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Sequence homogeneity of internal transcribed spacer rDNA in *Mikrocytos mackini* and detection of *Mikrocytos* sp. in a new location

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ABSTRACT: *Mikrocytos mackini* is a microcell parasite of Pacific oysters only known to occur on the Pacific coast of North America. It is the only described species in the genus, although a genetically divergent *Mikrocytos* sp. organism has been reported once in both the Atlantic Ocean and China. We developed methods for sequencing the internal transcribed spacer (ITS) of rDNA for the purpose of characterizing extant diversity within *M. mackini* throughout its known geographic range, and surveying for other evidence of *Mikrocytos* sp. organisms. Our specific aims were to examine relatedness of *M. mackini* among sites to make inferences about its recent evolutionary history, and to provide baseline data for future development of a species-specific molecular detection method. We found a total lack of genetic variation within *M. mackini* across the complete ITS1-5.8S-ITS2 array in over 70 samples collected throughout its range. We hypothesize that this could be a result of a founder effect if the parasite had been introduced into its known range alongside its host, which was imported from Asia beginning around 1914 to about 1961. We detected a single divergent sequence at a short stretch of 18S that was identical to the *Mikrocytos* sp. detected elsewhere, which adds to the recent and growing body of evidence that *Mikrocytos* is much more broadly distributed than the limited range of *M. mackini* suggests. A 1903 bp section of rDNA from *Mikrocytos* sp. was generated that contained regions of high divergence from *M. mackini* (in ITS1 and ITS2) that could be exploited for molecular diagnostics.

KEY WORDS: Microcell oyster parasite · Pacific oyster · Denman Island disease · Mikrocytosis · Genetic diversity · Internal transcribed spacer

INTRODUCTION

Several protistan parasites are known to induce serious mortality and cause substantial economic losses in commercially important oyster species. Among these are 2 genera of intracellular parasites called 'microcells', *Bonamia* and *Mikrocytos*, which have caused significant mortality to oyster populations around the world (Carnegie & Cochennec-Laureau 2004). Species of *Bonamia* are members of the eukaryotic phylum Haplosporidia (Carnegie et al. 2000); however, multiple lines of evidence suggest against a close relationship between *Mikrocytos* and *Bonamia* (Carnegie et al. 2003, Carnegie & Cochennec-Laureau 2004). The phylogenetic relationships of *Mikrocytos* are unknown.

Mikrocytos mackini causes mikrocytosis, or 'Denman Island disease', which is characterized by focal green lesions within the body wall or on the surface of the labial palps or mantle of infected oysters (Bower et al. 1994). Mortality rates in Pacific oysters *Crassostrea gigas* typically range up to about 30% (Quayle 1982), and non-lethal cases of the disease render oysters unmarketable (Bower et al. 2005). Long term annual survey data collected from Denman Island, British Columbia, Canada between 1960 and 1994 found that the prevalence of disease (characterized by typical

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green lesions) among Pacific oysters varied from 11 to 48% (Hervio et al. 1996). The known geographic range of *M. mackini* is restricted to British Columbia (BC) and adjacent areas in Washington State, USA, on the Pacific northwest coast of North America (Bower et al. 2005).

Mikrocytos mackini is the only described species in the genus. However, scant infections by a *Mikrocytos* sp. have been detected in European flat oysters *Ostrea edulis* collected in the Atlantic Ocean on the east coast of Canada (Gagné et al. 2008), and in Pacific oysters from the Yellow Sea, China (Wang et al. 2010). These 2 geographically disparate *Mikrocytos* sp. were identical to one another at a short fragment of 18S, and were 89% identical to *M. mackini.* The evolutionary history of *Mikrocytos* is entirely unknown. Phylogenetic analysis of 18S rDNA suggested that *M. mackini* is not closely related to any known protistan taxon and may be evolutionarily basal to other eukaryotes (Carnegie et al. 2003). However, a long-branch attraction artifact may explain this basal positioning; it can cause taxa to group together due to high evolutionary rates and convergent evolution and/or unequal taxon sampling (see review by Bergsten 2005). The tendency for outgroup taxa to form long branches creates a hazard towards misplacing long branched ingroup taxa as they are pulled towards the outgroup (Bergsten 2005). Although branch lengths are not shown in Carnegie et al. (2003), inspection of 18S alignments clearly show *M. mackini* to be exceptionally divergent from other protistan taxa.

