

Description of *Binucleospora elongata* gen. et sp. nov. (Microspora, Caudosporidae), a Microsporidian Parasite of Ostracods of the Genus *Candona* (Crustacea, Cyprididae) in Sweden

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SUMMARY

The microsporidium *Binucleospora elongata* n.g., n. sp., a parasite of ostracods of the genus *Candona* in Sweden, is described based on light microscopic and ultrastructural characteristics. Merogonial stages and sporonts are diplokaryotic. Rounded merogonial plasmodia produce at least 8 diplokaryotic merozoites by plasmotomy. Sporogonial plasmodia, with 16–32 diplokarya, are initially rounded, but the shape changes to ribbon-like prior to the release of sporoblasts. The close association between the nuclei breaks at the end of the sporogony. The fragile sporophorous vesicle breaks when the spores are mature. The binucleate spores are rod-shaped, slightly curved, and measure approximately $2 \times 5\text{--}6.8 \mu\text{m}$ in living condition. The exospore is two-layered with an internal double membrane-like layer and a cover of granular material. The isofilar polar filament is arranged in 11–14 irregular coils in the posterior half of the spore. The polaroplast has three regions: narrow, closely packed lamellae anteriorly, wider lamellae in the median region, and posterior tubuli. The nuclei are enveloped in multiple layers of smooth endoplasmic reticulum. The microsporidium is compared to the microsporidia of Ostracoda, and its possible relations to the genera *Octosporea*, *Pseudopleistophora* and *Perezia* are discussed. The new genus is provisionally included in the family Caudosporidae.

Abbreviations

AD	= anchoring disc
AL	= anterior polaroplast lamellae
C	= chromatin
D	= diplokaryon
DL	= double-layer of the exospore
E	= exospore
EN	= endospore
ER	= endoplasmic reticulum
GL	= granular layer of the exospore
HN	= host nucleus
M	= muscles
ML	= median polaroplast lamellae
N	= nucleus
P	= plasma membrane
PS	= polar sac
PT	= posterior polaroplast tubules
PV	= posterior vacuole
V	= sporophorous vesicle

Introduction

Ostracods are a group of minute crustaceans, where most species are about 1 mm long or even smaller. They live in a wide range of aquatic habitats. Although the ostracods constitute one of the largest classes of Crustacea, a phylum associated with numerous microsporidian parasites, surprisingly few microsporidia have been found in these hosts. The first microsporidian infection was reported from an ostracod in 1984 [14]. The next report originates from a find in 1989, when two new microsporidian species were found together in the same ostracod population in southern Sweden. One species was described as *Flabelliforma ostracodae* [1]. The other species, which is described herein, exhibits unique characteristics which make the establishment of a new genus necessary. A fourth microsporidian parasite of ostracods has recently been reported from Senegal [4].

Material and Methods

Ostracods of the genus *Candona* Baird, 1846 were collected in water-filled depressions on a meadow, on January 8th 1989. The meadow is situated close to the river Höje å, at Esarp in the south of Sweden and the depressions are only filled with water for a few weeks at the end of winter. The genus *Candona* contains numerous species and at least 30 of them are found in Scandinavia. Many of the species are inadequately described, and taxonomic confusion is great. It was not possible for us to have the host identified to species level.

Animals with anomalous white coloration were selected and punctured with a needle. The haemolymph was smeared on agar glasses according to the technique described by Hostounský and Zizka [6], and examined unfixed using phase contrast microscopy and dark field illumination. Specimens with proven infections were then cut in half.

One half was squashed between microscope slides. Slides were air dried lightly, fixed in Bouin-Duboscq-Brasil solution overnight, and stained using Giemsa solution or Heidenhain's iron haematoxylin [11]. Permanent preparations were mounted in D.P.X. (BDH Chemicals Ltd).

The other half was fixed for transmission electron microscopy using 2.5% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) at 4 °C for 27 h. After being washed in cacodylate buffer, the piece was postfixed in 2% (w/v) osmium tetroxide in cacodylate buffer at 4 °C for 1 h, washed in buffer, dehydrated in an ascending series of buffer-acetone solutions to absolute acetone, and embedded in epon. Sections were stained using uranyl acetate and lead citrate [10].

Results

Prevalence and Pathology

The population of ostracods harboured at least two microsporidian species: *Flabelliforma ostracodae* [1], and the species treated herein. Mixed infections were not found. Both microsporidia induced identical discolouration, and the prevalence of both was less than 1%.

The infected tissues were seen through the transparent cuticle as anomalous white coloration. The musculature, the frequent site of development for microsporidia of crustaceans, was undisturbed, and the infection was restricted to the adipose and connective tissues and to the haemocytes, which appeared filled with spores (Figs. 1–2).

Presporal Stages

All stages of the life cycle had paired nuclei. In merogonial stages and sporonts the nuclei were coupled as

diplokarya, while the nuclei of the late sporogonial stages and the spores were not closely associated.

As normally seen when a microsporidium is studied using field collected material, the merogonial part of the reproduction was nearly finished. A few rounded merogonial plasmodia, measuring up to 24 µm in diameter, were found in the squash preparations (Fig. 3). The mature plasmodium contained at least 8 diplokarya, which measured approximately 3 µm perpendicularly to the line of apposition. There was also a small number of merogonial plasmodia and merozoites in the ultrathin sections. They were limited by a plasma membrane, an approximately 8 nm thick unit membrane without external reinforcements. They had large and irregularly shaped diplokarya, and the cytoplasm contained numerous free ribosomes (Fig. 4). Rosette-like dividing stages were not observed, and plasmotomy appeared to be the normal method of merozoite production.

