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Molecular characterisation of a haplosporidian parasite infecting rock oysters *Saccostrea cucullata* in north Western Australia

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Abstract

A haplosporidian parasite was identified in rock oysters (*Saccostrea cucullata* Born, 1778) from the Montebello Islands (latitude −20.4′S longitude 115.53′E) off the northern coast of Western Australia by histopathological examination, PCR amplification and DNA sequencing of a segment of the SSU region of the parasite's rRNA gene. An oligonucleotide probe was constructed from the parasite's SSU rRNA gene in order to confirm its presence by *in situ* hybridisation. The parasite was disseminated throughout the gonad follicles of the host and to a lesser extent in the gills. The only parasite life stages thus far observed in this study were a uninucleate naked cell assumed to be a precursor to multinucleate plasmodial stages and a binucleate plasmodial stage. Whilst no parasite spores were detected in affected rock oysters, a phylogenetic analysis of the SSU region of the parasite's rRNA gene indicates the parasite belongs to the genus *Minchinia*. A PCR and *in situ* hybridisation assay for the *Minchinia* sp. was used to identify haplosporidians described by Hine and Thorne [Hine, P.M.., Thorne, T., 2002. *Haplosporidium* sp. (Haplosporidia: Haplosporidiidae) associated with mortalities among rock oysters *Saccostrea cucullata* in north Western Australia. Dis. Aquat. Organ. 51, 123–13], in archived rock oyster tissues from the same coastline.

Keywords: Haplosporidia; *Saccostrea cucullata*; *Minchinia*; Rock oyster; SSU rRNA gene; Parasite; Aquaculture; *In situ* hybridisation

1. Introduction

Haplosporidians are a phylum of obligate protozoan parasites, infecting several invertebrate groups. Haplosporidians can be amongst the most pathogenic of all bivalve pathogens particularly where naïve hosts are exposed to the parasite. They have received considerable attention since they are known to infect several commercially important bivalve species.

A *Haplosporidium* sp. parasite has previously been associated with mortality (up to 80%) in rock oysters (*Saccostrea cucullata*) in Western Australia (Hine and Thorne, 2002). Attempts to diagnose the parasite following the development of a specific probe for *Haplosporidium nelsoni* were unsuccessful (Hine and Thorne, 2002). Rock oysters (or milky oysters) are currently commercially harvested only in the Australian state of Queensland and this parasite would probably be a major impediment to the development of tropical rock oyster aquaculture in northern Western Australia (Anon, 2004).

The development of molecular tools provides a means to detect the cryptic life stages of haplosporidian parasites and to identify them. The small ribosomal subunit (SSU) region of the rRNA gene can be used for this purpose since it has both conserved and variable regions. This study describes the detection of a haplosporidian parasite in rock oysters from north Western Australia by successful amplification of the SSU region of the parasite's

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rRNA gene and provides evidence of the identity of this parasite by using *in situ* hybridisation (ISH). The parasite detected is compared to the parasite described by Hine and Thorne (2002) using the molecular tools developed in this study.

2. Materials and methods

2.1. Oyster sampling

Adult rock oysters ($n = 26$) were obtained from the Montebello Islands (latitude −20.4′S longitude 115.53′E) off the north-west coast of Western Australia in July 2005. Oysters were sampled from locations that appeared to be undergoing higher mortality than surrounding sites. Each oyster was processed by removing the soft tissue and cutting it into two pieces along the sagittal plane. A sagittal section was used so that a piece of gill tissue and digestive organ were present in each oyster section. Accordingly, half of each oyster was collected into 10% sea water buffered formalin and allowed to fix for 24 h before being transferred into 70% ethanol. This tissue was processed for paraffin embedding and histological sectioning using conventional histological techniques. The other half of each oyster was collected into 100% ethanol for genomic DNA extraction and subsequent PCR analysis. By using this method it was possible to correlate histologically positive results with PCR and ISH test results. In order to assess the specificity of the ISH assay unstained *Haplosporidium costale* and *Minchinia teredinis* sections were obtained from the Virginia Institute of Marine Science (VIMS). Representative histologically positive formalin fixed paraffin embedded rock oysters infected with the parasite described by Hine and Thorne, 2002 were obtained from the Western Australian Department of Fisheries for DNA extraction and ISH.

2.2. DNA extraction

Two different methods of DNA extraction were used. Genomic DNA from 26 ethanol stored rock oysters was extracted from approximately 5 mg of gill tissue using a Masterpure™ DNA purification kit (Epicentre Technologies, Sydney) and was performed according to the manufacturer's protocol.

