

**A new microsporidium *Berwaldia singularis* gen. et sp.nov.  
from *Daphnia pulex* and a survey of microsporidia described  
from Cladocera**

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SUMMARY

A new microsporidian parasite of a freshwater cladoceran from southern Sweden is described using light and electron microscopical methods. Development comprises 2 merogonial sequences, the first resulting in a cluster of 8 merozoites, the second in a chain of 4 merozoites. Each secondary merozoite develops into a sporont which divides into 2 sporoblasts, each of which develops into a spore. The spores are broadly oval and in fresh smears measure about 6 µm in length, with a single nucleus and a posterosome. The polar filament is about 40 µm long, of even thickness throughout, and appears as 15–18 coils in a single layer. The anchoring disc is small and the polaroplast is composed of 2 lamellar parts. Outside the plasma membrane of the sporont a 5-layered, electron-dense substance is produced, which further differentiates into endo- and exospore, an electron-dense substance occurring patchily on the exospore and a pansporoblast membrane. During development the sporoblasts and the young spores are connected by a dense substance. Mature spores appear single or paired. The pansporoblast membrane is composed of 2 structurally different layers, namely a thin outer, single membrane and an inner layer composed of tubular structures. It is connected to the spore coat by patches of the dense substance. The new microsporidium is considered to belong to a new genus of the family Telomyxidae, and its systematic relationship with this and the related family Tuzetiidae is discussed. A survey of microsporidia from Cladocera is included.

INTRODUCTION

*Daphnia pulex* (de Geer), collected in a pool in the south of Sweden in the spring and autumn of 1978, were found to be infected with microsporidia. It was a species with oval spores measuring approximately 6 µm in length in fresh smears, and especially infecting the fat body. Light microscopical observations were supplemented with studies of ultrastructure. On the spore wall of this microsporidium were secretions of electron-dense material, which evidently had a dual function: it connected many spores in pairs along their long axes, and it also served as attachment for a pansporoblast membrane of special structure which surrounded the spores in a loosely folded fashion. This microsporidium could not be identified with any species so far described, neither could it be included in any of the existing genera. A new genus is erected for the new species, which is named *Berwaldia singularis*.

The first record of microsporidia from Cladocera was made by Moniez (1887) who described 5 new species of the genus *Microsporidia* and, based on their spore shape, named these: *M. obtusa*, *M. ovata*, *M. elongata*, *M. acuta* and *M. incurvata*. At the present time 22 named species have been described from cladoceran hosts, but only 2 of these have been investigated ultrastructurally. About half of the descriptions are more than 50 years old and most are very brief. It is quite possible that the greater part of these older microsporidia do in fact represent distinct species, but diagnoses consisting of not much more than shape and length of the spore are difficult to compare with observations made with the aid of modern techniques. In addition, the generic position is doubtful for several of these older microsporidia. Green (1974) made a survey of parasites of Cladocera in which he listed 13 previously described microsporidia and gave brief descriptions of 2 new species. For each species diagnostic characters were given but he made no distinction between characters taken from the original description of the species and substitutional characters based on his own experience. These are sometimes so different from the original characters (for example, concerning spore morphology and dimensions) that it is doubtful if they represent the same species. The systematics of the microsporidia was revised by Sprague (1977) and in this monograph 14 species described from Cladocera are treated. Since then 4 new species have been described, 2 by Loubès & Akbarieh (1977, 1978) and another 2 by Voronin (1977). So far 22 named and 5 unnamed species of microsporidia have been described from Cladocera. These are listed below, classified according to Sprague's system (1977), except that 3 of them have been transferred to the collective genus *Microsporidium*, since the characteristics of the original descriptions do not justify the present generic assignment. Each species is accompanied by a brief diagnosis taken from the original description.

#### MATERIALS AND METHODS

Infected specimens of *Daphnia pulex* were collected from a pool at Saxtorp, Scania, Sweden, in the spring and autumn of 1978. The microsporidia were studied in fresh and stained smears, in paraffin-sectioned whole specimens and in ultrathin sections of fat body cells.

Infected specimens were smeared on microscope slides, lightly air-dried and fixed in Bouin–Duboscq–Brasil solution for at least 1 h. The smears were stained in Giemsa solution after hydrolysis in hydrochloric acid (Weiser & Briggs, 1971), in Heidenhain's iron haematoxylin or by the Feulgen method (Romeis, 1968), and mounted in DePex. Whole specimens were fixed in Bouin–Duboscq–Brasil solution overnight. After washing and dehydration in an ascending series of ethanols and clearing in butanol, the specimens were embedded in Paraplast. Sections were cut sagittally at a thickness of 5 and 10 µm and stained in Giemsa solution or Heidenhain's iron haematoxylin. Measurements were made with an eye-piece micrometer at 1000 $\times$ .

For electron microscopy the shells were removed and the specimens halved. The tissue pieces were fixed in 5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4 °C for 2–5 h. After washing in cacodylate buffer and post-fixation in 2% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4 °C, the pieces were

dehydrated in an ascending ethanol series. They were then stained in a solution of 1% (w/v) phosphotungstic acid and 0·5% (w/v) uranyl acetate in absolute ethanol for 45 min and, after washing in styrene, embedded in vestopal. The sections were stained with lead citrate and uranyl acetate.

#### OBSERVATIONS

##### *Pathological changes in the host*

Primarily, infection was restricted to fat body cells, which could be hypertrophied and completely filled with spores. In advanced stages of infection the adipose tissue was completely destroyed (Pl. 1 A) and infection could also be found in ovaries (Pl. 1 B) and hypoderm. At this stage infected material was easily recognized by its yellowish-white colour. Infection was never found in the intestinal epithelium, musculature or nerve tissue, and infected specimens of *D. pulex* could not be distinguished from uninfected due to locomotory disturbances.

##### *Merogonial sequences*

There were 2 merogonial sequences in the development of this microsporidium. The merozoites of the first and second cycles were distinguished by differences in morphology, and in stained smears, especially after staining in Giemsa solution, by different staining properties. The merozoites produced by the first merogony had small compact nuclei, measuring about 0·9  $\mu\text{m}$  in diameter, surrounded by a thin layer of cytoplasm (Pl. 1 C). Their nuclei were intensely stained. From each meront 8 merozoites were produced in a dense cluster. In the second merogony each meront formed 4 merozoites, usually in a short chain (Pl. 1 D–E). They had larger nuclei, measuring approximately 1·8  $\mu\text{m}$  in diameter which did not stain as intensely, and they seemed to be surrounded by a thicker layer of cytoplasm. In the material processed for electron microscopy the macronuclear merozoites were found together with different stages of sporogony, but there were no micronuclear merozoites. For this reason micronuclear merogony is considered to precede macronuclear merogony.

##### *Sporogony*

Each secondary merozoite developed into a sporont, which divided further, producing 2 sporoblasts. These developed in close association and the 2 spores thus formed were primarily connected along their length (Pl. 1 C–D). Mature spores also occurred singly. In smears, the unpaired spores could have been separated during preparation, but they were also present in ultrathin sections, in which no mechanical strain had been put on the material prior to fixation. There was no indication that sporonts could differentiate into sporoblasts without undergoing division. In ultrathin sections apparently single sporoblasts could be found. Studies of serial sections revealed that the sporoblasts were joined, but they appeared single as a result of the way the sections were cut. The single spores could not be explained by the sectioning and there is reason to believe that joined spores can separate during spore maturation. Since mature spores were regularly found

in paired condition, maturation did not necessarily lead to a separation between the spores.

