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Ultrastructural description of the life cycle of *Nosema monorchis* n. sp. (Microspora, Nosematidae), hyperparasite of *Monorchis parvus* (Digenea, Monorchiidae), intestinal parasite of *Diplodus annularis* (Pisces, Teleostei)

Céline Levron[a,*], Sonia Ternengo[a], Bhen Sikina Toguebaye[b], Bernard Marchand[a]

[a]*Laboratoire Parasites et Ecosystèmes Méditerranéens, Faculté des Sciences et Techniques, Université de Corse, B.P. 52, F-20250 Corte, France*
[b]*Laboratoire de Parasitologie, Département de Biologie animale, Faculté des Sciences et Techniques, Université C.A. Diop, Dakar, Sénégal*

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Abstract

Life cycle stages of a new species of the genus *Nosema* Naegeli, 1857 (Microspora, Nosematidae), were examined by light and electron microscopy. It parasitizes the gut and the uterus of the digenean *Monorchis parvus* (Monorchiidae), in *Diplodus annularis* (Pisces, Teleostei). All stages were in close contact with the cytoplasm of the host cell and were probably all diplokaryotic. The divisions of meronts and sporonts were recognizable by the formation of spindle plaques at the surface of the nucleus. Spores were oval, measured $3.2 \pm 0.3 \times 2.5 \pm 0.2$ µm on ultrathin sections, and had a polar filament with 16–17 coils. The polaroplast presented two parts: an anterior region with closely packed lamellae and a posterior part with wider lamellae. This *Nosema* species is compared with the other microsporidian parasites of digeneans. This new species is named *Nosema monorchis* n. sp., after the generic name of its host.

Keywords: Microsporidia; Nosematidae; *Nosema monorchis* n. sp.; Hyperparasite; Digenea

Introduction

The Microsporidia Balbiani, 1882 are unicellular parasites reported from every major group of animals and belong to the Phylum Microspora Sprague, 1977 (Larsson 1999). They are present in all invertebrate phyla (Canning 1990), including the Platyhelminthes, and hyperparasitism of Trematoda has been reported on numerous occasions. These microsporidian hyperparasites, belonging to the genera *Nosema* Naegeli, 1857, *Pleistophora* Gurley, 1893, *Unikaryon* Canning, Lai and Lie, 1974 and *Microsporidium* Balbiani, 1884, have been reviewed by Canning (1975). Ultrastructural data are available only for *Nosema eurytremae* (see Colley et al. 1975), *N. lepocreadi* (see Canning et al. 1983a), *N. diphterostomi* (see Levron et al. 2004), *Unikaryon legeri* (see Azevedo and Canning 1987) and *Unikaryon slaptonleyi* (see Canning et al. 1983b).

The parasitic fauna of Mediterranean fish and in particular of *Diplodus annularis* Linnaeus, 1758 (Pisces, Teleostei, Sparidae) was collected and studied. *Monorchis parvus* Looss, 1902 (Digenea, Monorchiidae) is an

*Corresponding author. Tel.: +33 4 95 45 00 29; fax: +33 4 95 45 00 29.
E-mail address: levron@univ-corse.fr (C. Levron).

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intestinal parasite of *D. annularis*. When studying the ultrastructure of *M. parvus*, we found some specimens, which were hyperparasitized by a microsporidium of the genus *Nosema*. The developmental stages are described as seen in light and electron microscopy and compared with other *Nosema* hyperparasites of digeneans.

Materials and methods

Several specimens of *D. annularis* were collected in the Bonifacio Strait Marine Reserve (41.20°N; 9.15°E, Mediterranean Sea, France) during the summers 2001 and 2002. After dissection and extraction, specimens of the digenean *M. parvus* were kept alive in a 0.9% NaCl solution.

Digeneans were fixed in cold (4 °C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2.

For light microscopy, fresh smears of infected specimens were examined under a microscope. Spores were photographed.

