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Mikrocytos roughleyi taxonomic affiliation leads to the genus *Bonamia* (Haplosporidia)

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ABSTRACT: Microcell-type parasites of oysters are associated with a complex of diseases in different oyster species around the world. The etiological agents are protists of very small size that are very difficult to characterize taxonomically. Associated lesions may vary according to the host species, and their occurrence may be related to variations in tissue structure. Lesion morphology cannot be used to distinguish the different agents involved. Ultrastructural observations on *Mikrocytos roughleyi* revealed similarities with *Bonamia* spp., particularly in regard to the presence of electron-dense haplosporosomes and mitochondria, whose absence from *M. mackini* also indicate that *M. roughleyi* and *M. mackini* are not congeneric. A partial small subunit (ssu) rRNA gene sequence of *M. roughleyi* was determined. This partial sequence, 951 nucleotides in length, has 95.2 and 98.4 % sequence similarities with *B. ostreae* and *B. exitiosus* ssu rDNA sequences, respectively. Polymorphisms among the ssu rDNA sequences of *B. ostreae*, *B. exitiosus* and *M. roughleyi* allowed identification of restriction enzyme digestion patterns diagnostic for each species. Phylogenetic analysis based on the ssu rDNA data suggested that *M. roughleyi* belongs in the phylum Haplosporidia and that it is closely related to *Bonamia* spp. On the basis of ultrastructural and molecular considerations, *M. roughleyi* should be considered a putative member of the genus *Bonamia.*

KEY WORDS: *Mikrocytos roughleyi* · *Saccostrea glomerata* · Bonamiosis · Microcell · Taxonomy · Small subunit rDNA

INTRODUCTION

Among the 10 OIE (Office International des Epizooties: the World Organization for Animal Health)-listed diseases of molluscs, 4 are caused by non-spore-forming organisms given the collective name of 'microcell' (Farley et al. 1988). Of these, 1 disease, bonamiosis, is caused by at least 2 haplosporidian protozoans that infect the haemocytes of flat oysters. *Bonamia ostreae* infects the European flat oyster *Ostrea edulis* in Europe and in North America (Pichot et al. 1980, Bannister & Key 1982, Polanco et al. 1984, Elston et al. 1986, McArdle et al. 1991, Barber & Davis 1994), *B. exitiosus* infects the New Zealand dredge oyster *T. chilensis* (Dinamani et al. 1987, Hine 1991, Hine et al. 2001b), and *Bonamia*-like organisms also infect the flat oysters *O. angasi* in Australia (Hine 1996) and *T. chilensis* in Chile (Kern 1993, Campalans et al. 2000) (present Table 1). A second disease, mikrocytosis, is caused by 2 other 'microcell' protozoans: *Mikrocytos mackini* causes Denman Island disease in the Pacific oysters *Crassostrea gigas* in British Columbia (Quayle 1961,

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Table 1. Comparisons among pathogen species, natural and experimental hosts, gross signs, histological features and parasite location. nd: disease experimental reproduction not done

	Bonamia ostreae	*Bonamia exitiosus*	*Bonamia* sp.	*Mikrocytos roughleyi*	*M. mackini*
Natural hosts	*Ostrea edulis*	*Tiostrea chilensis*	*O. angasi* *T. chilensis*	*Saccostrea glomerata*	*Crassostrea gigas*
Experimental hosts	*O. puelchana* *O. concaphila* *O. angasi* *T. chilensis* *C. rivularis*	nd	nd	nd	*C. virginica* *O. edulis* *O. concaphila*
Gross signs	Gill and mantle perforations or indentations	No sign	No sign	Abscesses and pustules on mantle or muscle	Abscesses and pustules on mantle or muscle
Histological features	Global haemocyte infiltration	Global haemocyte infiltration	Global haemocyte infiltration	Focal haemocyte infiltration	Focal haemocyte infiltration
Location	Haemocytes and cells epithelial	Haemocytes	Haemocytes	Haemocytes	Haemocytes, myocytes, connective cells

Farley et al. 1988), and *M. roughleyi* causes winter mortality in the Sydney rock oyster *Saccostrea glomerata* in SE Australia (Roughley 1926, Farley et al. 1988). Experimentally, *Bonamia* spp. can infect *Ostrea* spp. (Grizel et al. 1983, Le Borgne & Le Pennec 1983, Bougrier et al. 1986, Pascual et al. 1991) and *C. rivularis* (Cochennec et al. 1998). *M. mackini* has been shown to infect *C. gigas*, *C. virginica*, *O. edulis* and *O. concaphila* (Bower et al. 1997) (present Table 1). Therefore, *Bonamia* spp. and *Mikrocytos* spp. cannot be distinguished solely by the hosts which they infect. However, to date the geographic distributions of the 2 *Mikrocytos* species appear to be distinct, with *M. mackini* having been reported only in western Canada and *M. roughleyi* only in eastern Australia.