#### *Ultrastructure of sporogonic stages*

Primary merozoites could not be found in the material processed for ultrastructural studies and the youngest stages observed were secondary merozoites. They appeared as slightly irregular cells, usually measuring 2–4  $\mu\text{m}$  in diameter, surrounded by a unit membrane of about 8 nm in thickness (Pl. 2A). The finely granular nucleus was voluminous, with an irregular outline, and it was located in the centre of the cell. The cytoplasm was also granular, due to the presence of numerous free ribosomes and contained concentric strands of lamellar endoplasmic reticulum, surrounding the nucleus.

The cell border of the sporont was more complicated than that of the merozoites. Outside the plasma membrane of the merozoite, electron-dense material was gradually deposited (Pl. 2B), and it spread from a small number of foci to surround the sporont. It was differentiated into layers of different electron density, and appeared as a 5-layered coat (Pl. 2C).

The nucleus of the sporont was more elongated and spindle apparatuses were regularly found (Pl. 2B). The centriolar plaque appeared as a double disc with a diameter of about 200 nm, with the more electron-dense layer superimposed. The plaque was localized on the outer side of the nuclear envelope, but with the region of the plaque more or less invaginated into the nucleus (Pl. 2D). Radiating spindle tubules were not seen, but clusters of transversely sectioned, intranuclear tubules suggested their presence (Pl. 2E). Electron-dense areas presumed to be chromosomes were evident in the nucleus during this phase.

Two sporoblasts were formed from each sporont by nuclear and later cytoplasmic division, and the two cells remained together at least during the greater part of the further development (Pl. 3A–B). Each sporoblast was elongated with a central elongated nucleus, surrounded by concentric layers of endoplasmic reticulum (Pl. 3C). At one pole 2 vesicular areas developed (Pl. 3C, F) which are assumed to be posterosomes, structures concerned in the formation of the polar filament. At the other pole of the sporoblasts, whorls of membranes and enclosed tubules appeared laterally (Pl. 3C), which could be the initial stages of the polaroplast.

#### *The spore*

The spores were broadly oval, often wider at one end, with a slightly eccentric nucleus (Pl. 1C–D). Spores could be stained without difficulty using conventional histological methods. Most spores were uniform in size, measuring in fresh smears 5·5–6·5  $\times$  3·0  $\mu\text{m}$  and in fixed and stained preparations 3·0–3·5  $\times$  1·5  $\mu\text{m}$ . In stained smears larger spores measuring 4–5  $\mu\text{m}$  in length, were found in low numbers. The spores had no appendages, but their surfaces appeared shrunken or irregular. The reason for this was not obvious under the light microscope, but the study of ultra-thin sections revealed that it was due to the arrangement of the pansporoblast membrane.

The fixation procedure used was not suitable for young spores, and only a small number of these appeared correctly fixed. In young spores the cytoplasmic

structures were still lamellar but appeared more electron-dense than in the sporoblasts. They could be seen concentrically arranged around the nucleus, which had rounded up (Pl. 4 A). Development of the polar filament was initiated at the posterior pole, and the number of coils increased during maturation. The diameter of the filament was successively reduced, transverse sections appeared more rounded and the inner organization became more complex. In the spore wall the endospore layer thickened and changed from a thin layer of moderate electron density to a voluminous structureless layer. During this process the pansporoblast membrane differentiated and separated partially from the spore wall.

Ultrathin sections through mature spores revealed the different organelles (Pl. 4 B), although the thick endospore made fixation difficult. There was a rounded posterosome rather than a vacuole in the posterior region (Pl. 4 C). The polar filament was long, measuring about 40  $\mu\text{m}$  when everted, and appeared as 15–18 coils in a single layer, each coil at an angle of 60–70 degrees to the long axis of the spore. The diameter of the filament was uniform throughout its whole length and measured 110–130 nm. Sections through the filament revealed a sub-structure of concentric layers of different electron densities (Pl. 4 D): surrounding the filament were 3 thin layers, a very electron-dense, a structureless and another very dense layer. Beneath these were 2 layers of moderate electron density and about half-way to the centre a less dense layer, which appeared to be composed of longitudinal fibrils; the central part, with a diameter measuring approximately 60 nm, was moderately dense and had 1 or 2 more dense concentric sub-structures.

In the anterior quarter of the spore the filament was straight, and was connected to the anchoring disc by a swollen plug-like structure with a diameter of about 130 nm (Pl. 4 E). The swollen attachment seemed also to be composed of concentric sub-structures. The anchoring disc was relatively small, with a diameter of about 0·5  $\mu\text{m}$ , and was surrounded by an outer sheath of the same construction as the 3 thin outer layers of the polar filament. It had a sub-structure of superimposed layers of different electron densities.

The anterior part of the polar filament was surrounded by a polaroplast, which extended back about one third of the spore length. It was composed of lamellae overlain by a surface layer which had the same sub-structure as the sheath of the anchoring disc and the polar filament: 2 thin electron-dense layers surrounding a structureless one. In the anterior part the lamellae were thin and arranged regularly and compactly. In the posterior part the lamellae were wider apart and less regularly arranged. Strands of ribosomes surrounded the nucleus and the polaroplast (Pl. 4 B, E). Their arrangement suggested that they were located on membranes, but these could not be recognized.

#### *The spore-wall and the pansporoblast membrane*

The cytoplasmic structures of the spore were surrounded by a plasma membrane, a unit membrane measuring approximately 10 nm in thickness, then a structureless endospore and an outer irregular electron-dense exospore which was about 20 nm thick (Pl. 4 E). The thickness of the endospore varied between 60 and 200 nm, except for the region of the anchoring disc, where it was considerably thinner, sometimes as thin as 20 nm. The structures of the spore-coat and the surrounding pansporoblast membrane were formed from the 5-layered dense

material outside the plasma membrane of the sporont (Pl. 2C). The moderately dense layer outside the plasmalemma widened to become the endospore. The exospore was formed from the next, more dense layer. The following 2 layers, which completely surrounded the sporoblasts, retracted, and were represented by patches of electron-dense material on the coat of mature spores (Pl. 4E). This material connected the 2 sporoblasts formed by each sporont and persisted as a cementing substance between the spores. The spores were connected along their long axes (Pls 1C and 4F), and were oriented in the same or opposite directions. The areas of dense material also formed attachments for the pansporoblast membrane, which developed from the outermost moderately electron-dense layer of the sporont (Pl. 3D).

The pansporoblast membrane was a special structure composed of 2 layers. The outer layer was a single membrane, measuring 5 nm in thickness, and inside this a layer of tubules was formed (Pl. 3D, E). Each tubule had a diameter of about 20 nm, and a sub-structure could be seen as concentric layers of different electron density. The structure of the membrane hardly changed during spore maturation. The only difference was the more regular arrangement of the tubular layer in mature spores (Pl. 3E). The pansporoblast membrane occurred as loose folds surrounding diplospores and enclosing single spores (Pl. 4F). Threadlike or granular inclusions could be seen in the space between the exospore and the pansporoblast membrane.

#### MICROSPORIDIA PREVIOUSLY DESCRIBED FROM CLADOCERA

Twenty-two named species of microsporidia have been described from Cladocera. These are listed, together with 5 unnamed species, according to the classification system proposed by Sprague (1977). The diagnoses, hosts and collecting areas are taken from the original descriptions of the species. Three species have been transferred to the collective genus *Microsporidium* until supplementary information makes their taxonomic position clear.

#### *Family Pleistophoridae Stempell, 1909*

##### *Pleistophora intestinalis* Chatton, 1907

Spores pyriform,  $2 \times 3 \mu\text{m}$ , produced in a distinct pansporoblast. Stained spores with a central girdle of chromatin and clear spaces at the two poles: at the blunt end a vacuole, at the pointed end 'l'emplacement de la capsule polaire', which might be the polaroplast. In the midgut epithelium of *Daphnia pulex* and *D. magna*. France.

