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Microsporidium novacastriensis n. sp., a Microsporidian Parasite of the Grey Field Slug, *Deroceras reticulatum*[1,2]

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ABSTRACT. A new species of microsporidium (phylum Microspora), *Microsporidium novacastriensis* n. sp., from the grey field slug, *Deroceras reticulatum*, is described on the basis of light and electron microscope studies. Meronts are spherical at first, then become irregular as nuclear number increases. Sporonts are tubular or ribbon-like and divide unevenly to produce sporoblasts and then spores of varying lengths. Sporogonial stages are enclosed in a vesicle by a subpersistent membrane of uncertain origin. Fresh spores measure 3.5 by 2.08 μm and are produced in clusters of 12 to 120. The parasite infects only the intestinal epithelium of the slug. The new species is compared to microsporidia of other gastropod molluscs and to other microsporidia of similar developmental pattern and morphology.

A microsporidian parasite was commonly observed in the grey field slug, *Deroceras reticulatum* (Müller, 1774), during a study to find and evaluate possible biological control agents of slugs. Identification of the parasite was necessary before any further detailed study. There is only a single earlier record of microsporidia in slugs, a multisporous species from *D. reticulatum* assigned to the genus *Pleistophora* Gurley, 1893 by Brooks (1), but he made only preliminary observations and did not name the species. The genus *Pleistophora* has recently been revised and split into several new genera (4), and the basic system of microsporidian classification is currently under review. The aim of this study is to determine the life cycle and morphology of the parasite and to present a formal description of the species.

MATERIALS AND METHODS

The life cycle was studied in slugs collected from the field. Smears of healthy and infected tissues were studied either alive by phase-contrast illumination or after fixation in methanol and staining in Giemsa. Spore nuclei were stained by hydrolysis of stained smears in 1 N HCl at 60°C for 1 min followed by restaining in Giemsa for 45 min. For sectioning, whole slugs or pieces of infected tissue were fixed in alcoholic Bouin's fluid, dehydrated in ethanol, and embedded in Paraplast wax (melting pt., 56–57°C) under vacuum. Sections were cut at 6 μm and stained with Giemsa or Heidenhain's hematoxylin and eosin. For transmission electron microscopy, infected tissues were fixed in 2.5% (v/v) glutaraldehyde in Na cacodylate buffer, pH 7.2, and, after washing in the buffer, were postfixed in buffered osmium tetroxide. The material was embedded in Emix low viscosity resin (Emscope), and ultra-thin sections were cut with a glass knife. Sections were stained with uranyl acetate and lead citrate before examination with a Kratos Cora 60 kV electron microscope. Measurements of the stages of the microsporidium were made either with a filar micrometer or by the photographic method. All attempts to induce the spores to hatch artificially failed; these included the wet-dry method, treatment with alkali and acid, and pressure. Therefore the filament was measured from hatched spores after passage through the gut.

RESULTS

Light microscopy. (All measurements are in μm unless otherwise specified and all appropriate measurements of developmental stages are means.) The earliest stage of the life cycle recognized was the spherical uninucleate meront (Fig. 1) diameter 6 (±0.25, n = 35), binucleate meronts were also observed of diameter 8 (±0.26, n = 42) (Fig. 2). Meronts continued nuclear division forming irregular plasmodia, measuring up to 20 by 15, containing up to 24 nuclei (Fig. 3). At this stage the meront divided to form tubular sporonts (Fig. 4) with up to 32 nuclei. Sporonts measured up to 130 long. Most sporont ribbons contained 2–16 nuclei (Fig. 5) and were wrapped around themselves to form a bundle (Fig. 6). Smearing caused the bundle to break apart revealing its contents (Fig. 7). As sporonts matured, nuclei became more evenly spaced along their lengths (Fig. 8) and the formation of sporoblasts began. The sporont constricted around each nucleus, producing a loosely connected chain of sporoblasts (Figs. 9, 10) still wrapped in a tight bundle. Complete separation occurred and the sporoblasts matured separately into spores. The spores were produced in clusters containing between 12 and 120 spores (Fig. 12). Spore dimensions (±standard error) were 3.5 (±0.1, n = 100); however, uneven division of the sporont ribbon produced spores of varying lengths and long spores up to 7 by 2.1 were regularly observed (Fig. 11). The naturally extruded polar filament measured 45 to 55 (n = 30).

