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A hyperparasitic microsporidian infecting the salmon louse, *Lepeophtheirus salmonis*: an rDNA-based molecular phylogenetic study

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Abstract

The sea louse, *Lepeophtheirus salmonis*, is an obligate ectoparasitic copepod that lives on the external surface of salmonid fish. It is the most common ectoparasite of marine cage-reared salmonids, causing major economic loss to the aquaculture industry. During a sea louse monitoring programme, samples of *L. salmonis* were found to harbour an unreported microsporidian parasite. The microsporidian was observed in pre-adult and adult stages of both male and female copepods, with a prevalence of up to 5%. Unfixed spores were slightly pyriform in shape measuring 2.34 μm by 1.83 μm (±0.01 μm) and were not observed to be enclosed by a sporophorous vesicle. The microsporidian infection was observed in all areas of the copepods' body, xenoma-like cysts forming directly under the cuticle in the epidermal tissue layer. In the present study, rDNA (530f–580r) sequence data gathered from the unidentified microsporidian parasite isolated from infected sea lice were compared with equivalents available in the databases in an attempt to identify its systematic position. The microsporidian was found to group within the phylogenetic clade containing the family Enterocytozoonidae, being most similar to members of the intranuclear genus *Nucleospora*. This is the first report of a hyperparasitic microsporidian infecting a caligid copepod.

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Introduction

The salmon louse, *Lepeophtheirus salmonis*, is a major pathogen of farmed and wild Atlantic salmon, *Salmo salar* L., in Scotland and is a cause of major economic loss to the aquaculture industry. Hyperparasites have previously been reported from sea lice infecting Atlantic salmon; Gresty & Warren (1993) and Minchin & Jackson (1993) reported ciliated protozoa and the monogenean, *Udonella caligorum* (Johnston), respectively, both of which are found on the external surfaces of sea lice.

Microsporidians are obligate intracellular, spore-forming, protist parasites that infect every major animal group, especially insects, fish and mammals (Sprague & Vavra 1977). They are sufficiently unique to be classified in a separate phylum, the Microspora (Sprague & Vavra 1977); and, although previously considered to represent primitive eukaryotes, are now recognized as highly evolved organisms related to fungi (Mathis 2000; Van de Peer, Ben Ali & Meyer 2000).

Records of microsporidian infections in crustaceans include members of the Amblyosporidae and Thelohanidae that utilize freshwater copepods as intermediate hosts and mosquitoes as final hosts (Sweeney, Doggett & Piper 1993; Vossbrinck, Andreadis & Debrunner-Vossbrinck 1998). *Nosema*, *Ameson*, *Pleistophora*, *Thelohania* and an unidentified microsporidian species are parasitic in crabs and shrimp (Vivares, Loubes & Bouix 1976; Zhu,

Wittner, Tanowitz, Cali & Weiss 1994; Azevedo, Corral & Vivares 2000; Cheney, Lafranchi-Tristem & Canning 2000; Bell, Aoki & Yokoyama 2001). *Ordospora* and *Microsporidium* species have been found infecting cladocerans (Refardt, Canning, Mathis, Cheney, Lafranchi-Tristem & Ebert 2002). Little is known about their pathogenicity; however, microsporidia are responsible for causing 'cotton shrimp disease', a highly pathogenic muscle-infecting condition found in a number of shrimp genera (Ramasamy, Jayakumar & Brennan 2000) and *Nosema* spp. are reported to be responsible for feminization in gammarids (Dunn & Rigaud 1998; Terry, Smith, Bouchon, Rigaud, Duncanson, Sharpe & Dunn 1999).

Reports of hyperparasitic microsporidians are less abundant, being limited primarily to *Nosema*, *Unikaryon*, *Amphiacantha*, *Metchnikovella* and *Microsporidium* species in endoparasitic myxosporeans, acanthocephalans, platyhelminthes and gregarines (Caullery & Mesnil 1914; Vinckier, Devauchelle & Prensier 1970; Canning 1975; Shigina & D'iakonov 1977; Azevedo & Canning 1987; Larsson 2000; Tun, Yokoyama, Ogawa & Wakabayashi 2000), but have not been previously reported from parasitic copepods of marine fish.

Contemporary studies have revealed large discrepancies between microsporidian systematics based on molecular and traditional characteristics. These differences have resulted in confusion as to the specific taxonomic value of certain developmental and morphological features (see Baker, Vossbrinck, Maddox & Undeen 1994; Baker, Vossbrinck, Didier, Maddox & Shadduck 1995; Weiss & Vossbrinck 1998). Consequently, molecular-based analyses may reveal relationships that were not previously apparent. Weiss, Zhu, Cali, Tanowitz & Wittner (1994) reviewed the usefulness of microsporidian rRNA in diagnosis and phylogeny and concluded that the 530f–580r region of the gene extending from within the small subunit (SSU), through the internal transcribed spacer (ITS) region and into the large subunit (LSU), contained sufficient information to be useful for molecular phylogenetic studies of the Microspora. Initial rDNA-based analyses were typically restricted to a few genera (Baker *et al.* 1994) or focused on those species isolated from humans (Vossbrinck, Baker, Didier, Debrunner-Vossbrinck & Shadduck 1993; Weiss *et al.* 1994; Zhu *et al.* 1994; Baker *et al.* 1995). Recently, however, the amount of microsporidian rDNA sequence data has increased markedly. Nilsen, Endresen & Hordvik (1998), Muller, Trammer, Chioralia, Seitz, Diehl & Franzen (2000), Nilsen (2000) and Lom & Nilsen (2003) utilized these data to re-examine the phylogenetic status of established species, whereas Bell *et al.* (2001) and Yokoyama, Lee & Bell (2002) investigated the systematic placement of unclassified *Microsporidium* spp. infecting the musculature of marine fish.

In the present study, rDNA (530f–580r) sequence data gathered for an unidentified microsporidian isolated from the sea louse, *L. salmonis*, were compared with equivalents available in the databases in an attempt to identify the systematic position of this previously unrecorded microsporidian. A broader taxonomic perspective was achieved by analysing partial SSU sequences of 38 species belonging to at least 22 different genera.

