

Paranucleospora theridion n. gen., n. sp. (Microsporidia, Enterocytozoonidae) with a Life Cycle in the Salmon Louse (*Lepeophtheirus salmonis*, Copepoda) and Atlantic Salmon (*Salmo salar*)

STIAN NYLUND,^a ARE NYLUND,^a KUNINORI WATANABE,^a CARL E. ARNESEN,^b and EGIL KARLSBAKK^c

^aDepartment of Biology, University of Bergen, 5020 Bergen, Norway, and

^bFirda Seafood AS, 5966 Eivindvik, Norway, and

^cInstitute of Marine Research, P.O. Box 1870, Nordnes, 5817 Bergen, Norway

ABSTRACT. *Paranucleospora theridion* n. gen., n. sp., infecting both Atlantic salmon (*Salmo salar*) and its copepod parasite *Lepeophtheirus salmonis* is described. The microsporidian exhibits nuclei in diplokaryotic arrangement during all known life-cycle stages in salmon, but only in the merogonial stages and early sporogonial stage in salmon lice. All developmental stages of *P. theridion* are in direct contact with the host cell cytoplasm or nucleoplasm. In salmon, two developmental cycles were observed, producing spores in the cytoplasm of phagocytes or epidermal cells (Cycle-I) and in the nuclei of epidermal cells (Cycle-II), respectively. Cycle-I spores are small and thin walled with a short polar tube, and are believed to be autoinfective. The larger oval intranuclear Cycle-II spores have a thick endospore and a longer polar tube, and are probably responsible for transmission from salmon to *L. salmonis*. Parasite development in the salmon louse occurs in several different cell types that may be extremely hypertrophied due to *P. theridion* proliferation. Diplokaryotic merogony precedes monokaryotic sporogony. The rounded spores produced are comparable to the intranuclear spores in the salmon in most aspects, and likely transmit the infection to salmon. Phylogenetic analysis of *P. theridion* partial rDNA sequences place the parasite in a position between *Nucleospora salmonis* and *Enterocytozoon bieneusi*. Based on characteristics of the morphology, unique development involving a vertebrate fish as well as a crustacean ectoparasite host, and the results of the phylogenetic analyses it is suggested that *P. theridion* should be given status as a new species in a new genus.

Key Words. Electron microscopy, *Enterocytozoon bieneusi*, merogony, *Nucleospora salmonis*, parasite, phylogeny, sporogony.

MANY genera of microsporidia contain species that infect fish, but only three species with intranuclear development have been named: *Microsporidium rhabdophilia*, *Nucleospora salmonis*, and *Nucleospora secunda* (Hedrick, Groff, and Baxa 1991; Lom and Dykova 2002; Modin 1981). In addition, intranuclear development of unnamed *Nucleospora* spp. has been described from lymphocyte-like cells in the lumpfish, *Cyclopterus lumpus* (Mullins et al. 1994), and from lymphoblasts in farmed Atlantic halibut, *Hippoglossus hippoglossus* (Nilsen, Ness, and Nylund 1995). A *Nucleospora*-like intranuclear microsporidian was also detected in inflammatory cells in X-cell pseudotumours in the English sole, *Pleuronectes vetulus* (Gresoviac et al. 2007). Intranuclear development has been observed in *Enterospora* spp., microsporidians infecting the hepatopancreatocytes of marine decapod crustaceans (Stentiford and Bateman 2007; Stentiford et al. 2007). Also, a *Nucleospora*-related microsporidian has been detected in *Lepeophtheirus salmonis*, a copepod ectoparasite of salmonid fish (Freeman, Bell, and Sommerville 2003). None of the intranuclear microsporidians has been associated with gill and skin disease in fish. Important microsporidian gill parasites in salmonids belong to the genus *Loma* (Wittner and Weiss 1999).

Production of Atlantic salmon in Norway has for many years suffered from several diseases resulting in inflammation of different tissues like gills, heart, and skeletal muscle. These diseases have been named proliferative gill inflammation (PGI), pancreas disease (PD), heart and skeletal muscle inflammation (HSMI), and cardiac myopathy syndrome (CMS) (Ferguson, Poppe, and Speare 1990; Kongtorp et al. 2004; Kvællestad et al. 2005; Watanabe et al. 2006). It has been shown that a salmonid alphavirus plays an important role in the development of PD (McLoughlin et al. 1996), and it is believed that other viruses may be responsible for CMS, HSMI, and some cases of PGI (Kongtorp et al. 2004; Watanabe et al. 2006). However, recently we discovered that a microsporidian infection in salmon is normally present in cases with these diseases, in high concentrations in some tissues with pathology commonly ascribed to those diagnoses. In this study we

describe a new species of microsporidian associated with cases of CMS, HSMI, PD, and PGI in farmed Atlantic salmon from western Norway. This microsporidian is also present in the copepod ectoparasites *L. salmonis* and *Caligus elongatus* on the fish. This paper describes the development of the parasite in Atlantic salmon and *L. salmonis*, and presents the tissue tropism for the new microsporidian in Atlantic salmon.

MATERIALS AND METHODS

Materials. Tissue samples of skin, gills, heart, kidney, and spleen of Atlantic salmon *Salmo salar* and salmon lice (*L. salmonis*, *C. elongatus*) from Atlantic salmon were collected in Farms D and G in western Norway, which suffered heavy losses due to an unidentified disease (Table 1). The fish showed increased mucus production on skin and gills, slightly pale gills, and heavy melanization in the visceral cavity (possibly a response to vaccination); a few fish had petechia on the liver. The material from 2006/2007 to 2008/2009 represents different generations separated by a fallowing period from June 2007 until May 2008 (10 mo). Both farms were situated in the same county in western Norway. They were treated for salmon lice several times in 2006 and 2007. The 2008/2009 generation was treated for lice in January 2009.

Histology. Collected tissue samples of gills, heart, head-kidney, kidney, skin, and spleen were fixed by immersion at 6 °C in a modified Karnovsky's fixative where the distilled water was replaced by a Ringer's solution (Nylund et al. 1995). The fixative contained 4% (w/v) sucrose. Before embedding in EMBED-812 (Electron Microscopy Sciences, Hatfield, PA) the tissues were post-fixed in 2% (w/v) OsO₄. Semi- and ultrathin sections were cut on Reichert-Jung Ultracut E (Leica, Milton, Keynes, UK). The ultrathin sections (30–40 nm) were stained for 1.5 h in 5% (w/v) aqueous uranyl acetate solution and then stained with lead citrate. Semithin sections, 0.5 μm, were stained with toluidine blue.

DNA/RNA extraction. DNA was extracted from tissues using the DNeasy DNA Tissue kit (Qiagen, Hilden, Germany) as recommended by the manufacturer, while the RNA extractions from tissues were performed as described by Devold et al.

Corresponding Author: S. Nylund, Department of Biology, University of Bergen, N- 5020, Norway—Telephone number: +47 55 58 44 06; e-mail: stian.nylund@bio.uib.no

Table 1. Material included in the study.

Locality	N	Date	Weight (g)	Length (cm)	<i>Paranucleospora theridion</i> Range (C_t)	<i>Paranucleospora theridion</i> Mean (C_t) ± SD
Generation 2006						
Farm D	34	26.10.2006	363.5	37.2	14.0–26.7	19.5 ± 3.1
Farm G	10	31.10.2006	600.0	—	18.6–21.6	20.0 ± 1.2
Farm D	10	16.11.2006	572.5	37.3	15.8–23.3	20.7 ± 2.3
Farm D	10	07.02.2007	693.4	40.8	24.2–29.2	26.7 ± 1.9
Farm G	5	19.06.2007	2,390.0	61.6	21.3–26.6	25.3 ± 2.3
Farm D	5	19.06.2007	438.0	39.9	17.1–30.2	21.4 ± 5.2
Generation 2008						
Farm D	2	29.09.2008	504.5	38.9	12.3–13.1	12.7 ^a
Farm G	16	29.09.2008	515.7	37.6	11.5–25.3	16.3 ± 4.2
Farm D	10	06.11.2008	680.3	41.1	8.0–19.6	12.2 ± 3.5
Farm G	10	06.11.2008	492.8	37.7	9.5–17.7	12.9 ± 3.5
Farm D	5	08.01.2009	1,219.2	49.4	12.1–19.0	16.0 ± 2.8
Farm G	6	08.01.2009	1,045.5	47.4	14.3–18.2	15.8 ± 1.8
Farm G	10	27.02.2009	1,135.1	51.3	17.4–23.9	21.0 ± 3.1

The Atlantic salmon were kept at Farm D and G. Range and mean C_t values obtained from real time screening of gill tissues for presence of *Paranucleospora theridion*.

^aOnly two specimens.

(2000). Elution of DNA was performed twice in 100 µl of 10 mM Tris-HCl, pH 8.5 and the DNA was stored at –20 °C.

Polymerase chain reaction (PCR) and real-time PCR. Extracted DNA from tissues of salmon and salmon lice were used to obtain the partial small subunit (SSU) rRNA and large subunit rRNA and the complete internal transcribed spacer (ITS) sequence from the new species of microsporidian (GenBank accession nos. FJ389667 and FJ594969–FJ594990). Primers targeting the rRNA genes of *N. salmonis* (GenBank accession no. U78176) were used to obtain the first sequences of the new species (Table 2).

The PCR reaction mixture (50 µl) contained 1 × ThermoPol reaction buffer (New England BioLabs, Ipswich, MA), 10 mM of each dNTP (Promega, Madison, WI), 0.4 µM of each primer (Invitrogen, Carlsbad, CA), 1 U *Taq* DNA polymerase (New England BioLabs), and approximately 200–300 ng of DNA template. Amplification was performed in a GeneAmp PCR System 9700 machine (Applied Biosystems, Foster City, CA) at 95 °C for 5 min, 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 72 °C for 2 min followed by extension at 72 °C for 10 min, and a short storage at 4 °C. All PCR products were purified with EZNA PCR cycle pure (Omega Biotech, Norcross, GA), and sequenced using the Big Dye terminator sequencing kit (Applied Biosystems). Sequencing was performed at the sequencing facility at the University of Bergen (<http://seqlab.uib.no/>). Sequence data were analysed and assembled using VectorNTI software (Invitrogen, Carlsbad, CA). Sequences obtained in the present study were submitted to the GenBank.

Extracted RNA from tissues of salmon was tested by Taqman real time RT PCR (1-Step QRT-PCR, Verso, Thermo Scientific, Waltham, MA) for presence of the new microsporidian species *Paranucleospora theridion* n. gen., n. sp., using real-time primers and probes (Table 2). This assay has a repeatable Cycle-threshold (C_t) at 37. This level was set based on 10 replicates of 10-fold dilutions of a DNA-stock, and denotes the mean C_t value of the highest dilution for which the 10 replicates were positive. A standard curve was generated using a 10-fold serial dilution of DNA in three parallels. Regression analysis, standard curve slopes s of C_t vs. log quantity DNA, and amplification efficiency E where $E = [10^{1/(s-slope)}] - 1$ were calculated. The coefficient of determination, R^2 , was 0.99. The slope for the assay was –3.17, and the amplification efficiency E was 1.07.

During the real time RT PCR screening a house-keeping gene, elongation factor 1 alpha (EF1A), was used as an internal control (Olsvik et al. 2005). Each run consisted of 45 cycles and the sam-

ples were considered positive when the fluorescence signal increased above a set threshold of 0.003.

Relative quantification. The tissue distribution of *P. theridion* was examined using the Nuc-assay. The archaeal *Halobacterium salinarum* added before RNA extraction was used as an exogenous control (i.e. SAL-assay, courtesy of Linda Andersen, University of Bergen, Table 2) and the EF1A assay as endogenous control. When the controls indicated that suboptimal conditions had occurred during the RNA-extraction process, RT reaction or PCR, the processing was repeated and the samples rerun. The exogenous and endogenous controls were used for calculation of the mean normalized expression (MNE) of SSU rRNA from *P. theridion* in selected tissues (e.g. gills, skin from the side, skin taken behind the adipose fin, heart, kidney, spleen, hind gut, liver, and central nervous system [CNS]) using the Microsoft Excel-based computer software Q-Gene (Muller et al. 2002). The MNE

Table 2. Overview over primers and probes used for characterization and screening for *Paranucleospora theridion* (accession no. FJ59481) and the exogenous control (*Halobacterium salinarum*, GenBank accession no. AB219965).

Name	Sequence (5'-3')	Position (base)
Nuc- F ^a	CGGACAGGGAGCATGGTATAG	522–542
Nuc- Probe ^a	TTGGCGAAGAATGAAA	544–559
Nuc- R ^a	GCTCCAGGTTGGTCTTGAG	580–561
Sal-F ^b	GGAAATCTGTCGCTTAACG	541–561
Sal-probe ^b	CCGGTCCCAAGCTGAACA	565–578
Sal-R ^b	AGGVGTCCAGCGGA	599–582
Mic-F	CAGCAGGTTGATTCTGCCTGAC	1–22
Mic-R	GGTCCTGTTCAAGACGGG	1,885–1,868 ^c
Nuc- F1	GCGATGATCTGCTCTAGTTGTG	100–121
Nuc- R1	CTCTAGCCTTCGTCCTTGATCC	638–617
Nuc- F2	GGATCAAGGACGAAGGCTAGAG	617–638
Nuc- R2	GCTAACCTACTCATCCGTAAGC	1,091–1,069
Nuc- F3	GCTTACGGATGAGTAGGATTAGC	1,069–1,091
Nuc- R3	GCAGTGCTTACAACAGTGC	1,634–1,614
Nuc- F10	GGGCAGGAAAAGAAACCAACTG	1,537–1,558
Nuc- R10	GGTTGCGTCTCATCTAGTC	221–202

^aPrimers and probes for Realtime analysis (Nuc-assay).

^bPrimers and probes for Realtime analysis (Sal-assay).

^cNot all bases included in primer sequence are present in the GenBank sequence.

was calculated from values obtained from samples taken from individual fish, and not based on triplicates of the same sample. Templates for quantification were prepared by homogenizing selected tissues in sterile water, using a weight-to-water ratio of 1:2. For RNA purification, 50 µl of these homogenates, spiked with 2 µl of *H. salinarum* suspension were used. This made it possible to quantify the amount of *P. theridion* in relation to the volume of the different tissues and in relation to the exogenous control.