Transmission electron microscopy. Few meronts were observed and no multinucleate meronts were seen. Uninucleate meronts were seen but were most unusual in structure (Fig. 13). A central mass of constricted chromosomes formed a nucleolar-like structure at the center of the meront. A double membrane that appeared to be studded on both inner and outer layers with ribosome-like material surrounded the perimeter of the meront (Fig. 13, inset). Sporonts were tubular, corresponding to the shape observed with light microscopy, limited by a thick electron-dense cell wall, and contained nuclei and extensive membrane complexes (Fig. 14). Although sections of sporonts containing more than four nuclei were not observed, the sporont ribbon was almost certainly longer but was coiled and only a portion lay in the plane of any one section. Sporonts lay in a vesicle bounded by a weak sub-persistent membrane of unknown origin, which appeared to be similar in structure and in close contact with host endoplasmic reticulum. Several sporonts appeared to lie in one vesicle; this was because one sporont coiled in and out of the section plane producing several apparently unconnected sporonts. Sometimes the vesicle membrane fragmented, connecting several vesicles and developmental stages together (Fig. 15). Division of the sporonts into sporoblasts was not observed. Nuclei were separate in all stages and all divisions were presumed to be mitotic as no evidence of synaptonemal complexes were found.

The next stages observed were the mature spores, and as many as 64 spores were observed in one plane of a vesicle. The membrane surrounding large vesicles was rarely complete. Internal detail of the spores was not well preserved due to poor fixation, but the important features could be seen. Each spore was enclosed by a thin exospore layer and a thick electron-lucent endospore. The polar filament had 4–9 coils (Fig. 17) arranged in one or two rows, the minimum angle of tilt (3) measured was 30. The membrane complex of the polarplast was seen, with