##### *Pleistophora daphniae* (Weiser, 1945) Sprague, 1977

*Pleistophora daphniae* Weiser, 1945. Spores ovoid, ellipsoid or elongated ellipsoid,  $5-6 \times 2.5 \mu\text{m}$ . Most stained spores with a spherical granule at the posterior end. Schizonts often rounded, with single nuclei, give rise to multinucleate plasmodia, which divide into rosettes of sporoblasts, which develop into mature spores. 5–10 spores are produced from each plasmodium. In the haemocoel of *D. pulex*. Czechoslovakia.

*Family Duboscqiidae Sprague, 1977**Duboscqia sidae* (Jírovec, 1942) Sprague, 1977

*Dubosquia sidae* Jírovec, 1942. Spores oval or slightly narrowed at one pole,  $3 \times 1.2\text{--}1.6 \mu\text{m}$ , produced in pansporoblasts with 16, rarely 8 spores. Infected specimen coloured white. In the haemocoele of *Sida crystallina*. Czechoslovakia.

*Family Thelohaniidae Hazard & Oldacre, 1975**Thelohania chydoricola* Green, 1974

Spores pyriform,  $4.5\text{--}5.0 \times 2.0\text{--}2.5 \mu\text{m}$ , curved in one plane. Development in octosporous pansporoblasts with a diameter of about  $18 \mu\text{m}$ . In the fat body of *Chydorus sphaericus* and *Scapholeberis mucronata*. England. It is not clear that this is a *Thelohania* species since the generic assignment within the family Thelohaniidae depends on ultrastructural characteristics. However, microsporidia of the genus *Thelohania* as defined by Hazard & Oldacre (1975) are parasites of Crustacea, so it could be a species of this genus although it has not been shown.

*Thelohania* sp. 1 Voronin, 1977

Spores ovoid with one end strongly pointed, dimensions in stained preparations  $4.3(3.9\text{--}4.5) \times 2.8(2.5\text{--}3.1) \mu\text{m}$ . In fresh smears the spores were found in octosporous pansporoblasts with a diameter of  $10 \mu\text{m}$ . Extruded polar filament  $36\text{--}42 \mu\text{m}$ . In hypodermis of *Simocephalus vetulus*. U.S.S.R. The species was considered to be distinct from previously known *Thelohania* species but could not be described due to scarcity of material. However, the generic assignment is not clear without knowledge of the ultrastructure.

*Toxoglugea* sp. Voronin, 1977

Spores elongated, curved. Length  $4 \mu\text{m}$  or more, diameter  $1.1\text{--}1.6 \mu\text{m}$ . The height of the bent spore  $3.2\text{--}3.5 \mu\text{m}$ . One end of the spore with a metachromatic granule. In smears practically all spores were found in groups of 4. Infected tissue unknown. *Ceriodaphnia reticulata*. U.S.S.R. The species could not be described due to scarcity of material.

*Family Gurleyidae Sprague, 1977**Gurleya tetraspora* Doflein, 1898

Spores oval, with one end pointed the other rounded. No report of spore size. Spore surface with longitudinal grooves. The rounded end of the spore with a large, clear vacuole. Figure shows pansporoblasts with 4 spores. In hypodermal tissues of *D. maxima*. Infected specimens with brownish patches on the cuticle. Germany.

*Gurleya vavrai* Green, 1974

Spores oval, slightly tapered towards one end,  $5.5\text{--}6.0 \times 2.8\text{--}3.0 \mu\text{m}$ . Pansporoblasts with a diameter of about  $8 \mu\text{m}$  containing 4 spores. No report of tissue specificity. Described from *D. longispina*. England.

*Family Unikaryonidae Sprague, 1977*

*Nosemoides simocephali* Loubès & Akbarieh, 1977

Spores ovoid,  $2.5-3 \times 1.5-1.75 \mu\text{m}$ , developing in direct contact with the host cytoplasm. A sporogonial plasmodium gives rise to a group of sporoblasts with single nuclei, each developing into a spore. Pansporoblasts and diplokarya absent. In the intestinal epithelium of *Simocephalus vetulus*. Collecting area not reported. The description was based on ultrastructural studies.

*Family Caudosporidae Weiser, 1958*

*Octosporea bayeri* Jírovec, 1936

Spores elongated with rounded ends, slightly bent or straight,  $5.5-9 \times 1.5-2.5 \mu\text{m}$ , usually  $7 \times 2 \mu\text{m}$ . Fresh spores with a refractile vacuole at the anterior end. Dry smears stained in Giemsa solution revealed a metachromatic granule at the posterior pole. Feulgen preparations showed 2 nuclei in each spore. Infected specimen opaque, not as white as in other types of microsporidian infections. Induces cellular hypertrophy. In the fat body of *D. magna*. Czechoslovakia.

*Octosporea diaphanosomae* Voronin, 1977

Spores of 2 types. Small to medium sized spores,  $3.5 \times 1.1 \mu\text{m}$ , are rod-shaped, macrospores,  $6.8 \times 2.8 \mu\text{m}$ , ellipsoid. The lower the number of spores produced by each sporont the greater the spore dimensions. Line drawings of developmental stages show 1 sporont with a diplokaryon. In ovaries of *Diaphanosoma brachyurum*. U.S.S.R.

*Family Nosematidae Weiser, 1958*

*Nosema polyphemii* Voronin, 1977

Spores ellipsoid. Dimensions in fresh smears  $3.5 (3.2-4.0) \times 1.6 (1.5-1.8) \mu\text{m}$ , in stained preparations  $3.2 (2.8-3.5) \times 1.6 (1.4-1.8) \mu\text{m}$ . There is no surrounding mucus layer. 2 spores are produced by each sporont. In the ovaries of *Polyphemus pediculus*. U.S.S.R. The ranking into *Nosema* is based only on the disporoblastic sporogony. Since the occurrence of diplokarya is not mentioned in the description neither is clearly shown in the illustrations, it is doubtful that this species could be included in the family Nosematidae.

*Systematic position uncertain*

*Baculea daphniae* Loubès & Akbarieh, 1978

Spores uninucleate, rod-shaped, thin,  $2.8-3 \times 0.20 \mu\text{m}$ . 2-4 spores produced from each sporont. Development in a parasitophorous vacuole with a variable number of spores. In the digestive epithelium of *D. pulex*. France. Description based on ultrastructural investigations.

*Unclassified microsporidia**Microsporidium acutum* (Moniez, 1887) *hoc loco*

*Microsporidia acuta* Moniez, 1887. Spores pointed,  $5 \times 2 \mu\text{m}$ . Described from *D. pulex*. No report of collecting area. Schröder (1914) considered this species to belong to the genus *Thelohania*, although nothing in the short description was indicative of development in octosporous pansporoblasts. Hazard & Oldacre (1975) did not consider this species to belong to the family Thelohaniidae.

*Microsporidium cladocera* (Pfeiffer, 1895) Sprague, 1977

*Glugea cladocera* II Pfeiffer, 1895. Spores 2–3  $\mu\text{m}$  long, developing in ‘sporoblasts’, here considered to be pansporoblasts, with a diameter of 3–4  $\mu\text{m}$ ; each pansporoblast producing 8, 16 or more spores. Infected specimen completely filled with spores. In the fat body and hypodermis of *D. magna* and *Limnetis* sp. Germany.

*Microsporidium daphniae* (Jírovec in Weiser, 1947) comb. n.

