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Myospora metanephrops (n. g., n. sp.) from marine lobsters and a proposal for erection of a new order and family (Crustaceacida; Myosporidae) in the Class Marinosporidia (Phylum Microsporidia) ☆

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ABSTRACT

In this study we describe, the first microsporidian parasite from nephropid lobsters. *Metanephrops challengeri* were captured from an important marine fishery situated off the south coast of New Zealand. Infected lobsters displayed an unusual external appearance and were lethargic. Histology was used to demonstrate replacement of skeletal and other muscles by merogonic and sporogonic stages of the parasite, while transmission electron microscopy revealed the presence of diplokaryotic meronts, sporonts, sporoblasts and spore stages, all in direct contact with the host sarcoplasm. Analysis of the ssrDNA gene sequence from the lobster microsporidian suggested a close affinity with *Thelohania butleri*, a morphologically dissimilar microsporidian from marine shrimps. Whilst morphological features of the lobster parasite are consistent with members of the family Nosematidae, molecular data place the parasite closer to members of the family Thelohanidae. Due to the contradiction between morphological and molecular taxonomic data, we propose the erection of a new genus in which the lobster parasite is the type species (*Myospora metanephrops*). Furthermore, we recommend the erection of a new family (Myosporidae) and a new order (Crustaceacida) to contain this genus. The taxonomic framework presented could be further applied to the re-classification of existing members of the Phylum Microsporidia.

1. Introduction

The Microsporidia are a phylum of obligate intracellular parasites thought to be derived from the Fungi (Edling et al., 1996; Keeling and Doolittle, 1996). They are widely distributed across numerous hosts from the major animal phyla and can be distinguished from other pathogen groups by the presence of a polar capsule, polar filament and the unique diplokaryon stages in one or more life history stages. Traditionally, taxonomy has used rapidly changing characteristics to classify lower phylogenetic levels (such as species and genus), with conservative characters used for classifying higher levels such as class, order and family (Vossbrinck and Debrunner-Vossbrinck, 2005). A recent review of microsporidian taxonomy based upon developmental and molecular characteristics has suggested that pathogen characters that change their evolutionary state more rapidly, or are homoplasious, such as the number of nuclei per cell and the presence of sporophorous vesicles, has led to contradictions in the classification of the phylum (Vossbrinck and Debrunner-Vossbrinck, 2005).

The confusion in classification within large families is demonstrated by the family Thelohaniidae, which contains pathogens infecting terrestrial, freshwater and marine hosts. The emergence of the genus *Thelohania* in particular as a large and polyphyletic group is partially attributable to a lack of suitable comparisons (both in terms of morphology and molecular phylogeny) of 'new' species with the type species *Thelohania giardi* Henneguy and Thélohan, 1892 from the shrimp *Crangon crangon* (Linneaus, 1758), and to more recent 'revisions' of the genus that include those species forming sporogonial plasmodia (Hazard and Oldacre, 1975; Sprague, 1977). The more inclusive nature of this description compared to the relatively prescriptive definition of the original genus appears to be at least partially responsible for creating the confusion. Recently, Brown and Adamson (2006) highlighted this issue by demonstrating that *Thelohania butleri* Johnston, Vernick and Sprague, 1978 from the pink shrimp (*Pandalus jordani* Rathbun, 1902), a potentially close relative to the type species *T. giardi*, is rather distant in terms of molecular sequence data from

☆ Note: *Myospora metanephrops* ssrDNA gene sequences are registered in GenBank under accession numbers HM140491–HM140499.

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other members of the genus *Thelohania* described from freshwater crayfish and from terrestrial insects. Brown and Adamson (2006) concluded with a proposition that these latter species are not in fact members of the genus *Thelohania*, and that the genus requires revision, ideally with a redescription using ultrastructure and molecular phylogeny of the type species, *T. giardi*.

Similar taxonomic discussions have occurred within the genus *Nosema*. Baker et al. (1994) and Vossbrinck and Debrunner-Vossbrinck (2005), utilising lsrRNA sequences, have suggested that the genus *Nosema* was likely composed of 'true *Nosema*' spp. (similar to the type species *Nosema bombycis* Nägeli, 1857), and numerous unrelated '*Nosema*' spp. that only share the common character of a diplokaryotic stage in the life cycle (Vossbrinck and Debrunner-Vossbrinck, 2005). The general ultrastructure of members of the genus *Nosema* has been described by Cali (Cali, A., 1971. Morphogenesis in the genus *Nosema*. In: Proc. IVth. Int. Colloq. Insect Pathol. Maryland, 1970, pp. 431–438) and Iwano and Ishihara (1991). During merogony, diplokaryotic stages undergo binary fission while plasmodial stages (containing multiple diplokarya) undergo multiple fission to form diplokaryotic sporonts. During sporogony, binary fission of sporonts leads to two diplokaryotic sporoblasts that eventually divide into spores. In the type species, *N. bombycis*, the spores can be of two types, a pyriform type with few polar coils formed during early infection and an ovoid type with a larger number of coils formed later in infection. The former are considered to be involved in cell–cell transmission (auto-infection) within the host with the latter responsible for between-host transmission (Canning and Vavra, 2000). Members of the genus *Nosema* are generally considered as insect pathogens, a feature highlighted by their placement in the Class Terresporidia by Vossbrinck and Debrunner-Vossbrinck (2005). However, several authors have also reported members of the genus in crustacean hosts based on ultrastructural characteristics. These species are *Nosema stenocrypsis*, *Nosema granulosus* and *Nosema artemiae* (see Diarra and Toguebaye, 1996; Terry et al. 1999 and Ovcharenko and Wita, 2005, respectively). A *Nosema* sp. was also reported from the marine decapod *Callinectes sapidus* (Weidner, 1970), but this was later moved to the genus *Ameson* (see Sprague et al., 1992). No examples of *Nosema* have been described infecting fish, although they have been described as hyperparasites within myxozoan parasites infecting fish (Lom and Dyková, 1992). Furthermore, sister genera within the family Nosematidae include *Hirsutosporus* (a parasite of Diptera (Batson 1983), *Ichthyosporidium*, a parasite of fish (Casal and Azvedo, 1995), and *Wittmannia*, a hyperparasite of a dicyemidan in squid (Czaker, 1997). Each of these shares the basic feature of the Nosematidae, the lack of a sporophorous vacuole, and diplokaryotic stages throughout merogony and sporogony, although monokaryotic meronts are also reported in *Wittmannia* (Canning and Vavra, 2000). However, as stated by Vossbrinck and Debrunner-Vossbrinck (2005), diplokaryotic stages throughout the life cycle may not necessarily be an indication of relatedness between these genera, particularly when considering the wide ecological range over which they exist. For this reason, Vossbrinck and Debrunner-Vossbrinck (2005) have suggested that genetic analyses applied to the Microsporidia may be used to divide the phylum into groups that reflect habitat and host. In doing so, three large Classes have been proposed: the Aquasporidia (parasites in hosts with links to freshwater habitats), the Marinosporidia (in hosts with links to marine habitats) and the Terresporidia (in hosts with links to terrestrial habitats). Because these groupings are based first on genotypic similarity, and subsequently on host type (e.g. fish, crustacean, insect) and even type of tissue infected (e.g. muscle), the simplified classification appears to offer an objective means to classify currently known and newly discovered microsporidian parasites and as such provides a modern, scientifically-based framework for taxonomy of this group.

Nephropid lobsters of the genera *Metanephrops*, *Nephrops* and *Homarus* are commercially exploited in global crustacean fisheries. The Auckland Islands, to the south of New Zealand, supports one of the main *Metanephrops* fisheries, and contributes approximately one-third of the total annual landings for New Zealand of about 900 tonnes. The fishery takes place on the muddy seabed of the upper shelf slope between 350 m and 550 m depth. As part of trawl surveys to assess the extent of the stock during March 2007, a low percentage of *Metanephrops challengeri* with an unusual external appearance were observed. During a similar survey during March 2008, samples of affected lobsters were collected for histology, electron microscopy and nucleic acid analysis. Laboratory analyses revealed a novel microsporidian parasite causing extensive damage to the skeletal and heart muscles of affected lobsters. We provide a histopathological and ultrastructural description of the disease caused by this pathogen and use life cycle features to compare it with existing members of the phylum Microsporidia. Apparent mismatch between these ultrastructural features and the molecular phylogeny generated by analysis of the ssrDNA sequences highlight the current confusion in some elements of the taxonomy of this pathogenic group. Consequently, classification of the lobster pathogen is problematic as ultrastructural features are consistent with the Nosematidae (lacking a sporophorous vacuole) and molecular taxonomy is consistent with some members of the Thelohanidae (possessing a sporophorous vacuole in which sporogony occurs). This is, the first reported case of a microsporidian parasite infection in the clawed marine lobsters (Decapoda; Nephropidae) and to our knowledge is the first attempt to classify a new taxon by utilising the higher-level classification system proposed by Vossbrinck and Debrunner-Vossbrinck (2005). To otherwise classify this novel species based solely upon morphological characteristics would contribute towards the existing confusion in the taxonomy of this large group. In addition to the erection of a new genus and species, we erect a new order and family to contain this taxon. The framework presented here could be further applied to the re-classification of existing members of the phylum Microsporidia for which appropriate morphological and molecular data are available and further, for novel microsporidian parasites discovered in a wide range of host phyla from marine, freshwater and terrestrial habitats.

2. Materials and methods

2.1. Animal capture and tissue preparation

Lobsters, *M. challengeri*, were captured during March 2008 using 30 min tows of a commercial twin-rig trawl on the upper shelf slope between 350 and 550 m depth from the vicinity of the Auckland Islands, to the south of New Zealand (Fig. 1). Lobsters displaying atypical external appearance (e.g. altered colouration, increased carapace opacity, Fig. 2) and some apparently healthy control animals were separated from the catch, anaesthetised by chilling to 4 °C, and processed for histology, electron microscopy and molecular biology.

