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Ultrastructural Description of the Life Cycle of *Nosema diphterostomi* sp. n., a Microsporidia Hyperparasite of *Diphterostomum brusinae* (Digenea: Zoogonidae), Intestinal Parasite of *Diplodus annularis* (Pisces: Teleostei)

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Summary. The life cycle of a new microsporidium, *Nosema diphterostomi* sp. n. is described. This parasite infects the epithelial gut and connective tissue of a trematode *Diphterostomum brusinae* (Digenea: Zoogonidae), intestinal parasite of *Diplodus annularis* (Pisces: Teleostei). All development stages are in close contact with the host cell cytoplasm and have paired nuclei (diplokaryon). Matures spores measure about 2.1 × 1.4 µm. They possess a polar filament with 6-7 coils, a posterior vacuole and a polaroplast made up of an outer part of dense and closely spaced lamellae encircling an inner part of widely spaced lamellae. All morphological and ultrastructural features indicate that the described microsporidium belongs to the genus *Nosema*. In comparison with the other *Nosema* of trematodes, this parasite is a new species. We propose the name *Nosema diphterostomi* sp. n. according to the generic name of its host.

Key words: *Diphterostomum brusinae*, *Diplodus annularis*, hyperparasite, Microsporidium, *Nosema diphterostomi* sp. n., Nosematidae.

INTRODUCTION

Diphterostomum brusinae (Stossich, 1888) Stossich, 1903 (Digenea, Zoogonidae) is a parasite of the digestive tract of the Sparid fish *Diplodus annularis* (Linnaeus, 1758). During an ultrastructural study of this platyhelminth, we discovered a microsporidium which develops in different tissues of this parasite.

Hyperparasite microsporidia of trematodes have been reported on numerous occasions. They belong to the genus *Nosema* Naegeli, 1857, *Pleistophora* Gurley, 1893 and *Unikaryon* Canning, Lai *et* Lie, 1974 as well as to the collective group of *Microsporidium* Balbiani, 1884 (Canning 1975, Canning and Madhavi 1977, Sprague 1977, Canning and Olson 1980, Canning *et al.* 1983, Azevedo and Canning 1987). The species, found in *D. brusinae*, belongs to the genus *Nosema*. In this study, the development cycle of this new species is described

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and compared with that of the other *Nosema* from trematodes.

MATERIALS AND METHODS

Several teleostean specimens *Diplodus annularis* (Linnaeus, 1758) (Sparidae) were collected in the "Bonifacio Strait Marine Reserve" (Mediterranean Sea, France) during 2001 and 2002 summers. After necropsy and extraction, the specimens of the trematode *Diphterostomum brusinae* (Stossich, 1888) Stossich, 1903 (Zoogonidae) were kept alive in a 0.9 % NaCl solution. Different adults specimens were fixed in cold (4°C) 2.5 % glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2, rinsed for one night in a 0.1 M sodium cacodylate buffer at pH 7.2, postfixed in cold (4°C) 1 % osmium tetroxide in the same buffer for 1 h, dehydrated with ethanol and propylene oxide, embedded in Epon and then polymerised at 60°C for 48 h. Ultrathin sections (60 to 90 nm thick) were cut on a LKB 8800A ultramicrotome, placed on copper grids, and stained with uranyl acetate and lead citrate according to Reynolds (1963).

The grids were examined in a Hitachi H600 electron microscope at 75 kV in the laboratory "Parasites and Mediterranean Ecosystems" in the University of Corsica (Corte, France).

During examination of sections of *D. brusinae*, hyperparasite microsporidia were found in connective tissue and intestinal cells.

RESULTS

Site of infection (Figs 1, 2)

The microsporidium infects intestinal cells (Fig. 1) and connective tissue (Fig. 2). All development stages are in close contact with the cytoplasm of the host cell.

Development stages (Figs 3-14)

Meronts (Fig. 3): They are more or less spherical cells with two or four nuclei in diplokaryotic arrangement. The meronts are surrounded by a single plasma membrane. Their cytoplasm is electron-light and contains some endoplasmic reticulum cisternae and free ribosomes. In longitudinal section, the diplokaryon occupies three quarters of the cell. The nuclear envelope is rather difficult to observe. Mostly, the nuclear envelope appears clearly only where the two nuclei of the diplokaryon are adjacent. However, the nucleoplasm, electron-dense, always allows us to distinguish the diplokaryon of the cytoplasm which surrounds it.