2.3. DNA extraction: archived samples

Genomic DNA was extracted from the formalin fixed paraffin embedded rock oysters by following a freeze thaw extraction method adapted from Sydney University's (EMAI) Johne's Disease Laboratory Manual under "Preparation of paraffin embedded tissues for PCR for *Mycobacterium paratuberculosis*". The procedure was performed as follows. For each sample three 12 μm sections were cut from the block and placed in a sterile 1.5 ml centrifuge tube. Considerable care was taken to ensure that no DNA could be transferred between samples with each block being processed on separate occasions and new sterile blades being used. The tube was centrifuged at 16,100*g* for 1 min in order to pellet the tissue. Two hundred microliters of sterile water with 0.5% v/v Tween 20 was added followed by 10 min boiling. The sample was then frozen in liquid nitrogen for 10 min. The freeze thaw process was repeated another two times and followed by a 20 min centrifuge at 4500 rpm. The supernatant was removed to a fresh 1.5 ml eppendorf. Another eppendorf containing a 1:5 dilution of the extract was also produced. The quality of the genomic DNA and absence of inhibitory factors from the extractions was verified with bivalve primers 16 R3 5′-GCT GTT ATC CCT RNR GTA-3′ and Proto 16′F 5′-AWK WGA CRA GAA GAC-3′ (Chase et al., 1998).

2.4. Amplification by polymerase chain reaction

Originally, degenerate haplosporidian primers (Hap F1 and Hap R3; Table 1) were used in the polymerase chain reaction (PCR; Mullis, 1983) to detect a haplosporidian species in ethanol preserved rock oyster tissue. These primers amplify a 344 bp section of the parasite's small subunit (SSU) region of the rRNA gene and were chosen as previous studies, such as those of Renault et al. (2000), have used this primer set to amplify DNA from undescribed haplosporidians. *Haplosporidium nelsoni* DNA was used as a positive control in the initial PCRs.

Each PCR was performed using reaction mixtures that had a total volume of 50 μL and contained reaction buffer (67 mM Tris–HCl, 16.6 mM $[NH_4]_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin, and 0.2 mM dNTP's), 2 mM of $MgCl_2$, 40 pmol of each primer, and 0.55 U of *Taq* polymerase and template DNA. Each of the reaction mixtures was

Table 1
Polymerase chain reaction primer sequences employed in the study

Primer	Sequence (5′–3′)	Position	Reference
Hap F1	gttctttcwtgattctatgma	1144	Renault et al. (2000)
Hap R3	akrhrttcctwgttcaagayga	1467	Renault et al. (2000)
Minch F1B	ctcgcgggctcagctt	1273	Present paper
Minch R2B	ggcgctttgcagattcccca	1417	Present paper
FSSUF	ctcaaagattaagccatgcatgtccaagtata	1[a]	Present paper
16 sb	gatcccttccgcaggttcacctac	1682	Medlin et al. (1988)
In situ probe: SSR 69	agcccaaaaccaacaaaacgtccacatgcg	757	Present paper

[a] FSSUF primer starts at base 15 of most haplosporidian sequences in GenBank.

subjected to: (i) an initial denaturation phase of 5 min at 94 °C, (ii) 35 amplification cycles, each cycle consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 48 °C, 4 min of extension at 68 °C and (iii) a final 7 min extension at 68 °C.

The success of each PCR was determined by loading a 10 μL aliquot of the PCR products on a 2% agarose gel and electrophoresing it for 20 min at 46 mA. Detection of the PCR products was performed using ethidium bromide staining.

2.5. DNA sequencing

The PCR products were cloned into a plasmid vector PCR2.1 using the TA cloning kit (Invitrogen Life Technologies) following the manufacturer's protocol. Three cloned DNA inserts were sequenced using an ABI 377 XL Automated Sequencer (Applied Biosystems, 2001) using Big Dye 3.1 sequencing chemistry according to manufacturer's protocol. Forward and reverse sequences were aligned and any alignment errors or base misreads were corrected by comparing to the alternative sequence. The primer sequences were removed from each end of the resulting consensus sequence. The position and direction of the rRNA PCR products were verified by BLAST searches of the GenBank database: (http://www.ncbi.nlm.nih.gov/blast/).

Sequences were aligned for further primer development to host 18 s and other haplosporidian SSU sequences using the CLUSTALW algorithm within the MEGA 3.1 software. As a result, synthetic oligonucleotides were designed to more precisely target the haplosporidian parasite DNA in PCR. These primers were designated Minch F1B and Minch R2B (Table 1) and amplify a 166 bp region of the SSU rRNA gene sequence. The Minch primers were used to amplify parasite DNA from histologically positive formalin fixed rock oyster samples containing the parasite described by Hine and Thorne (2002). The PCR products of four reactions were cloned and sequenced using the method outlined above.