The lack of information on the taxonomic affiliations of *Mikrocytos mackini* has presented challenges to the development of a specific molecular assay for detection purposes; the *Mikrocytos* sp. mentioned above cross-reacts with the PCR reaction originally designed by Carnegie et al. (2003) to be specific to *M. mackini* (Gagné et al. 2008, Wang et al. 2010). Although *M. mackini* was recently de-listed by the World Organisation for Animal Health's (OIE) Aquatic Animal Health Code, it continues to be subject to regulatory testing in Canada. Domestic disease control is considered important because of the susceptibility of commercially important *Crassostrea virginica* in Atlantic Canada and the interest in *C. gigas* aquaculture in northern BC, both being cool water regions where *M. mackini* is anticipated to be more pathogenic.

This paper describes a molecular diversity study of *Mikrocytos* across the only geographic area where it is reliably found using a newly-isolated genetic marker—the internal transcribed spacer regions (ITS1 and ITS2) and the intervening 5.8S gene of rDNA. ITS regions evolve faster than the neighboring 18S sequence (Hillis & Dixon 1991) and are therefore usually superior for characterizing intraspecific diversity. Also, if suitable conserved regions are present within the species, ITS regions are a good target for species level detection because they are expected to be more divergent from close relatives than the 18S sequence and therefore should be less susceptible to false positives. Indeed, molecular diagnostic assays based on ITS are abundant in the plant pathology and human medical literature (see review by Iwen et al. 2002), and have been developed for pathogens of aquatic species including bivalves (e.g. Audemard et al. 2004, Maloy et al. 2005, Liu et al. 2009), crustaceans (e.g. Small et al. 2007) and finfish (e.g. Chen et al. 2008). The specific aims of the present study were to characterize genetic diversity within *M. mackini* and to survey for the presence of other genetically divergent *Mikrocytos* sp. organisms that may co-occur in its endemic range. Information gained provides insight into possibilities for the recent evolutionary history of *M. mackini* and prerequisite data for the future development of improved molecular detection tools.

MATERIALS AND METHODS

Oyster sampling and histology. In the present study we used Pacific oyster *Crassostrea gigas* samples infected with *Mikrocytos mackini* or *Mikrocytos* sp. (see next paragraph) that were collected between 2002 and 2008 for a variety of purposes, including to address research questions about the life cycle and seasonality of *M. mackini*, and for routine disease surveillance. We analyzed 74 *M. mackini*-infected adult Pacific oysters and 1 *Mikrocytos* sp.-infected juvenile oyster (see next paragraph) obtained from 7 general locations along the south coast of BC, Canada, and from Puget Sound, Washington, USA (Table 1, Fig. 1). In one instance, tissues from 4 oysters were combined into a single tube of 95% ethanol for DNA analysis and it is unknown whether lesions were present. In all other cases, except for the oyster in which *Mikrocytos* sp. was found (see next paragraph), lesions that were suggestive of infection with *M. mackini* were observed in the mantle, labial palps and/or adductor muscle. Between 1 and 3 lesions from each of these oysters were excised and preserved in 95% ethanol. Thus, a total of 72 genetic samples were obtained for analysis from 75 oysters.

One oyster included in this study was sampled as a juvenile (~6 mo old) on the west coast of Vancouver Island, Canada (Site B, Fig. 1) and did not exhibit any lesions. The oyster was shucked and the anterior end (mantle, palps, and a small portion of digestive gland) was transferred to 95% ethanol. The rest of the oyster was pool fixed in Davidson's solution (Shaw & Battle 1957) with 2 to 3 other oysters for 24 to 72 h and processed using routine histological techniques. Histolog-

Table 1. Sampling locations, dates, and sample sizes of Pacific oysters infected with *Mikrocytos mackini* analyzed in the present study. Geographic coordinates are approximate only as multiple local sites were sampled within some sampling areas (see Fig. 1). n: no. of samples