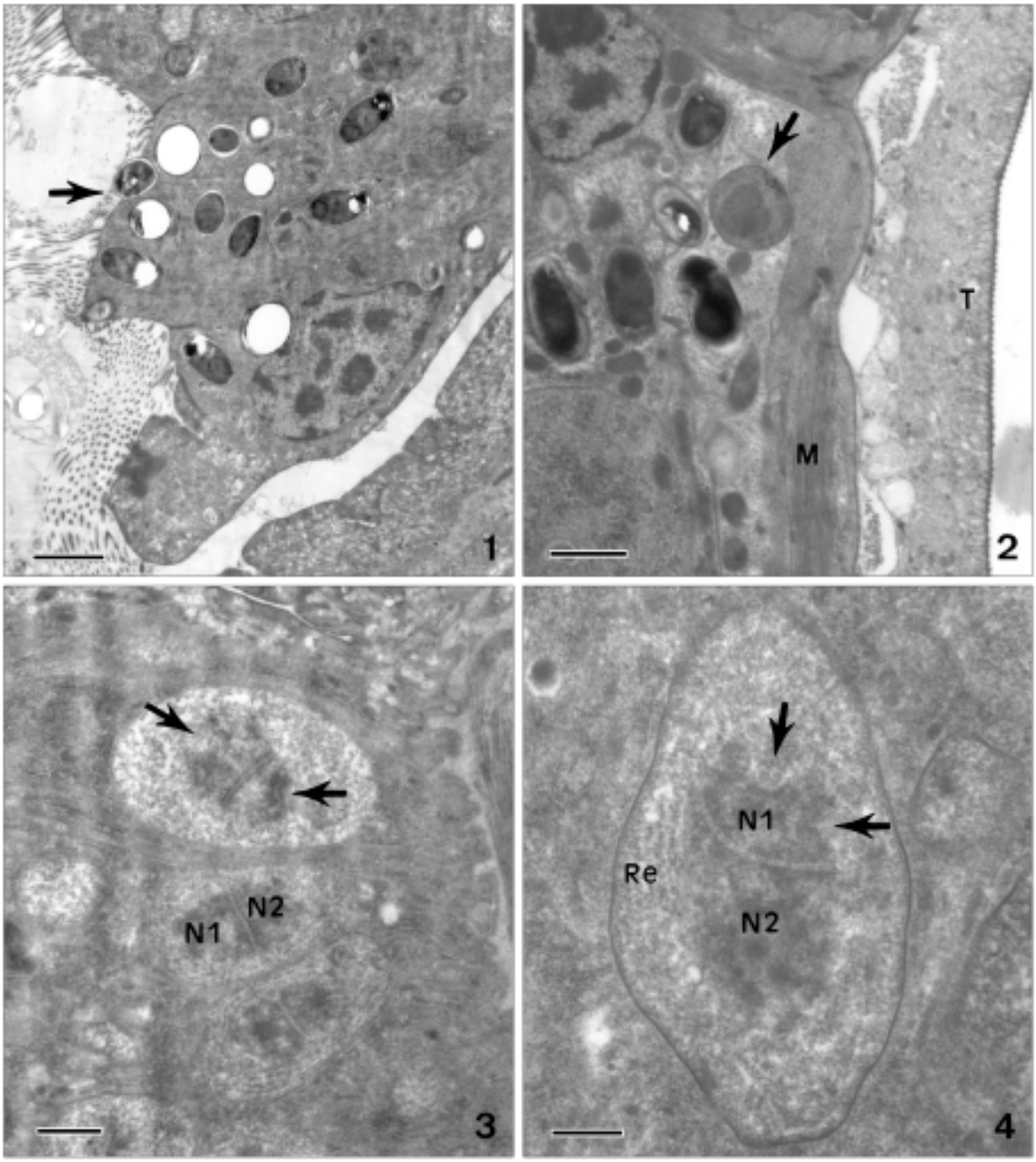
During the meront division, both nuclei constituting the diplokaryon divide simultaneously. The first demonstration of this division is the appearance of one then two spindle plaques on the surface of the nuclear envelopes (Fig. 3). These spindle plaques appear in the shape of a superficial depression of the nuclear envelope underlined by an internal layer of electron-dense material. Prior to the division of the cytoplasm, the spindle plaques migrate bringing about the elongation then the division of diplokaryon. So, it appears temporarily meronts with two diplokarya.