*Glugea daphniae* Jírovec in Weiser, 1947. Spores egg-shaped,  $4.5 \times 2.5 \mu\text{m}$ , produced in pairs. In the fat body of *D. pulex*. Czechoslovakia. No illustrations. The genus *Glugea* Thélohan, 1891, contains disporoblastic parasites of fish, where the microsporidia develop in symbiotic relationship with the host cell. This microsporidium obviously does not belong to *Glugea*. This was shown by Vávra (1978) in an electron microscopic investigation of a species which he supposed to be identical with Jírovec's *G. daphniae*. It was found that each sporont produced 4 sporoblasts. These were coupled into doublets and the spores matured pair-wise connected by an electron-dense material. The pansporoblast membrane was thin and fragile and dissolved after the death of the host and then the still joined spores were set free. According to Vávra (1978) the species has affinities to the genus *Gurleya* Doflein, 1898, but he does not transpose it to that genus. The production of 4 spores enclosed by a pansporoblast membrane is characteristic for the family Gurleyidae Sprague, 1977. The 2 genera of this family, *Gurleya* Doflein, 1898, and *Pyrotheca* Hesse, 1935, are imperfectly known (Sprague, 1977) and they are distinguished only by small differences in the shape of the spores. This microsporidium does probably belong to the family Gurleyidae, but the generic assignment could hardly be done for the moment. It is provisionally transferred to *Microsporidium*.

*Microsporidium elongatum* (Moniez, 1887) Sprague, 1977

*Microsporidia elongata* Moniez, 1887. Spores elliptical,  $5 \times 2 \mu\text{m}$ . Described from *S. vetulus*. Locality not reported.

*Microsporidium holopedii* Frič & Vávra, 1894

The microsporidium was provisionally named without description. A few illustrations show some developmental stages. There are rounded to pyriform, irregular cells, which might be interpreted as spores. From the magnification indicated, their length could be calculated to be 12–15  $\mu\text{m}$ . There are also structures which could be plasmodia with several nuclei, and morula-like groups

of cells. The infection was observed in *Holopedium gibberum*. Infected animals were chalky white, and infection was found in tissues surrounding the heart and the intestine, with branches into the head, antennae and legs. Czechoslovakia.

*Microsporidium incurvatum* (Moniez, 1887) Sprague, 1977

*Microsporidia incurvata* Moniez, 1887. Spores slightly bent,  $5 \times 2 \mu\text{m}$ , the 2 ends of nearly equal width. Each spore with at least 1 clear spot. Described from *D. pulex*. Collecting area not reported.

*Microsporidium leydigii* (Pfeiffer, 1895) Sprague, 1977

*Glugea leydigii* Pfeiffer, 1895. Spores pyriform. No data on dimensions. Sporocyst with 8, 12, or 24 spores. Infection could spread from the fat body to most tissues, including hypodermis. Infected specimen chalky white and slow moving. Described from *D. pulex*. Germany.

*Microsporidium obtusum* (Moniez, 1887) Sprague, 1977

*Microsporidia obtusa* Moniez, 1887. Spores blunt, with the posterior end swollen, and with an asymmetrical clear spot,  $4 \times 2.5 \mu\text{m}$ . Described from *D. reticulata* and *S. vetulus*. No report of collecting area.

*Microsporidium ovatum* (Moniez, 1887) Sprague, 1977

*Microsporidia ovata* Moniez, 1887. Spores oval, not longer than  $3 \mu\text{m}$ , a clear spot seldom visible. Described from *S. vetulus* and *C. sphaericus*. Locality unknown.

*Microsporidium porterae* (Weiser, 1961) *hoc loco*

*Spiroglugea porterae* Weiser, 1961. This species was originally reported by Fantham & Porter (1958) as *Spiroglugea* sp.. They did not describe it as a new species due to insufficient material, but they considered it to be a new species. However, they published a brief report of their observations. Weiser's description is based on the characteristics published by Fantham & Porter (1958). His enquiries for the original slides were fruitless so he was not able to re-evaluate that material. The following characteristics are taken from the diagnose by Fantham & Porter (1958). Spores tubular with rounded ends, of uniform diameter and slightly spirally curved. One end with a vacuole the other with the polar capsule, here interpreted as the polaroplast. Infection was reported from *Daphnia longispina* and from larvae of *Calliphora erythrocephala* and some other Diptera. The paper is based on material collected in Canada and England without specification. There is no report of spore size from *Daphnia* but spores from Diptera larvae measured  $12-13.5 \times 1-1.5 \mu\text{m}$ . On a few occasions 2 minute oval nuclei were observed in the spores. There is no report of octosporous pansporoblasts which is characteristic for the genus *Spiroglugea* Léger & Hesse, 1924. In this genus the spores are uninucleate. It must be considered as a *Microsporidium* species.

*Microsporidium schaefernai* (Jírovec, 1937) Sprague, 1977

*Plistophora schäfernai* Jírovec, 1937. Spores oval, with both ends rounded, and with a refractile vacuole in the anterior part. Length  $3.8-4.2 \times 1.6-2 \mu\text{m}$ , average  $4 \times 2 \mu\text{m}$ . Macrospheres uncommon,  $7 \times 2.5 \mu\text{m}$ . 16 or more uninucleate spores

produced by each plasmodium. No pansporoblast membrane. Extruded polar filament composed of 2 different parts, a proximal part, 1–20 µm long, easily stained with Giemsa stain, and a distal thinner part, 30–40 µm, weaker stained. Infected animals grey-green. In the ovaries of *D. pulex*. Induced cellular hypertrophy. Czechoslovakia.

#### *Microsporidium* sp. III<sub>10</sub> Loubès, 1979

Spores pyriform with narrow anterior end, 4–4.5 × 2.5 µm. Uninucleate sporoblasts found in irregular number. Exospore of mature spores with fine hairs. In hypodermis and fat body of *D. pulex*. France. The species has been ultrastructurally investigated.

#### *Microsporidium* sp. III<sub>11</sub> Loubès, 1979

Spores pyriform, 2.5–3 × 1.5 µm. Exospore of mature spores with fine hairs. In the straight part of the polar filament paramural bodies were observed. Sporoblasts and spores were produced in a morula-like cluster of 12 or more. In hypodermis of *S. vetulus*. France. This species has been ultrastructurally investigated.

#### *Microsporidium* sp. III<sub>12</sub> Loubès, 1979

Spores oval, 3 × 1.6 µm. All stages with single nuclei. Polaroplast with piled granular masses. Spore wall without hairs. 4 uninucleate spores produced in a pansporoblast. In the fat body and hypodermis of *C. sphaericus*. France. The microsporidium has been ultrastructurally investigated.

*Pyrotheca cyclopis* (Leblanc, 1930) Poisson, 1953, which was described in Belgium from *Cyclops albidus*, has been reported from *Daphnia* spp. by Fantham & Porter (1958). The spores are long, 16.5 × 3 µm, slightly curved, with a pointed anterior end. Spores are formed in groups of 4, all directed in the same way, but not enclosed in a membrane. *Bacillidium daphnaiae* Jírovec, 1942, described from the haemocoele of *D. pulex* and *D. magna*, has been shown by Vávra (1959) to belong to the fungal genus *Monosporella*.

#### DISCUSSION

Among the microsporidia described from cladoceran hosts 8 species complete their sporogony inside a pansporoblast membrane, and the number of spores produced by each sporont is 4 or more. There are also some multisporous species where the spores are found in groups, although an enclosing pansporoblast membrane has not been observed. All these species are clearly different from *Berwaldia singularis*, and so are the few ultrastructurally investigated species devoid of a pansporoblast membrane. A number of species could be excluded by their pyriform or rod-shaped spores. There is, however, one species which appears to have characters in common with *B. singularis*. In the description of *Microsporidium daphnaiae* (Jírovec in Weiser, 1947) it is mentioned that the spores are produced in pairs. This species, however, has been ultrastructurally investigated by Vávra (1978) and it has been clearly shown that from each sporont 4 sporoblasts are produced. These are grouped into 2 doublets where the spores are joined by an electron-dense material. It was also noted that the pansporoblast membrane

is fragile and disappears upon death of the host. Nothing is mentioned about the fine structure of this membrane, possibly indicating that it is a thin structure like most pansporoblast membranes. These characters differentiate clearly *M. daphniae* from *B. singularis* and it may be concluded that *B. singularis* is not identical with any microsporidium so far described from Cladocera.