2.2. Histology

For histopathology, the hepatopancreas, gill, heart, midgut and skeletal muscles from the abdomen, cephalothorax and claw were removed from apparently healthy lobsters and from those displaying the external appearance described above (Fig. 2). Excised samples were placed into Davidson's seawater fixative for 24 h before transfer to 70% industrial methylated ethanol for transportation. All samples were processed within 14 days of collection. Fixed samples were processed to wax in a vacuum infiltration processor

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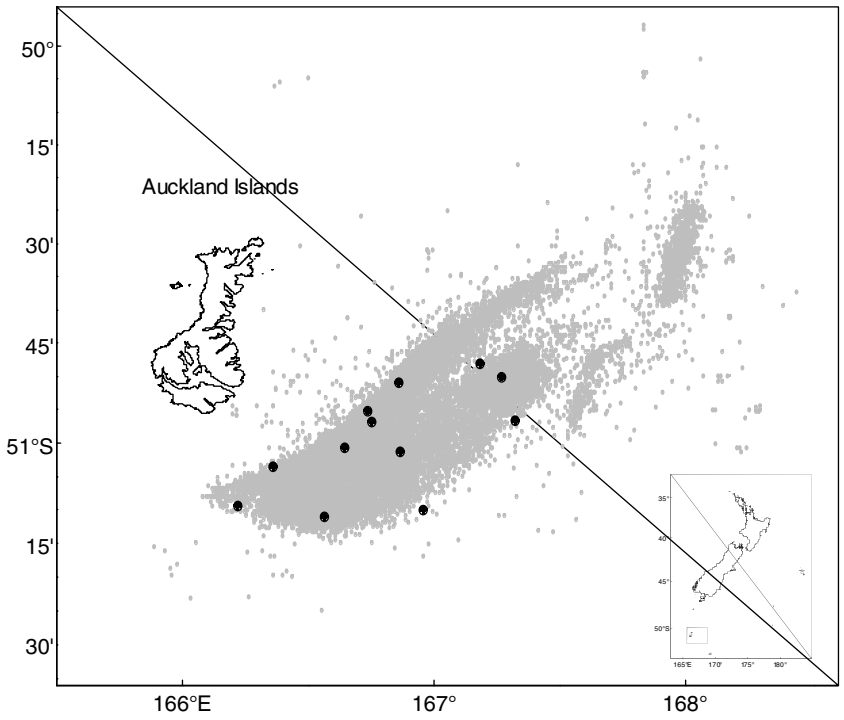

Fig. 1. Location of *Metanephrops challengeri* fishery in the Auckland Islands area (grey dots represent midpoints of commercial tows), and survey trawl stations from 2008 (black dots). Location of Auckland Islands relative to New Zealand shown in inset.

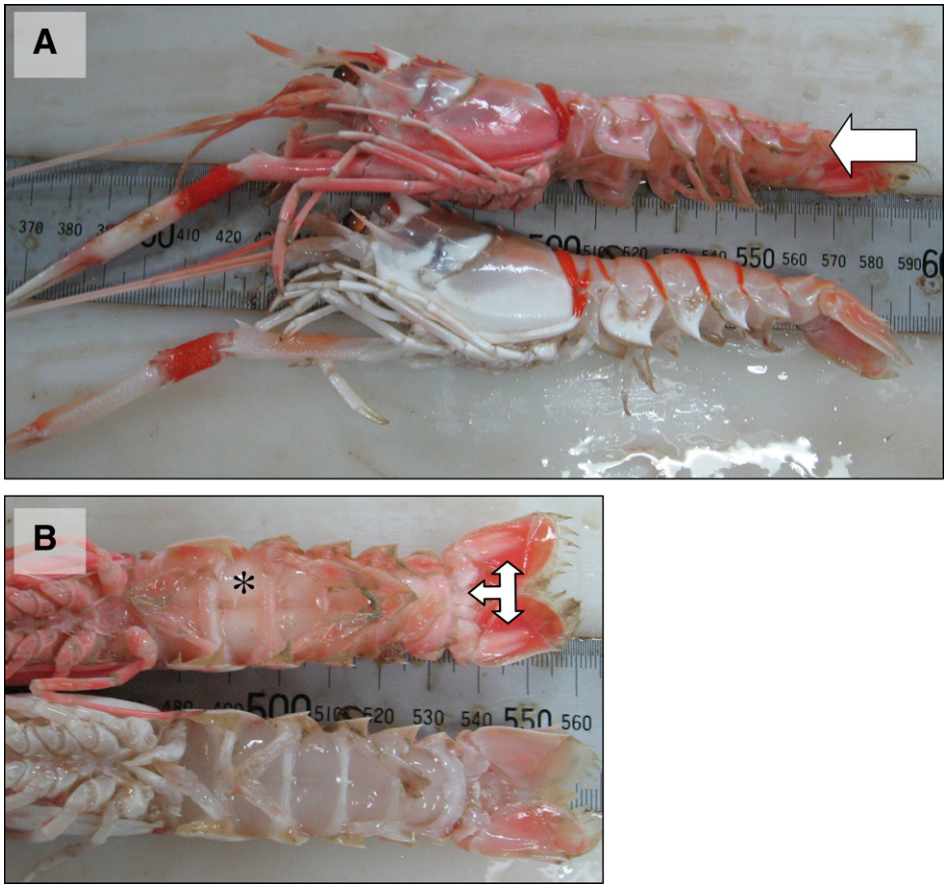

Fig. 2. *Myospora metanephrops* infected and non-infected *Metanephrops challengeri*. (A) Infected lobster (arrow) appears differentially pigmented with increased opacity in all body sections relative to non-infected lobster. (B) Infection is most apparent in major flexor muscles (asterisk) and telson muscles (arrow) of infected lobster compared with non-infected lobster.

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using standard protocols (see Stentiford et al., 2007). Sections were cut at a thickness of 3–5 μm on a rotary microtome and were mounted onto glass slides before staining with H&E. Stained sections were examined by light microscopy (Nikon Eclipse E800) and digital images were taken using the Lucia™ Screen Measurement System (Nikon).

2.3. Electron microscopy

For electron microscopy, small pieces (~2 mm^3) of skeletal muscle displaying a milky white appearance and opacity were fixed in a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), with 1.75% sodium chloride for 2 h at room temperature. Fixed tissue samples were transported to the Cefas laboratory, Weymouth, UK, prior to rinsing in 0.1 M sodium cacodylate buffer with 1.75% sodium chloride (pH 7.4) and post-fixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4 °C. Specimens were washed in three changes of 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series; they were then embedded in Agar 100 epoxy (Agar Scientific, Agar 100 pre-mix kit medium) and polymerised overnight at 60 °C. Semi-thin (1–2 μm) sections were stained with Toluidine Blue for viewing with a light microscope to identify suitable target areas. Ultrathin sections (70–90 nm) of target areas were mounted on uncoated copper grids and stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

2.4. DNA extraction

Abdominal muscle samples were removed from lobsters displaying the external appearance described above (Fig. 2) and preserved in 95% ethanol. Total DNA was isolated from three lobsters infected with the microsporidian using a modified protocol consisting of glass beads to rupture the parasite cysts, and recovery of DNA using a Tissue Kit (Qiagen Inc., Valencia, CA, USA). Briefly, samples of the ethanol-preserved muscle tissues (~50–100 mg) were placed in sterile deionized water to facilitate the removal of residual ethanol. Tissue samples were minced with a sterile scalpel blade and added to 300 μl buffer AE (Qiagen) and 0.3 g of 0.1 mm glass beads prior to disruption using a Fast Prep FP120 homogenizer (Thermo Savant) for three rounds of 30 s at full speed. The tubes were centrifuged at 12,000*g* to sediment the glass beads and cell debris, and DNA was recovered from the supernatants using a Tissue Kit (Qiagen) following the manufacturer's recommendations. The DNA was quantified using a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) and stored at −20 °C.

2.5. PCR amplifications

A fragment of the ssrRNA gene from the microsporidian infecting the lobsters was amplified in duplicate from the three genomic DNA samples using previously described primers (Tourtip et al., 2009): forward primer MF1 5′ CCGGAGAGGGAGCCTGAGA 3′ and reverse primer MR1 5′ GACGGGCGGTGTGTACAAA 3′. Amplification reactions were carried out in a DNA Engine thermocycler (MJ Research Inc., Waltham, MA, USA) and contained 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM each of dNTP, 2.5 μM of each primer, 1 unit of Taq polymerase (Applied Biosystems, Foster City, CA, USA), and 20–50 ng genomic DNA in a total volume of 20 μl. Thermocycling conditions were as follows: an initial denaturation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, primer annealing at 52 °C for 1 min, chain extension at 72 °C for 2 min, with a final extension at 72 °C for 5 min. Amplified products (5 μl aliquots) were visualized by agarose gel electrophoresis (1.5% w/v), stained with ethidium bromide and viewed under a UV light source. Amplification products selected for sequence analysis (approximately 1,000 bp and 1,200 bp) were excised from the agarose gels using a sterile scalpel blade, combined for each fragment size and sample, and purified using a Qiaquick Gel Extraction Kit (Qiagen).

2.6. Cloning and sequencing

Cloning and sequencing were performed according to methods described previously (Small et al., 2007). Briefly, purified PCR products were cloned into the plasmid pCR®4-TOPO® (Invitrogen, Carlsbad, CA, USA) and transformed into competent *Escherichia coli* using a TOPO TA Cloning® Kit (Invitrogen) following the manufacturer's protocols. Transformed bacterial colonies were screened for inserts using a PCR-based screening reaction using M13 primers. Aliquots of 5 μl of all PCR products were analysed on an agarose gel as described above and products from clones containing the correct insert size were treated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) (Amersham Biosciences, Piscataway, NJ, USA) prior to sequencing. PCR fragments were bi-directionally sequenced using the Big Dye Terminator kit (Applied Biosystems) with M13 sequencing primers and one-quarter of the recommended concentration of Big Dye. Aliquots of 10 μL of each sequencing reaction product were electrophoretically separated on an ABI 3100 Genetic Analyzer (Applied Biosystems). Three clones were sequenced for each sample and fragment size.

2.7. Sequence analysis

Sequences were imported into Sequencher 4.5 (Gene Codes Corporation, Ann Arbor, MI, USA) for trimming of vector and primer sequences. Consensus sequences were subjected to Basic Local Alignment Search Tool (BLAST) searches of the National Center for Biotechnology (NCBI) database (http://www.ncbi.nlm.nih.gov/). Taxa from the microsporidian small subunit BLAST analysis with the highest similarity scores were noted and sequences for these, as well as 63 other microsporidian taxa and two outgroup taxa (fungi) were downloaded from GenBank for phylogenetic comparison (see Table 1). Sequences were aligned first using the CLUSTAL-W program in the MacVector sequence analysis package (MacVector Inc., Cary, NC, USA) using default gap settings for multiple and pairwise alignment. The MacVector-generated alignment was then imported into MAFFT (Katoh et al., 2002, 2005) in order to determine and apply an alternative/better algorithm for the alignment. The L-INS-i algorithm was applied and a new alignment was generated. This alignment was imported into SEAVIEW (Galtier et al., 1996) so that individual regions of the alignment could be subjected to further alignment methods. A total of seven local alignments were performed using the MUSCLE algorithm. The full alignment was 1,472 bases in length. Maximum parsimony analysis was performed using Paup v.4.0b10. Gaps in the alignment were treated as informative. Five hundred bootstrap replicates were performed with 100 random additions. Analyses using the fully aligned dataset, as well as with the exclusion of parts of the dataset with more ambiguous alignment results (six regions, totalling 565 characters) were performed.

3. Results

3.1. Field observations

Following trawl capture, affected lobsters were clearly distinguishable from their non-affected counterparts due to an apparent

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Table 1
List of taxa included in the phylogenetic analysis. *Basidobolus ranarum* and *Conidiobolus coronatus* are fungi used as outgroup taxa for the analysis.