For electron microscopy the digeneans were rinsed overnight in 0.1 M sodium cacodylate buffer at pH 7.2, post-fixed for 1 h in cold (4 °C) 1% osmium tetroxide in the same buffer. After dehydration in ethanol and propylene oxide, the specimens were embedded in Epon and then polymerized at 60 °C for 48 h. Ultrathin sections (60–90 nm thick) were cut on an ultramicrotome (LKB 8800A), placed on copper grids and stained with uranyl acetate and lead citrate, according to Reynolds (1963). The grids were examined in an electron microscope (Hitachi H600) at 75 kV.

Results

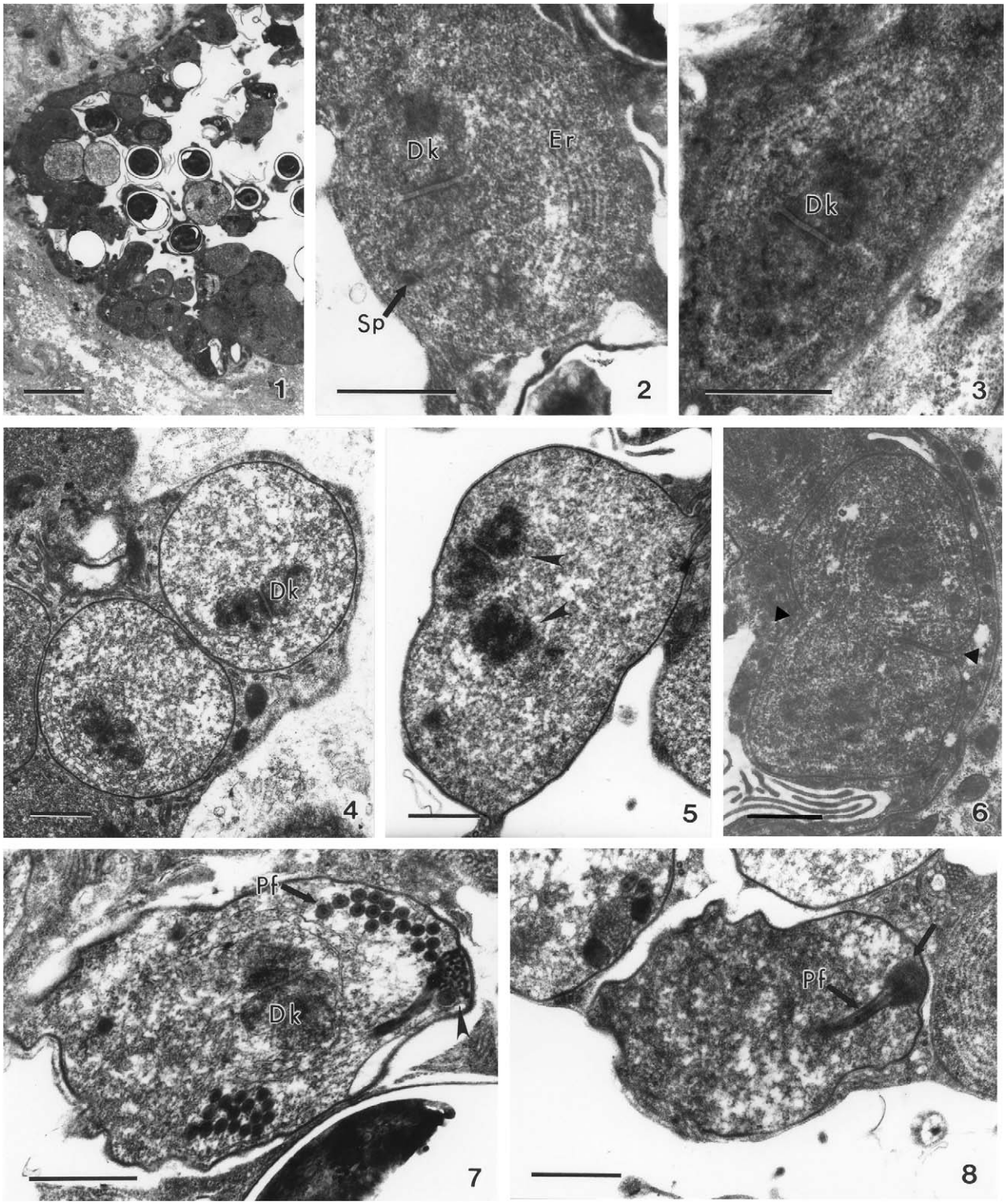
Among 16 specimens of *M. parvus* examined, only three were parasitized by the microsporidia. Examination of infected specimens revealed the presence of spores in digestive and uterine cells (Fig. 1). In some specimens, the infected tissues were hypertrophied and there remained only digenean eggs and microsporidian spores. All development stages of the parasite were in close contact with the host cell cytoplasm.