Site	Sampling area	Latitude (°N)	Longitude (°W)	Month/year sampled (n)	Total n
A	Nuchatlitz Island	49.80	126.99	03/2002 (12), 04/2007 (1)	13
B	Lemmen's Inlet	49.18	125.88	02/2004 (4), 04/2004 (1), 09/2004 (1)[a], 04/2005 (16), 06/2007 (2)	24
C	Desolation Sound	50.06	124.89	11/2006 (1), 05/2007 (2), 03/2008 (5)	8
D	Nelson Island	49.70	124.19	06/2007 (4)	4
E	Baynes Sound	49.65	124.91	05/2007 (3), 05/2008 (6), 06/2008 (3)	12
F	Ladysmith	48.98	123.79	04/2008 (2), 05/2008 (2)	4
G	Puget Sound	47.87	122.60	05/2005 (4), 05/2007 (1), 04/2008 (5)	10
	Total				75

[a]Single sample containing *Mikrocytos* sp. from British Columbia, Canada (*Mikrocytos* sp.-BC), not *M. mackini*

ical examination of this sample was performed because the detected *Mikrocytos* 18S sequence was atypical (see 'Results'). Deparaffinized 5 µm thick tissue sections were stained with Harris's modified hematoxylin and 0.5% alcoholic eosin (H&E) and also stained using a digoxigenin-labeled DNA probe *in situ* hybridization (DIG-ISH) technique (Meyer et al. 2005). The probe was originally developed to be *M. mackini* specific (Carnegie et al. 2003), but its sequence also matches perfectly with the 18S sequence of the *Mikrocytos* sp. found in the study area.

PCR confirmation of *Mikrocytos mackini* infection. The PCR test for *Mikrocytos mackini* in the 18S sequence using primers Mikrocytos-F and Mikrocytos-R as described by Carnegie et al. (2003) was used to confirm presence of the pathogen in all samples included in the present study. Reaction conditions followed Carnegie et al. (2003) except that a 50 µl reaction volume was used. These fragments were sequenced as an early check for any divergent forms, as this PCR amplified *Mikrocytos* sp. parasites in earlier studies: herein referred to as '*Mikrocytos* sp.-Atl', to reflect its origin in *Ostrea edulis* from the Atlantic Ocean (Gagné et al. 2008), and '*Mikrocytos* sp.-YS', to reflect its origin in *C. gigas* from the Yellow Sea, China (Wang et al. 2010).

PCR primer development and sequencing of ITS-rDNA. DNA was extracted from Pacific oysters containing *Mikrocytos mackini* using a DNeasy Tissue Kit (Qiagen). The final volume of PCR reactions used was 10 to 25 µl and each reaction contained 5 to 50 ng total DNA, 200 µM dNTP, 0.2 µM primers (forward and reverse), and 0.5× TITANIUM™ *Taq* DNA Polymerase (Clontech Laboratories) in 1× PCR amplification buffer. Thermocycler conditions were 3 min at 95°C; a 'touchdown' step for 5 cycles of 30 s at 95°C, 30 s at 65°C to 53°C (dropping 3°C per cycle), 2.5 min at 72°C; 30 cycles of 30 s at 95°C, 30 s at 50°C, 2.5 min at 72°C; and one final cycle of 3 min at 72°C. PCR products were visualized on 1.5% agarose gels stained with SYBR Safe (Invitrogen) and, when appropriate, 5 µl of each was purified with ExoSAP-IT (USB Corporation) for sequencing. Sequencing reactions used Applied Biosystems' BigDye Terminator v3.1 with 1 µl of purified PCR product and 1.6 pmol of primer in a 10 µl reaction volume. Sequencing reactions were purified using the Dye EX 2.0 Spin Kit (Qiagen) and run on an ABI 3130*xl* genetic analyzer.