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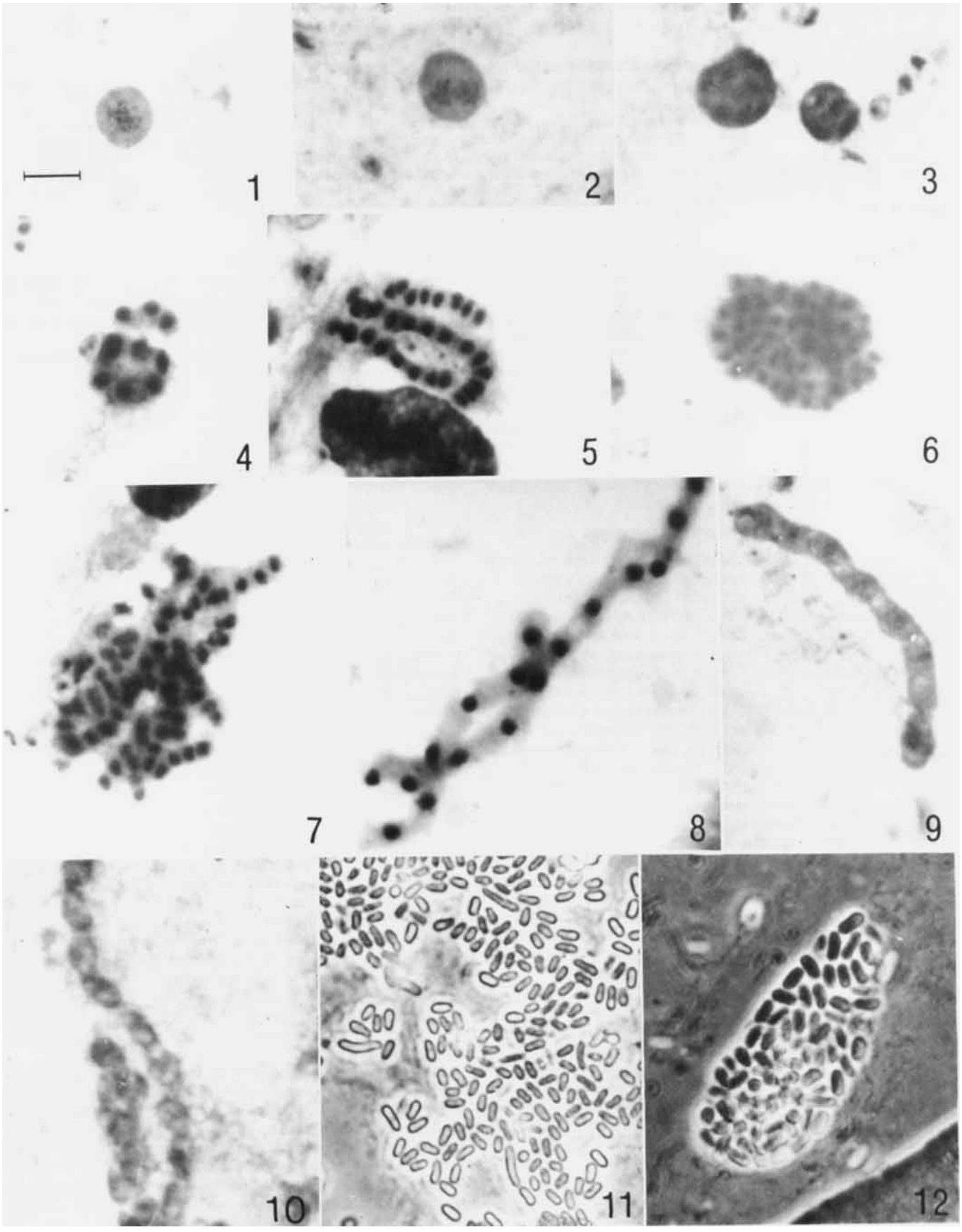
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the polar cap at the anterior end of the polarplast lamellae (Fig. 16).

Pathology. The parasite was found in 2–100% of populations of *D. reticulatum* throughout Britain. Infected individuals tend to be quiescent, rarely moving or feeding and the parasite eventually killed the host. All body tissues were examined in stained sections of whole slugs, but the only tissues attacked were the intestinal epithelial cells between the stomach and rectal ceca. Parasitized tissue appeared speckled and milky rather than the translucent grey of healthy gut. Infected cells became completely filled with spores and in severe infections the gut became fragile and might completely break down. Spores were passed out in the feces and infected a new host when ingested. It was noted in laboratory cultures that young slugs reared from eggs became infested by the parasite despite their environment being free from spores. Washing slug eggs greatly reduced subsequent infection and since no stages of the parasite were observed in Giemsa-stained smears of washed eggs, we concluded that infection was being transmitted to young slugs by ingestion of spores contaminating the surface of the egg coat. Newly emerged slugs were frequently observed feeding on egg coats. The parasite attacks a section of the host gut concerned with secretion of mucus and absorption of assimilates: host death appears to result from a combination of starvation and gut damage allowing entry of contaminants into the hemocoel of the host.

DISCUSSION

Microsporidium novacastriensis exhibits an unusual morphology and development compared to many microsporidia already described. The early stages of development, particularly meronts, were infrequently observed because mature infections of the parasite from field-collected slugs were used in this study; however, comparisons between non-infected and infected tissue suggested that the structures shown in Fig. 13 were meronts. Meronts exhibited unusual morphology. The chromosomes were present in a tight bundle, forming a nucleolar-like structure, previously unobserved in other microsporidia. A double membrane studded with ribosome-like particles lay at the perimeter of the meront. At first this was thought to be the nuclear envelope, but its structure is atypical of animal cell nuclei. Therefore, it seems likely that the nuclear envelope is absent in the meronts observed and the double membrane originates from the endoplasmic reticulum or cell membrane of the parasite. The membrane may be beginning to secrete the thick cell wall of the sporonts.

