

# Molecular detection of the oyster parasite *Mikrocytos mackini*, and a preliminary phylogenetic analysis

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**ABSTRACT:** The protistan parasite *Mikrocytos mackini*, the causative agent of Denman Island disease in the oyster *Crassostrea gigas* in British Columbia, Canada, is of wide concern because it can infect other oyster species and because its life cycle, mode of transmission, and origins are unknown. PCR and fluorescent *in situ* hybridization (FISH) assays were developed for *M. mackini*, the PCR assay was validated against standard histopathological diagnosis, and a preliminary phylogenetic analysis of the *M. mackini* small-subunit ribosomal RNA gene (SSU rDNA) was undertaken. A PCR designed specifically not to amplify host DNA generated a 544 bp SSU rDNA fragment from *M. mackini*-infected oysters and enriched *M. mackini* cell isolates, but not from uninfected control oysters. This fragment was confirmed by FISH to be *M. mackini* SSU rDNA. A *M. mackini*-specific PCR was then designed which detected 3 to 4× more *M. mackini* infections in 1056 wild oysters from Denman Island, British Columbia, than standard histopathology. *Mikrocytos mackini* prevalence estimates based on both PCR and histopathology increased (PCR from 4.4 to 7.4%, histopathology from 1.2 to 2.1%) when gross lesions were processed in addition to standard samples (i.e. transverse sections for histopathology, left outer palp DNA for PCR). The use of histopathology and tissue imprints plus PCR, and standard samples plus observed gross lesions, represented a 'total evidence' approach that provided the most realistic estimates of the true prevalence of *M. mackini*. Maximum parsimony and evolutionary distance phylogenetic analyses suggested that *M. mackini* may be a basal eukaryote, although it is not closely related to other known protistan taxa.

**KEY WORDS:** Denman Island disease · *Mikrocytos mackini* · PCR validation · Fluorescent *in situ* hybridization

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## INTRODUCTION

The protistan parasite *Mikrocytos mackini* (Farley et al. 1988) causes Denman Island disease of wild and cultured *Crassostrea gigas* oysters in British Columbia, Canada. Each April and May, oysters with this disease develop focal green lesions in the mantle, labial palps, and adductor muscle; many do not recover. Mortality due to Denman Island disease reached 35% in one *C. gigas* population (Quayle 1961, Bower 1988, Bower et

al. 1994), and other oysters (e.g. *C. virginica*, *Ostrea edulis*, and *O. conchaphila*) may be even more susceptible (Bower et al. 1997). The parasite itself is a small (3 µm), amitochondriate protist of unknown taxonomic affiliation from vesicular connective tissue cells, heart and adductor muscle myocytes, and hemocytes of infected oysters (Hervio et al. 1996, Hine et al. 2001). It has only been documented in British Columbia, yet the Office International des Epizooties (OIE) considers *M. mackini* a serious threat to oyster aquaculture globally

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(OIE 2000) because it is pathogenic to several oyster species and because its life cycle, mode of transmission, and origins are unknown.

*Mikrocytos mackini* might be understood more thoroughly and managed more effectively if specific molecular diagnostic assays existed to complement conventional histopathological techniques. However, *M. mackini* small-subunit ribosomal DNA (SSU rDNA) eluded identification for a decade, presumably because 'universal' eukaryotic PCRs preferentially amplified the oyster DNA predominating in host-parasite mixtures, and because pure isolates of *M. mackini* are not available. We describe here: successful amplification of *M. mackini*'s SSU rDNA gene using a primer pair designed specifically *not* to amplify host DNA; proof of the identity of this gene using fluorescent *in situ* hybridization (FISH); design and validation of a PCR assay specific for *M. mackini* SSU rDNA; and a preliminary SSU rDNA phylogenetic examination of *M. mackini*'s affinities.

## MATERIALS AND METHODS

**Amplification of presumptive *Mikrocytos mackini* SSU rDNA.** A rapid PCR cloning approach was used to isolate a presumptive *M. mackini* SSU rDNA gene fragment for sequencing. First, genomic DNA was extracted from the labial palps of one uninfected and one laboratory-infected *Ostrea edulis* using a DNeasy Tissue Kit (QIAGEN). The infection status of each oyster was determined using standard histopathology. DNA was also extracted from cryopreserved *M. mackini* cells that had been purified in 1999 from another laboratory-infected *O. edulis* (Joly et al. 2001). The amount of DNA in all samples was quantified using a Genequant *pro* RNA/DNA Calculator (Biochrom).

