nuclei (FN) in the sporulation involving meiosis. (Bar = 500 nm).

Fig. 6. Synaptonemal complexes appeared in both nuclei of the binucleate stage. N, nucleus; SC, synaptonemal complexes. (Bar = 500 nm).

gonial plasmodium (Fig. 2d–g, Figs. 7–9). Occasionally, bundles of folded tubules appeared in the cytoplasm of the sporogonial plasmodium (Fig. 8). The granular metabolites decreased as maturation proceeded and eventually there were few metabolites left in vesicles which contained mature spores (Figs. 8 and 9). Cytokinesis gave rise to eight uninucleate sporoblasts (Fig. 2h, Figs. 8 and 9). Tubules extending from the surface of sporoblasts were common during this stage of the

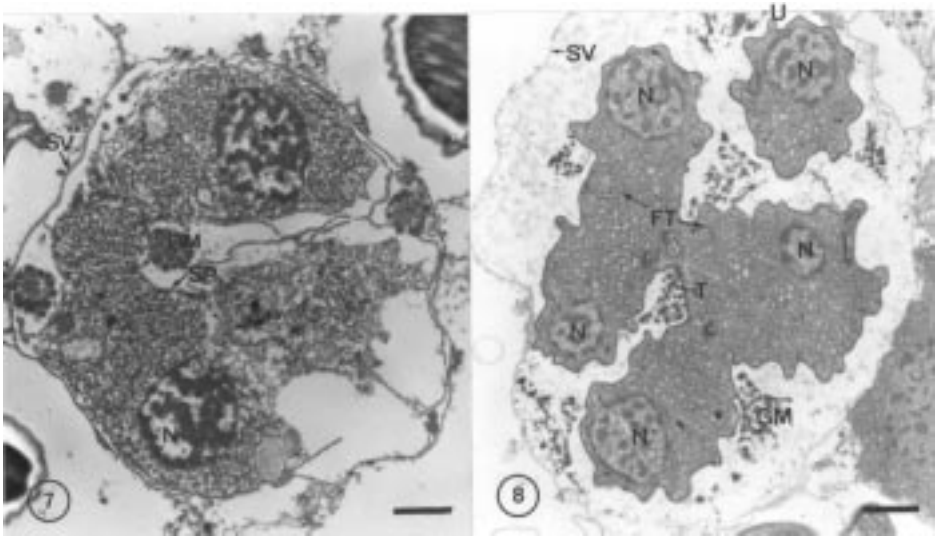

Fig. 7. The binucleate sporont with incomplete cytokinesis within the sporophorous vesicle. GM, granular metabolites; N, nucleus; SP, sporont plasmalemma; SV, sporophorous vesicle. (Bar = 1 μm).
Fig. 8. Bundles of folded tubules in the cytoplasm of the sporogonial plasmodium. FT, folded tubules; GM, granular metabolites; N, nucleus; SV, sporophorous vesicle; T, extended tubules. (Bar = 1 μm).

sporont (Fig. 9). Eight uninucleate meiospores were formed within the sporophorous vesicle (Fig. 2i, Fig. 10).

3.3. Ultrastructures of the meiospore

The immature sporoblasts of the meiospore were uninucleate and irregular in shape (Fig. 10). The plasmalemma of each sporoblast evolved to be a bi-layered thick spore wall. The inner electron-translucent layer was essentially the endospore; whereas the outer layer was the thinner, electron-dense exospore (Fig. 11). There was usually a posterior vacuole, which may be collapsed during the fixation process, in the mature spore (Fig. 11).

The mature spore was uninucleate. Fresh spores measured $7.38 \pm 0.63 \times 4.59 \pm 0.47$ μm ($n = 25$); Giemsa-stained spores were $6.20 \pm 0.58 \times 4.32 \pm 0.58$ μm ($n = 25$). The polar filament was of the anisofilar type, consisting of 3–4 large and 5–6 small coils (Fig. 11). Ultrastructurally, the polar filament was essentially a multi-layered tube (Fig. 11). A large well-constructed polaroplast was located in the anterior portion of the mature spore (Figs. 11 and 12). It consisted of two parts, an upper part of close-packed lamellae merged into a vesicular network (Fig. 12). A distinct anchoring disc was at the anterior end of the straight portion of the polar filament (Fig. 12).