GenBank No.	Pathogen name	Host name	Environment
AY090056	*Ambylospora canadensis*	*Aedes canadensis*	Freshwater
AY090058	*Ambylospora cinerei*	*Acanthacyclops vernalis*	Freshwater
L15741	*Ameson michaelis*	*Callinectes sapidus*	Marine
AF104087	*Bacillidium* sp.	*Lumbriculus* sp.	Freshwater
AJ581995	*Bacillidium vesiculoformis*	*Nais simplex*	Freshwater
AY973624	*Caudospora simulii*	*Prosimulium mixtum*	Freshwater
AF027683	*Culicosporella lunata*	*Culex pilosus*	Freshwater
AJ438957	*Dictyocoela berillonum*	*Echinogammarus berilloni*	Freshwater
AF397404	*Dictyocoela deubenum*	*Gammarus deubeni*	Freshwater
AJ438955	*Dictyocoela meulleri*	*Gammarus deubeni celticus*	Freshwater
L39107	*Encephalitizoon cuniculi*	*Homo sapiens*	Human
L39108	*Encephalitzoon hellem*	*Homo sapiens*	Human
AF056014	*Glugea americanus*	*Lophius americanus*	Marine
AF056016	*Glugea anomala*	*Gasterosteus aculeatus*	Marine
GAU15987	*Glugea atherniae*	*Atherina boyeri*	Marine
AY090038	*Glugea* sp.	*Epinephelus awoara*	Marine
AF056015	*Glugea stephani*	*Pleuronectes americanus*	Marine
AY090067	*Hazardia milleri*	*Culex quinquefasciatus*	Freshwater
AF387331	*Heterosporis anguillarum*	*Anguilla japonica*	Freshwater
AF356225	*Heterosporis sp.*	*Perca flavescens*	Freshwater
AF356222	*Kabatana takedai*	*Oncorhynchus masu*	Marine
EU709818	*Liebermannia sp. Laprid*	*Covasacris pallidinota*	Terrestrial
AJ252951	*Loma acerinae*	*Gymnocephalus cernuus*	Freshwater
AF320310	*Loma embioticia*	*Cymatogaster aggregata*	Marine
AF104081	*Loma* sp.	*Encelyopus cimbrius*	Marine
AJ438962	*Microsporidium* sp. JES2002G	*Gammarus chevreuxi*	Marine
AY033054	*Microgemma caulleryi*	*Hyperoplus lanceolatus*	Marine
AJ252952	*Microgemma* sp.	*Taurulus bubalis*	Marine
AF151529	*Microsporidium prosopium*	*Prosopium williamsoni*	Freshwater
AY530532	*Myosporidium merluccius*	*Merluccius* sp.	Freshwater
AY958070	*Nedelospora canceri*	*Cancer magister*	Marine
DQ073396	*Nosema antherae*	*Antheraea pernyi*	Terrestrial
L39111	*Nosema bombycis*	*Bombyx mori*	Terrestrial
AY960987	*Nosema plutellae*	*Plutella xylostella*	Terrestrial
AY211392	*Nosema spodopterae*	*Helicoverpa armigera*	Terrestrial
AF356223	*Ovipleistophora mirandellae*	*Gymnocephalus cernuus*	Freshwater
AY305325	*Paranosema grylli*	*Gryllus bimaculatus*	Terrestrial
AF027682	*Parathelohania anophelis*	*Anopheles quadrimaculatus*	Freshwater
AY090065	*Parathelohania obesa*	*Anopheles crucians*	Freshwater
AJ252959	*Perezia nelsoni*	*Litopenaeus setiferus*	Marine
AJ252953	*Pleistophora hippoglossoideos*	*Hippoglossoides platessoides*	Marine
AF104085	*Pleistophora mirandellae*	*Rutilus rutilus*	Freshwater
AJ438985	*Pleistophora mulleri*	*Danio rerio*	Freshwater
AJ252955	*Pleistophora ovariae*	*Notemigonus crysoleucas*	Freshwater
AJ252956	*Pleistophora typicalis*	*Myoxocephalus scorpius*	Marine
EU534408	*Potasphora morhaphis*	*Potamorhaphis guianensis*	Freshwater
AF322654	*Pseudoloma neurophilia*	*Danio rerio*	Freshwater
AJ749819	*Schroedera airthreyi*	*Plumatella* sp.	Freshwater
L39113	*Septata instestinalis*	*Homo sapiens*	Terrestrial
AF056013	*Spraguea lophii*	*Lophius americanus*	Marine
AF364303	*Tetramicra brevifilum*	*Scophthalmus maximus*	Marine
DQ417114	*Thelohania butleri*	*Pandalus jordani*	Marine
AM261747	*Thelohania contejeani*	*Astacus fluviatilis*	Freshwater
AY183664	*Thelohania montirivulorum*	*Cherax destructor*	Freshwater
AF294781	*Thelohania parastaci*	*Cherax destructor*	Freshwater
AF031538	*Thelohania solenopsae*	*Solenopsis invicta*	Terrestrial
AJ002605	*Trachipleistophora hominis*	*Homo sapiens*	Human
DQ403816	*Trachipleistophora sp. AH2006a*	*Hemicentetes semispinosus*	Terrestrial
AF033315	*Vairimorpha lymantriae*	*Lymantria dispar*	Terrestrial
DQ996241	*Vairimorpha necatrix*	*Pseudaletia unipuncta*	Terrestrial
AJ252961	*Vavraia culicis*	*Aedes albopictus*	Terrestrial
X74112	*Vavraia oncoperae*	*Wiseana cervinata*	Terrestrial
L39112	*Vittaforma corneae*	*Homo sapiens*	Terrestrial
AY635841	*Basidiobolus ranarum*	Outgroup	Terrestrial
AF296753	*Conidiobolus coronatus*	Outgroup	Terrestrial

alteration in the normal pigmentation and translucency of the carapace of the cephalothorax, abdomen and limbs. Normal lobsters displayed a translucent carapace with a bright white pigmentation along the anterior-ventral cephalothorax and a distinctive orange-red posterior border to the cephalothorax and abdominal segments. In contrast, affected lobsters displayed an apparent loss of the orange-red posterior border to the thoracic segments with hyperpigmented uropods compared with unaffected lobsters. In addition, the cephalothorax and walking limbs of affected lobsters displayed a distinctive pink colouration compared with that found in uninfected animals. Ventrally, the large abdominal flexor muscles of affected lobsters were clearly visible through the arthrodial

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membranes, while muscles extending into the uropods were imparted with a distinctive white and opaque appearance which clearly contrasted the hyperpigmented telson carapace (Fig. 2). Affected lobsters were lethargic and did not display the typical tail flipping behaviour observed in unaffected specimens.

3.2. Histopathology

Affected lobsters had a wide-spread infection of the skeletal and heart musculature in addition to the longitudinal and circular muscles surrounding the hepatopancreatic tubules and the mid- and hind-gut. In heavily infected lobsters, muscle fibres and constituent myofibrils of the major abdominal flexor muscles of the abdomen were largely replaced by xenomas filled with spores of the parasite. Large oval to elliptical xenomas formed adjacent to apparently unaffected myofibrils (Fig. 3A), with masses of apparently unicellular, basophilic and refractile parasite stages (Fig. 3B). Xenomas were widely distributed throughout the muscle sections and in some cases replaced a large proportion of the normal musculature. Necrosis and rupture of heavily infected muscle fibres was accompanied by infiltration of host haemocytes, leading to large areas where normal fibrillar structure was replaced with necrotic cell debris and haemocytic aggregates (Fig. 3C). Similar pathological manifestations were observed in skeletal muscles of the cephalothorax and limbs (not shown). Melanisation of haemocyte aggregates in nodules was not observed. Parasites were observed in the circular and longitudinal muscles of the gut (Fig. 3D).

Despite the pronounced infection of the skeletal muscles, the most severely infected organ appeared to be the cardiac muscle. The myofibres of the myocardium were massively hypertrophic with the sarcoplasm virtually replaced with masses of parasite cells (Fig. 3E). The expansion of the infected muscle fibres led to an apparent occlusion of haemal spaces (c.f. thrombosis) within the myocardium. Where rupture of the myofibres was apparent, pronounced infiltration of host haemocytes led to the appearance of large haemocytic granulomas containing liberated spore stages of the parasite at their core. Once again, melanisation of these aggregates was not observed (Fig. 3F). In general, the spongy tissue

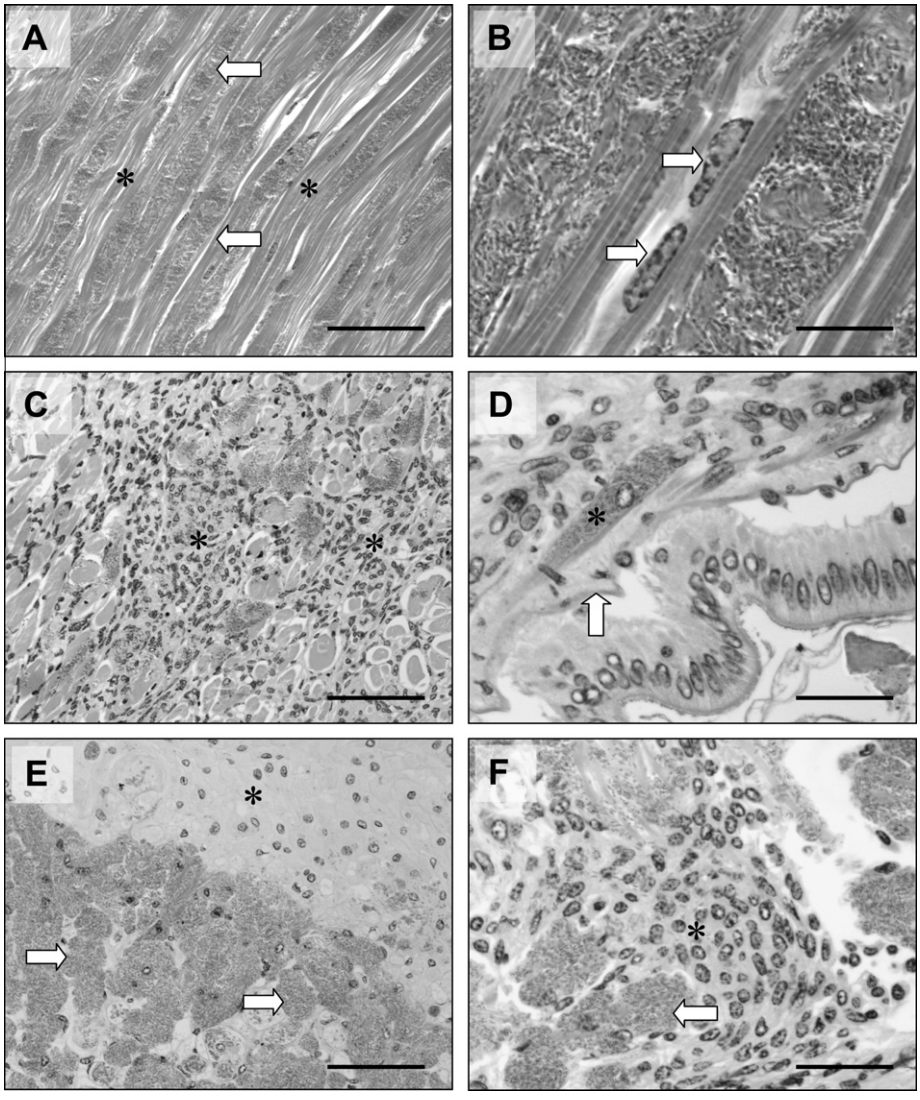

Fig. 3. Histopathology of *Myospora metanephrops* infection of *Metanephrops challengeri*. (A) Cysts containing microsporidian parasites (arrows) apparently displacing or replacing normal myofibrils (asterisks) within the abdominal musculature. Scale bar = 100 μm. (B) Higher power image showing parasitic cysts containing refractile parasite stages between myofibrils. Host muscle nuclei shown (arrows). Scale bar = 20 μm. (C) Infiltration of infected myofibres by host haemocytes (asterisk) in the claw musculature. Scale bar = 100 μm. (D) Infected gut-associated circular muscle cell (asterisk) adjacent to the basement membrane of the midgut (arrow). Scale bar = 50 μm. (E) Heavily infected myofibres within myocardium of heart (arrows). Pericardium appears largely unaffected (asterisk). Scale bar = 100 μm. (F). Haemocyte aggregation with apparently internalised parasite stages (asterisk) in the heart. Heavily infected myofibres adjacent (arrow). Scale bar = 100 μm.