Sporonts (Figs 4, 5): The transformation of meronts into sporonts is realized by the appearance of a wall outside the plasma membrane. The sporonts are thus recognizable by an electron-dense thick envelope (≈ 300 Å) and an elongated shape. They always contain reticulum endoplasmic cisternae, free ribosomes and one or two diplokarya.

The division process of sporonts is similar to that of the meronts. Two spindles plaques appear on the nuclear envelope of each nucleus (Fig. 4). Nucleus elongates simultaneously then divides by central constriction prior to the cytoplasm division (Fig. 5). So, it forms in a moment sporonts with two diplokarya.

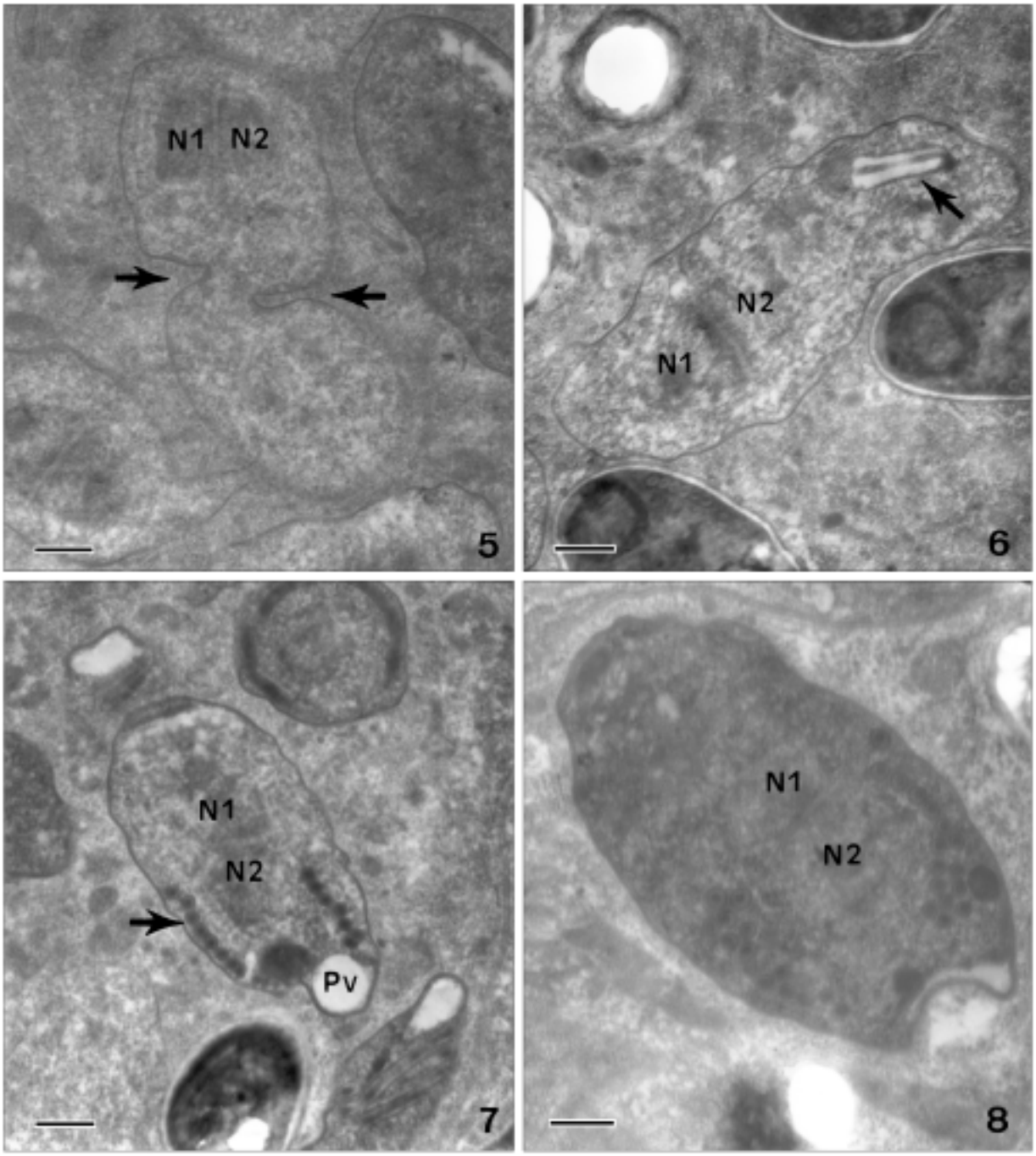
Sporoblasts (Figs 6-8): The division of sporonts gives rise to two sporoblasts. Each sporoblast, like the other stages of development, possesses a diplokaryon. They are elongated and surrounded by a thick envelope of about 350 Å. Their cytoplasm is electron-dense. The evolution of the sporoblast begins with the genesis of the polar filament and with the appearance of a vacuole, in contact to a dictyosome. This vacuole serves as matrix for the internal parts of the polar filament (Fig. 6). In transverse section, the young polar filament presents a thick envelope, a clear layer and an electron-dense central axis. In longitudinal section, the old sporoblasts possess a polar filament with 7 to 8 coils around the diplokaryon (Fig. 7). However, some abnormal sporoblasts are observed with a polar filament arranged in 13 to 16 coils (Fig. 8).