Meronts are the earliest stages observed in the life cycle of microsporidia. They were round or oval in shape and had one or two diplokarya (Figs. 2 and 3). They were not easy to recognize in the host cell because their electron opacity was identical to that of the surrounding host cytoplasm and their plasma membrane was very thin. Nevertheless, the greater opacity of the diplokaryon allowed us to distinguish them. Signs of the division of the diplokarya were observed in the form of depressions of the nuclear envelope, that were underlined by an internal layer of electron-dense nucleoplasm forming the spindle plaques (Fig. 2). Initially, two spindle plaques appear on each nucleus, near the contact zone between the two nuclei. These two spindle plaques migrate on the outer surface of the nucleus, then initiate the division of the diplokaryon. Meronts with two diplokarya are thus formed. The meront cytoplasm contains endoplasmic reticulum cisternae and numerous free ribosomes.

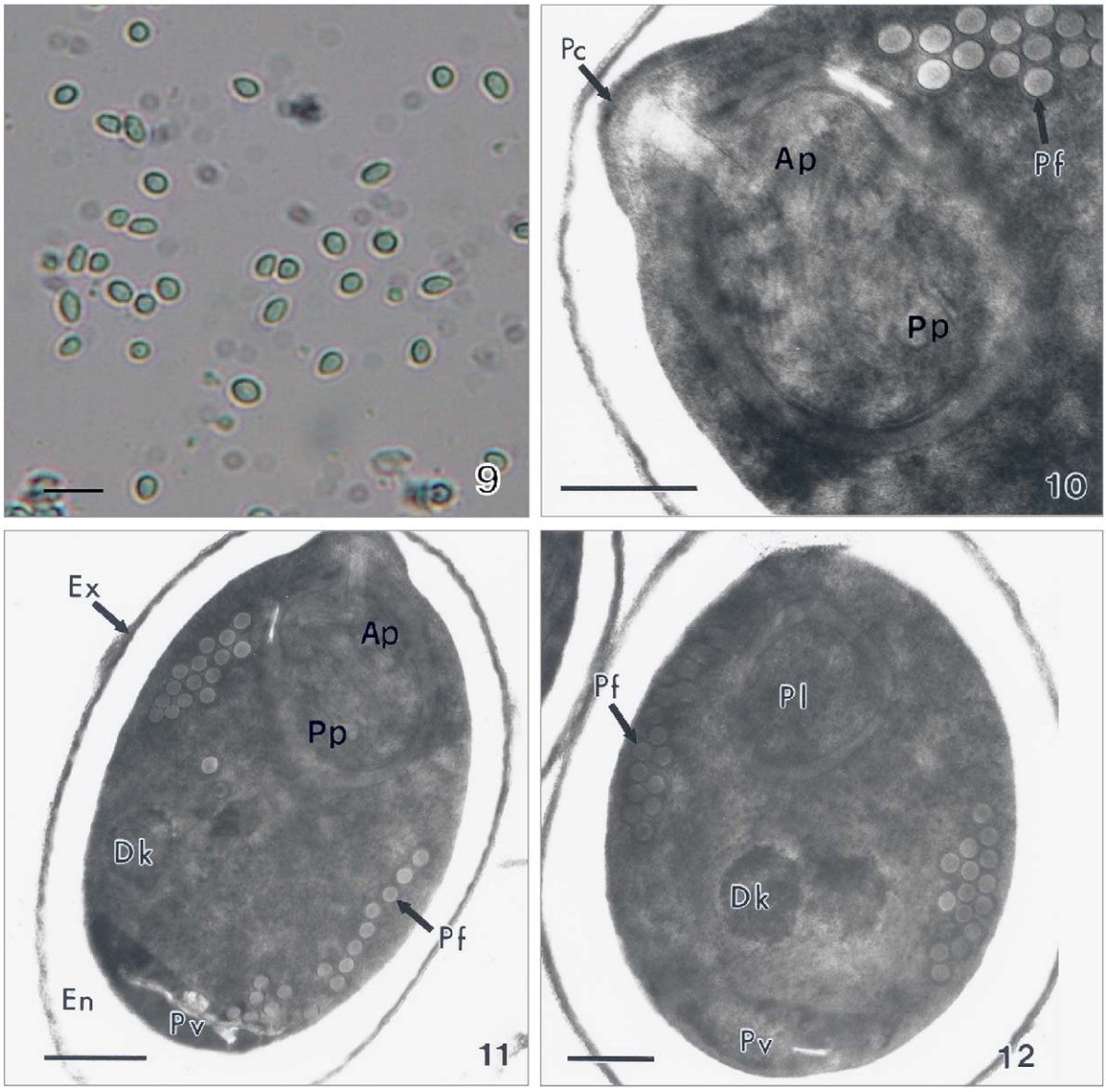
The sporonts (Figs. 4–6) have a wall, which is formed around their plasma membrane so that their limit appears thicker than that of meronts. One or two diplokarya, endoplasmic reticulum cisternae and free ribosomes are present in the sporont cytoplasm. The process of division is similar to that of the meronts. Spindle plaques are formed on the nuclear envelope of each nucleus, and are involved in the division of the diplokaryon. Before the final division, sporonts are formed in chains (Fig. 5). Thereafter, the sporonts, having two diplokarya (Fig. 5), undergo, by constriction, a final division of the cytoplasm (Fig. 6) giving rise to two sporoblasts.