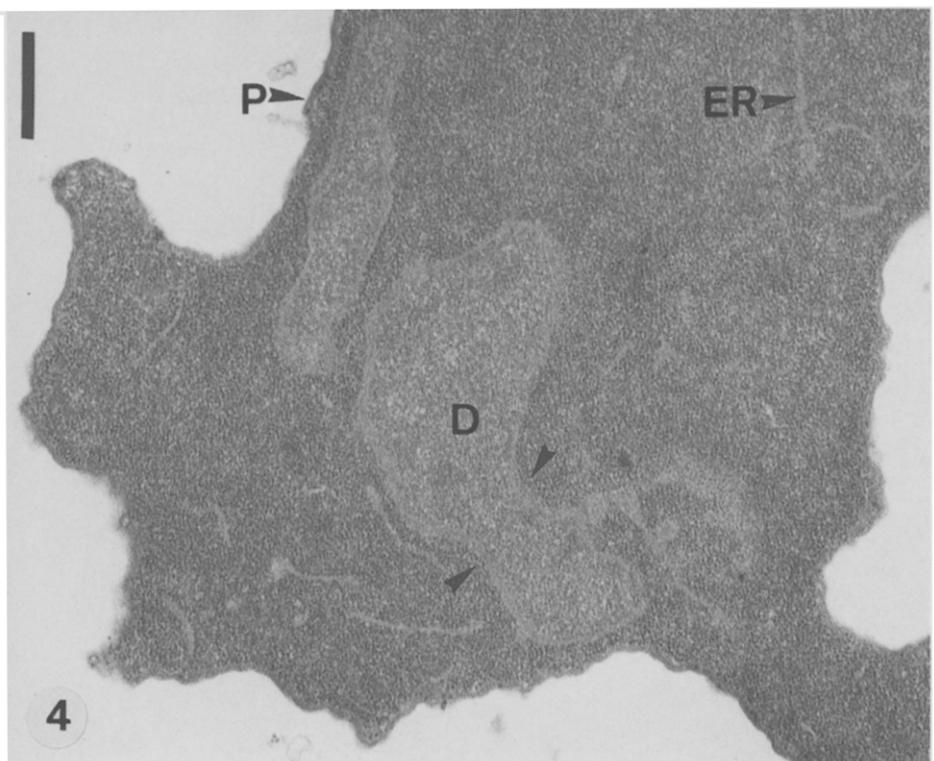
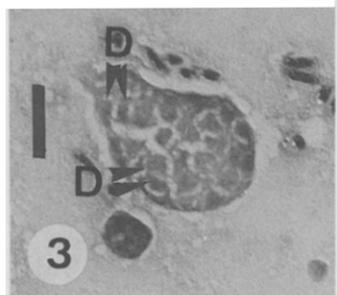
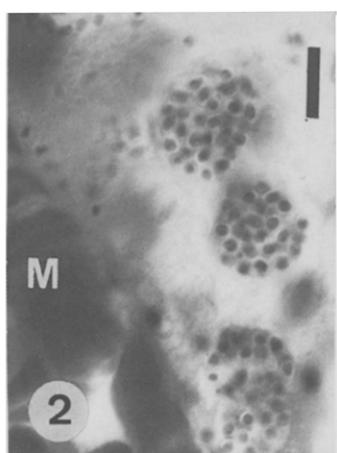
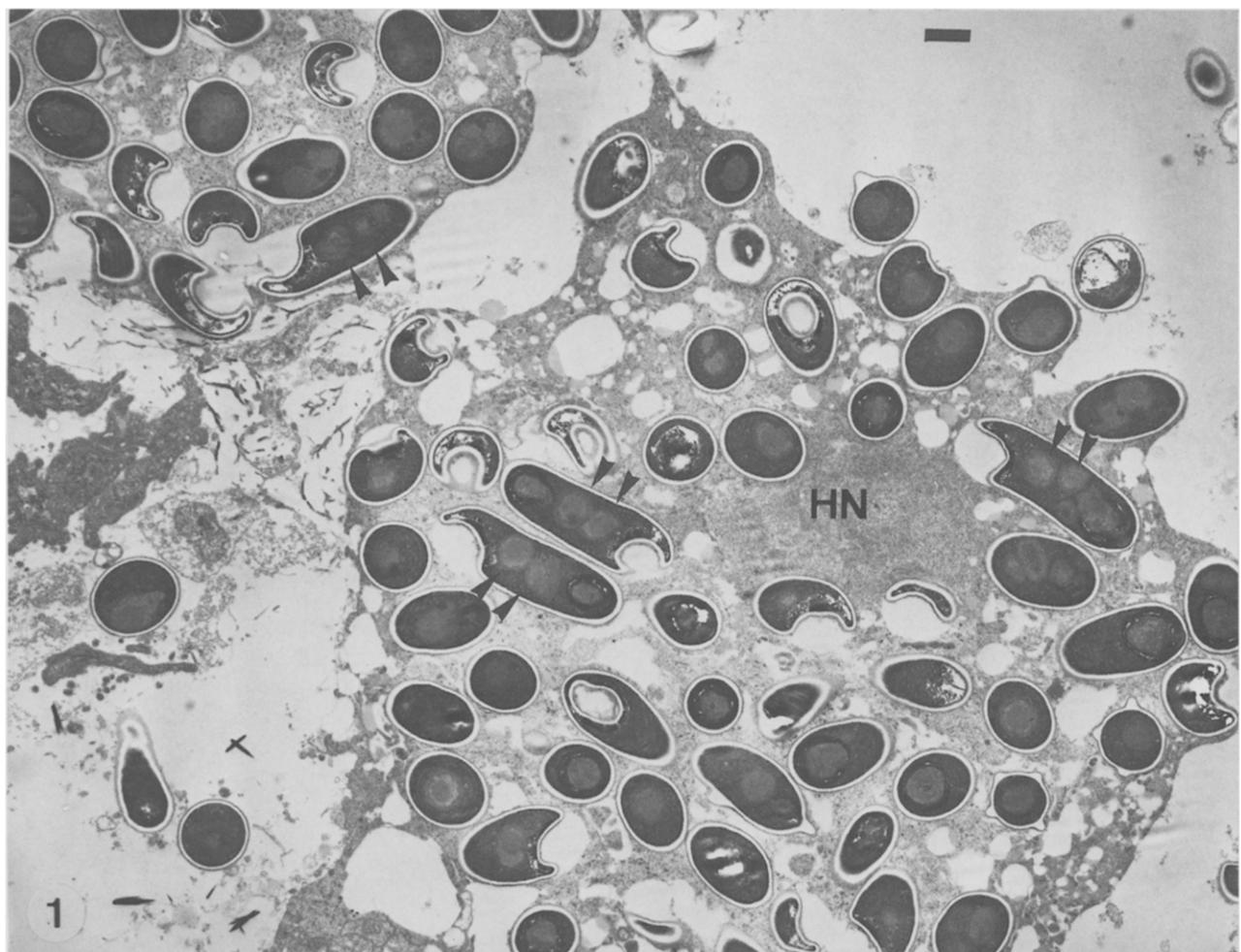
The last, or possibly only, generation of merozoites matured to sporonts, which grew to more or less rounded plasmodia, containing 16–32 diplokarya (Fig. 5 a–c). Rounded mature sporogonial plasmodia were of approximately the same size as the merogonial plasmodia. They were distinguished by their distinctly smaller diplokarya, approximately 2 µm wide. During division of the sporogonial plasmodia, ribbon-like formations of binucleate sections of cytoplasm were formed prior to their separation as sporoblasts (Figs. 10–11).