Tuzet, Maurand, Fize, Michel & Fenwick (1971) proposed a new classification of the microsporidia, where the forms with sporoblasts developing inside a pansporoblast membrane were grouped together in a special sub-order, Pansporoblastina. Originally only 3 families were included in this sub-order: Monosporidae Tuzet *et al.* 1971, Telomyxidae Léger & Hesse, 1910 and Polysporidae Tuzet *et al.* 1971. The last family was a collective taxon for microsporidian genera with 4 to many spores produced from each sporont, each genus characterized by a distinct number. In 1977 two new concepts of the taxonomy of microsporidia were published by Sprague and by Weiser. Weiser's (1977) system is not based on the presence or absence of a pansporoblast membrane and thus differs from that of Tuzet *et al.* (1971) while that proposed by Sprague (1977) is a modification of the system of Tuzet *et al.* (1971) and splits Polysporidae into 5 families, thus restoring 1 older family, including 1 family erected by Hazard & Oldaere (1975) after the proposal of the system by Tuzet *et al.* (1971) and creating 3 new families. The 2 other families are retained although with the name Tuzetiidae Sprague, Tuzet & Maurand in Sprague, 1977, as an emendation for Monosporidae.

The family Telomyxidae is characterized by the occurrence of diplospores (Codreanu, 1961). Two sporoblasts are produced within a pansporoblast membrane. Each of these develops into a spore, and the 2 spores remain glued together in a common matrix. So far only 1 genus has been described in this family: *Telomyxa* Léger & Hesse, 1910, with 4 species. Only *T. glugeiformis*, the type species, has been investigated in enough detail to allow clear identification, and it is the only species where ultrastructural details are known (Codreanu & Vávra, 1970; Larsson, 1981). Diplokarya have not been observed in any developmental stage of this species.

The family Tuzetiidae is characterized by the pansporoblast membrane simultaneously dividing with its contents, so that each spore is enclosed in a pansporoblast membrane of its own. The only genus in this family is *Tuzetia* Maurand, Fize, Fenwick & Michel, 1971, created for a species previously known as *Nosema infirmum* Kudo, 1921. Maurand (1973) further characterized the genus *Tuzetia* from ultrastructural studies of 2 species from copepods, 1 of which had been reported previously (Maurand, Fize, Michel & Fenwick, 1972). Maurand (1973) listed as generic characters that there are 3–6 sporoblasts produced by each sporont, nuclei in diplokaryan arrangement in vegetative stages and a single nucleus in the spore. *Plistophora debaisieuxi* Jírovec, 1943, was ultrastructurally investigated by Loubès & Maurand (1976), and it was transferred to *Tuzetia*. Loubès (1979) added *Nosema cyclopis* Kudo, 1921, and an unnamed species III<sub>7</sub>. Codreanu & Codreanu-Balcescu (1975) named another 2 species, but did not give sufficient information for their identification. So far the hosts of *Tuzetia* are found in Copepoda, Ephemeroptera and Diptera.

If the number of spores enclosed by the pansporoblast membrane is used to differentiate microsporidian families, the genus *Berwaldia* appears intermediate

between Telomyxidae and Tuzetiidae. However, the development indicates a closer affinity to Telomyxidae. In both *Berwaldia* and *Telomyxa* diplokarya are absent, 2 sporoblasts are produced by each sporont and the conjoined sporoblasts remain together during maturation. In *Tuzetia* merozoites are diplokaryotic, more sporoblasts are produced and the spores mature singly. For these reasons it seems most correct to include *Berwaldia* in the family Telomyxidae.

A comparison of structural details reveals differences between the 3 genera. In *Telomyxa* the substance connecting the spores is voluminous, enclosing both spores, and the pairing is permanent. In *Berwaldia* the cementing layer is restricted to a strand between the spores, they are never enclosed by it, and the attachment between the spores is weaker and could be broken. The arrangement of the 2 spores in diplospores is also different. In *Telomyxa* they appear in the same plane but at an angle to the long axis of the dipospore, or in different planes, one perpendicular to the other (Larsson, 1981). In *Berwaldia* the 2 spores are located in the same plane with their long axes parallel.

The connecting substance is also formed differently in *Telomyxa* and *Berwaldia*. The plasma membrane of the sporont of *Telomyxa* is locally duplicated, and electron-dense material is secreted between the membranes. In the sporont of *Berwaldia*, dense material in several strata is formed outside the plasma membrane and from this the adhesive substance between the spores is formed. In *Tuzetia* a supplementary cell coat is formed outside the plasma membrane of the sporont, but this has been described as a composite layer of 3 membranes (Maurand, 1973).

The pansporoblast membrane is differently formed in the 3 genera. In *Telomyxa* the outer of the 2 membranes, formed by the duplication of the plasma membrane, partially or completely loses its attachment to the cementing layers and becomes a pansporoblast membrane. In *Tuzetia* this membrane is formed from the plasma membrane of the sporont before differentiation of the sporoblasts. In *Berwaldia* the membrane is formed of the outermost layer of dense secretions.

Usually the pansporoblast membrane of microsporidia is considered to be a thin membrane of unit type, often with a rough outer surface (Vávra, 1976). In the space between the membrane and the surface layer of the spore or the sporoblast, tubular structures have been found in different microsporidia. Overstreet & Weidner (1974) revealed their occurrence in *Inodosporus spraguei* and they were reported from *Tuzetia debaisieuxi* by Loubès & Maurand (1976). In the last mentioned species the tubules were attached to the exospore of the mature spores and they were not connected to the pansporoblast membrane (Loubès & Maurand, 1976). In *Berwaldia* the tubules are intimately connected to the pansporoblast membrane even when it surrounds mature spores.

*Telomyxa glugeiformis* has so far only been found as a parasite of Ephemeroptera, and the other, somewhat dubious, species of that genus are known from Diptera, Trichoptera and Ciliophora. No species has been reported from Crustacea. The *Tuzetia* species are reported from 2 insect orders and from Copepoda. *B. singularis* is a parasite of the phyllopod *Daphnia pulex*; its infectivity has not been tested on other crustaceans in the laboratory. However, different copepods and the isopod *Asellus aquaticus*, collected together with infected *D. pulex*, were brought into the laboratory for inspection. None was infected with *B. singularis*.

Issi & Voronin (1979) described a new microsporidium from *Chironomus*

*plumosus* and created the genus *Neoperezia* for this species. In this genus the sporogony occurs inside a pansporoblast membrane. From each sporont 2 sporoblasts are formed, each of which develops into a spore. The 2 spores are permanently joined by the pansporoblast membrane or by a plasmatic bridge. It is apparent that these are common characteristics for both *Neoperezia* and *Berwaldia*, but a further comparison will show that there are also several differences.

In the diagnose of *Neoperezia*, Issi & Voronin (1979) state that the nuclei occur singly during the greater part of the developmental cycle. A plausible interpretation would be that at least in some stage diplokarya could be found. There is also one illustration indicating that. Merogony results in a lobed sporogonial plasmodium where the nuclei appear to be arranged as diplokarya. In the development cycle of *Berwaldia* there are 2 merogonial sequences and diplokarya are totally absent. In the second merogony 4 sporonts are formed arranged in a short chain. The illustrations of *Neoperezia* show that the merogony results in a sporogonial plasmodium which appears stellate in smears and from this several sporonts are formed.

