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ORIGINAL PAPER

Ultrastructure, development, and host–parasite relationship of a new species of the genus *Pleistophora*—a microsporidian parasite of the marine fish *Epinephelus chlorostignei*

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Abstract The life cycle of a new microsporidian of the genus *Pleistophora* is described. This parasite infects the epithelial cells of the gut and the peritoneal cavity of the Red Sea fish, *Epinephelus chlorostignei.* All stages develop within a special structure, the sporophorocyst, which is covered by a thick dense wall. This wall grows along with the growth of the parasites inside. Meronts are uni- to binucleate, which divide and constantly give rise to sporonts. During transition to sporonts, the cell border of the meronts increases its thickness, temporarily featuring thick irregular projections. Eventually, a uniform thick sporont wall is formed; then, the sporont cells detach themselves from the wall (future wall of the sporophorous vesicle, SPV) and start a series of divisions to produce sporoblasts. The SPV wall is compact, has no pores, and consists of two layers. Mature spores measure about 2.0× 1.8 μm. They possess a polar filament with 20–28 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 microsporidian parasite belongs to the genus *Pleistophora*.

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Introduction

The term microsporidia refers to a group of obligate intracellular protists that belong to the protozoan phylum microspora. These pathogens have been noted for many years to cause striking deformations in infected fish and other species and are serious pests of silkworms and honey bees (Weber et al. 2000; Wittner and Weiss 1999). More than 1,200 species of microsporidia are known infecting members of almost each major phylum of the animal kingdom and entering a variety of cell types (Weber et al. 1994).

About 156 species belonging to 14 genera of the phylum microsporidia (Balbiani 1882) have been described from fishes, and several cause severe disease. These parasites are widely distributed by both host species and geographical location. Whereas, most fish microsporidia are host specific, at least at the genus level, few show broad host specificity (Schubert 1969; Morrison and Sprague 1981; Faye et al. 1991, 1996; Hedrick et al. 1991; Lom et al. 2000a).

Comprehensive lists of fish hosts and microsporidian species appear in Canning and Lom (1986); Lom and Dyková (1992); Dyková (1995); and Lom (2002).

The generic and specific differentiation of these parasites are often aided by ultrastructural data (Canning and Lom 1986). Keys for generic differentiation were prepared by Weiser (1989) and Pekkarinen et al. (2002).

Microsporidia have been known to produce xenomas in many invertebrate animals and ectothermia vertebrates,

especially fishes. The study of microsporidia causing xenomas in fish offers an insight into cell pathology and is of interest since many of them are important agents of diseases in commercial fish (Lom and Dyková 2005).

Microsporidian parasites of the genus *Pleistophora* (Gurley 1893) are important parasites of fish and crustacean causing serious disease in marine and fresh water fish. This genus is originally erected for a species described from fish muscles (Matthews and Matthews 1980; Lom and Nilsen 2003).

The key feature of the genus *Pleistophora* is the presence of a sporophorocyst, which represents a dense, rather solid wall enclosing, in all developmental stages of the parasite, i.e., meronts, sporonts, and sporophorous vesicles with sporoblasts and spores (Lom and Dyková 1992, 2005). Microsporidia are now recognized as significant contributors to mortality in fishes (Kent and Poppe 1998; Shaw and Kent 1999).

The present light and ultrastructural study describes some aspects of xenomas (containing developmental stages) and the events of spore maturation of an apparently new microsporidian species, the morphological characteristics of which are discussed comparatively with special reference to the ultrastructural aspects of the extrusion of the polar tube.

Materials and methods

Freshly caught living specimens of the fish, *Epinephelus chlorostignei*, were collected from boat landing sites or sometimes from the market places of Suez and Hurghada at the Gulf of Suez and Red Sea, respectively. A total of 180 fishes was examined. Descriptions and measurements of spores were done according to the guidelines of Lom and Arthur (1989). Measurements were based on 30 spores, and the range of the data is presented here. Infection was determined by the presence of xenomas located along the wall of the abdominal cavity being recognizable by the naked eye. Measurements of xenomas and fresh spores were made in wet mount preparations. After crushing the xenoma, native spores and Giemsa stained probes were examined under the light microscope. For transmission electron microscopy, the xenomas and surrounding tissues were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 20–24 h, rinsed overnight in the same buffer at 4°C, and postfixed in 2% OsO_4 in the same buffer at 4°C for 4 h. After dehydration in an ascending ethanol series (70%, 80%, 90%, 95%, and 100% staying 2 h in each stage) and in propylene oxide (two changes for 3 h each), the infected tissues were embedded in Epon (staying 10–12 h in each change). Semithin sections were stained with toluidine blue. Meanwhile, ultrathin sections were examined with a Zeiss 902A transmission electron microscope.