Spores (Figs 9-14): Fresh spores are not observed. Our study was exclusively realized in electron microscopy. Spores appear ovoid in shape and possess a diplokaryon (Fig. 9). On 28 spores measured in cross and longitudinal sections, the average size is 2.1 ± 0.3 µm with a minimum of 1.4 µm and a maximum of 2.6 µm in length and 1.4 ± 0.2 µm with a minimum of 1.1 µm and a maximum of 2.1 µm in width. The spore envelope is composed of an electron-dense exospore of 220 ± 80 Å thick and an electron-lucent endospore of 475 ± 225 Å thick. Several elements are recognizable inside the spore: a diplokaryon, a polar filament with an anchoring disc, a polaroplast and a posterior vacuole (Figs 9-11).



Figs 1-4. Electron micrographs of *Nosema diphterostomi* sp. n. **1** - gut epithelial cells of *D. brusinae* infested by *N. diphterostomi* (arrow); **2** - connective tissues containing *N. diphterostomi* (arrow); **3** - meronts showing the spindle plaques (arrows) and diplokaryon (N1 and N2); **4** - sporont with the electron-dense membrane and the spindle plaques (arrows). M - muscle, N1, N2 - diplokaryon, Re - endoplasmic reticulum, T - tegument. Scale bars 2.5 µm (1); 1.5 µm (2); 1 µm (3); 0.5 µm (4).