3.4. Sporulation sequence and ultrastructures of the lanceolate spore

Mature lanceolate spores were sparsely distributed within the haemocoel of infected larvae. The spore was measured 8.14 ± 0.42 μm $\times$ 4.41 ± 0.28 μm ($n = 10$) in fresh preparations. The sporulation to form lanceolate spores, according to the present observations, proceeded from an uninucleate sporont (Fig. 13a). Two resulting lanceolates were formed via nuclear dissociation (Fig. 13b–d). The binucleate sporont was confined in a sporophorous vesicle (Fig. 14). Light-stained metabolites were in the space between the parasite plasmalemma and the sporophorous vesicle (Fig. 14). There was rough endoplasmic reticulum distributing in the cytoplasm (Figs. 14 and 15).

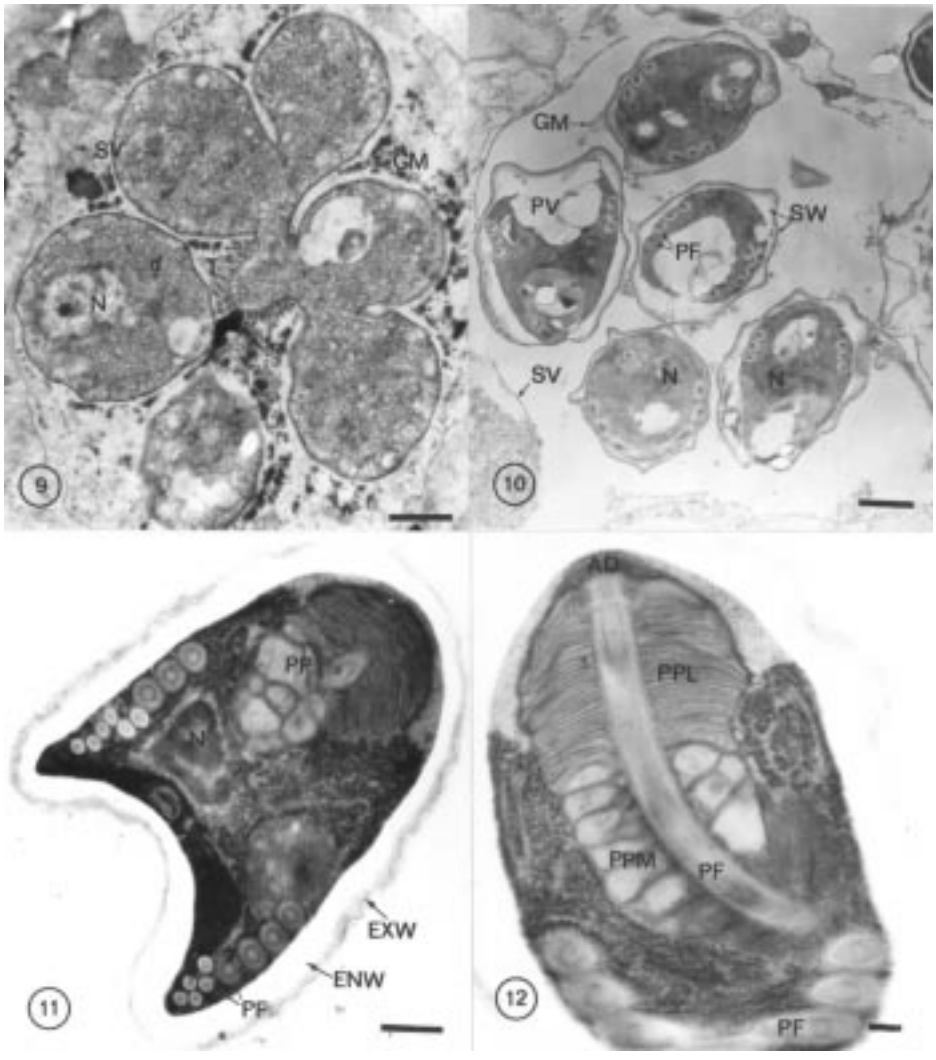

Fig. 9. The octonucleate sporont. Tubules extended from the surface of sporoblasts were common during this stage. GM, granular metobolites; N, nucleus; SV, sporophorous vesicle; T, extended tubules. (Bar = 1 μm).
Fig. 10. Eight uninucleate irregular immature spores were formed within the sporophorous vesicle. GM, granular metabolites; N, nucleus; PF, polar filament; PV, posterior vacuole; SV, sporophorous vesicle; SW, spore wall. (Bar = 1 μm).
Fig. 11. A mature meiospore with collapsed posterior end. ENW, endospore; EXW, exospore; N, nucleus; PF, polar filament; PP, polaroplast. (Bar = 500 nm).
Fig. 12. The anterior portion of a mature spore. PF, polar filament; PPL, anterior lamellate polaroplast; PPM, posterior vesicular polaroplast. (Bar = 200 nm).