Results

Light microscopic studies

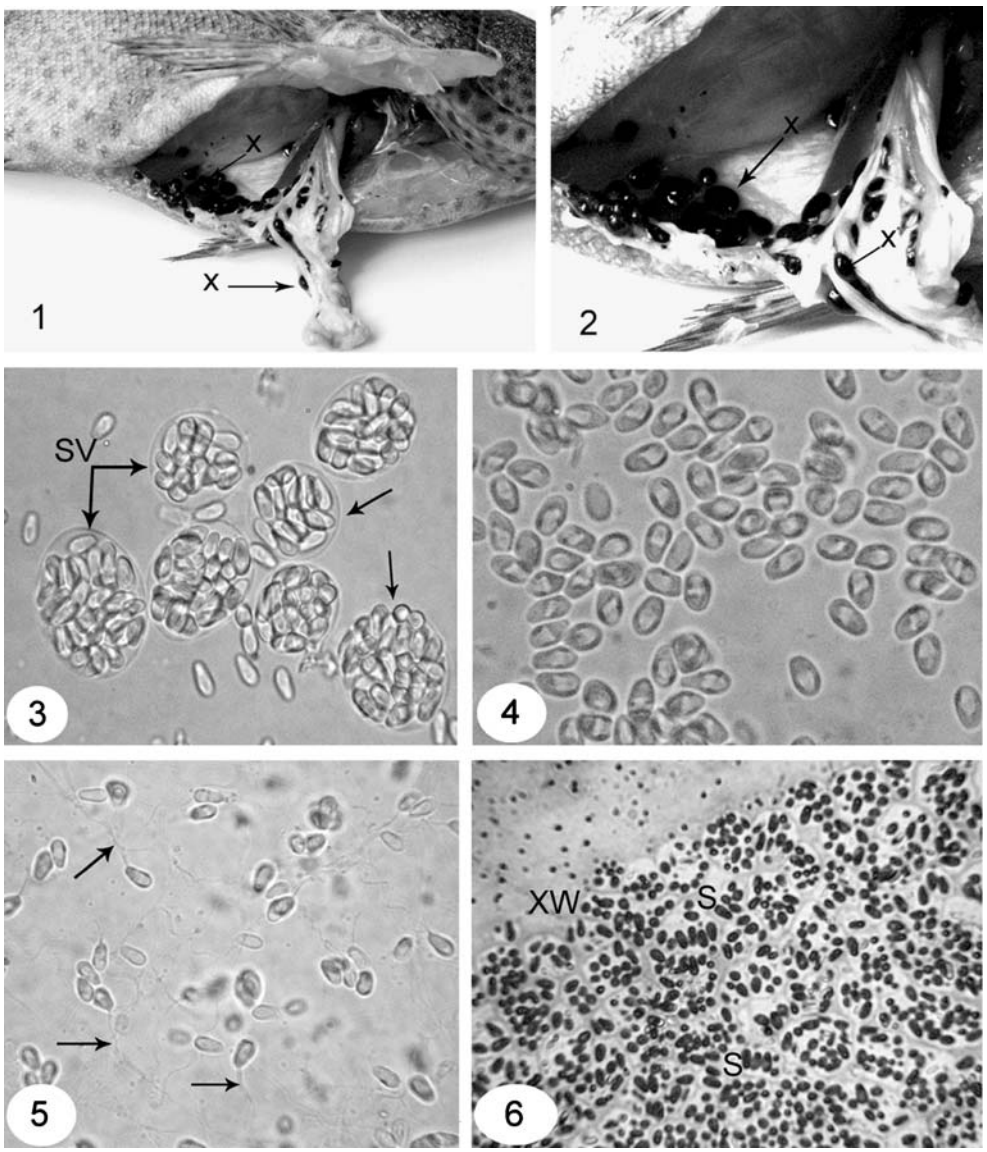
Numerous macroscopic, black cysts (xenomas)—ranging in size from 3 to 5 mm—were observed throughout the peritoneal cavity of the infected fish being embedded in different organs (Fig. 1 (1, 2)). These xenomas appeared spherical in shape. At high magnification, it was observed that the xenomas were limited by a thick wall encircling numerous spores within the cytoplasm of the hypertrophic host cell. After dissection and rupture of the xenoma, secondary xenomas where the spores are found within the sporophorous vesicle were observed. Free ellipsoidal spores were identified to belong to the phylum Microsporidia (Fig. 1 (3, 4)). Histological observations showed that the parasitic foci were encapsulated by a host-derived fibrous membrane being filled with mature spores (Fig. 1 (6)).

