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The Diversity of Microsporidia in Parasitic Copepods (Caligidae: Siphonostomatoida) in the Northeast Pacific Ocean with Description of *Facilispora margolisi* n. g., n. sp. and a new Family Facilisporidae n. fam.

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ABSTRACT. Three distinct microsporidia were identified from parasitic copepods in the northeast Pacific Ocean. Sequencing and phylogenetic analysis of a partial small subunit ribosomal RNA gene (SSU rDNA) sequence identified a genetically distinct variety of *Desmozoon lepeophtherii* from *Lepeophtheirus salmonis* on cultured Atlantic salmon *Salmo salar*, and this was confirmed by transmission electron microscopy. Phylogenetic analysis resolved the SSU rDNA sequence of the second organism in a unique lineage that was most similar to microsporidia from marine and brackish water crustaceans. The second occurred in *L. salmonis* on Atlantic, sockeye *Oncorhynchus nerka,* chum *O. keta* and coho *O. kisutch* salmon, in *Lepeophtheirus cuneifer* on Atlantic salmon, and in *Lepeophtheirus parviventris* on Irish Lord *Hemilepidotus hemilepidotus*. Replication occurred by binary fission during merogony and sporogony, diplokarya were not present, and all stages were in contact with host cell cytoplasm. This parasite was identified as *Facilispora margolisi* n. g., n. sp. and accommodated within a new family, the Facilisporidae n. fam. The third, from *Lepeophtheirus hospitalis* on starry flounder *Platichthys stellatus*, was recognized only from its unique, but clearly microsporidian SSU rDNA sequence. Phylogenetic analysis placed this organism within the clade of microsporidia from crustaceans.

Key Words. *Lepeophtheirus*, marine fishes, microsporidian taxonomy, sea lice.

THE phylum Microsporidia includes obligate, spore-forming parasites, considered now to be fungi (Keeling and Fast 2002). Microsporidia have been detected in most animal taxa with an emphasis on economically important invertebrates and fishes, in which these parasites cause diseases (Shaw and Kent 1999). Approximately 50 species belonging to nine genera have been reported from copepods (Bronnvall and Larsson 2001; Vávra et al. 2005). Freeman et al. (2003) reported microsporidian parasites from Scottish specimens of *Lepeophtheirus salmonis* (Siphonostomatoida: Caligidae), a copepod parasite of salmonid fishes in oceans of the northern hemisphere. The parasite from *L. salmonis*, based on ribosomal RNA gene sequences and on patterns of development, was later determined to belong to a new genus and named *Desmozoon lepeophtherii* (Freeman and Sommerville 2009). This parasite was also shown to be related to members of the genus *Nucleospora*, which occur in marine fish. *Desmozoon lepeophtherii* also occurs in numerous tissues from Atlantic salmon, *Salmo salar* that are reared in netpens and infested with *L. salmonis* (Freeman 2002; Nylund et al. 2010). The microsporidian undergoes two cycles of development in the salmon and one in the copepod. However, transmission mechanisms between the salmon and copepod hosts were not identified. Nylund et al. (2010) proposed the name *Paranucleospora theridion,* which is herein considered a junior synonym of *D. lepeophtherii* (see also Freeman and Sommerville 2011).

The management of parasitic copepods on marine netpen-reared Atlantic salmon on the Pacific coast of Canada and concern about the negative impacts of copepod infections on wild salmon has stimulated considerable research (e.g. Jones and Hargreaves 2009). In addition, recent evidence suggested that *L. salmonis* is associated with the epidemiology of amoebic gill disease among farmed Atlantic salmon in Washington State (WA), USA (Nowak et al. 2010). Microscopic examination of stained histological preparations of *L. salmonis*, collected to further explore the latter observations, revealed xenoma-like structures below the cuticle similar to those originally described by Freeman et al. (2003). This paper reports the results of a more thorough surveillance of marine parasitic copepods for the occurrence of microsporidia. Light and electron microscopy combined with analyses of partial small subunit ribosomal gene sequences (SSU rDNA) were used to document the first description of *D. lepeophtherii* in *L. salmonis* from the Pacific Ocean and to describe a new microsporidian species in *L. salmonis*, *Lepeophtheirus cuneifer*, and *Lepeophtheirus parviventris*, all copepod parasites of marine fishes in the northeastern Pacific Ocean. A novel SSU rDNA microsporidian sequence was also obtained from *Lepeophtheirus hospitalis*.

MATERIALS AND METHODS

Collection of parasitic copepods. Adults, preadults, and chalimi of *L. salmonis* were collected from Atlantic salmon cultured in marine netpens in WA and in British Columbia (BC), Canada and from wild Pacific salmon (*Oncorhynchus* spp.) in BC and WA. Specimens of *L. cuneifer* were also obtained from cultured Atlantic salmon in BC and from Pacific herring *Clupea pallasi* in BC and Alaska, USA. Other specimens collected from BC included *L. parviventris* from Irish Lord *Hemilepidotus hemilepidotus*, *Lepeophtheirus bifidus* from starry flounder *Platichthys stellatus*, *Lepeophtheirus oblitus* from kelp greenling *Hexagrammos decagrammus*, *Lepeophtheirus hospitalis* from *P. stellatus*, and *Caligus clemensi* from Atlantic salmon, sablefish *Anoplopoma fimbria*, Pacific salmon, and the threespine stickleback *Gasterosteus aculeatus*. Freshly collected copepods were preserved in 95% (v/v) ethanol, 4% (v/v) neutral buffered formaldehyde (NBF) or 3% (v/v) glutaraldehyde in Sörensen's buffer. Some larger specimens of *L. salmonis* were sagittally bisected and the halves preserved in 95% ethanol and either NBF or glutaraldehyde, the latter following further subdivision into pieces of 1 or 2 mm. The adult copepods were identified using established morphological criteria (Kabata 1974, 1988).

Preparation of copepods for light and electron microscopy. Copepods fixed in NBF and previously shown by polymerase chain reaction (PCR; see below) to be positive for microsporidia were processed for routine histological examination. Sections of 3–5 μm were mounted onto glass slides, stained with haematoxylin and eosin, Giemsa or Gram's stain and coverslipped for light microscopy. Specimens preserved in glutaraldehyde were washed with 0.1 M Sörenson's buffer, post-fixed 1 h with 1% (w/v) OsO_4, washed with 50% (v/v) ethanol, and

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stained for 1 h with 5% (w/v) uranyl acetate. Tissues were dehydrated through an alcohol gradient and two changes of absolute alcohol into acetone. Tissues were incubated overnight in a 50:50 mixture of acetone and epoxy resin (Jembed 812; Canmeco Inc., Quebec, Canada) followed by an incubation of 6–7 h in 100% resin at 60 °C.

Electron microscopy. Tissues were processed from specimens of *L. salmonis* previously shown by PCR to be positive for microsporidia. Tissue blocks were trimmed and cut on a Reichert-Jung Ultracut E ultramicrotome (Leica Microsystems, Richmond Hill, Ontario, Canada). Semithin sections of 0.5 μm were mounted on glass slides and stained with 1% (w/v) toluidine blue for light microscopy. Ultrathin sections of less than 100 nm were stained with 5% (w/v) uranyl acetate and lead Sato stain (i.e. a mixture of lead acetate, lead nitrate and lead citrate; Sato 1968), mounted onto 200-mesh copper super-grids and viewed at 80 kV using a Hitachi TEM 7500 (Nissei-Sangyo, Rexdale, Ontario, Canada). Digital images were captured using an AMT HR40 camera (Advanced Microscopy Techniques, Danvers, MA, USA).