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comprising the pericardium of the heart was not infected (see Fig. 3E).

3.3. Ultrastructure

Ultrastructurally, infected muscle fibres revealed multiple stages of a microsporidian parasite in different stages of development. Merogony involved the development of the earliest observed stages, diplokaryotic meronts and quadri-nucleate (two diplokaryotic nuclear sets) to octo-nucleate (four diplokaryotic nuclear sets) plasmodia. All developmental stages were in direct contact with the cytoplasm of host muscle cells, a feature of the Nosematidae. The smallest diplokaryotic meronts contained two large and closely apposed nuclei surrounded by abundant endoplasmic reticulum and other cytoplasmic membranes. Apparent myelin bodies were also present within the cytoplasm, with all components bound by a simple plasmalemma (Fig. 4A). A centriolar plaque, with associated microtubules, appeared to form simultaneously at the periphery of each paired nucleus within the diplokaryotic meront, with the plaque forming an invagination that contained the microtubules prior to nuclear division (Fig. 4B and inset). Division of the diplokaryotic meront resulted first in a quadri-nucleate meront (two diplokaryotic nuclear sets) (Fig. 4C) and finally an octo-nucleate meront (four diplokaryotic nuclear sets) reminiscent of the genus *Thelohania* (Fig. 4D). Each of these stages retained a simple bounding plasmalemma, although the extent of membrane development within the plasmodial cytoplasm was reduced. A sporophorous envelope or vacuole (or any other form of interfacial membrane) was not present. Prior to plasmotomy of the octo-nucleate meront, large vacuoles were apparent within the meront cytoplasm, particularly at the periphery of the diplokaryotic nuclei (Fig. 4D). Plasmotomy involved an apparent cytokinesis of individual diplokaryotic nuclear sets within the octo-nucleate meront plasmodium (Fig. 4E), resulting in the production of short chains of four closely apposed early sporonts, each containing a diplokaryotic nuclear set. Early sporonts remained closely apposed in these chains prior to their eventual isolation (Fig. 4F).

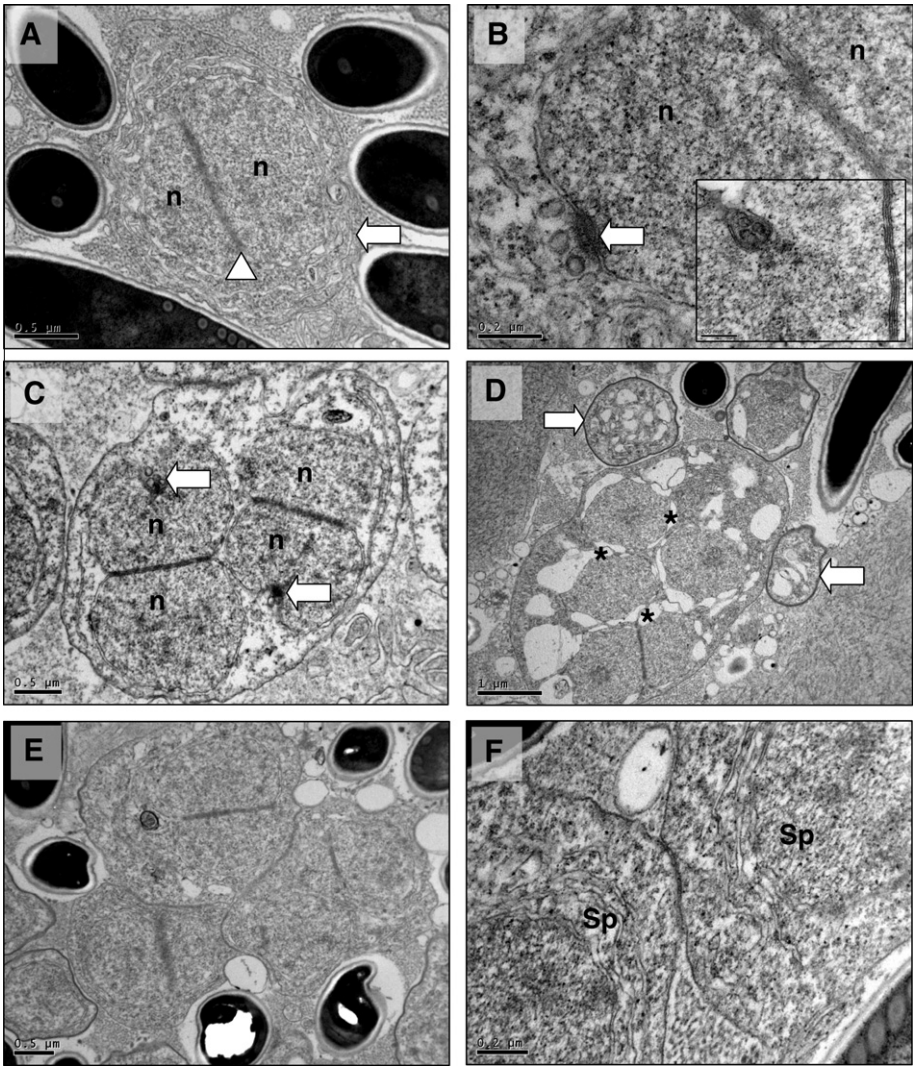

Fig. 4. *Myospora metanephrops* merogony. (A) Earliest stage bi-nucleate (diplokaryotic) meront (arrow). Closely apposed nuclei (n) are identified in this and subsequent stages by a separating line produced by the adjacent nuclear membranes (arrow head). Scale bar = 0.5 µm. (B) Early spindle apparatus formation within a diplokaryotic meront (arrow) and invagination of nuclear membrane to contain the spindle apparatus (inset). Scale bar = 0.2 m. (C) Quadri-nucleate meront with two diplokaryotic nuclear sets (n) and synchronous spindle apparatus formation (arrows). Scale bar = 0.5 µm. (D) Likely octo-nucleate meront with four diplokaryotic nuclear sets (not all visible). Vacuolation of nuclear membranes is apparent (asterisks). Closely apposed sporonts are visible at the periphery of the meront plasmodium (note thicker wall sporonts) (arrows). Scale bar = 1 µm. (E) Apparent plasmotomy of an octo-nucleate meront plasmodium into four diplokaryotic sporonts. Scale bar = 0.5 µm. (F) Detail of adjacent early sporonts formed following meront plasmotomy. Two closely apposing early sporonts (Sp) prior to thickening of wall. Scale bar = 0.2 µm.

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Sporogony involved development of the early diplokaryotic sporont into a quadri-nucleate sporont (two diplokaryotic nuclear sets) that underwent fission to form two diplokaryotic sporoblasts. Sporoblasts developed directly into individual diplokaryotic spores. Once again, all developmental stages occurred in direct contact with the cytoplasm of the host muscle cell. Diplokaryotic sporonts were initially bound by a simple plasmalemma that appeared to be progressively reinforced by the deposition of an electron-dense substance to form a distinct sporont wall (Fig. 5A). At this stage, diplokaryotic sporonts could be distinguished from early sporonts and all meront stages, which were bound by a less electron-dense plasmalemma (Fig. 5B). The four early sporonts from each meront plasmotomy event appeared to remain closely apposed in a chain formation during development of the sporont wall. Individual sporonts appeared capable of undergoing at least the initial preparations for nuclear division to form the quadri-nucleate sporont (Fig. 5C). Quadri-nucleate sporonts were differentiated from quadri-nucleate meronts by a relatively electron-dense cell wall (Fig. 5D). At this stage, the two diplokaryotic nuclear sets located at the poles of the sporont presumably undergo cell fission to form two diplokaryotic sporoblasts.

Sporoblasts, the products of sporont fission, occurred individually and in their early stages were difficult to distinguish from diplokaryotic sporonts (Fig. 6A). However, their development was accompanied by distinct crenulation of the sporoblast wall and a lengthening of the cell (Fig. 6B). Of the organelles observed in mature spores, formation of polar filament precursors within the sporoblasts appeared to occur first (Fig. 6C). These precursors then aligned along the inner surface of the sporoblast cell wall to form linear arrays that culminated in a precursor to the anchoring disk (Fig. 6D). Further development to the mature spore involved the formation of an increasingly electron-dense cytoplasm and a loss in crenulation of the sporoblast wall. Mature spores were rod-shaped, diplokaryotic and contained a distinct anchoring disk at the apical end of a coiled isofilar polar filament that aligned in single ranks of 11 coils along the inner membrane of the spore wall. An indistinct posterior vacuole was observed at the distal end of the spore, a region that was slightly constricted relative to the apical region. The spore was surrounded by a thick electron-lucent endospore overlaid with a thinner electron-dense exospore. No projections were observed on the spore surface (Fig. 6E and inset). A lamellar polaroplast was found at the apical end of the spore. This was composed of ordered concentric membranes that surrounded the polar filament (Fig. 6F). Mature spores fixed for transmission electron microscopy measured approximately 3.4×0.9 μm in size. A schematic representation of the life cycle as deduced from ultrastructural observations is given in Fig. 7.