In wet mounts, fresh spores appeared mostly ovoid to pyriform in shape reaching a size of 2.0±0.5 (1.8–3.0) µm×1.8±0.2 (1.5–2.1) µm. In addition, they possessed a large vacuole at the posterior end (Fig. 1 (4)). Fresh spores were able to eject spontaneously or under pressure their filament (Fig. 1 (5)). These spores were located within a sporophorous vesicle which was bound by a dense envelope—a thick amorphous wall. This sporophorous wall became enlarged during the growth and the division of the parasites inside.

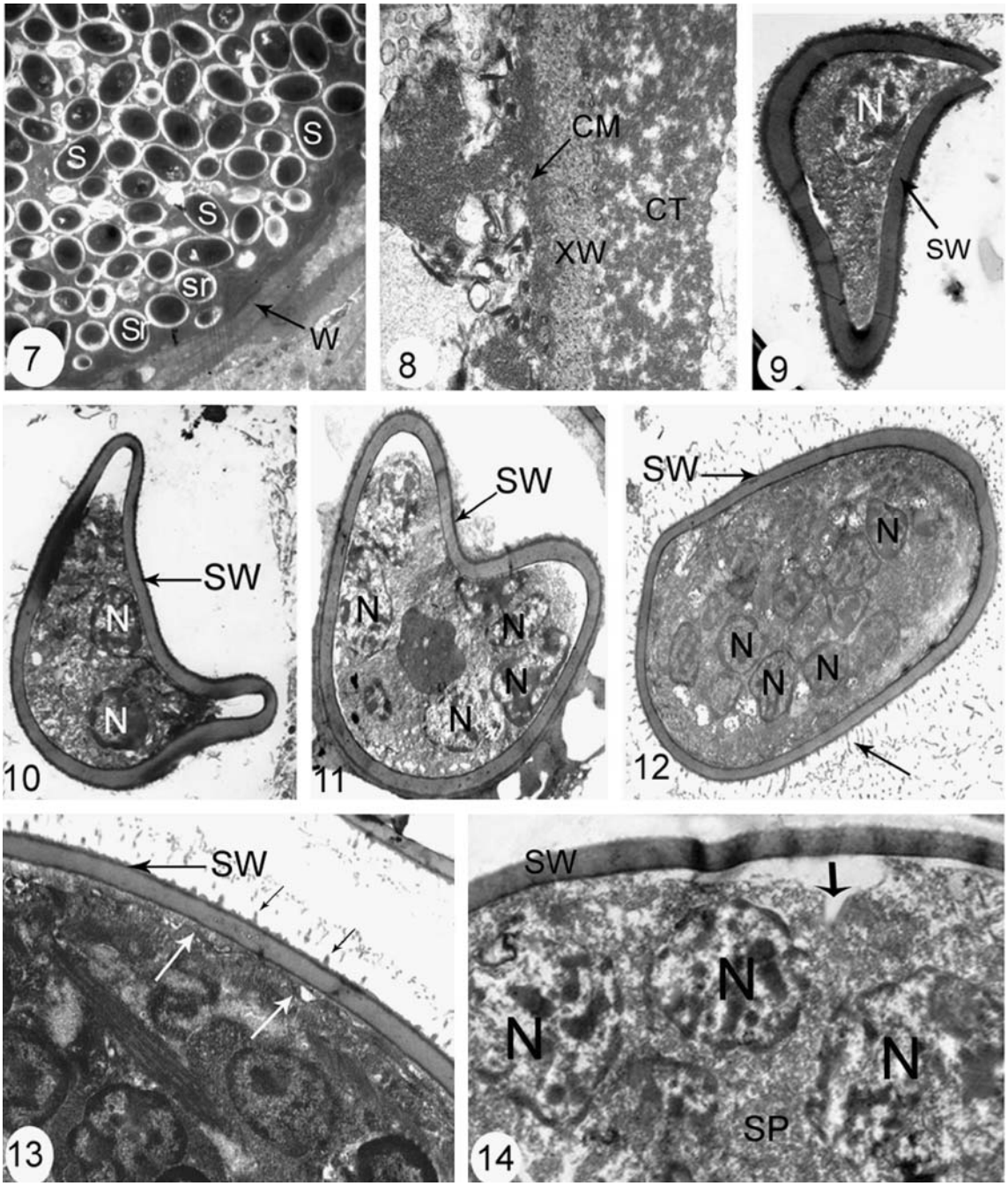
Electron microscopic studies

The first stage of parasite development observed was the uninuclear meront with a spherical nucleus (Fig. 2 (9)), which divides to produce binucleated meronts with two spherical nuclei (Fig. 2 (10)). Additional multinucleated meronts with six to 12 irregularly shaped nuclei arose by plasmotomy constantly producing new sporonts (Fig. 2 (11, 12)). The next step was the detachment of the plasmalemma of the sporont from the sporophorous vesicle (SPV) wall (Fig. 2 (13)). This space became filled with a fine granular substance containing strands of the endoplasmic reticulum. The sporont segmented into separate sporoblasts (Figs. 2 (14) and 3 (15, 16)). Prior to cell division, uninucleated cells marked by the presence of the centriolar plaque at the nuclear envelope of the cell were observed (Fig. 3 (17)). The multinucleated sporont or sporogonial plasmodium produced several sporoblasts by plasmotomy (Fig. 3 (18)). The development proceeded by the formation of advanced stages of sporo-