Materials and methods

Lepeophtheirus salmonis were collected during routine Atlantic salmon harvests from fish farms on the west coast of Scotland. The sea lice were screened microscopically for the presence of microsporidian infection, which appeared grossly as opaque internal inclusions throughout the body.

When microsporidian infections were observed, infected tissues were dissected out and fresh tissue squashes performed using physiological saline (0.85%). Spore dimensions were calculated ($n = 100$) using a compound microscope and Zeiss KS 300 ver.3.0 (Imaging Associates Ltd, Thame, UK) image analysis software using a JVC KY-F30B 3CCD camera (JVC, Yokohama, Japan) mounted on an Olympus BH-2 compound microscope (Olympus, Tokyo, Japan), with an interfacing 2.5× top lens and a 100× oil immersion lens (accuracy 0.01 μm). In addition, dissected tissue specimens were fixed in 2.5% glutaraldehyde for 2 h and washed in cacodylate buffer (0.1 M, pH 7.2) overnight. Samples were then postfixed in 1% osmium tetroxide for 1 h, before dehydration through a graded acetone series. After dehydration, samples were embedded in Spurr resin before being polymerized at 60 °C for 48 h. Semi-thin 1 μm sections were cut using a glass knife on a Reichert Ultracut E ultramicrotome (Reichert-Jung, Wien, Austria) and visualized by staining with 1% alcian blue and examined using standard light microscopy.

Whole infected adult female sea lice were washed in sterile sea water and homogenized in 0.5 mL high concentration urea buffer (TNES urea: 10 mM Tris–HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 0.5% SDS; 4 M urea – modified from Asahida, Kobayashi, Saitoh & Nakayama 1996), and vortexed (in a manner to maximize shearing directions) for 1 min with 0.4 g of 0.5 mm silica beads to facilitate the mechanical disruption of mature spore walls. Proteinase K was added to a concentration of 100 $\mu g\ mL^{-1}$ and digestion allowed to occur overnight at 56 °C. DNA was subsequently extracted with Tris-saturated phenol and phenol:chloroform:isoamylalcohol (25:24:1), treated with RNase A, further extracted with phenol:chloroform:isoamylalcohol (25:24:1) and diethyl ether, and then precipitated at −80 °C with 2.5× volume of 95% cold ethanol and 0.1× volume 4 M sodium acetate, and finally resuspended in MilliQ water. DNA concentrations and purities were checked on a Pharmacia GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Little Chalfont, UK). Targeted DNA (*c.* 1400 BP) was amplified using the polymerase chain reaction (PCR) primers described by Vossbrinck *et al.* (1993): forward primer 530f (5′-GTGCCATC-CAGCCGCGG-3′), reverse primer 580r (5′-GG-TCCGTGTTTCAAGACGG-3′). A region of approximately 800 bp in length, located within the 530f–580r rDNA target was also amplified from the original genomic DNA extractions using the additional primers, forward primer (5′-GTCT-GTGGATCAAGGACGAA-3′) and reverse primer (5′-ACTGATATGCTTAAGTTCAGG-3′). Each 25 μL PCR reaction contained ∼10 ng of genomic DNA, 25 pmol of each primer and utilized Ready-To-Go™ PCR Beads (Amersham Pharmacia Biotech) which comprise ∼1.5 units of *Taq* DNA polymerase, 10 mM Tris–HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP and stabilizers including BSA. After an initial denaturation at 95 °C for 5 min, samples were subjected to 30 cycles of amplification [denaturation at 95 °C for 30 s, primer annealing at 43 °C (55 °C for the internal amplicon) for 30 s, and extension at 72 °C for 1 min], followed by a 10-min terminal extension at 72 °C. All amplifications were performed on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler (Perkin-Elmer Corporation, Norwalk, CT, USA). The PCR product obtained was visualized in an ethidium bromide-stained 1% agarose gel, the DNA band excised and purified using GFX spin columns (Amersham Pharmacia Biotech).

Sequences were determined directly from the PCR products. Cycle sequencing reactions using the BigDye Terminator™ Sequencing Kit (Perkin-Elmer Corporation) and incorporating the same primers as those used in the initial PCR were performed according to the manufacturer's instructions. Sequencing products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corporation). Sense and anti-sense strands were sequenced for all products (full 530f–580r region and internal amplicon) and four replicates (PCR products amplified from separate DNA extractions) performed for each gene region. CLUSTAL W (Thompson, Higgins & Gibson 1994) was used for initial sequence alignments with default settings for gap and weighting values. Regions of ambiguous alignment were identified visually and removed prior to analyses. Alignment files were converted into distances by the Kimura 2 parameter and trees constructed using the neighbour-joining (NJ) algorithm (Saitou & Nei 1987) within the Phylogeny Inference Package (PHYLIP version 3.57; Felsenstein 1993). In addition, cladograms were produced from the alignment files using the maximum-likelihood (ML) algorithm within PUZZLE (Olsen, Matsuda, Hagstrom & Overbeek 1994; Strimmer & von Haeseler 1996). Numerical values at the nodes indicate either 1000 (NJ) or percentage of 500 (ML) bootstrap replicates that support the observed tree. *Amblyospora stimuli* (Andreadis) that occupies a basal phylogenetic position within the Microspora and *Tritrichomonas foetus* (Levine) a distantly related protozoan parasite of cattle, were used as outgroups in the cladistic analyses.

For ease of interpretation, sequence comparisons were made by percentage identities rather than by distance matrices.