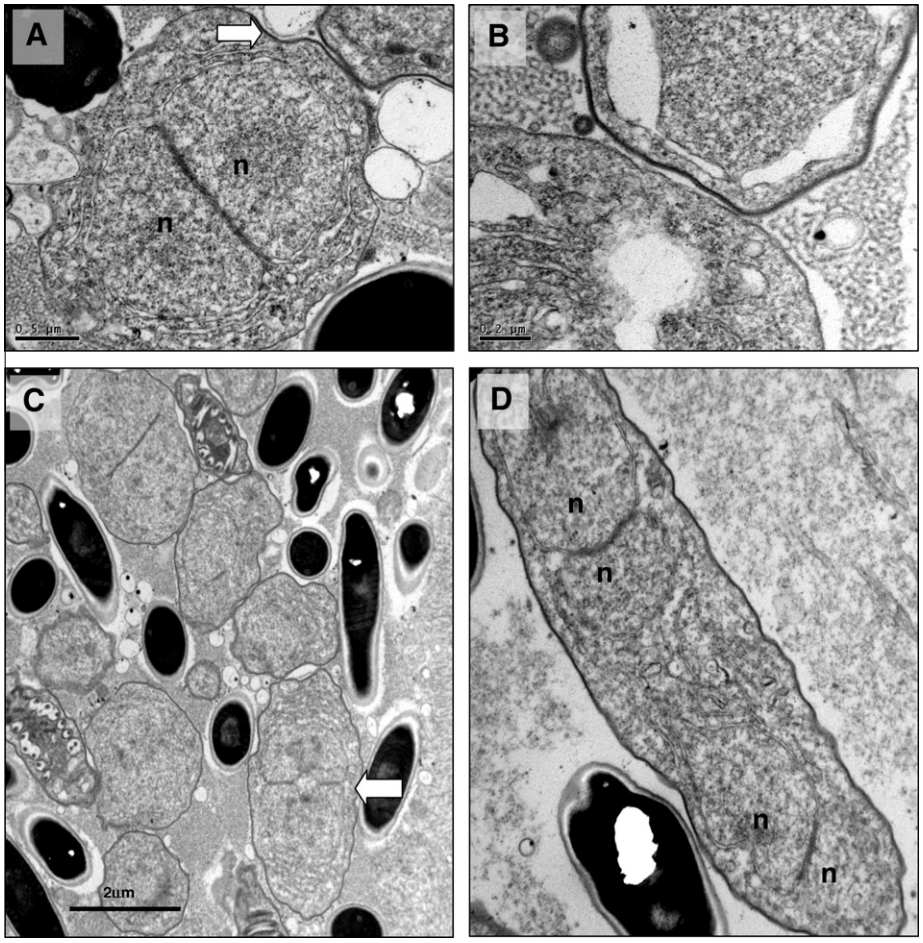

Fig. 5. *Myospora metanephrops* early sporogony. (A) Closely apposed early diplokaryotic (n) sporonts following plasmotomy of meront plasmodium. The wall of the early sporont is beginning to thicken in some regions (arrow). Scale bar = 0.5 μm. (B) Closely apposed early sporont (thin walled) and late sporont (thick walled) Scale bar = 0.2 μm. (C) Chain of four diplokaryotic sporonts following plasmotomy of octo-nucleate meront plasmodium. The cell walls of the sporonts have thickened and at least one (arrow) appears to be undergoing nuclear separation prior to karyotomy to form the quadri-nucleate sporont. Scale bar = 2 μm. (D) Quadri-nucleate sporont with two diplokaryotic (n) nuclear sets, prior to binary fission to produce two diplokaryotic sporoblasts. Scale bar = 0.2 μm.

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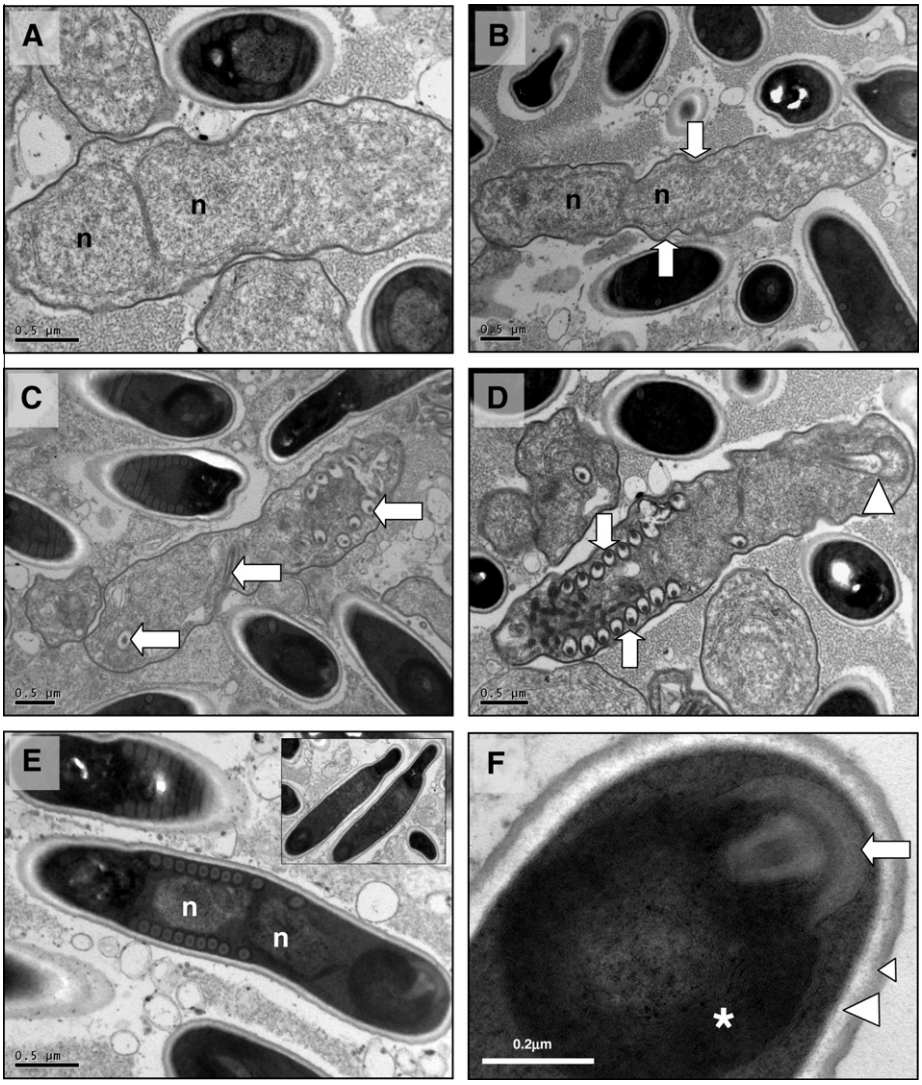

Fig. 6. *Myospora metanephrops* late sporogony. (A) Early diplokaryotic (n) sporoblast (product of fission of quadri-nucleate sporont) showing initiation of elongated form. Scale bar = 0.5 µm. (B) Early diplokaryotic (n) sporoblast displaying initial signs of crenulation of the wall (arrows). Scale bar = 0.5 µm. (C) Elongated diplokaryotic sporoblast with crenulated wall and development of polar filament precursors (arrows). Scale bar = 0.5 µm. (D) Developing diplokaryotic (n) sporoblast with polar filament formed and linearly aligned along inner wall (arrow) and terminating in an apical anchoring disk (arrowhead). (E) Mature diplokaryotic (n) spore with characteristic elongated form, apical anchoring disk at the terminal end of a polar filament, arranged in 11 coils in linear ranks along the inner wall of the spore. A slightly constricted region is present at the distal end of mature spores (inset). Scale bar = 0.2 µm. (F) Apical region of mature spore showing anchoring disk (arrow), lamellar polaroplast (asterisk), electron-lucent endospore (large arrowhead) and electron-dense exospore (small arrowhead). Scale bar = 0.2 µm.

3.4. Molecular phylogeny

The BLAST results for the larger ssrDNA amplification products indicated that the sequences were of host origin as they demonstrated a 99% identity to ssrDNA sequences from the type species of the sister genera to *Metanephrops*, *Nephrops norvegicus* Linnaeus, 1758. These larger products served as positive controls for PCR amplification. The BLAST results for the smaller ssrDNA amplification products confirmed our assumption that the parasite found in *M. challengeri* was a microsporidian, with *T. butleri*, a microsporidian from the smooth pink shrimp, *P. jordani*, demonstrating the highest score in the BLAST search of GenBank. The microsporidian from *M. challengeri* and *T. butleri* had a maximum identity of 86% while the top 10 taxa returned from the BLAST search had a maximum identity ranging from 85% to 89%. The ssrDNA sequences of the parasite from *M. challengeri* had low intra-specific variation (0.00–0.003 uncorrected "*p*" distance). The microsporidian ssrDNA sequences obtained from infected *M. challengeri* (NZ4C/D/E, NZ5B/C/D, and NZ6A/B/C) were deposited in GenBank with accession numbers to be assigned.

Two sets of phylogenies were derived from the molecular data. Phylogenies resulting from use of the whole data set versus a data set trimmed of regions of questionable alignment were similar in topology. For parsimony analysis only the reduced dataset was used for further analysis (Fig. 8). The resulting phylogeny was similar to that reported by Vossbrinck and Debrunner-Vossbrinck (2005). In general, one large clade contained primarily microsporidians infecting fish and crustaceans (99% bootstrap support). Within this clade, however, are included *Trachipleistophora hominis* (infecting humans), *Trachipleistophora* sp. AH2006a, (infecting terrestrial mammals), and *Vavraia culiculis* (infecting mosquitoes) and *Vavraia oncoperae* (infecting moths). In this phylogeny, the type species of the genus *Nosema*, *N. bombycis*, groups with other *Nosema* spp. and is sister to the genus *Vairimorpha* (lacking a sporophorous vacuole). Additionally, species of *Encephalitozoon* (lacking a sporophorous vacuole, infecting humans) are sister to the *Nosema*/*Vairmorpha* clade.

In the analysis using the reduced data set, the microsporidian from *M. challengeri* was sister to a clade containing both *T. butleri* and an unnamed microsporidian (*Microsporidium* sp.) from gamm-

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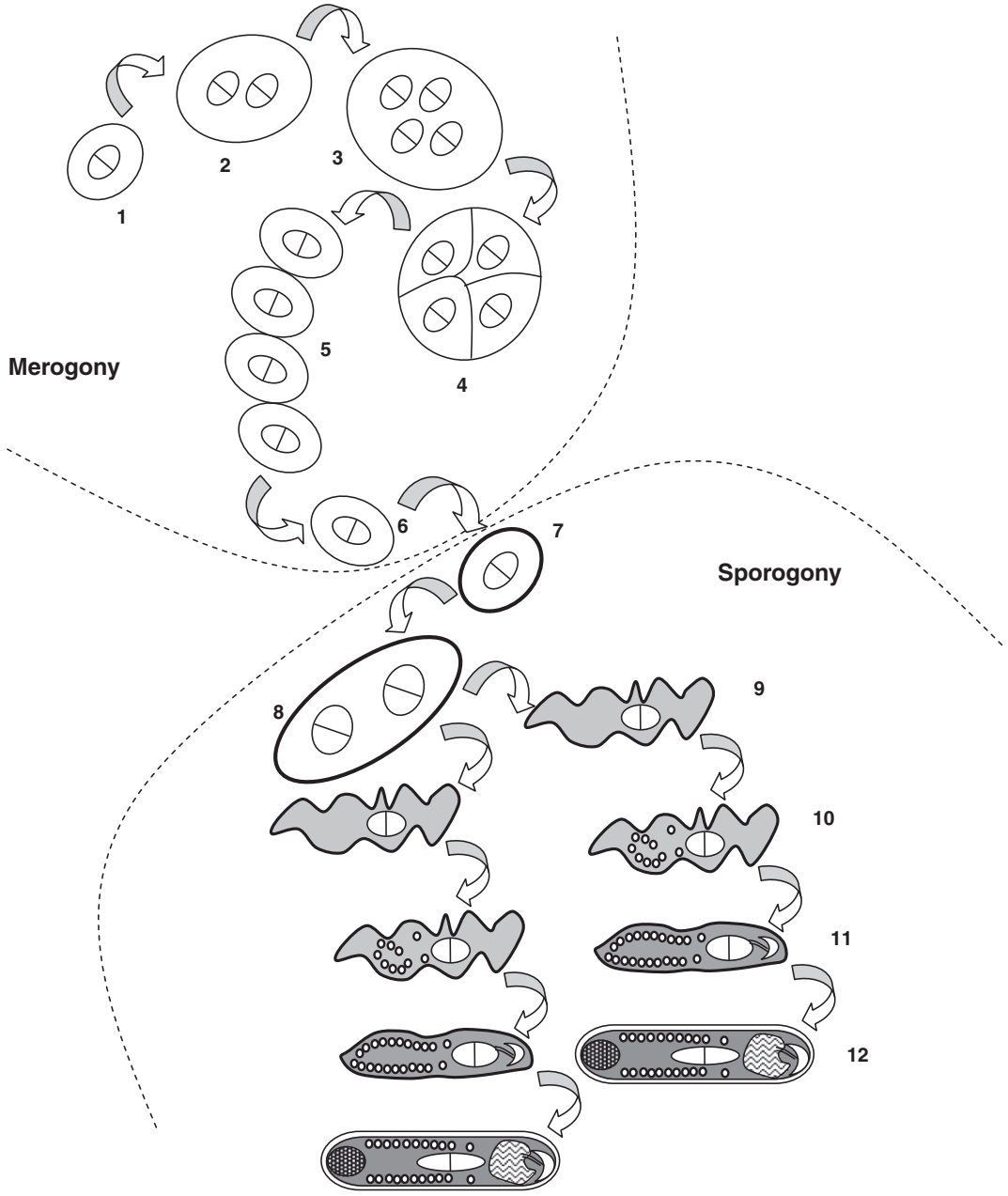