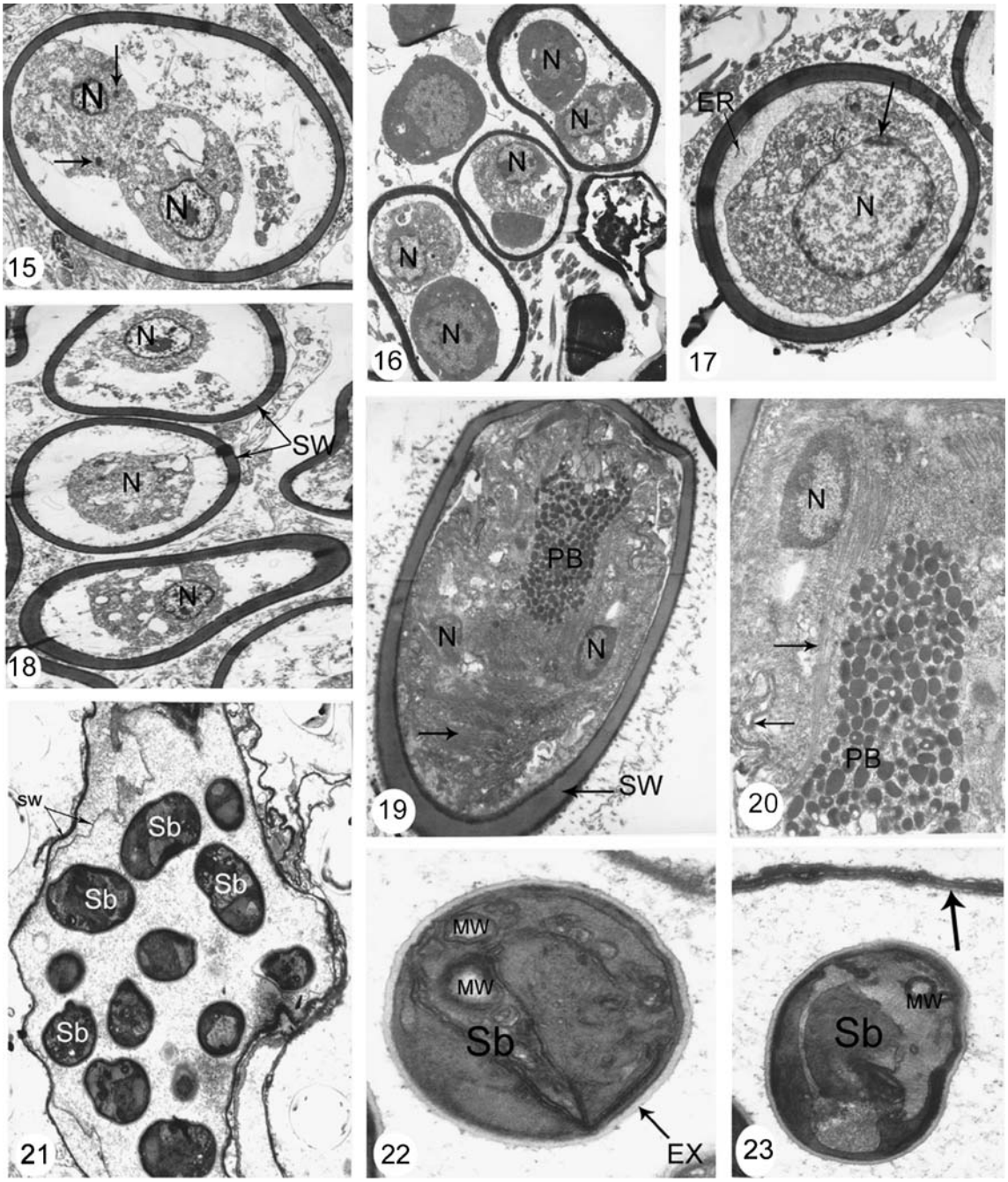
blasts containing already most of the typical structures of spores including the exospore (Fig. 3 (19, 20)). The latter appeared electron pale. It became separated from the sporoblast to build a dense exospore around the mature spore (Fig. 3 (21, 22, 23)). An amazing feature of the present *Pleistophora* species is the presence of large membrane-bound vacuoles containing electron dense material. These vacuoles are called paramural bodies and were evenly distributed in the spore cavity (Fig. 4 (24)). Furthermore dense substances were irregularly distributed in the matrix of the spore. These substances and several membranous structures may participate in the formation of the endospore (Fig. 3 (20)). These dense substances may fuse at the outer side of the immature spore contributing to the formation of the endospore (Fig. 4 (25, 26)). Such structures were not observed in the mature stages of the spore (Fig. 4 (27, 28, 29)). Mature spores appeared electron dense, uninucleate, and were ellipsoidal in shape (Fig. 4 (28, 29)). At the anterior end of the spore, the anchoring disk was found in a central position (Fig. 4 (30)). There was a definite number (23–28) of turns of the polar tube. These turns circled around the large future posterior vacuole (Fig. 4 (28)). The polaroplast consisted of an anterior region of closely packed membranes and a posterior region comprising