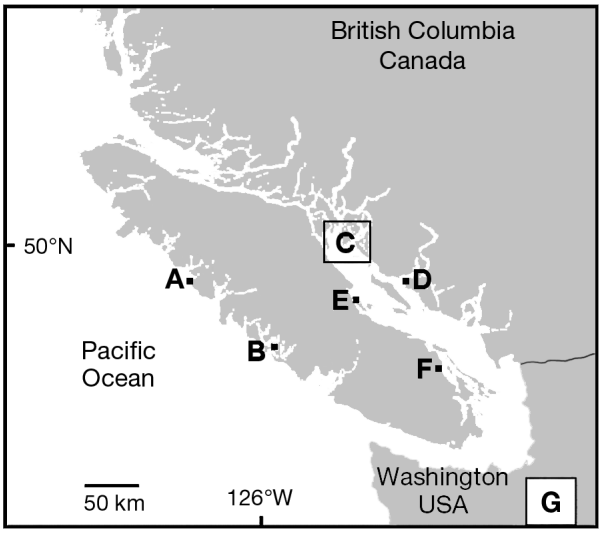

Fig. 1. Approximate locations of sampling sites in British Columbia, Canada (A to F), and Puget Sound, Washington, USA (G), of Pacific oysters *Crassostrea gigas* infected with *Mikrocytos mackini* analyzed in this study. Oysters sampled at Sites C and G were sampled from within the squares shown. Samples from other sites (■) were less geographically dispersed. Sample sizes and dates are listed in Table 1. The single *Mikrocytos* sp. sample found in British Columbia (*Mikrocytos* sp.-BC) was collected at Site B

Sequence, target organism or group, and citation information for all primers used in this study are listed in Table 2. Primers to amplify ITS in *Mikrocytos mackini* were developed *de novo* as they were not available

Table 2. Primers used in this study to amplify rDNA from *Mikrocytos mackini* (Mm) and *Mikrocytos* sp.-BC from British Columbia (M. sp.). rDNA location was determined based on the full length *M. mackini* consensus rDNA sequence (see 'Results'). Primers were designed in this study except as otherwise indicated. See Fig. 2a for map of primer locations

Primer	Sequence (5′– 3′)	Specificity	Location (bp)	Fig. 2a label
Mm18S_120F	AAAGGGGGATATCCACGGTA	Mm (M. sp. unknown)	67–86	1
Mikrocytos-F[a]	AGATGGTTAATGAGCCTCC	Mm/M. sp.	318–336	2
Mm18SF1	GACGGCAGGAGTATTGTTTGACGA	Mm/M. sp.	682–705	3
Mikrocytos-R[a]	GCGAGGTGCCACAAGGC	Mm/M. sp.	847–863	4
Mm18SF3	AGAGTATGCCCGCAAGAGTGAAAC	Mm/M. sp.	923–946	5
Mm18S_1128F	TGGTTAATTCCGGTAATCG	Mm/M. sp.	1132–1150	6
Mm18S_1403R	GAGACGCGCTTACGAGAAG	Mm/M. sp.	1381–1399	7
Mm18S_1450R	TGACGGACAGTGTGAACAAGTC	Mm/M. sp.	1427–1448	8
Mm18S_1435F	GTTCACACTGTCCGTCATACAC	Mm/M. sp.	1432–1453	9
BCF1a	CTTCTGTGCTCTAGCGATAGTG	M. sp.	~1487–1508	10
18S-EUK1776-R[a]	CGGAAACCTTGTTACGAC	Universal non-metazoan	1547–1564	11
Mm28SR2	CCTAGGGACACCACAACTCTC	Mm/M. sp.	2006–2026	12
Mm28SR1	CGGTTTAGTCAGCCTTCACAG	Mm/M. sp.	2075–2095	13
Mm28SR3	CCAACCACTGCTACTCATACC	Mm/M. sp.	2134–2154	14
Mm28SR5	AGACATACGTTTCTTTAGGTG	Mm/M. sp.	2184–2024	15
pro28S-R	TACTTGTTYGCTATCGGTCTC	Universal protistan	2027–2227	16

[a]From Carnegie et al. (2003)

from the literature. A 'universal' protozoan reverse primer (pro28S-R) that excludes bivalves was designed near the 5′ end of 28S using an alignment of a taxonomically broad set of protozoan and oyster sequences from GenBank. The whole ITS region for *M. mackini* was amplified as a single fragment by using pro28S-R with a forward primer at the 3′ end of the 18S sequence (Mm18S_1435F). Sequencing with PCR primers confirmed that the ~800 bp product indeed represented the target region; the 5′ end matched identically with the published partial 18S gene for *M. mackini* (GenBank accession no. AF477623). This sequence allowed primers putatively specific to *M. mackini* to be designed in 28S (see next paragraph).

The complete ITS region between 18S and 28S genes was amplified and sequenced in *M. mackini* samples using rDNA primers designed to be specific to the parasite to preclude amplification of host material (Table 2). When possible, a ~2000 bp fragment was amplified using forward primer Mm18S_120F, positioned 67 bp from the 5′ end of the published partial 18S gene for *M. mackini* from GenBank (AF477623) and a reverse primer near the 5′ end of the 28S gene (Mm28SR1). For sequencing, 3 internal primers were used to 'walk-through' the fragment: Mm18S_1403R, Mm18S_1128F, and Mikrocytos-F. When amplification of the 2000 bp fragment was problematic, a ~700 to 800 bp fragment was generated instead, using Mm18S_1435F paired with either an alternative specific reverse primer (Mm28SR3) or pro28S-R, both located near the 5′ end of the 28S sequence. These shorter products were sequenced using PCR primers only.