The remaining SSU sequence of the rRNA gene was obtained by (i) using a new forward primer (FSSUF) for the sequence at the 5′ end of the SSU rDNA paired with the Minch R2B primer and (ii) pairing the Minch F1B

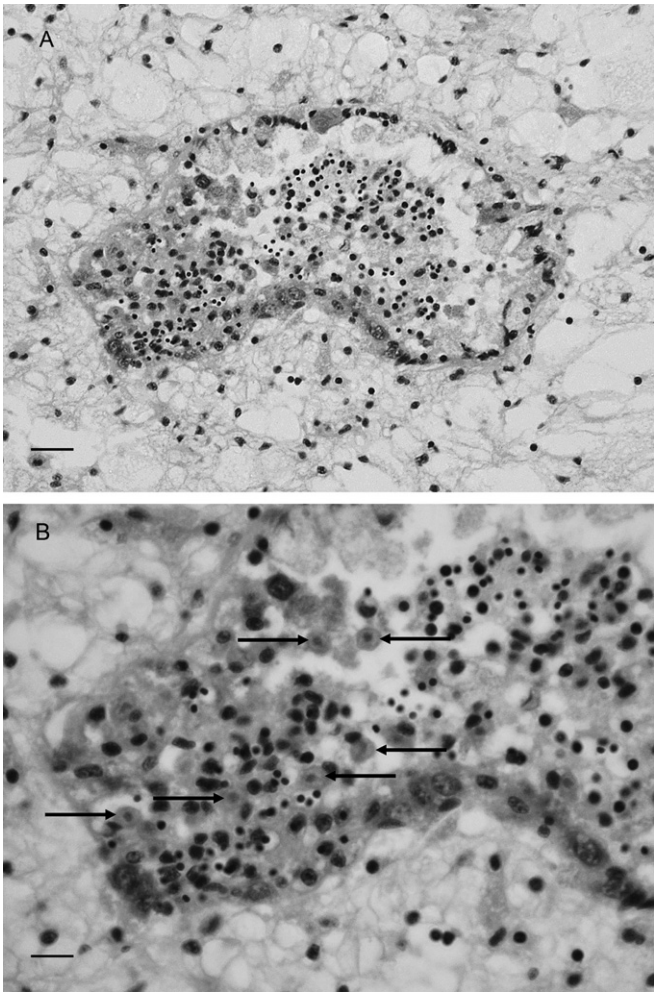

Fig. 1. (A) Rock oyster reproductive tissue containing haplosporidian parasites in a hematoxylin-eosin stained section. Scale bar = 25 μm. (B) Higher magnification view of the same section. Parasites are identified with an arrow. Scale bar = 15 μm.

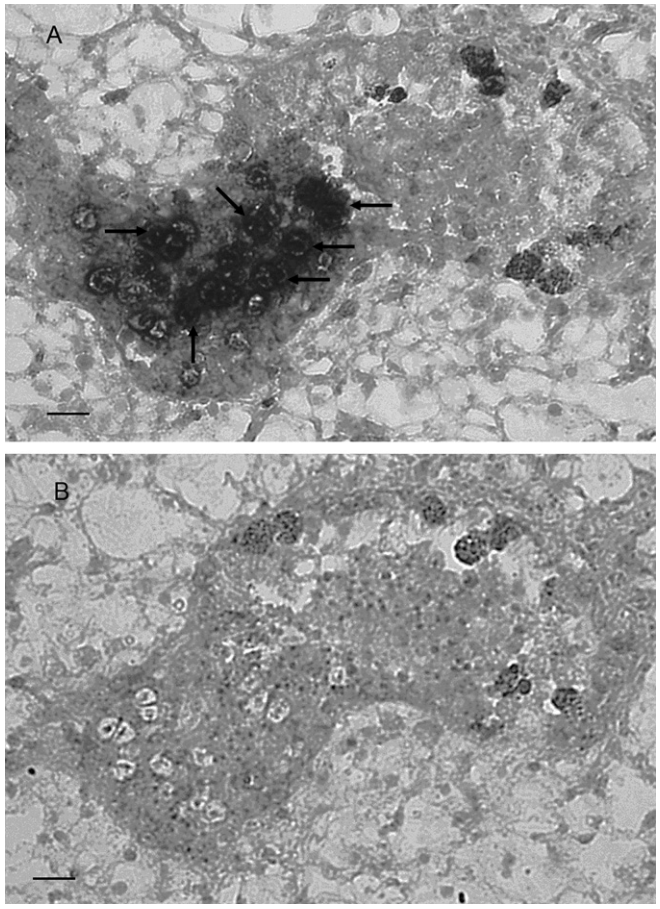

Fig. 2. (A) Haplosporidian parasites (arrows) identified in an *in situ* hybridisation of rock oyster gonad follicles. Parasites are identified by a darker colouration and are indicated by an arrow. (B) Negative control serial section from the same *in situ* hybridisation. Sections are counterstained in a Brazilian hematoxylin. Scale bars = 10 μm.