The primers and probes used were obtained from Applied Biosystems, and the amounts of probes and primers were optimized for each assay. For the Nuc-assay, a final concentration of 600 nM of each primer was used and 175 nM of the probe, and for the EF1A (Olsvik et al. 2005), a primer concentration of 900 nM of each primer and 200 nM of probe. For the Sal assay, optimal concentrations were 300 nM for the forward primer, 900 nM for the reverse primer, and 200 nM for the probe. All assays were run in a total volume of 12.5 µl with 2 µl of isolated total RNA as the template. For signal detection the samples were run 45 cycles in an ABI 7500 Sequence Detection System real-time thermocycler as recommended by the manufacturer (Applied Biosystems). The threshold was fixed for all runs: the C_t value refers to the cycle number at which the change in fluorescence exceeded a fixed threshold during a real time PCR. Samples above the threshold were considered positive. Numerous controls (i.e. RNA extraction controls lacking RNA, and no template controls) were included to avoid false positives.

Phylogenetic analysis. The SSU rRNA sequence from a *P. theridion* isolate (GenBank accession no. FJ594981) was aligned with homologous sequences from a selected number of micro-

poridia (Table 3), based on BLAST searches and the phylogeny of Vossbrinck and Debrunner-Vossbrinck (2005). To perform pairwise comparisons between the different SSU rRNA sequences, the multiple sequence alignment editor GeneDoc (<http://www.psc.edu/biomed/genedoc>) was used. Polymorphic regions were manually aligned. Gaps in the alignment were deleted (the alignment can be obtained from the corresponding author).

Phylogenetic analyses of the data sets were performed using PAUP* version 4.0 (Swofford 1998) and Tree-Puzzle 5.2 (<http://www.tree-puzzle.de>). Tree-Puzzle reconstructs phylogenetic trees from molecular data by maximum likelihood, and computes maximum likelihood distances and branch lengths. In this study 10,000 quartet puzzling (QP) steps were carried out. The QP tree search estimates support values for each internal branch. The model best suited to the dataset was identified as GTR+Γ by the Akaike information criterion using the Modeltest 3.6 script (Posada and Crandall 1998). To test the robustness of the phylogeny, the phylogenetic relationship based on the SSU rRNA sequences of the selected microsporidians was also analysed by parsimony using PAUP with 1,000 bootstrap replicates. This gave the same topology (results not shown). The phylogeny presented in this study is the result of analysis using the GTR+Γ substitution model in TREE-PUZZLE. Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

Gross pathology in Atlantic salmon, *Salmo salar*. All Atlantic salmon in two farms suffering from gill disease were positive

Table 3. Overview of the microsporidians included in the phylogenetic study.

Species	Host	Accession no.	Country
<i>Cystosporogenes operophterae</i>	Lepidoptera	AJ302320	
<i>Cystosporogenes legeri</i>	Lepidoptera	AY233131	
<i>Endoreticulatus schubergi</i>	Lepidoptera	L39109	
<i>Endoreticulatus</i>	Lepidoptera	AY502945	Bulgaria
<i>Endoreticulatus bombycis</i>	Lepidoptera	AY009115	
<i>Enterocytozoon bieneusi</i>	Mammals	L07123	
<i>Enterocytozoon bieneusi</i>	Mammals	AF023245	
<i>Enterocytozoon bieneusi</i>	Mammals	AF024657	
<i>Glugoides intestinalis</i>	Cladocera	AF394525	
<i>Nucleospora salmonis</i>	Salmonidae	U78176	
<i>Nucleospora salmonis</i>	Salmonidae	AF185987	USA
<i>Nucleospora salmonis</i>	Salmonidae	AF185989	USA
<i>Nucleospora salmonis</i>	Salmonidae	U10883	USA
<i>Nucleospora salmonis</i>	Salmonidae	AF185998	Canada, Nova Sc.
<i>Nucleospora salmonis</i>	Salmonidae	AF186001	Canada, New Fo.
<i>Nucleospora salmonis</i>	Salmonidae	AF185996	Canada, BC
<i>Nucleospora salmonis</i>	Salmonidae	AF185999	Canada
<i>Nucleospora salmonis</i>	Salmonidae	AF185992	Canada, BC
<i>Nucleospora salmonis</i>	Salmonidae	U78176	
<i>Nucleospora salmonis</i>	Salmonidae	AF185993	Chile
<i>Nucleospora salmonis</i>	Salmonidae	AF185992	Chile
<i>Nucleospora</i> sp. I	Pleuronectidae	AF201911	USA
<i>Nucleospora</i> sp. II	Copepoda	AJ431366	Scotland
<i>Paranucleospora theridion</i>	Copepoda	FJ594981	Norway
<i>Paranucleospora theridion</i>	Salmonidae	FJ389667	Norway
<i>Orthosomella operophterae</i>	Lepidoptera	AJ302316	
<i>Orthosomella operophterae</i>	Lepidoptera	AJ302317	
<i>Vittaforma corneae</i>	Human	L39112	
<i>Microsporidium</i> sp.	Cladocera	AF394528	
<i>Microsporidium</i> sp.	Gammarus	AJ438964	UK
<i>Microsporidium</i> sp.		AJ871393	UK
<i>Encephalitozoon</i> sp.	Homonidae	L16866	
<i>Ordozpora colligata</i>	Cladocera	AF394529	

The species represent a subsample from clade IV (Vossbrinck and Debrunner-Vossbrinck 2005).

for *P. theridion* n. gen., n. sp. in four successive years including two different generations (Table 1). Some of the fish had skin haemorrhages, loss of scales, and slightly pale gills with a high production of mucus compared with healthy fish. The gills in most fish were strongly pigmented at the base of the primary lamellae. The gill epithelium showed hypertrophy and hyperplasia, with necrosis and invasion of leucocytes (i.e. inflammation). Inflammation was also observed in the other examined tissues: kidney, heart, spleen, gut, and exocrine pancreas. The pathology in all tissues was associated with the presence of *P. theridion*, revealed by histology and real-time PCR, and confirmed by PCR and sequencing: skin (GenBank accession nos. FJ594972, FJ594974, FJ594984), gills (FJ594969, FJ594971, FJ594982, FJ594983, FJ594988, and FJ594989), pseudobranch (FJ594978), heart (FJ594973), kidney (FJ594977, FJ594986), liver (FJ594975), spleen (FJ594976, FJ594985), and hind-gut (FJ594970).

Development of *Paranucleospora theridion* n. gen., n. sp. in salmon. Two presumed developmental cycles with the production of two different spore types were inferred in Atlantic salmon, with diplokarya in all observed stages. In Developmental Cycle I, a thin-walled spherical to subspherical spore with a short polar tubule is presumed to form in the cytoplasm of polymorphonuclear leucocytes, macrophage-like cells, and blood vessel endothelial cells in most tissues and in epithelial cells in skin and gills. In Developmental Cycle II, the second spore type is thick-walled and ellipsoidal with a longer polar tubule, and develops in the nucleus of epidermal cells of gills and skin (Fig. 1).

Developmental Cycle I. The developmental cycle producing thin-walled spherical/oval spores occurs in the cytoplasm, but not in the nucleus of cells in all salmon tissues examined by histology

and transmission electron microscopy. The first developmental stages observed in infected cell cytoplasm are small, 0.8–1.8 µm in diameter, diplokaryotic meronts surrounded by a unit membrane in direct contact with the host cell cytoplasm (Fig. 2). The meront cytoplasm at this stage contains abundant ribosomes and usually a single prominent endoplasmic reticulum (ER) lamellum. Meront growth and multiplication of diplokarya results in plasmodia, 1.9–4.6 µm in length, with two to more than 12 diplokarya (Fig. 3–5). The nuclei of diplokarya in merogonal plasmodia are elongated and associated in a coffee-bean like manner, often with lobed ends (Fig. 3). These meronts show an undulating outline with short, rounded, projections (Fig. 3, 4). Plasmotomy was observed and infected cells contained up to three plasmodia in the plane of a section. Developing plasmodia were juxtanuclear, sometimes in an invagination (Fig. 3). Plasmodia were surrounded by host cell mitochondria, but there was no deposition of dense material on the surface of the plasmalemma during the transition from meront stage to the sporont stage. The sporonts are distinguished from the meronts by the appearance of dense discs associated with the formation of the polar tube (Fig. 6–8). Sporonts measured 1.7–5.5 µm in length. Sporont diplokarya were eventually located to the periphery of the sporogonal plasmodia, and were rounded with short zones of nuclear apposition (Fig. 6). The precursors for the polar tube mainly develop in the centre of the sporonts (Fig. 6, 8). Assembly of the sporoblast components occurs in the sporogonal plasmodia, around peripherally placed anchoring discs in polar caps associated with bulbs on the sporont surfaces (Fig. 8). A short polar tube and a vesicular polaroplast precursor are associated with each polar cap element while diplokarya appear to be located lateral to the forming extrusion ap-

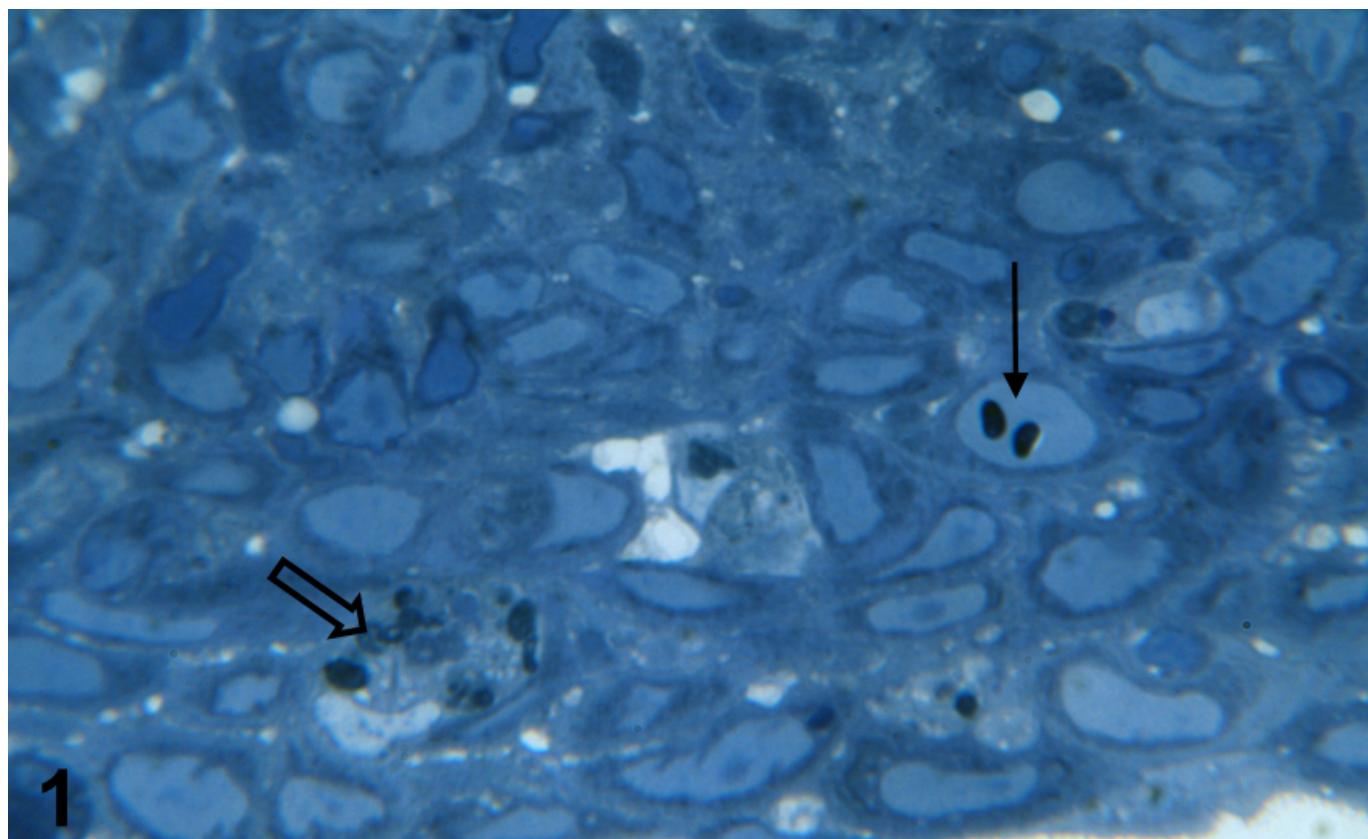


Fig. 1. Section through skin epithelial cells from Atlantic salmon *Salmo salar*. Cell nucleus containing two spores of *Paranucleospora theridion* n. gen., n. sp. (arrow). Necrosis of epithelial cells (open arrow).

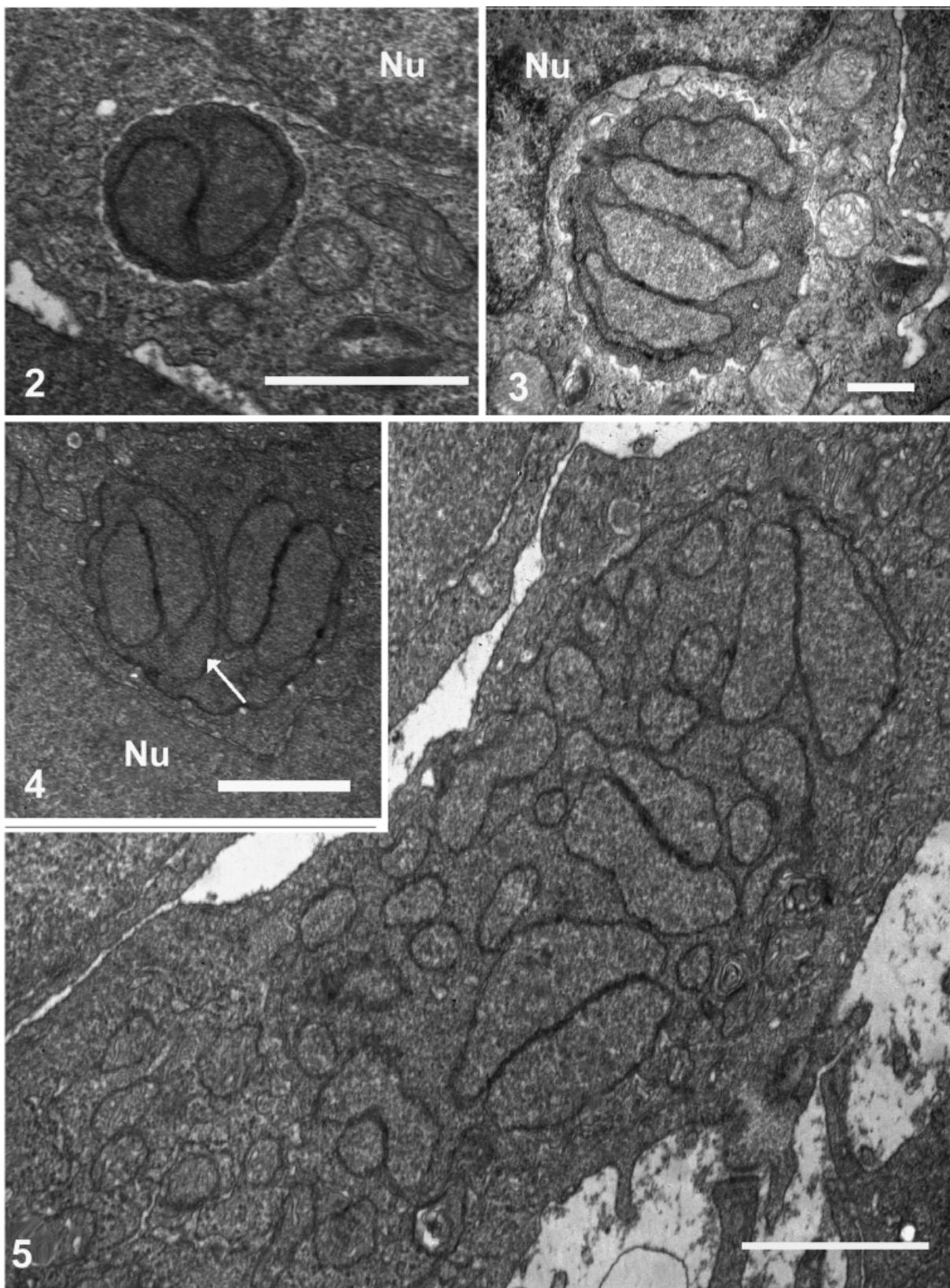


Fig. 2–5. Merogonal stages in Developmental Cycle I of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. 2. Early merontal stage, containing a single diplokaryon, in cell cytoplasm of host tissue. Scale bar = 1.0 μm . 3. Early merontal stage containing two diplokarya. Scale bar = 0.5 μm . 4. Meront showing two diplokarya and a double membrane (arrow) separating them. The membrane is continuous with the plasmalemma of the meront. Scale bar = 1.0 μm . 5. Large plasmodium (meront) containing several diplokarya. Scale bar = 1.0 μm . Nu, host cell nucleus.