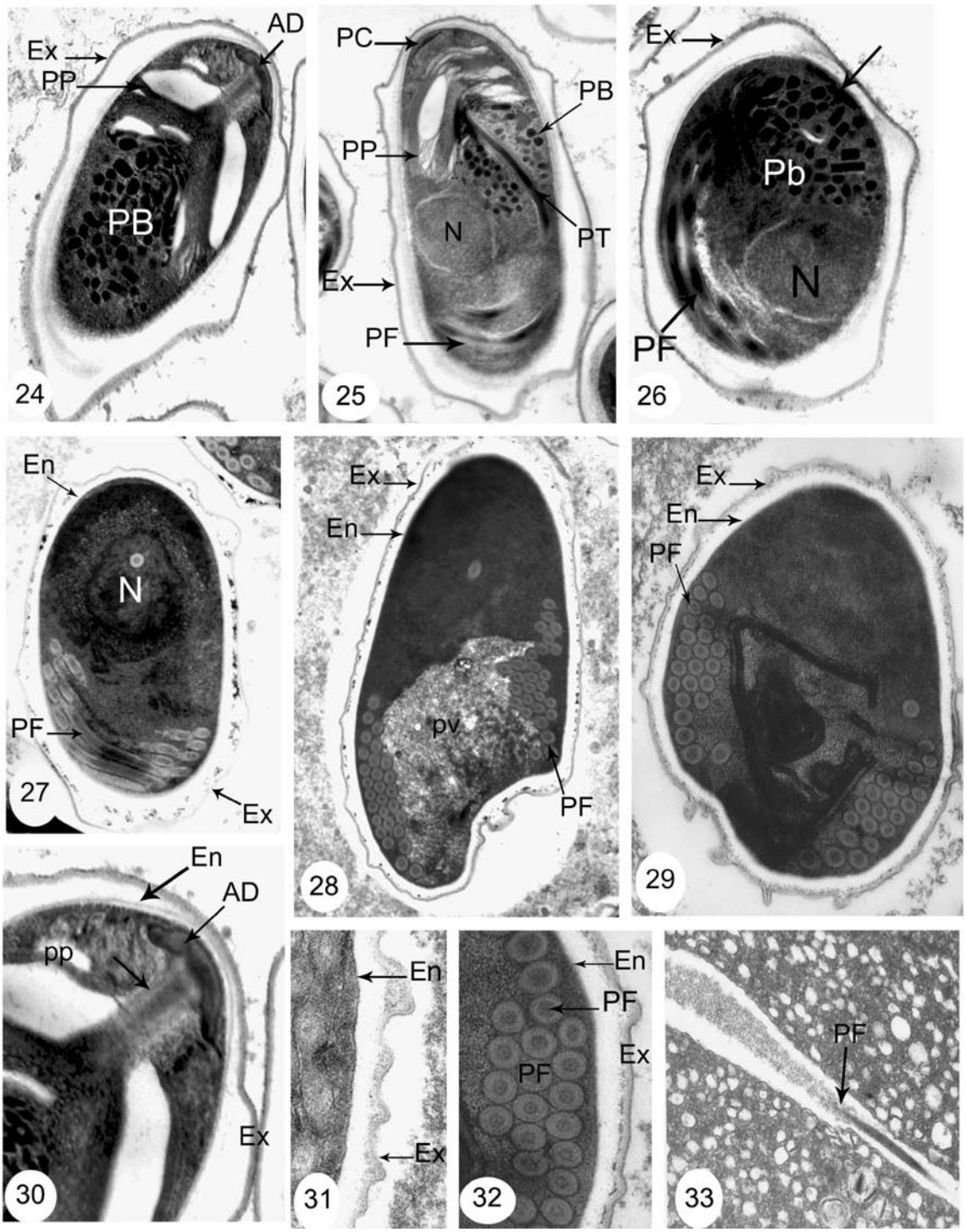
Figs. 1–6 *1*, *2* Photographs showing the large xenomas (*X*) of *Pleistophora* sp. in the body cavity of infected fish. *3*, *4* Photomicrographs of fresh spores after rupture of the xenoma showing the secondary xenomas (*arrows*) where the spores are still found within the *SV* (*3*) or after the release of the spores from the *SV* (*4*); ×2,500. *5* Photomicrograph of fresh spores showing the ejected polar filament (*arrows*); ×2,300. *6* Photomicrograph of a semithin section of a part of the xenoma filled mainly with spores (*S*) and surrounded with xenoma wall (*XW*); ×2,000. *SV* sporophorous vesicle



Figs. 7–14 *7* Transmission electron micrograph showing the periphery of a xenoma showing the sporont (*Sr*), spores (*S*), and the xenoma wall (*XW*); ×5,000. *CT* a layer of connective tissue as manifestation of the host response, *XW* refractile xenoma wall is composed of layers of the cell coat, *CM* cell membrane of the xenoma. *8* Transmission electron micrograph showing the periphery of a xenoma showing the surrounding layers; ×30,000. *9*, *10* Transmission electron micrographs showing meronts of *Pleistophora* sp. with one (uninucleate meront) or two (binucleate meront) nuclei (*N*) being each surrounded with a sporophorous wall (*SW*); ×4,400. *11*, *12* Transmission electron micrographs showing multinucleated meronts with 4–12 nuclei surrounded with sporophorous wall (*SW*). Note the temporarily thick irregular projections (*arrow*); ×7,000 and ×4,400. *13* Transmission electron micrograph showing the plasmalemma (*arrows*) of the sporont detached from the sporophorous wall (*SW*). Note the temporarily thick irregular projections (*black arrows*); ×20,000. *14* Transmission electron micrograph showing early phase of division of the sporogonial plasmodium (*SP*) surrounded by sporophorous wall (*SW*) with many nuclei (*N*) and undergoing cytoplasmic cleavage (*arrow*) to form binucleated cells; ×30,000