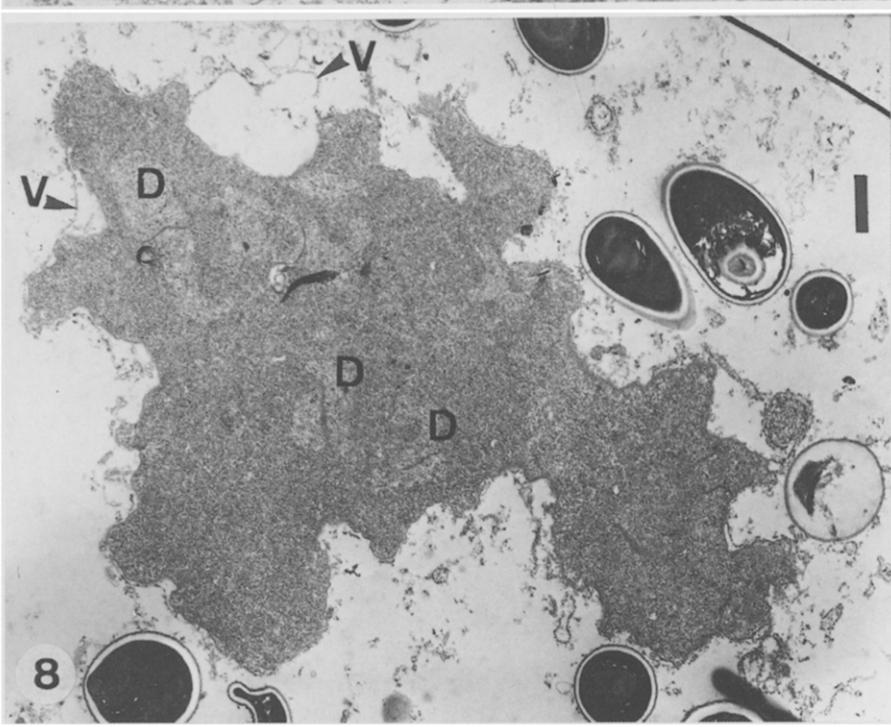
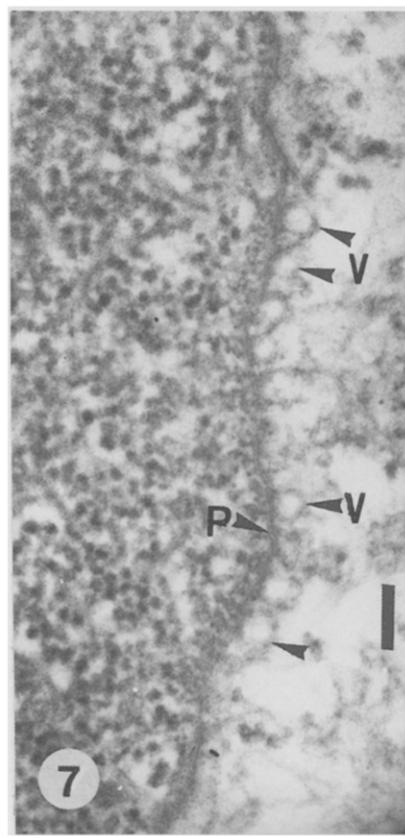
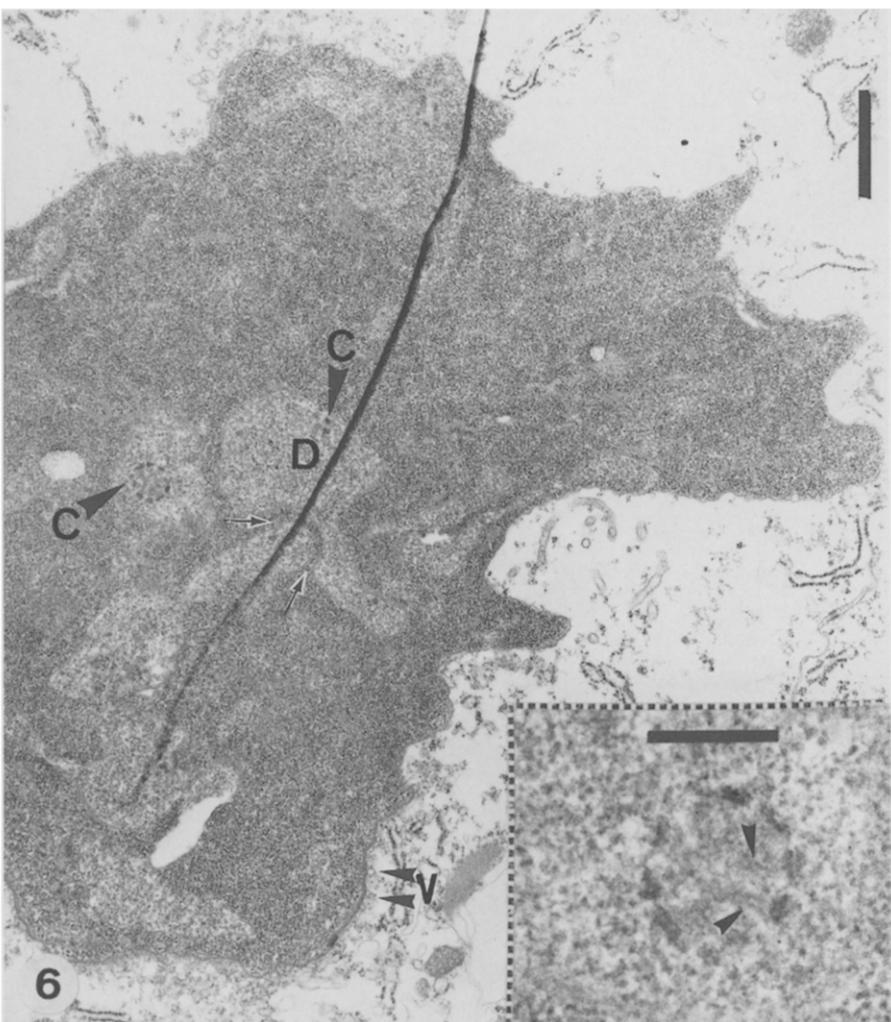
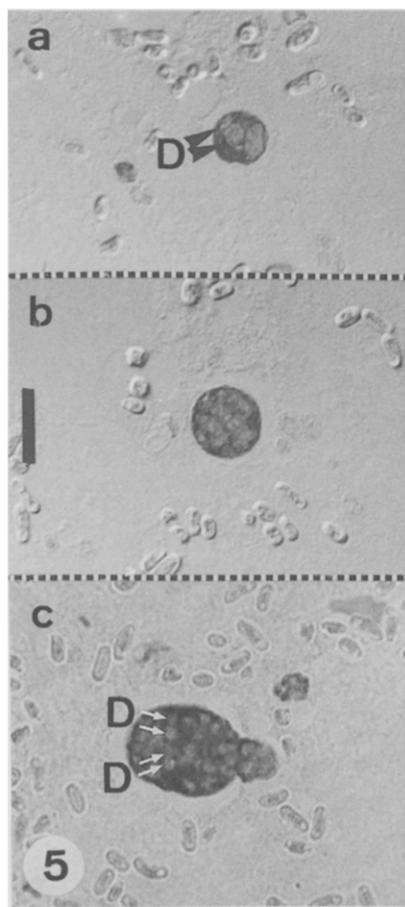
The cytoplasm of merogonial and sporogonial plasmodia had numerous free ribosomes. Traces of smooth endoplasmic reticulum were visible in merogonial plasmodia (Fig. 4), and the amount of smooth reticulum increased towards the end of the sporogony (Fig. 9). There were no distinct signs of rough endoplasmic reticulum at any stage of the sporogony.