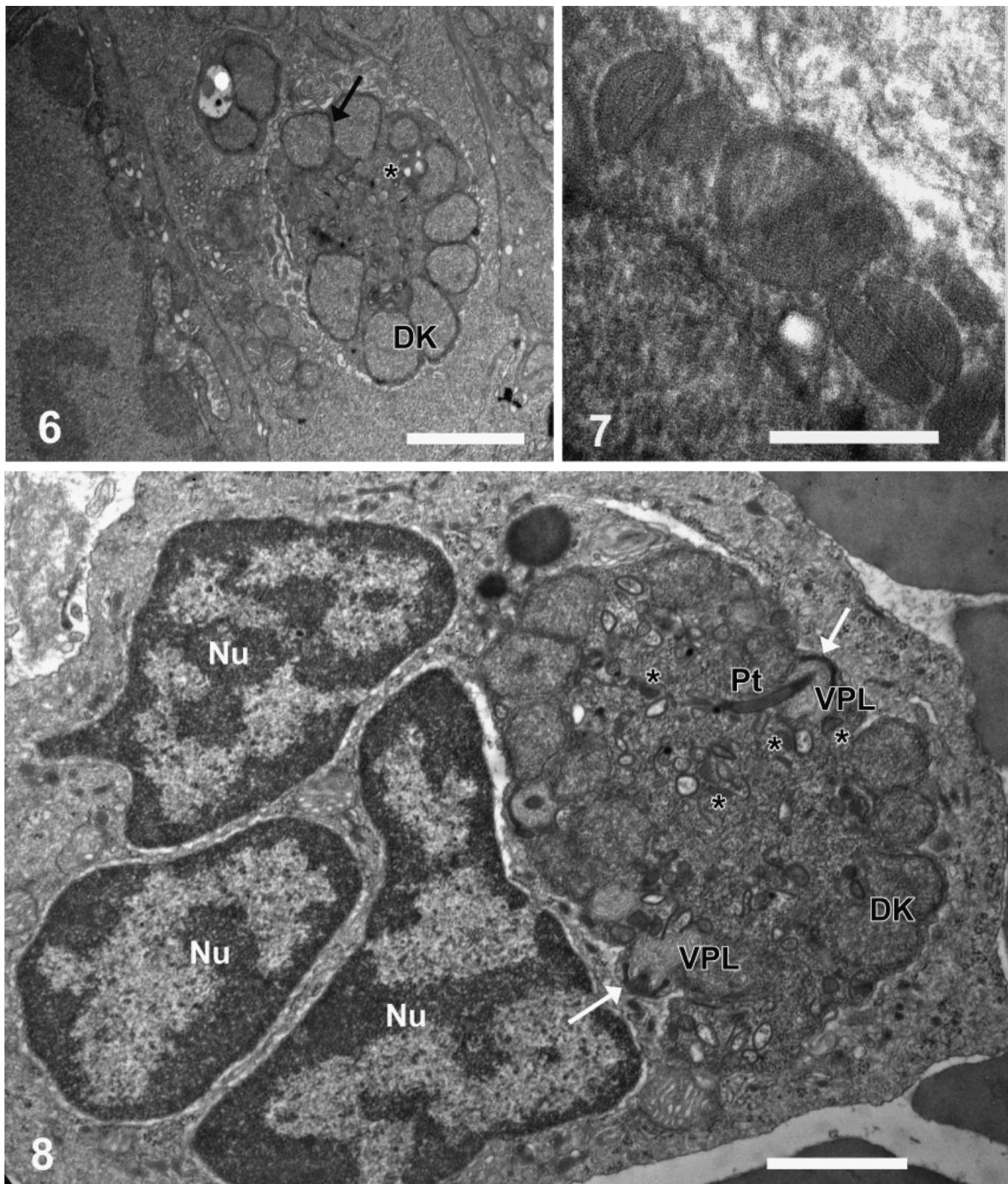


Fig. 6–8. Sporonts in Developmental Cycle I of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **6.** Two sporogonial plasmodia in the host cell cytoplasm. The diplokarya (DK) in the large plasmodia are located towards the plasmalemma of the parasite. A short zone of nuclear apposition is indicated (arrow). Dense discs associated with polar tube formation are indicated by asterisks. Scale bar = 0.2 μm. **7.** Discs formed during the development of the sporont. Scale bar = 1.0 μm. **8.** Late sporont before schizogony showing peripherally placed anchoring discs in polar caps associated with bulbs on sporont surfaces (arrows). Polar tube (Pt) and vesicular polaroplast (VPL) precursors are indicated. Nu, host cell nucleus. Scale bar = 1.0 μm.

paratus at the periphery of the sporont (Fig. 6, 8). Up to 14 sporoblasts were observed forming in sections of sporogonal plasmodia. Schizogonic division produces diplokaryotic sporoblasts in direct contact with the host cell cytoplasm (Fig. 9, 11). A 20-nm-thick layer of amorphous material is deposited on the sporoblast surface, apparently derived from cytoplasmic vesicles (Fig. 10). The sporoblasts develop into spherical, thin-walled spores 1.1 (0.9–1.2) µm in diameter in direct contact with the host cell cytoplasm (Fig. 12, 13). The polar tube of these spores is short, about 1.1 µm in length. The polaroplast appears to be composed of loosely arranged lamellae. A posterior vacuole is present, with dense granular content (Fig. 12). Up to 30 spores and sporoblasts were seen in sections of a single infected cell.

Developmental Cycle II. The developmental cycle producing thick-walled ellipsoidal spores in the host cell nucleus apparently occurs only in epidermal cells of gills and skin (Fig. 1). In histological sections from gills and skin tissues a total of 221 cells containing, intranuclear spores were observed; 132 skin epithelial cells, 85 gill epithelial cells, and four chloride cells from gills. In these 75.1% (166/221) of the cell nuclei contained one spore only, 23.1% (51/221) contained two spores, and 1.8% (4/221) contained three spores. A single nucleus contained four spores (Fig. 20).

The first developmental stages, the meronts observed in host cell nuclei, were single diplokaryotic stages surrounded by a unit membrane in direct contact with the host cell nucleoplasm (Fig. 14, 15). The meront cytoplasm contains abundant ribosomes and usually a single ER lamellum (Fig. 16). Meronts contained a maximum of two diplokarya (Fig. 16). Sporogonic stages were distinguished by the appearance of dense barrel-shaped elements measuring about 80 nm in diameter (Fig. 17–19). At this stage possible polar sac primordia were also recognizable (Fig. 18). There is no deposition of dense material on the surface of the plasmalemma during transition to sporogony. Membrane whorls occur peripherally in both meronts and sporoblasts (Fig. 16, 18). The sporogonic stages with polar tube precursors develop two sets of the extrusion apparatus before division, giving rise to two sporoblasts (Fig. 19). Sporoblasts develop into mature, ellipsoidal, diplokaryotic spores in direct contact with the host cell nucleoplasm (Fig. 20–22). The mature spores are about 2.4–2.7 µm long × 2.0–2.1 µm wide. The anisofilar polar tube show five to six coils, with four to five coils of normal diameter (i.e. 150 nm) and with one coil or less with reduced filament diameter (i.e. 82–110 nm). The polaroplast is rounded, with a peripheral dense lamellar part surrounding a less dense lamellar region surrounding a central vesicular part (Fig. 21). A large vacuole with amorphous contents is located posterolateral to the polaroplast and opposed to the nucleus (Fig. 20–22). The exospore is thin (i.e. 28 nm) and the endospore relatively thick (i.e. 100–130 nm) (Fig. 22).

Occurrence in the salmon louse *Lepeophtheirus salmonis*. *Paranucleospora theridion* n. gen., n. sp. was detected in all developmental stages of the salmon louse *L. salmonis*: an adult male (GenBank accession no. FJ594987), adult females (FJ594981, FJ594990), and egg strings (FJ594980). All lice ($N = 50$) from both farms, with the exception of a few lice from farm D ($N = 6$), were positive for the parasite after screening with real time PCR. In addition, four specimens of *C. elongatus*, collected from salmon in farm G, were also positive for presence of this microsporidian (FJ594979). Egg strings from *C. elongatus* were strongly positive with *C_t* reaching 21.3. Developmental stages of *P. theridion* in *L. salmonis* were observed in epithelial cells, connective tissue cells, gonadal cells, satellite cells, and unidentified haemocytes. In lightly infected lice only a few infected cells with fewer than 20 spores were present, while in heavily infected lice most of the cephalothorax, gonadal segment, and abdomen were filled with different developmental stages of this microsporidian (Fig. 23, 24). Spores and other developmental stages of

P. theridion were also found in the extremities and in the upper and lower lip of the mouth tubule. The parasite was never found in nervous tissues, in the gut epithelium, gut lumen, or in somatic muscle fibres of *L. salmonis*.

Development of *Paranucleospora theridion* in *Lepeophtheirus salmonis*. The dominant spores in fresh smears from *L. salmonis* were spherical to slightly subspherical 2.4 (2.2–2.5, $N = 30$) µm in diameter (Fig. 25), and about 1.6–2.0 µm in ultrathin sections. In addition, macrospores, measuring about 5.0 µm in diameter, were observed in some sections (Fig. 36, 37).

Infected cells in *L. salmonis* varied from slightly, to strongly hypertrophic, containing large numbers of spores and other developmental stages of *P. theridion* (Fig. 23, 24). The earliest stages discerned in the developmental sequence in the salmon louse were diplokaryotic meronts in direct contact with the host cell cytoplasm (Fig. 26). Host cells with a single meront were observed, but in most cells patches with merogonial stages occurred among other developmental stages (i.e. sporonts, sporoblasts, and spores). Merogonial stages varied in size and morphology from more or less spherical meronts with one or two diplokarya to rarer, large, multilobed plasmodia with more than eight diplokarya. The diplokaryotic nuclei are large and rounded and the cytoplasm contains a few ER profiles and moderate numbers of free ribosomes. The multinucleate merogonial plasmodia up to 9.1 µm in length divide by schizogony in which nuclei accumulate at the periphery of the plasmodia while lobes are formed in the same number as there are nuclei. Sporonts are directly derived from merogonial plasmodia, and are recognized by addition of dense material on the surface of the plasma membrane (Fig. 27–29). These plasmodia are diplokaryotic or with a mixture of mono- and diplokarya. It has not been established if the monokaryotic stage is a result of nuclear fusion or a result of dissociation of the twin nuclei. Uninuclear cells produced by sporont schizogony are of similar size and shape as the meronts, but with one monokaryon (Fig. 29). In addition to free ribosomes the uninuclear cells contain rough ER and the plasmalemma is covered by dense material about 20 nm thick (Fig. 29). These Stage I cells vary from round to multilobed 2.7–5.7 µm in length, and are actively dividing. Smaller Stage II cells, 2.0–2.7 µm in length, are produced that can be distinguished by more prominent ER and the occurrence of early elements of the developing extrusion apparatus (Fig. 30, 31). Early Stage II cells contain a polar sac primordium, closely apposed to the nucleus, which includes a translucent vacuole and a large dense body, 0.45–0.55 µm in diameter (Fig. 30). The polar sac primordium appears to be the origin of thread-like, dense Golgi elements that are in contact with the polar tube primordium (Fig. 31, 32). Stage II cells are still dividing. Following formation of several coils in the polar tube (Fig. 31, 32); the anchoring disc and polar cap occur peripherally in the cell, subtended by a manubrial primordium and a vesiculate polaroplast (Fig. 33). Cells at this stage were not seen to divide, and are assumed to be sporoblasts, 2.3–2.6 µm in diameter. Vacuoles appear in the Stage I cells and persist in the sporoblasts as a large vacuole posterior to the forming polaroplast. The anisofilar polar filament in mature spores consists of four to six coils normally in single rows, with one coil being clearly thinner (Fig. 34, 35). The spore wall consists of a one-layered dense exospore of a uniform 28 nm thickness and a lucent endospore, 42–56 nm thick. The endospore is considerably thinner over the anchoring disc (Fig. 34, 35).

A few very large spores occurred in most of the xenoma-like infected cells in sea lice. These spores were thick walled, with several sets of the extrusion apparatus and with an exceptionally high number of irregularly arranged polar tube profiles (Fig. 36, 37).