PCR primers were created to selectively amplify *Mikrocytos mackini* SSU rDNA from an oyster-parasite DNA mixture. Primer design for this part of the project (and primer and probe design for subsequent parts) was based on an alignment (Se-Al v. 1, see <http://evolve.zoo.ox.ac.uk/software/Se-Al/main>; Department of Zoology, University of Oxford, UK) of oyster SSU rDNA gene sequences with those of numerous protists and a fungus (oysters: *Crassostrea virginica* [GenBank accession no. X60315] and *Ostrea edulis* [U88709]; protists: *Acanthamoeba griffini* [S81337], *Acanthamoeba* sp. [AF063240], *Alexandrium minutum* [U27499], *Bonamia ostreae* [AF262995], *Cafeteria roenbergensis* [L27633], *Coccolithus pelagicus* [AJ246261], *Collozoum pelagicum* [AF091146], *Cyanophora paradoxa* [X68483], *Entamoeba coli* [AF149915], *Euglena acus* [AF090871], *Euglypha rotunda* [X77692], *Giardia ardeae* [Z17210], *Haplosporidium nelsoni* [X74131], *Marteilia refringens*

[AJ250699], *Orbulina universa* [Z83962], *Oxytricha nova* [X03948], *Paramecium tetraurelia* [X03772], *Perkinsus marinus* [X75762], *Pyrenomonas salina* [X55032], *Spongopora subterranea* [AF245217], *Spraguea lophii* [AF033197], *Tetramitus rostratus* [M98051], *Toxoplasma gondii* [U03070], *Trichomonas vaginalis* [U17510], *Vannella anglica* [AF099101], and the clam parasite QPX [AF155209]; fungus: *Saccharomyces* sp. [AB040997]). Both forward and reverse primers (18S-EUK581-F: 3-GTGCCAGCAGCCGCG-5 and 18S-EUK1134-R: TTT AAGTTTCAGCCTTGCG, respectively) were specific for highly conserved sequences. The reverse primer, however, was mismatched to the oyster target at 2 positions near the 3' end (underlined above). It was expected to amplify oyster rDNA inefficiently, if at all.

PCR reaction mixtures of 25  $\mu$ l included primers at 0.05  $\mu$ M and template DNA (genomic DNA from either the *Mikrocytos mackini*-infected or -uninfected *Ostrea edulis*, the cryopreserved *M. mackini* cells, or a no-template water control) at 1 ng  $\mu$ l<sup>-1</sup>; PCR buffer at 1 $\times$  concentration; MgCl<sub>2</sub> at 2.5 mM; deoxynucleotides (dNTPs) at 0.2 mM; and Platinum *Taq* polymerase at 0.05 U  $\mu$ l<sup>-1</sup>. All PCR reagents, including primers and PCR-grade water, were purchased from Invitrogen Canada. The temperature profile included an initial denaturation at 94°C for 10 min; 35 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. Products were electrophoresed on a 1.5% agarose (in 1 $\times$  Tris-borate-EDTA) gel containing 0.1  $\mu$ g ml<sup>-1</sup> ethidium bromide and were visualized using UV light. They were cloned using a TOPO TA Cloning Kit (Invitrogen Canada) and sequenced using the original PCR primers and an ABI 377 (Applied Biosystems) automated DNA sequencer.

A second and third PCR from *Mikrocytos mackini*-infected *Ostrea edulis* and cryopreserved *M. mackini* cells amplified most of the remainder of a non-oyster SSU rDNA gene—presumably *M. mackini* SSU rDNA—partially sequenced above. Two new primer pairs were designed. The first pair was 18S-EUK18-F (ACCTGGT-TGATCCTGCC; non-specific) and 18S-PRESMACK-R (GGTCCTTGGCTGACGAG; presumptive *M. mackini*-specific); the second was 18S-PRESMACK-F (AGAAG-TTCGACGGCAGG; presumptive *M. mackini*-specific) and 18S-EUK1776-R (CGGAAACCTTGTTACGAC; non-specific). Reaction conditions were as before, except the MgCl<sub>2</sub> concentration was 1.5 mM and the annealing temperature was 50°C. Products were again cloned and sequenced. A Sequencher (Gene Codes) contig was constructed comprising these and the earlier presumptive *M. mackini* sequence, and the consensus sequence was included in the alignment above for FISH probe design. (The partial *M. mackini* SSU rDNA sequence was deposited in GenBank under accession no. AF477623.)