Sporonts were long and ribbon-like, enclosed by a thin vesicle membrane, which was incomplete or broken. The structure of the vesicle membrane was very different from the thick persistent membranes of *Pleistophora typicalis* (Gurley, 1893) and *Vavraia culicis* (Weiser, 1947) (4), which are known to be of parasite origin, but closely resembles those observed in *Pleistophora* sp. (8) considered to be of host rather than parasite origin. The simple structure, similarity, and intimacy of the vesicle membrane of the slug parasite with host endoplasmic reticulum suggests that it too is derived from host rather than parasite tissue; however, more detailed studies are needed, perhaps using fluorescent or radioactive labels, to identify the origin of the membrane of the vesicle surrounding the microsporidium in the slug.

The ribbon-like appearance of the sporonts is also an unusual feature of the slug microsporidium but has been found in other microsporidia. Very similar sporonts are observed in *Orthosoma operophterae* Canning et al., 1983 (5), but this microsporidium differs from the slug parasite in two important ways: *O. operophterae* possesses diplokarya in its early stages of development and the parasite lies free in the host tissue not enclosed by any membrane. *Pleistophora* sp., a parasite of the larch sawfly (8), produces tubular sporonts very similar to the slug microsporidium and the sporonts are enclosed by a similar vesicle membrane though the early sporont of *Pleistophora* sp. shows nuclei in diplokaryon arrangement and differs in this character from the slug parasite. The most similar microsporidium known to the authors is a parasite of the argentine stem weevil (*Listronotus bonariensis* Küshel, 1955) in New Zealand, which has a morphology and development almost identical to the slug parasite (Malone, pers. commun.).

Microsporidia have been described from several gastropod molluscs (Table I) but only three species are multisporous. *Steinhausia brachynema* Richards, 1973 occurs in *Biomphalaria glabrata* (Say) (9) and attacks the gut epithelium as does the slug parasite but the other two differ because sporulation and spore production of *S. brachynema* is from a multinucleate plasmodium, producing spherical spores. *Steinhausia* belongs to an order of microsporidia, Chytridiopsida, with an unusual spore structure differing greatly from the classical forms of the order Microsporida. *Pleistophora husseyae* (Michelson, 1961), which occurs in planorbid snails (7), differs from the slug microsporidium on several points: the slug microsporidium infects only the intestinal epithelium whereas *P. husseyae* attacks all tissues: spore formation of *P. husseyae* occurs by division of a multinucleate sporogonial plasmodium and sporont ribbons were not mentioned; spores of *P. husseyae* are larger than those of the slug microsporidium. The only record of microsporidia in slugs is of a *Pleistophora* species described by Brooks (1) in the USA and assigned to the genus on limited observations. When we compared our observations and slide material with his, they proved to be the same species.

As more microsporidia are found and described, it is becom-

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Figs. 1–12. *Microsporidium novacastriensis.* **1–10**, Giemsa-stained smears; **11**, **12**, fresh material viewed with phase contrast. **1–10**, **12**, scale bar = 6 μm; **11**, scale bar = 10 μm. **1**. Uninucleate meront. **2**. Binucleate meront. **3**. Multinucleate meront. **4**. Meront splitting into sporont. **5**. Early sporont ribbons. **6**. Bundle of sporont ribbons. **7**. Bundle of sporont ribbons broken by smearing. **8**. Mature sporont ribbons, nuclei spaced evenly along length. **9**. Mature sporont begins to form sporoblasts. **10**. Chain of loosely connected sporoblasts. **11**. Spores in saline, showing varying lengths of spores produced. **12**. Cluster containing more than 60 spores.