Phylogeny. There was no variation in the rRNA sequence of isolates of *P. theridion* n. gen., n. sp. from different Atlantic

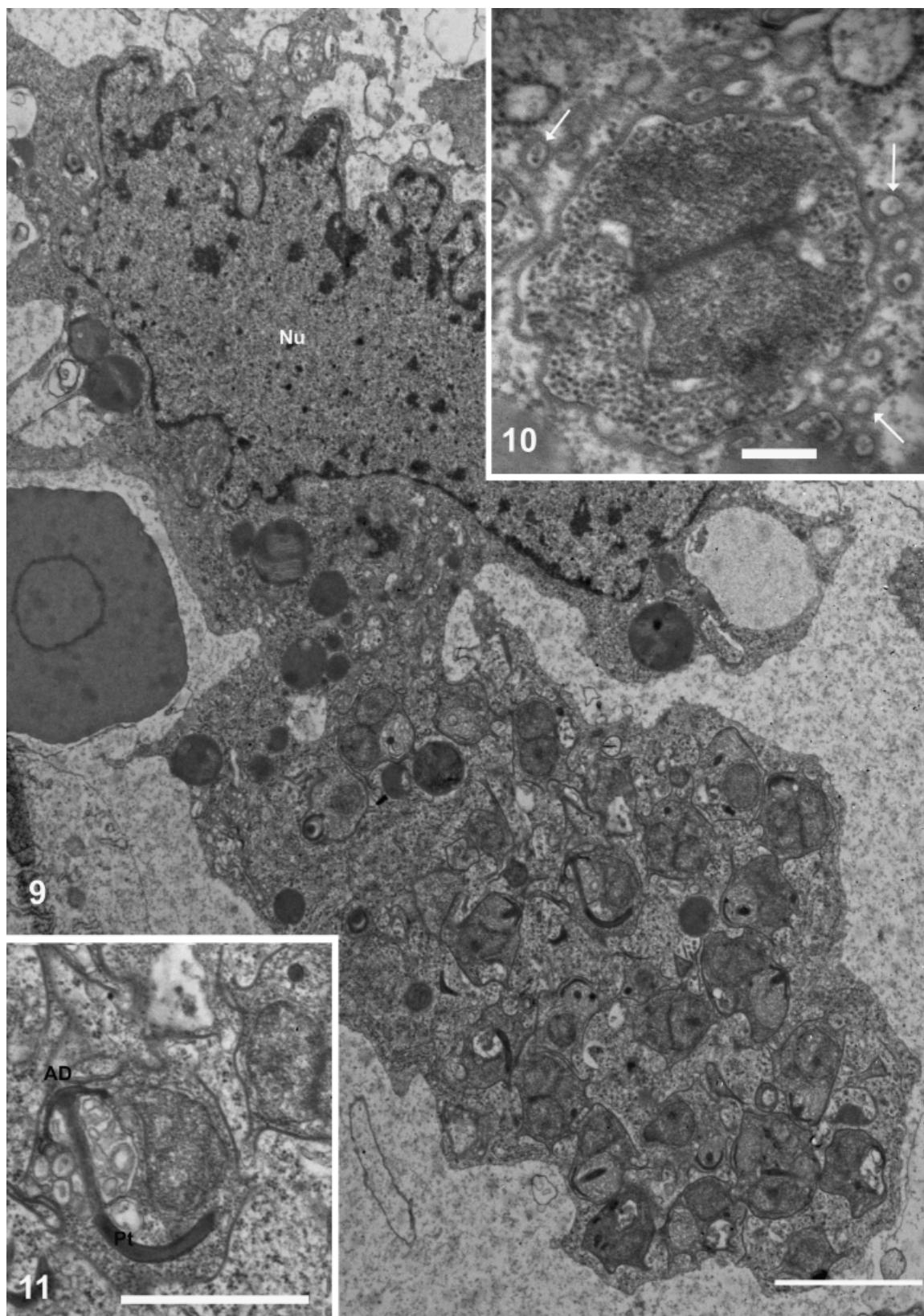


Fig. 9–11. Sporoblasts in Developmental Cycle I of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **9.** Endothelial cell of host containing sporoblasts and spores in the cytoplasm. Nu, host cell nucleus. Scale bar = 2.0 μm . **10.** Deposition of dense material on the surface of a sporoblast. Vesicles of amorphous material (arrows). Scale bar = 0.2 μm . **11.** Sporoblast showing anchoring disc (AD) and polar tube (Pt) (magnification of Fig. 9). Scale bar = 1.0 μm .

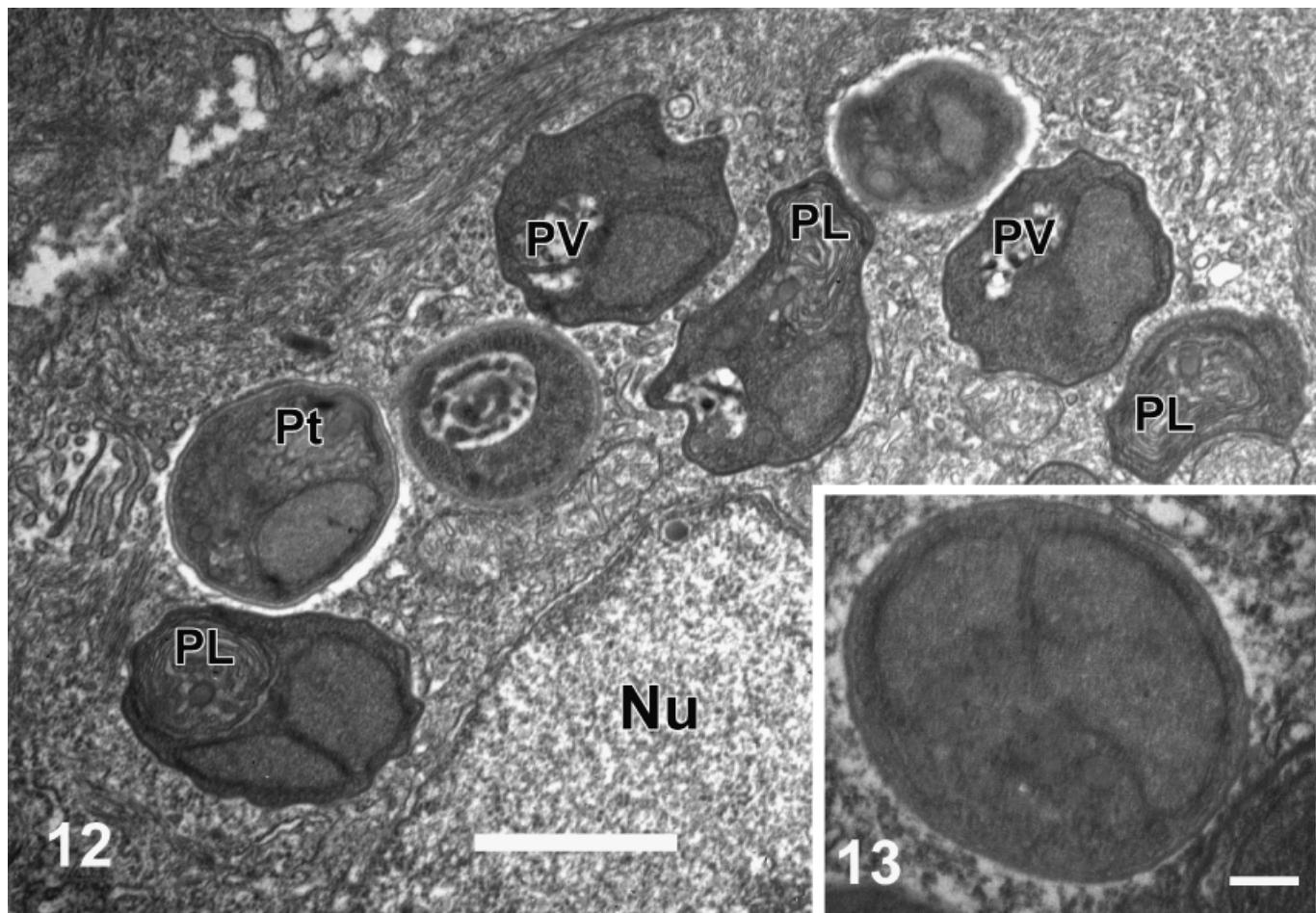


Fig. 12,13. Diplokaryotic spores in Developmental Cycle I of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **12.** Thin-walled diplokaryotic spores in the cytoplasm of an infected host leucocyte. A polar tube (Pt), lamellar polaroplasts (PL), and posterior vacuoles (PV) are indicated. Nu, host cell nucleus. Scale bar = 1.0 µm. **13.** Diplokaryotic spore. Scale bar = 0.2 µm.

salmon tissues, rainbow trout, *L. salmonis* or *C. elongatus* ($N = 1$ sample). The rRNA sequences from *P. theridion* are 99.4% (eight substitutions in 1,412 nucleotides) similar to a shorter sequence from a microsporidian collected from *L. salmonis* in Scotland (GenBank accession no: AJ431366). The phylogeny was obtained by analysis of 1,174 nt of rRNA from a selection of related microsporidians (Table 3, Fig. 38) and shows that the closest relative to *P. theridion* is an undescribed enterocytozoonid microsporidian species from the flatfish *P. vetulus*, collected in Washington State, USA. *Nucleospora salmonis* and *Enterocytozoon bieneusi* are the closest described relatives to *P. theridion* (Fig. 38). The partial SSU rRNA sequences of the undescribed enterocytozoonid microsporidian (AF201911), *N. salmonis* (NSU78176), and *E. bieneusi* (AF023245) show 91-, 88-, and 82% identity with *P. theridion*, respectively.

Tissue distribution in salmon. The tissue distribution of *P. theridion* n. gen., n. sp. in Atlantic salmon at the sampling date in November shows that the parasite is present in all tissues in more or less equal amounts in both farms (Fig. 39).

DISCUSSION

This study describes the presence of developmental stages of a microsporidian parasite that uses Atlantic salmon (*S. salar*) and salmon louse, *L. salmonis* as hosts. The parasite has also been detected in rainbow trout (*Oncorhynchus mykiss*) and *C. el-*

ongatus. This parasite has been associated with up to 80% mortality in commercial farms producing Atlantic salmon (data not shown). However, the importance of this parasite in causing disease has to be confirmed by challenge experiments.

Paranucleospora theridion n. gen., n. sp. shares many developmental features with members of the family Enterocytozoonidae. Phylogenetic analysis of rRNA (partial 16S) also suggest a close relationship between the genera *Nucleospora*, *Enterocytozoon*, and *P. theridion*. An undescribed enterocytozoonid detected in epidermal pseudotumours of the English sole *P. vetulus* (Khattra et al. 2000; accession no. AF201911) now appears as a possible member of the genus *Paranucleospora*. The microsporidian found in Scottish *L. salmonis*, phylogenetically related to *Nucleospora* (see Freeman et al. 2003) is now identified with *P. theridion*. The SSU rRNA sequence divergence between *P. theridion* and *N. salmonis* (12%) or *E. bieneusi* (18%), type species in their respective genera, far exceeds that between other microsporidian genera (see Lom and Nilsen 2003), and support the erection of a separate genus.

The cytoplasmic developmental sequence of *P. theridion* is very similar to that of *E. bieneusi*. Shared characteristics are development in direct contact with the host cell cytoplasm, juxtapositional positioning, sporogonial plasmodia, very elongate nuclei in meronts, early appearance of polar tube precursors as dense discs, and assembly of the components of the extrusion apparatus before division of the plasmodium to form sporoblasts (Cali and

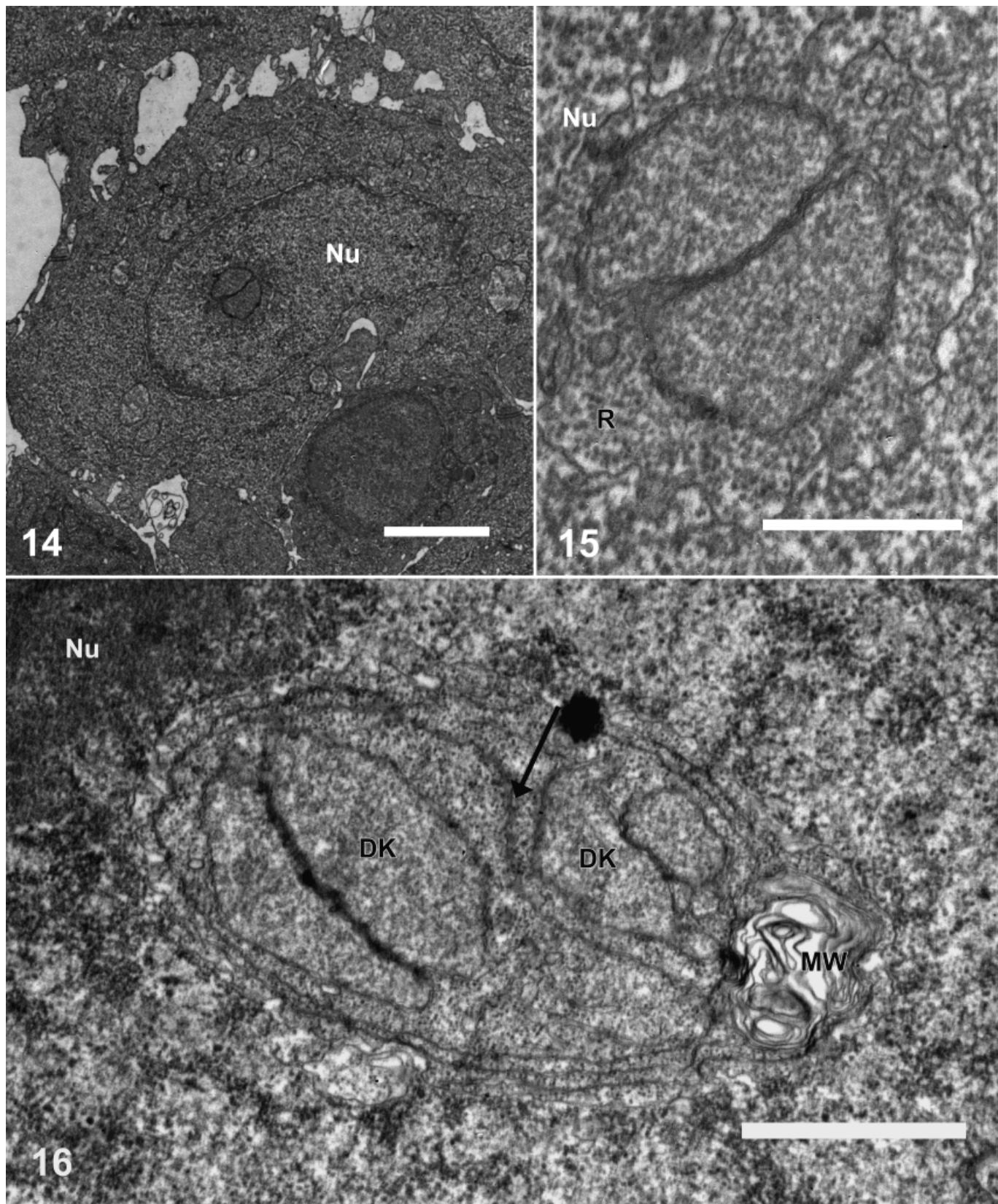


Fig. 14–16. Merogonial stages in Developmental Cycle II of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **14.** Meront with diplokaryon in host cell nucleoplasm. Scale bar = 2.0 μm . **15.** Detailed view of meront with ribosomes (R). Scale bar = 0.5 μm . **16.** Intranuclear meront containing two diplokarya (DK) partly separated by a double membrane (arrow). A peripheral membrane whorl (MW) is indicated. Scale bar = 0.5 μm . Nu, host cell nucleus.

