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Ultrastructure of *Nucleospora secunda* n. sp. (Microsporidia), parasite of enterocytes of *Nothobranchius rubripinnis*

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A new intranuclear microsporidium, *Nucleospora secunda* n.sp., infecting intestinal epithelial cells of *Nothobranchius rubripinnis* Seegers, 1986, is described. Developmental stages and spores are isolated from one another in the karyoplasm. Merogony proceeds from simple uninucleate meronts to multinucleate meronts. Transformation into sporogonial plasmodia is marked by the appearance of ellipsoidal dark bodies, comparable to polar tube precursors, which later coalesce to form the turns of the polar tube. Primordia of the extrusion apparatus are already established in the plasmodium before it starts to cleave into separate sporoblasts. Ellipsoidal mature spores are of average size 1.6 × 0.8 µm and have 4 to 5 turns of the polar tube. The polaroplast consists of an outer region of closely spaced lamellae, enclosing a region of widely spaced lamellae or loose flat cisternae. Tubular inclusions appear in the karyoplasm of infected nuclei.

Key words: *Nucleospora secunda*; Microsporidia; Nuclei of enterocytes; Ultrastructure; *Nothobranchius rubripinnis*.

Introduction

Microsporidia which live in the nuclei of host cells are extremely scarce in fish hosts. *Microsporidium rhabdophilia* Modin, 1981 was the first species to be reported from this site (Modin 1989). It was found in the nuclei of rodlet cells from four salmonid species in California (*Oncorhynchus tschawytscha*, and also *O. kisutch, O. mykiss gairdnerii* and *O. mykiss irideus*). Its suspected identity with the later-described *Nucleospora salmonis* Hedrick, Groff and Baxa, 1991 cannot be confirmed as the description (Modin 1989) did not offer any ultrastructural data. However, the larger spore size (2.6 to 3.5 µm) seems to contradict this.

Nucleospora salmonis develops primarily within the nuclei of haemoblasts (lymphoblasts and plasmablasts). Since the first observation of this species by Elston et al. (1987), *N. salmonis* was found to be associated with plasmacytoid leukemia, a neoplastic condition involving massive lymphoproliferation. It has not yet been decided unequivocally whether this condition is due to a concurrent viral infection of the host, to *Nucleospora*, or to both of these. The infection is widespread; it is common along the Pacific coast of America in *Oncorhynchus tschawytscha*, occurs also in *O. mykiss gairdnerii* and *O. nerka* (see Shaw and Kent 1999) and was even found in *Salmo salar* in Chile (Bravo

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1996). Gresoviac et al. (2000) presume that all salmonid species may be susceptible. *N. salmonis* was described under two different names in the same year, as *N. salmonis* Hedrick, Groff and Baxa, 1991 and as *Enterocytozoon salmonis* Chilmonczyk, Cox and Hedrick, 1991, in view of its close resemblance to the parasite of humans, *Enterocytozoon bieneusi*. The prevailing current opinion is in favour of the name *Nucleospora salmonis*, the status of a different genus being supported by 20% genetic divergence in the 16S and 28S genes (Docker et al. 1997). Nevertheless, both genera are close enough to be in one family Enterocytozoonidae (Cali and Owen 1990), being different from all other microsporidia in that in both the primordia of the extrusion apparatus are already formed at the stage of the sporogonial plasmodium. The manner in which polar tube precursors (PTP) arise from electron dense discs is an especially striking phenomenon.

Microsporidia very similar to *N. salmonis* have been found in lymphocyte-like cells of Atlantic lumpfish, *Cyclopterus lumpus* (Mullins et al. 1994) and in farmed Atlantic halibut *Hippoglossus hippoglossus* (Nilsen et al. 1995). Both groups of authors refrained, however, from identifying their parasites as *N. salmonis* because of their incomplete ultrastructural data.

While studying lethal infections in the aquarium fish *Nothobranchius rubripinnis* provoked by a microsporidian species similar to *Glugea anomala*, which produced xenomas in the intestine and throughout various body organs, sections of the intestinal wall revealed a striking infection of enterocyte nuclei. Microsporidia with minute spores were found and their developing stages were reminiscent of those of *Nucleospora salmonis.* The present communication deals with the morphology and taxonomic position of this microsporidian species.