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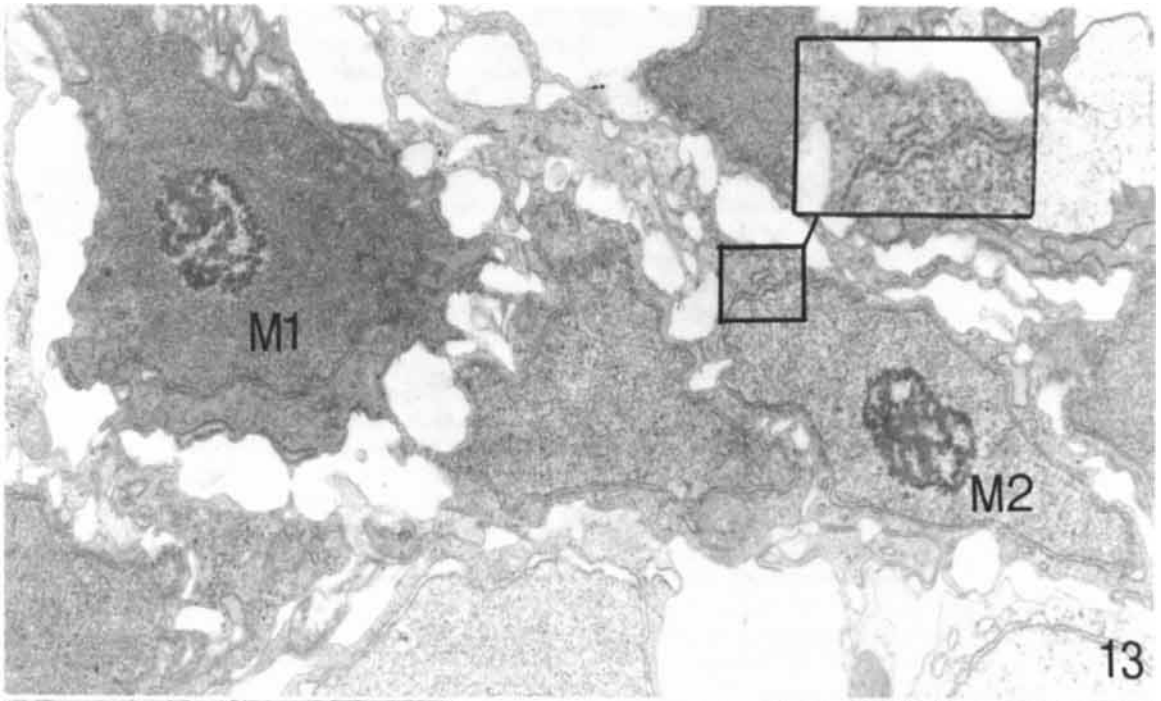
Figs. **13**, **14**. Electron micrographs of life cycle stages. **13**. Group of meronts, ×30,000; inset, double membrane structure, ×50,000. M1, M2, individual meronts. **14**. Section of sporont ribbon containing three nuclei, ×36,000. N, nucleus; CW, sporont cell wall.