Polymerase chain reactions and sequencing. Deoxyribonucleic acid (DNA) was extracted from alcohol-preserved copepods by following the DNeasy animal tissue protocol (Qiagen, Mississauga, Ontario, Canada) as previously reported (Jones and Prosperi-Porta 2011). DNA solutions were stored at −20 °C prior to use as template in PCR. Microsporidian generic and type-specific oligonucleotide primers were used to amplify partial small subunit (SSU), internal transcribed spacer (ITS), and partial large subunit (LSU) rDNA sequences (Table 1). Amplification methods follow those described earlier (Freeman et al. 2003; Pomport-Castillon et al. 1997; Vossbrinck et al. 1993). PCR products were visualized by UV transillumination following electrophoresis in 1.5% agarose and staining with Sybr Safe (Invitrogen, Burlington, Ontario, Canada). The products were cleaned using ExoSap-IT (USB Corporation, Cleveland, OH, USA) and sequencing reactions were performed using BDT V3.1 (Applied Biosystems, Foster City, CA, USA). Reaction products were purified using Qiagen Dye-Ex 2.0 kits and sequences were obtained using a 16 capillary 3130xl Genetic Analyzer (Applied Biosystems). The sequences were edited and assembled in Sequencher 4.9 (Gene Codes Corporation, Ann Arbor, MI, USA) and contigs were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine identity with archived sequences.

Table **1.** Generic and type-specific oligonucleotide primers used to screen copepods by polymerase chain reactions for microsporidian ribosomal RNA gene sequences.

Name	5′–3′ sequence	Reference[a]
530-F	GTGCCAGC(A/C)GCCGCGG	a
580-R	GGTCCGTGTTTCAAGACGG	a
F[b]	GTCTGTGGATCAAGGACGAA	b
R[b]	ACTGATATGCTTAAGTTCAGG	b
B-F[c]	CCGGAGGCGAAGGCGTTACC	c
B-R[c]	GGGGTCGTCTGGGTCTCCCC	c
C-F[d]	TTTGGGCTGCACGCGCACTA	c
C-R[d]	GCTTTTCCTTGTTCACTCGCCGC	c
A-1R	AGTTTTCCCGTGTTGAGTCAAAT	c
BC-1R	TTAAGCCGCACACTCCACTC	c
F	GGTTGATTCTGCCTGACGT	d
R	GACGGGCGGTGTGTACAAAG	e

[a]a, Vossbrinck et al. 1993; b, Freeman et al. 2003; c, present study; d, Baker et al. 1994; e, Pomport-Castillon et al. 1997.
[b–d]Specific for sequence Types A, B, and C, respectively.

Phylogenetic analysis. Archived partial SSU rDNA sequences were selected from 26 microsporidia and fungi (Table 2) based on the results of the BLAST analysis and published literature. Microsporidian sequences were aligned using Muscle 3.7 with default parameters (Edgar 2004). Pairwise genetic distances and associated standard errors were calculated from this alignment in MEGA 5.05 by using the Kimura-2 parameter method with a gamma distribution of 1 (Tamura et al. 2011). Secondly, a maximum likelihood phylogenetic analysis using GTR with gamma distribution as the nucleotide substitution model was conducted from the same alignment in MEGA 5.05. The analysis only considered positions in the alignment with 95% or greater site coverage and was run for 1,000 bootstrap replications. *Conidiobolus coronatus* and *Basidiobolus ranarum* were used as outgroup sequences in the analysis.

RESULTS

Molecular biology. Three distinct microsporidian rDNA sequences (i.e. partial SSU, ITS, and partial LSU) occurred among 294 of the 530 copepods belonging to four *Lepeophtheirus* spp. that were tested (Table 3). The first, designated Type A (GenBank accession number, HM800847), was 1,826 bp and by BLAST analysis most closely resembled *Paranucleospora theridion* (FJ594990, 97% identity over 100% coverage), *Desmozoon lepeophtherii* (AJ431366, 97% and 97%), and Enterocytozoonidae gen. sp. (AF201911, 88% and 99%). Whereas FJ594990 and AJ431366 were identical to each other, HM800847 differed from AJ431366 at 38 of 1,787 sites. Within the SSU rDNA sequence, differences occurred at 5 of 1,223 sites. The second sequence, designated Type B (HM800848, HM800849, HM800850, HM800851 and HM800852), ranged from 909–1,897 bp and most closely resembled *Microsporidia* sp. (GQ868443, 70% and 91%), *Kabatana* sp. (FJ843105, 82% and 75%), and *Spraguea* sp. (AB623034, 89% and 82%). The third sequence, designated Type C (HM800853), was 1,774 bp and most closely resembled *Cucumispora dikerogammari* (GQ246188, 89% and 89%), *Microsporidium* sp. (AJ438962, 90% and 83%), and *Microsporidium* sp. (FN434092, 93% and 70%).

Multiple alignment of 29 taxa (Table 2) resulted in a dataset of 2,005 positions within the SSU rRNA gene. The genetic distances of the Type A sequence (HM800847) to related sequences ranged from 0.004 for *D. lepeophtherii* (AJ431366) to 0.750 for *Ameson michaelis* (L15741). The genetic distances of the Type B sequence (HM800849) ranged from 0.359 for *Thelohania butleri* (DQ417114) to 0.661 for *Thelohania parastaci* (AF294781), and that of Type C (HM800853) from 0.082 for *Microsporidium* sp. (AJ438962) to 0.605 for *D. lepeophtheri* (FJ594987). Maximum likelihood analysis of the 915 phylogenetically informative SSU rDNA positions showed that Type A was included in a well-supported clade along with sequences obtained from microsporidia that are parasites of *L. salmonis* or salmonid fishes (Fig. 1). The Type B sequence, while most closely associated with a small clade derived from microsporidia of marine crustaceans, failed to resolve with any sequences used in this analysis (Fig. 1). The Type C sequence occurred within a well-supported clade of sequences from microsporidia that are parasitic in a variety of crustaceans of marine or brackish waters and was most closely related to sequences from microsporidia of gammarid crustaceans (Fig. 1). A similar tree topology was obtained by maximum parsimony (not shown).

Microsporidian rDNA sequence types did not occur uniformly among *Lepeophtheirus* spp. The Type A sequence

Table **2.** List of microsporidia examined, with associated accession numbers for ribosomal RNA gene sequences, host, habitat, and geographic locality.

GenBank	Organism	Host	Habitat	Location
AF294781	*Thelohania parastaci*	Crustacean, *Cherax destructor*	F	Australia
GQ246188	*Cucumispora dikerogammari*	Crustacean, *Dikerogammarus villosus*	F	France
GQ258752	*Cucumispora dikerogammari*	Crustacean, *Dikerogammarus villosus*	F	Poland
AF397404	*Dictyocoela duebenum*	Crustacean, *Gammarus deubeni*	F	UK
AJ438985	*Pleistophora mulleri*	Crustacean, *Gammarus duebeni celticus*	F	Ireland
AF387331	*Heterosporis anguillarum*	Fish, *Anguilla japonica*	F	China
FJ843105	*Kabatana sp.*	Fish, *Gymnorhamphichthys rondoni*	F	Brazil
AJ252955	*Pleistophora ovariae*	Fish, *Notemigonus crysoleucas*	F	USA
L15741	*Ameson michaelis*	Crustacean, *Callinectes sapidus*	M	USA (Atlantic)
AY958070	*Nadelspora canceri*	Crustacean, *Cancer magister*	M	USA (Pacific)
AJ438962	*Microsporidium sp.*	Crustacean, *Gammarus chevreuxi*	M	UK
HM800853	TYPE C	Crustacean, *Lepeophtheirus hospitalis*	M	Canada (Pacific)
AJ431366	*Desmozoon lepeophtherii*	Crustacean, *Lepeophtheirus salmonis*	M	Scotland
FJ594987	*Paranucleospora theridion*[a]	Crustacean, *Lepeophtheirus salmonis*	M	Norway
HM800847	TYPE A (*Desmozoon lepeophtherii*)	Crustacean, *Lepeophtheirus salmonis*	M	Canada/USA (Pacific)
HM800849	TYPE B (*Facilispora margolisi* n. g., n. sp.)	Crustacean, *Lepeophtheirus salmonis*	M	Canada (Pacific)
AJ252959	*Perezia nelsoni*	Crustacean, *Litopenaeus setiferus*	M	USA (Atlantic)
HM140491	*Myospora metanephrops*	Crustacean, *Metanephrops challengeri*	M	New Zealand
DQ417114	*Thelohania butleri*	Crustacean, *Pandalus jordani*	M	Canada (Pacific)
AF056016	*Glugea anomala*	Fish, *Gasterosteus aculeatus*	M	France
AF056014	*Glugea americanus*	Fish, *Lophius americanus*	M	France
AF056013	*Spraguea lophii*	Fish, *Lophius americanus*	M	France
GQ868443	*Microsporidia sp.*	Fish, *Lophius gastrophysus*	M	Brazil
AF356222	*Kabatana takedai*	Fish, *Oncorhynchus masu*	M	Japan
U10883	*Nucleospora salmonis*	Fish, *Oncorhynchus tshawytscha*	M	USA (Pacific)
AF056015	*Glugea stephani*	Fish, *Pleuronectes americanus*	M	France
AB623034	*Spraguea sp.*	Fish, *Seriola dumerili*	M	Japan
AY635841	*Basidiobolus ranarum*	Outgroup		
AF296753	*Conidiobolus coronatus*	Outgroup		

[a]*Paranucleospora theridion* is a junior synonym of *Desmozoon lepeophtherii*.
F, freshwater; M, marine.