Early screening for divergent *Mikrocytos* sp. organisms using PCR, as described above, uncovered one such sample, hereafter referred to as '*Mikrocytos* sp.-BC', to reflect its collection location in BC, Canada. The rest of the 18S gene and ITS was amplified and sequenced in this sample using PCR primers different from those used for *M. mackini*. Forward primer Mm18SF1 was designed near the 3′ end of the Mikrocytos-F/Mikrocytos-R product, and paired with 'universal' reverse primer 18S-EUK1776-R. This ~900 bp fragment was sequenced using internal primers Mm18SF3 and Mm18S_1450R. To amplify and sequence ITS, forward primer BCF1a, located near the end of the 18S gene and designed to be specific to *Mikrocytos* sp.-BC, was paired with either of 2 reverse primers in the 5′ end of the 28S sequence: Mm28SR2 or Mm28SR5. Sequencing of ITS in this sample used PCR primers. As the 18S sequence for this sample turned out to be identical to the 486 bp available for *Mikrocytos* sp.-Atl (see 'Results'), we tried without success to extend the available sequence for the latter. We attribute the PCR failures to the low quality of the old DNA sample.

Sequence analysis. Sequence data were viewed and edited using Sequencher v4.8 (Gene Codes). An alignment of 18S-28S for *Mikrocytos mackini* and *Mikrocytos* sp.-BC was done in BioEdit (Hall 1999) using the Clustal W multiple alignment option with default settings and was checked by eye. We tried to annotate these sequences with approximate start/stop points of the various rDNA regions. MegAlign (Lasergene; DNASTAR) was used in attempts to align *Mikrocytos* sequences with GenBank sequences from all 5 eukary-

otic supergroups described by Keeling et al. (2005), because the taxonomic affiliations of *M. mackini* are unknown. This was not highly successful, as high divergence made these alignments difficult. As such, we relied heavily on information gleaned from divergence levels between *M. mackini* and *Mikrocytos* sp.-BC along the length of the entire sequence to putatively assign approximate start/stop points for some rDNA regions, as evolutionary rates are known to vary among the various rDNA regions (see 'Discussion'). For regions of good alignment, sequence divergence between *M. mackini* and *Mikrocytos* sp.-BC was summarized using percent similarity, which was calculated in BioEdit and represents the number of variable bases (including gaps) divided by the total number of bases sampled.

RESULTS

rDNA sequencing and analysis

In this study, we developed methods to amplify and sequence the rDNA region between the 18S and 28S genes in *Mikrocytos mackini* and a newly-sampled *Mikrocytos* sp.-BC organism, using many newly-developed primers (Table 2, Fig. 2). For *M. mackini*, these data extend the published sequence of 1457 bp of the 18S gene (Carnegie et al. 2003, GenBank accession no. AF477623) by a further 749 bp. Although annotation was difficult (described in next paragraph), this additional 749 bp spans from the 3′ end of the 18S to the 5′ end of the 28S sequence and hence contains the ITS1-5.8S-ITS2 region, hereafter called the 'ITS array'. A total of 71 *M. mackini* samples were isolated and sequenced from 74 oysters (due to pooling in 1 case as described in 'Materials and methods', and that 1 oyster contained *Mikrocytos* sp.-BC and not *M. mackini*). Of these samples, 26 were sequenced at ca. 1500 to 2000 bp, using forward primers Mm18S_120F or Mikrocytos-F with reverse primers Mm28SR1 or Mm28SR3 (Table 2, Fig. 2). The remaining 45 samples were sequenced at ca. 700 bp from the end of the 18S gene to the start of the 28S gene, using forward primer Mm18S_1435F with Mm28SR1 or Mm28SR3 (Table 2, Fig. 2). Hence, the whole ITS array was sequenced in all 71 samples; however, no variable positions were found. A reference sequence for the 2107 bp of rDNA generated here for *M. mackini* from genes 18S to 28S was deposited in GenBank (accession no. HM563060).