The completion of cytokinesis led to the formation of sporoblasts (Figs. 15–17). The early stage of the sporoblasts was irregular in shape (Fig. 16). Two lanceolate spores were actually formed within the sporophorous vesicle (Fig. 13d). As usual, metabolites were present within the vesicle and these remained until the sporoblasts were nearly mature (Figs. 15–17). At an early stage in sporoblast formation, the sporophorous vesicle broke down to liberate them and spore maturation was

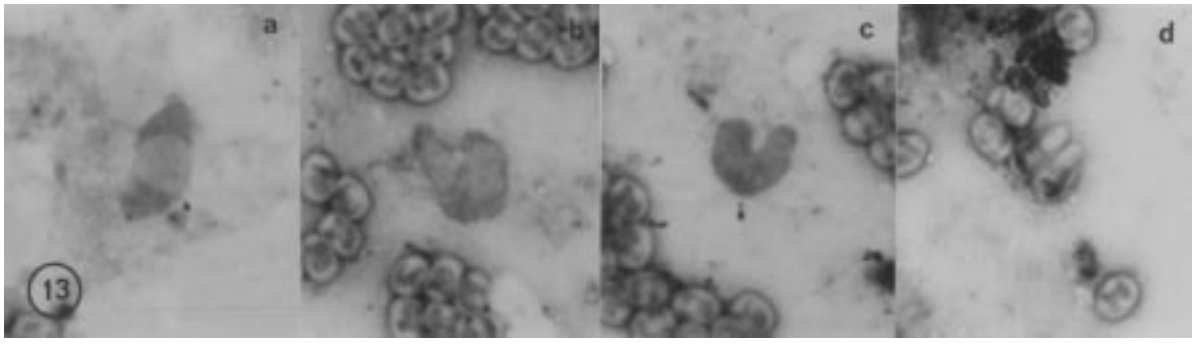

Fig. 13. Sporulation involving nuclear dissociation (1000 ×). (a) Uninucleate sporont. (b) Early binucleate sporont. (c) Dividing binucleate sporont. (d) Mature lanceolate spores lying in apposition.

completed free in the haemocoel (Figs. 16 and 17).

The mature lanceolate spores, occasionally curved or bent, were uninucleate with a similar cell content as the meiospore. It must be noticed that the polar filament was not formed until the spore became mature (Figs. 17 and 18). The polar filament was of the isofilar type and consisted of at least 5–6, or perhaps up to 8–9 coils (Fig. 18). There was usually a median-sized posterior vacuole (Fig. 18). The cytoplasm of the spore was full of rough endoplasmic reticulum (Fig. 18). The large polaroplast was composed of two parts, including the upper multi-chambered and the lower reticulate part (Fig. 18). Exospore and endospore wall were relatively thin when compared with the meiospore (Fig. 18). An anchoring disc covering the top of the straight portion of the polar filament was like that in the meiospores (Fig. 19).

4. Discussion

The microsporidium infecting *C. fuscanus* was here considered to be a new species. The development of the meiospore started by undergoing repeated binary fissions of diplokaryotic meronts to produce more meronts. Karyogamy and subsequent restoration of the diplokaryon to form the earliest stage of the sporont were observed as seen in many species of *Amblyospora* [14,17]. In *Amblyospora*, an octosporoblast was known to be derived from an uninucleate sporont via meiosis and subsequent mitosis [14,17,18]. However, for the new isolate, it seems that two nuclei of the diplokaryotic sporont independently underwent meiosis based on the evidence of existing synaptonemal complexes in both nuclei of the sporont [19,20]. No mingling stage was found in this microsporidium. Thus, additional mitosis may not occur during the sporulation to form an octosporoblast [14,17]. Massive metabolites which appeared circular in the young octosporoblast became smaller and fewer; eventually disappeared during its maturation process. It seems that those granules were utilized for forming the spore wall [8]. One of characteristic structures in meiospores was bundles of folded tubules which appeared in the cytoplasm of the sporont. This unique structure has been found in spores formed in the alternate copepod host of *A. californica* and was reported to be associated with the formation of polar filaments [11].