primer with the 16 s universal eukaryotic SSU rRNA gene primer for the sequence at the 3′ end (Medlin et al., 1988). The sequence generated in these reactions was compared to the overlapping sequence obtained from the HAP PCR reactions to ensure the correct organism and species was sequenced. Each of these PCR reactions was performed using the same method as for the HAP primers except the final concentration of $MgCl_2$ in the reactions was lowered to 1.5 mM and the annealing temperature was 61 °C for the FSSUF/Minch R2B reactions and 48 °C for the Minch F1B/16 sb reactions. Extension temperatures were also raised to 72 °C. The success of each PCR reaction was determined using the same method described for the PCR utilising HAP primers. The cloning and sequencing of these PCR products was performed using the same method as the HAP primer reactions. The SSU rRNA gene sequence was obtained from the clones of three positive oysters each sequenced three times to give nine sequences. Each sequence was verified as belonging to a haplosporidian parasite using BLAST searches of the GenBank database.

2.6. In situ Hybridisation

In order to identify the parasite in histological sections, the SSU rRNA gene sequence obtained was compared to other haplosporidian sequences and a region with high sequence variability was identified. This region was used to design a 30 bp oligonucleotide probe for use with *in situ* hybridisation. The probe was labelled with digoxigenin at the 5′ end using a DIG Oligonucleotide Tailing Kit (Roche Diagnostics) according to the manufacturer's instructions. The probe was designated SSR 69 and its sequence is given in Table 1.

Labelling efficiency was assessed by spotting 1 μL serial aqueous dilutions of probe on a positively charged nylon membrane (Boehringer Mannheim). The DNA was fixed by baking at 120 °C for 30 min, washed in buffer 1 (0.1 M Tris pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, and 0.05% Triton X-100) for 2 × 5 min and incubated for 30 min in antibody (alkaline-phosphatase conjugated anti-digoxigenin Fab fragments (Roche Applied Science) diluted 1:5000 in buffer 1). After two 10 min washes in buffer 1, and 5 min equilibrium in buffer 3 (0.1 M Tris pH 9.5, 0.1 M NaCl, and 50 mM $MgCl_2$), the reaction was developed overnight in BM purple (Roche Applied Science).

Formalin fixed oyster tissue was embedded in paraffin blocks. Unstained sections were cut 6 μm thick and placed on aminoalkylsilane-coated slides. Sections were dewaxed with xylene and rehydrated in an ethanol series. They were then rinsed and immersed in pure water. The sections were digested in a Proteinase K solution (Proteinase K 0.5 μg/ml, 0.5 mM Tris–HCl, pH 7.6) for 45 min and immersed in pure