Fig. 7. Proposed lifecycle of *Myospora metanephrops* based upon ultrastructural observations. Key: (1) diplokaryotic meront; (2) quadri-nucleate meront; (3) octo-nucleate meront; (4) plasmotomy of octo-nucleate stage; (5) chain of diplokaryotic pre-sporonts; (6) isolated diplokaryotic sporont prior to wall thickening; (7) diplokaryotic sporont with thickened wall; (8) quadri-nucleate sporont prior to division to two sporoblasts; (9) very early diplokaryotic sporoblast produced from binary fission of quadri-nucleate sporont; (10) early diplokaryotic sporoblast with polar filament precursors; (11) late diplokaryotic sporoblast with polar filaments in linear ranks and appearance of defined anchoring disk; (12) mature diplokaryotic spore with linear ranks of polar filament (11 coils), anchoring disk, lamellar polaroplast, posterior vacuole and well developed exospore and endospore.

arid amphipods (92% bootstrap support). The *M. challengeri* microsporidian was also sister to *T. butleri* using the full data set only (52% support). However, an unnamed amphipod microsporidian was basal to the clade containing *T. butleri* and the microsporidian from *M. challengeri*. In both analyses, *Perezia nelsoni*, *Nadelospora canceri* and *Ameson michaelis*, all parasites of the musculature of marine crustaceans, were basal to the larger clade containing mostly parasites of freshwater and marine fishes and crustaceans.

While our data suggest that at least a new genus should be erected for the parasite from *M. challengeri*, the morphological characters suggest that the parasite should be placed into the Suborder Apansporoblastina (lacking a sporophorous vacuole), while the molecular data indicates placement within the Suborder Pansporoblastina (possessing a sporophorous vacuole). As such, placing a new species, genus or family, based upon this contradictory evidence would lead to further confusion in the current taxonomy of the group. Instead, we propose the erection of a new higher level taxonomy to contain the new genus, based upon habitat, host type and tissue location within the host.

4. Taxonomic summary

Phylum Microsporidia (Balbiani, 1882).

Class Marinosporidia (Vossbrinck and Debrunner-Vossbrinck, 2005). Microsporidian parasites infecting hosts from the marine environment.

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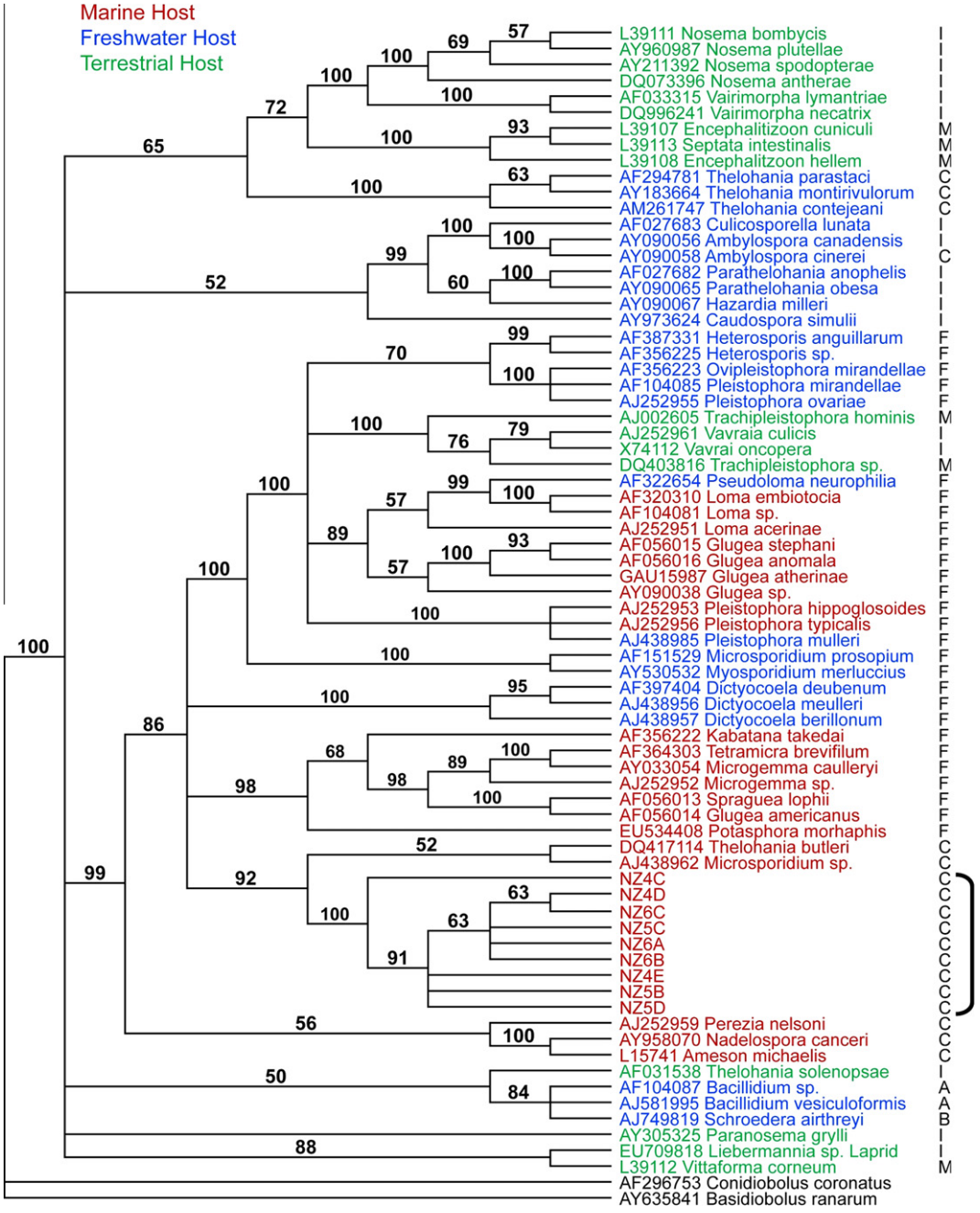

Fig. 8. Phylogenetic analysis including ssrDNA sequences of the microsporidian isolated from *Metanephrops challengeri* (depicted by side bracket: NZ4C/D/E, NZ5B/C/D, and NZ6A/B/C). Maximum parsimony bootstrap support values for each clade are given above the lines. Taxa are colour-coded based on host environment (marine = red, freshwater = blue, terrestrial = green). Host identifiers are listed to the right of each taxa. (I) insect, (M) mammal, (C) crustacean, (F) fish, (B) bryozoan, (A) annelid. AF296753 and AY635841 are fungi used as outgroups for the analysis.

Order Crustaceacida new order. Microsporidian parasites, diplosporoblastic, diplokaryotic, monomorphic, associated with host cell cytoplasm. Spores rod-shaped to cylindrical, approximately 4–6 × 2 μm with 9–11 polar filament coils in a single rank. Infect crustacean hosts from marine and brackish water environments.

Family Myosporidae new family. Microsporidian parasites, diplosporoblastic, diplokaryotic, monomorphic, associated with host cell cytoplasm. Spores rod-shaped to cylindrical, approximately 4–6 × 2 μm with 9–11 polar filament coils in a single rank. Infect the musculature of marine and brackish water crustacean hosts. Type genus *Myospora* n.gen.

Genus Myospora n. gen.

Definition. Spores rod-shaped to cylindrical, approximately 4–6 × 2 μm with 9–11 polar filament coils in a single rank. Merogonic and sporogonic stages occur within the sarcoplasm of muscle cells. Parasite life stages in direct contact with the host cell cytoplasm. Life cycle with diplokaryotic meront stage and tetra- and octo-nucleate meront plasmodia, which form chains of diplokaryotic pre-sporonts by plasmotomy. Diplokaryotic sporonts undergo nuclear division to form tetra-nucleate sporonts that divide to form two diplokaryotic sporoblasts. Sporoblasts develop directly to form diplokaryotic spores. Infecting crustacean hosts from brackish water and marine environments.

Type Species: Myospora metanephrops n. sp.

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Description: Spores elongate, 4.3–6 × 1.7–2.3 μm in size in tissue fixed for electron microscopy. Possesses 9–11 polar filament coils in a single rank. All life stages are diplokaryotic and in direct contact with the muscle cell sarcoplasm.

Type host: Metanephrops challengeri Balss, 1914.

Type locality: Upper shelf slope, between 350 and 550 m depth, from the vicinity of the Auckland Islands, to the south of New Zealand.

Site of infection: Sarcoplasm of muscle cells.

Etymology: The generic name refers to the location of the parasite in the musculature of the host. The specific name refers to its infection in the lobster host *M. challengeri*.

Type material: Syntype slides of histological sections stained with H&E and transmission electron microscopy resin blocks have been deposited in the Registry of Aquatic Pathology (RAP) at the Cefas Weymouth Laboratory, UK. *Myospora metanephrops* ssrDNA sequences have been deposited in GenBank under accession numbers to be assigned.

5. Discussion

This is the first description of a microsporidian parasite from clawed lobsters (Decapoda; Nephropiidae). Microsporidians are known to infect a variety of other crustaceans, but lobsters have not previously been reported as hosts. This is significant because lobsters have few reported parasites whereas other crustaceans, including brachyuran crabs, harbour a plethora of parasitic agents (Shields and Overstreet, 2007; Stentiford, 2008).