Simultaneously with the nuclear divisions, blisters of electron dense material, secreted on the plasma membrane, were released as primordia of a sporophorous vesicle (Figs. 6–7). The separation of the vesicle from the plasmodium was practically complete before the thick wall of the sporogonial plasmodium was initiated (Fig. 8). This layer was also formed by uniform, electron dense material, secreted on the surface of the plasma membrane. The production of the wall material was apparently a rapid process, and the thick wall was completed almost simultaneously over the whole plasmodium (Fig. 9).

Rounded configurations of electron-dense spots were observed in nuclei of sporogonial plasmodia (Fig. 6). They were apparently chromosomes sec-

Figs. 1–4. Pathogenicity and early development of *Binucleospora elongata* gen. et sp. nov. – Figs. 1–2. Ultrastructural (1) and light microscopic (2) appearance of host cells, probably haemocytes, filled with microsporidia (arrowheads point at the two separated nuclei). – Fig. 3. Merogonial plasmodium. – Fig. 4. Ultrastructural detail of a thin-walled merogonial plasmodium with numerous free ribosomes and traces of endoplasmic reticulum (arrowheads indicate the border between the components of the diplokaryon). Scale bars: Fig. 1 = 1 µm, Figs. 2–3 = 10 µm, Fig. 4 = 0.5 µm. Fig. 2. Haematoxylin, Fig. 3. Giemsa staining.





tioned through the mitotic spindle. Until the sporogonial plasmodium became ribbon-like, prior to the release of sporoblasts, the two components of the diplokaryon were uniformly electron-dense and in close contact with each other (Figs. 8–9). Simultaneously with the change of shape of the plasmodium, the nuclei of the diplokarya became more elongated and the density of the nucleoplasm decreased (Figs. 10–12). Later, in sporoblasts and spores, a sheath of numerous layers of membranes was built around each nucleus, which separated the two components of the original diplokaryon from each other (Figs. 1, 15, and 20).

The mature spores were binucleate and originated within the fragile sporophorous vesicle produced by the sporont (Fig. 6). Reductive division was not observed.

The Mature Spore

Mature spores were rod-shaped and slightly curved. Living spores (Fig. 13) measured $5\text{--}6.8 \times \text{c. }2 \mu\text{m}$, fixed and stained spores (Fig. 14) $3.7\text{--}5.8 \times \text{c. }2 \mu\text{m}$. In living spores the great posterior vacuole was distinctly seen, and the anchoring apparatus at the opposite pole appeared as a dark spot (Fig. 13). The two nuclei were distinct in stained spores (Fig. 14).

The spore wall measured 85–105 nm. It had the usual three components: a 14–17 nm thick electron-dense exospore, a lucent endospore, with considerably reduced thickness above the anchoring disc, and an approximately 8 nm thick plasma membrane (Figs. 15, 18). The exospore had an internal double-layer, approximately of the same thickness as the plasma membrane, and a slightly thicker external cover of granular, moderately electron dense material (Fig. 18).