Material and methods

Specimens of *Nothobranchius rubripinnis* Seegers, 1986 (Cyprinodontiformes) were obtained from an ornamental fish dealer in the Czech Republic, who bought the fish abroad from another importer; the fish came from unspecified localities in Tanzania. Small tissue samples were fixed overnight in 2% osmium tetroxide in 0.1 M cacodylate buffer. After washing, samples were dehydrated using a graded series of acetone and embedded in Spurr's medium. Ultrathin sections were double stained with uranyl acetate and lead citrate. Sections were examined in a JEOL JEM 1010 electron microscope operated at 80 kV.

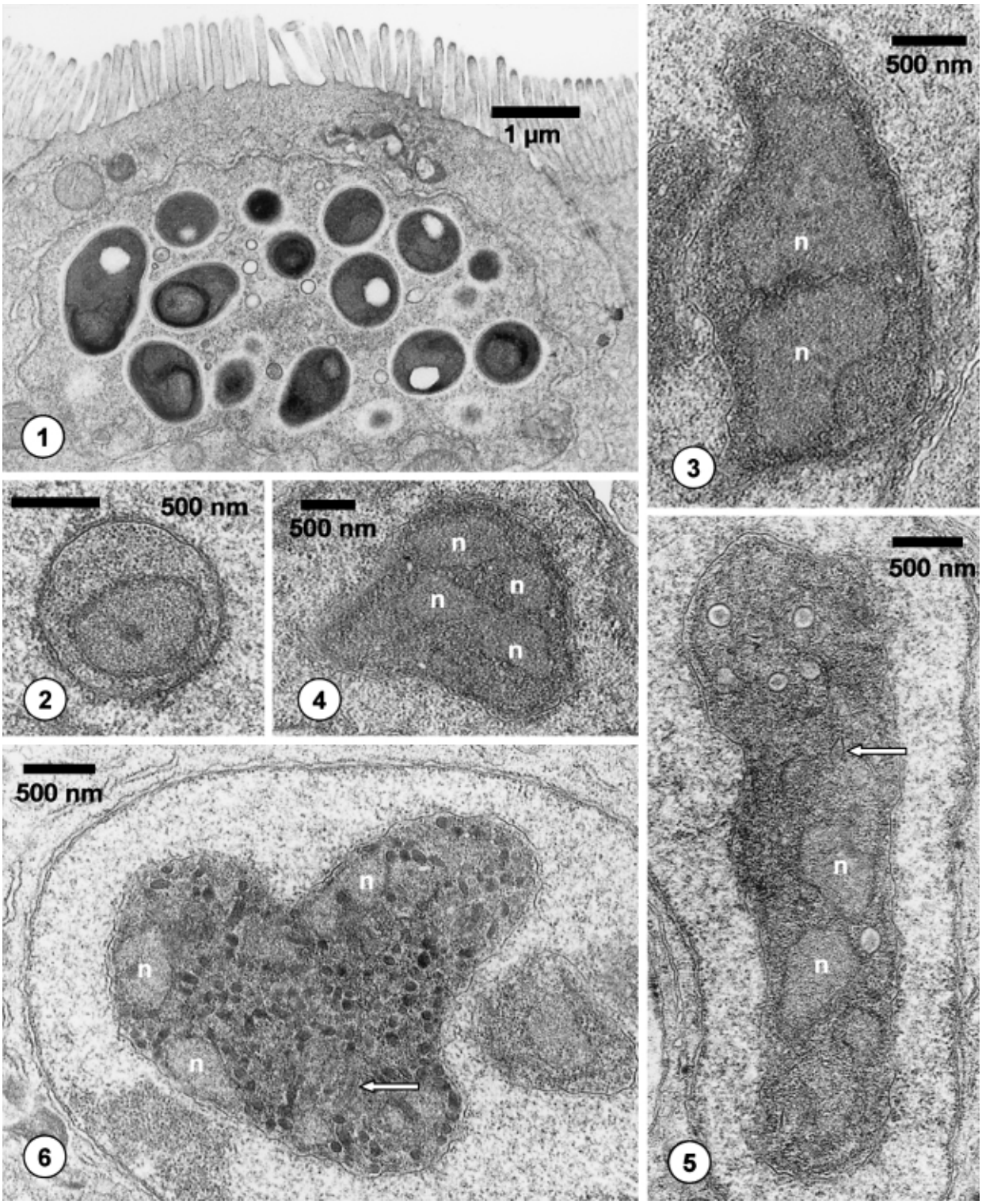
Results

Developmental stages and spores were found isolated from one another in the enterocyte nuclei (Fig. 1). There were one or several parasite cells at different stages of development within one