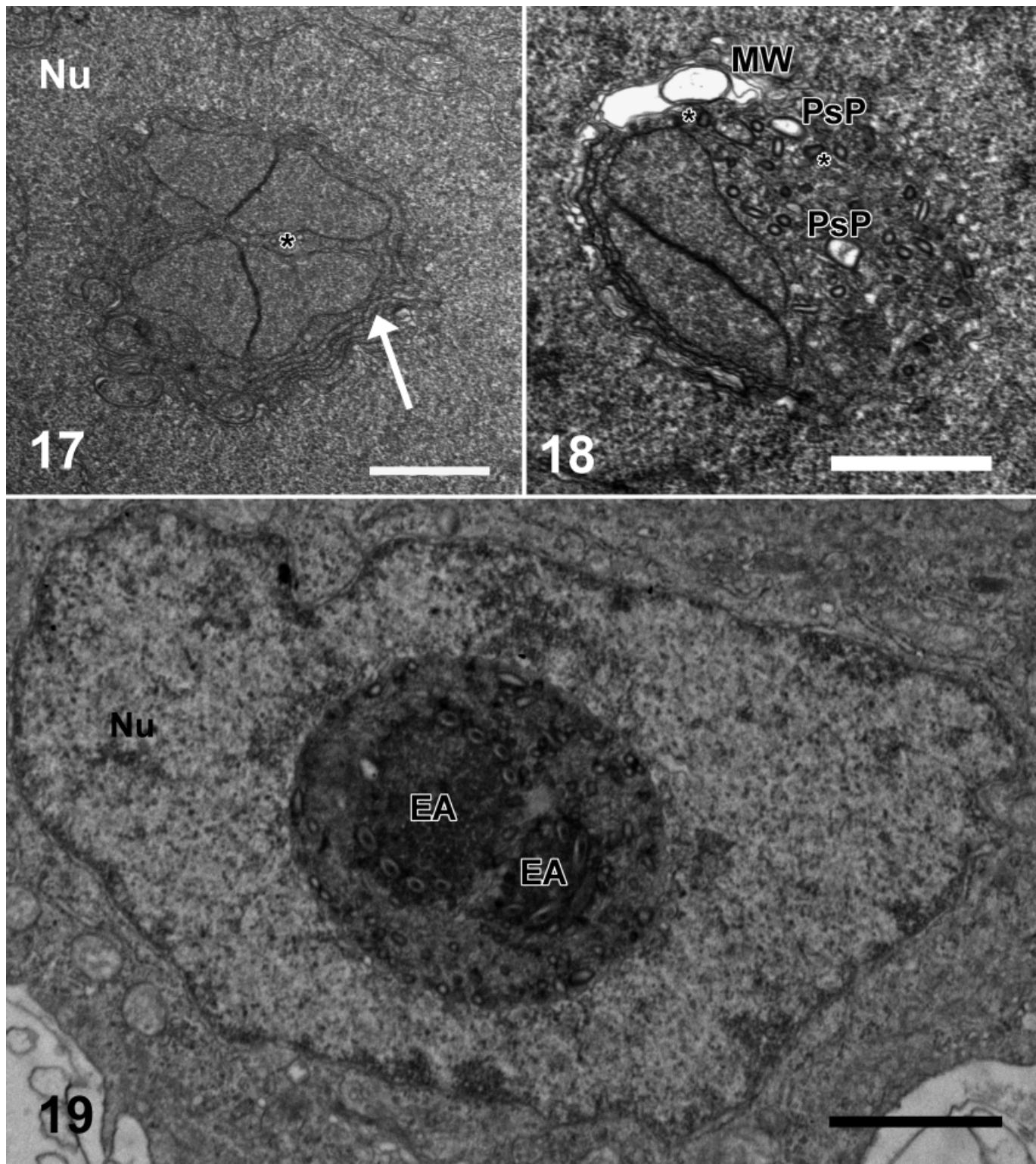


Fig. 17–19. Sporonts and sporoblasts in Developmental Cycle II of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **17.** Early sporont stage with abundant rough endoplasmic reticulum (arrow) and barrel-shaped elements (asterisks). Scale bar = 1.0 μm . **18.** Sporont containing polar sac primordia (PsP). A membrane whorl (MW) is also indicated. Scale bar = 1.0 μm . **19.** Sporoblast mother cell with two sets of extrusion apparatus (EA). Scale bar = 1.0 μm . Nu, host cell nucleus.

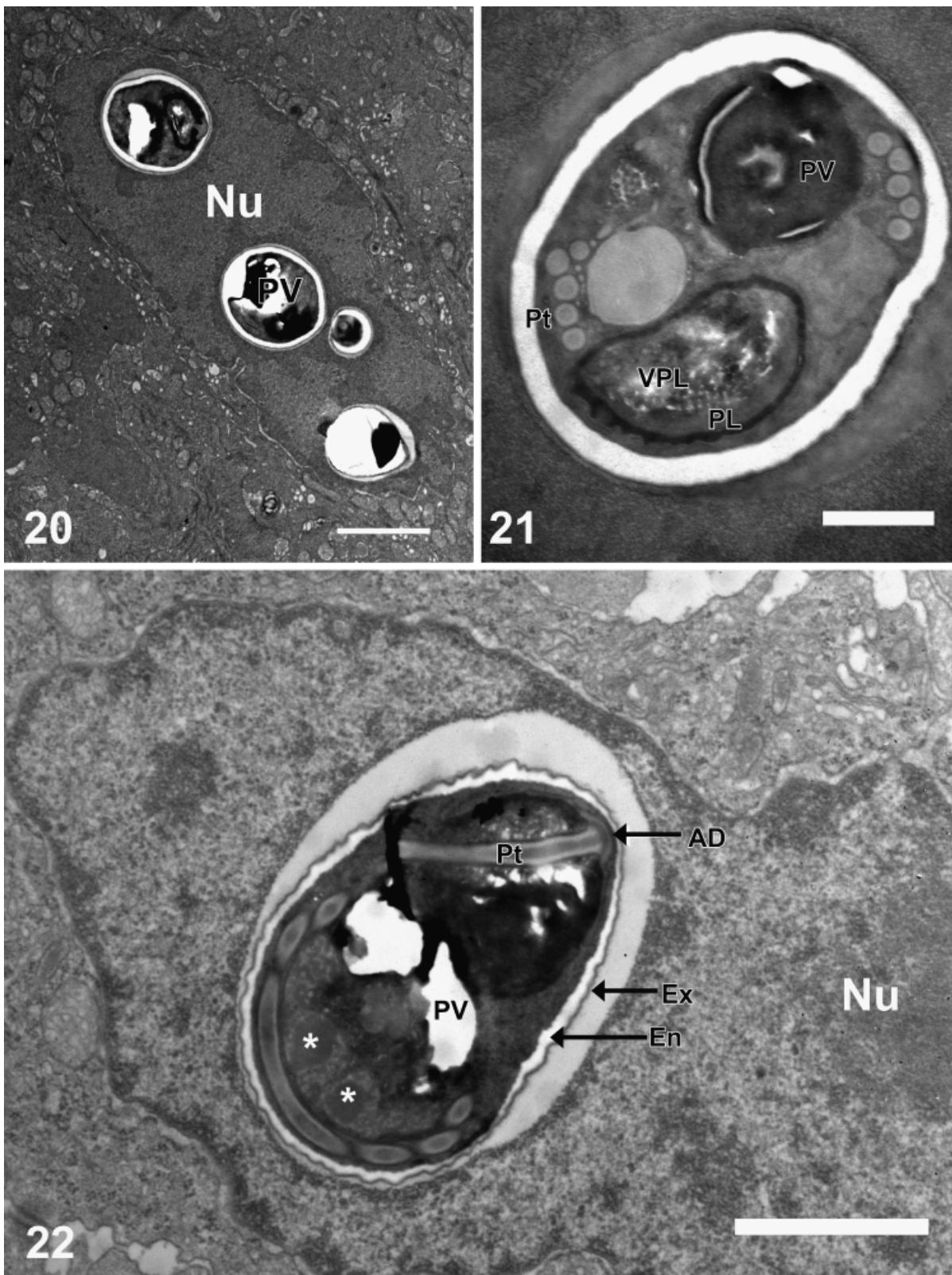


Fig. 20–22. Spores in Developmental Cycle II of *Paranucleospora theridion* n. gen., n. sp. of the Atlantic salmon host *Salmo salar*. **20.** Epithelial cell containing four spores in the nucleus (Nu). Scale bar = 2.0 μm . **21.** Mature spore. The lamellar polaroplast (PL), vesicular polaroplast (VPL), posterior vacuole (PV) and the polar tube (Pt) are indicated. Scale bar = 1.0 μm . **22.** Mature diplokaryotic spore in host cell nucleus. The diplokaryon (asterisks), anchoring disc (AD), polar tube (Pt), posterior vacuole (PV), exospore (Ex), and endospore (En) are indicated. Scale bar = 1.0 μm .

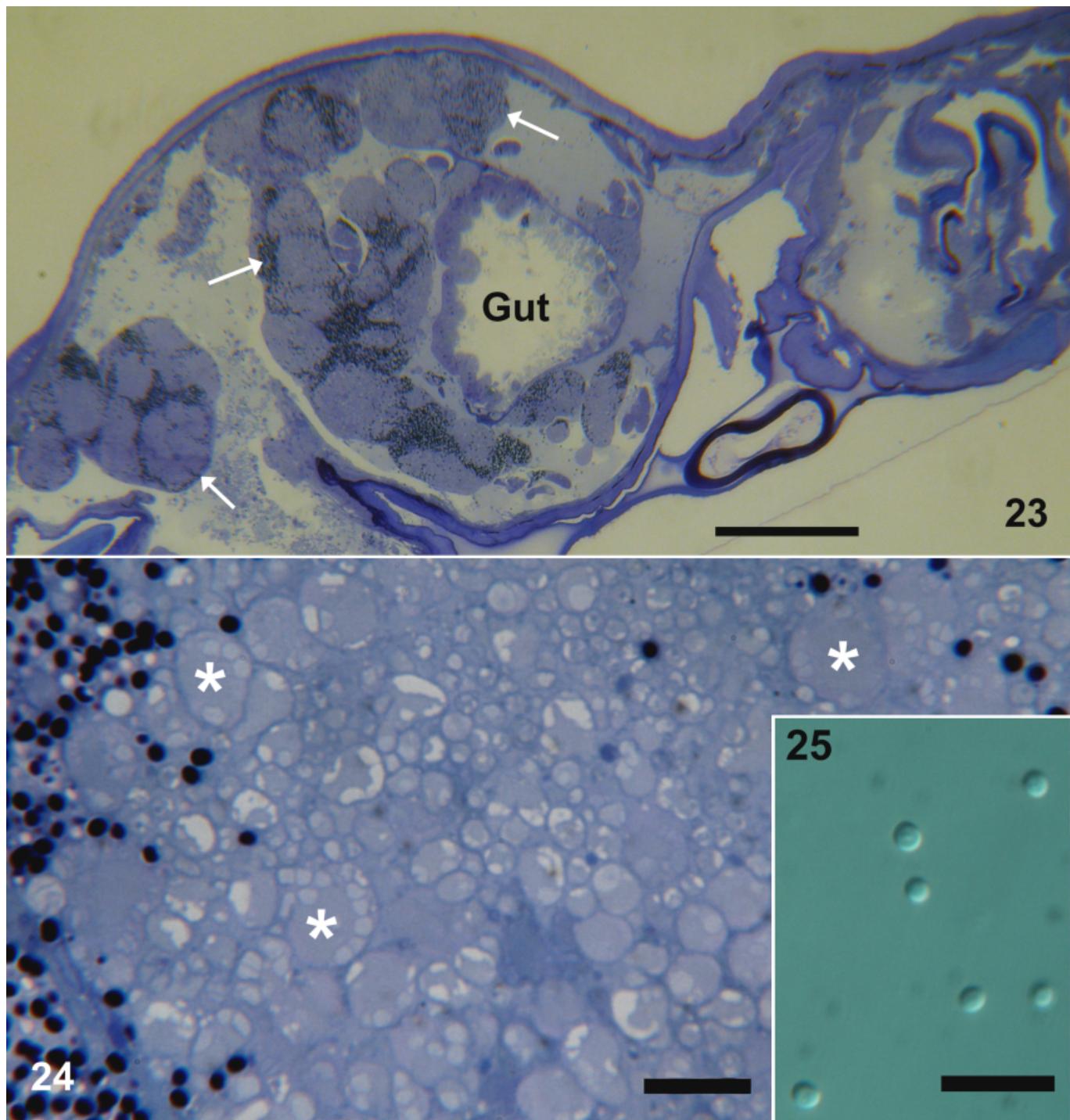


Fig. 23–25. *Paranucleospora theridion* n. gen., n. sp. in the salmon louse *Lepeophtheirus salmonis*. 23. Section through the cephalothorax of an adult *L. salmonis*. Infected tissues (arrows). Scale bar = 200 µm. 24. Section through an infected cell in *L. salmonis*. Plasmodia (asterisks). Scale bar = 10 µm. 25. Live spores of *P. theridion*. Scale bar = 10 µm.

Owen 1990; Cali and Takvorian 1999; Desportes et al. 1985). Both develop very small thin-walled spores (Cali and Owen 1990; Desportes et al. 1985). *Paranucleospora theridion* n. gen., n. sp. differs by its diplokaryotic state and a very short polar tube, which are characteristics not shared with any member in the Family Enterocytozoonidae. Another fish microsporidian, *Microfilum lutjani* (Faye, Toguebaye, and Bouix 1991), also shows a very short polar tube, about 0.5 µm in length, and develops in direct contact with

the leucocyte host-cell cytoplasm. However, other characteristics, such as tetrasporoblastic development with sporogonial plasmodia forming rosettes, larger (4.5 × 2.6 µm) and thick-walled spores, and formation of xenomas up to 0.78 mm long are very different (see Faye et al. 1991).