The sporulation sequence of the lanceolate spore in most species of *Amblyospora* was reported to be diplophasic [4]. However, it was apparently not the case for the present species. Although the sporulation was observed to start with nuclear dissociation of a binucleate sporont, it could be actually derived from direct nuclear dissociation of the diplokaryotic meront as that in *A. trinus* [15].

Though this microsporidium was morphologically similar to *A. trinus* which infects another predacious mosquito *Culex halifaxi* [15], we did

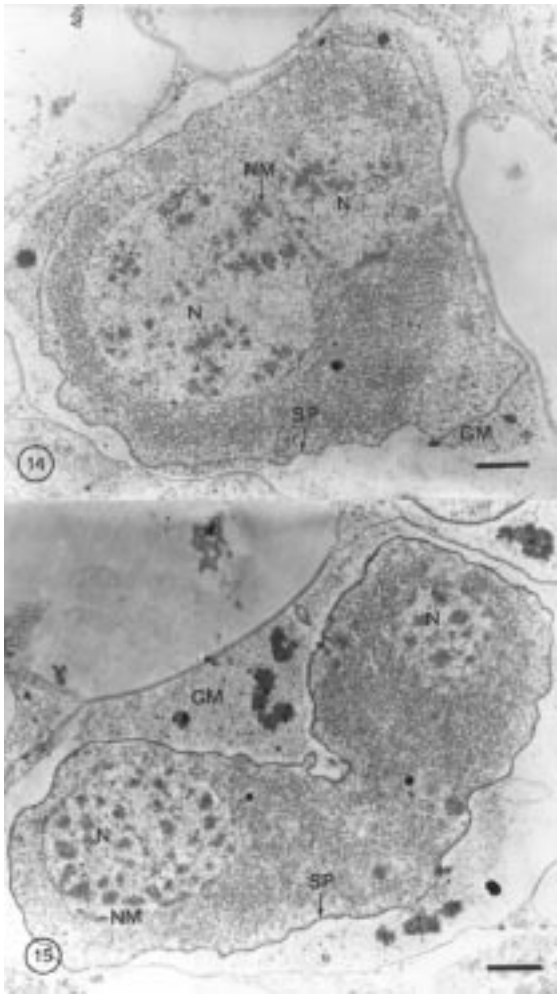

Fig. 14. The binucleate sporont in the sporulation to form lanceolate spores. N, nucleus; NM, nuclear membrane; GM, granular metabolites; SP, sporont plasmalemma. (Bar = 1 μm).
Fig. 15. Dividing of a binucleate sporont. GM, granular metabolites; N, nucleus; NM, nuclear membrane; SP, sporont plasmalemma. (Bar = 1 μm).