The polar filament was isofilar, and the 11–14 coils were arranged in an irregular single row in the posterior half of the spore (Fig. 15). However, sometimes some of the median coils were displaced and arranged in double rows (Figs. 15, 20). The angle of tilt of the anterior coil to the long axis of the spore was approximately 60° . The diameter of the filament was 106–128 nm, but it increased slightly in the proximity of the anchoring disc. Transverse sections of the polar filament revealed layers of different electron density and thickness (Fig. 17: a–d). The center (a) was approximately 70 nm thick and moderately electron dense. In direction outwards followed: an about 10 nm thick, electron dense internal ring (b); an approximately 5 nm thick, moderately electron dense external

ring (c); and an approximately 6 nm thick unit membrane cover (d).

Perfect longitudinal sections through the mid-line of the anterior part of the spores were not obtained. The detailed construction of the anchoring disc is therefore unknown. The largest sectioned disc measured approximately 212 nm in diameter (Fig. 16).

The polaroplast occupied the anterior third of the spore, surrounding the straight part of the polar filament. It was a slightly oval body, and it was divided into three regions, in which the compartments were formed by 6 nm thick unit membranes, continuous with the membrane that covered the polar filament (Figs. 15, 16). The anterior bell-shaped region covered approximately the anterior 3/4 of the two posterior parts of the polaroplast. It consisted of extremely electron dense, closely packed lamellae, spaced with a period of 4–5 nm. The following median region had moderately electron dense, 10–18 nm wide lamellae, and the final section was composed of tubuli measuring 75–100 nm in diameter. The polar sac was filled with moderately electron dense material. It enclosed the anchoring disc, and the peripheral fold extended backwards to enclose the anterior half of the polaroplast (Fig. 16).

The nuclei were situated in the center of the spore. The largest sectioned nucleus measured 780 nm in diameter. The nuclei had a moderately electron dense center and a less dense periphery. Both nuclei were surrounded by conspicuous layers of smooth endoplasmic reticulum, which prevented close contact between them (Fig. 15). The membrane folds were about 20 nm apart, and they looked exactly like the membranes of the nuclear envelope. Similar concentric membrane folds were sometimes seen in the cytoplasm close to the nuclei (Fig. 20). In addition, small rounded structures, possibly projections of the membrane folds, were found in the posterior half of the spore (Figs. 15, 20). The cytoplasm was densely packed with free ribosomes, but there were no signs of polyribosomes. The membrane of the posterior vacuole collapsed following fixation, and the vacuole area was seen as an irregular electron-lucent cavity with peripheral membrane fragments (Fig. 15).

The Sporophorous Vesicle

The sporophorous vesicle originated with blister formation (Fig. 7), and at the end of the sporogony, it followed the outline of the enclosed sporoblasts (Fig. 12). Occasionally mature spores were enveloped by the ves-

◀ Figs. 5–8. Early sporogony of *B. elongata*. – Fig. 5 a–c. Three stages in the development of the sporogonial plasmodium; signs of beginning plasmotomy are visible in c. – Fig. 6. Ultrathinly sectioned sporogonial plasmodium with initiation of the sporophorous vesicle (arrows point at the borders between the components of the diplokaryon); the circularly arranged intranuclear structure is probably a transversely sectioned mitotic spindle (inserted detail shows a magnification, arrowheads indicate spindle tubules). – Fig. 7. Magnified surface detail of the same cell showing the blister-like vesicle primordia. – Fig. 8. Sporogonial plasmodium with diplokarya and developing sporophorous vesicle. Scale bars: Fig. 5 (with common bar on b) = 10 μm , Figs. 6 and 8 = 1 μm , inset on 6 = 300 nm, Fig. 7 = 100 nm. Fig. 5. Giemsa staining.

icle (Fig. 19). The envelope was about 10 nm thick and uniformly electron dense. Thin fibrils connected it to the surface layer of the sporogonial plasmodium, sporoblasts and newly formed spores. The vesicle was very delicate, and disappeared while the parasites were still within the host tissue.

Discussion

Cytology

The microsporidium described herein conforms in most respects with the normal cytology of microsporidia and only three details of the cytology need comment: the membranous envelopes separating the nuclei of the spore, the unusual construction of the polaroplast, and the absence of polyribosomes in the spore.

Microsporidia have nuclei of the normal eukaryotic type, where the nuclear envelope is composed of two unit membranes. Normally there are no additional membranes surrounding the nuclei, like the arrangement seen in this species (Figs. 15, 20). However, additional membranes are found in at least one more microsporidium: in the spores of the unnamed *Amblyospora* species, described by Darwish and Canning, where the single nucleus is enveloped by concentric layers of the endoplasmic reticulum [3]. However, to our knowledge it is unique that the two components of a diplokaryon are separated by membrane stacks.

Most microsporidia have polaroplasts with two lamellar regions: anteriorly closely packed and narrow, posteriorly wider and less regularly arranged. A polaroplast with two lamellar regions followed by a final section with tubules has previously been described for *Agglomerata sidae* [7]. However, the polaroplast of *A. sidae* differs from the polaroplast of the species described herein by the arrangements of the lamellae: the wide lamellae are found in the anterior zone while the narrow lamellae lie in the median zone.

Strands of polyribosomes are normally a characteristic component of the newly produced but fully mature microsporidian spore, but they are reduced during the ageing of the spore. Polyribosomes occur in the proximity of the nucleus, the polaroplast, and the spore wall, as seen in *Octosporea muscaedomesticae* (Fig. 7 in [8]). The mature spores of the species described herein have numerous ribosomes, but polyribosomes were never observed (Figs. 15, 20). As the spores

were studied in animals collected alive, the absence of polyribosomes could not be explained as a phenomenon of ageing.