Several studies have included observations of the ultrastructure of *Bonamia ostreae* (Comps et al. 1980, Pichot et al. 1980, Bréhelin et al. 1982, Grizel et al. 1983, Grizel 1985, Chagot et al. 1992, Montes et al. 1994) and *B. exitiosus* (Dinamani et al. 1987, Hine 1991, Hine & Wesney 1994a,b, Hine et al. 2001b), but there are none on *Bonamia* sp. in *Ostrea angasi* in Australia or *Tiostrea chilensis* in Chile. A recent study on the ssu rDNA gene sequences of *B. ostreae* (Carnegie et al. 2000, Cochennec et al. 2000) and *Bonamia* sp. from New Zealand showed that they are congeneric, and a new species, *B. exitiosus*, was created (Hine et al. 2001b). An unpublished study on the ssu rDNA gene sequences of *Bonamia* sp. from Australia and *B. exitiosus* from New Zealand suggests that these 2 parasites are conspecific, despite showing very different pathology in their respective hosts (R. D. Adlard pers. comm.).

The ultrastructure of *Mikrocytos mackini* confirms that this parasite is different from *Bonamia* spp. because of the lack of organelles in most eukaryotic cells including mitochondria or their equivalents (Farley et al. 1988, Hine et al. 2001a). There have been no published studies on the ultrastructure of *M. roughleyi*. The only *Mikrocytos* sp. for which sequences from the ssu rDNA gene cluster have been published is *M. roughleyi* (Adlard & Lester 1995).

Certification of stocks as free from specific disease agents prior to movement is the basis of disease control in member countries of the Office International des Epizootis. For this to be effective, listed etiological agents must be reliably detectable and identifiable. Heavy infection by microcells may be detected by light microscopy, but identification cannot be carried out with any certainty. Also, light infection cannot reliably be detected by light or electron microscopy. There is an urgent need to characterize each 'microcell' species, and identify gene sequences that allow them to be distinguished from each other.

The aim of this study was the characterization of *Mikrocytos roughleyi* by means of ultrastructural observation and ssu rDNA gene sequence determination to clarify taxonomic relationships with other microcell parasites.

MATERIALS AND METHODS

Electron microscopy. 4% glutaraldehyde (in 0.2 M cacodylate buffer) fixed tissues from infected *Saccostrea glomerata* were provided by R. Hand, New South Wales Australia Fisheries Department. They were post-fixed for 1 h in 1% osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer. Tissues were then cleared in propylene oxide and embedded in epon resin. Ultra-

thin sections were made using copper grids and double-stained with uranyl acetate and lead citrate. These were then examined in a Jeol JEM 1200 EX transmission electron microscope.

DNA extraction. Genomic DNA was extracted from ethanol-fixed, individually infected oysters. Tissues were suspended in 10 volumes of extraction buffer (NaCl 100 mM, EDTA 25 mM, pH 8, SDS 0.5%) with Proteinase K (100 µg ml^{-1}). Following overnight incubation at 50°C, DNA was extracted using a standard phenol/chloroform protocol followed by ethanol precipitation (Sambrook et al. 1989).

Polymerase chain reaction. The partial ssu rDNA was amplified using the universal primer Suni and the *Bonamia ostreae* specific primer Bo-Boas (Cochennec et al. 2000). PCR was performed in 50 µl volume containing about 10 ng of DNA with 5 µl of 10× PCR buffer, 5 µl of 25 mM $MgCl_2$, 5 µl of 2 mM dNTP, 0.5 µl of each 100 µM primer and 0.25 µl (1 U) of Taq DNA polymerase (Promega). Samples were denatured for 5 min at 94°C and amplified by 30 cycles: 1 min at 94°C for denaturation, 1 min at 55°C for primer annealing, and 1 min at 72°C for elongation in a thermal cycle apparatus (Appligene). Polymerization at 72°C was extended for 10 min to ensure complete elongation of the amplified products. After amplification, 5 µl of the PCR products were analyzed by electrophoresis on ethidium bromide-staining agarose gel (2% in Tris acetate EDTA buffer).