nucleus – meronts, sporogonial plasmodia and mature spores. Early uninucleate meronts (Fig. 2) of about 1.2 µm in size, had a round nucleus occupying a great part of the cell volume, leaving a rather narrow space around it. There was a tiny nucleolus. The cytoplasm showed no distinct cisternae of endoplasmic reticulum (ER) or vesicles, just free ribosomes. The plasmalemma was coated with a thin layer of granular material from the host nucleus.

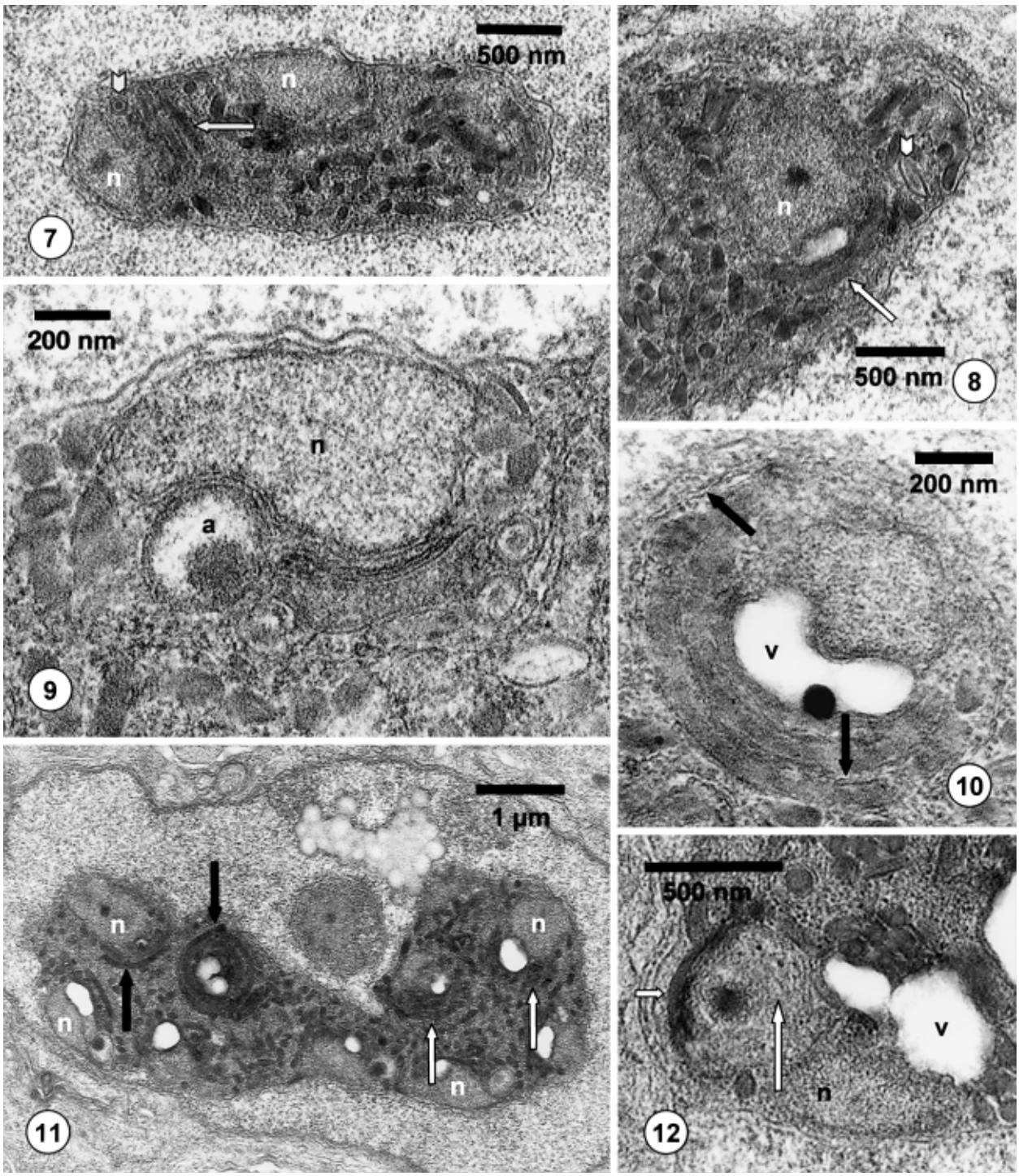
In a growing, binucleate meront (Fig. 3) the nuclei assumed an irregular shape, while the nucleus to cytoplasm ratio was maintained and the cytoplasm occupied only the periphery of the cell. In meronts with three nuclei (Fig. 4) of irregular shape the cytoplasm became more important, already displaying scarce ER cisternae and some vesicles. Meronts could proliferate by plasmotomy.