In *Neoperezia* the spores are permanently joined by plasmatic bridges. The structure of these bridges is not described and they could not be seen in the illustrations. The pansporoblast is voluminous and the membrane is separated from the spores by a big space. The pansporoblast membrane is fragile and disappears at spore maturation. In *Berwaldia*, both free and joined spores are surrounded by the pansporoblast membrane which is intimately associated with the spore wall. The pansporoblast membrane of *Neoperezia* is not described and the species has not been ultrastructurally investigated. It could be suspected to be a thin structure since it disappears at the end of the sporogony. The pansporoblast membrane of *Berwaldia* is a thick specialized structure.

The disporoblastic sporogony is a character shared by more genera than *Neoperezia* and *Berwaldia*, and the differences concerning the occurrence of diplokarya, the number of merogonial sequences, the arrangement of the pansporoblast membrane and possibly also the ultrastructure of the pansporoblast membrane clearly indicate that these genera are different. The information available for *Neoperezia* is not sufficient to include it in the family Telomyxidae.

#### DESCRIPTION

##### *Berwaldia* gen. nov.

*Diagnosis:* 2 merogonies, merozoites with single nuclei. The sporont forms 2 sporoblasts, each of which develops into a spore. Spores with single nuclei. The 2 spores are connected by a cementing substance. Mature spores occur singly or paired, surrounded by a 2-layered pansporoblast membrane, with an outer single membrane and an inner layer of tubules.

*Derivation of name:* after Franz Berwald (1796–1868), the Swedish composer.

##### *B. singularis* sp.nov.

*Type host:* *Daphnia pulex* (de Geer) (Phyllopoda: Daphniidae).

*Infection sites:* fat body, ovaries, hypoderm.

*Type locality:* a pool at Saxtorp, Scania, Sweden.

*Types:* holotype (Pl. 1C) and paratypes on slide no. 781029-C-1 RL.

*Deposition of types:* the type slide in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, USA.

*Merogonial stages:* 2 merogonial sequences occur, the first producing a cluster of 8 merozoites with small single nuclei, diameter about 0·9  $\mu\text{m}$ , surrounded by a thin layer of cytoplasm. Both nuclei and cytoplasm stain intensely with Giemsa solution. The second merogony produces a chain of 4 merozoites, with large single nuclei, diameter about 1·8  $\mu\text{m}$ , surrounded by a thicker layer of cytoplasm. Both nuclei and cytoplasm stain less intensely with Giemsa solution.

*Sporogonial stages:* as for the genus with the following additions. The developing spores, and a part of the mature spores, are connected along their longitudinal axes by the cementing substance. The outer single layer of the pansporoblast membrane about 5 nm thick, the tubules in the inner layer with a diameter of about 20 nm. *Spore:* broadly oval, size 5·5–6·5  $\times$  3·0  $\mu\text{m}$  in fresh smears, 3·0–3·5  $\times$  1·5  $\mu\text{m}$  in fixed and stained preparations. Spore wall usually 160–200 nm thick, refractive, thinner in the region of the anchoring disc than round the rest of the spore. The surface of the spore without sculpture and appendages. Polar filament about 40  $\mu\text{m}$  long, of equal width, 110–130 nm, throughout the whole length, with 15–18 coils in a single row, each coil at an angle of 60–70 degrees to the long axis of the spore.

*Derivation of name:* *singularis* in the sense strange or characteristic, alluding to the pairing of the spores and the structure of the surrounding pansporoblast membrane.

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## KEY TO LETTERING OF PLATES

- a* anchoring disc
- cp* centriolar plaque
- er* endoplasmic reticulum
- en* endospore
- ex* exospore
- f* fat body lobe
- g* gut
- h* holotype
- m<sub>1</sub>* primary merozoite
- m<sub>2</sub>* secondary merozoite
- n* nucleus
- o* ovariole
- p* polaroplast
- p<sub>1</sub>* anterior part of the polaroplast
- p<sub>2</sub>* posterior part of the polaroplast
- pf* polar filament
- pl* plasmalemma
- pm* pansporoblast membrane
- pmi* inner layer of the pansporoblast membrane
- pmo* outer layer of the pansporoblast membrane
- ps* posterosome
- r* ribosomes
- sb* sporoblast
- sm* mature spore
- sy* young spore
- t* tubules
- v* vesicular area
- w* whorl of membranes

## EXPLANATION OF PLATES

## PLATE 1

Light microscopic appearance of *Berwaldia singularis*.

A. and B. Fat body lobes (A) and ovarioles (B) filled with spores. Sagittal sections 5 µm. Heidenhain's iron haematoxylin.

C-E. Different developmental stages of the microsporidium. C-D. Slide no. 781029-C-1 RL with the holotype indicated. The stages which could be clearly identified are primary and secondary merozoites, sporoblasts, young and mature spores. Heidenhain's iron haematoxylin.

E. A chain of secondary merozoites. Giemsa stain.

## PLATE 2

Differentiation of the sporont.

A. Secondary merozoite.

B. Early sporonts with deposition of electron-dense material on the cell border (arrows).

C. Detail of the cell border of the sporont with patches of stratified electron-dense material outside the plasmalemma.

D. The centriolar plaque is seen as a 2-layered disc lying in an invagination of the nuclear envelope.

E. Transversely sectioned intranuclear tubules.

## PLATE 3

Development of the sporoblasts.

A. Sporont dividing to produce 2 sporoblasts.

B. Later stages of sporoblast formation; each sporoblast enclosed by an electron-dense coat.

C. Longitudinally sectioned sporoblast. The developing pansporoblast membrane has partially separated from the cell border.

D. Detail of the cell border of the sporoblast. The outer layer of the 5-layered coat outside the plasmalemma gives rise to the pansporoblast membrane. The inner tubular layer is not completely developed. The layers of the sporoblast wall lie internal to the tubules.

E. Cell border of a young spore. Pansporoblast membrane is completely developed with a continuous tubular layer. In the spore wall plasmalemma, endospore and exospore are visible.

F. Posterior part of a sporoblast with 2 areas interpreted as being posterosomes.

## PLATE 4

Ultrastructure of the spore.