DNA sequencing. Amplicons (= PCR products) were cloned using the TA Cloning kit (Invitrogen) following the manufacturer's protocol. Two recombinant plasmids with putative ssu rDNA inserts were sequenced by Appligene (Laboratory Qbiogen). Sequences were compared for similarity with public databases lodged in GenBank and EMBL using BLAST (Altschul et al. 1990).

Restriction fragment length polymorphism (RFLP). A PCR was performed with primers designed for amplification of *Bonamia ostreae*: Bo-Boas (Cochennec et al. 2000). Template DNA was obtained from *Mikrocytos roughleyi*, infected *Saccostrea glomerata*, *B. exitiosus*-infected *Tiostrea chilensis* and *B. ostreae*, infected *Ostrea edulis*. RFLP analysis was performed by separate digestions of 10 µl of PCR product with *Bgl*I and *Hae*II (Promega, France). The resulting fragment patterns were analyzed electrophoretically on 2% agarose gel.

Phylogenetic analysis. Phylogenetic analyses were conducted by aligning the *Mikrocytos roughleyi* ssu rDNA sequence with 53 other protistan ssu rDNA sequences, including those of dinoflagellates (*Amphidinium carterae:* AF009217; *Pfiesteria piscicida:* AF077055; *Prorocentrum minimum:* Y16238; *Alexandrium fundyense:* U09048; *Gonyaulax spinifera:* AF052190; *Symbiodinium corculorum:* L13717); apicomplexans (*Babesia bovis:* L19078; *Theileria parva:* L02366; *Eimeria tenella:* U40264; *Sarcocystis hominis:* AF006470; *Toxoplasma gondii:* U03070); ciliates (*Euplotes crassus:* AY007438; *Oxytricha nova:* X03948; *O. trifallax:* AF164121; *Entodinium caudatum:* U57765; *Paramecium tetraurelia:* X03772; *Tetrahymena pyriformis:* M98021; *T. thermophila:* X56165); fungi (*Ajellomyces capsulatus:* Z75307; *Neurospora crassa:* X04971; *Kluyveromyces lactis:* X51830; *Saccharomyces cerevisiae:* J01353; *Candida glabrata*, X51831; *Pneumocystis carinii:* S83267); stramenopiles (*Fucus distichus:* M97959; *Ochromonas danica:* M32704; *Achlya bisexualis:* M32705; *Phytophthora megasperma:* X54265; QPX; AF261664; *Ulkenia profunda:* L34054); haplosporidians (*Bonamia ostreae:* AF192759; *B. exitiosus:* AF337563; *Minchinia teredinis:* U20319; *Haplosporidium nelsoni:* U19538; *H. costale:* AF387122; *H. louisiana:* U47851; *Urosporidium crescens:* U47852); *Perkinsus* spp. (*P. marinus:* AF042708; *P. atlanticus:* AF140295; *P. chesapeaki:* AF042707); and other protozoans (*Emiliania huxleyi:* M87327; *Chlamydomonas reinhardtii:* M32703; *Nitella flexilis:* U05261; *Acanthamoeba palestinensis:* L09599; *Dictyostelium discoideum:* X00134; *Naegleria fowleri:* U80059; *Physarum polycephalum:* X13160; *Trypanosoma cruzi:* X53917; *Euglena gracilis:* M12677; *Encephalitozoon cuniculi:* L17072; *Chondrus crispus:* Z14140; *Vairimorpha necatrix:* Y00266; *Giardia lamblia:* M54878). The sequences were aligned using the CLUSTAL-W algorithm (Thompson et al. 1994) in the MacVector 7.0 DNA sequence analysis software package (Oxford Molecular) with gap penalties of 8 for gap insertions and 3 for gap extensions in both pairwise and multiple alignment phases. Parsimony jack-knife analysis was performed using PAUP*4b10.0 (Swofford 2002) with 100 random additions of 100 jack-knife replicates.