Figs. **15–17**. Electron micrographs of life cycle stages. **15**. Section of sporont ribbon, with sporonts and mature spores in same vesicle, ×18,000. **16**. Section of mature spore, ×60,000. **17**. Section of mature spore showing six coils of the polar filament, ×36,000. N, nucleus; VM, vesicle membrane; S, mature spore; EX, exospore; EN, endospore; PM, polarplast membrane.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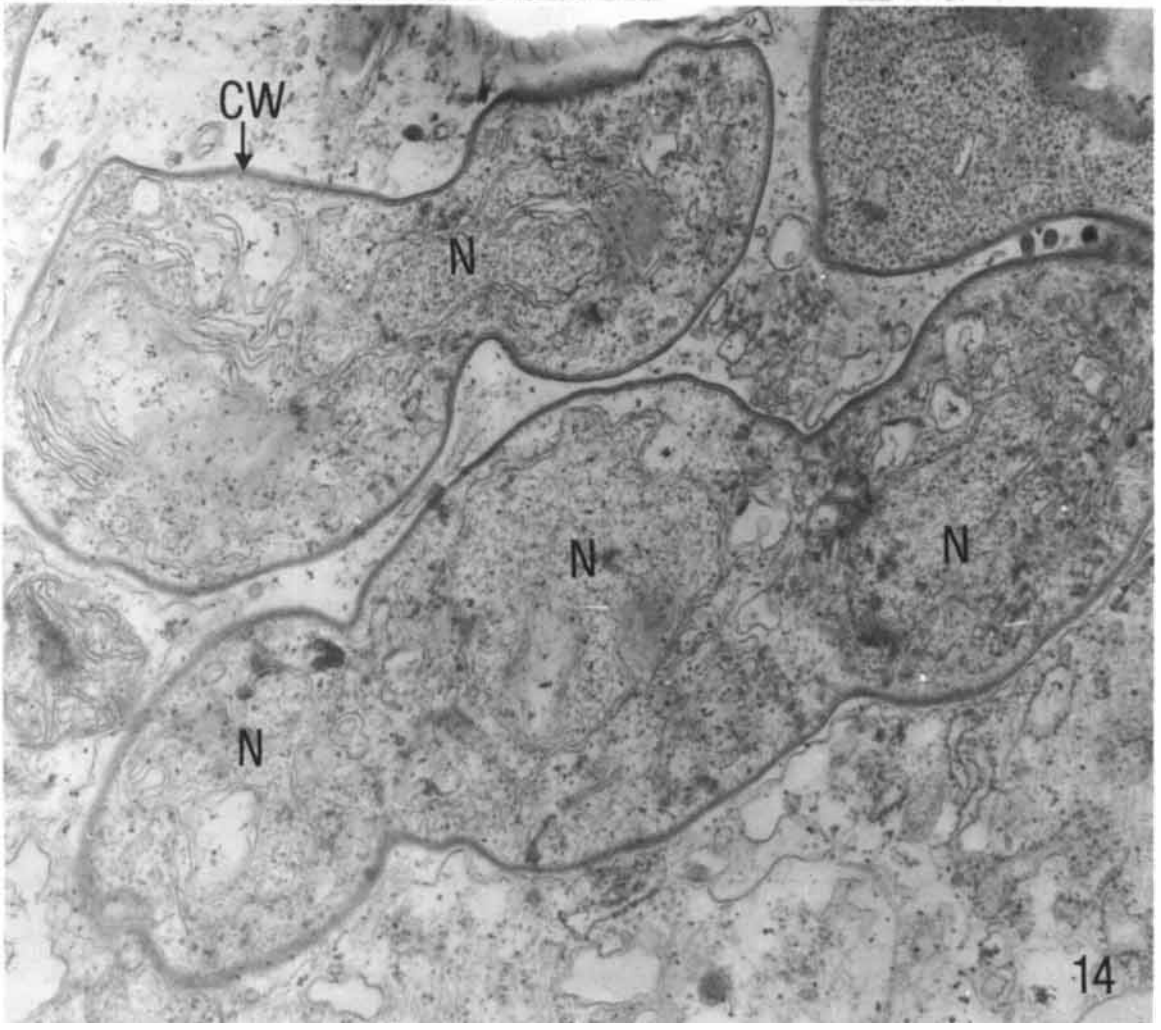