Figs. 15–23 *15* Transmission electron micrograph showing binucleated sporont undergoing cytoplasmic cleavage to form further uninucleated sporonts. Note the starting of sporogenesis by the appearance of dense bodies (*arrows*) within the sporont; ×12,000. *16* Transmission electron micrograph showing uninucleated cells undergoing another cell division before becoming sporoblasts. Some of these cells completed the division and divided into two sporonts and others still with cytoplasmic connection; ×6,000. *17* Transmission electron micrograph showing uninucleated cell prior to cell division, which is marked by the presence of the centriolar plaque at the nuclear envelope of the cell (*arrows*); ×15,000. *18* Transmission electron micrograph showing uninucleated sporonts surrounded with a sporophorous wall (*SW*) being ready for the start of sporogenesis; ×12,000. *19* Transmission electron micrograph showing early sporoblast containing two nuclei (*N*) with the appearance of paramural bodies (*PB*) and membranous structures (*arrow*) as precursors of the spore membranes and polar filament coils; ×20,000. *20* Transmission electron micrograph showing enlarged part of the early sporoblast showing the dense bodies (*PB*) and membranes (*arrows*); ×20,000. *21* Transmission electron micrograph showing overview of a part of a sporophorocyst of *Pleistophora* sp. with at least 13 sporoblasts (*Sb*) in different phases of maturation being surrounded by a sporophorous wall (*SW*) composed of a double layer. Note the double layers of sporophorous wall (*SW*; *arrows*); ×7,000. *22*, *23* Transmission electron micrographs showing enlarged sporoblasts (*Sb*) in an advanced stage of development showing the future exospore (*Ex*) appears pale. Note that the endospore and the filament coils are not yet formed; ×20,000. *N* nuclei, *ER* endoplasmic reticulum, *N* nucleus. *MW* membrane whorls



a series of loosely packed membranes (Fig. 4 (25)). The possibility of an autoinfection of the same host exists when the polar tube becomes extruded. Fully formed xenomas were surrounded by a thick osmiophilic electron dense wall enclosing numerous sporonts and mature spores. This wall was surrounded by thick layer of connective tissue as manifestation of the host response (Fig. 2 (7, 8)).

Discussion

The above-described stages of the present *Pleistophora* species differs from previously known ones in some aspects, but they resemble them with respect to the presence of a sporophorous vesicle, to the appearance of the wall, and the procedure of development (Pekkarinen 1996; Lom and Dyková 2005).

◀ **Figs. 24–33** *24* Transmission electron micrograph showing an immature spore surrounded by the exospore (*Ex*) showing the anchoring disk (*AD*) and the paramural bodies (*PB*) that appeared in the matrix of the spore; ×20,000. *25* Transmission electron micrograph showing the ultrastructure of a nearly mature uninucleate spore with the polaroplast (*PP*) that reveals two portions—the anterior one consists of tightly arranged membranes and the posterior one consisting of loosely arranged membranes and filament coils arranged in two rows. The polar cap (*PC*), the polar filament (*PF*), and the polar tube (*PT*) are shown. Note that some of the paramural bodies (*PB*) begin to move toward the membrane being ready to fuse with the membrane for the formation of the endospore; ×20,000. *26* Transmission electron micrograph showing the ultrastructure of a nearly mature uninucleate spore. Some of the bodies already fused with the spore membrane (*arrows*); ×20,000. *27* Transmission electron micrograph showing an *oblique* section through the mature uninucleate spore with polar filament coils (*PF*); ×20,000. *28*, *29* Transmission electron micrographs showing *longitudinal* section of a spore showing the spore wall, the polar filament (*PF*) with 23–28 turns, and the posterior vacuole (*PV*); ×20,000. *30* Transmission electron micrograph showing the anchoring disk (*AD*) at the spore apex showing the lucent layer of the shaft (*arrow*) of the future polar tube; ×30,000. *31* Transmission electron micrograph showing details of the spore wall, exospore (*Ex*), and endospore (*En*); ×30,000. Note the multilayered cover of the exospore (*Ex*). *PF* polar filament. *32* Transmission electron micrograph showing details of the polar filament coils of a spore with a long polar filament (*PF*); ×30,000. *33* Transmission electron micrograph showing fresh spores showing the extruded polar filament (*PF*); ×20,000. *PP* polaroplast, *Ex* exospore, *N* nuclei, *PB* paramural bodies, *En* endospore