RESULTS

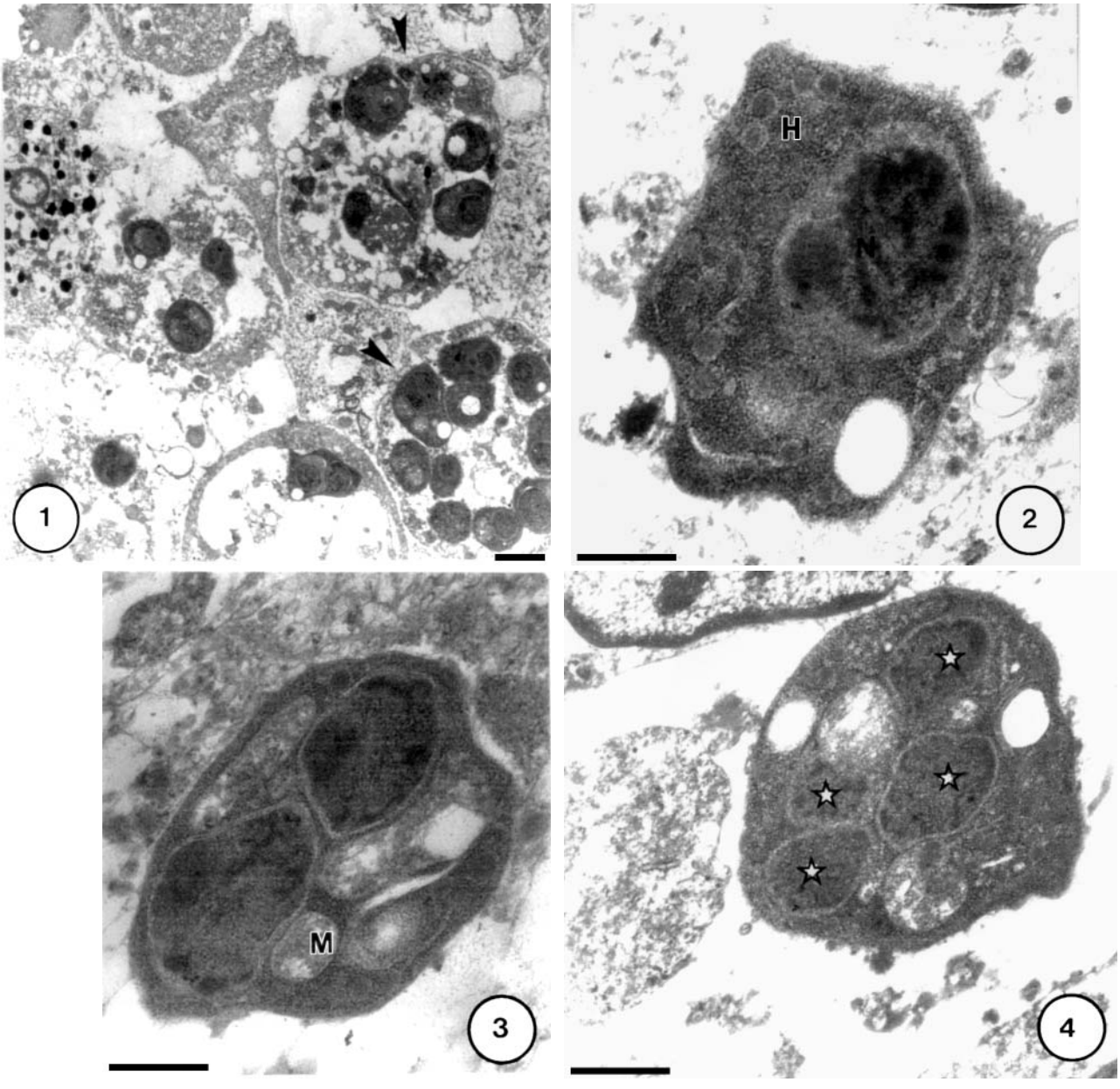
Ultrastructural studies

Mikrocytos roughleyi cells were found in haemocytes within the gills and digestive gland. Infected haemocytes generally contained several parasites. The structure of some haemocytes was only slightly affected, while others were more profoundly altered and displayed vacuolization (Fig. 1). The simplest form of the parasite was ovoid, about 3 to 5 µm in diameter, and bound by a unit membrane. The nuclear envelope consisted of an inner and an outer membrane. A peripherally located nucleolus was sometimes present. The cytoplasm was densely packed with ribosomes and contained 1 or 2 mitochondrial profiles in section.