**FISH.** To determine the identity of the above consensus sequence, FISH was used. Four oligonucleotide probes specific for the sequence were designed and purchased with 5' Oregon Green labels (MACKINI-1-OG: AGCCACAGCCTTCAC; MACKINI-2-OG: CCGCCCTTCTCAGGTC; MACKINI-3-OG: CGAAA-GTGGTAGCTAAAG; and MACKINI-4-OG: AGTAG-CCTGCTTCCACT; all from Invitrogen Canada). They were hybridized to 5  $\mu\text{m}$  sections of *Ostrea edulis* that had been infected with *Mikrocytos mackini* in laboratory passages (Hervio et al. 1996). The base FISH methodology was derived from published procedures (Dubilier et al. 1995, Stokes & Bureson 1995, Stokes et al. 1995). Tissue sections adhered to aminoalkylsilane-coated slides (Silane-Prep; Sigma Aldrich) were deparaffinized in xylene ( $3 \times 2$  min), rehydrated through a descending isopropanol series ( $3 \times 100, 80, 50$ , and  $30\%$  for 30 s each, followed by 1 min in tap water), and equilibrated in phosphate-buffered saline (PBS, 1 min). The sections were then digested with Proteinase K ( $100 \mu\text{g ml}^{-1}$  in PBS for 10 min at  $37^\circ\text{C}$ , followed by a wash in PBS plus  $0.2\%$  glycine for 5 min); acetylated using acetic anhydride ( $5\%$  [v/v] in  $0.1\text{M}$  triethanolamine-HCl [pH 8.0] for 10 min at room temperature, followed by a wash in PBS for 5 min; see Schwarzscher & Heslop-Harrison 2000); and equilibrated in  $5 \times$  SET ( $750 \text{ mM NaCl}$ ,  $6.4 \text{ mM EDTA}$ ,  $100 \text{ mM Tris}$  base; 5 min at room temperature). Excess SET was drained off, and each section was flooded with prehybridization buffer ( $5 \times$  SET,  $0.02\%$  bovine serum albumin,  $0.025\%$  SDS). After incubation for 1 h at  $42^\circ\text{C}$ , the prehybridization buffer was drained off and replaced with 20 to 25  $\mu\text{l}$  of prehybridization buffer containing the appropriate oligonucleotide(s). The sections were covered with parafilm coverslips and incubated overnight at  $42^\circ\text{C}$ . They were washed the next day with  $0.2 \times$  SET ( $3 \times$  at  $42^\circ\text{C}$  for 2.5 min total), air dried, mounted with a glycerol-in-PBS medium, and covered with glass coverslips. Slides were examined ( $1000 \times$  magnification) on a Zeiss epifluorescent microscope with a dual fluorescein-rhodamine filter system.

Four experimental conditions were used. The *Mikrocytos mackini*-specific treatment included 4 Oregon Green-labeled, presumptive *M. mackini*-specific oligonucleotides, each at  $10 \text{ ng } \mu\text{l}^{-1}$ . A competitive negative control treatment included these probes at  $10 \text{ ng } \mu\text{l}^{-1}$  plus unlabeled versions of the same probes, each at  $200 \text{ ng } \mu\text{l}^{-1}$ . A competitive positive control treatment included the 4 labeled, presumptive *M. mackini*-specific probes at  $10 \text{ ng } \mu\text{l}^{-1}$  plus a *Nocardia crassostreae*-specific reverse PCR primer at  $1 \mu\text{g } \mu\text{l}^{-1}$ . (*Nocardia crassostreae* is an actinomycete bacterial pathogen of oysters [Friedman et al. 1998] that was not present in these tissue sections.) This control was designed to demonstrate that only a hyperconcentra-

tion of *specific* oligonucleotides (i.e. of sequences matching those of the labeled probes, as in the competitive negative control) would disrupt hybridization. Finally, a no-probe treatment included no oligonucleotides, and was used to indicate the level of background fluorescence in the absence of labeled probes.

***Mikrocytos mackini*-specific PCR assay design and validation.** Two *M. mackini*-specific PCR primers were designed based on the eukaryotic SSU rDNA alignment described earlier: MIKROCYTOS-F (AGATG-GTTAATGAGCCTCC) and MIKROCYTOS-R (GCG-AGGTGCCACAAGGC). *M. mackini*-specific PCR reaction mixtures ( $15 \mu\text{l}$ ) were optimized to include PCR buffer at  $1 \times$  concentration,  $\text{MgCl}_2$  at  $1.25 \text{ mM}$ , dNTPs at  $0.2 \text{ mM}$ , primers at  $0.05 \mu\text{M}$ , Platinum *Taq* polymerase at  $0.05 \text{ U } \mu\text{l}^{-1}$ , and template DNA at  $1 \text{ ng } \mu\text{l}^{-1}$ . The cycling program began with initial denaturation at  $94^\circ\text{C}$  for 10 min, was followed by 40 cycles of  $94^\circ\text{C}$  for 1 min,  $60.5^\circ\text{C}$  for 1 min, and  $72^\circ\text{C}$  for 1 min, and ended with final extension at  $72^\circ\text{C}$  for 10 min. Products were electrophoresed as above on  $1.5\%$  agarose (in  $1 \times$  TBE) gels containing  $0.1 \mu\text{g ml}^{-1}$  ethidium bromide and were visualized using UV light. A 546 bp product was diagnostic for *M. mackini*.

For the validation phase of this study, a total of 1056 *Crassostrea gigas* were collected at an intertidal aquaculture site at Henry Bay, Denman Island, British Columbia ( $50^\circ\text{N}$ ,  $127^\circ\text{W}$ , in NW quarter) from January to December 2001 ( $\leq$ ca. 100 oysters  $\text{mo}^{-1}$ ). From each oyster, a transverse section was obtained and processed for standard histopathological diagnosis of *Mikrocytos mackini* as described by Hervio et al. (1996). Also from each oyster, a distal portion (about  $2 \text{ mm}^3$ ) of the left outer labial palp was excised and fixed in  $95\%$  ethanol for subsequent genomic DNA extraction (using a DNeasy Tissue Kit, as above) and PCR amplification using the *M. mackini*-specific PCR. (Palp samples were used for PCR because oyster palps contain extensive areas of the vesicular connective tissue that *M. mackini* infects.) When the greenish focal lesions typical of *M. mackini* infections (though typical also of *Nocardia crassostreae* infections; Elston et al. 1987, Friedman et al. 1998) were observed, as many as 3 per oyster were processed as follows: one physical half of each pustule was excised and processed for standard histopathological diagnosis of *M. mackini*; the other half was blotted dry and imprinted on a glass microscope slide, then fixed in  $95\%$  ethanol for DNA extraction and *M. mackini*-specific PCR analysis. The tissue imprints were quick-stained with a modified Wright-Giemsa staining procedure (Hemacolor<sup>®</sup>, EM Science) and examined microscopically for the presence of *M. mackini* cells as described by Hervio et al. (1996).