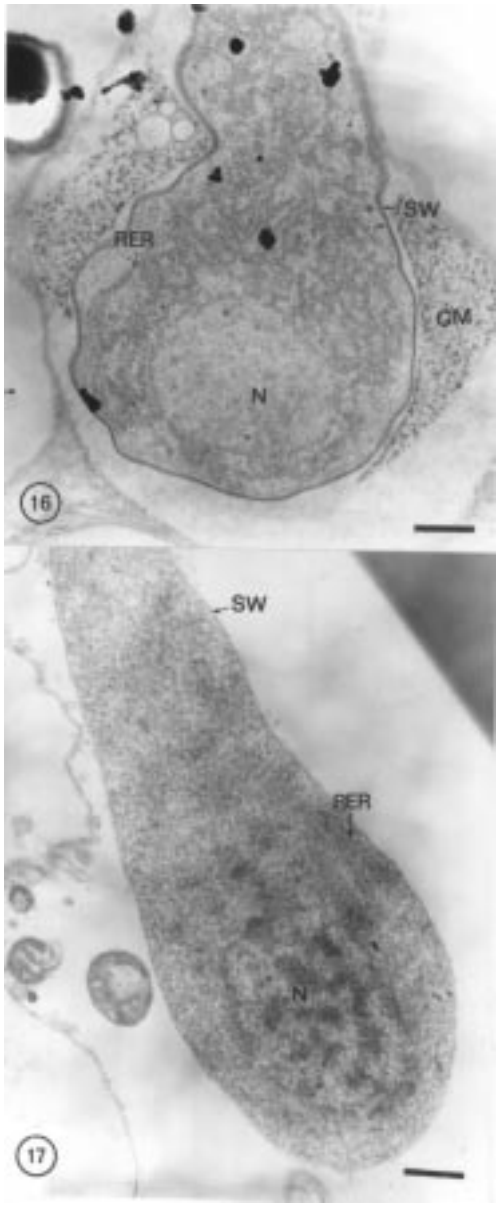

Fig. 16. The young sporoblast shaped as irregular. GM, granular metabolites; N, nucleus; RER, rough endoplasmic reticulum; SW, spore wall. (Bar = 1 μm).
Fig. 17. Elongate sporoblast of the lanceolate spore. N, nucleus; RER, rough endoplasmic reticulum; SW, spore wall. (Bar = 500 nm).

not consider them to be the same due to the consideration of host specificity of *Amblyospora* sp. [21]. In fact, this is the first microsporidium to be described in *C. fuscanus*. In addition, the spore size of the new isolate was significantly smaller than the species infecting *C. halifaxi*, although they were similar in shape [15]. They also differed in the number of coiled turns of the polar filament of meiospores: eight in *A. trinus* and nine in the present species. In addition, *Amblyospora* is typically known to have a more complicated life cycle [12]. In general, *Amblyospora* has three

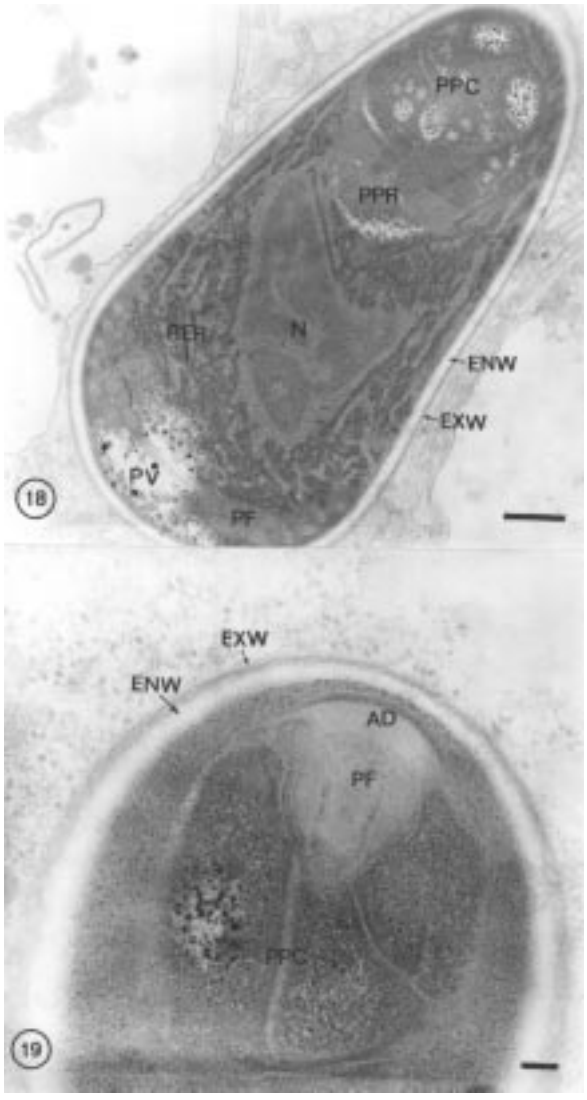

Fig. 18. A mature lanceolate spore. ENW, endospore wall; EXW, exospore; PF, polar filament; PPC, multi-chambered polaroplast; PPR, reticulate polaroplast; PV, posterior vacuoles; RER, rough endoplasmic reticulum. (Bar = 500 nm).
Fig. 19. The anchoring disc at the anterior end of the straight portion of the polar filament. PPC, multi-chambered polaroplast; AD, anchoring disc; ENW, endospore; EXW, exospore. (Bar = 100 nm).

sporulations occurring in two or three different host stages to give rise to three types of spores, i.e. oval meiospores in larval mosquitoes, lanceolate spores in copepods, and binucleate spores produced in adult females [15]. It has been shown for several species that a copepod serves as an intermediate host [6,7,11,12,22]. Nevertheless, we did not find any spores related to the intermediate host were involved in the developmental cycle since no copepods have been collected in larval breeding sites of the mosquitoes. Thus, it was still not clear whether an intermediate copepod was involved in the transmission of this microsporidium. Binucleate spores which typically form in adult females and are responsible for transovarial transmission were not observed in either *C. fuscanus* or *C. halifaxi* [15]. However, aberrant binucleate meiospores may occasionally be seen during the sporulation (data not shown). The evidence suggested that this species differs from the typical species belonging to *Amblyospora* and related species in genera *Parathelohania, Edhazardia*, *Culicospora*, *Culicosporella*, *Duboscqia*, *Cristulospora* and *Tricornia* [9,10,23–25]. All these microsporidia are heterosporous as well, but their life cycles normally require two successive host generations to complete [10,25,26].