In addition, 1 to 11 haplosporosomes, displaying an electron-dense matrix, were observed (Fig. 2). Single cytoplasmic lipid droplets were rarely observed. Plasmodial forms with 2 to 4 nuclei were rarely observed (Figs. 3 & 4). These cells were larger and their nuclei and cellular shapes were more irregular than the uninucleate cells. They had cytoplasmic haplosporosomes, as did the smaller cells, but more numerous mitochondria. Small strands of smooth endoplasmic reticulum were sometimes observed.

DNA sequence

An amplicon of 951 bp was obtained. (GenBank Accession No. = AF508801). BLAST analysis of this sequence against public databases confirmed that it is an ssu rRNA gene sequence and revealed similarities with ssu rRNA gene sequences of taxa in the phylum Haplosporidia, particularly with those in the genus *Bonamia*. *B. ostreae* and *B. exitiosus* sequence similarities were 95.2 and 98.4 %, respectively.



Figs. 1 to 4. *Saccostrea glomerata* naturally infected with *Mikrocytos roughleyi*. Electron microscopy. Fig. 1. Infected oyster showing numerous parasites within hemocyte cytoplasm (arrow) (scale bar = 3 µm). Fig. 2. Parasite showing nucleus with excentric nucleolus (N), electron-dense haplosporosomes (H) (scale bar = 200 nm). Fig. 3. Diplokaryotic cell showing mitochondria (m) (scale bar = 200 nm). Fig. 4. Multinucleate plasmodial stage with 4 nuclei (stars) (scale bar = 500 nm)

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B.exitiosus     CCATTTAATTGGTCGGGCCGCTGGTCCTGATCCTTTACTTTGAGAAAATTAAAGTGCTCA
M.roughleyi     CCATTTAATTGGTCGGGCCGCTGGTCCTGATCCTTTACTTTGAGAAAATTAAAGTGCTCA
B.ostreae       CCATTTAATTGGTCGGGCCGCTGGTCCTGATCCTTTACTTTGAGAAAATTAAAGTGCTCA
                                                                  HaeII
                                                                    ▼
B.exitiosus     AAGCAGGCTCGCGCCTGAATGCATTAGCATGGAATAATAAGACACGACTTCGGCGCCGCC
M.roughleyi     AAGCAGGCTCGCGCCTGAATGCATTAGCATGGAATAATAAGACACGACTTCGGCACCGCC
B.ostreae       AAGCAGGCTCGCGCCTGAATGCATTAGCATGGAATAATAAGACACGACTTCGGCGCCGCC

B.exitiosus     ACTCGTGGCGGGTGTTTTGTTGGTTTTGAGCTGGAGTAATGATTGATAGAAACAATTGGG
M.roughleyi     ACTCGTGGCGGGTGTTTTGTTGGTTTTGAGCTGGAGTAATGATTGATAGAAACAATTGGG
B.ostreae       ---- TC----GGCGGTTGTTTTGTTGGTTTTGAGCTGGAGTAATGATTGATAGAAACAATTGGG
                  ▲ BglI

B.exitiosus     GGTGCTAGTATCGCCGGGCCAGAGGTAAAATTCTTTAATTCCGGTGAGACTAACTTATGC
M.roughleyi     GGTGCTAGTATCGCCGGGCCAGAGGTAAAATTCTTTAATTCCGGTGAGACTAACTTATGC
B.ostreae       GGTGCTAGTATCGCCGGGCCAGAGGTAAAATTCTTTAATTCCGGTGAGACTAACTTATGC

B.exitiosus     GAAAGCATTCACCAAGCGTGTTTTCTTTAATCAAGAACTAAAGTTGGGGGATCGAAGACG
M.roughleyi     GGAAGCATTCACCAAGCGTGTTTTCTTTAATCAAGAACTAAAGTTGGGGGATCGAAGACG
B.ostreae       GAAAGCATTCACCAAGCGTGTTTTCTTTAATCAAGAACTAAAGTTGGGGGATCGAAGACG