This study is the third incidence of an organism similar to but genetically divergent from *Mikrocytos mackini*. The first, called *Mikrocytos* sp.-Atl here, was reported by Gagné et al. (2008), and the second, called *Mikrocytos* sp.-YS here, was reported by Wang et al. (2010). For our newly discovered *Mikrocytos* sp.-BC sample, we sequenced a total of 1903 bp (GenBank accession no. HM563061) that span the majority of the 18S gene, the entire ITS array, and into the 28S gene. This sequence was compared with a shorter 18S sequence for *Mikrocytos* sp.-Atl from GenBank (accession no. DQ237912), which Wang et al. (2010) reported to be 100% identical to the 18S sequence for *Mikrocytos* sp.-YS. This yielded a 486 bp alignment of 18S (excluding priming sites), and no variable bases were found.

Annotation was tried to allow divergence estimates to be made for individual rDNA regions as evolutionary rates are known to vary among them, but this was hampered by difficulties aligning *Mikrocytos*

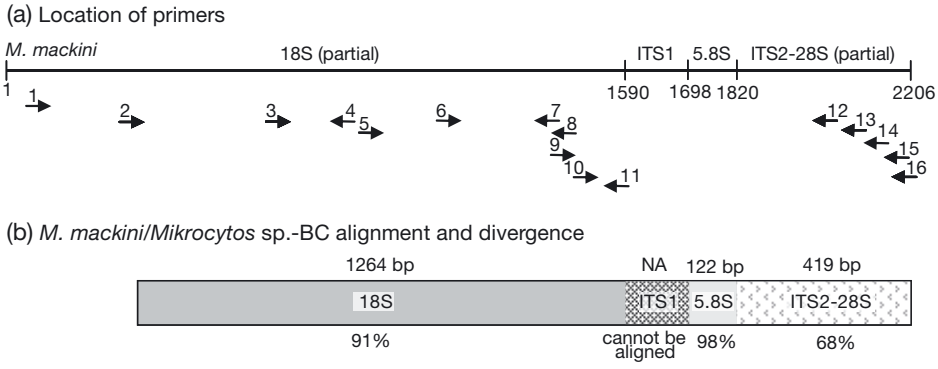

Fig. 2. *Mikrocytos mackini* and *Mikrocytos* sp. Schematic depiction of (a) annealing locations of primers (shown as numbered arrows) in relation to *M. mackini* rDNA, with full primer details given in Table 2; and (b) the length of the *M. mackini* and *Mikrocytos* sp.-BC (*Mikrocytos* sp. from British Columbia) sequence alignment for each rDNA region and percent identity estimates. NA: length of the ITS1 alignment is not available because this region could not be aligned. The first 99 bp of *M. mackini* sequence depicted in (a), including the sequence used to design primer 1, were from GenBank (accession no. AF477623); the remaining 2107 bp were generated in this study and have been deposited in GenBank (accession no. HM563060). Lengths of alignments depicted here are drawn approximately to scale; accurate lengths of rDNA regions for *M. mackini* and *Mikrocytos* sp.-BC are given in 'Results'

sequences with those from other protists listed in GenBank. However, in most cases, junctions between regions of high versus low sequence homology between *M. mackini* and *Mikrocytos* sp.-BC were very clear and when considered as possible start/stop points of the rDNA regions; sections were putatively identified as the 18S gene, ITS1 and 5.8S gene of lengths and relative levels of sequence diversity consistent with those found in the literature. Details about this annotation and associated descriptive statistics on divergence between *M. mackini* and *Mikrocytos* sp.-BC are presented here, but we suggest that the annotation be considered tentative only.

Substantial levels of divergence were observed between rDNA sequences generated for *Mikrocytos mackini* and *Mikrocytos* sp.-BC. The full alignment of available rDNA sequence data for each organism from the 18S to the 28S gene was of length 2298 bp; details on lengths and divergence of each rDNA region are summarized in Fig. 2. The 18S sequences for *M. mackini* and *Mikrocytos* sp.-BC were 1253 and 1238 bp, respectively, and were 91% identical. The much shorter ITS1 regions were 108 and 159 bp for *M. mackini* and *Mikrocytos* sp.-BC, respectively, but were too divergent to be aligned. The 5.8S sequence was 122 bp in both organisms and had 3 variable bases with no gaps, which corresponds to an identity of 97.5%. The junction between the ITS2 and 28S sequence was not distinguishable (see 'Discussion') and this precluded separate divergence estimates for these 2 regions. The ITS2-28S fragments were similar in length for *M. mackini* and *Mikrocytos* sp.-BC, at 387 and 384 bp, respectively, but were only 68% similar. The first 86 bp after the end of the 5.8S gene, which therefore represents the first portion of ITS2, were highly variable, containing 54 bp from *M. mackini* and 86 bp from *Mikrocytos* sp.-BC with 44% similarity.