The sequence determined in the current study was submitted to GenBank and assigned the accession number: AJ431366. GenBank accession numbers of additional sequences utilized in the analyses are: *Amblyospora stimuli* (AF027685), *Ameson michaelis* (L15741), *Encephalitozoon cuniculi* (L39107), *Encephalitozoon (=Septata) intestinalis* (L19567), *Endoreticulatus shubergi* (L39109), *Enterocytozoon bieneusi* (AF023245 and AF024657), *Glugea anomala* (AF044391), *Glugea plecoglossi* (AJ295326), *Glugea stephani* (AF056015), *Heterosporis anguillarum* (Kamaish T. 1996),

Ichthyosporidium sp. (L39110), *Loma salmonae* (U78736), *Microgemma* sp. (AJ252952), *Microsporidium seriolae* (AJ295322), *Microsporidium* sp. (RSB) (AJ295323), *Microsporidium* sp. (D) (AF394528), *Nosema apis* (U97150), *Nosema granulosis* (AJ011833), *Nosema trichoplusiae* (U09282), *Nucleospora* spp. (U78176, AF186001, AF201911, AF186007, AF185992 and AF185989), *Ordospora colligata* (AF394529), *Pleistophora ehrenbaumi* (AF044392), *Pleistophora finisterrensis* (AF044393), *Pleistophora hippoglossoideos* (AF044388), *Pleistophora mirandellae* (AJ295327), *Pleistophora* sp. (Tb) (PSP252957), *Pleistophora* sp. (Pa) (PSP252958), *Pleistophora* sp. (Ls) (AJ252959), *Pleistophora* sp. I (AFO44394), *Pleistophora* sp. II (AF044389), *Pleistophora* sp. III (AF044390), *Pleistophora typicalis* (AF044387), *Pseudoloma neurophilia* (AF322654), *Spraguea lophii* (AF033197), *Trachipleistophora hominis* (THAAJ2605), *Tritrichomonas foetus* (M81842), unidentified micro (GHB) (AJ295324), unidentified micro Mj (AJ295328), unidentified micro Tr (AJ295329), *Vairimorpha necatrix* (M24612), *Vairimorpha lymantriae* (AF141129), *Vavraia oncoperae* (X74112), and *Vittaforma corneum* (L39112).

Results

The microsporidian infection was observed in pre-adult and adult stages of both male and female copepods, with a prevalence of up to 5%. Parasitized individuals appeared opaque at the sites of infection which were observed throughout the body and heavily infected female lice were often observed with malformed or disproportionately extruded egg strings (Fig. 1).

Microscopic examination of fresh tissue squashes revealed free uniform spores that were not observed within a sporophorous vesicle, subspherical (slightly pyriform) in shape and measuring 2.34 μm by 1.83 μm (±0.01 μm).

Histological sections from the abdomen of infected sea lice revealed xenoma-like cysts from a few microns in size up to 300 μm in section diameter. These cysts developed directly under the cuticle or along the haemocoelic separations (structures separating haemal sinuses/lacunae) of the haemocoel and originated from the epidermal (hypodermal) tissue layer. Large cysts were seen completely filling the haemocoelic cavity between the cuticle and the centrally located gut, however the microsporidian was never observed inside intestinal tissues (Fig. 2). Developing prespore stages of the parasite were visible as poorly stained areas of both small and large cysts; they were not seemingly restricted to specific areas of the cyst and were located either centrally or at the periphery (Fig. 2).

Partial rRNA genes were successfully sequenced from microsporidia taken from four infected sea lice

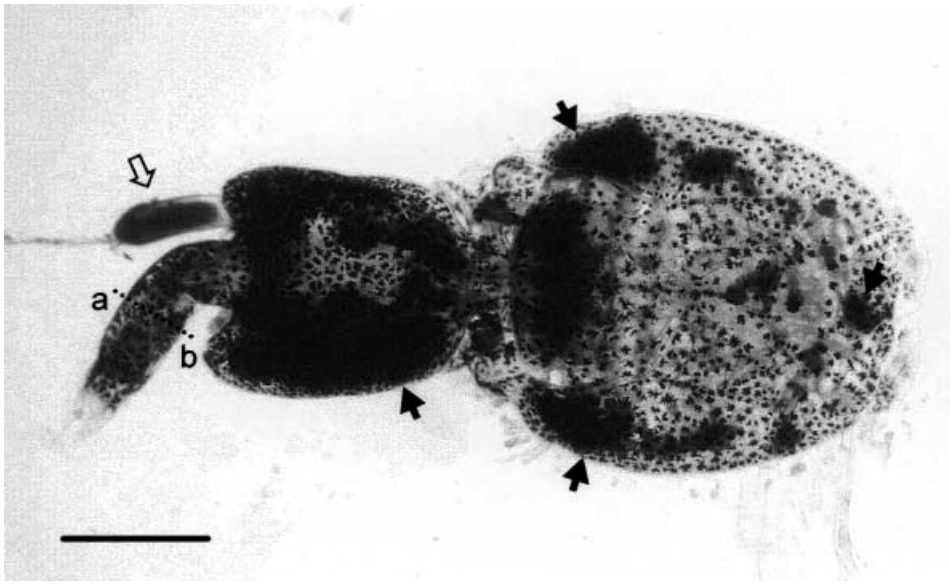

Figure 1 Adult female *Lepeophtheirus salmonis* with the microsporidian infection visible as opaque inclusions (filled arrows), note aborted egg string (open arrow); line a–b represents the histological section detailed in Fig. 2 (bar = 2 mm).

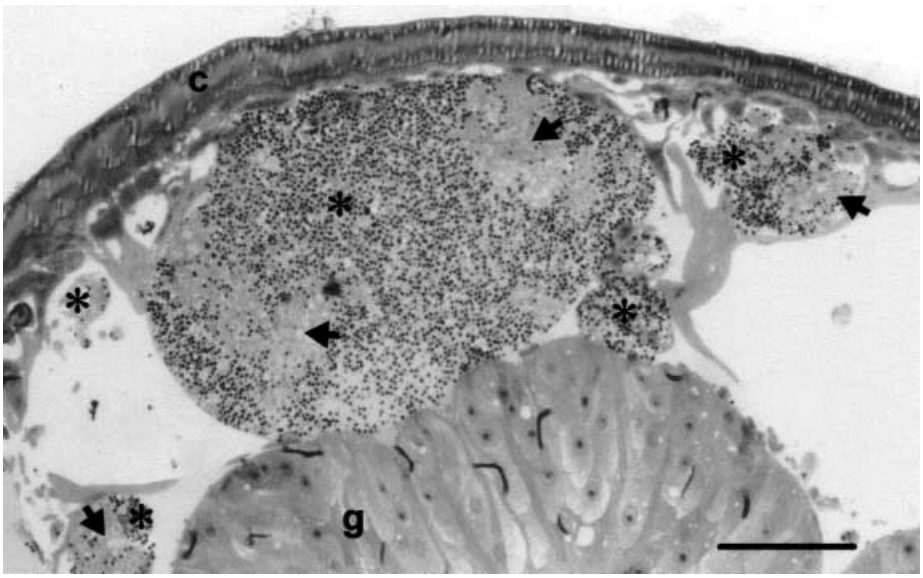

Figure 2 Transverse semi-thin section through the abdomen of an infected adult female sea louse. Xenoma-like cysts (*) are developing between the cuticle (c) and the centrally positioned gut (g). Poorly stained areas of the cysts (filled arrows) represent developing prespore stages of the microsporidian (bar = 100 μm).