B.exitiosus     ATCAG
M.roughleyi     ATCAG
B.ostreae       ATCAG
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Fig. 5. *Bonamia ostreae*, *B. exitiosus* and *Mikrocytos roughleyi* Bo-Boas sequence alignment (CLUSTALW). Positions of recognition sites of restriction enzymes *Bgl*I and *Hae*II indicated by arrowheads

Assessment of ssu rDNA polymorphism

CLUSTAL-W multiple alignment program showed a polymorphic region of 6 base pairs. This region was located within the specific *Bonamia* genus Bo-Boas primer amplified sequence (Fig. 5). Results of RFLP analysis of *B. ostreae*, *B. exitiosus* and *Mikrocytos roughleyi* PCR products are given in Fig. 6. The *B. ostreae* profile consisted of 2 bands (120 and 180 pb) for *Bgl* I and 2 bands (115 and 185 pb) for *Hae* II, while the *B. exitiosus* profile consisted of 1 band (304 pb) and 2 bands (117 and 187 pb), respectively. The *M. roughleyi* profile consisted of a unique band (304 pb) for both *Bgl* I and *Hae* II.

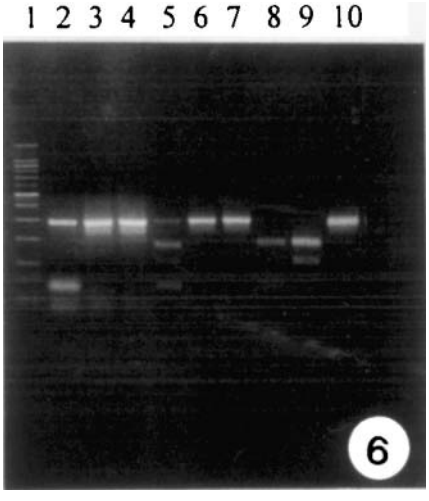

Fig. 6. Two % agarose gel electrophoresis. Lane 1: 100 bp molecular weight marker. Lanes 2 to 4: specific *Bonamia* genus PCR; Lane 2: 300 bp product obtained with *B. ostreae* DNA; Lane 3: 304 bp product obtained with *B. exitiosus* DNA; Lane 4: 304 bp product obtained with *Mikrocytos roughleyi* DNA; Lanes 5 to 10: RFLP results; Lanes 5 and 8: *B. ostreae* profile showing 2 bands both for *Bgl*I and *Hae* II; Lanes 6 and 9: *B. exitiosus* profile showing 1 band for *Bgl*I and 2 bands for *Hae* II; Lanes 7 and 10: *M. roughleyi* profile showing 1 band for both *Bgl*I and *Hae* II

Phylogenetic analysis

Phylogenetic relationships of *Mikrocytos roughleyi* were inferred by parsimony analysis of ssu rDNA sequences from *M. roughleyi* and 53 other eukaryotic taxa. The *M. roughleyi* sequence grouped with the sequences for taxa in the phylum Haplosporidia, particularly with the sequences from *Bonamia ostreae* and

B. exitiosus. The bootstrap support value for grouping *B. exitiosus* with *M. roughleyi* was 69 and 100% for the *B. ostreae*/*B. exitiosus*/*M. roughleyi* clade (Fig. 7). This clade is found nested within the Haplosporidia, which is monophyletic with a support value of 100%.

DISCUSSION

The taxonomy of *Bonamia* spp. and *Mikrocytos* spp. remains unclear, and their relationships with other protistans are poorly understood. Initially, *M. roughleyi* was considered to be a distinct genus from *Bonamia* on the basis of gross signs of the diseases each causes, associated histological lesions and host species affected (Farley et al. 1988). There is some evidence that gross signs and lesions depend on the species of host, rather than the species of parasite. *B. exitiosus* in *Tiostrea chilensis* in New Zealand closely resembles *Bonamia* sp. in *Ostrea angasi* in Australia, and they may be conspecific. *B. exitiosus* only infects haemocytes, does not cause gill lesions, and heavy