The sporoblasts (Figs. 7 and 8) are more or less elongated and surrounded by a thick wall. They are distinguished from the sporonts by the progressive formation of the various components of the spore. The polar filament extends gradually from an electron-dense and compact apparatus, which correspond to the primordium of the polar cap (Fig. 7). This material forms a central axis and an electron-dense peripheral tube separated by a less electron-dense zone (Fig. 7). At the level of the anterior extremity of the sporoblast, the polar cap of the filament is elaborated (Fig. 8).

The spores are the last development stage of the microsporidia. They are oval (Fig. 9) and measure $3.2 \pm 0.3 \times 2.5 \pm 0.2\,\mu m$ ($n = 20$) in ultrathin sections; spores measured on the light micrographs of fresh spores were slightly larger. The spore wall is composed of two parts: an endospore and an exosopore. The electron lucent endospore is 180 nm thick, and the electron-dense exospore is 40 nm thick. The spores contain a diplokaryon, a polar filament, a polaroplast, a posterior vacuole and endoplasmic reticulum cisternae (Figs. 10–12). The polar filament is isofilar and arranged as 16–17 coils around the diplokaryon (Figs. 11 and 12). It is 90 nm in diameter. The polar filament is coiled with a sharp angle of tilt so that in some sections one group of coils lies close to the polaroplast (Figs. 10 and 11). The anterior extremity of the polar filament emerges at the level of the polar cap (Fig. 10). The polaroplast is situated in the anterior part of the spore. It presents two parts (Figs. 10 and 11): an anterior part with closely packed lamellae and a posterior part with wider or more irregularly arranged lamellae. The posterior region of the spore is characterized by the presence of a vacuole with an amorphous electron-dense content (Figs. 11 and 12).