Table **3.** Occurrence of three microsporidian small subunit rDNA sequence types among parasitic copepods (*Lepeophtheirus* spp., *Caligus clemensi*) from fishes in the Pacific Ocean off Washington (WA), British Columbia (BC) and Alaska (AK).

Copepod				Percent positive		
Species	*N*	Fish host	Location	Type A	Type B	Type C[a]
L. salmonis	88	F	WA	14.8	43.2	0 (n = 58)
	12	Ws,c	WA	0	75.0	n.d.
	161	F	BC	1.4	65.2	0 (n = 35)
	20	Wm	BC	0	60.0	n.d.
	12	Ws,c	BC	0	50.0	n.d.
	99	Wx	BC	0	58.6	n.d.
	40	Wm	BC	0	60.0	n.d.
	20	Wm	BC	0	90.0	n.d.
L. cuneifer	20	F	BC	0	50.0	0
	4	H	BC	0	50.0	0
	2	H	AK	0	0	0
L. parviventris	20	IL	BC	0	30.0	0
L. oblitus	3	KG	BC	0	0	0
L. hospitalis	4	SF	BC	0	0	25.0
L. bifidus	1	SF	BC	0	0	0
C. clemensi	2	F	WA	0	0	n.d.
	2	A	BC	0	0	n.d.
	20	Wx,B	BC	0	0	n.d.

[a]Number of copepods examined for Type C in brackets if different from number examined for Types A and B; n.d., not determined.
A, sablefish; B, 3-spine stickleback; F, farmed Atlantic salmon; H, herring; IL, Irish lord; KG, kelp greenling; SF, starry flounder; W, wild Pacific salmon (sockeye, coho, chum, x – multiple species).

occurred only in *L. salmonis* from Atlantic salmon (Table 3). The prevalence was approximately 15% in copepods from Washington (WA) and 1.5% in those from British Columbia (BC) (Table 3). In contrast, the Type B sequence was detected in *L. salmonis* from Atlantic and Pacific salmon and the prevalence ranged from 43% to 90% (Table 3). The Type B sequence was also detected in *L. cuneifer* and *L. parviventris* (Table 3). Type A and Type B sequences were both amplified from approximately 10% of *L. salmonis*. There was no difference between WA and BC in the percent of *L. salmonis* that were positive for Type A or B ($\chi^2 = 2.13$, $P = 0.129$ or $\chi^2 = 1.45$, $P = 0.229$, respectively). Combining all *L. salmonis* specimens, the percent positive for the Type A sequence was significantly lower than for Type B ($\chi^2 = 21.63$, $P < 0.001$). In addition, the Type A sequence was detected in one of 17 (5.9%) *L. salmonis* chalimus stages. The Type C sequence was detected in one of four specimens of *L. hospitalis,* but not in 143 specimens belonging to five other copepod species (Table 3).

Histology in *Lepeophtheirus salmonis*. Developmental stages and mature spores of Type A were observed within connective tissue cells below the cuticle in the cephalothorax, genital segment, abdomen, and larger appendages. Some parasite clusters were partitioned by membranes, indicating the involvement of adjacent host cells (Fig. 2, 3). Microsporidian developmental stages were not observed in striated muscle or in other organs.

Infections with Type B ranged from light, in which mature spores and other developmental stages were observed in connective tissue cells below the cuticle in the cephalothorax, genital segment, abdomen, and larger appendages, to severe, in

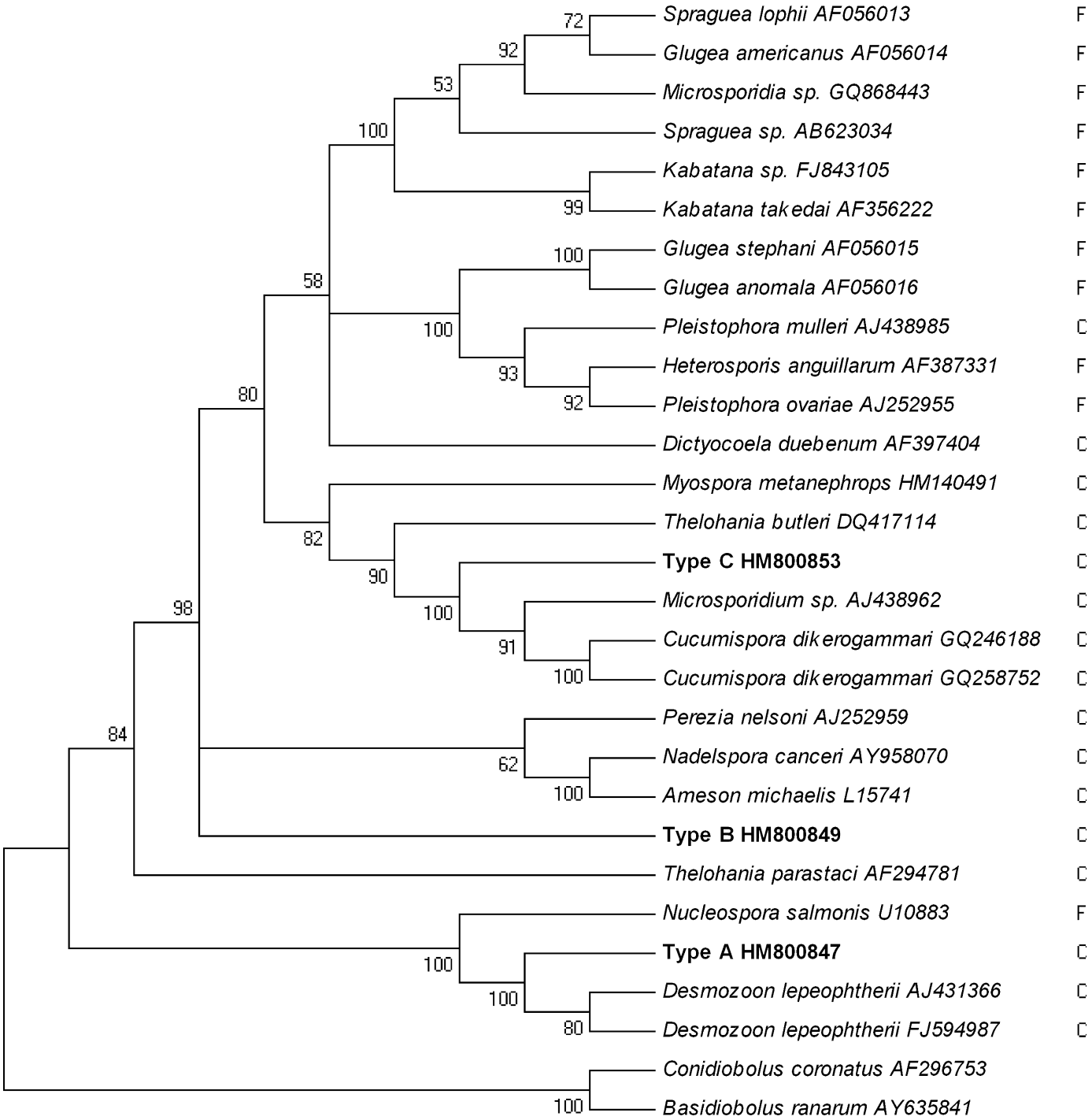

Fig. **1.** Maximum likelihood analysis of partial small subunit ribosomal RNA gene sequences from *Lepeophtheirus* spp. and from 26 other microsporidia based on an alignment of 915 informative positions. Branch nodes show bootstrap support values. Sequences from *Conidiobolus coronatus* and *Basidiobolus ranarum* are outgroups. Host taxa are depicted to the right (F, fish; C, crustacean).

which clusters of spores and other developmental stages or free spores occurred within striated muscle fibres and cells of the digestive gland, gut, and gonads (Fig. 4, 5).