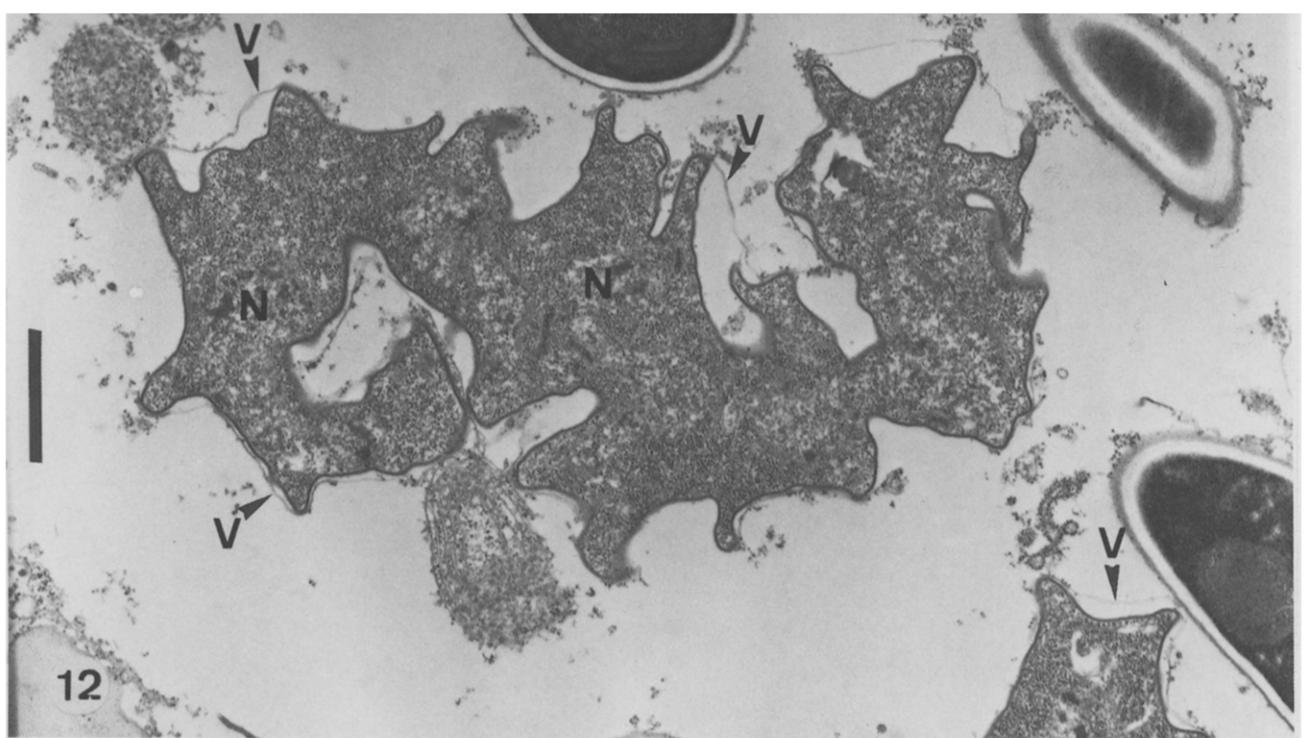
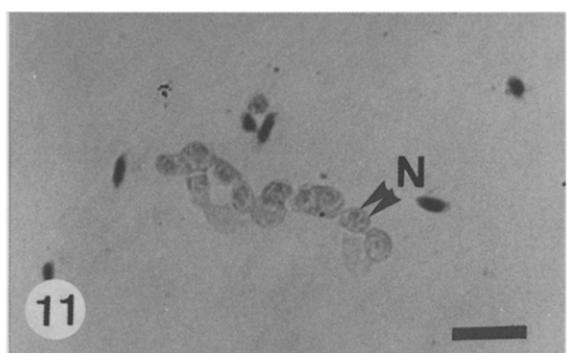
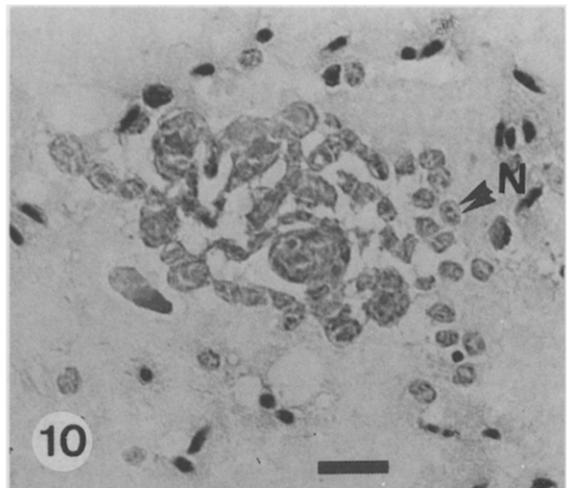
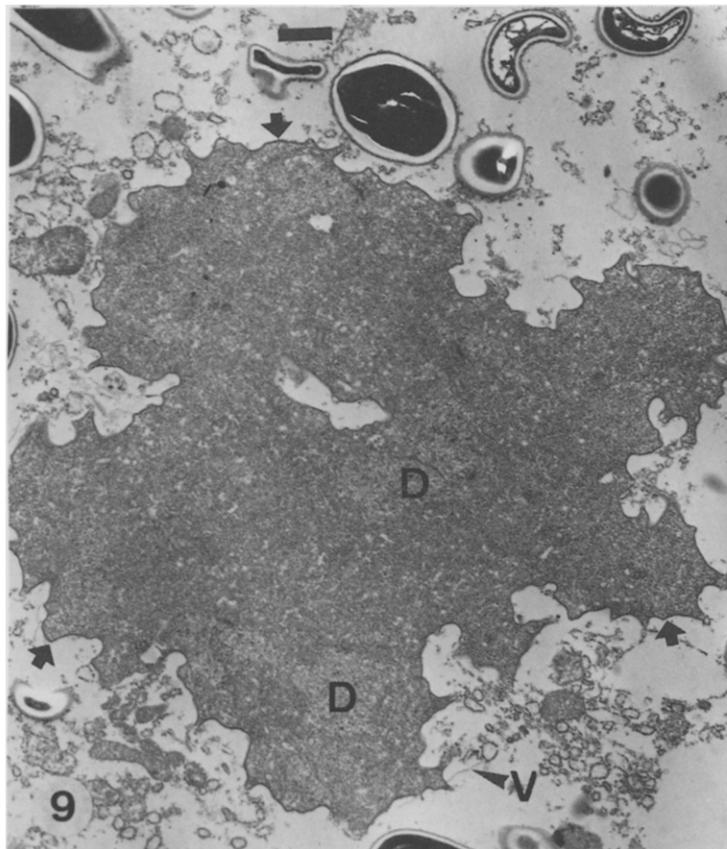
Taxonomy