Table 1 Percentage identity of the sea lice microsporidian to selected microsporidia over the 530f–580r region of the rRNA gene (gaps excluded). Host type and estimated length of gene region are also given

Microsporidian	Host	Estimated length of 530f–580r region	Percentage identity over number of bases
Present study	Crustacea sea louse	1490–1500	100 (1411)
Nucleospora sp.	Fish English sole	1487	87.7 (1410)
Nucleospora salmonis	Fish chinook salmon	1496	83.9 (1406)
Enterocytozoon bieneusi	Mammal rhesus macaque	1631	76.2 (1396)
Loma salmonae	Fish chinook salmon	1396	66.2 (1316)
Glugea anomala	Fish stickleback	1434	65.3 (1318)
Pleistophora typicalis	Fish shorthorn sculpin	1437	65.1 (1322)
Nosema apis	Insect honeybee	1392	64.4 (1289)
Microsporidium sp.	Crustacea metapenaeid shrimp	1381	64.2 (1297)

each containing approximately 10^8 spores, with 100% homology over a region of 1411 bases from within the 530f–580r region of the gene. Due to direct sequencing of the PCR products, the exact length of the entire 530f–580r region is unknown, but is estimated to be between 1480 and 1500 bp in length after suitable comparisons were made with available sequences in GenBank.

Table 1 shows percentage identities of the *L. salmonis* microsporidian sequence over the number of available bases from the 530f–580r region for eight microsporidians from seven different genera. The estimated length of 1490–1500 bases is similar to the two *Nucleospora* spp. infecting fish. Indeed, these two species, *Nucleospora* sp. infecting English sole, *Pleuronectes vetulus* (Girard), and *N. salmonis* infecting salmonids, demonstrate the highest percentage identities (87.7 and 83.9%, respectively) to the sea louse-infecting microsporidian. The human pathogen *E. bieneusi* (Desportes) demonstrates 76.2% sequence identity with the sea louse-infecting microsporidian but has a large insertion in the ITS region and is significantly longer at 1631 bases. *Loma salmonae* (Putz, Hoffman & Dunbar), *Glugea anomala* (Moniez) and *Pleistophora typicalis* (Gurley) infecting fish all have shorter 530f–580r regions and lower percentage identities of 66.2, 65.3 and 65.1%, respectively. The only complete 530f–580r microsporidian sequences available from other arthropod hosts, *Nosema apis* (Zander) from the honeybee and *Microsporidium* sp. (Bell *et al.* 2001) from a metapenaeid shrimp, were more

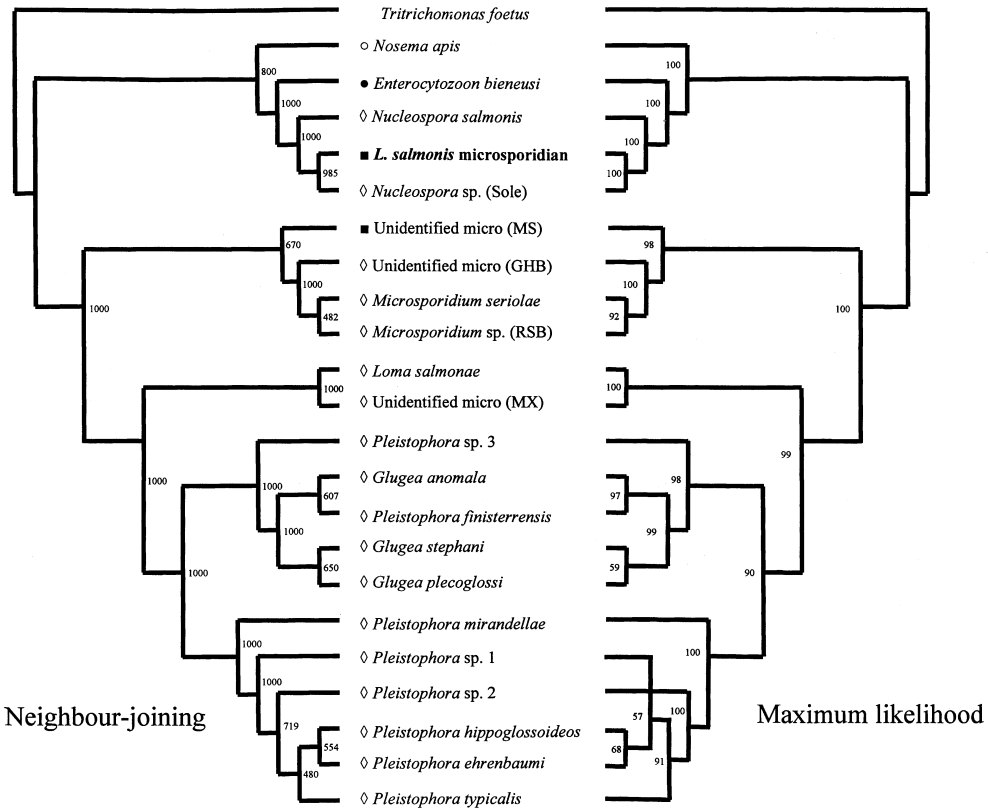

Figure 3 Cladograms of 22 microsporidians based on the complete 530f–580r region of the rRNA gene (*c.* 1400 bp, see Table 1). Trees constructed used neighbour-joining (NJ) or maximum-likelihood (ML) algorithms. Figures at nodes represent 1000 bootstrap replicates (NJ) and 500 (ML) (shown as percentages for ML). ●, Mammalian host; ○, insect host; ■, crustacean host; ◇, fish host. MS, metapenaeid shrimp; GHB, gilthead sea bream; RSB, red sea bream; MX, myxosporean.

distantly related with 64.4 and 64.2% identities, respectively.