Microsporidian parasites lacking a sporophorous vacuole (or parasitophorous vacuole) and known to be diplokaryotic during at least some phases of their life cycle have been described from the following genera: *Ambylospora, Anncallia, Auraspora, Bacillidium, Binucleospora, Burenella, Campanulospora, Caudospora, Cristulospora, Culicospora, Culicosporella, Edhazardia, Evlachovaia, Hazardia, Hirustosporus, Hrabeyia, Ichthyosporidium, Jirovecia, Merocinta, Mrazekia, Nosema, Parathelohania, Parastempellia, Pilosporella, Rectispora, Ringueletium, Spraguea, Vairimorpha, Vittaforma, Weiseria* and *Wittmannia*. Furthermore, those possessing a sporophorous vacuole and diplokaryotic during one or more phases of their life cycle reside within the genera *Culicosporella*, *Heterovesicula, Issia, Octosporea, Pseudopleistophora* and *Scipionospora* (Canning and Vavra, 2000). Several of these are pathogens of insects that undergo at least part of their life cycle in freshwater aquatic habitats while others are pathogens of freshwater oligochaetes. Two genera (*Ichthyosporidium* from the family Nosematidae and *Spraguea* from the family Spraguidae) are parasites of marine teleosts while another three genera have at least part of their life cycles associated with freshwater crustaceans: *Binucleospora* (Family Caudosporidae), *Mrazekia* (Family Mrazekidae) and *Parathelohania* (Family Ambylosporidae).

Therefore, the discovery, described here, of a diplokaryotic microsporidian parasite which develops in direct contact with the host cell cytoplasm of a marine decapod represents a significant difference from known genera with similar morphological features in freshwater and terrestrial hosts. The key finding is that the lobster parasite (*M. metanephrops*) is diplokaryotic throughout its (observed) life cycle. Following the definition of Lom and Dyková (1992), the Family Nosematidae is 'diplokaryotic throughout its life cycle, diplosporoblastic and monomorphic', with 'oval spores with an isofilar polar tube'. The lobster parasite certainly possesses the first three of these characteristics and has an isofilar polar tube although the spores are rod-shaped to cylindrical rather than oval. Within this family, *Nosema* is the dominant genus with a type species from a terrestrial insect (*Bombyx mori*). The genus has been generally considered to contain pathogens of insects (Vossbrinck and Debrunner-Vossbrinck, 2005). Several studies have nonetheless described apparent *Nosema* spp. from crustacean hosts (e.g. Ovcharenko and Wita, 2005) even though these do not generally follow the strict generic descriptor of the production of two spore types (one type apparently for auto-infection and the other for transmission) and the presence of uninucleate stages in at least some parts of the life cycle (see Canning and Vavra, 2000). Since the genus *Nosema* has expanded to contain pathogens that span a wide taxonomic host range (terrestrial insects to marine decapod crustaceans), and may in fact contain organisms with little taxonomic affinity with the type species, it has been suggested that the genus has become a 'holding group' for inadequately described species that require re-classification based upon not only ultrastructural features but also analysis of genomic data (Brown and Adamson, 2006). However, one study of note has demonstrated a very close similarity between ssrRNA gene sequences of *N. granulosus* (from freshwater gammarid crustaceans) and those of the type species *N. bombycis*, even though the former species appeared to display a monomorphic sporulation compared with the dimorphic pattern in the type species (Terry et al., 1999). Strict comparison of non-molecular characters of *M. metanephrops* with those of the insect pathogen *N. bombycis* discriminates it on the grounds that the former lacks dimorphism in the spores, has larger cylindrical spores with a different arrangement of the polar filament, and is present in a marine crustacean host. These morphological characters would not place *M. metanephrops* in *Nosema*.

Other genera within the Nosematidae have largely been grouped based upon morphological characters and include *Hirsutosporus* (Batson, 1983), *Ichthyosporidium* (Casal and Azvedo, 1995) and *Wittmannia* (Czaker, 1997). Each shares the basic feature of the Nosematidae: diplokaryotic stages throughout most stages of merogony and sporogony (Canning and Vavra, 2000). *Ichthyosporidium* (type species *Ichthyosporidium giganteum*) is a xenoma-forming parasite of marine teleosts. While diplokaryotic stages persist through merogony and sporogony, it differs from *M. metanephrops* by its larger spore shape and size (with multiple ranks of 32 polar filament coils) and its occurrence in marine teleost hosts. *Hirsutosporus* (type species *Hirsutosporus austrosimulii*) is a diplokaryotic pathogen of freshwater aquatic insect larvae of the genus *Austrosimulium*. While diplokaryotic stages persist through early development of the parasite, it differs from *M. metanephrops* by its larger, unikaryotic spore (with single and disordered ranks of 18–20 polar filament coils and the appearance of surface 'tufts' emerging from the exospore), and its presence in freshwater aquatic insect larvae. Finally *Wittmannia* (type species *Wittmannia antarctica*) is a hyperparasite in the dicyemidan parasite of squid, *Parelodone turqueti*. The merogonic stages are unikaryotic and diplokaryotic, and give rise to diplokaryotic sporonts that divide to produce two sporoblasts. The spores are diplokaryotic, 4.3–6 × 1.7–2.3 μm in size and possess nine to 11 polar filament coils in a single rank. Spores (and earlier developmental stages) possess a distinctive myelinosome that locates to the region of the posterior vacuole. While *Wittmannia* has some morphological similarity to *M. metanephrops*, it can be differentiated by the presence of unikaryotic meronts, a larger spore size, the characteristic persistence of myelinosomes throughout development, and its presence in a hyperparasite host of a marine cephalopod mollusc. Together, the morphological features of *M. metanephrops*, while appearing in several ways to be similar to those of some members of the family Nosematidae, are not entirely consistent with those of any type species of the representative genera within this family. Based upon morphological criteria alone, these distinctions appear to support the erection of a new genus within the Nosematidae with the lobster parasite as the type species of this new genus.

Vossbrinck and Debrunner-Vossbrinck (2005), using a molecular phylogenetic approach based upon ssrDNA gene analysis, have

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demonstrated that features such as the presence of diplokaryotic status may not necessarily indicate relatedness between sister genera, particularly when they inhabit a wide ecological and host taxonomic range. It is therefore feasible to assess the molecular phylogeny of *M. metanephrops* in relation to existing genera for which genetic data are available. In this way, it is possible to compare phylogenetic similarity against relatedness based upon morphology or habitat (e.g. terrestrial, freshwater). In this study, the ssrDNA sequence from the lobster microsporidian was compared with ssrDNA sequences of several microsporidian genera that either lack or possess a sporophorous vacuole (or parasitophorous vacuole). Using this approach, *M. metanephrops* displays closest affinity to the microsporidian parasite *T. butleri* from the smooth pink shrimp *P. jordani*. *Thelohania butleri*, considered a close relative to the understudied type species *T. giardi*, has recently been shown (via ssrDNA sequencing) to be distant from other described members of the genus *Thelohania* that are from terrestrial (e.g. ants) and freshwater (crayfish) habitats (Brown and Adamson, 2006). Using purely morphological criteria, the close molecular affinity between *M. metanephrops* and *T. butleri* appears remarkable. However, in ecological terms, both hosts (lobster and shrimp, respectively) are marine decapod crustaceans, and both parasites cause progressive replacement of host musculature with masses of early and late life stages. In the molecular phylogenetic study of Brown and Adamson (2006), *T. butleri* from marine shrimps was most closely aligned with an undescribed microsporidian from *Gammarus* sp., and occurred as a sister clade to other microsporidians from marine shrimps including *P. nelsoni* from penaeid shrimps (Canning et al., 2002) and *A. michaelis* from the blue crab *C. sapidus* (Sprague, 1965, 1970; Overstreet, 1977). Interestingly, both *Perezia* and *Ameson* are also described as pathogens of the musculature of marine decapods (Vivarès and Azevedo, 1988; Canning et al., 2002).

Sprague et al. (1992) defined the Phylum Microsporidia as consisting of two Classes; the Dihaplophasea and the Haplophasea depending on the manner of nuclear splitting during 'haplosis'. Two general types are known, one involving diplokaryotic stages (as seen in the parasite from lobsters in the current study) and the other, apparently haplophasic. This feature has been used to define the two major classes and, as stated above, is based solely upon morphological criteria, identifiable using transmission electron microscopy. In families, genera and species comprising the Class Dihaplophasea, the diplokaryotic phase is initiated by the pairing of gametes, which eventually form the first diplokaryotic meront. Within the Dihaplophasea, there is further division into two orders, the Meiodihaplophasida and the Dissociohaplophasida; the former undergoing haplosis by meiosis and the latter undergoing a simpler form of nuclear dissociation. The Thelohanidae (containing *T. butleri*) and the Perezidae (containing *P. nelsoni* and *A. michaelis*) are classified into the Class Dihaplophasea, Order Meiohaplophasida. In contrast, the Nosematidae, while also residing in the Class Dihaplophasea, falls within the Order Dissociohaplophasida. Using ultrastructural features, *M. metanephrops* would be excluded from the Thelohanidae and Perezidae by the absence of an 'interfacial membrane' in the former. In contrast, the presence of a bi-nucleate (diplokaryotic) meront, sporont and spore and the absence of an 'interfacial membrane' would appear to place *M. metanephrops* into the Superfamily Nosematoidea, and further within the Nosematidae.

Due to the contradictory evidence presented, it is useful to consider other life history and habitat features of the lobster parasite that may assist in its classification. Vossbrinck and Debrunner-Vossbrinck (2005) have suggested that the Class Marinosporidia contains *A. michaelis* (Family Perezidae, Superfamily Thelohanoidea) and, with the exception of the Genus *Icthyosporidium* (Family Ichthyosporidae), no other members of the Superfamily Nosematoidea (i.e. the Families Nosematidae, Caudosporidae, Pseudopleistophoridae and Mrazekidae). In the current study, a similar higher-level classification based upon habitat type from which the host was collected revealed that *M. metanephrops* was a close match (based upon ssrDNA sequence) to *P. nelsoni*, *A. michaelis*, *N. canceri* and *T. butleri*; all morphologically distinct parasites infecting the musculature of marine crustaceans. In addition, ssrDNA sequence phylogeny placed this 'group' distantly from other *Thelohania* spp. infecting freshwater crustaceans. A previous study of ssrDNA sequences of *T. butleri* also placed this species distant to other *Thelohania* spp. infecting freshwater crustaceans and aquatic insects (Brown and Adamson, 2006). In that analysis, *T. butleri* was once again found to group closest to microsporidians infecting the musculature of marine crustacean hosts (including *P. nelsoni* and *A. michaelis*). Taken together, and despite their morphological dissimilarity, this evidence suggests that *T. butleri*, *P. nelsoni*, *N. canceri*, *A. michaelis*, the unassigned '*Microsporidium* sp.' from an amphipod, and now, *M. metanephrops*, are a closely related group. Their close relationship suggested by the molecular phylogeny appears to offer a feasible means for their assemblage under clade III in the Class Marinosporidia as proposed by Vossbrinck and Debrunner-Vossbrinck (2005). Our findings clearly support the work of these latter authors.