Electron microscopy in *Lepeophtheirus salmonis*. All developmental stages of Type A were in direct contact with host cell cytoplasm. The earliest observed stages were meronts possessing a diplokaryon (Fig. 6–10). Cytokinesis among diplokaryotic meronts was not observed. Instead, dissociation of the diplokaryon was mediated by well-developed mitotic spindles (Fig. 6–10), followed by a series of nuclear divisions. Numerous lobate processes extended from the resulting multi-nucleated plasmodium (Fig. 11) and the terminus of each process contained a single nucleus (Fig. 12, 13). The cytoplasm of early meronts was granular and somewhat dense, whereas that of the plasmodium contained more endoplasmic reticulum (ER) and was comparatively lucent.

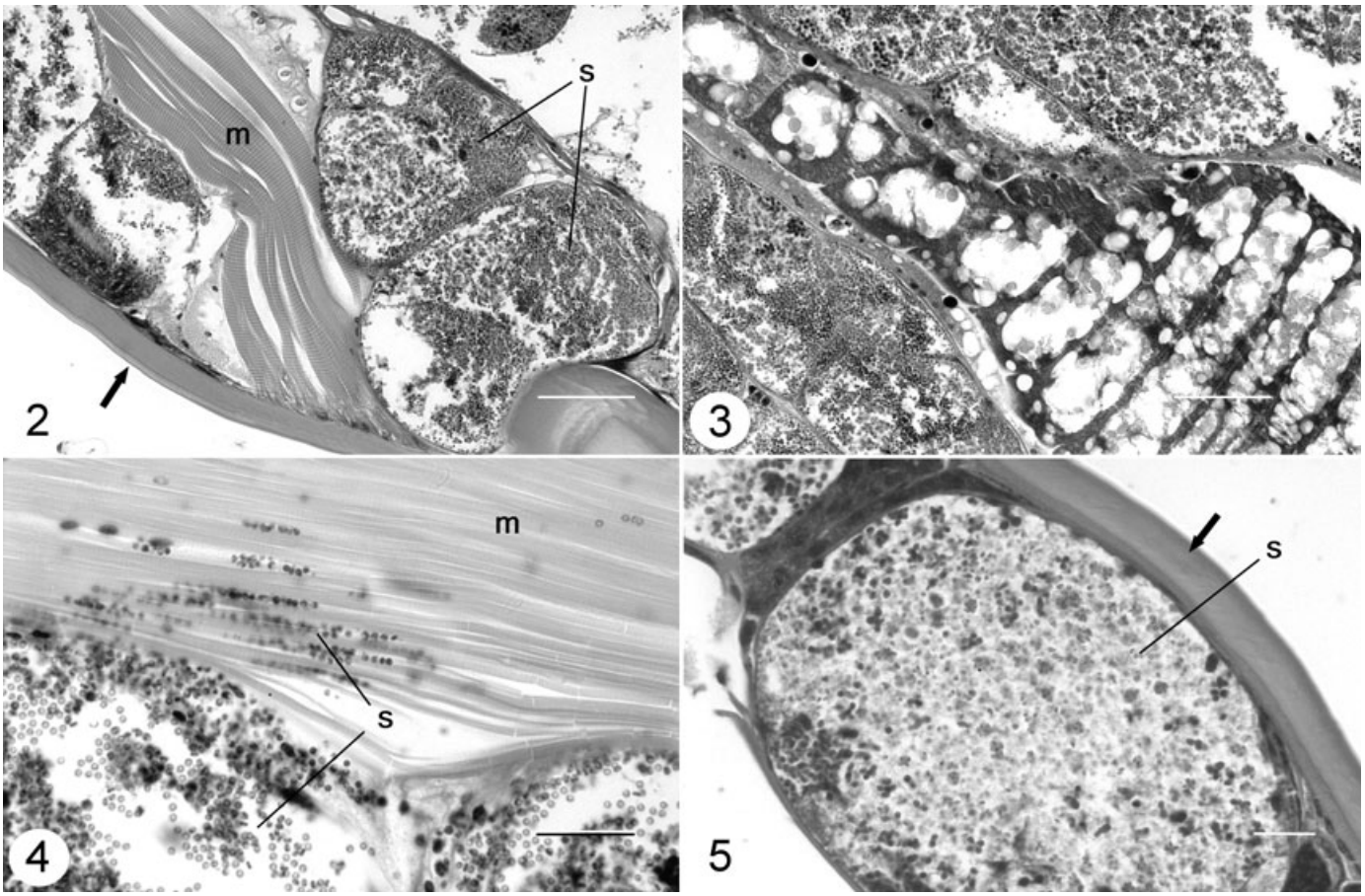

Fig. **2–5.** Histological sections of microsporidian infections in *Lepeophtheirus salmonis* from the Pacific Ocean. **2, 3.** *Desmozoon lepeophtherii* (Giemsa stain), **4, 5.** *Facilispora margolisi* n. gen., n. sp. (**4,** Giemsa stain; **5,** H&E). Labelled structures include striated muscle (m), microsporidian spores (s), host cuticle (arrow). Scale bars are 50 μm (**2–4**) and 20 μm (**5**).

Uninucleate sporoblasts, formed by schizogonous cleavage from the tips of the plasmodial lobes, underwent one or more division cycles (Fig. 14) followed by the development of polar filament apparatus (Fig. 15, 16). Maturation of the spore wall was associated with the coalescence of dense plaques on the outer surface of the cell membrane. The sporoblast cell membrane had a corrugated appearance beneath the spore wall (Fig. 14, 15). Formation of the polar filament (PF) was initiated by the organization of cytoplasmic vacuoles into rough ER-like cisternae. Ribosome-like structures remained on the cytoplasmic surface of these cisternae during development of the PF. The mature spore was subspherical, measuring (mean ± standard error) 2.05 ± 0.04 × 1.80 ± 0.04 μm (n = 20, range: 1.80–2.57 × 1.39–2.25 μm). The spore wall consisted of a well-defined and relatively thick lucent endospore and a thin, dense exospore. Mature spores were surrounded by a clear zone. The PF was isofilar and made seven coils surrounding and slightly posterior to the polaroplast (Fig. 17). The manubrium extended from the anchoring disc, the latter two structures being adjacent to and anterior to the polaroplast, respectively (Fig. 17).

All developmental stages of Type B were in direct contact with host cell cytoplasm or nucleoplasm and there was no evidence of diplokarya during merogony or sporogony. An intimate association between meronts and host cell mitochondria frequently occurred (Fig. 18–22). Meronts underwent a series of cell divisions by binary fission (Fig. 23, 25, 28); the number of division cycles was not determined. Nuclear division preceded cell division and no more than two nuclei were observed per dividing cell. Non-dividing meronts measured 3.34 ± 0.10 × 2.78 ± 0.08 μm (n = 12, range: 2.91–3.94 × 2.36–3.38 μm) and had well-developed rER. Meront division was usually equal, but occasionally resulted in daughter cells of unequal size. The appearance of dense plaques associated with the cell membrane marked the initiation of sporogony (Fig. 24, 25), although precocious development of the PF was also evident (Fig. 21). Coalescence of these plaques indicated formation of the spore wall and the early sporoblast had a scalloped or irregular profile (Fig. 26). Sporoblast division by binary fission resulted in two immature spores containing the PF primordium (Fig. 27, 28). Rarely, infections were observed within the nucleus (Fig. 30) of connective tissue cells.