Twenty-one 530f–580r microsporidian sequences from seven different genera and 38 SSU sequences from 22 different genera were aligned and used in phylogenetic analyses using the ML and NJ methodologies (Figs 3 & 4). The *L. salmonis* microsporidian groups within the family Enterocytozoonidae, independent of the tree building method used, and is supported by high bootstrap scores in both cases. Within the Enterocytozoonidae, the sea louse-infecting microsporidian groups within the clade containing the genus *Nucleospora* and is most closely related to the *Nucleospora* sp. infecting the English sole, again supported by high bootstrap scores irrespective of the method used. This clade forms a sister group to that containing the genus *Enterocytozoon.*

Discussion

The size and shape of spores and lack of a sporophorous vesicle from the microsporidian in the present study are important taxonomic features which have not been reported from microsporidian species infecting other members of the Copepoda. However, no microsporidian species have been previously recorded from marine copepods. Other marine Crustacea have been recorded with microsporidian infections, but the spore dimensions and taxonomic features are again different from those of the present study.

The microsporidian infection was only grossly observed in pre-adult and adult stages of *L. salmonis*; however, earlier copepodid and chalimus lice stages were not sampled during this study and may also potentially harbour the infection. Furthermore, utilizing sensitive PCR detection techniques with the specific primers described above will detect early infections not yet visible by eye. Therefore, the prevalence of 5% recorded during this study may in fact prove to be a significant underestimate. Whether the malformed egg strings seen in infected female lice represents a degree of host castration or simply that senescent lice no longer able to produce

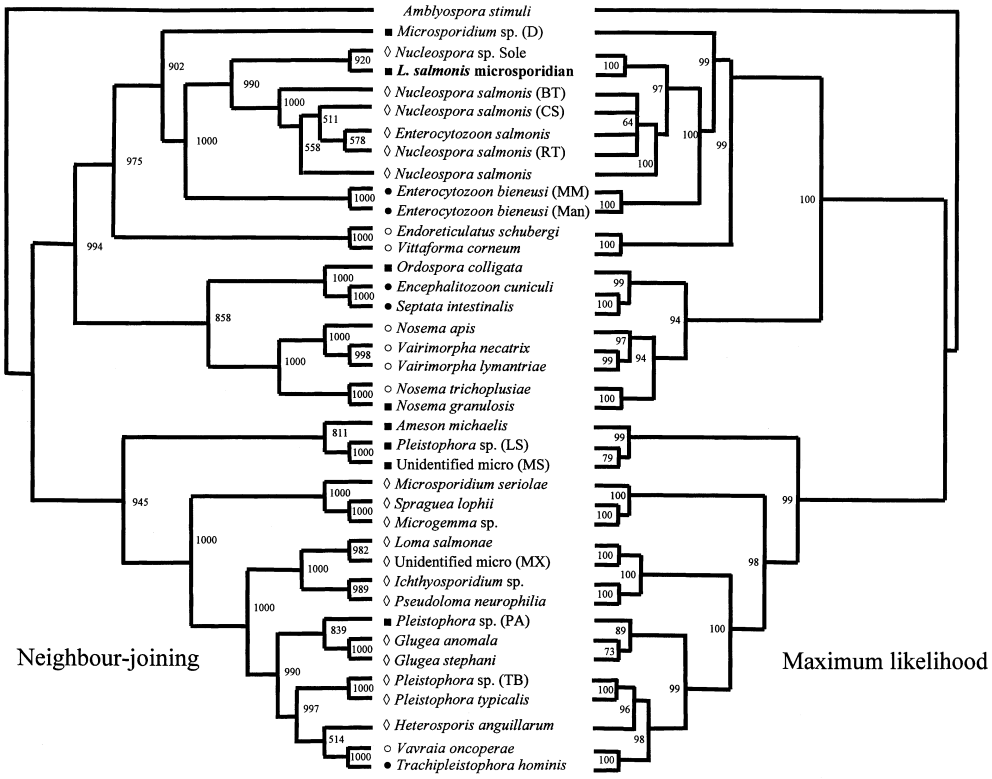

Figure 4 Cladograms of 39 microsporidians based on the SSU region of the rRNA gene (*c.* 900 bp). Trees constructed used neighbour-joining (NJ) or maximum-likelihood (ML) algorithms. Figures at nodes represent 1000 bootstrap replicates (NJ) and 500 (ML) (shown as percentages for ML). ●, Mammalian host; ○, insect host; ■, crustacean host; ◇, fish host. D, daphnia; BT, brook trout; CS, chinook salmon; RT, rainbow trout; MM, *Macaca mulatta*; LS, *Litopenaeus setiferus*; MS, metapenaeid shrimp; MX, myxosporean; PA, *Penaeus aztecus*; TB, *Taurulus bubalis*.

egg strings become infected remains unknown. Pathogenicity may also result from the large xenoma-like cysts occupying the coelomic cavity and further histological studies are required to confirm that there is no other tissue location for this parasite.

The microsporidian from *L. salmonis* groups within the clade containing the family Enterocytozoonidae and the genera *Enterocytozoon* and *Nucleospora*. The *Nucleospora* spp. are the only known fish-infecting microsporidians that group away from the monophyletic clade containing all other fish-infecting microsporidian parasites (Nilsen 2000; Bell *et al.* 2001). The grouping of this sea louse-infecting microsporidian within this clade is supported by 100% bootstrap values.