In advanced, multinucleate meronts a few ER cisternae were distributed throughout the cytoplasm. In some of these meronts, numerous bodies were visible; some of them were circular, and some were fusiform in cross section and encased flocculent, moderately dense material (Fig. 5) bounded by a wall. The latter were reminiscent of PTP.

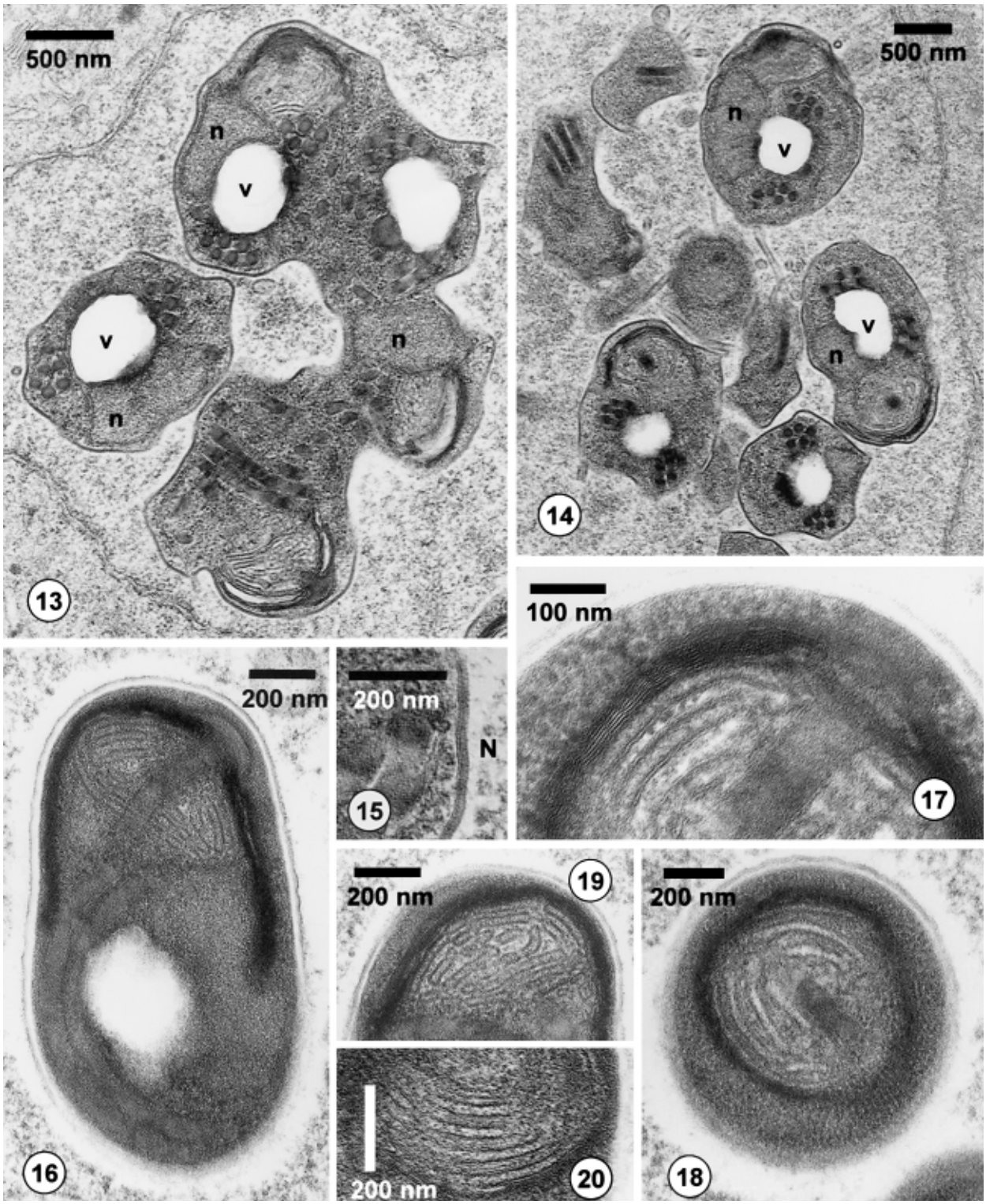
Transformation of grown meronts into sporogonial plasmodia was marked by the appearance of numerous ellipsoidal dark bodies. These bodies, about 80 × 240 nm in size (Fig. 6) looked as if they were advanced stages of the bodies seen in small number in Fig. 5 (arrow). They were structures composed of more dense material in which, eventually, a dense wall differentiated around a central core. These bodies then appeared to coalesce into what seemed to be primordial stretches of the future turns of the polar tube (Fig. 6, arrow; Figs. 7, 8). There was no intervention of the intermediate stage of electron dense discs known in *Enterocytozoon bieneusi* (Desportes-Livage et al. 1996). Within the mass of dense bodies were clusters of vesicles. Gradually, primordia of anchoring discs appeared in their typical positions, i.e., in a depression of the nuclear surface (Fig. 9). This marks the stage of assembly, close to the nucleus, of the extrusion apparatus. The opaque bodies revealed dif-