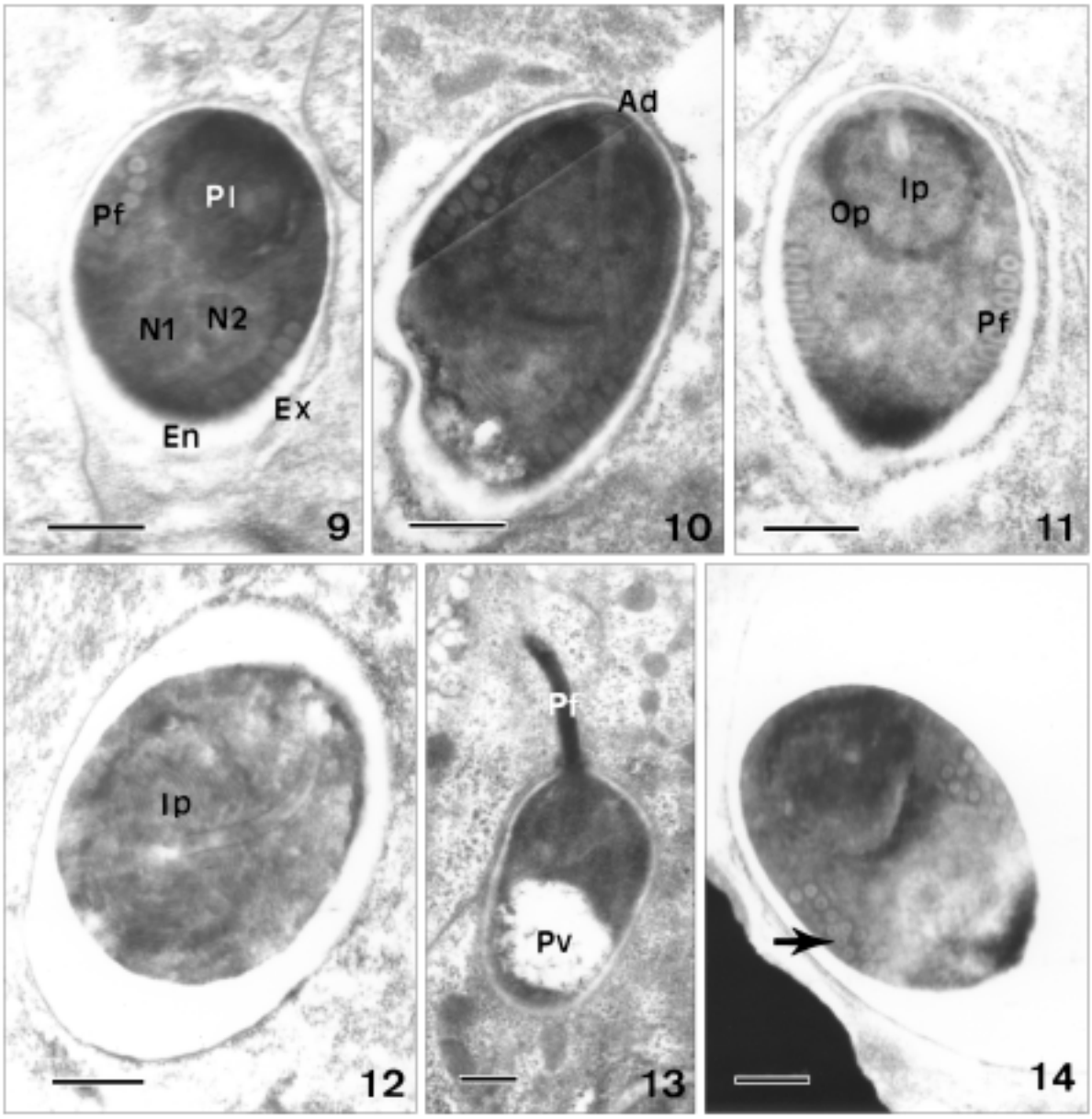
The polar filament is about 100 nm in diameter. It is isofilar, possesses a short rectilinear proximal part followed by an important helical distal part describing 6 to 7 coils around the diplokaryon. In its proximal part, the polar filament expands slightly before fusing with the anchoring disc (Fig. 10).



Figs 5-8. Electron micrographs of sporogony and sporogenesis of *N. diphterostomi* sp. n. **5** - division of sporonts with the strangulation of the cytoplasm (arrows); **6** - young sporoblast showing the formation of the polar filament (arrow); **7** - mature sporoblast containing polar filament with 7 to 8 coils (arrow); **8** - abnormal sporoblast with 13 to 16 coils of polar filament. N1, N2 - diplokaryon, Pv - posterior vacuole. Scale bars 0.5 µm.

The polaroplast (Figs 10-12) presents two lamellar parts: an outer electron-dense part made up of closely arranged membranes encircling a less electron-dense inner part constituted of more loosely arranged membranes.