The family Enterocytozoonidae was created by Cali & Owen (1990) after completing the description of the developing stages of the previously reported pathogen infecting immuno-compromised humans, *Entercytozoon bienuesi* (Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Lavergne, Ravisse & Modigliani 1985). There are currently two genera within this family, *Nucleospora* and *Enterocytozoon*; however, the actual number of species assigned to each genus remains unclear. The intranuclear microsporidian, *N. salmonis*, was originally described from chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), by Hedrick, Groff & Baxa (1991), but was shortly thereafter renamed as *E. salmonis* by Chilmonczyk, Cox & Hedrick (1991). Subsequently, two other intranuclear microsporidian parasites of fish, *Enterocytozoon* sp. from Atlantic halibut, *Hippoglossus hippoglossus* (L.) (see Nilsen, Ness & Nylund 1995), and *Enterocytozoon* sp. from lumpfish, *Cyclopterus lumpus* L. (see Mullins, Powell, Speare & Cawthorn 1994), were also assigned to the genus *Enterocytozoon*. However, more recently, Docker, Kent, Hervio, Khattra, Weiss, Cali & Devlin (1997) suggested that, in the absence of significant reasons for the suppression of the generic name *Nucleospora*,

the original name, *N. salmonis* is valid, as supported by the International Code of Zoological Nomenclature. Furthermore, Docker *et al.* (1997) suggested that the intranuclear *Enterocytozoon* spp. from Atlantic halibut and lumpfish also be reassigned to the genus *Nucleospora*. A second species of *Nucleospora* has recently been described, *N. secunda* (Lom & Dykova 2002), which is also an intranuclear parasite of fish, which leaves only one nonintranuclear member in the family, the type species of the genus *Enterocytozoon*, *E. bieneusi*. It is worth noting that according to Dengjel, Zahler, Hermanns, Heinritzi, Spillman, Thomschke, Loscher, Gothe & Rinder (2001) 14 genotypes of *E. bieneusi* exist infecting mammals other than humans.

Nucleospora salmonis has been detected in at least five species of salmonids from three different continents. SSU rDNA studies of isolates representing these distant geographical locations and different salmonid hosts suggests that they represent a single species (Gresoviac, Khattra, Nadler, Kent, Devlin, Vivares, De La Fuente & Hedrick 2000). However, the *N. salmonis*-like specimen from the non-salmonid host, English sole, was sufficiently different, with a variation of 12.72% with respect to the SSU rDNA when compared with that of the genotype from chinook salmon, to be assumed a separate species (Gresoviac *et al.* 2000).

One other intranuclear microsporidian species, *Microsporidium rhabdophilia*, has been described from rodlet cells of salmonid fish (Modin 1981). As *N. salmonis* has since been observed in rodlet cell nuclei by Chilmonczyk *et al.* (1991); Lom & Dykova (1992) suggested that they could be the same species. Modin however, performed thorough histological examinations and reported that the parasite showed a great degree of host cell specificity being present in the nuclei of rodlet cells only. No signs of disease were ever observed with *M. rhabdophilia* infections, unlike *N. salmonis* infections which are associated with acute anaemia (Elston, Kent & Harrell 1987), lymphoblastosis (Morrison, MacConnell, Chapman & Westgard 1990) and leukaemia (Hedrick, Groff, McDowell, Willis & Cox 1990). However, due to the lack of DNA and ultrastructural studies on *M. rhabdophilia* further classification is not possible.

The Enterocytozoonidae represents an intriguing group of microsporidian parasites, consisting of only two described genera and four species. These parasites infect humans, other mammals, fish and possibly a crustacean, demonstrating the diverse host range and possible opportunistic nature of these organisms. To date, *N. salmonis* and *N. secunda* remain the only true species attributed to the genus *Nucleospora*, as the precise location of the species infecting English sole has not yet been determined (Khattra, Gresoviac, Kent, Myers, Hedrick & Devlin 2000). There are presently, limited rRNA gene data available for members of established genera that are closely related to *Nucleospora* within the Enterocytozoonidae. Consequently, it remains unclear as to the percentage variation that might be expected within a species or between species of this genus. Thus, it seems possible that the 2.14% variation described by Gresoviac *et al.* (2000) between putative *N. salmonis* isolates may actually represent a number of closely related *Nucleospora* species, especially given the environmental and geographical isolation of several of the fish hosts. These uncertainties emphasize the need, highlighted by Cheney, Lafranchi-Tristem, Bourges & Canning (2001), to examine additional, more rapidly evolving protein coding genes when investigating intrageneric and sibling species relationships within the Microspora and the problems associated with assigning species status to parasites in general (Kunz 2002).

The partial rRNA gene molecular phylogeny of this microsporidian parasite of *L. salmonis*, places it convincingly within the clade containing the family Enterocytozoonidae. The sea lice microsporidian groups most closely with the intranuclear microsporidians (*Nucleospora* spp.) infecting fish, and more distantly to the human parasite, *E. bieneusi*, isolated from the intestinal epithelium. The microsporidian spores isolated from *L. salmonis* are of a similar shape and only marginally larger to those described from other members of the Enterocytozoonidae. However, the large xenoma-like cysts forming in sea lice are not a characteristic of the Enterocytozoonidae and the infection in sea lice is neither intranuclear nor in the intestinal epithelium. The family Enterocytozoonidae was originally created because of the unique polar filament development in *E. bieneusi*, a feature also seen in *Nucleospora* spp. Therefore, further detailed information on developmental stages and ultrastructure are required before this microsporidian can be assigned to an existing genus within the Enterocytozoonidae or a new genus be established.

References

Asahida T., Kobayashi T., Saitoh K. & Nakayama I. (1996) Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffers containing high concentrations of urea. *Fisheries Science* **62,** 727–730.

Azevedo C. & Canning E.U. (1987) Ultrastructure of a microsporidian hyperparasite, *Unikaryon legeri* (Microsporida), of trematode larvae. *Journal of Parasitology* **73,** 214–223.

Azevedo C., Corral L. & Vivares C.P. (2000) Ultrastructure of the microsporidium *Inodosporus octospora* (Thelohaniidae), a parasite of the shrimp *Palaemon serratus* (Crustacea, Decapoda). *Diseases of Aquatic Organisms* **41,** 151–158.

Baker M.D., Vossbrinck C.R., Maddox J.V. & Undeen A.H. (1994) Phylogenetic relationships among *Vairimorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. *Journal of Invertebrate Pathology* **64,** 100–106.

Baker M.D., Vossbrinck C.R., Didier E.S., Maddox J.V. & Shadduck J.A. (1995) Small subunit rRNA phylogeny of various microsporidia with emphasis on AIDS related forms. *Journal of Eukaryotic Microbiology* **42,** 564–570.

Bell A.S., Aoki T. & Yokoyama H. (2001) Phylogenetic relationships among microsporidia based on rDNA sequence data, with particular reference to fish-infecting *Microsporidium* Balbiani 1884 species. *Journal of Eukaryotic Microbiology* **48,** 258–265.