**Phylogenetic analysis.** Following preliminary neighbor joining and maximum parsimony analyses using

the SSU rDNA alignment produced above for *Mikrocytos mackini*-specific PCR primer design, the consensus *M. mackini* SSU rDNA sequence was aligned with SSU rDNA sequences from representatives of 6 groups: Diplomonadida (*Giardia microti* [AF006677] and *Hexamita inflata* [L07836]), Parabasalidea (*Calonympha* sp. [X97976], *Pentatrachomonas hominis* [AF124609], and *Tritrichomonas foetus* [U17509]), Kinetoplastids and Euglenoids (*Euglena acus* [AF090871] and *Trypanosoma cruzi* [AF359495]), Entamoebae (*Entamoeba coli* [AF149915] and *E. dispar* [Z49256]), Heterolobosea (*Tetramitus rostratus* [m98051] and *vahlkampfia damariscottae* [AJ224891]), and Microspora (*Ameson michaelis* [L15741], *Antonosporea scoticae* [AF024644], *Bacillidium* sp. [AF104087], *Endoreticulatus schubergi* [L39109], *Enterocytozoon bieneusi* [AF024657], *Microsporidium* sp. [AF397404], *Pleistophora* sp. [AJ252959], and *Visvesaria acridophagus* [AF024658]). Sequences from 2 Archaea, *Halobacterium volcanii* (K00421) and *Ignicoccus* sp. (AJ318042), were included as an outgroup. The alignment was created using Clustal W (European Bioinformatics Institute, available at [www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)) and refined by eye in Se-Al v. 1. Regions of uncertain positional homology were discarded.

Evolutionary distance and maximum parsimony analyses were performed using PAUP v. 4.0b10 (Swofford 2002). For evolutionary distance, starting trees were obtained by neighbor joining. Distances were uncorrected. For maximum parsimony, starting trees were obtained by simple stepwise addition of sequences, with *Hexamita inflata* as the reference and one tree held at each step. For both analyses, the branch swapping algorithm was tree-bisection-reconnection, steepest descent was not in effect but 'MulTrees' was, and topological constraints were not enforced. A 50% majority rule consensus tree of 1000 bootstrap replicates was constructed for each analysis.

## RESULTS

### Amplification of presumptive *Mikrocytos mackini* SSU rDNA

PCR produced a single 544 bp amplicon from the cryopreserved *Mikrocytos mackini* cell DNA sample and the *M. mackini*-infected *Ostrea edulis* palp DNA sample, but not from uninfected *O. edulis*. A GenBank Blast search revealed its sequence to be that of a unique eukaryotic SSU rDNA gene fragment, presumably *M. mackini* SSU rDNA. Subsequent PCR's with primer pairs 18S-EUK18-F/18S-PRESMACK-R and 18S-PRESMACK-F and 18S-EUK1776-R expanded this sequence to 1457 bp.

## FISH

Presumptive *Mikrocytos mackini*-specific probes hybridized strongly to structures in 5  $\mu\text{m}$  sections that appeared to be *M. mackini* cells (Fig. 1A), and hybridized to these structures alone. The pattern of fluorescent staining conformed very closely to that expected for *M. mackini* cells: intense green rings or loops  $\sim 3 \mu\text{m}$  in exterior diameter (representing the *M. mackini* cell cytoplasm, containing rRNA to which the probes hybridized) surrounding dark central areas (the unstained *M. mackini* cell nuclei). These stained structures were distributed systemically in laboratory-infected oysters (typical of *M. mackini* in laboratory infections) but were particularly prevalent in vesicular connective tissue, and in and between fibers in the adductor muscle (in these laboratory-infected oysters, the site to which *M. mackini* cells were introduced). Hematoxylin & eosin (H&E)-stained histopathological serial sections of the same laboratory-infected oysters showed that *M. mackini* cells were distributed precisely as the fluorescent-stained structures. The stained structures were *M. mackini* cells.

Hybridization of the *Mikrocytos mackini*-specific probes at  $10 \text{ ng } \mu\text{l}^{-1}$  was blocked (fluorescence was extinguished) by a mixture of the same probes, but without labels, at  $200 \text{ ng } \mu\text{l}^{-1}$  (the competitive negative control; Fig. 1B); the  $20\times$  concentration of unlabeled probes appeared to outcompete the labeled probes for the same, specific binding sites. Confirmation was provided by the competitive positive control treatment in which labeled probe binding (and thus fluorescence) was restored, despite the presence of  $1 \mu\text{g } \mu\text{l}^{-1}$  of a non-specific oligonucleotide (the *Nocardia crassostreae* primer; Fig. 1C).