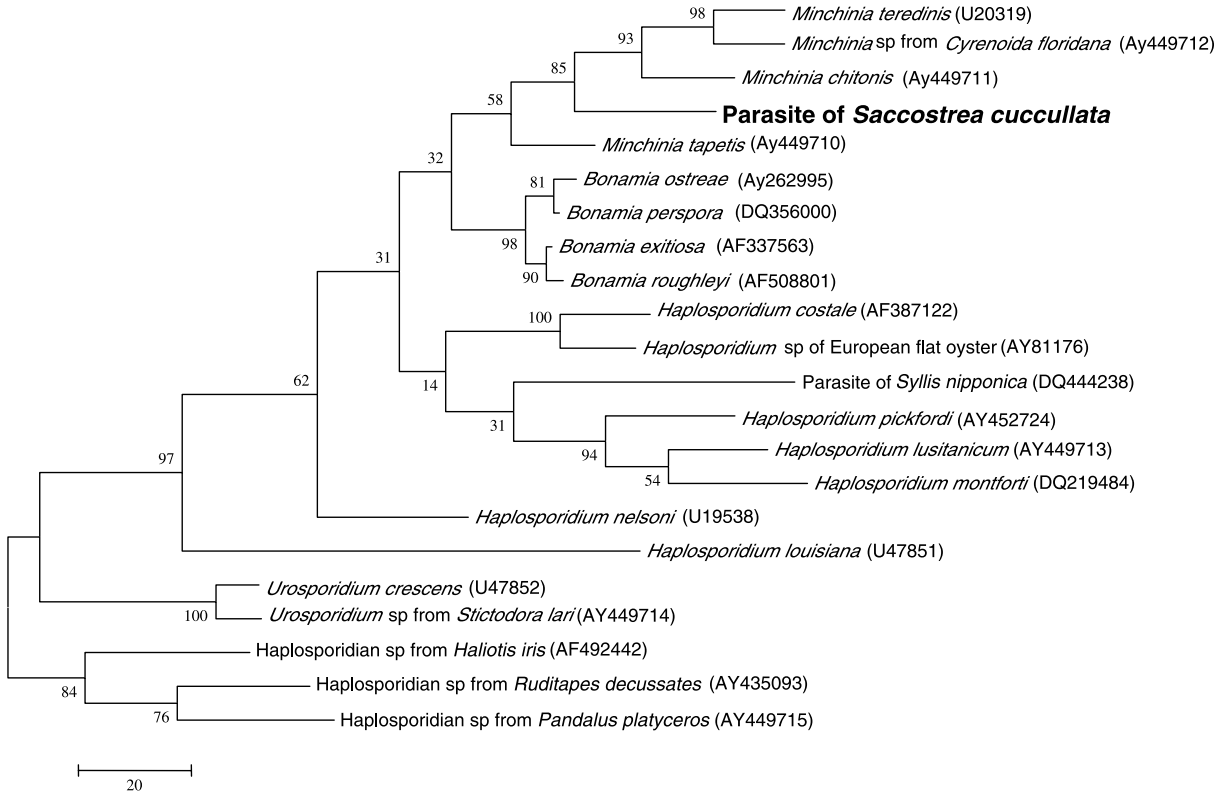

Fig. 3. A maximum parsimony tree illustrating the relationships of the SSU rRNA gene sequences within phylum haplosporidia including the rock oyster parasite. The analysis was conducted using 2218 aligned nucleotide positions ith the previously reported basal haplosporidians as an outgroup. The analysis was supported by 300 replicate bootstrap analysis. Numbers at nodes are percentages out of bootstrap analysis. Bar represents the equivalence between the distance and the number of changes.

water at 4 °C (2 × 10 min). Post fixing was performed using 0.4% paraformaldehyde in 1× PBS pre cooled to 4 °C for 20 min. Sections were then washed in pure water.

The sections were then incubated in a pre-hybridisation solution: 1 mM Tris–HCl pH 7.4, 20 mM standard saline citrate (SSC), 0.1 mM EDTA, 5% (w/v) dextran sulfate, and 50% (v/v) formamide for 1 h at 42 °C. Slides were then covered in 50 μL of hybridisation solution: 1 μg labelled probe with 1 mM Tris–HCl pH 7.4, SSC 20 mM, 0.1 mM EDTA, 5% dextran sulfate (w/v) and 50% (v/v) formamide, and denatured for 15 min at 95 °C in an oven. The slides were transferred to a 42 °C oven and incubated overnight.

The hybridisation solution was drained off and the slides were rinsed and immersed in 40 mM SSC (2 × 20 min). This was followed by a 30 min incubation in a solution containing 0.1% SSC, 2 mM $MgCl_2$, 0.1% Triton X-100 at 42 °C. Slides were then covered in Tris-buffered saline containing 3% bovine serum albumin and 0.1% Triton X-100 (TBSBT) for 5 min.

Hybridisation of the probes was detected by incubating the sections in anti-digoxigenin alkaline-phosphatase conjugate prediluted 1:600 in TBSBT for 30 min. Sections were then washed in Tris-buffered saline and alkaline-phosphatase substrate buffer for 5 min each. Slides were then stained with BM purple (Roche Applied Science) overnight. Slides were finally counterstained with Brazilian hematoxylin and coverslipped. Negative controls included uninfected sections and incubation with an identical hybridisation mix but without any labelled PCR product. The specificity of the probe was assessed using *H. costale* and *M. teredinis* sections in multiple assays.

2.7. Phylogenetic analysis

The SSU rRNA gene sequences employed in the phylogenetic analysis were obtained from GenBank. Sequences were aligned using CLUSTALW (Thompson et al., 1994) in MEGA 3.1 software (Kumar et al., 2004). Alignments were visually checked and an opening gap penalty of 10 and a gap extension penalty of 3 for both pairwise and multiple alignments were found to be the most effective. Phylogenetic analysis was conducted using MEGA3.1 with the haplosporidian parasites of *Haliotis iris* (AF492442), *Ruditapes decussatus* (AY435093) and *Pandalus platyceros* (AY449715) as an outgroup. Phylogenetic tree construction was performed using a maximum parsimony analysis with the close neighbour interchange (CNI) heuristic option with a search factor of 2 and random initial trees addition of 2000 replicates (Azevedo et al., 2006). Bootstrap values were also calculated over 300 replicates.

3. Results

A protozoan parasite identified as a haplosporidian was detected by PCR in rock oysters (*S. cuccullata*) from northwest Australia using degenerate HAP primers. Of the twenty six oysters processed nine were positive. These nine samples also tested positive by histology and *in situ* hybridisation.