Mature spores (Fig. 31, 32) were ovoid and measured 2.66 ± 0.07 × 1.59 ± 0.06 μm (n = 22, range: 2.14–3.21 × 1.00–2.06 μm). The exospore was ~ 16 nm thick and consisted of inner and outer electron layers sandwiching a somewhat lucent inner zone (Fig. 34). The endospore was ~ 37 nm thick and was lucent with a granular appearance. Between three and four coils of the PF were visible in sections of virtually all mature spores; rarely, five coils were observed (Fig. 33). Sections of the PF revealed a laminated profile including two closely dense outer layers, a lucent middle zone, and a dense core (Fig. 34). The PF was isofilar with a mean diameter of 101.3 nm. The manubrium had a mean diameter

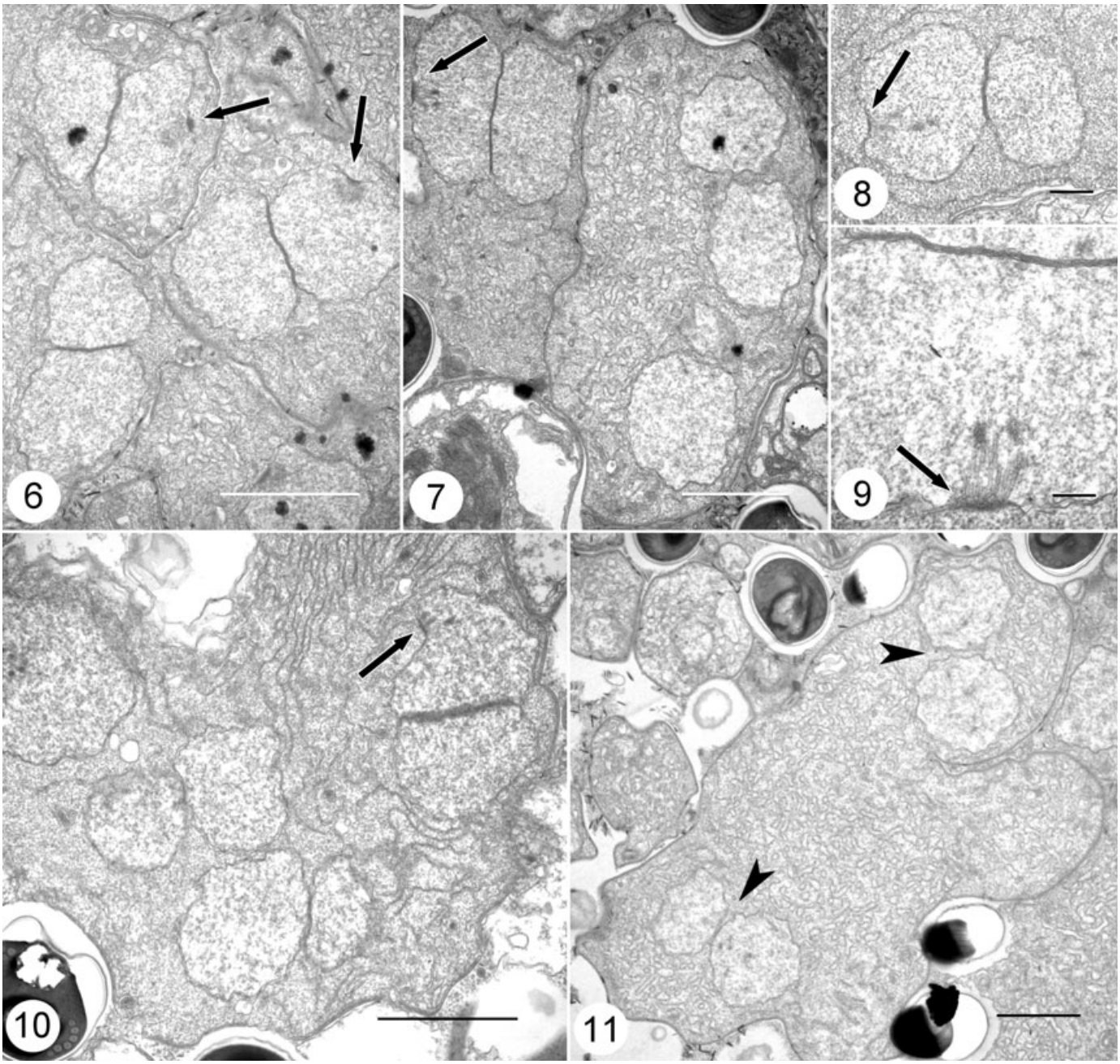

Fig. **6–11.** *Desmozoon lepeophtherii* in *Lepeophtheirus salmonis* from the Pacific Ocean. Electron micrographs of merogonic development. **6–10**. Diplokaryotic meronts with detail of mitotic spindles in diplokaryon. **7, 10, 11**. Multinucleate meronts with early development of plasmodial lobes. Dissociated diplokarya (arrowheads) and centriolar plaques with associated microtubules (arrows) are depicted. Scale bars are 250 nm (**9**), 500 nm (**8**) or 2,000 nm (**6, 7, 10, 11**).

of 121.5 nm shortly after arising from the anchoring disc and had a highly stratified cortex with a relatively lucent central zone. Occasionally, incomplete division of the sporoblast resulted in conjoined spores, each possessing a sporoplasm and extrusion apparatus (Fig. 35).

DISCUSSION

Systematics within the phylum Microsporidia Balbiani, 1882 has been transformed by the application of rDNA sequences as phylogenetically informative characters, as reviewed by Weiss and Vossbrinck (1999) and more recently by Corradi and Keeling (2009). Traditional microsporidian taxonomy is based on characters associated with structure, patterns of development, and/or interactions with the host visualized by electron microscopy (Sprague 1977; Sprague et al. 1992; Tuzet et al. 1971; Vávra and Larsson 1999). Phylogenetic inferences based on the molecular data have tended to not fully support the traditional taxonomy (Refardt et al. 2008) and a consensus has not been reached on the extent to which molecular and traditional data may be integrated in the formulation of a unified microsporidian taxonomy. In addition, the rDNA sequence