An electron-lucent vacuole occupies the posterior end of the spore (Fig. 13). A few spores, in the process of emergence, were observed emptying of their content (Fig. 13). In this case, the envelope, which conserved its initial organization, presents a distinct fusion between its



Figs 9-14. Electron micrographs of spores of *N. diphterostomi* sp. n. **9** - spore showing the diplokaryon (N1, N2), the exospore (Ex), the endospore (En), the polar filament (Pf) and the polaroplast (Pl); **10** - mature spore with anchoring disc (Ad); **11** - spore showing the inner (Ip) and the outer parts (Op) of the polaroplast. Pf: polar filament; **12** - section of the spore showing the lamellae of the inner (Ip) part of the polaroplast; **13** - extrusion of the polar filament (Pf) in the host cell. Pv - posterior vacuole; **14** - abnormal spore with 13 to 14 polar tube coils (arrow). Scale bars 0.5 µm.

endospore and its exospore at the spore opening level. The polar filament, turned like a finger glove, is then observed in the cytoplasm of the host cell. Inside the spore, the posterior vacuole swells, causing the extrusion of the spore content along the canal formed by the polar filament.

Abnormal spores presenting a polar filament with 13 to 14 coils were observed (Fig. 14). These spores are bigger than the most numerous spores considered as normal. They measure 3.5×2.2 µm.

DISCUSSION

The ultrastructural characteristics and the developmental cycle of the microsporidium, parasite of the

Table 1. *Nosema* species hyperparasites of trematodes.

Species	Type host	Hyperparasite	Spore mesurations	Localities	References
Nosema dollfusi	*Bucephalus cuculus*	*Crassostrea virginica*	3 × 1.7 µm	Maryland USA	Sprague 1964
Nosema eurytremae	*Eurytrema pancreaticum*; *Postharmostomum gallinum*	*Bradybaena similaris*	3.94 × 2.26 µm	Malaysia	Colley *et al.* 1975
Nosema gigantica	*Allocreadium fasciatusi*	*Aplocheilus melastigma*	7.9 × 4.9 µm	India	Canning and Madhavi 1977
Nosema xiphidiocercariae	Plagiorchiidae	*Lymnae palustris*	4.5 × 2.3 µm	Moscow Russia	Sprague 1977
Nosema lepocreadii	*Lepocreadium manteri*	*Leuresthes tenuis*	3.5 × 1.5 µm	San Diego USA	Canning and Olson 1980; Canning *et al.* 1983
Nosema diphterostomi sp. n.	*Diphterostomum brusinae*	*Diplodus annularis*	2.1 × 1.4 µm	Corsica, France	Present study

trematode *D. brusinae*, present all criteria of the genus *Nosema* Naegeli, 1857 described by Sprague (1977). All ultrastructural features of spores of the genus *Nosema* were specified by Sato *et al.* (1982) and Canning and Vavra (2000).

To our knowledge, 16 microsporidia species were described in trematodes, but among these species, only five belong to the genus *Nosema* (Table 1). They are *Nosema dollfusi* Sprague, 1964, *Nosema eurytremae* Canning, 1972, *Nosema xiphidiocercariae* Voronin, 1974, *Nosema gigantica* Canning *et* Madhavi, 1977, and *Nosema lepocreadii* Canning *et* Olson, 1980.

Nosema dollfusi was described in the sporocysts of *Bucephalus cuculus*, parasite of the oyster *Crassostrea virginica* from Maryland in USA. These spores are binucleate and measure 3 × 1.7 µm in stained preparations (Sprague 1964). The merogony and the sporogony were not described. This *Nosema* differs from the present species by the spore size and by its host which, in larval stage, parasitizes a mollusc. Nevertheless, this trematode, in adult stage, parasitizes a fish (Gibson *et al.* 2001). Thus, it is very likely that this microsporidium is also present in adult trematode when it is parasite of fish. Probably, there is a transovarial transmission.

Nosema eurytremae presents a wide host spectrum, but was first described as a hyperparasite of larvae of the trematodes *Eurytrema pancreaticum* and *Postharmostomum gallinum* in the land snail *Bradybaena similaris* in Malaysia. It was studied in electron microscopy by Colley *et al.* (1975). According to these authors, fresh spores measure 3.94 × 2.26 µm. The spore wall is about 250 nm thick except at the level of the anchoring disc where it is approximately 80 nm thick. The polar filament makes 11 to 12 coils. The polaroplast presents an anterior part composed of laminated and dense membranes and a posterior part formed by flattened sacs. This *Nosema* is different from the present species by the size and ultrastructure of its spores but also by the fact that it lives in larva parasite of land snail. It must be also present in adult parasites of land animals (birds, mammals).