The parasite measured 7 μm in diameter and was disseminated throughout the gonad follicles (Fig. 1) and to a lesser extent the gills of the infected oyster. No parasites were observed in the connective (Leydig) tissue surrounding the follicles or infecting any other tissue in the oyster. The only parasite life stages observed were uninucleate naked cells (Fig. 1) and binucleate plasmodia.

There appeared to be little in the way of a defensive response from the oyster to the parasite. Indeed, there appeared to be few hemocytes in the vicinity of the parasites or in the Leydig tissue surrounding the oyster reproductive tissue. No phagocytosis of parasite cells was observed.

The parasite was identified in hematoxylin-eosin stained sections using *in situ* hybridisation by designing a 30 bp oligonucleotide probe from the parasite's SSU rRNA gene. The oligonucleotide probe was labelled with dioxigenin and applied to unstained tissue sections in *in situ* hybridisation. The probe produced strong hybridisation signals with little background staining (Fig. 2A). This signal was not reproduced in tissues processed from uninfected oysters or in negative control material (Fig. 2B).

Spores were not detected in this study so the parasite was not able to be identified using the established morphological criteria (Burreson, 2001). In order to carry out a

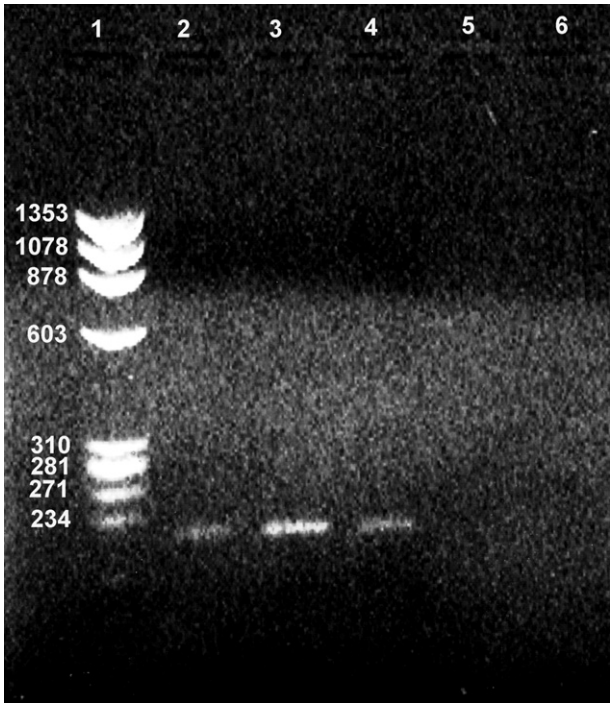

Fig. 4. Agarose gel electrophoresis of the Minch PCR products from archived rock oyster tissues containing the haplosporidian parasite described by Hine and Thorne (2002). Samples were electrophoresed in a 2% agarose gel for 45 min at 46 milliamps. Lane 1: Phi/HaeIII molecular weight markers. The size of each marker in base pairs is indicated to the left. Lanes 2–4: PCR products from archived tissues. Lane 5: negative control (no DNA).

phylogenetic analysis, additional primers were developed to sequence the remaining sections of the parasite's SSU rRNA gene (Table 1). The PCR products generated were cloned and sequenced. The 1682 bp SSU rRNA gene sequence was found to be unique, and consequently it was submitted to GenBank (Accession No. EF 165631).

The parasite SSU rRNA gene sequence obtained in this study falls inside the genus *Minchinia* and is sister taxon to a clade composed of *M. chitonis*, *M. teredinis*, and *Minchinia* sp. Overall, bootstrap support for the monophyly of the genus *Minchinia* is at 58% (Fig. 3). Monophyly for the genus *Bonamia* is strongly supported (98%) while support for the *Haplosporidium* excluding *H. nelsoni* and *Haplosporidium louisiana* is weakly supported at 14% (Fig. 3).

The Minch primers also were used to amplify a 166 bp section of formalin fixed archived samples of the parasite described by Hine and Thorne (2002). The Minch primers encompass a variable section of the SSU region of the parasite's rRNA gene. The Minch reactions produced consistently positive results with no indication of contamination (Fig. 4). When compared to the parasite detected, in this study the sequences were within the levels expected for intraspecific variation. Overall, two sites within the 166 bp SSU sequence produced by the archive rock oyster parasites were different to that produced by the parasite detected in this study. At site 1489 the archived samples contained a T instead of a C, while at site 1459 the archived samples contained a G instead of an A.