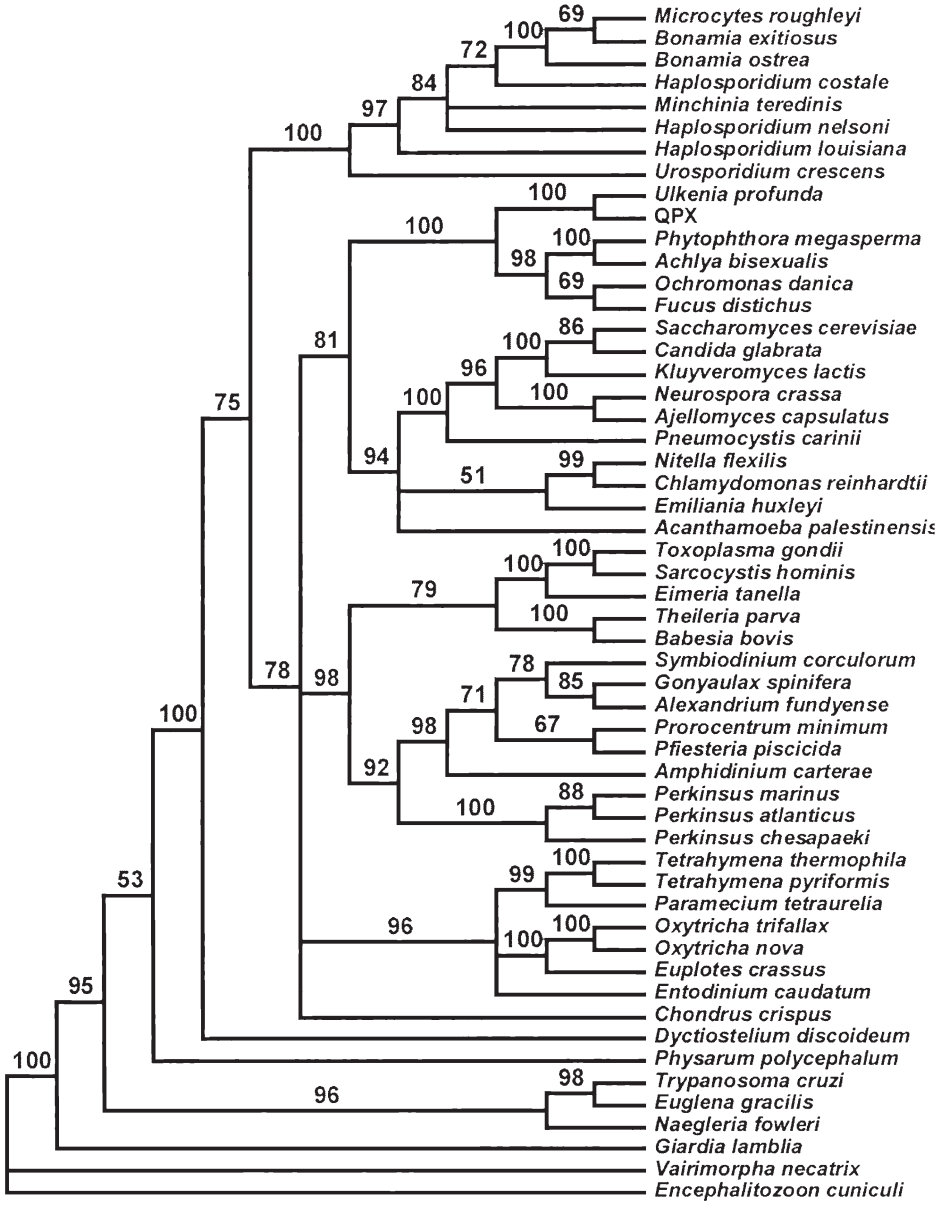

Fig. 7. Parsimony bootstrap consensus tree generated by phylogenetic analysis of the *Mikrocytos roughleyi* ssu rDNA sequence with 53 other representative, taxonomically diverse, eukaryote ssurDNA sequences. Bootstrap support values above 50 are shown on branches. Analysis was done with 100 bootstrap replicates of 100 random additions and gaps were treated as missing data

parasite burdens are associated with this disease. Australian *Bonamia* sp. infects gill epithelial cells and haemocytes, causes gill lesions, and a relatively small number of these parasites are capable of causing severe disease. *B. ostreae* is intermediate in that it infects both haemocytes and epithelial cells, it causes gill lesions, but only heavy parasite burdens are associated with disease (Comps et al. 1980, Pichot et al. 1980, Balouet et al. 1983, Montes et al. 1994). Absence of gill lesions in *B. exitiosus*-infected *T. chilensis* may be due to lack of infection of gill epithelium or to the greater collagen content of *T. chilensis* gills compared to other *Tiostrea* species; this in turn may be related to the long larval brooding period of 3 wk between the gills (Hine 1991, Hine & Wesney 1994a,b).