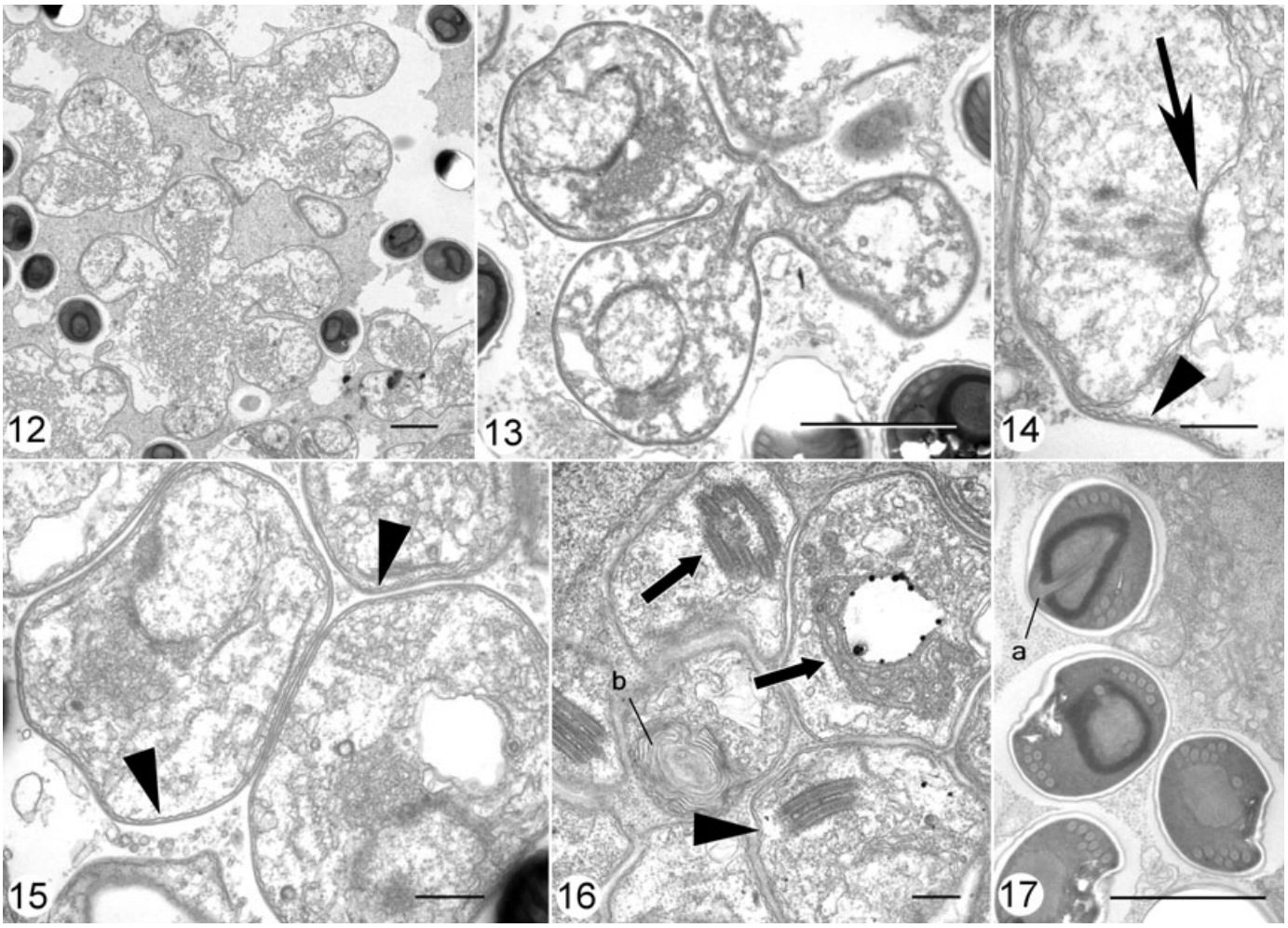

Fig. **12–17.** *Desmozoon lepeophtherii* in *Lepeophtheirus salmonis* from the Pacific Ocean. Electron micrographs of sporogonic development. **12, 13**. Plasmodia showing well-developed lobes with apical nucleus. **14**. Centriolar plaque and mitotic microtubules (large arrow) within nucleus of sporoblast. **15, 16**. Postdivisional sporoblasts with development of filament apparatus. Rough endoplasmic reticulum-like cysternae (small arrows) and corrugated appearance of cell membrane beneath sporoblast spore wall (arrowheads) are depicted. **17**. Mature spores. The developing laminate polaroplast (b) and anchor disc (a) are depicted. Scale bars are 500 nm (**14–16**) or 2,000 nm (**12, 13, 17**).

data suggest that the host and its habitat are also useful in the determination of higher level microsporidian taxonomy (Vossbrinck and Debrunner-Vossbrinck 2005). It is in this context that we report evidence of three distinct microsporidian rDNA sequences obtained from parasitic copepods belonging to the genus *Lepeophtheirus* in the northeast Pacific Ocean. Based on an alignment of partial SSU rDNA sequences, spore morphology, merogony and sporogony, the Type A microsporidian is concluded to be *D. lepeophtherii*, first reported from salmon lice *L. salmonis* and Atlantic salmon *S. salar* in Scotland and Norway (Freeman 2002; Freeman and Sommerville 2009; Freeman et al. 2003; Nylund et al. 2010). In contrast, the Type B organism is sufficiently dissimilar to known microsporidia and is concluded to be a new species, described herein. The Type C rDNA sequence is novel and the formal designation of this organism as a new species is deferred pending the availability of additional material.

The occurrence of *D. lepeophtherii* in *L. salmonis* from the Pacific Ocean expands the known geographic range of this microsporidian. In the Pacific region of North America, the salmon louse occurs on wild Pacific salmon *Oncorhynchus* spp. and on Atlantic salmon that are reared for aquaculture in seawater in open netpens. Only *L. salmonis* collected from Atlantic salmon were infected with *D. lepeophtherii* with a prevalence of about 15% in copepods from WA and 1.5% in those from BC. In Scotland, 5% of salmon lice were infected (Freeman et al. 2003). Spore and meront morphology, merogony, and sporogony of the Pacific form of *D. lepeophtherii* were similar to those reported earlier (Freeman and Sommerville 2009; Nylund et al. 2010). In the copepod, diplokarya in meronts dissociated into individual nuclei and underwent karyokinesis to form multinucleated plasmodia. One nucleus occupied the tip of each of several plasmodial processes, from which sporoblasts cleaved by schizogony (Freeman and Sommerville 2009). The sporoblasts underwent at least one subsequent cycle of cell division. Phylogenetic analysis showed that the Pacific sequence clustered closely with those obtained from the Scottish and Norwegian isolates of *D. lepeophtherii*. Further confirming the earlier work, the Pacific form of *D. lepeophtherii* was closely related to the intranuclear salmon parasite *Nucleospora salmonis*, supporting the placement of the copepod parasite within the family Enterocytozoonidae (Freeman and Sommerville 2009; Nylund et al. 2010). *Desmozoon lepeophtherii* is also a parasite of Atlantic salmon (Freeman