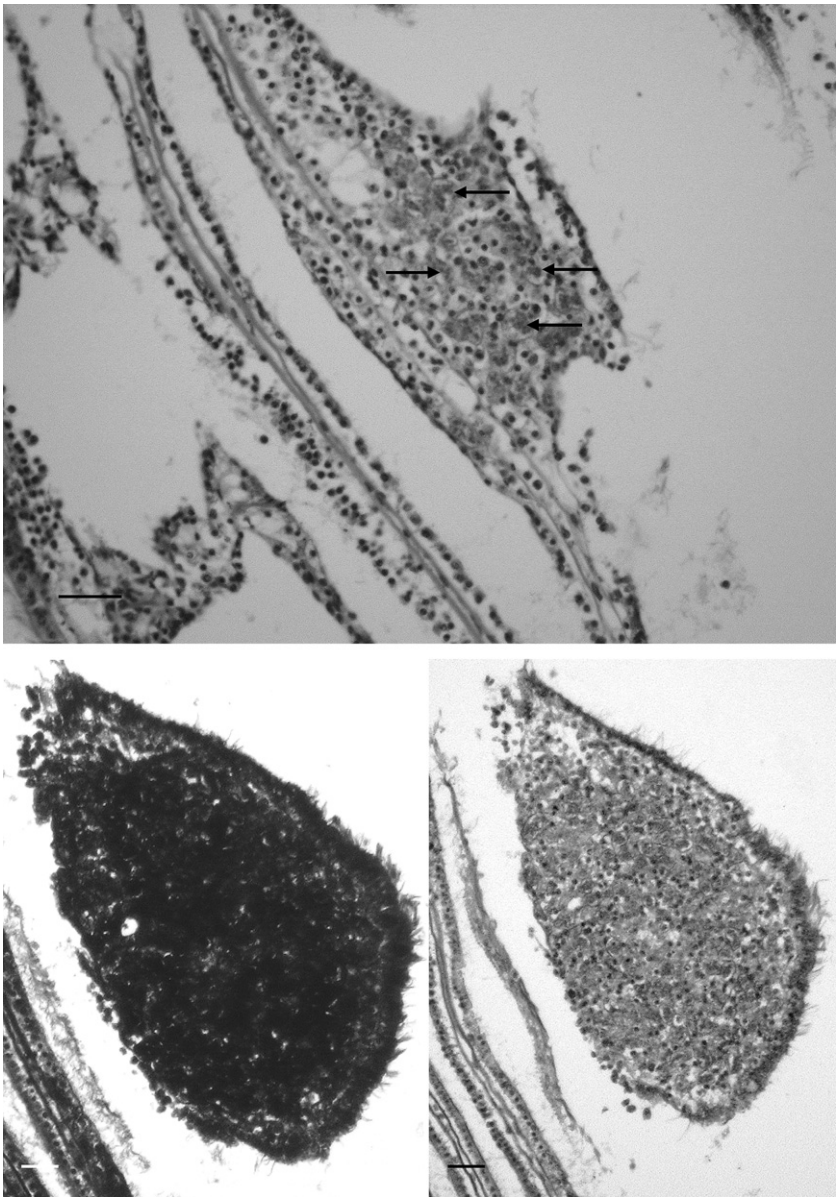

Fig. 5. Serial sections of rock oyster gill tissue containing the parasite described by Hine and Thorne (2002). Top: a hematoxylin-eosin stained section. Parasites are indicated with an arrow. Bottom left: an *in situ* hybridisation assay containing the SSR 69 probe. Bottom right: an *in situ* hybridisation assay with no probe in the hybridisation solution (negative control). Parasites are identified by a darker colouration. Sections are counterstained in a Brazilian hematoxylin. Scale bars = 10 μm.

In order to ascertain whether the oligonucleotide probe reacted with the parasite described by Hine and Thorne (2002) an *in situ* hybridisation was performed on archived tissue used in that study. The *in situ* hybridisation produced strong hybridisation signals with little background staining (Fig. 5). *Haplosporidium costale* and *M. teredinis* sections were included in the assay and did not produce a reaction (Fig. 6).

4. Discussion

Haplosporidian parasites associated with oyster mortality have been infrequently detected on the north Western Australian coastline. However, the geographic isolation of the region and difficulties in locating the parasites by techniques such as traditional histology has hampered further research into the biology of these parasites (Hine and Thorne, 2002).

The results of the present paper demonstrate through PCR and *in situ* hybridisation the presence of a cryptic haplosporidian parasite in rock oysters.