### *Mikrocytos mackini*-specific PCR assay design and validation

In validation trials, *Mikrocytos mackini* was detected more frequently by PCR than by standard histopathological analysis or by microscopic analysis of stained tissue imprints (Table 1). When PCR from palp DNA was compared against the histopathological analysis of a standard transverse section, *M. mackini* was estimated to be  $3.7\times$  more prevalent by PCR (4.4%) than by histopathology (1.2%). Of 1056 oysters, 46/1056 were *M. mackini*-positive by PCR, but only 13/1056 were *M. mackini*-positive by histopathology (Table 1, Column A). While 7/1056 (0.7%) were *M. mackini*-positive by both techniques, 1004/1056 (95.1%) were negative by both methods. There were  $6.5\times$  more *M. mackini*-positive by PCR alone (39/1056) than by histopathology alone (6/1056).



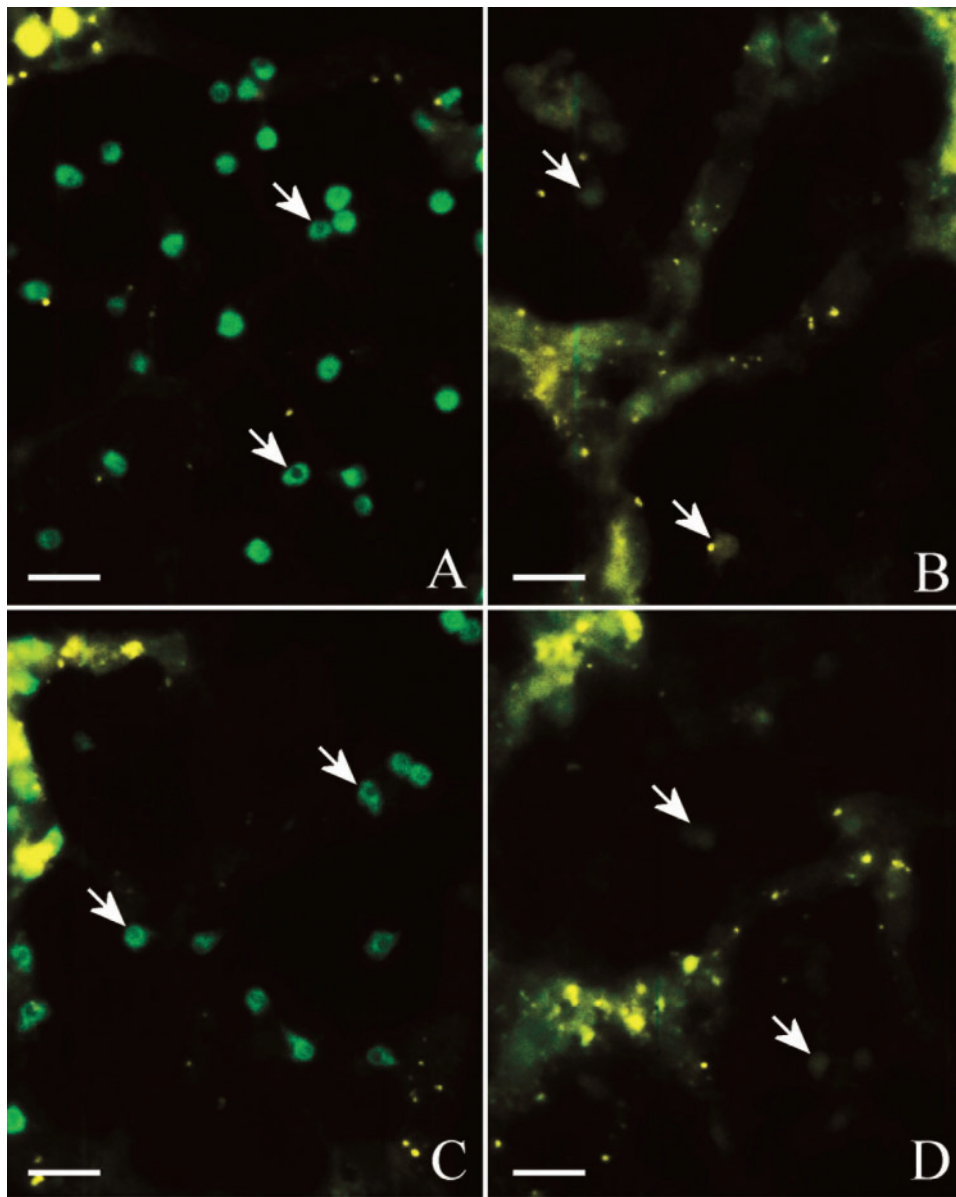


Fig. 1. Fluorescent *in situ* hybridization (FISH) to consecutive *Mikrocytos mackini*-infected *Ostrea edulis* sections (scale bars = 10  $\mu\text{m}$ ). Arrows denote *M. mackini* cells infecting *O. edulis* vesicular connective tissue. (A) *M. mackini*-specific probes (each at 10  $\text{ng } \mu\text{l}^{-1}$ ) alone. (B) *M. mackini*-specific probes at 10  $\text{ng } \mu\text{l}^{-1}$  plus unlabeled, *M. mackini*-specific competitive probes at 200  $\text{ng } \mu\text{l}^{-1}$ . (C) *M. mackini*-specific probes at 10  $\text{ng } \mu\text{l}^{-1}$  plus unlabeled *Nocardia crassostreae*-specific oligonucleotides at 1  $\mu\text{g } \mu\text{l}^{-1}$ . (D) No-probe condition, illustrating the level of background autofluorescence in the absence of labeled probes