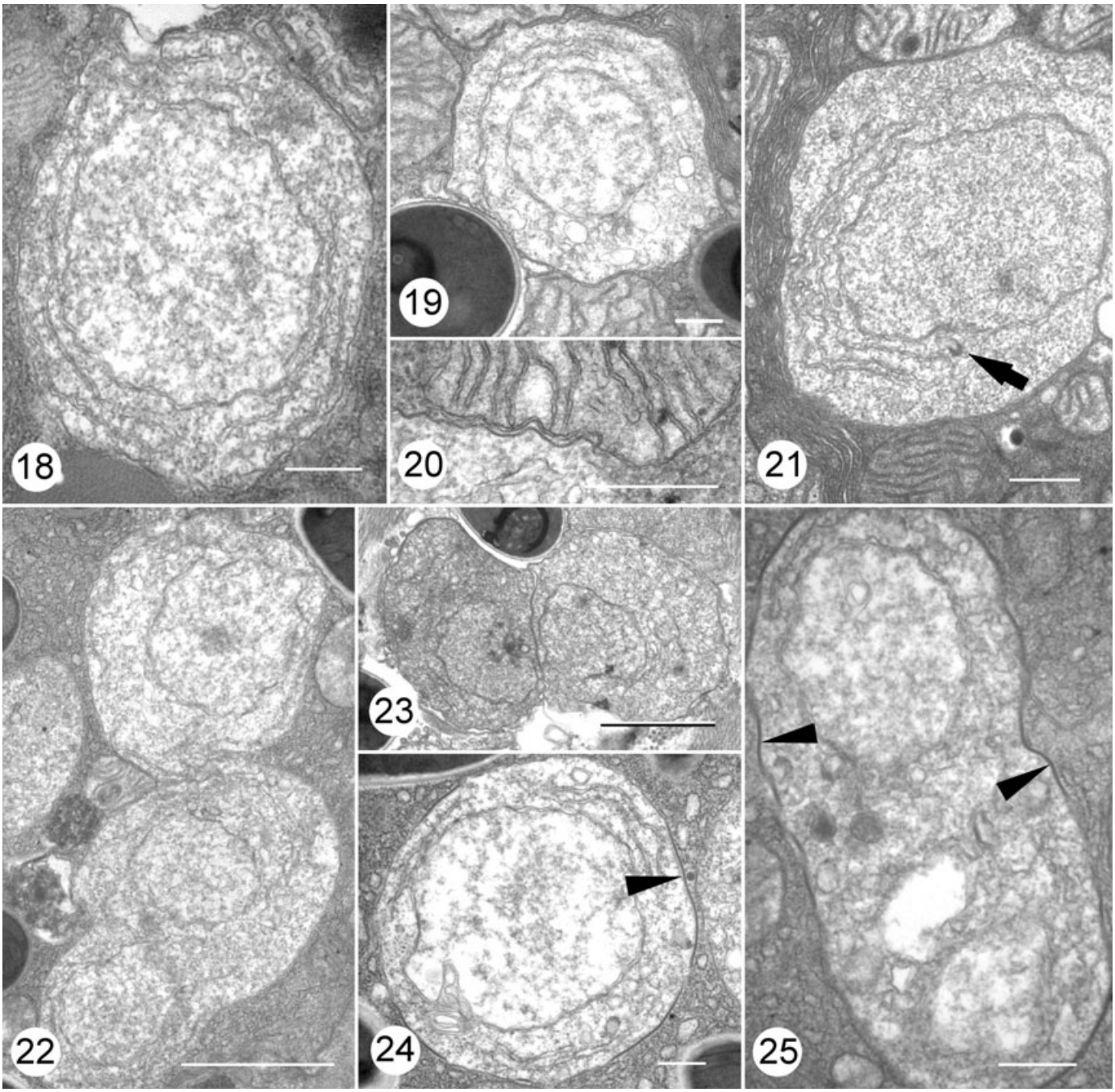

Fig. **18–25.** *Facilispora margolisi* n. gen., n. sp. in *Lepeophtheirus salmonis* from the Pacific Ocean. Electron micrographs of merogonic and early sporogonic development. **18–21.** Meronts containing a single nucleus in close association with host mitochondria. **21.** Endoplasmic reticulum and possible anchor disc primordium (arrow), represents precocious development of the infection apparatus. **22, 23.** Bi-nucleated meront preceding and following cytokinesis. **24.** Early sporoblast with plaque-like areas of electron density on the cell membrane (arrowhead). **25.** Sporoblast with two nuclei prior to cytokinesis, also showing plaques on cell membrane (arrowhead). Scale bars are 500 nm (**18–22**) or 2,000 nm (**23–25**).

2002; Nylund et al. 2010) and the ectoparasitic lifestyle of *L. salmonis* may facilitate transmission of the microsporidian between the copepod and fish hosts.

Desmozoon lepeophtherii undergoes two distinct cycles of development in the salmon: in the cytoplasm of cells from skin, gill, heart, kidney, and spleen and also in the nucleus of epidermal cells of skin and gills. The parasite has been implicated as a possible primary agent of proliferative gill disease among Atlantic salmon in Norway (Nylund et al. 2011). The present epizootiological data support a role of Atlantic but not Pacific salmon in the development of *D. lepeophtherii*. However, no PCR evidence of *D. lepeoptherii* was found in skin and gill samples from 60 Atlantic salmon from a netpen site where specimens of *L. salmonis* were infected with the

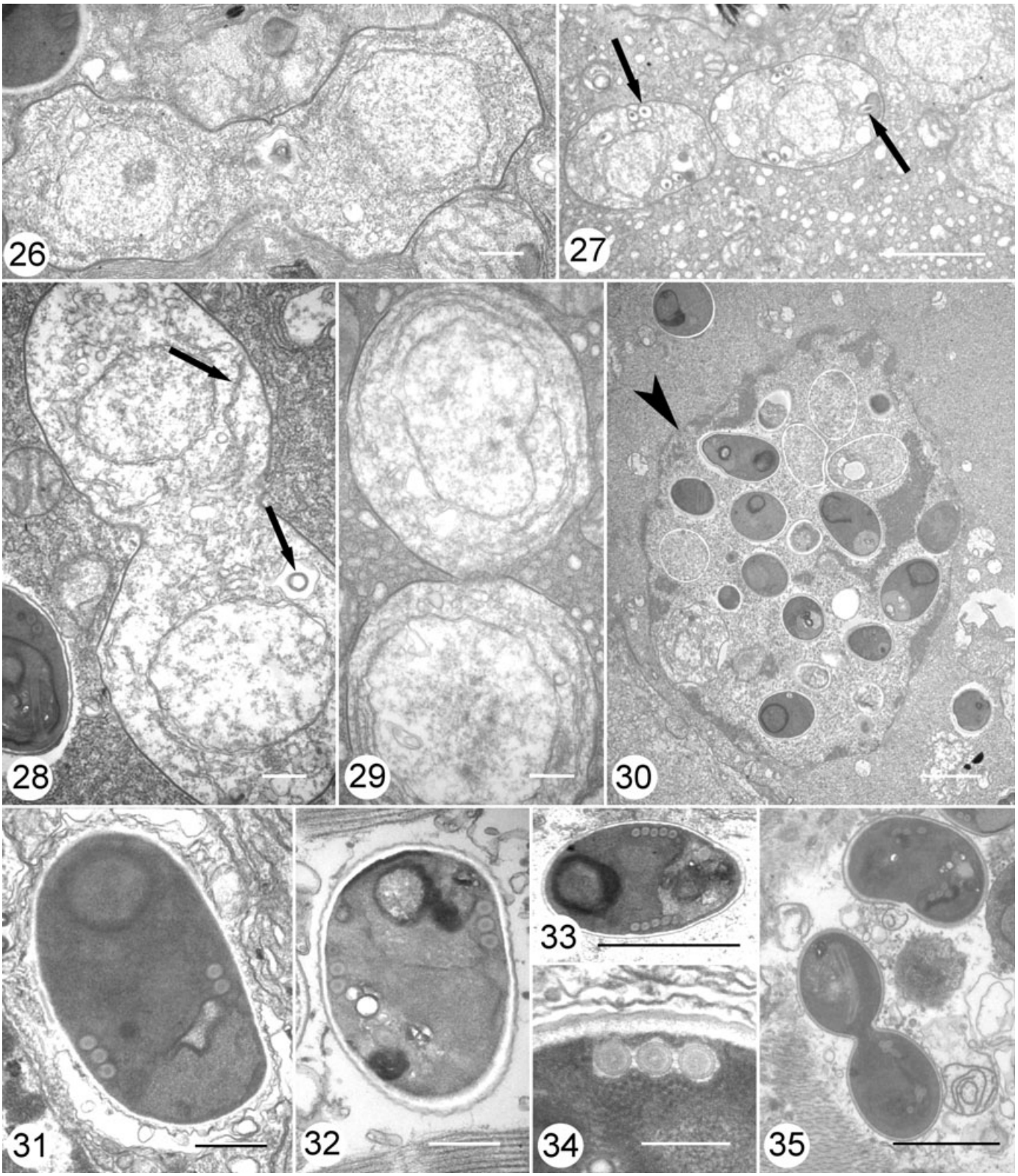

Fig. **26–35.** *Facilispora margolisi* n. gen., n. sp. in *Lepeophtheirus salmonis* from the Pacific Ocean. Electron micrographs of sporogonic development and structure of mature and aberrant spores. **26–29.** Sporoblasts during and following cytokinesis, showing polar filament primordium (arrow). **30.** Intranuclear infection with late sporoblasts and mature spores, showing nuclear membrane (arrowhead). **31, 32.** Mature spores with polar filament (PF) making three coils. **33.** Spore with five coils of PF. **34.** Fine structure of spore wall and PF. **35.** Aberrant conjoined spores following incomplete division of sporoblast. Scale bars are 250 nm (**34**), 500 nm (**26, 28, 29, 31, 32**) or 2,000 nm (**27, 30, 33, 35**).

microsporidian (data not shown). Atlantic salmon is an exotic species on the Pacific coast of North America and further work is required to determine whether in this region, a salmonid other than Atlantic salmon, along with *L. salmonis*, serve as hosts to *D. lepeophtherii*.

The morphological and developmental similarity evident between Atlantic and Pacific specimens of *D. lepeophtherii* was not reflected in the partial SSU rDNA sequence data, which showed these to be distinct geographic varieties. The complete identity of sequences between Scottish and Norwegian specimens of *D. lepeophtherii* contrasted with a difference of 0.4% between either of these and the homologous sequence obtained from Pacific specimens. Voucher specimens of the Pacific variety of *D. lepeophtherii* have been deposited in the Parasitology Collection of the Canadian Museum of Nature, Ottawa, Ontario, with catalogue numbers CMNPA 2011-0005 and CMNPA 2011-0006.