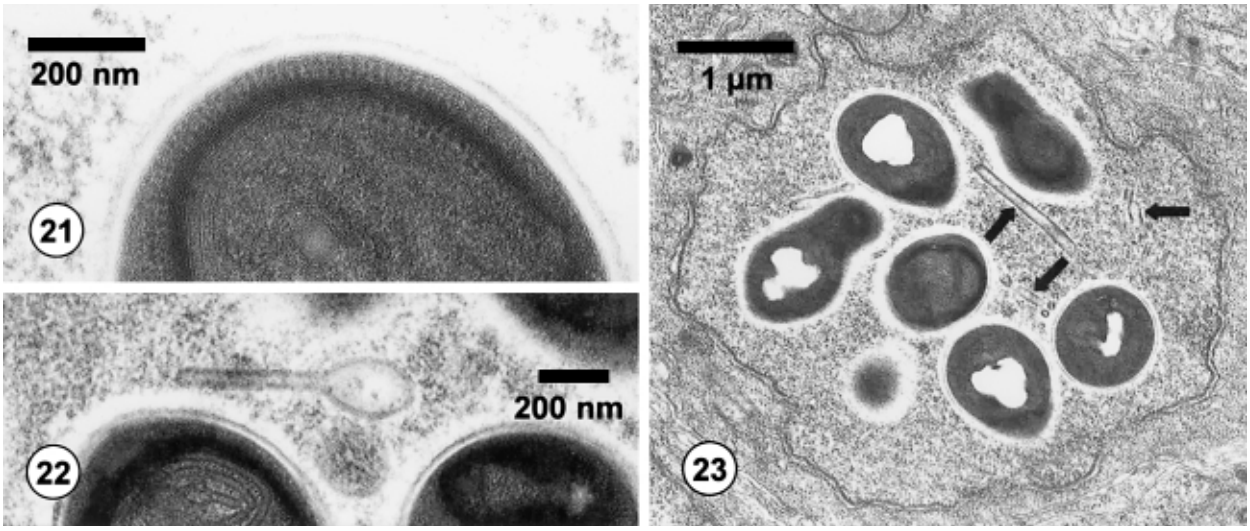
Figs. 1–6. *Nucleospora secunda.* **1.** A group of mature spores within the nucleus of an enterocyte. Lucent posterior vacuoles are clearly visible. **2.** An early meront. **3.** Meront with two nuclei (n). **4.** A meront with four nuclei (n) seen in the section. **5.** Late meront transforming into sporogonial plasmodium. Note the circular cross sections of intracytoplasmic bodies; spindle-shaped section marked by arrow. **6.** An early sporogonial plasmodium with numerous PTP. n – nuclei of future sporoblasts, arrow points at stretches of primordial polar tube.