Figs. 1–8. Electron micrographs of *Nosema monorchis* n. sp. **1.** Section of the gut epithelium of *Monorchis parvus* showing different development stages of the microsporidia. **2.** Spherical meront with one diplokaryon (Dk). Er: Endoplasmic reticulum, Sp: Spindle plaque. **3.** Oval meront with one diplokaryon (Dk). **4.** Sporonts with one diplokaryon (Dk). **5.** Sporont with two diplokarya (arrow heads). **6.** Sporont showing the constriction of the membrane (arrow heads). **7.** Sporoblast showing the primordium of the polar cap (arrow head). Dk: Diplokaryon, Pf: Polar filament. **8.** Sporoblast with the polar cap (arrow). Pf: Polar filament. Scale bars 5 μm in Fig. 1 and 1 μm in Figs. 2–8.



Figs. 9–12. Light and electron micrographs of mature spores of *Nosema monorchis* n. sp. **9.** Light micrograph of fresh spores. **10.** Longitudinal section of the anterior part of spore showing the organization of the polaroplast. Ap: Anterior part of the polaroplast, Pc: Polar cap, Pf: polar filament, Pp: Posterior part of the polaroplast. **11.** Longitudinal section of mature spore. Ap: Anterior part of the polaroplast, Dk: Diplokaryon, En: Endospore, Ex: Exospore, Pf: Polar filament, Pp: Posterior part of the polaroplast, Pv: Posterior vacuole. **12.** Longitudinal section of mature spore. Dk: Diplokaryon, Pf: Polar filament, Pl: Polaroplast, Pv: Posterior vacuole. Scale bars 10 μm in Fig. 9 and 0.4 μm in Figs. 10–12.

Discussion

The microsporidium examined in this study presents most the characters of the genus *Nosema* Naegeli, 1857: nuclei are diplokaryotic throughout the life cycle, all stages are in direct contact with the host cell cytoplasm, without a sporophorous vesicle, merogony is by binary or multiple fission and sporogony is disporoblastic (Sprague 1977, 1978; Vávra et al. 1981; Larsson 1986). Members of genus *Nosema* are an important and widely distributed group of microsporidia (Tsai et al. 2003), which have been reported in many hosts including digeneans (Larsson 1999).

Seven species of *Nosema* have been found in digeneans. They are *N. dollfusi* Sprague, 1964, *N. eurytremae* Canning, 1972 (syn. *Perezia helminthorum* Canning and Basch, 1968), *N. gigantica* Canning and Madhavi, 1977, *N. lepocreadii* Canning and Olson, 1980,

N. strigeoideae Hussey, 1971, *N. xiphidiocercariae* Voronin, 1974 and *N. diphterostomi* Levron, Ternengo, Toguebaye and Marchand, 2004. Three of these species were reported in the digeneans of fish. They are *N. gigantica*, hyperparasite of *Aplocheilus melastigma* (Cyprinodontiformes, Aplocheilidae in freshwater), *N. lepocreadii*, hyperparasite of *Leuresthes tenuis* (Atheriniformes, Atherinidae in salt water) and *N. diphterostomi*, hyperparasite of *D. annularis* (Perciformes, Sparidae in salt water) (Table 1).

Nosema gigantica is a parasite of *Allocreadium fasciatusi* (Allocreadiidae) and *N. lepocreadii* lives in *Lepocreadium album* (Lepocreadiidae). The hosts and hyperhosts of these two species are taxonomically distant from the host and hyperhost of the species studied here. *N. diphterostomi* and the species described here possess the same hyperhost and the same geographical localization. However, their hosts are different. *Nosema diphterostomi* lives in *Diphterostomum brusinae* (Zoogonidae) and the *Nosema* described in this study infects *M. parvus* (Monorchiidae).