Cali A. & Owen R.L. (1990) Intracellular development of *Enterocytozoon*, a unique microsporidian found in the intestine of AIDS patients. *Journal of Protozoology* **37,** 145–155.

Canning E.U. (1975) The microsporidian parasites of Platyhelminthes: their morphology, development, transmission and pathogenicity. *Commonwealth Agricultural Bureaux Special Publication* **2,** 1–32.

Caullery M. & Mesnil F. (1914) Sur les Metchnikovellidae et autres protistes parasites des gregarines d' annelids. *Comptes rendus de la Societe de Biologie* **77,** 527–532.

Cheney S.A., Lafranchi-Tristem N.J. & Canning E.U. (2000) Phylogenetic relationships of *Pleistophora*-like microsporidia based on small subunit ribosomal DNA sequences and implications for the source of *Trachipleistophora hominis* infections. *Journal of Eukaryotic Microbiology* **47,** 280–287.

Cheney S.A., Lafranchi-Tristem N.J., Bourges D. & Canning E.U. (2001) Relationships of microsporidian genera, with emphasis on the polysporous genera, revealed by sequences of the large subunit of RNA polymerase II (RPBI). *Journal of Eukaryotic Microbiology* **48,** 111–117.

Chilmonczyk S., Cox W.T. & Hedrick R. (1991) *Enterocytozoon salmonis* n. sp.: an intranuclear microsporidium from salmonid fish. *Journal of Protozoology* **38,** 264–269.

Dengjel B., Zahler M., Hermanns W., Heinritzi K., Spillman T., Thomschke A., Loscher T., Gothe R. & Rinder H. (2001) Zoonotic potential of *Enterocytozoon bieneusi*. *Journal of Clinical Microbiology* **39,** 4495–4499.

Desportes I., Le Charpentier L., Galian A., Bernard F., Cochand-Priollet B., Lavergne A., Ravisse P. & Modigliani R. (1985) Occurrence of a new microsporidian: *Enterocytozoon bieneusi* n.g., n. sp., in the enterocytes of a human patient with AIDS. *Journal of Protozoology* **32,** 250–254.

Docker M.F., Kent M.L., Hervio D.M.L., Khattra J.S., Weiss L.M., Cali A. & Devlin R.H. (1997) Ribosomal DNA sequence of *Nucleospora salmonis* (Hedrick, Groff and Baxa, 1991) (Microsporea: Enterocytozoonidae): implications for phylogeny and nomenclature. *Journal of Eukaryotic Microbiology* **44,** 55–60.

Dunn A.M. & Rigaud T. (1998) Horizontal transfer of parasitic sex ratio distorters between crustacean hosts. *Parasitology* **117,** 15–19.

Elston R.A., Kent M.L. & Harrell L.H. (1987) An intranuclear microsporidium associated with acute anemia in the chinook salmon, *Oncorhynchus tshawytscha*. *Journal of Protozoology* **34,** 274–277.

Felsenstein J. (1993) *PHYLIP: Phylogeny Inference Package, Version 3.5c*. University of Washington, Seattle, WA.

Gresoviac S.J., Khattra J.S., Nadler S.A., Kent M.L., Devlin R.H., Vivares C.P., De La Fuente E. & Hedrick R.P. (2000) Comparison of small subunit ribosomal RNA gene and internal transcribed spacer sequences among isolates of the intranuclear microsporidian *Nucleospora salmonis*. *Journal of Eukaryotic Microbiology* **47,** 379–387.

Gresty K.A. & Warren A. (1993) Incidence of ciliate epibionts on *Lepeophtheirus salmonis* from salmon in Japan and Scotland: a scanning electron microscopic study. In: *Pathogens of Wild and Farmed Fish: Sea Lice* (ed. by G.A. Boxshall & D. Defaye), pp. 356–363. Ellis Horwood Ltd, Chichester.

Hedrick R.P., Groff J.M., McDowell T.S., Willis M. & Cox W.T. (1990) Hematopoietic intranuclear microsporidian infections with features of leukaemia in chinook salmon *Oncorhynchus tshawytscha*. *Diseases of Aquatic Organisms* **8,** 189–197.

Hedrick R.P., Groff J.M. & Baxa D.V. (1991) Experimental infections with *Enterocytozoon salmonis* Chilmonczyk, Cox & Hendrick (Microsporea): an intranuclear microsporidium from chinook salmon *Oncorhynchus tshawytscha*. *Diseases of Aquatic Organisms* **10,** 103–108.

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 microsporidium from English sole (*Pleuronectes vetulus*) affected by X-cell pseudotumors. *Journal of Parasitology* **86,** 867–871.

Kunz W. (2002) When is a parasite species a species? *Trends in Parasitology* **18,** 121–124.

Larsson J.I. (2000) The hyperparasitic microsporidian *Amphiacantha longa* Caullery and Mesnil, 1914 (Microspora: Metchnikivellidae), description of the cytology, re-description of the species, amended diagnosis of the genus *Amphiacantha* and establishment of the new family Amphiacanthidae. *Folia Parasitologica* **47,** 241–256.

Lom J. & Dykova I. (1992) *Protozoan Parasites of Fishes. Developments in Aquaculture and Fisheries Science*, Vol. 26, pp. 125–154. Elsevier, Amsterdam.

Lom J. & Dykova I. (2002) Ultrastructure of *Nucleospora secunda* n. sp. (Microsporidia), parasite of enterocytes of *Nothobranchius rubripinnis*. *European Journal of Protistology* **38,** 19–27.

Lom J. & Nilsen F. (2003) Fish microsporidia: fine structural diversity and phylogeny. *International Journal of Parasitology* **33,** 107–127.

Mathis A. (2000) Microsporidia: emerging advances in understanding the basic biology of these unique organisms. *International Journal of Parasitology* **30,** 795–804.

Minchin D. & Jackson D. (1993) *Udonella caligorum* Johnston, 1835 (Platyhelminthes: Udonellidea) associated with caligid copepods on farmed salmon. In: *Pathogens of Wild and Farmed Fish: Sea Lice* (ed. by G.A. Boxshall & D. Defaye), pp. 346–355. Ellis Horwood Ltd, Chichester.

Modin J.C. (1981) *Microsporidium rhabdophilia* n. sp. from rodlet cells of salmonid fishes. *Journal of Fish Diseases* **4,** 203–211.