Nosema xiphidiocercariae is hyperparasite of sporocyst, cercaria, metacercaria in Plagiorchiidae parasite of *Lymnaea palustris*, freshwater mollusc in Russia. These fresh spores measure 4.5 × 2.3 µm and the spores, stained with Giemsa, measure 4.0 × 2.3 µm (Sprague 1977). This *Nosema* differs from the present species by the spore size and by the fact that its host lives in a freshwater mollusc. The microsporidium must be present in the adult trematode, parasite of freshwater fish.

Nosema gigantica infests adult of *Allocreadium fasciatusi*, parasite of *Aplocheilus melastigma* a freshwater fish in India. The ultrastructural data of its devel-

opment stages are not available. Its spores stained with Giemsa measure 7.9 × 4.9 µm (Canning and Madhavi 1977). It differs from the present species by the spore size and by the fact that its host lives in a freshwater animal.

Nosema lepocreadii was described in adult of *Lepocreadium manteri*, parasite of *Leuresthes tenuis* a marine fish in San Diego (USA). It was studied in electron microscopy by Canning *et al.* (1983). Its sporogony is disporoblastic but sometimes polysporoblastic. Its fresh spores measure 3.5 × 1.5 µm, possess a polar filament with 10 coils and a polaroplast with an anterior granular part and a lamellar posterior part (Canning and Madhavi 1977, Canning *et al.* 1983). It is different from our species by its sporogony, the size and the ultrastructure of its spores.

Other microsporidia, initially classified in the genus *Nosema* and living in adult trematodes parasites of marine animals, were described. There are *Microsporidium distomi* Lutz *et* Splendore, 1908 (Canning 1975) and *Microsporidium spelotremae* Guyénot, Naville *et* Ponse, 1925 (Canning 1975).

Microsporidium distomi was described in adult trematode *Distomum lingulata*, parasite of the amphibian *Bufo marinus*. These spores measure 2.0 × 0.8-1µm (Sprague 1977). It was transferred by Canning (1975) in the *Microsporidium* group because it forms cysts and its development stages were not described.

Microsporidium spelotremae is hyperparasite of *Spelotrema carcini* parasite of the crab *Carcinus maenas*. Its fresh spores measure 3.5 × 1.5 µm and fixed measure 2.6-3 × 1.3-1.5 µm (Sprague 1977). It was moved by Canning (1975) in the *Microsporidium* group because its spores are sometimes grouped and its development stages were not described.

The present *Nosema* parasitizes essentially the epithelial gut. According to Larsson (1999), the digestive tissue is the first reached by the microsporidia. Next, the spores are going to extrude their polar filament to infect new tissues (Berrebi 1978). This polar filament extrusion is possible by an osmotic pressure increase inside the spore (Lom and Vavra 1963, Weidner and Byrd 1982). This hatching process of the spore was described at length by Toguebaye and Marchand (1987) in *Nosema couilloudi*.

An ultrastructural peculiarities of this new *Nosema* is the polaroplast organization. Such a polaroplast was observed in *Ameson atlanticum* parasite of the crab *Cancer pagurus* (Vivares and Azevedo 1988).

The *Nosema*, described here, differs from all *Nosema* species known in the trematodes. This species is new and we name it *Nosema diphterostomi* sp. n. after the type host, *Diphterostomum brusinae*.

Taxonomic summary

Type host: *Diphterostomum brusinae* (Digenea, Zoogonidae).

Type locality: Bonifacio Strait Marine Reserve, Corsican Mediterranean Coast.

Sites of infection: Intestinal cells and connective tissue.

Life cycle stages: Diplokaryotic. In close contact with the host cell cytoplasm.

Spores: Ovoid. 2.1 × 1.4 µm. Isofilar polar filament with 6-7 coils. Polaroplast with an outer part and a lamellar inner part. Posterior vacuole with amorphous electron-dense content.

Material deposited: In the laboratory "Parasites and Mediterranean Ecosystems" in the University of Corsica (France) No 72928, 10/03.

Etymology: Specific name alludes to the host genus.

Remarks: *N. diphterostomi* is a hyperparasite of the fish *Diplodus annularis* (Sparidae).

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