The morphology, dimensions and ultrastructure of spores are useful criteria used in microsporidian classification (Larsson 1986). As seen in Table 1, the *Nosema* described here differs from previous ones by many features: hosts, hyperhosts, localities, dimensions of spores, number of coils of the polar filament and structure of the polaroplast. However, the study technique influences the description of the spore. It is thus difficult to compare our results with results from optical microscopy. Only three species have been examined by electron microscopy: *N. diphterostomi* (Levron et al. 2004), *N. eurytremae* (Colley et al. 1975) and *N. lepocreadii* (Canning et al. 1983a). In these species, the coils of filament are bunched in a single rank, contrary to our species, which possesses the highest number of polar filament coils. Moreover, in the present species one group of coils can be found near to the polaroplast as in *U. slaptonleyi* (Canning et al. 1983b). The subdivision of the polaroplast into anterior and posterior parts that occurs in our species is also seen in all three of the other species studied by electron microscopy, but the arrangement in the remaining species is not known.

We consider therefore that this species is new and we propose to name it *N. monorchis* n. sp. after the generic name of its host.

Taxonomic summary

Type host: *Monorchis parvus* (Digenea, Monorchiidae).

Type locality: Bonifacio Strait Marine Reserve, Corsican Mediterranean Coast. (41.20°N; 9.15°E).

Sites of infection: gut and uterus.

Table 1. *Nosema* species described from digeneans

Nosema species	Host	Hyper-host	Spore size (µm)	Number of polar filaments	Internal organization	Locality	References
N. diphterostomi	*Diphterostomum brusinae*	*Diplodus annularis*	2.1 × 1.4 (TEM section)	6–7	Polaroplast with two regions	Corsica France	Levron et al. (2004)
N. dollfusi	*Bucephalus cuculus*	*Crassostrea virginica*	3 × 1.7 (LM fixed)			Maryland USA	Sprague (1964)
N. eurytremae	*Eurytrema pancreaticum*; *Postharmostomum gallinum*	*Bradybaena similaris*	3.94 × 2.26 (TEM section)	11–12	Polaroplast with two regions; no posterior vacuole	Malaysia	Colley et al. (1975)
N. gigantica	*Allocreadium fasciatusi*	*Aplocheilus melastigma*	7.9 × 4.9 (LM fixed)			India	Canning and Madhavi (1977)
N. lepocreadii	*Lepocreadium manteri*	*Leuresthes tenuis*	3.5 × 1.5 (TEM section)	10	Polaroplast with two regions	San Diego USA	Canning and Olson (1980), Canning et al. (1983a)
N. monorchis n. sp.	*Monorchis parvus*	*Diplodus annularis*	3.2 × 2.5 (TEM section)	16–17		Corsica France	Present study
N. strigeoidea	Twelve species	Ten species snails	4.7 × 3.1 (LM fresh)			Michigan USA	Hussey (1971)
N. xiphidiocercariae	Plagiorchiidae	*Lymnaea Palustris*	4.5 × 2.3 (LM fresh)			Moscow Russia	Sprague (1977)

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

Spores: Ovoid. $3.2 \pm 0.3 \times 2.5 \pm 0.2\,\mu m$ in TEM sections. Isofilar polar filament with 16–17 coils. Polaroplast with an anterior part and a posterior part. Posterior vacuole with amorphous electron-dense content.

Material deposited: In the laboratory "Parasites and Mediterranean Ecosystems" in the University of Corsica (France). Grids No. 70,912.

Etymology: Specific name alludes to the host genus.

Remarks: *Nosema monorchis* is a hyperparasite of the fish *Diplodus annularis* (Sparidae).

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