When 145 individual gross focal lesions were examined (from 88 oysters; Table 1, Column B), *Mikrocytos mackini* was detected in 70/145 (48.3%) by PCR but in only 23/145 (15.9%) by tissue imprints and 18/145 (12.4%) in histopathological sections. All but one positive diagnosis of *M. mackini* by histopathology or tissue imprints was confirmed by PCR. However, PCR detected 47 and 53 *M. mackini* infections that tissue imprints and histopathology, respectively, did not reveal.

All data—standard histopathology and tissue imprints and PCR from both palp DNA and gross lesions—were integrated in Table 1, Column C. Oysters were classified as Hist/Imprint positive if *Mikrocytos mackini* cells were found either in standard transverse sections, or by histopathological or tissue imprint analysis of gross lesions. Oysters were PCR positive if *M. mackini* SSU rDNA was amplified from either palp or lesion DNA. *M. mackini* was estimated to be 3.5 $\times$  more prevalent by PCR (7.4%) than by histopathology (2.1%).

Table 1. Validation of the *Mikrocytos mackini*-specific PCR against standard histopathology and tissue imprints. Hist: histopathology

	A Histopathology vs. PCR from palp tissue			B Individual pustules: Histopathology vs. PCR			C All data integrated		
	Hist +ve	Hist -ve	Total	Hist +ve	Hist -ve	Total	Hist/ Imprint +ve	Hist/Imprint -ve	Total
PCR +ve	7	39	46	17	53	70	18	60	78
PCR -ve	6	1004	1010	1	74	75	4	974	978
Total	13	1043	1056	18	127	145	22	1034	1056
				Individual pustules: tissue imprints vs. PCR					
				Imprint +ve	Imprint -ve	Total			
				PCR +ve	47	70			
				PCR -ve	75	75			
				Total	122	145			

While 78/1056 oysters were *M. mackini*-positive by PCR, only 22/1056 were positive by histopathology or tissue imprints. Overall, there were 15× more *M. mackini*-positive oysters by PCR alone (60/1056) than by classical techniques alone (4/1056).

*Nocardia crassostreae* was observed in 2/1056 oysters. One oyster was *N. crassostreae*-positive by histopathology but *Mikrocytos mackini*-negative by both histopathology and PCR. The second oyster was heavily infected by *N. crassostreae*, with *N. crassostreae* colonies abundant in both the transverse histopathological section and a gross lesion. *M. mackini* was not observed microscopically in the transverse section or the gross lesion, yet the lesion generated a positive PCR signal. Subsequent FISH analysis of the lesion revealed *M. mackini* cells, thus indicating a dual infection.

### Phylogenetic analysis

Evolutionary distance (Fig. 2A) and maximum parsimony (Fig. 2B) analyses generated phylogenetic trees that were similar to each other and generally supportive of earlier SSU rDNA phylogenetic analyses (e.g. Simpson et al. 2002). Sequences from the Diplomonadida and Parabasalidea were basal to the Kinetoplastids and Euglenoids, Entamoebae, and Heterolobosea. Both evolutionary distance and maximum parsimony placed *Mikrocytos mackini* alone at the base of the eukaryotic tree. Bootstrap support for this placement was weak (51%) in the case of maximum parsimony, but stronger (77%) using evolutionary distance.

### DISCUSSION

Characterization of the SSU rDNA of unknown or cryptic protistan parasites by PCR is difficult because

'universal' eukaryotic PCR primers amplify host DNA as well as parasite DNA. Host DNA can predominate in bulk mixtures, and its amplification can swamp the amplification of parasite DNA. *Mikrocytos mackini* SSU rDNA was successfully amplified directly from the genomic DNA of an infected oyster because the reverse PCR primer (18S-EUK1134-R) was mismatched to the oyster target sequence in places that prevented oyster SSU rDNA amplification. Mismatches corresponded to Positions 14 and 19 in the 19-base primer. Failure of oyster SSU rDNA to amplify from either the infected or the uninfected *Ostrea edulis* samples suggests that these mismatches disrupted the amplification of oyster DNA. Mismatches to *M. mackini* SSU rDNA at Positions 12 and 13, discovered only after sequencing, were less disruptive. Amplification of *M. mackini* SSU rDNA was weak but succeeded, as detection of this sequence in *M. mackini* cells by FISH ultimately demonstrated.