The intranuclear development of *P. theridion* in salmon epidermal cells resembles that of members of the enterocytozoonid genus *Nucleospora*. *Nucleospora* spp. show intranuclear sporo-

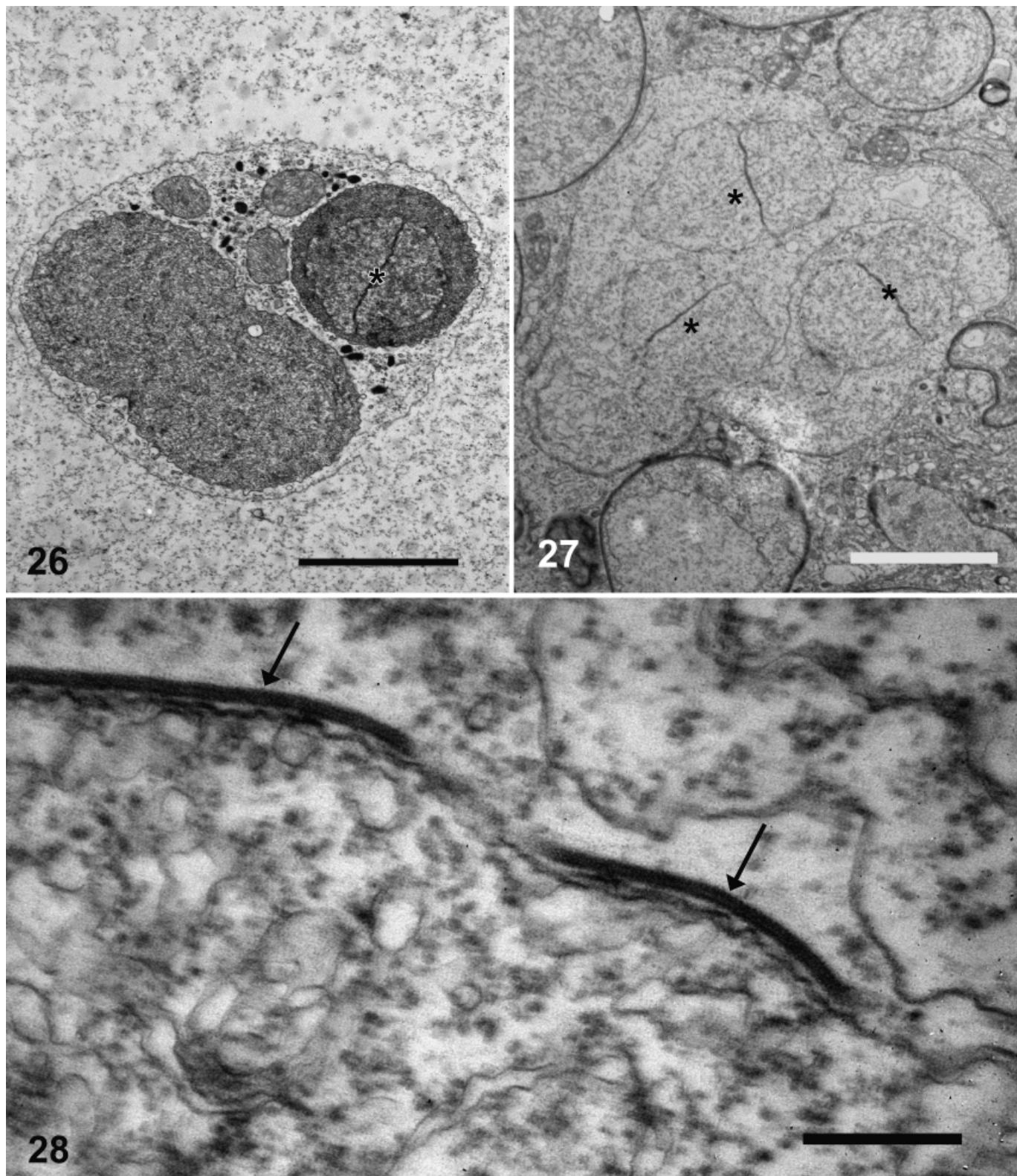


Fig. 26–28. Merogonal and sporont stages of *Paranucleospora theridion* n. gen., n. sp. in the salmon louse *Lepeophtheirus salmonis*. 26. Haemocyte with early meronts with diplokaryon (asterisk). Scale bar = 2.0 μ m. 27. Diplokaryotic meront/sporont. Diplokaryon (asterisks). Scale bar = 2.0 μ m. 28. Addition of dense material (arrows) on the surface of transitional stages between meronts and sporonts. Scale bar = 0.2 μ m.

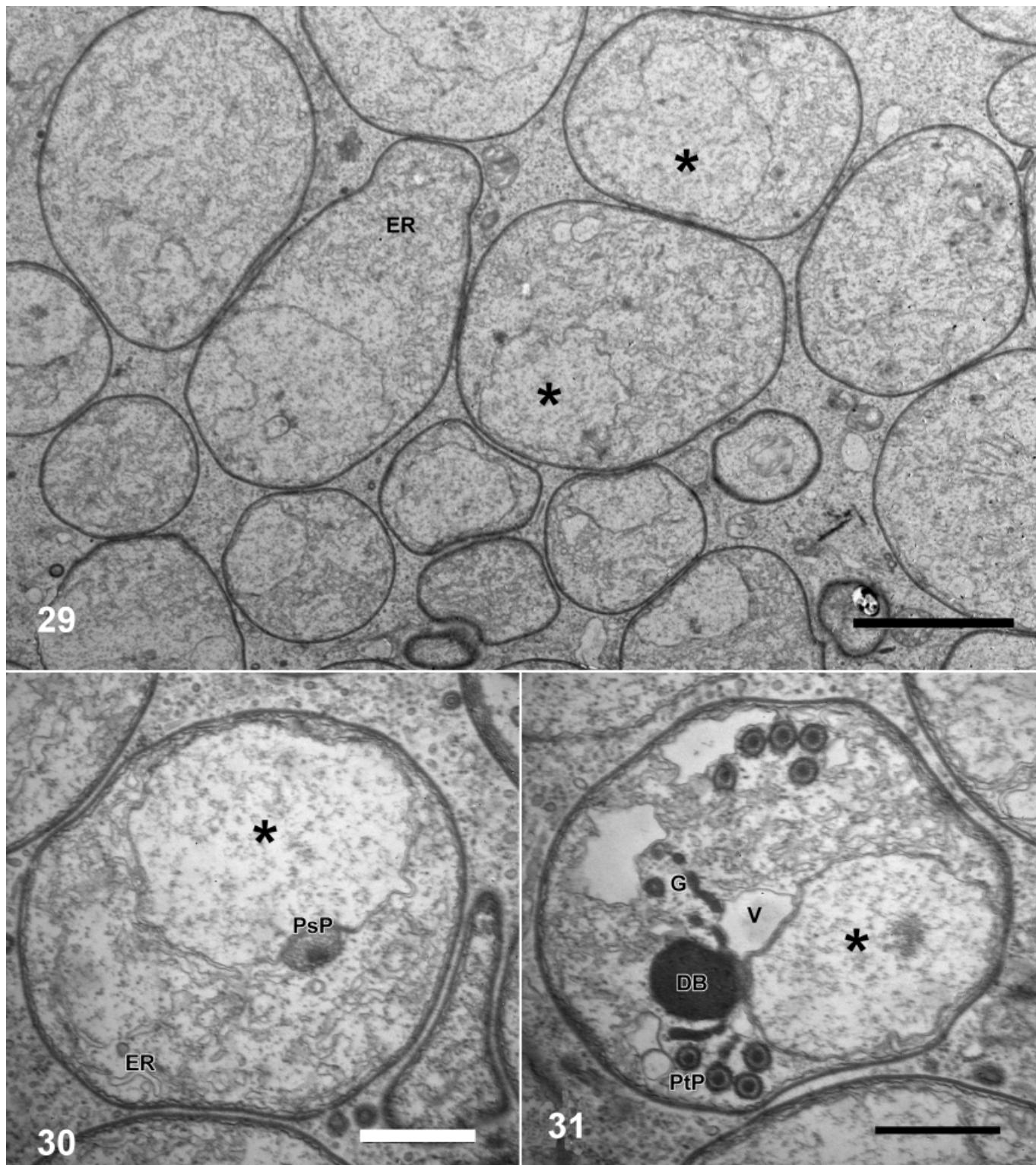


Fig. 29–31. Sporonts of *Paranucleospora theridion* n. gen., n. sp. in the salmon louse *Lepeophtheirus salmonis*. 29. Early monokaryotic sporonts with rough endoplasmatic reticulum (ER) and dense material on the surface of the plasmalemma. Monokaryon (asterisk). Scale bar = 2.0 μm . 30. Sporont with polar sac primordium (PsP). Scale bar = 2.0 μm . 31. Sporont containing polar tube primordium (PtP), a dense body (DB), translucent vacuole (V), and Golgi elements (G). Scale bar = 2.0 μm .

gonial plasmodia in direct contact with the nucleoplasm, and develop polar tube precursors and other elements of the extrusion apparatus before fission and sporoblast formation (Lom and Dyk-

ova 2002). The main differences in the intranuclear development of genus *Paranucleospora* and *P. theridion* to genus *Nucleospora* are the diplokaryotic vs. monokaryotic development and the

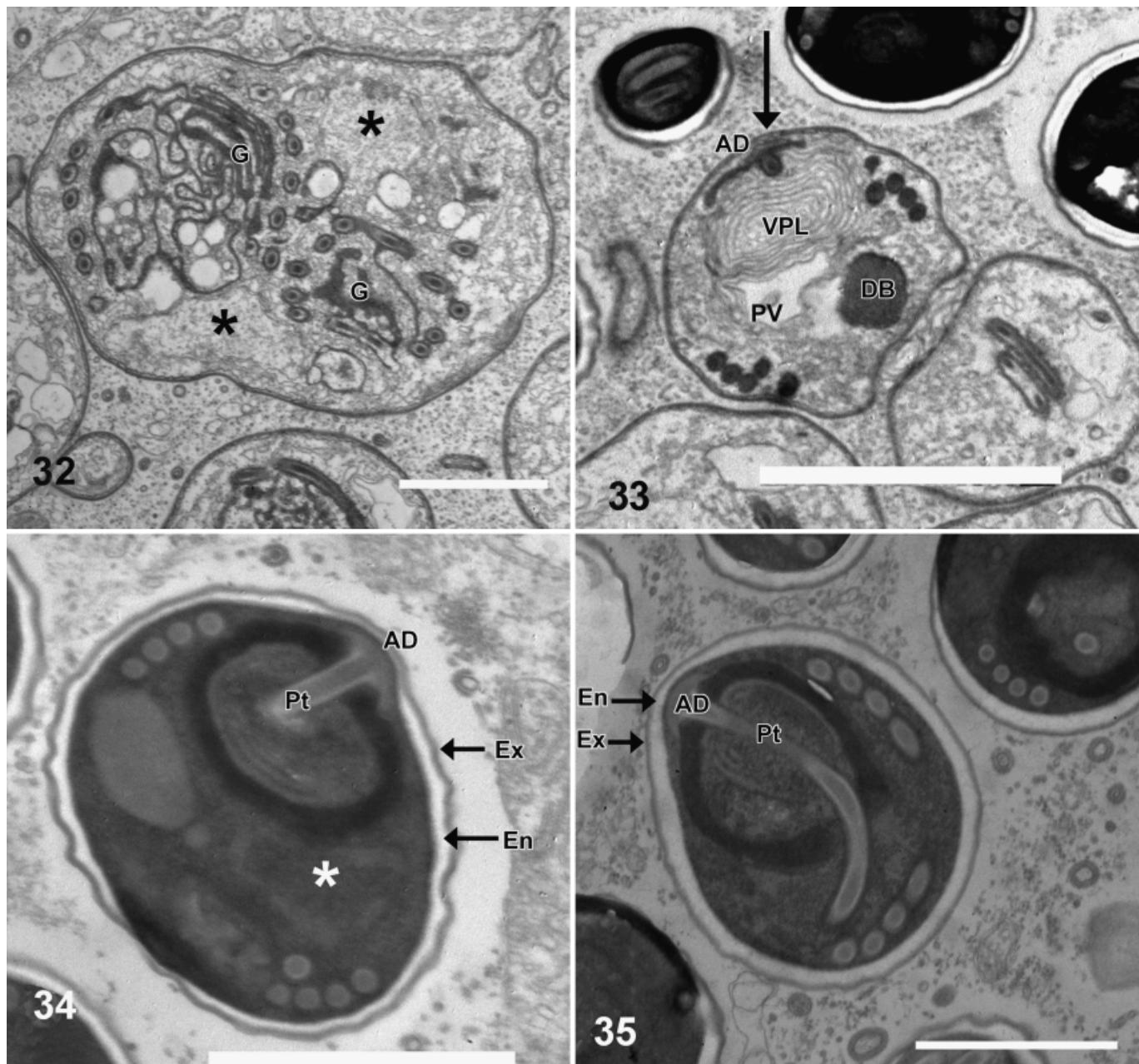


Fig. 32–35. Sporoblasts and spores of *Paranucleospora theridion* n. gen., n. sp. in the salmon louse *Lepeophtheirus salmonis*. **32.** Mother sporoblast before binary fission. Monokaryotic nuclei (asterisks) and Golgi elements (G) are indicated. Scale bar = 1.0 µm. **33.** Sporoblasts (arrow) with anchoring disc (AD), vesicular polaroplast (VPL), posterior vacuole (PV), and dense body (DB). Scale bar = 2.0 µm. **34.** Mature spore. The spore nucleus (asterisk), polar tube (Pt), anchoring disc (AD), exospore (Ex), and endospore (En) are indicated. Scale bar = 1.0 µm. **35.** Mature spore. Scale bar = 1.0 µm.

number of spores produced in sporogony. The occurrence of diplokarya in microsporidian development has been considered an important character in classification (reviewed by Sprague, Bechner, and Hazard 1992), but is now considered a plesiomorphic trait of lower weight (Larsson 1986). Genus *Nucleospora* contain, in addition to *N. salmonis* and *N. secunda*, several undescribed species (see Freeman et al. 2003; Lom 2002; Lom and Dykova 2002). So far, only intranuclear development is known for *Nucleospora* spp., and it is always mononuclear. *Paranucleospora theridion* seems to produce only two spores in the nuclei of infected cells; very rare occurrences of up to four spores are interpreted as double infections. *Nucleospora* spp. generally (always?) produces

more than 2, with normally 6 (Nilsen et al. 1995), 1–18 (Chilmonczyk, Cox, and Hedrick 1991), 1–6 (Mullins et al. 1994), and 7–18 (Lom and Dykova 2002, in images). All these observations are based on sections and hence represent minima. In addition, *P. theridion* spores differs from those of *Nucleospora* spp. in showing anisofilar polar tubes, and by generally containing a large vacuole in addition to the posterior vacuole, with contents of a density comparable to that of the polar tube (cf. Canning et al. 2005).