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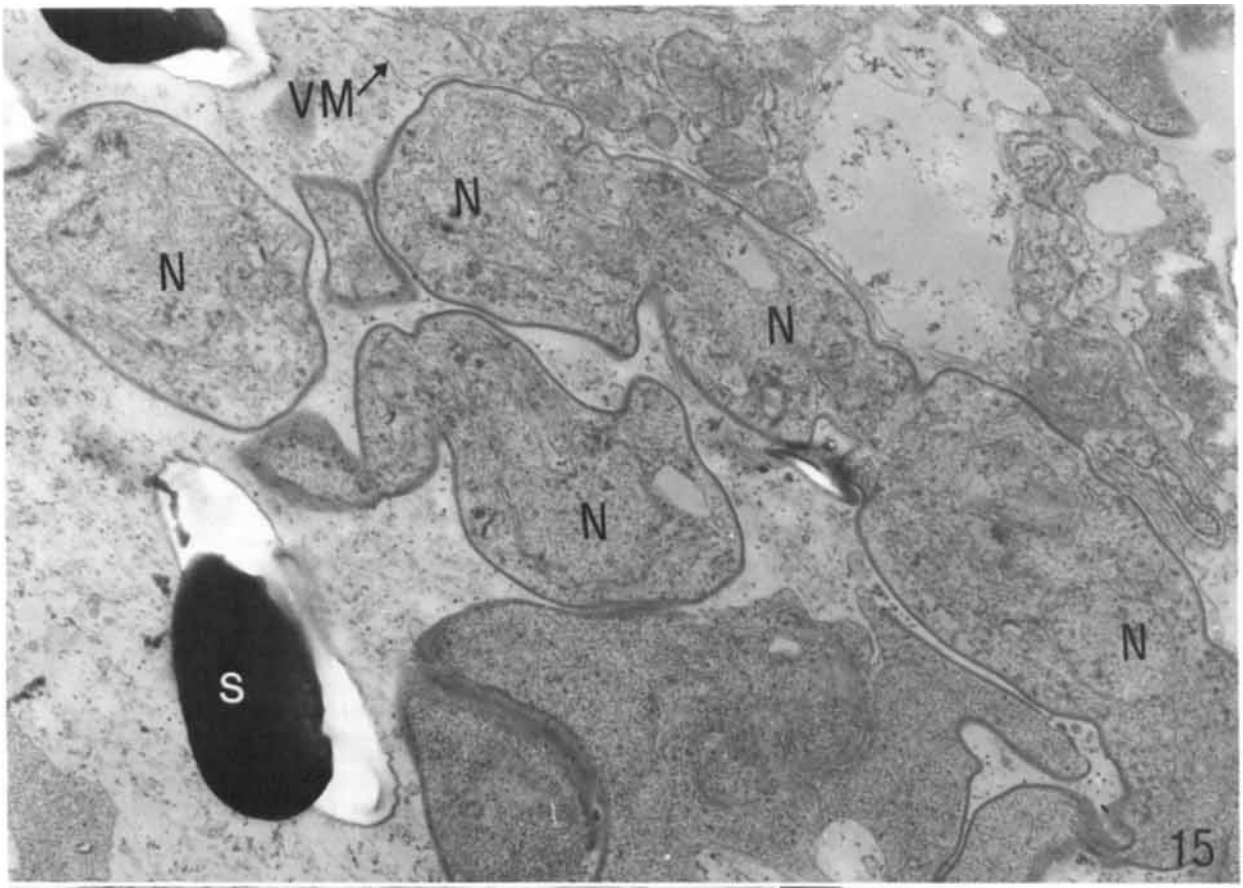
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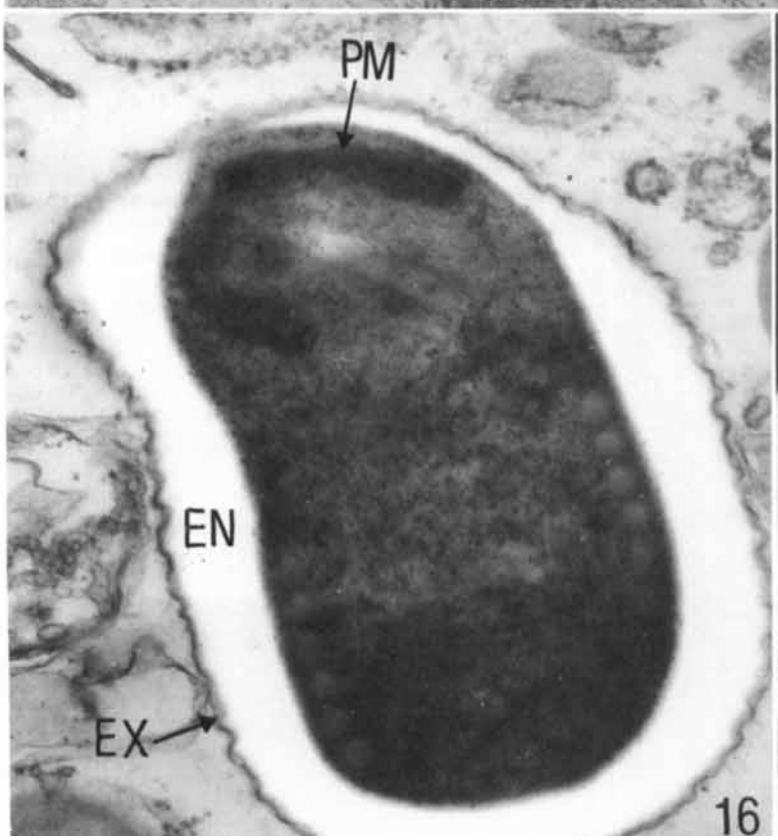
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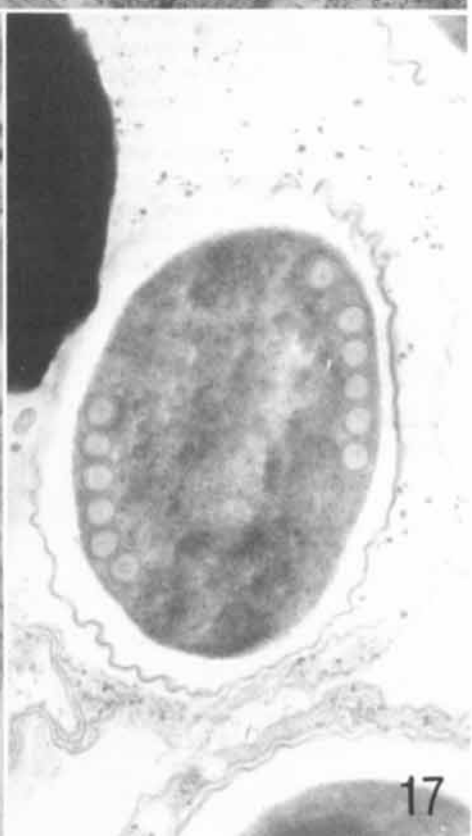
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TABLE I. *Records of microsporidia in gastropods.*