Vossbrinck and Debrunner-Vossbrinck (2005) in their analyses of 125 species of Microsporidia from across a wide taxonomic and ecological host range appear to have provided a new impetus for re-classification of existing members of the phylum and a framework for those attempting to classify new species. Using such an approach, molecular taxonomy (e.g. based upon ssrDNA sequences) rather than morphology provides the primary means to classify organisms, with environment type (e.g. marine) and host type (e.g. crustacean) providing variables against which the classification can be tested. In this respect, they have devised three broad classes based upon those pathogens found in marine, freshwater and terrestrial hosts. Vossbrinck and Debrunner-Vossbrinck (2005) discuss this issue by stating that 'unfortunately, the characters which are used to determine the higher levels of classification in the Microsporidia (number of nuclei/cell, presence of a membrane surrounding the parasite (sporophorous vesicle), and type of nuclear division) appear to be characters which change states quickly at the genus, species and population levels'. In the current study, application of this approach to *M. metanephrops* has highlighted the issue by demonstrating a close genomic similarity to *T. butleri*, a morphologically distinct pathogen, but with a similar host ecology and pathogenesis. Additional support is provided by relative genetic similarity to other morphologically distinctive microsporidians (*P. nelsoni, A. michaelis, N. canceri*) causing muscle infections in several other marine decapod crustaceans. It is important to note that utilising purely morphological criteria would have placed these individual pathogens in at least three different existing families.

Another consideration is that while the genera *Myospora*, *Nadelospora*, *Ameson*, and *Perezia* appear to be relatively closely related in molecular terms, there is considerable plasticity in their morphologies. That is, while there may have been conserved phylogenic sequences between these genera, the crustacean musculature did not impose a limitation on the development of widely divergent morphological characters. Indeed, such morphological variances now appear to be the rule rather than the exception within the Marinosporidia, if not the Microsporidia, as proposed by Vossbrinck and Debrunner-Vossbrinck (2005).

Based upon the ecological classes proposed by Vossbrinck and Debrunner-Vossbrinck (2005), the genus *Myospora* would be the type genus within a new family (Myosporidae). Considering ecology, host type, tissue type and, to a certain extent, molecular phylogeny, we expect that the family Myosporidae would also contain

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the aforementioned *T. butleri*, *P. nelsoni*, *A. michaelis* and *N. canceri* as parasites infecting the musculature of marine crustaceans but refrain from formal designation of new combinations until full ultrastructural and molecular data on these species is forthcoming. Other (novel) muscle-infecting microsporidian genera from marine crustaceans may also be predicted to fall into this family as DNA sequence information becomes available for these pathogens.

The family Myosporidae would also require the erection of a new Order (Crustaceacida) within the Class Marinosporidia (Vossbrinck and Debrunner-Vossbrinck, 2005). The classification supports the viewpoint that host group and habitat are basic indicators of relatedness for most microsporidian genera and higher taxa, and further, provides a framework for the re-classification of other microsporidians that currently reside in distant taxa based purely on morphological grounds. Adherence to guidelines laid down in the International Code for Zoological Nomenclature (ICZN) requires the prescription of morphological characteristics for the proposed new family (Myosporidae), and by extension, the new order (Crustaceacida). At present, these features are essentially extensions from the specific and generic description of *M. metanephrops*. However, due to the suggested plasticity in morphology of even closely related taxa, the familial and ordinal descriptors as proposed here are likely to require further revision as the philosophy concerning the placement of taxa based upon morphological and molecular characteristics is re-assesed. Similar subdivisions of the three class structure proposed by Vossbrinck and Debrunner-Vossbrinck (2005) could be envisaged for other host groups within the marine environment (e.g. fish) and for the other major environment types (terrestrial and freshwater). The new classification provides clear guidance and encourages informed placement of newly discovered organisms into appropriate higher level taxa. Those taxa which cannot be clearly classified into a particular environment/host type will be highlighted by such an approach and may be discussed with regards to evolutionary or host-specific cross-over between habitat types, and the potential for cross-species infectivity or presence of multi-environment and host life cycles.

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References

Baker, M.D., Vossbrinck, C.R., Maddox, J.V., Undeen, A.H., 1994. Phylogenetic relationships among *Variomorpha* and *Nosema* species (Microspora) based on ribosomal RNA sequence data. J. Invertbr. Pathol. 64, 100–106.

Batson, B.S., 1983. A light and electron microscopical study of *Hirsutosporus austrosimulii* gen. n., sp. n., (Microspora: Nosematidae), a parasite of *Austrosimulium* sp. (Diptera: Simuliidae) in New Zealand. Protistologica 19, 263–280.

Brown, A.M.V., Adamson, M.L., 2006. Phylogenetic distance of *Thelohania butleri* Johnston, Vernick, and Sprague, 1978 (Microsporidia; Thelohaniidae), a parasite of the smooth pink shrimp *Pandalus jordani*, from its congeners suggests need for a major revision of the Genus Thelohania Henneguy, 1892. J. Eukaryot. Microbiol. 53, 445–455.

Canning, E.U., Curry, A., Overstreet, R.M., 2002. Ultrastructure of *Tuzetia weidneri* sp. n. (Microspora: Tuzetiidae) in skeletal muscle of *Litopenaeus setiferus* and *Farfantepenaeus aztecus* (Crustacea: Decapoda) and new data on *Perezia nelsoni* (Microsporidia: Pereziidae) in *L. setiferus*. Acta Protozool. 41, 63–77.

Canning, E.U., Vavra, J., 2000. Phylum Microsporida. In: Lee, J.L., Leedale, G.F., Bradbury, P. (Eds.), The Illustrated Guide to the Protozoa, second ed. Society for Protozoologists, Allen Press, USA, pp. 39–126.

Casal, G., Azvedo, C., 1995. New ultrastructural data on the microsporidian *Ichthyosporidium giganteum* infecting the marine teleostan fish *Ctenolabrus rupestris* (L.). J. Fish Dis. 18, 191–194.

Czaker, R., 1997. *Wittmannia Antarctica* N.G., N. Sp., (Nosematidae), a new hyperparasite in the Antarctic dicyemid mesozoan *Kantharella antarctica*. J. Eukaryot. Microbiol. 44, 438–446.

Diarra, K., Toguebaye, B.S., 1996. Ultrastructure of *Nosema stenocrypsis* Diarra & Tougebaye, 1994, a microsporidian parasite of *Stenocrypsis major* (Crustacea, Ostracoda, Cyprididae). Arch. Protistenkd. 146, 363–367.

Edling, T.D., Li, J., Visvesvara, G.S., Vodkin, M.H., McLaughlin, G.L., Katiyar, S.K., 1996. Phylogenetic analysis of beta-tubulin sequences from amitochondrial protozoa. Mol. Phylogenet. Evol. 5, 359–367.

Galtier, N., Gouy, M., Gautier, C., 1996. SEAVIEW and PHYLO_WIN, two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. 12, 543–548.

Hazard, E.I., Oldacre, S.W., 1975. Revision of the Microsporidia (Protozoa) close to Thelohania, with description of one new family, eight new genera and thirteen new species. U.S. Dept. Agric. Tech. Bull. 1530, 104pp.

Henneguy, F., Thélohan, P., 1892. Myxosporidies parasites des muscles chez quelques crustacés décapodes. Ann. Microgr. 4, 617–641.

Iwano, H., Ishihara, R., 1991. Dimporphism of spores of *Nosema* spp. in cultured cell. J. Invertebr. Pathol. 57, 211–219.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucl. Acids Res. 33, 511–518.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucl. Acids Res. 30, 3059–3066.

Keeling, P.J., Doolittle, W.F., 1996. Alpha-tubulin from early-diverging eukaryotic lineages and the evolution of the tubulin family. Mol. Biol. Evol. 13, 1297–1305.

Lom, J., Dyková, I., 1992. Protozoan Parasites of Fishes. Elsevier, Amsterdam, London, New York, Tokyo. pp. 125–157.

Ovcharenko, M., Wita, I., 2005. The ultrastructural study of *Nosema artemiae* (Codreanu, 1957) (Microsporidia: Nosematidae). Acta Protozool. 44, 33–41.

Overstreet, R.M., 1977. Microsporosis of blue crabs. In: Sindermann, C.J., Lightner, D.V. (Eds.), Disease Diagnosis and Control in North American Marine Aquaculture. Elsevier, New York, pp. 200–203.

Reynolds, E.S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17, 208–212.

Small, H.J., Shields, J.D., Hudson, K.L., Reece, K.S., 2007. Molecular detection of the *Hematodinium* sp. infecting the blue crab, *Callinectes sapidus*. J. Shell. Res. 26, 131–139.

Sprague, V., 1965. *Nosema* sp. (Microsporita, Nosematidae) in the musculature of the crab *Callinectes sapidus*. J. Protozool. 12, 66–70.

Sprague, V., 1970. Some protozoan parasites and hyperparasites in marine decapod crustaceans. In: Snieszko, S.F. (Ed.), A Symposium on Diseases of Fish and Shellfishes. Amer. Fish. Soc, Spec. Publ., pp. 416–430.

Sprague, V., 1977. Systematics of the Microsporidia. In: Bulla, L.A., Cheng, T.C. (Eds.), Comparative Pathobiology, vol. 2. Plenum Press, New York and London, pp. 1–463.

Sprague, V., Becnel, J.J., Hazard, E.I., 1992. Taxonomy of the Phylum Microspora. Crit. Rev. Microbiol. 18, 285–395.

Shields, J.D., Overstreet, R.M., 2007. Parasites, symbionts, and diseases. In: Kennedy, V., Cronin, L.E. (Eds.), The Blue Crab *Callinectes sapidus*. University of Maryland Sea Grant College, College Park, Maryland, pp. 299–417.

Stentiford, G.D., Bateman, K.S., Feist, S.W., 2007. *Enterospora canceri* n.gen., n.sp., an intranuclear microsporidian infecting European edible crab (*Cancer pagurus*). Dis. Aquat. Org. 75, 61–72.

Stentiford, G.D., 2008. Diseases of the European edible crab (*Cancer pagurus*): a review. ICES J. Mar. Sci. 65, 1578–1592.

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

Tourtip, S., Wongtripop, S., Sritunyalucksana, K., Stentiford, G.D., Bateman, K.S., Sriurairatana, S., Chayaburakul, K., Chavadej, K., Withyachumnarnkul, B., 2009. *Enterocytozoon hepatopenaei* sp. nov. (Microspora: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): fine structure and phylogenetic relationships. J. Invertebr. Pathol. 102, 21–29.

Vivarès, C.P., Azevedo, C., 1988. Ultrastructural observations of the life cycle stages of *Ameson atlanticum* sp., nov., a microsporidian parasitizing *Cancer pagurus* L.. J. Fish Dis. 11, 379–387.

Vossbrinck, C.R., Debrunner-Vossbrinck, B.A., 2005. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitolog. 52, 131–142.

Weidner, E., 1970. Ultrastructural study of microsporidian development I. *Nosema* sp. Sprague 1965 in *Callinectes sapidus* Rathbun. Z. Zellforsch. 105, 33–54.

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