Figs. 7–12. 7, 8. Sporogonial plasmodium with PTP-like bodies coalescing into primordial turns of the polar tube. n – nuclei of the future sporoblasts, the arrow points at stretches of the primordial polar tube; the arrowhead points at its transverse section in Fig. 7; and in Fig. 8, at a fusiform section of a body identical with that in Fig. 5. **9.** Primordium of the anchoring disc (a) leaning against the depression in the nucleus (n). Some of the transverse sections show where the PTP has fused into the structure approaching that of the polar tube. **10.** Initial stretches of the polar tube around the future posterior vacuole (v). Arrows point at the ER cisternae. **11.** Advanced sporogonial plasmodium with several primordia of the future extrusion apparatus (arrows). n – nuclei. **12.** The area of a future sporoblast still within the plasmodium. Lamellar part (short arrow) and vesicular part (long arrow) of the polaroplast. n – nucleus, v – posterior vacuole.



Figs. 13–20. 13. Sporogonial plasmodium in the process of segmentation into separate sporoblasts with parts of closely and widely spaced lamellae of the polaroplast distinctly seen. n – nucleus, v – posterior vacuole. **14.** A group of recently separated sporoblasts. n – nucleus, v – posterior vacuole. **15.** Enlargement of the cell wall of a sporoblast at the stage seen in Fig. 14. N – host nucleoplasm. **16.** An almost longitudinal section through a mature spore. **17.** Apex of the spore; lamellar and vesicular part of the polaroplast can be seen. **18.** Within the outer region of the polaroplast, comprised of closely packed lamellae, is the region of widely separated lamellae attached to the straight stretch of the polar tube. **19, 20.** Slightly different appearances of the inner widely spaced polaroplast lamellae; in Fig. 20, a middle lamella can be seen between the regularly spaced lamellae.



Figs. 21–23. 21. Polyribosome ornamentation on the surface of the polaroplast. **22, 23.** Ampoule-like and tubular structures (arrows) within the infected host cell nucleus.

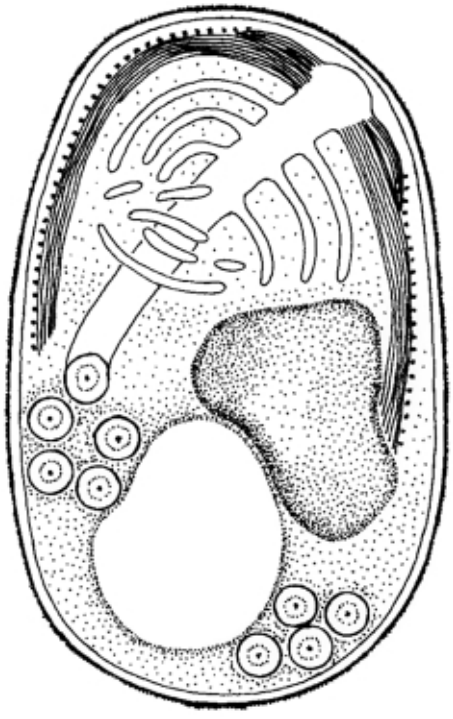


Fig. 24. Diagrammatic representation of a *Nucleospora secunda* spore.

ferentiation into the double dense walls and seemed to be arranged in spaces between the few cisternae of the ER. In sections close to the nucleus, they displayed a wall enclosing a dense core (Fig. 9). In spaces between sections of these polar tube primordia, ER membranes extended in the direction in which the primordial turns of the PT would run (Fig. 10). In stages where the polar tube became quite distinct, there was a lucent area occupied by the future posterior vacuole (Fig. 10). In advanced sporogonial plasmodia, numerous primordia of the extrusion apparatus were present (Fig. 11), in which the assembly of different regions of the polaroplast proceeded (Fig. 12, arrows). In cross sections, the final number of turns of the polar tube was already visible.