The development of *P. theridion* in *L. salmonis* resembles that of species of *Microgemma*, *Neonosemoides*, and *Kabatana* in being apansporoblastic and in direct contact with the host cell

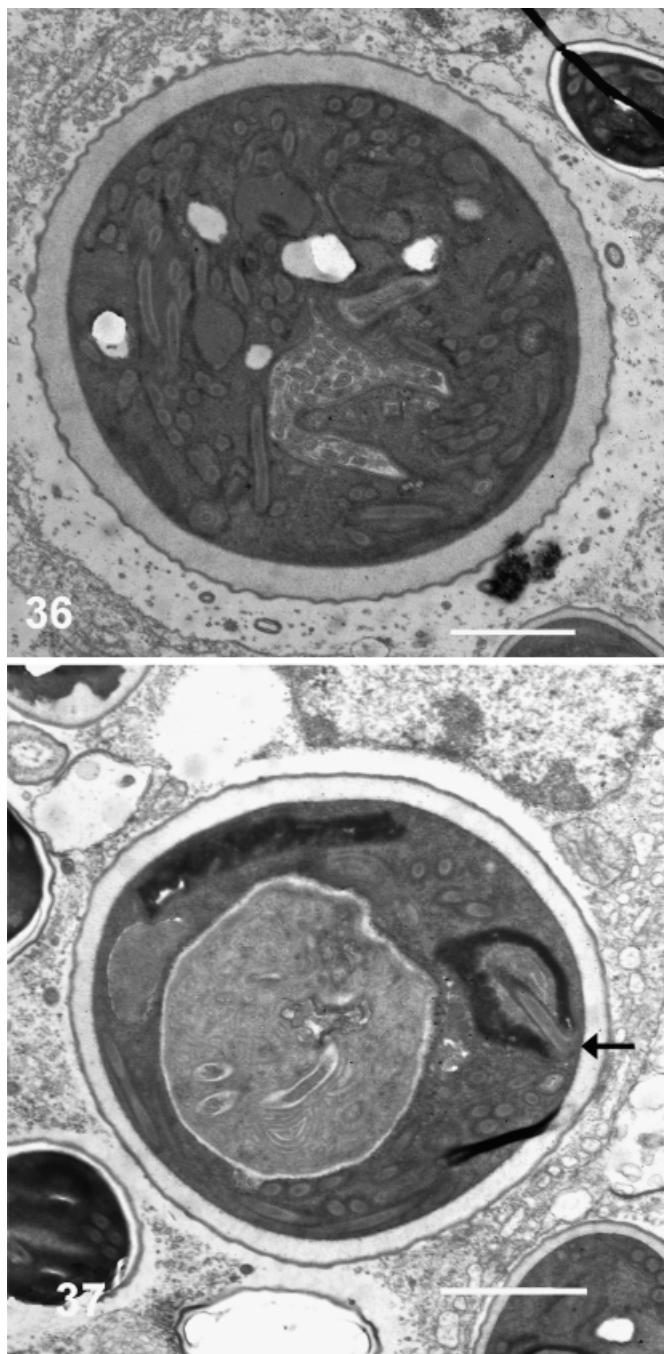


Fig. 36,37. Macrospheres of *P. theridion* in *L. salmonis*. 36. Macrospore. Scale bar = 1.0 μm . 37. Macrospore. Anchoring disc (arrow). Scale bar = 1.0 μm .

cytoplasm (Lom 2002). *Microgemma* spp. differ by having monokaryotic merogonial stages enclosed by ER, have free sporonts, and produce sporoblast mother cells (Canning et al. 2005; Lom 2002). *Neonosemoides tilapiaie*, like *P. theridion*, show diplokaryotic merogony, but the diplokarya dissociate, merogony proceeds as monokaryotic, and sporoblast mother cells are not known (Faye, Toguebaye, and Bouix 1996). The overall development in *Kabatana* spp. is very similar to that of *P. theridion* and includes sporoblast mother cells, but diplokarya do not occur and *Kabatana* spp. never produce xenomas (Lom 2002; Lom, Dyková,

and Shaharom 1990). None of these three genera shows polar tube assembly in still proliferating sporogonial stages. Furthermore, *Microgemma* spp. and *Kabatana* spp. for which rDNA sequences are available, have proven unrelated to the Enterocytozoonidae (Vossbrinck and Debrunner-Vossbrinck 2005).

The very large spores regularly produced by *P. theridion* in *L. salmonis* have no functional counterpart among the microsporidia and may have resulted from anomalous development. A life cycle involving three (known) cycles of sporogony is unique for fish-infecting microsporidia, but does occur in some microsporidia infecting arthropods. The development of *P. theridion* seems to follow the developmental Cycle IV pattern of Larsson (1986), but with two types of spores produced in each of two very different hosts. All development in the salmon is diplokaryotic and presumably haplophasic, while sexual processes (e.g. karyogamy and meiosis in sporonts) likely occur in the salmon louse.

The known merogony of the intracytoplasmic spores in salmon is limited to nuclear proliferation and plasmotomy of the merogonial plasmodia, with direct transition to sporogony. As limited numbers of spores are produced in each infected cell, the systemic occurrence of *P. theridion* in salmon and high density of the parasite in many organs suggest that autoinfection is important in the proliferation of the parasite. Microsporidia may produce autoinfective spores, which become activated and germinate within the same host in which they were produced (Iwano and Kurtti 1995). The species that produce autoinfective spores in addition to environmental spores usually have different numbers of polar tube coils within their spores (Iwano and Ishihara 1989, 1991) where the autoinfective spores have the shorter polar tubules (Iwano and Kurtti 1995). We therefore consider the cytoplasmic spores of *P. theridion* to be autoinfective, i.e. promoting proliferation and spreading within the host. The differing functional roles of these spores are also supported by the fact that the spore wall of these cytoplasmic spores is much thinner compared with that of the intranuclear spores: this can be explained if these spores deposit their sporoplasm within the same host experiencing minimal environmental stress. Because host cells with *P. theridion* spores in the cytoplasm were observed to degenerate, this will release spores in the tissues and body fluids, which may be effectively spread the infection throughout the organism. The mode of *P. theridion* entry into epidermal cells is unknown.

The parasite proliferation resulting from nuclear development appears very limited, since only stages with two diplokarya were observed producing two spores, exceptionally four. However, because the turnover ratio of epidermal cells may be high (Lester and Adams 1974), a possibility is that division of epithelial cells with intranuclear meronts is also accompanied by simultaneous meront division. Desportes-Livage et al. (1996) observed coincidental divisions of the host cell nucleus and merogonic bipartitioning of uninucleate stages of *N. salmonis*, and suggested merogony could proceed in this manner. The mature spores of *P. theridion* produced in nuclei of epidermal cells are thick walled and resistant, and may be released to the water through the normal sloughing of epidermal cells. However, salmon lice feeding on the epidermis of infected salmon must devour large numbers of these spores, rendering it feasible that the lice are infected per os.

Vertical (i.e. transovarial) transmission occurs in the life cycle of a number of species of Microsporidia: *Amblyospora* spp. (Andreadis and Hall 1979; Dickson and Barr 1990), *Nosema* spp. (Han and Watanabe 1988; Raina et al. 1995), and *Edhazardia aedis* (Becnel et al. 1989). Because we repeatedly found the egg strings of *L. salmonis* to be highly positive for *P. theridion* with real time PCR, and were able to sequence targeted rDNA of *P. theridion* from them, it is likely these contain parasite stages. However, we have not been able to detect the parasite in positive egg strings using light and transmission electron microscopy,

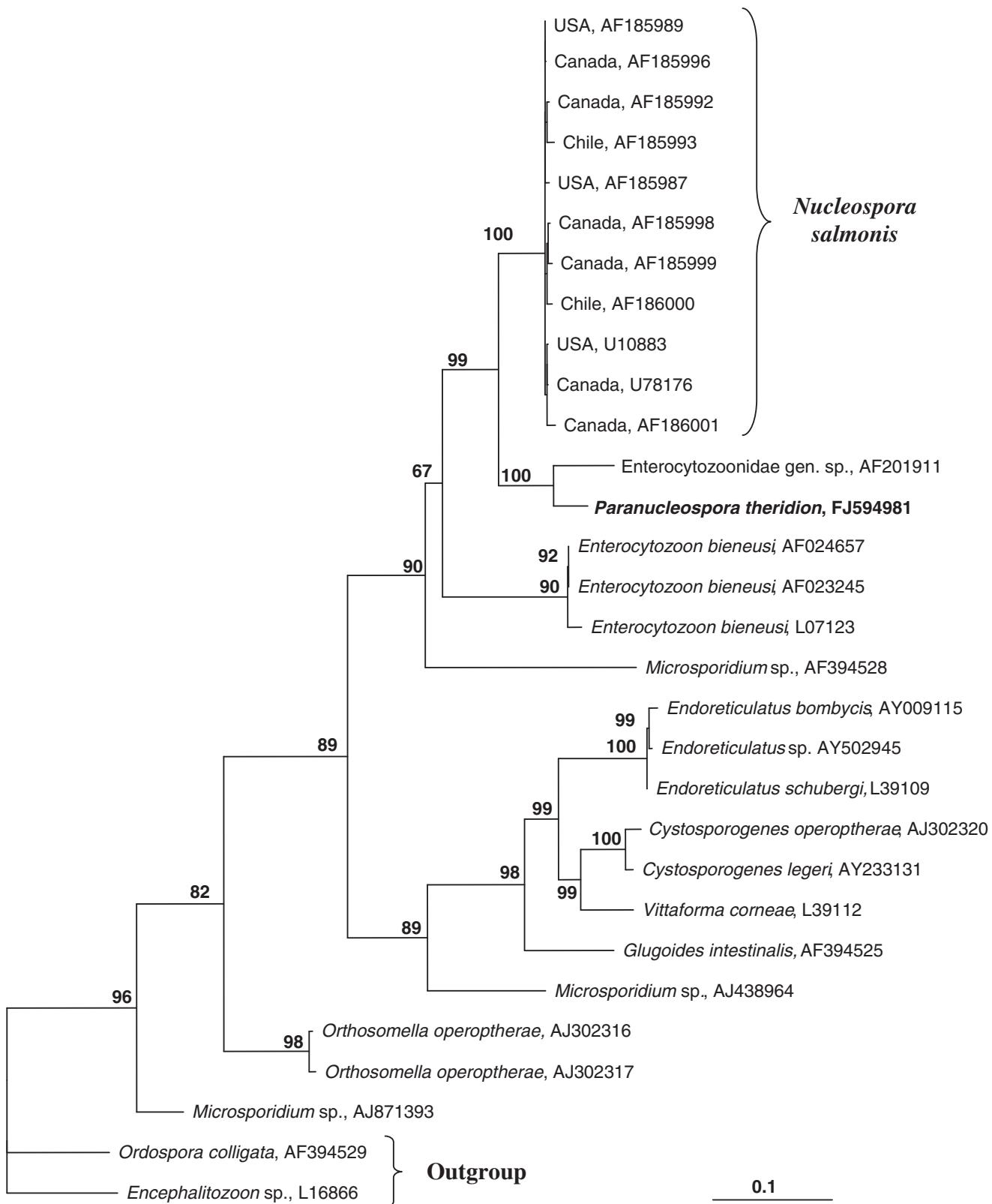


Fig. 38. The phylogenetic position of *Paranucleospora theridion* n. gen., n. sp. in relation to a selection of other microsporidia. The relationships are presented as a maximum likelihood tree derived by Quartet puzzling and based on an alignment small subunit rDNA sequences. Branch lengths represent relative phylogenetic distances according to maximum likelihood estimates. See Table 3 for names and database accession numbers.

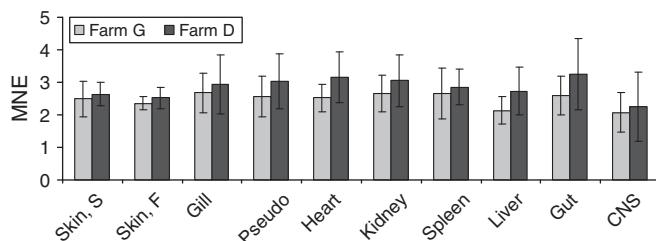


Fig. 39. Tissue distribution of *Paranucleospora theridion* in Atlantic salmon *Salmo salar* collected on 6 November 2008 in two farms in western Norway. The mean normalized expression (MNE) describes the amount of *P. theridion* detected in tissue samples in relation to an exogenous control based on RNA.

which are far less sensitive methods (data not shown). Sokolova, Lange, and Fuxa (2008) presented evidence that particularly large spores, referred to as megaspores were responsible for the transovarial transmission of *Kneallhazia solenopsae* in fire ant queens (Sokolova et al. 2008). Such a role is also a possibility for the extremely large *P. theridion* spores in the salmon louse. However, because at least some of these contained several sets of the extrusion apparatus, these spores may be anomalous and non-functional.

Both Atlantic salmon and salmon lice may function as natural reservoirs for *P. theridion*. In addition, there could be reservoirs in other caligids (i.e. *C. elongatus*) and fish species (i.e. *Salmo trutta*) including marine fish.

Taxonomic summary

Phylum Microspora Sprague, 1977

Class Microsporea Delphy, 1963

Order Microsporidia Balbiani, 1892

Suborder Apansporoblastina Tuzet, Maurand, Fize, Michel & Fenwick, 1971

Family Enterocytozoonidae Cali and Owen, 1990

Genus *Paranucleospora* n. gen.

Diagnosis. Monotypic genus. Two-host life cycle. Three different developmental cycles leading to the production of three different types of spores. All development in direct contact with host cell cytoplasm or nucleoplasm. Spores small with anisofilar polar tubes.