The Type B organism was dissimilar to *D. lepeophtherii* in its morphology and pattern of merogonic and sporogonic development. The lack both of diplokarya and presporogonic proliferation, when combined with cell division that occurred only by binary fission, suggested that the Type B organism shares an affinity with members of the Order Chytridiopsida within the Class Haplophasea (Sprague et al. 1992). However, unlike the Type B organism, members of the chytridiopsid genera *Chytridioides*, *Chytridiopsis*, *Nolleria*, *Buxtehudea*, *Jiroveciana*, and *Enterocytozoon* are polysporous or display polysporoblastic sporogony (Sprague et al. 1992). Infections in *L. salmonis* involved striated muscle, digestive gland, ovary, egg string, and connective tissue and the prevalence was usually greater than 50%, regardless of whether the copepod infected Pacific or Atlantic salmon. Unlike *D. lepeophtherii*, the microsporidian was also observed in *L. cuneifer* from Atlantic salmon and Pacific herring and was detected by PCR in *L. parviventris*, a copepod parasite of non-salmonid fish. The relatively short polar filament of three to four coils in most mature spores, combined with the involvement of most tissues raised the possibility of autoinfection, as suggested for *Nosema apis* (de Graaf et al. 1994). In addition, infection in the genital segment, including the observation of spores within developing embryos (not shown) indicated that vertical transmission may also occur. In contrast, the occurrence of spores in which the polar filament makes five coils indicates spore dimorphism, possibly facilitating horizontal transmission among copepods (de Graaf et al. 1994). However, the rarity of this spore type suggests that the frequency of this mode of transmission is low. The consequences of microsporidian infections on the behaviour or survival of the copepod hosts are not known.

The Type B rDNA sequence (i.e. partial SSU, ITS, partial large subunit) was, by BLAST analysis, most similar to sequences from parasites of fishes. However, although an association with microsporidia of Crustacea in marine or brackish water habitats was suggested from phylogenetic analysis of the partial SSU rDNA sequence, the uniqueness of this sequence was emphasized by its failure to resolve within a phylogeny that was otherwise topologically similar to that of Stentiford et al. (2010). Here and in the latter study, *Myospora metanephrops* resolved as a sister group to *Thelohania butleri* and an unnamed microsporidian of *Gammarus chevreuxi*. The Type C sequence from *L. hospitalis* and *Cucumispora dikerogammari* from *Dikerogammarus villosus* were well-supported within this clade. Similar to Stentiford et al. (2010), a clade of sequences from *Ameson michaelis*, *Nadelspora canceri*, and *Perezia nelsoni*, parasites of decapod crustaceans, was basal to the larger aforementioned clade, which also included sequences from microsporidia of fishes. The Type B sequence was also basal to this larger clade. The Type B organism, as with *C. dikerogammeri*, *T. butleri*, *M. metanephrops*, *A. michaelis*, *N. canceri*, *P. nelsoni*, and the unnamed parasite of *G. chevreuxi*, produces mature spores in host skeletal muscle. However, the absence of diplokarya throughout the developmental cycle of the Type B organism distinguished it from most of these myotropic parasites of crustaceans (Canning et al. 2002; Johnston et al. 1978; Ovcharenko et al. 2010; Stentiford et al. 2010; Weidner 1970). However, among these, diplokarya were also absent in *N. canceri*, a parasite of the Dungeness crab *Cancer magister* named for its needle-like spores (Olson et al. 1994). Mature spores of the Type B organism are clearly not needle-shaped. Thus, the unique combination of morphological and developmental data supports the designation of the Type B organism as a new species within a new genus: *Facilispora margolisi* n. gen., n. sp. Stentiford et al. (2010) erected the family Myosporidae to accommodate *M. metanephrops* and suggested that pending corroboration with additional data, *A. michaelis*, *N. canceri*, *P. nelsoni*, and *T. butleri* may also be assigned to this family. However, *F. margolisi* n. gen., n. sp., because of its unique combination of developmental characteristics and host species along with the novel rDNA sequence, should be placed within a new family, the Facilisporidae n. fam. By accommodating Myosporidae within the Order Crustaceacida in the Class Marinosporidia, Stentiford et al. (2010) sought to align microsporidian molecular phylogenetic characters with those of host and habitat, as suggested earlier (Baker et al. 1997; Vossbrinck and Debrunner-Vossbrinck 2005). The present phylogeny supported the concept of Crustaceacida and although the taxon is not strictly monophyletic, inclusion therein of Facilisporidae n. fam. along with Myosporidae is for the time being advised given host and apparent phylogenetic affinities. Accordingly, the definition of Crustaceacida is emended to accommodate monokaryotic microsporidia belonging to Facilisporidae n. fam. Nevertheless, the validity of Crustaceacida will continue to be tested and its definition refined with additional data from among microsporidia of marine and brackish water crustaceans. In agreement with Stentiford et al. (2010), it is prudent to delay familial assignments of other species presently aligning themselves within the Crustaceacida, pending the availability of sequence data, perhaps from microsporidian genetic loci other than SSU rDNA. In particular, the analysis of partial SSU rDNA sequences from *Thelohania parastaci* and *Thelohania butleri* provided further support for a polyphyletic relationship among *Thelohania* spp. and reaffirmed the need to revise the genus (Brown and Adamson 2006).

TAXONOMIC SUMMARY

Phylum Microsporidia Balbiani, 1982.

Class Marinosporidia Vossbrinck and Debrunner-Vossbrinck 2005.

Order Crustaceacida Stentiford et al. 2010.

Emended diagnosis. Microsporidian parasites of Crustacea from marine or brackish waters: diplosporoblastic, monokaryotic or diplokaryotic, direct association with host-cell cytoplasm. Spores ovoid, to rod-shaped, with 3–11 polar filament coils in a single rank.

Family Facilisporidae n. fam.

Diagnosis. Microsporidia of parasitic marine copepods in which all developmental stages are monokaryotic. Merogonic and sporogonic replication by binary fission. Developmental stages in direct contact with host cell cytoplasm. Diplosporoblasts produce ovoid spores. Polar filament with 3–5 coils in one rank. Infections in skeletal muscle and other organs and tissues.

Type Genus. *Facilispora* n. gen.

Facilispora n. gen.

Diagnosis. With characteristics of the family.

Type species. *Facilispora margolisi* n. sp.

Etymology. The genus name refers to the simple binary fission-based pattern of development utilized by the type species.

Facilispora margolisi n. sp.

Diagnosis. With characteristics of the genus. Meronts subspherical, ~ 2.8 × ~ 3.3 μm, with granular cytoplasm and rough endoplasmic reticula. Mature spores ovoid, ~ 1.6 × ~ 2.7 μm. Exospore trilaminate, endospore lucent with granular texture. Polar filament isofilar, ~ 101 nm in diameter. Infections in cells of the subcuticular connective tissue, skeletal muscle, ovary, and digestive gland, and in all body segments containing these tissues.

Prevalence. From 30% to 90% among host species and localities.

Type host. *Lepeoptheirus salmonis* Krøyer.

Type locality. Queen Charlotte Sound, British Columbia Canada (50° 41′N, 126° 41′W).

Type material. Hapantotype and parahapantotype slides, containing Gram-stained histological sections of infected *L. salmonis*, have been lodged in the Parasitology Collection of the Canadian Museum of Nature with catalogue numbers CMNPA 2011-0003 and CMNPA 2011-0004, respectively.

Other hosts. *Lepeophtheirus cuneifer*, *Lepeophtheirus parviventris*.

Other localities. Puget Sound, Strait of Georgia, Strait of Juan de Fuca.

Etymology. The species is named after Dr. Leo Margolis, who made important contributions to the parasitology of marine organisms in Western Canada.

Molecular sequences. Sequences of the small subunit ribosomal RNA gene have been deposited in GenBank with accession numbers HM800848, HM800849, HM800850, HM800851, and HM800852.

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