Visualisation of *Mikrocytos* sp.-BC

Visual evidence of the presence of parasites was not found upon histological examination of the H&E stained tissue sections from the single *Mikrocytos* sp.-BC sample. DIG-ISH staining was conducted twice and results were inconclusive both times.

DISCUSSION

During our study on rDNA sequence diversity targeting *M. mackini* infecting Pacific oysters on the northwest coast of North America, a divergent *Mikrocytos* sp. organism was detected by PCR and sequencing that has not been previously reported from this geographic region. Although our ability to characterize this parasite was severely limited by our lack of success in visualizing it using histology or *in situ* hybridization, the fact that we were able to sequence a much larger portion of the rDNA cistron (1903 bp from the 18S to 28S gene)—compared to previous findings of *Mikrocytos* sp. organisms (486 bp of 18S; Gagné et al. 2008, Wang et al. 2010)—allows some preliminary investigation into its evolutionary relationship to *M. mackini*. The use of 18S rDNA sequence data for phylogenetic studies of eukaryotic taxa has been prolific since the 1980s (cf. Hillis & Dixon 1991), leading to the accumulation of a wealth of publicly available data for this genetic region. Although not universally accepted among taxonomists, 18S sequence data have been used extensively to help delineate species boundaries in protistan groups with few or conserved morphological features (Caron et al. 2009 and references therein). In analyses involving ca. 2500 pairwise comparisons of complete 18S sequences from a taxonomically broad range of morphologically defined protistan species, Caron et al. (2009) calculated an average inter-specific and intra-specific sequence similarity of 87 and 98%, respectively. Hence the 91% sequence similarity observed here between *M. mackini* and *Mikrocytos* sp.-BC in the 18S sequence is consistent with inter-specific divergence levels within other protistan genera.

Three geographically disparate *Mikrocytos* sp. samples have been reported to date; in addition to the one found here, one was found in the Atlantic Ocean (Gagné et al. 2008) and another in China (Wang et al. 2010). The short (ca. 500 bp) stretch of 18S rDNA sequence data available for each of these samples is identical; however, these data are insufficient to conclude that the organisms themselves are indeed genetically identical. Sequencing a longer portion of rDNA including both the 18S gene and the more quickly-evolving ITS regions would be more informative and so is recommended here for future characterization of *Mikrocytos* sp. organisms. As *M. mackini* is the only described species in the genus and has only been detected in the geographic range sampled here, the apparently widespread occurrence of *Mikrocytos* sp. organisms is all the more intriguing. The facts that (1) protistan diversity is generally very high; (2) the small size of microcell oyster parasites makes them very difficult to detect visually; and (3) *Mikrocytos* is not closely related to other known shellfish pathogens, making its incidental detection during molecular surveys for other taxa unlikely, all combine to make new *Mikrocytos* discoveries unlikely events.

Mikrocytos mackini is typically detected only in oysters over 2 yr of age between the months of March and May (Bower 1988, Quayle 1988). Indeed, all but one of

the 71 *M. mackini* samples analysed here came from adult oysters within the expected seasonal range, the exception being a single sample collected in the month of November. Interestingly, the *Mikrocytos* sp.-BC organism was detected in 1 sample derived from a batch of 60 juvenile oysters that had been sampled opportunistically during an unrelated study, and were sampled outside of the normal *M. mackini* season (mid-September).