Separation of individual sporoblasts occurred at the stage where all constituents of the spore were already laid down (Fig. 13). At this stage the cell wall consisted of a thin (about 17 nm) cell coat covering the plasmalemma (Fig. 15), unlike the usually much thicker cell wall of sporonts in other microsporidia. There was already a definite number (4 to 5) of polar tube turns; these turns circled around the large future posterior vacuole. The turns were slanted at an angle of almost 50° to the future antero-posterior axis of the spore (Fig. 14).

Mature spores measured 1.5–1.8 × 0.77–0.87 µm (in situ in the ultrathin sections). They were of ellipsoidal shape and, unlike developmental stages, which were in direct contact with the karyoplasm, they lay within a narrow halo of shrunken karyoplasm. The endospore was about 15–24 nm thick, the exospore was 10–15 nm thick. The anchoring disc was shifted sideways to a sublateral position (Fig. 16) and its "umbrella", unusually about three lamellae thick, covered the apical part of the polaroplast. The outer part of the polaroplast consist-

ed of about 8 laminae closely apposed (at an interval of about 4–5 nm, Fig. 17) to each other and extended as a cup-like structure well below the midspore length. The inner part of the polaroplast consisted of widely spaced lamellae, separated by about 24 nm, which in some sections appeared as flat double-walled cisternae. Their lamellae arose from the outer membrane covering the proximal part of the polar tube (Fig. 18). These flat cisternae/vesicles had different appearance in some sections (Fig. 19) and some had a middle lamina (Fig. 20).

All around above the polaroplast laminae and also on other parts of the spore there were rows of polyribosomes spaced at regular intervals (Fig. 21). The single nucleus (Figs. 16, 24) was shifted laterally and touched from below by the vesicular part of the polaroplast. The 4 to 5 turns of the polar tube coils circled at an oblique angle around the posterior vacuole (Fig. 24).

Developmental stages as well as spores were free in the nucleoplasm and were never attached to the nuclear envelope of the host cell. Granular material was always accumulated around the stages. Signs of conspicuous pathology of the nucleus of the host cell were missing. However, strange structures appeared among the developmental stages and/or spores. These included formations like bunches of grapes (Fig. 11) and, more frequently, ampoule- (Fig. 22) or tube-like (Fig. 23) shapes which were believed not to be extruded polar tubes. These structures might represent reactions of the host nuclei to the presence of parasites.

Discussion

There are several points in which the above-described *Nucleospora* sp. differs from previously known species. Differences from *Enterocytozoon bieneusi* are quite evident; the species in question infects nuclei and not cytoplasm, it has a fish and not a mammalian host, 4 to 5 rather than 4 to 7 turns of the polar tube, and the nuclei in late stages of plasmodial sporonts tend to be peripheral rather than scattered throughout the cytoplasm. Developmental stages of *Enterocytozoon* as described by Desportes et al. (1985), Cali and Owen (1990) and especially by Desportes-Livage et al. (1996) also differ from *Nucleospora salmonis.* The parasite described here more closely resembles *Nucleospora* than *Enterocytozoon*.

A comparison of our findings with features described for *N. salmonis* reveals several differences:

(a) the site of infection, i.e., nuclei of enterocytes instead of nuclei of haemopoietic cells, or, rather rarely, epithelial cells of urinary tubules and mesangial cells of the glomeruli;
(b) there are 4 to 5 turns of the polar tube instead of 8 to 12 turns of the polar tube in *N. salmonis*;
(c) in early meronts the nucleus occupies – as seen on the cross section – at least half of the cell volume, rather than about one third in *N. salmonis*;
(d) the ER system is not as well developed as in *N. salmonis*, a difference which persists even at later stages, when the polar tube precursors appear among the ER cisternae;
(e) there is also not such a well developed "granular body" as that described by Desportes-Livage et al. (1996) close to the nuclei of sporogonic plasmodia in *N. salmonis*;
(f) the host is a warm-water African fish with no possible links or contact with a salmonid from a different region; hosts of both species are quite different at the genetic level (order Cyprinodontiformes vs. Salmoniformes).