Sporogony I. Cytoplasmic diplokaryotic minute, thin-walled spores with short polar tube. Merogony limited (plasmotomy); spore components produced before division of sporogonial plasmodia; dense plasmalemma coat added during fission of plasmodium. **Sporogony II.** Intranuclear diplokaryotic development. Merogony by binary fission; sporogonial proliferation limited; one or two spores produced in each nucleus. **Sporogony III.** Merogony diplokaryotic, diplokaryotic sporonts develop dense plasmalemma coating and become monokaryotic; divide schizogonically and produce monokaryotic cells. The monokaryotic sporogonial cells (i.e. Stage I cells) proliferate by binary or multiple fission, producing smaller cells (i.e. Stage II cells). Stage II cells develop components of the extrusion apparatus but continue binary fission. Sporoblasts recognized by apical polar sac.

Etymology. *Para* (Gk) beside, *Nucleospora* (generic name) as this taxon occurs as the sister group of *Nucleospora* on the rDNA phylogeny. Gender of name is feminine.

Paranucleospora theridion n. sp.

Synonym. “microsporidian from *L. salmonis*” of Freeman et al. (2003).

Diagnosis. With characters of the genus. Two developmental cycles in Atlantic salmon *S. salar*, resulting in spores in the cytoplasm of reticulo-endothelial and epithelial cells and spores in

the nuclei of epithelial cells. Spherical autoinfective diplokaryotic spores, 0.9–1.2 µm in diameter, produced in the cytoplasm of reticuloendothelial and epithelial cells in Atlantic salmon. Environmental diplokaryotic spores, 2.4–2.7 × 2.0–2.1 µm, in nuclei of epithelial cells of gills and skin of Atlantic salmon. Spherical monokaryotic spores, 2.2–2.5 µm in diameter, in the cytoplasm of epithelial cells, connective tissue cells, gonadal cells, satellite cells, and unidentified haemocytes in the salmon louse *L. salmonis*.

Type definitive hosts. *Lepeophtheirus salmonis* Krøyer (Copepoda, Caligidae).

Type intermediate host. Atlantic salmon *S. salar* L. (Teleostei, Salmonidae).

Additional hosts. *Caligus elongatus* Nordmann (Copepoda, Caligidae), *O. mykiss* (Walbaum) (Teleostei, Salmonidae).

Type locality. Fensfjorden, western Norway (60°51'N, 04°56'E).

Type material deposition. Slides of histological sections from gills, kidney and skin stained with Diff- Quick® (Dade Behring AB, Switzerland) have been deposited in the Zoological Museum, University of Bergen (ZMUB no. 84838).

Prevalence. 98% in *L. salmonis* ($N = 50$); 100% in *S. salar* ($N = 103$) (type locality).

Site of infection. Cytoplasm of epithelial cells, connective tissue cells, haemocytes and satellite cells in *L. salmonis*; nucleus of epithelial cells and cytoplasm of leucocytes, endothelial cells, and epithelial cells of *S. salar*.

Etymology. *Theridion* (Gk) meaning little beast.

Gene sequences. Sequences of the 16S rRNA gene, ITS, and a portion of the 23S rRNA gene are deposited as GenBank accession nos: FJ594980, FJ594981, FJ594987, FJ594990 (Type definitive host—*L. salmonis*), FJ389667, FJ594969-FJ594978, FJ594982-FJ594986, FJ594988 (Intermediate host—*S. salar*), and FJ594979, FJ594989 (Additional hosts—*C. elongatus* and *O. mykiss*, respectively).

ACKNOWLEDGMENT

This work was financed by the Norwegian research counsel (NFR) on grant number 164832/S40.

LITERATURE CITED

- Andreadis, T. G. & Hall, D. W. 1979. Significance of transovarial infections of *Amblyospora* sp. (Microspora, Thelohaniidae) in relation to parasite maintenance in the mosquito *Culex salinarius*. *J. Invert. Pathol.*, **34**:152–157.
- Becnel, J. J., Sprague, V., Fukuda, T. & Hazard, E. I. 1989. Development of *Edhazardia aedis* (Kudo, 1930) n. g., n. comb. (Microsporida, Amblyosporidae) in the mosquito *Aedes aegypti* (L.) (Diptera, Culicidae). *J. Protozool.*, **36**:119–130.
- Cali, A. & Owen, R. L. 1990. Intracellular development of *Enterocytozoon*, a unique microsporidian found in the intestine of AIDS patients. *J. Protozool.*, **37**:145–155.
- Cali, A. & Takvorian, P. M. 1999. Developmental morphology and life cycle of the Microsporidia. In: Wittner, M. & Weiss, L. M. (ed.), *The Microsporidia and Microsporidiosis*. ASM Press, Washington, DC. p. 85–128.
- Canning, E. U., Feist, S. W., Longshaw, M., Okamura, B., Anderson, C. L., Tse, M. T. & Curry, A. 2005. *Microgemma vivaresi* n. sp. (Microsporidia, Tetramicridae), infecting liver and skeletal muscle of sea scorpions, *Taurulus bubalis* (Euphrasen, 1786) (Osteichthyes, Cottidae), an inshore, littoral fish. *J. Eukaryot. Microbiol.*, **52**:123–131.
- Chilmonczyk, S., Cox, W. T. & Hedrick, R. P. 1991. *Enterocytozoon salmonis* n. sp.: an intranuclear microsporidium from salmonid fish. *J. Protozool.*, **38**:264–269.
- Desportes, I., Lecharpentier, Y., Galian, A., Bernard, F., Cochandpriollet, B., Lavergne, A., Ravisse, P. & Modigliani, R. 1985. Occurrence of a

- new microsporidan—*Enterocytozoon bieneusi* n. g., n. sp., in the enterocytes of a human patient with AIDS. *J. Protozool.*, **32**:250–254.
- Desportes-Livage, I., Chilmonczyk, S., Hedrick, R., Ombrouck, C., Monge, D., Maiga, I. & Gentilini, M. 1996. Comparative development of two microsporidian species: *Enterocytozoon bieneusi* and *Enterocytozoon salmonis*, reported in AIDS patients and salmonid fish, respectively. *J. Eukaryot. Microbiol.*, **43**:49–60.
- Devold, M., Krossøy, B., Aspehaug, V. & Nylund, A. 2000. Use of RT-PCR for diagnosis of infectious salmon anaemia virus (ISAV) in carrier sea trout *Salmo trutta* after experimental infection. *Dis. Aquat. Org.*, **40**:9–18.
- Dickson, D. L. & Barr, A. R. 1990. Development of *Amblyospora campbelli* (Microsporida, Amblyosporidae) in the mosquito *Culiseta incidunt* (Thomson). *J. Protozool.*, **37**:71–78.
- Faye, N., Toguebaye, B. S. & Bouix, G. 1991. *Microfilum lutjani* n. g., n. sp. (Protozoa, Microsporida), a gill parasite of the Golden African snapper *Lutjanus fulgens* (Valenciennes, 1830) (Teleost Lutjanidae)—developmental cycle and ultrastructure. *J. Protozool.*, **38**:30–40.
- Faye, N., Toguebaye, B. S. & Bouix, G. 1996. Ultrastructure and development of *Neonosemoides tilapiaie* (Sakiti and Bouix, 1987) n. g., n. comb. (Protozoa, Microspora) from African cichlid fish. *Eur. J. Protistol.*, **32**:320–326.
- Ferguson, H. W., Poppe, T. & Speare, D. J. 1990. Cardiomyopathy in farmed Norwegian salmon. *Dis. Aquat. Org.*, **8**:225–231.
- Freeman, M. A., Bell, A. S. & Sommerville, C. 2003. A hyperparasitic microsporidian infecting the salmon louse, *Lepeophtheirus salmonis*: an rDNA-based molecular phylogenetic study. *J. Fish Dis.*, **26**: 667–676.
- Gresoviac, S. J., Baxa, D. V., Vivares, C. P. & Hedrick, R. P. 2007. Detection of the intranuclear microsporidium *Nucleospora salmonis* in naturally and experimentally exposed chinook salmon *Oncorhynchus tshawytscha* by *in situ* hybridization. *Parasitol. Res.*, **101**:1257–1264.
- Han, M. S. & Watanabe, H. 1988. Transovarial transmission of two microsporidia in the silkworm, *Bombyx mori*, and disease occurrence in the progeny population. *J. Invert. Pathol.*, **51**:41–45.
- Hedrick, R. P., Groff, J. M. & Baxa, D. V. 1991. Experimental infections with *Enterocytozoon salmonis* Chilmonczyk, Cox, Hedrick (Microsporea)—an intranuclear microsporidium from chinook salmon *Oncorhynchus tshawytscha*. *Dis. Aquat. Org.*, **10**:103–108.
- Iwano, H. & Ishihara, R. 1989. Intracellular germination of spores of a *Nosema* sp. immediately after their formation in cultured cell. *J. Invert. Pathol.*, **54**:125–127.
- Iwano, H. & Ishihara, R. 1991. Dimorphism of spores of *Nosema* spp. in cultured cell. *J. Invert. Pathol.*, **57**:211–219.
- Iwano, H. & Kurtti, T. J. 1995. Identification and isolation of dimorphic spores from *Nosema furnacalis* (Microspora, Nosematidae). *J. Invert. Pathol.*, **65**:230–236.
- Khattra, J. S., Gresoviac, S. J., Kent, M. L., Myers, M. S., Hedrick, R. P. & Devlin, R. H. 2000. Molecular detection and phylogenetic placement of a microsporidian from English sole (*Pleuronectes vetulus*) affected by X-cell pseudotumors. *J. Parasitol.*, **86**:867–871.
- Kongtorp, R. T., Kjerstad, A., Taksdal, T., Guttvik, A. & Falk, K. 2004. Heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.: a new infectious disease. *J. Fish. Dis.*, **27**:351–358.
- Kvellestad, A., Falk, K., Nygaard, S. M. R., Flesja, K. & Holm, J. A. 2005. Atlantic salmon paramyxovirus (ASPV) infection contributes to proliferative gill inflammation (PGI) in seawater-reared *Salmo salar*. *Dis. Aquat. Org.*, **67**:47–54.
- Larsson, R. 1986. Ultrastructure, function, and classification of microsporidia. *Prog. Protistol.*, **1**:325–390.
- Lester, R. J. G. & Adams, J. R. 1974. *Gyrodactylus alexanderi*—reproduction, mortality and effect on its host *Gasterosteus aculeatus*. *Can. J. Zool.*, **52**:827–833.
- Lom, J. 2002. A catalogue of described genera and species of microsporidians parasitic in fish. *Syst. Parasitol.*, **53**:81–99.
- Lom, J. & Dykova, I. 2002. Ultrastructure of *Nucleospora secunda* n. sp. (Microsporidia), parasite of enterocytes of *Nothobranchius rubripinnis*. *Eur. J. Protistol.*, **38**:19–27.
- Lom, J. & Nilsen, F. 2003. Fish microsporidia: fine structural diversity and phylogeny. *Int. J. Parasitol.*, **33**:107–127.
- Lom, J., Dykova, I. & Shaharom, F. 1990. *Microsporidium arthuri* n. sp., parasite of *Pangasius sutchi* (Pangasiidae, Siluroidea) in South-East Asia. *Dis. Aquat. Org.*, **8**:65–67.
- McLoughlin, M. F., Nelson, R. T., Rowley, H. M., Cox, D. I. & Grant, A. N. 1996. Experimental pancreas disease in Atlantic salmon *Salmo salar* post-smolts induced by salmon pancreas disease virus (SPDV). *Dis. Aquat. Org.*, **26**:117–124.
- Modin, J. C. 1981. *Microsporidium rhabdophilia* n. sp. from rodlet cells of salmonid fishes. *J. Fish. Dis.*, **4**:203–211.
- Muller, P. Y., Janovjak, H., Miserez, A. R. & Dobbie, Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques*, **32**:1372–1374, 1376, 1378–1379.
- Mullins, J. E., Powell, M., Speare, D. J. & Cawthron, R. 1994. An intranuclear microsporidian in lumpfish *Cyclopterus lumpus*. *Dis. Aquat. Org.*, **20**:7–13.
- Nilsen, F., Ness, A. & Nylund, A. 1995. Observations on an intranuclear microsporidian in lymphoblasts from farmed Atlantic halibut larvae (*Hippoglossus hippoglossus* L.). *J. Eukaryot. Microbiol.*, **42**:131–135.
- Nylund, A., Hovland, T., Watanabe, K. & Endresen, C. 1995. Presence of infectious salmon anemia virus (ISAV) in tissues of Atlantic salmon, *Salmo salar* L., collected during three separate outbreaks of the disease. *J. Fish. Dis.*, **18**:135–145.
- Olsvik, P. A., Lie, K. K., Jordal, A. E. O., Nilsen, T. O. & Hordvik, I. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Mol. Biol.*, **6**:21.
- Page, R. D. M. 1996. TreeView: an application to display phylogenetic trees on personal computers. *CABIOS*, **12**:357–358.
- Posada, D. & Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinform. Appl. Note*, **14**:817–818.
- Raina, S. K., Das, S., Rai, M. M. & Khurad, A. M. 1995. Transovarial transmission of *Nosema locustae* (Microsporida, Nosematidae) in the migratory locust *Locusta migratoria migratorioides*. *Parasitol. Res.*, **81**:38–44.
- Sokolova, Y. Y., Lange, C. E. & Fuxa, J. R. 2008. Phylogenetic relationships of *Heterovesicula cowani*, a microsporidian pathogen of Mormon crickets, *Anabrus simplex* (Orthoptera: Tettigoniidae), based on SSU rDNA sequence analyses. *J. Invert. Pathol.*, **99**:112–116.
- Sprague, V., Becnel, J. J. & Hazard, E. I. 1992. Taxonomy of phylum Microspora. *Crit. Rev. Microbiol.*, **18**:285–395.
- Stentiford, G. D. & Bateman, K. S. 2007. *Enterospora* sp., an intranuclear microsporidian infection of hermit crab *Eupagurus bernhardus*. *Dis. Aquat. Org.*, **75**:73–78.
- Stentiford, G. D., Bateman, K. S., Longshaw, M. & Feist, S. W. 2007. *Enterospora canceri* n. gen., n. sp., intranuclear within the hepatopancreatocytes of the European edible crab *Cancer pagurus*. *Dis. Aquat. Org.*, **75**:61–72.
- Swofford, D. L. 1998. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, MA.
- Vossbrinck, C. R. & Debrunner-Vossbrinck, B. A. 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. *Folia Parasitol.*, **52**:131–142.
- Watanabe, K., Karlsen, M., Devold, M., Isdal, E., Litlabo, A. & Nylund, A. 2006. Virus-like particles associated with heart and skeletal muscle inflammation (HSMI). *Dis. Aquat. Org.*, **70**:183–192.
- Wittner, M. & Weiss, L. M. 1999. The Microsporidia and Microsporidiosis. ASM Press, Washington, DC. p. 418–446.

Received: 07/03/09, 09/21/09; accepted: 08/06/09