Evolutionary rates among the different regions within the rDNA cistron are known to vary, with rRNA coding regions 18S, 5.8S, and 28S being more conserved overall than non-coding internal transcribed spacer regions (Hillis & Dixon 1991). However, among the coding regions, evolutionary rates within the 28S gene are more variable than within 18S and 5.8S genes due to the presence of several divergent domains (Hillis & Dixon 1991); and, within ITS2, there are 'subregions' that are more conserved (Coleman 2003 and references therein). Variable evolutionary rates within ITS2 and 28S are a likely explanation for our inability to annotate the junction between these 2 regions in our *Mikrocytos* sequences, as we relied heavily on divergence levels between *M. mackini* and *Mikrocytos* sp.-BC to substantiate our annotation because alignments with other taxa were too ambiguous. These variable rates presumably obscured the boundary between the generally less-conserved ITS2 and the generally more-conserved 28S regions. Indeed, the first divergent domain in the 28S sequence ('D1') occurs within ca. 100 to 200 bp of its start (Hassouna et al. 1984) and hence will have been included in our sequences here.

We found a complete absence of genetic variation at rDNA in *Mikrocytos mackini* although the whole ITS array was analyzed in a large number of infected oysters collected from across its known geographic range. Some degree of variation at ITS below species level is common; as noted earlier, ITS evolves more quickly than the 18S gene (Hillis & Dixon 1991). In an analysis of over 4000 fungal species each represented by an average of only 7 sequences, Nilsson et al. (2008) found an overall average variability at ITS of 2.5%, and that intraspecific variation was present in almost 80% of species analyzed. Unfortunately, the lack of ITS variability found in *M. mackini* in the present study does not provide sufficient evidence to make inferences about its mode of reproduction, which along with most other aspects of its life cycle is not well understood. Distinguishing sexual versus asexual reproduction requires estimates of linkage disequilibrium, using data from several gene regions (cf. Tibayrenc & Ayala 2002, e.g. Morehouse et al. 2003); low variation itself does not indicate clonality.

Evolutionary theory predicts that the highest levels of neutral genetic diversity within a species will occur within its geographic centre of origin, where genetic changes have been accumulating for the longest evolutionary time period. One hypothesis to account for the absence of rDNA diversity in *M. mackini* is that the coalescent time of the entire sample is relatively recent, possibly due to an introduction event followed by dispersal throughout its known range over too short a time period for molecular changes to have accumulated via either mutation or recombination. This hypothesis seems highly plausible for multiple reasons. First, the only known wild host species infected by *M. mackini* is the Pacific oyster, which is native to Asia and was first imported to BC around 1914 (Waldichuk et al. 2010). A founder effect associated with the introduction could have limited genetic diversity throughout its current known range. Second, that *M. mackini* has not been reported in Asia does not necessarily imply its absence. Detection is very difficult, as explained earlier for *Mikrocytos* in general, and the temperature dependence of *M. mackini* infections (Hervio et al. 1996, Bower et al. 1997) means that it could persist at sub-clinical levels in regions with warmer water temperatures than those found in its known range. Last, the recent first report of *Mikrocytos* sp. in China (Wang et al. 2010) establishes that the genus is indeed present in Asia.

An alternative hypothesis for sequence homogeneity of *Mikrocytos mackini* at ITS is that it is a true reflection of extant diversity; the species as a whole is indeed depauperate in ITS variation. The plausibility of this is supported by the 22% of fungal species that are invariable at this region (Nilsson et al. 2008), but note that this may be a substantial overestimate as an average of only 7 sequences were analyzed per species. Further, Pacific oysters from Asia were imported to BC repeatedly between 1914 and 1961, which may have provided ample opportunity for the introduction of multiple strains or clones of *M. mackini*; however, the likelihood of post-introduction establishment and subsequent spread is unknown. Multiple mechanisms causing extremely low ITS diversity in a benthic foraminifer (*Virgulinella fragilis*) on a global scale are discussed by Tsuchiya et al. (2009) and include rapid dispersal abilities, a zero fixation rate for new mutations, and a cryptically ubiquitous global population.

Results presented here invite multiple avenues for future research on *Mikrocytos mackini*. We observed multiple segments of extensive rDNA sequence divergence between *M. mackini* and *Mikrocytos* sp.-BC that could be exploited for the development of a specific molecular detection method for *M. mackini*. Most notable were the entire ITS1 and the 5′ end of ITS2. Surveying for the presence of this parasite in native populations of Pacific oysters in Asia using molecular detection methods might reveal as-yet undiscovered populations. Finally, the isolation of additional genetic regions of *M. mackini* could

further our knowledge of this parasite in several research areas, including its phylogenetic placement, mode of reproduction, and method of transmission.

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