The combination of these differences is clearly in favour of establishing our finding as a new species. Therefore we propose to designate it as *Nucleospora secunda* n.sp.

***Nucleospora secunda* n.sp.:**
Host: *Nothobranchius rubripinnis* Seegers, 1986 (Cyprinodontidae)
Site of infection: intestine, within the nuclei of enterocytes.

Developmental stages: Uninucleate meronts with large nuclei and scarce cytoplasmic organelles grow into multinucleate plasmodia. There are isolated nuclei throughout the cycle. Merogony stages can proliferate by plasmotomy. Appearance of dense ellipsoidal bodies marks the transformation into the sporogonial plasmodia. These bodies, comparable to the polar tube precursors, coalesce into the turns of the future polar tube. Gradually, primordia of components of the extrusion apparatus appear. When all of the spore constituents have been laid down, the plasmodium, still without a thick cell coat, divides into individual sporoblasts.

Ellipsoidal mature spores, of average size 1.65 × 0.82 µm, have a laterally shifted anchoring disc.

The polaroplast consists of a lamellar part of closely apposed membranes, forming a deep cup-like structure extending below the mid-spore length; the inner part consists of widely spaced membranes sometimes seen as conspicuously distended flat cisternae. There are 4 to 5 turns of the polar tube at a sharp angle to the longitudinal axis, circling around the posterior vacuole. The posterior vacuole is located posterior to the single nucleus.

Derivation of the name: it is the second nominal species described in the genus.

Microscopical slides with semithin sections of infected enterocytes were deposited in the collection of the Institute of Parasitology AS CR under the numbers PI-063 and PI-064.

The species is assigned to the family Enterocytozoonidae Cali and Owen, 1990.

Ultrastructural patterns of *Nucleospora secunda* are similar to those of *N. salmonis*. PTP seem generally more dense than those depicted by Desportes-Livage et al. (1996); however, sections through these bodies, especially the spindle-like profiles, are closely similar. Vesicular clusters which we found in advanced sporogonial plasmodia are reminiscent of clusters of vesicles considered to give rise to the inner part of the polaroplast in *N. salmonis* (Desportes-Livage 1996).

Nucleospora spp. previously described from non-salmonid hosts differ from *N. secunda*. Thus, although Mullins et al. (1994) did not record a complete series of developmental stages of *Nucleospora* sp. found in the nuclei of lymphocyte-like cells of *Cyclopterus lumpus*, it seems to correspond to *N. salmonis* and is certainly different from *Nucleospora* sp. The *Nucleospora* sp. from lymphoblasts of *Hippoglossus hippoglossus* (Nilsen et al. (1995) is in accord with *N. salmonis* in having 10 turns of the polar tube coil, which differentiates it from *N. secunda*. There are also minor differences between the two: spores of the *Nucleospora* sp. from *Hippoglossus* have a coil of electron dense material in the posterior vacuole, the electronlucent vesicles in sporogonial plasmodia are more pronounced and the PTP displays a less dense centre when compared with our findings.

Judging by the infection of haemoblast nuclei and the fish host species, the finding of Elston et al. (1987) should be considered as *N. salmonis*. However, in their pictures of a presumed young sporoblast which had separated from the sporogonial plasmodium, there are only 4–5 polar tube coils. This contrasts with the 9–11 turns already present in the future sporoblasts before their separation from the sporogonial plasmodium, described by Desportes-Livage et al. (1996). Nevertheless, Chilmonczyk et al. (1991) reproduced a micrograph of a sporoblast in the course of being separated from the plasmodium, with also just four turns of the tube; thus it may be that development proceeds at different pace in different populations.

Unlike both *Enterocytozoon bieneusi* and *Nucleospora salmonis*, *N. secunda* was not associated with any obvious retrovirus infection.

Due to technical problems – all our material was limited to embedded blocks – we could not perform the analysis of SSU rDNA and compare the sequence with those of the two already sequenced *Nucleospora* species. Elucidation of relationships of these species is an urgent task for the future. Continuing research may also show that intranuclear microsporidia are more frequent than one may think, but escape attention just due to their small size.

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