While the FISH assay described here provided unambiguous confirmation of the identity of the presumptive *Mikrocytos mackini* SSU rDNA sequence, its usefulness in routine diagnostics will be limited. FISH is much more time-consuming and labor-intensive than PCR, and while commensurate in time and labor cost with standard histopathology, FISH reveals less about the general health of the oyster and other infections than standard histopathology does. FISH would be most useful for confirming weak positive PCR signals in apparently healthy oysters, but in such cases FISH is *least* likely to provide positive confirmation and *most* likely to generate false negative results, as *M. mackini* infections are naturally focal (Farley et al. 1988) and faint or invisible lesions in very lightly infected oysters can easily go unsampled. However, FISH does reveal many more *M. mackini* cells than standard H&E staining in lesions where *M. mackini* can be identified by both methods. This is a function of

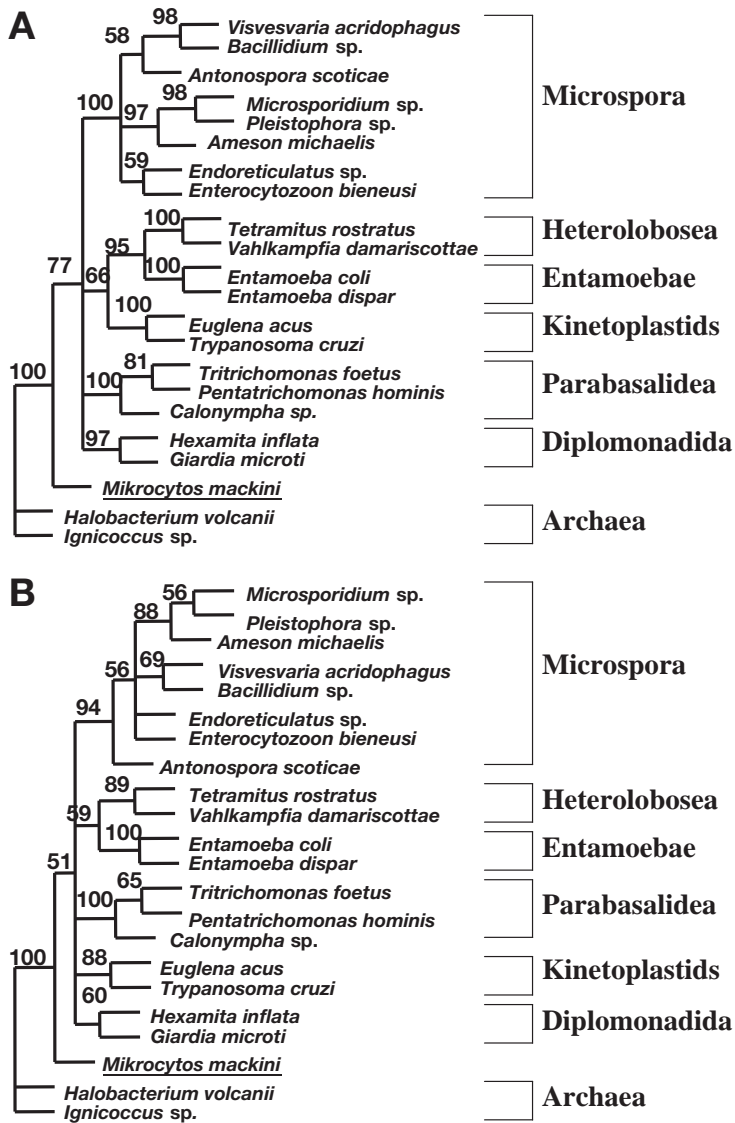


Fig. 2. Bootstrap 50% majority-rule consensus trees. Numbers represent percentages of 1000 bootstrap replicates. (A) Evolutionary distance. Minimum evolution score = 2.36018; (B) Maximum parsimony. Tree length = 1691

the specific staining of *M. mackini* cells in FISH versus the non-specific H&E staining of all tissue structures. FISH should therefore be most useful for detecting *M. mackini* cells in focal lesions identified using standard histopathology as suspicious but in which no H&E-stained *M. mackini* cells can be observed.

*Mikrocytos mackini*-specific PCR, on the other hand, is a powerful complement to standard techniques. Its primary strengths are high throughput and superior sensitivity. In all comparisons, the PCR described here was 3 to 4× more sensitive—revealed *M. mackini* prevalences 3 to 4× higher—than standard histopathology and tissue imprints. Its primary weakness would

be a susceptibility to false negative results attributable to sampling error, a flaw common to PCR diagnostic assays (Burreson 2000) but particularly troublesome when the parasite to be detected has a patchy or focal distribution in its host, as *M. mackini* does (Farley et al. 1988). The minimum frequency of false negative PCR results in this study could be estimated as 0.6%, the frequency in Table 1, Column A of Hist +ve/PCR -ve oysters. To this would be added the frequency of *double* false negatives, the oysters *M. mackini*-negative by both PCR and histopathology that in fact did carry the parasite. This value cannot be known. The susceptibility of this assay to false *positive* PCR results is more difficult to establish. The PCR primers could only be designed with regard to known sequences from described eukaryotic and prokaryotic taxa, but they were designed to bind to relatively unconserved SSU rDNA regions, and so should hybridize to *M. mackini* SSU rDNA alone. Genomic DNA samples from the bacterium *Nocardia crassostreae*, 3 microsporidians (*Loma salmonae* and unidentified isolates from shrimp *Pandalus platyceros* and *Pandalus jordanii*), and 2 other bivalve pathogens (*Bonamia ostreae* and *Perkinsus qugwadi*) were run as negative controls in numerous PCR trials and never amplified.