Morrison J.K., MacConnell E., Chapman P.F. & Westgard R.L. (1990) A microsporidium induced lymphoblastosis in chinook salmon *Oncorhynchus tshawytscha* in freshwater. *Diseases of Aquatic Organisms* **8,** 99–104.

Muller A., Trammer T., Chioralia G., Seitz H.M., Diehl V. & Franzen C. (2000) Ribosomal RNA of *Nosema algerae* and phylogenetic relationship to other microsporidia. *Parasitology Research* **86,** 18–23.

Mullins J.E., Powell M., Speare D.J. & Cawthorn R. (1994) An intranuclear microsporidian in lumpfish *Cyclopterus lumpus*. *Diseases of Aquatic Organisms* **20,** 7–13.

Nilsen F. (2000) Small subunit ribosomal DNA phylogeny of microsporidia with particular reference to genera that infect fish. *Journal of Parasitology* **86,** 128–133.

Nilsen F., Ness A. & Nylund A. (1995) Observations on an intranuclear microsporidian in lymphoblasts from farmed Atlantic halibut larvae (*Hippoglossus hippoglosossus* L.). *Journal of Eukaryotic Microbiology* **42,** 131–135.

Nilsen F., Endresen C. & Hordvik I. (1998) Molecular phylogeny of microsporidians with particular reference to muscle infecting species of fishes. *Journal of Eukaryotic Microbiology* **45,** 535–543.

Olsen G.J., Matsuda H., Hagstrom R. & Overbeek R. (1994) FastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *Computing and Applied Biosciences* **10,** 41–48.

Ramasamy P., Jayakumar R. & Brennan G.P. (2000) Muscle degeneration associated with cotton shrimp disease of *Penaeus indicus*. *Journal of Fish Diseases* **23,** 77–81.

Refardt D., Canning E.U., Mathis A., Cheney S.A., Lafranchi-Tristem N.J. & Ebert D. (2002) Small subunit ribosomal DNA phylogeny of microsporidia that infect *Daphnia* (Crustacea: Cladocera). *Parasitology* **124,** 381–389.

Saitou N. & Nei M. (1987) The neighbour joining method: a new method for reconstructing phylogenetic trees. *Molecular and Biological Evolution* **4,** 406–425.

Shigina N.G. & D'iakonov L.P. (1977) Ultrastructure of *Nosema diplostomi*: hyperparasite of trematodes of the genus *Diplostomum*. *Parazitologiia* **11,** 241–246.

Sprague V. & Vavra J. (1977) Systematics of the microsporidia. In: *Comparative Pathobiology 2* (ed. by L.A. Bulla & T.C. Cheng), pp. 1–510. Plenum, New York.

Strimmer K. & von Haeseler A. (1996) Quartet puzzling: a quartet maximum likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution* **13,** 964–969.

Sweeney A.W., Doggett S.L. & Piper R.G. (1993) Life cycle of a new species of *Duboscqia* (Microsporidia: Thelohanidae) infecting the mosquito *Anopheles hilli* and an intermediate copepod host, *Apocyclops dengizicus*. *Journal of Invertebrate Pathology* **62,** 137–146.

Terry R.S., Smith J.E., Bouchon D., Rigaud T., Duncanson P., Sharpe R.G. & Dunn A.M. (1999) Ultrastructural characterisation and molecular taxonomic identification of *Nosema granulosis* n. sp., a transovarially transmitted feminising (TTF) microsporidian. *Journal of Eukaryotic Microbiology* **46,** 492–499.

Thompson J.D., Higgins D.G. & Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Tun T., Yokoyama H., Ogawa K. & Wakabayashi H. (2000) Myxosporeans and their hyperparasitic microsporeans in the intestine of emaciated tiger puffer. *Fish Pathology* **35,** 145–156.

Van de Peer Y., Ben Ali A. & Meyer A. (2000) Microsporidia: accumulating molecular evidence that a group of amitochondriate and suspectedly primitive eukaryotes are just curious fungi. *Gene* **246,** 1–8.

Vinckier D., Devauchelle G. & Prensier G. (1970) *Nosema vivieri* n. sp. (Microspora, Nosematidae) hyperparasite of gregarina living in nemertina coelom. *Comptes Rendues Academie des Sciences Hebdomadaires Seances*. **27,** 821–823.

Vivares C.P., Loubes C. & Bouix G. (1976) Further cytochemical research on microsporidian parasites of the Mediterranean shore crab, *Carcinus mediterraneus* Czerniavsky, 1884. *Annales de Parasitologie Humaine et Comparee* **51,** 1–14.

Vossbrinck C.R., Baker M.D., Didier E.S., Debrunner-Vossbrinck B.A. & Shadduck J.A. (1993) Ribosomal DNA sequences of *Encephalitozoon hellem* and *Encephalitozoon cuniculi*: species identification and phylogenetic construction. *Journal of Eukaryotic Microbiology* **40,** 354–362.

Vossbrinck C.R., Andreadis T.G. & Debrunner-Vossbrinck B.A. (1998) Verification of intermediate hosts in the life cycles of microsporidia by small subunit rDNA sequencing. *Journal of Eukaryotic Microbiology* **45,** 290–292.

Weiss L.M. & Vossbrinck C.R. (1998) Microsporidiosis: molecular and diagnostic aspects. *Advances in Parasitology* **40,** 351–395.

Weiss L.M., Zhu X., Cali A., Tanowitz H.B. & Wittner M. (1994) Utility of microsporidian rRNA in diagnosis and phylogeny: a review. *Folia Parasitologica* **41,** 81–90.

Yokoyama H., Lee S-J. & Bell A.S. (2002) Occurrence of a new microsporidian in the skeletal muscle of the flying fish *Cypselurus pinnatibarbatus japonicus* (Exocoetidae) from Yakushima, Japan. *Folia Parasitologica* **49,** 9–15.

Zhu X., Wittner M., Tanowitz H.B., Cali A. & Weiss L.M. (1994) Ribosomal RNA sequences of *Enterocytozoon bieneusi*, *Septata intestinalis* and *Ameson michaelis*: phylogenetic construction and structural correspondence. *Journal of Eukaryotic Microbiology* **41,** 204–209.

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