Fish microsporidia are embedded directly in the host tissues, which they destroy by induction of an enormous hypertrophy of the infected cell. Infections with the present species were detected by the occurrence of black xenomas distributed in the body cavity of the infected fish. Similar observations have been recorded by Canning (1976) and Weissenberg (1976). Both authors stated that in fish, a peculiar type of host–parasite relationship occurs when microsporidia induce the development of xenomas. These results also were documented by Maurand et al. (1988) in *Pleistophora longifilis*, which infects the testes of *Barbus barbus*. Summerfelt (1964) described similar processes in *Pleistophora ovarial* which infect the ovary of *Pimephales promelas*. Thus it is a common feature that fish host form layers of membranes around the parasite xenoma. This term, introduced by Weissenberg (1949), characterizes a wall of host origin surrounding the dividing parasites in the parasitized host cell, which later becomes hypertrophic. In the cytoplasm of the hypertrophic host cell, the parasite divides repeatedly producing an enlarged xenoma with numerous spores and other life cycle stages (Morrison and Sprague 1981; Lom and Pekkarinen 1999). In a paper by Lom (2002), a complete list of microsporidians parasitic in fish was published. There are numerous listed microsporidian species, but only few cause xenomas in fish (Dyková and Lom 1978; Lom and Pekkarinen 1999; Azevedo and Matos 2002).

Both parasite and host seem to benefit from the formation of a xenoma. The parasite obtains optimal growth conditions and protection against host attacks when masking its surface with host components. The host gets benefits by confining the parasite and limiting the parasite's spread (Lom and Dyková 1992). The genus *Pleistophora* (Gurley 1893) comprises multinucleate meronts being produced by plasmotomy and by formation of a thick amorphous wall (Lom and Dyková 1992). At the onset of sporogony another—central layer—appears within the wall, which then separates from the sporont's surface and develops the thick wall (Lom and Dyková 1992, Lom et al. 2000a, b). Sporonts grow into multinucleated sporogonial plasmodia. The sporogony runs as a polysporoblastic process. Inside the SPV wall, the plasmodia divide stepwise into uninucleate sporoblasts, which finally mature into spores as recorded by Lom and Dyková (1992).

The present species is characterized by peculiar organelles—the so-called paramural bodies—which are associated with the inner side of the cell membrane. The presence of these bodies, which apparently assist in the formation of the sporoblast wall and the endospore, is one of the reasons to keep this species as a new one of the genus *Pleistophora*.

Sporonts that give rise to multinucleated plasmodia were also described by T'sui and Wang (1988). Inside the cyst, sporophorous vesicles or pansporoblasts occur in different stages of development. These observations are in accordance with the findings of Lom et al. (2000a, b). The SPV wall is detached from the parasite's surface, thus, forming an empty space. The new structure is called sporophorous vesicle (Lom and Dyková 1992).

A small posterior vacuole is seen in the present species. Similar observations were recorded by Canning and Nicholas (1980) in *Pleistophora typicalis*, where also a vacuole appears at both ends of the spore. The polaroplast is the first apparent vacuole. Similar structures were reported by Lom and Corliss (1997). The center of the filament is filled with an electron dense substance. A similar observation was reported by Kudo and Daniels (1963) from *Thelohania calefornia* and by Lom and Corliss (1997) from *Pleistophora hyphessobryconis*.

Only very few cases of autoinfection inside a host are known involving an extrusion of polar tubes within fish xenomas (Lom and Pekkarinen 1999). One of the first observations of sporoplasm extrusion in microsporidia was published by Lom and Vávra (1963). Later, some ultrastructural studies contributed to the understanding of this complex process (see Canning et al. 1992; Magaud et al. 1997; Cali and Takvorian 1999; Keohane and Weiss 1999; Shaw and Kent 1999).

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