A phylogenetic analysis of the parasite indicates a *Minchinia* species between *Minchinia tapetis* and *Minchinia chitonis* (Fig. 3). However, confirmation of this assessment, using spore ornamentation, is required. Spore ornamentation is the most accepted characteristic used to assess the taxonomic placement of haplosporidian parasites excepting the *Bonamia* species in which spores have not been detected (Burreson and Ford, 2004). The most accepted morphological characteristic used to separate the *Minchinia* from the *Haplosporidium* is the presence of an epispore cytoplasmic extension (ECE) within the spore ornaments, while the *Haplosporidium* possess ornamentation consisting of spore wall material (Azevedo et al., 2006; Burreson and Ford, 2004; Hine and Thorne, 1998). Consequently, if the genus *Minchinia* is genuinely monophyletic, one would expect the spores of this parasite to possess ECEs. A monophyletic *Minchinia* genus is suggested with bootstrap support at 58% (Fig. 3).

The samples used in the present study were obtained from within the geographic range of a haplosporidian parasite described by Hine and Thorne (2002) from the same host. The phylogenetic assessment of a *Minchinia* species is consistent with the parasite described by Hine and Thorne (2002) where a conclusive placement of the parasite in either *Minchinia* or *Haplosporidium* genera was not possible. An *in situ* hybridisation of archived rock oyster tissue infected with the parasite described by Hine and Thorne (2002) produced a positive reaction (Fig. 5). The probe did not recognise any of the other haplosporidian parasites assayed (Fig. 6). A 166 bp sequence was obtained from archived formalin fixed tissues containing the parasite described by Hine and Thorne (2002). The sequence encompasses a variable region of the parasite's SSU rRNA gene. However, the sequence differed by only two base pairs from the SSU rRNA gene sequence obtained from the rock oyster parasite detected in this study.

While only nine samples have tested positive to date, the parasite present in those samples appears unusual to other haplosporidians as the primary site of its detection is not connective tissue, gill or digestive gland epithelium but the gonad follicles of the host (Figs. 1 and 2). The association of the parasite with the host reproductive tissue may be subject to environmental or seasonal influence explaining the differences between the parasite detected in this study and the parasite described by Hine and Thorne (2002). Hine and Thorne (2002) noted the parasite had a higher

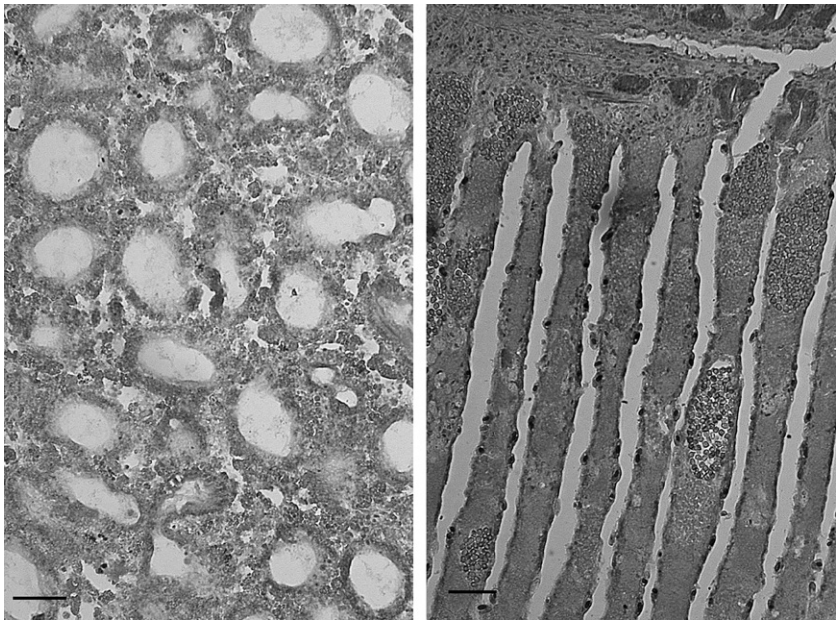

Fig. 6. Haplosporidian parasites used to test the specificity of the ISH assay. Left: an Eastern oyster (*Crassostrea virginica*) digestive gland containing *Haplosporidium costale*. Scale bar = 50 μm. Right: *Minchinia teredinis* in the gills of the shipworm *Teredo* sp. Sections are counterstained in a Brazilian hematoxylin. Scale bar = 12 μm.

prevalence of infection and more common sporogenesis in oysters with empty gonad follicles. All of the oysters used in this study were leading towards spawning.

Hine and Thorne (2002), who did find spores in their study, noted that the spore surface microtubules derived from the episporoplasm resemble similar microtubules in epispore cytoplasmic vacuoles of *Minchinia* in crabs except that the microtubules are unaligned and that only *Haplosporidium ascidiarum* from tunicates had similar unaligned microtubules. Thus, it is possible that *H. ascidiarum* may also be member of genus *Minchinia*.

It is hoped that the molecular approaches developed in this study will be used to clarify the relationship between this parasite and another haplosporidian parasite infrequently infecting pearl oysters (*Pinctada maxima*; Hine and Thorne, 1998) on the north Western Australian coast.

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