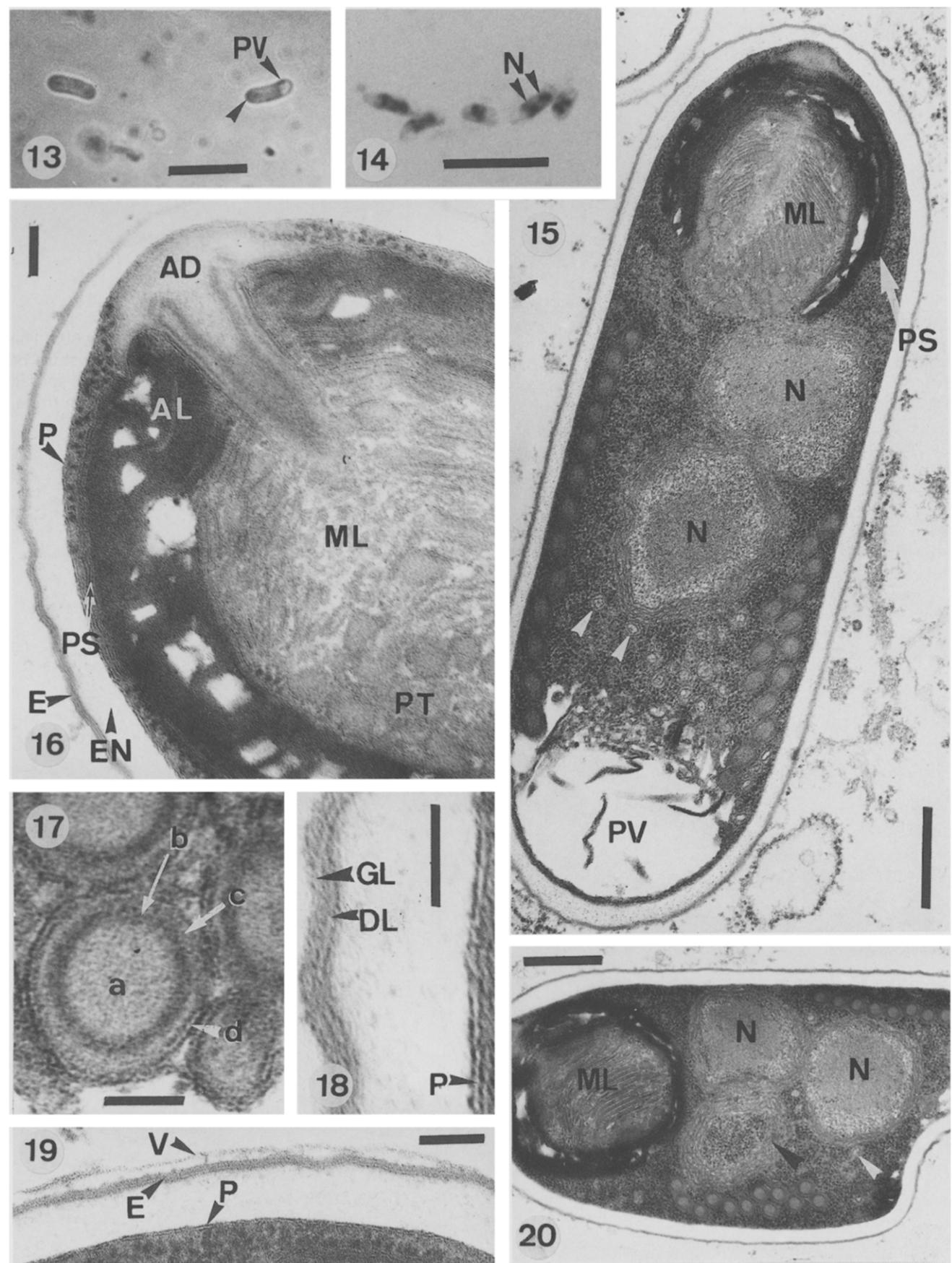
There are only three microsporidian species previously reported from ostracods: *Vavraia cycloctypris* [14], *Flabelliforma ostracodae* [1], and *Nosema stenocypris* [4]. The first two have isolated nuclei in all stages of their life cycles, and are thereby clearly different from the species treated herein, which is basically diplokaryotic: meronts and sporonts are truly diplokaryotic, but at the end of the sporogony the close association between the two components of the diplokaryon is broken. Sporoblasts and spores have two nuclei separated by stacks of endoplasmic reticulum (Figs. 1, 15, 20). The only description of *N. stenocypris* known to us is a brief congress abstract without illustrations [4], which makes a detailed comparison impossible. However, *N. stenocypris* is diplokaryotic throughout its life cycle. It is disporoblastic, producing spores with lamellar and vesicular polaroplast. The spores are slightly smaller, and the host is a different species of ostracod from a different continent. All these features indicate that also *N. stenocypris* is different from the species treated herein.

A diplokaryotic life cycle, combined with the production of elongated spores in sporophorous vesicles, is characteristic for the genera *Octosporea* and *Pseudopleistophora*. The *Octosporea* species, which are parasites of dipterans and crustaceans [2, 5, 8], are octosporoblastic, and the eight spores are produced by rosette-like budding. The polaroplast of the type species, *Octosporea muscaedomesticae*, is divided into two parts: an anterior part, which originates from sac-like structures but is described as granular in mature spores, and a posterior lamellar part [8]. The species studied by us produces 16–32 sporoblasts from a ribbon-like plasmodium, and the polaroplast is divided into three parts: two anterior lamellar parts and a posterior tubular region (Fig. 16). A further difference is the absence of polyribosomes in the spores of the species described herein, while characteristic layers of polyribosomes surround the diplokaryon of sporulated *O. muscaedomesticae*.