Species	Host	Tissue	Spore size	Reference
Microsporidium sp.	Planorbid snails	No data	No data	9
Nosema sp.	*Lymnea rubiginosa*	Digestive tract, heart, mantle	2.8 × 4.5 μm	6
Pleistophora husseyae	*Physa* sp. snails	All tissues	4.8–5.4 × 3.2 μm	7
Pleistophora sp.	*Deroceras reticulatum*	Intestinal epithelium	4.5 × 2.0 μm	1
Steinhausia brachyneema	*Biomphalaria glabrata*	Gut epithelium, mantle tissue	Spherical, 2.6 μm	10

ing clear that the present system of classification is not satisfactory and a new system based on features present or absent such as diplokarya, presence of sexual forms and meiosis is to be proposed (2). *Microsporidium novacastriensis* does not fit into any of the present genera and rather than create a new genus and add to the present confusion, the slug parasite will be described in the collective group *Microsporidium* created by Sprague (11) for microsporidia of uncertain identity, until a new classification system is published or further diagnostic information obtained.

TAXONOMIC SUMMARY

Microsporidium novacastriensis n. sp.

Diagnosis. Nuclei isolated throughout the life cycle with no evidence of meiosis. Meronts, spherical at first, but become irregular with variable numbers of nuclei and divide to form long, ribbon-like sporonts containing up to 120 nuclei, which are often coiled or folded in a bundle. Sporonts divide directly into sporoblasts, which form spores without further division. Sporonts and spores are enclosed by a thin membrane, which sometimes fragments. Spores are produced in clusters of up to 120, are ovoid, and have mean dimensions when fresh of 3.5 (±0.1, n = 30) by 2.1 (±0.03). There are between four and nine coils of the polar filament.

Type host. The grey field slug, *Deroceras reticulatum* (Müller, 1774).

Type locality. Leazes Park, Newcastle upon Tyne, England.

Infection sites. The intestinal epithelial cells; no other tissues are infected.

Type material. To be deposited in the Department of Zoology, The British Museum, London, England.

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