The use of (1) PCR from standard palp DNA samples and any observable gross lesions, (2) histopathological analyses of standard transverse sections and of gross lesions, and (3) tissue imprints of lesions together compose a 'total evidence' approach to *Mikrocytos mackini* diagnosis and a strategy for obtaining the truest estimates of the prevalence of this parasite in oyster populations. PCR of palp DNA alone, without regard to observable lesions, produced an *M. mackini* prevalence estimate of 4.4%. Including as many as 3 gross lesions per oyster in the PCR analysis increased the prevalence estimate to 7.4%, because *M. mackini* SSU rDNA amplified from lesions in many oysters that were *M. mackini*-negative in the palps. (In only a single oyster of the 18 from which 3 lesions were sampled was no *M. mackini* detected by PCR.) Adding histopathological data increased the prevalence further, but only by another 0.4% (to 7.8%) because the rate of false negative PCR diagnoses was low. Generally, PCR alone would be enough to obtain reasonable estimates of the prevalence of *M. mackini* in oyster populations. Histopathology would complement PCR by providing a slight improvement in prevalence estimates and general information concerning the health of the oysters in the population.

The usefulness of this *Mikrocytos mackini*-specific PCR is already being established. Previous histopathological work demonstrated that *M. mackini* infections were seasonal, with parasite prevalence and oyster mortality peaking annually in April and May and with *M. mackini* rarely observed before March and after June (Farley et al. 1988). Bower (1988) observed some *M. mackini* cells in January 1987, but only in association with tissue abscesses that are uncommon at that time of year (Farley et al. 1988). Using the *M. mackini*-specific PCR, *M. mackini* has now been detected in the palps of apparently healthy oysters in every month of the year, suggesting that oyster populations in fact harbor *M. mackini* year round (Meyer et al. unpubl. data). In addition, this assay will be useful in detecting *M. mackini* in oyster broodstock and seed before export, thus reducing the likelihood of accidental introduction of *M. mackini* to disease-free areas outside of British Columbia. It can also be used to identify areas within British Columbia, the Pacific Northwest of the US, and other locations around the world that are truly *M. mackini*-free.

Finally, this study has provided new insight into the phylogenetic affinities of *Mikrocytos mackini*. Farley et al. (1988) suggested that *Mikrocytos* spp. (including *Mikrocytos roughleyi*, a parasite of *Saccostrea commercialis* in Australia; Farley et al. 1988) and *Bonamia* spp. are closely related, all species being small (<5 µm) intracellular oyster parasites with eccentric, spherical nuclei. Hine et al. (2001), however, in transmission electron microscope images of *M. mackini*, found none of the haplosporosome-like structures present in the cytoplasm of *M. roughleyi* and *Bonamia* spp. (Pichot et al. 1980, Dinamani et al. 1987, Farley et al. 1988), and nothing resembling mitochondria; *M. mackini* thus resembles the other species at the light microscope level only. Furthermore, *M. mackini* alone among these 'microcell' protistans demonstrates a tissue specificity primarily for vesicular connective tissue and heart and adductor muscle myocytes (Farley et al. 1988, Hervio et al. 1996, Hine et al. 2001); *M. roughleyi* and *Bonamia* spp. primarily parasitize oyster hemocytes (Balouet et al. 1983, Dinamani et al. 1987, Farley et al. 1988). Hine et al. (2001) concluded that *M. mackini* was not a Haplosporidian, the group to which *Bonamia ostreae*, based on ultrastructural (Perkins 1990) and SSU rDNA phylogenetic (Carnegie et al. 2000) analyses, is purported to belong. The *M. mackini* SSU rDNA sequence in this study could be aligned unambiguously with Haplosporidian SSU rDNA only at highly conserved SSU rDNA regions, and distance and parsimony analyses showed that these sequences were only distantly related. The conclusion that *M. mackini* is not a Haplosporidian (Hine et al. 2001) is thus supported by these data.

SSU rDNA phylogenetic analyses provided no support for a close relationship of *Mikrocytos mackini* to any other described species or taxa, and indicated instead that *M. mackini* may be an early-branching eukaryote basal to all or most described eukaryotic taxa. This result would support the suggestion that *M. mackini* is an ancient eukaryote that lacks mitochondria because it arose before the acquisition of mitochondria by eukaryotic cells (Hine et al. 2001). The Microspora, however, once considered basal eukaryotes, are now thought to be derived fungi, secondarily amitochondriate and with genomes secondarily reduced in size and complexity as adaptations for a parasitic existence (Keeling et al. 2000, Mathis 2000, Van de Peer et al. 2000). *Giardia* spp. may also be more recently evolved and secondarily reduced (Lloyd & Harris 2002). Thus, in the absence of supporting phylogenetic evidence from other genes, the conclusion that *M. mackini* is a basal eukaryote must be regarded with some caution.

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