Pseudopleistophora szollosi, the only species of the genus, was found in eggs of a polychaete [12]. It is a polysporous species with characteristic sporogony: the early sporogonial plasmodium of *P. szollosi* interdigitates with the host cytoplasm, and the sporoblasts are formed by vacuolation of the sporogonial plasmo-

Figs. 9–12. Late sporogony of *B. elongata*. – Fig. 9. Lobed diplokaryotic sporogonial plasmodium with thick sporont wall (arrows). – Fig. 10. Fragmentation of the sporogonial plasmodium into sporoblasts; the components of the diplokaryon separate at this stage. – Fig. 11. Chain of sporoblasts. – Fig. 12. Filiform sporogonial plasmodium, prior to the release of sporoblasts, enclosed in a sporophorous vesicle. Scale bars: Figs. 9 and 12 = 1 µm, Figs. 10 and 11 = 10 µm. Figs. 10–11. Giemsa staining.





dium. Further, the sporophorous vesicle of *Pseudopleistophora* is a considerably thicker structure than the normal sporophorous vesicle of microsporidia, and it is prominently different from the fragile vesicle of the Swedish microsporidium (Figs. 8, 12, 19). It is apparent that neither *Octosporea* nor *Pseudopleistophora* can accomodate the microsporidium described herein.

A life cycle characterized by diplokarya in the early development, release of numerous sporoblasts from a ribbon-like plasmodium, and by elongated mature spores suggests a third possible genus: *Perezia*. However, the sporoblasts and spores of *Perezia lankesteriae*, the type species, which lives as a hyperparasite in gregarines of *Ciona intestinalis* (Tunicata), are uninucleate, and there is no production of sporophorous vesicles in *Perezia* [9]. So, *Perezia* can also be excluded, even if the genus previously contains one parasite of microcrustaceans: *Perezia diaphanosomae* – a species with a well known ultrastructure and obviously a distinct member of the genus [13].

Since none of the established genera can accomodate this new species, we see no alternative to creating a new genus. However, we hesitate to establish a new family. The life cycle is basically identical to the development expressed by the *Octosporea* and *Caudospora* species, and we therefore believe that the best solution will be to place the new genus preliminarily in the family Caudosporidae Weiser, 1958.

Description

Binucleospora gen. nov.

Diagnosis. Merogony diplokaryotic. Rounded merogonial plasmodium produces numerous merozoites by plasmotomy. A diplokaryotic sporont initially yields a rounded plasmodium with numerous diplokarya, which later changes shape to become ribbon-like. Simultaneously with the change of shape, the close association between the nuclei of the diplokarya breaks. Reductive division not observed, probably lacking. Binucleate sporoblasts are formed within a sporophorous vesicle produced by the sporont. Spores are rod-shaped and binucleate. Exospore two-layered with an internal double layer and a granular surface layer. Polaroplast divided into three regions: an anterior part with densely arranged, thin lamellae, a median part

with wider lamellae, and a posterior part with tubuli. Polar filament isofilar.

B. elongata sp. nov.

Merogony. As for genus, at least 8 merozoites are formed. The number of cycles of merogony unknown.

Sporogony. As for the genus; 16–32 sporoblasts are formed.

Spores. Rod-shaped and slightly curved. Unfixed spores measure $5.4\text{--}7.2 \times 2.0\text{--}2.3 \mu\text{m}$, fixed and stained spores $4.6\text{--}8.3 \times 1.8\text{--}2.8 \mu\text{m}$. Spore wall is 85–105 nm thick, with 14–17 nm thick exospore. Polar filament is 106–128 nm wide with 11–14 coils, arranged in a single irregular row (median coils sometimes forming a double row), in the posterior half of the spore. The angle of tilt is approximately 60° . The polaroplast is oval and occupies the anterior third of the spore. Two nuclei in the mid-region of the spore.

Sporophorous vesicle. A fragile, about 10 nm thick, electron-dense layer, which is disrupted while still within the host cell. Narrow fibrils connect the envelope with the wall of the sporogonial plasmodium.

Host tissue involved. Adipose and connective tissue, and haemocytes.

Type host. An unidentified species of the genus *Canadona* Baird, 1846 (Ostracoda, Cyprididae).

Type locality. Small temporary water bodies on a meadow close to the river Höje å, Esarp, Scania in the south of Sweden.

Types. Syntypes on slides No. 890108-R-(1–3).

Deposition of types. In the International Protozoan Type Slide Collection at Smithsonian Institution, Washington, D.C., USA (USNM 43219) and in the collection of the senior author.

Acknowledgements

The authors are greatly indebted to Ms. Lina Gefors, Ms. Birgitta Klefbohm and Ms. Inger Norling, Department of Zoology, University of Lund, for skilful technical assistance. The investigation was supported by research grants from the Swedish Natural Science Research Council and of Helge Ax:son Johnson's Foundation.

◀ Figs. 13–20. Mature spores of *B. elongata*. – Fig. 13. Living spores with anchoring apparatus (arrowhead) and posterior vacuole visible. – Fig. 14. Stained spores exhibiting the two nuclei. – Fig. 15. Longitudinally sectioned spore revealing the characteristic organelles; some of the median filament coils are displaced as an internal row of coils (white arrowheads indicate projections of membrane folds). – Fig. 16. Anterior pole of the spore exhibiting the layers of the spore wall and the polaroplast region. – Fig. 17. Transversally sectioned polar filament coil; the layers are indicated a–d in direction outwards. – Fig. 18. Detail of the spore wall showing the layers of the exospore. – Fig. 19. Surface of a spore enclosed in a sporophorous vesicle. – Fig. 20. Mature spore with concentrically arranged membrane folds around the nuclei and in the cytoplasm (arrowheads). Scale bars: Figs. 13 and 14 = 10 µm, Figs. 15 and 20 = 0.5 µm, Figs. 16 and 19 = 100 nm, Figs. 17 and 18 = 50 nm. Fig. 14. Giemsa staining.

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Key words: *Binucleospora elongata* gen. et sp. nov. – Microspora – Ultrastructure – Taxonomy – Ostracoda

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