In addition, we now know both two microsporidia which infect predacious mosquitoes exhibit simultaneous heterosporogony in the larva of their hosts. It was evidently distinct from the genus *Vairimorpha* which is also heterosporous producing two types of spore in primarily hymenopteran and lepidopteran insects [27,28]. In *Vairimorpha*, both *Nosema*-like (binucleate sequence) and *Thelohania*-like (uninucleate sequence) dimorphic forms are simultaneously produced in infected lepidopteran larvae [28]. All related data have revealed that it is not appropriate to place the present microsporidium in any of the above genera [3,4].

Therefore we proposed a new genus which will be established for microsporidian species developing in predacious mosquito larvae with concurrent sporulation sequences in one host. The type species of this newly established genus would be *Intrapredatorus* (= *Amblyospora*) *trinus* [15] from *C. halifaxi*. In turn, we here named *Intrapredatorus barri* n.g., n.sp. for this new parasite. A copepod host may or may not be involved in the horizontal transmission; it has not been collected in the breeding site thus far. *Culex quinquefasciatus* or *Chironomus* sp. probably played a role in

horizontal transmission as they were cohabitant with *C. fuscanus* and served as its prey [29]. In fact, cannibalism of infected individuals has been speculated as one of pathways for horizontal transmission in certain species of microsporidia [15]. In addition, transovarial transmission via an unobserved binucleate spores can not be excluded at this moment. However, all relevant assumptions remained for further verification.

5. Taxonomy

5.1. Intrapredatorus n.g.

Type species: *Intrapredatorus* (*Amblyospora*) *trinus* Bectel and Sweeny, 1990.

Hosts: Predacious mosquito larvae: *C. halifaxi* Theobald, 1903 (Diptera: Culicidae) for the type species; *C. fuscanus* Wiedemann, 1820 (Diptera: Culicidae) for the present species.

Development in host: Presporogonic proliferation by binary division of diplokaryotic cells (meronts). Two concurrent sporulation sequences occur in one infected larvae, both ending with uninucleate spores. In one sequence, haplosis is by meiosis and sporogony is octosporoblastic. The resulting mature meiopores are oval in shape, resembling the meiospore of most species of *Amblyospora* (numerous). In the other sequence, haplosis is thought to be by nuclear dissociation and sporogony may be disporoblastic. The shape of resulting mature spores are lanceolate (scant). Both spores develop in fat body tissues of larvae. Sub-persistent sporophorous vesicles derived from an interfacial envelope are produced in each sporulation sequence. The new genus is assigned to be a member of the family Amblyosporidae.

5.2. Intrapredatorus barri n.sp.

The specific name was after the last name of the professor emeritus of UCLA School of Public Health, Dr A. Ralph Barr who passed away in 1995.

Type host: *C. fuscanus* Wiedemann, 1820 (Diptera: Culicidae).

Type locality: Artificial tank in Liu-Chiu Islet, Ping-Tung, Taiwan.

Site of infection: Fat body of larvae.

Development in host: Two sporulation sequences occur concurrently in one mosquito larvae. Eight uninucleate meiospores are formed within a sporophorous vesicle in one sequence. The lanceolate spores are formed by nuclear dissociation to produce two mature spores in the other sequence.

Spore morphology: The oval meiospores (sometimes called octospores) measured $7.38 \pm 0.63 \times 4.59 \pm 0.47$ μm while the lanceolate spores measured $8.14 \pm 0.42 \times 4.41 \pm 0.28$ μm in fresh preparations. Polar filament coiled with nine turns about the posterior vacuole was anisofilar in meiospores; whereas isofilar in lanceolate spores.

Transmission: Not known; perhaps via cohabitant prey mosquitoes as the transmitting vehicle. Perhaps also transovarial via binucleate spores.

Type specimens: Holotype and paratype slides have been deposited at the Department of Parasitology, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan.

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