Ultrastructural studies showed that *Mikrocytos roughleyi* presents more similarities to *Bonamia ostreae* and *B. exitiosus* than to its congener *M. mackini*. Particularly notable are the presence of haplosporosomes and mitochondria in *Bonamia* spp. and *M. roughleyi*, and their absence from *M. mackini* (Hine et al. 2001a). Diplokaryotic plasmodia were rare in this study, but tetra-nucleate plasmodia were observed similar to those described in *B. ostreae* and *B. exitiosus* (Brehélin et al. 1982, Hine et al. 2001b). The large multinucleate stage that occurs in *Haplosporidium* spp. and *Minchinia* spp., and the characteristic spores of those genera, were not observed.

The lack of concordance between genotypic and phenotypic traits herein is not confined to haplosporidians and does not always mean that apparently ultrastructurally diverse species may be congeneric. Microsporidian parasites of the genus *Pleistophora* spp. appear ultrastructurally to be closely related, although they infect insects and fishes. However, rDNA sequencing has shown that the *Pleistophora* spp. infecting fishes are polyphyletic, and that *Pleistophora* spp. in insects are not related to the species in fishes (Nilsen et al. 1998). Ultrastructural characters considered important in microsporidian classification appear to have arisen several times during evolution. Similarly, the haplosporosomes and haplosporosome-like bodies that originally characterized ascetosporans only are now known to occur in paramyxeans and vegetative stages of a primitive clade of myxozoans (Anderson et al. 1999), and cannot be considered as characteristic of haplosporidians only.

The preliminary ultrastructural description provided here was not sufficient to elucidate relationships of *Mikrocytos roughleyi* with *Bonamia* spp. Therefore, the ssu rDNA gene was sequenced. This gene has been widely used in the taxonomic study of oyster pathogens including *B. ostreae* and *B. exitiosus* and *Haplosporidium* spp. (Fong et al. 1993, Ko et al. 1995, Stokes et al. 1995, Flores et al. 1996, Carnegie et al. 2000, Cochennec et al. 2000, Hine et al. 2001a). Adlard & Lester (1995) proposed a *M. roughleyi*-diagnostic method based on amplification of a segment of the internal transcribed spacer region (ITS) within the ribosomal gene cluster (rRNA). However, attempts to sequence the gene were unsuccessful. The ssu rDNA sequence of *M. roughleyi* obtained in this study showed strong similarity to those of *B. ostreae* and *B. exitiosus*, and also to *H. nelsoni*, *H. costale* and *M. teredinis*, suggesting that *M. roughleyi* shares homologies with the phylum Haplosporidia. These results confirm earlier studies indicating that *Bonamia* spp. are related to the genus *Haplosporidium* (Carnegie et al. 2000, Cochennec et al. 2000). In addition, some divergent regions among the *Bonamia* spp. and *M. roughleyi* were evident. This polymorphism was confirmed by RFLP analysis, and allowed a rapid molecular assay for the discrimination of these 3 parasites.

This additional genomic information, combined with that on *Bonamia ostreae* and *B. exitiosus*, suggests that *Bonamia* and *Mikrocytos roughleyi* should be included in the phylum Haplosporidia. Such results indicate the difficulty in explaining phenotypic features, including the lack of a known spore stage in *Bonamia* spp. and the presence of a vacuolated stage in *B. exitiosus* (Hine et al. 2001b), but its apparent lack in *B. ostreae* and *Haplosporidium* spp. Therefore, it is important to carry out further ultrastructural and genetic studies. Particularly, there is a need to complete the ssu rDNA sequence to eliminate possible analysis artefacts. Sequencing of other genes of phylogenetic interest will test our hypothesis (Baldauf et al. 2000). Such work, using 2 approaches, is essential as a first step towards elucidation of microcell relationships and taxonomy, and one of the benefits of such studies will be the development of specific identification tools for each parasite species.

In conclusion, based on our ultrastructural and molecular results, we propose a revision of *Mikrocytos rougheyi* taxonomy, and that *M. roughleyi* be considered as a putative member of the phylum Haplosporidia. More molecular or ultrastructural data are needed to confidently determine whether *M. roughleyi* should be placed in the genus *Bonamia*.

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