A. Young spore. The 3 layers of the spore coat are visible and the endospore (arrow) is still weakly developed.

B. Part of a fat body lobe with mature spores, arrows indicate strands of ribosomes.

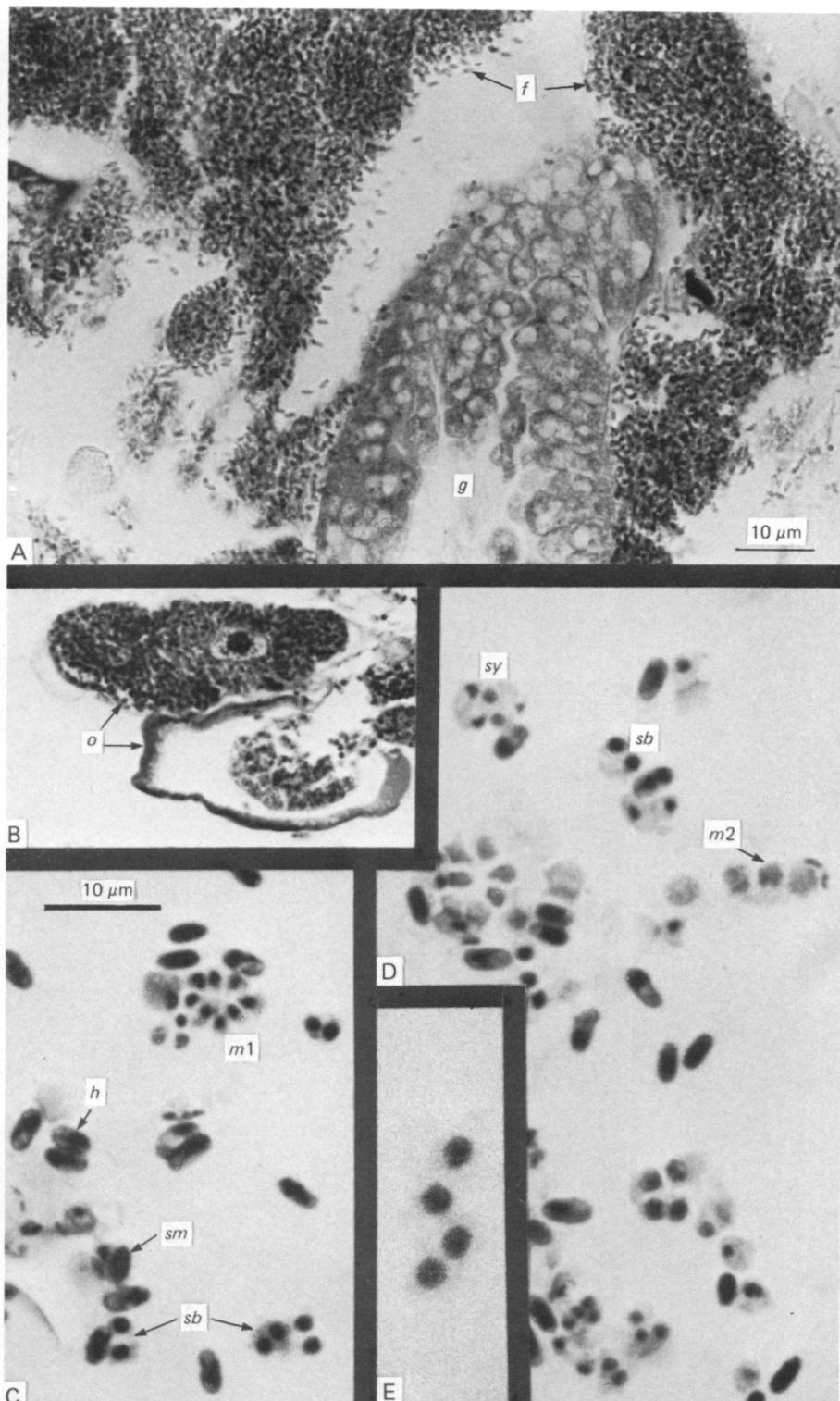
C. Longitudinally sectioned mature spore with the arrangement of the organelles visible.

D. Transverse sections through polar filament coils, arrows indicate the tubular or filamentous layer.

E. Anterior end of a mature spore showing a stratified anchoring disc and the attachment of the straight part of the polar filament. In the polaroplast the lamellae are differently arranged in the anterior and the posterior part (arrows). The pansporoblast membrane is attached at the patches of dense secretions outside the exospore.

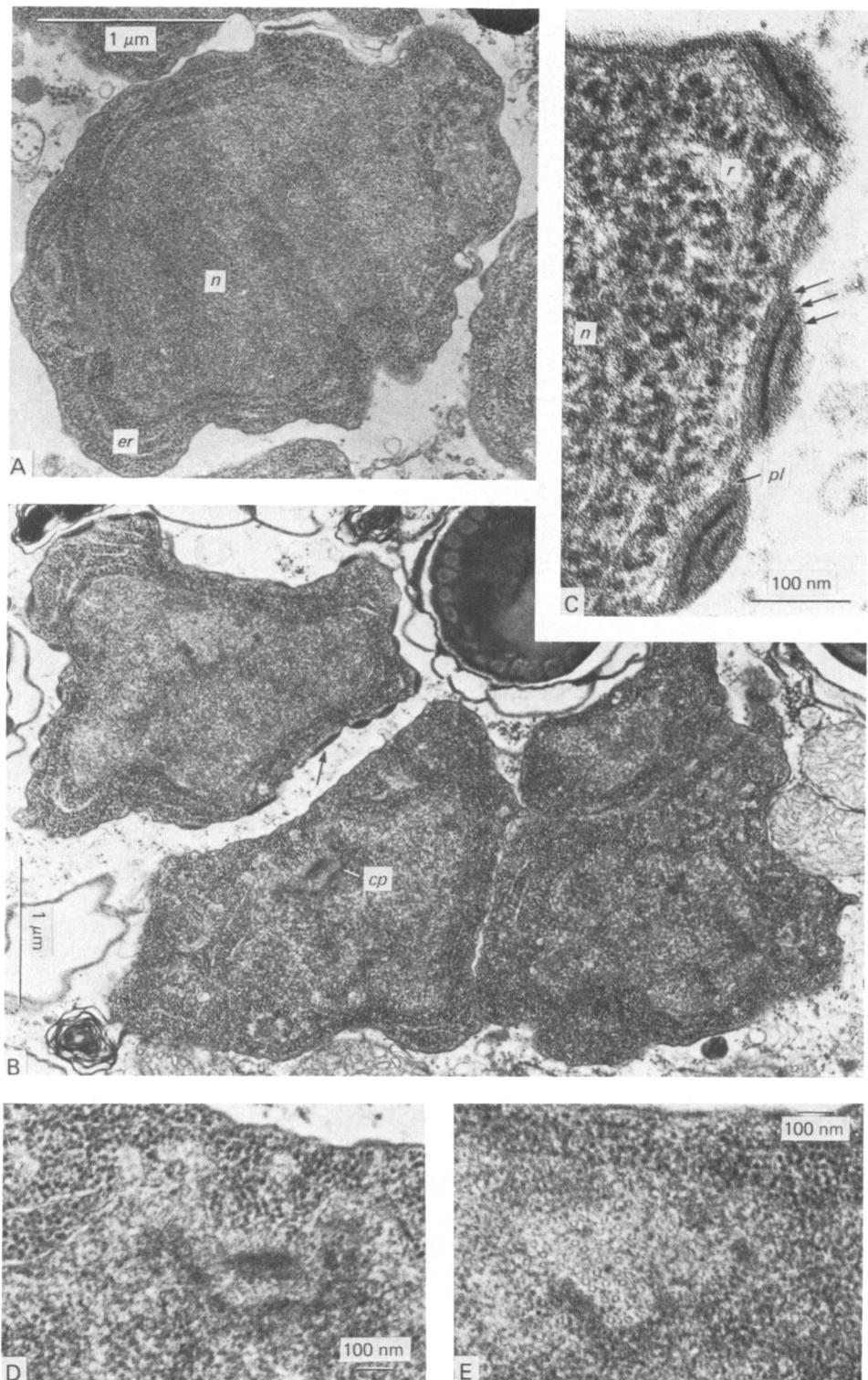
F. Longitudinal section through a diplospore, where the spores are connected by the dense patches of surface material. Note the folded appearance of the pansporoblast membrane due to the spot-wise attachments.

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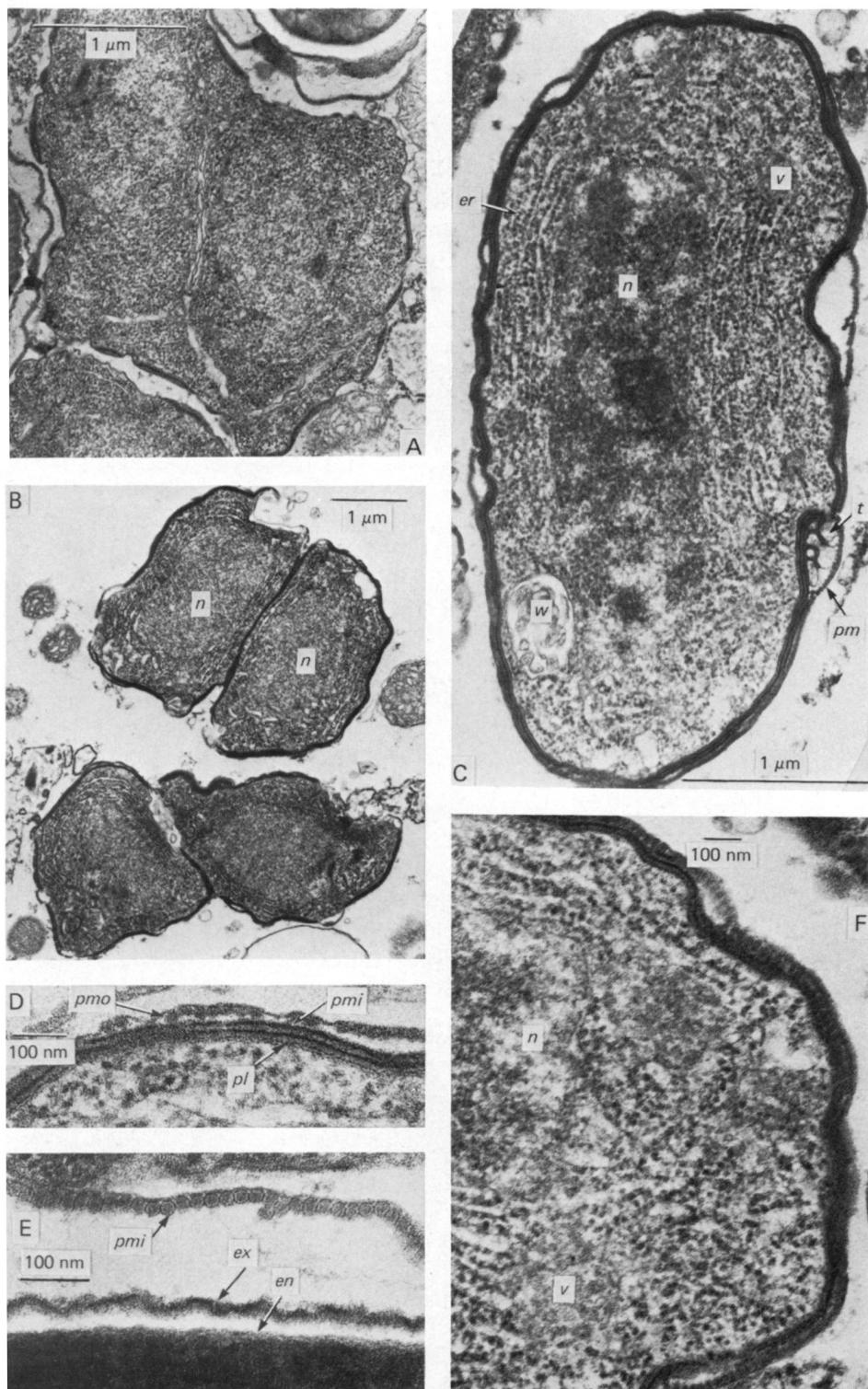


RONNY LARSSON

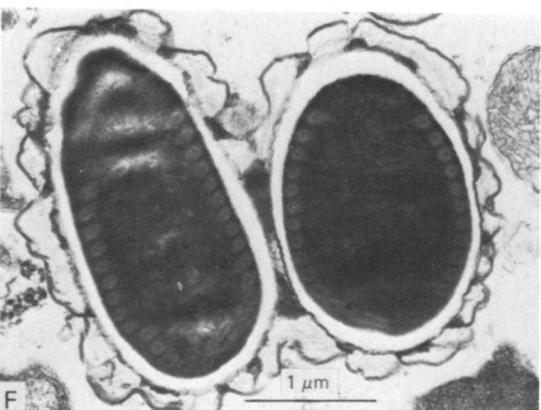
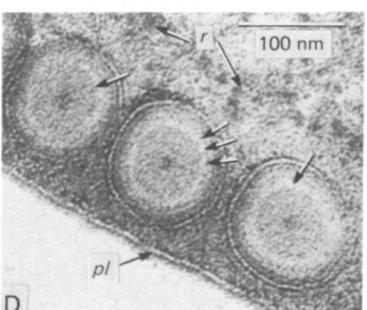
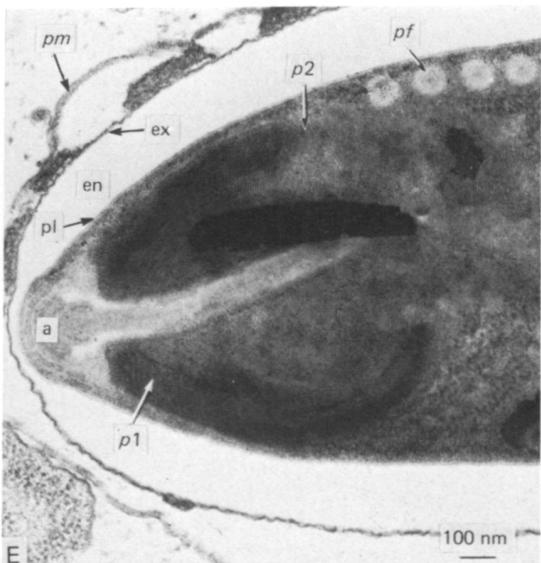
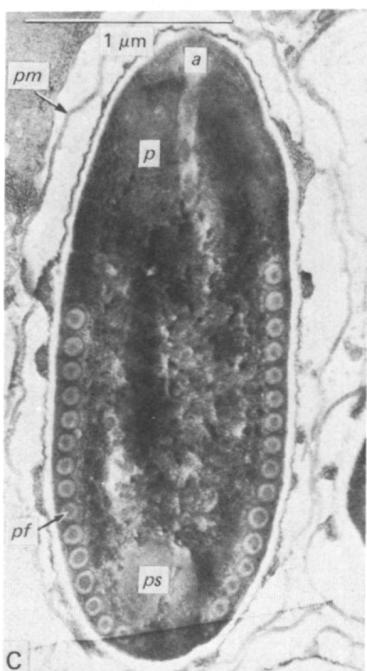
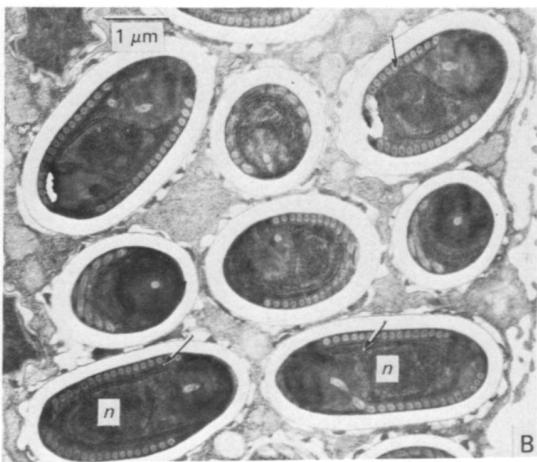
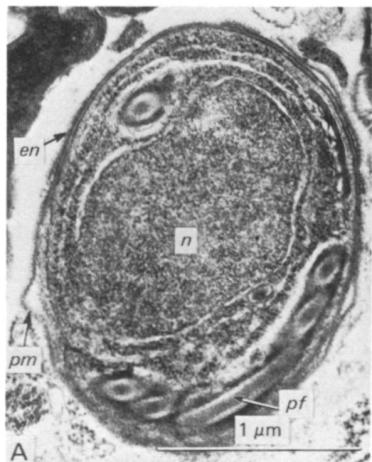
(Facing p. 342)



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