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Detection of *Minchinia occulta* in samples of pearl oysters *Pinctada maxima* infected by *Haplosporidium hinei*.

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Abstract

Objective To determine if juvenile pearl oysters (*Pinctada maxima*) infected with *Haplosporidium hinei* are also infected with another haplosporidian parasite, *Minchinia occulta*.

Design Archived samples of pearl oysters infected with *H. hinei* were examined using polymerase chain reaction (PCR) assays and in situ hybridisation (ISH) to analyse and identify haplosporidians. A 144-bp and 220-bp region of *Minchinia* DNA were targeted by PCR and amplified DNA from formalin-fixed *H. hinei*-infected pearl oyster samples was sequenced. A 25-bp oligonucleotide probe targeting a variable section of the parasite's small subunit rRNA gene was used in ISH.

Results The results of DNA-based diagnostic assays supported each other. The sequences obtained by PCR were found to be almost identical to *M. occulta* from rock oysters and the ISH assay demonstrated infection with *M. occulta* in affected pearl oysters. ISH indicated a prevalence of infection of 26.7% in one of the previous outbreaks.

Conclusion Pearl oyster spat are susceptible to infection by a *Minchinia* parasite, most likely *M. occulta*, which was recently identified in rock oysters within the pearl-producing zones of Western Australia and is associated with mortalities of up to 80% in this species. The occurrence of haplosporidian co-infections in pearl oysters suggests the immunocompetence of juvenile oysters may be an important factor in preventing infection and therefore preventing mortalities such as those occurring in the recent outbreaks of pearl oyster oedema disease.

List of abbreviations and acronyms

bp	Base pair(s)
ddH$_2$O	Double distilled water
dNTP	dATP, dCTP, dGTP or dTTP

DIG	Digoxigenin
H&E	Hematoxylin and Eosin
ISH	*In situ* hybridization
MSN	Minimum Spanning Network
min	Minutes
PCR	Polymerase Chain Reaction
Pearl Oyster	*Pinctada maxima*
Rock Oyster	*Saccostrea cuccullata*
s	Seconds
SEM	Scanning Electron Microscopy
SSC	Standard Saline Citrate
SSU	Small Subunit region of the rRNA gene
Spat	Juvenile pearl oysters
Sydney rock oyster	*Saccostrea glomerata*
TEM	Transmission Electron Microscopy

Introduction

Shellfish aquaculture produces nearly a quarter of world aquaculture output [1] and Australia's most valuable shellfish culturing industry is the pearling industry [2]. With an annual export value of nearly $A300 million[2] the Australian pearl oyster industry is the world's largest producer of the highly prized, south sea pearls that come from the silver lipped pearl oyster *Pinctada maxima* (Jameson, 1901). The product is marketed primarily to Japan, the United States of America, Hong Kong and Europe. In recent

times the industry has moved to hatchery production of juvenile pearl oysters rather than relying solely on wild caught adult oysters. The production of spat from hatcheries allows for the implementation of selective breeding programs to improve stock quality and has resulted in the intensification of the industry. However, the survival of hatchery raised spat can be low after they are deployed to the open ocean and the causes of spat mortalities are not well described[3]. Since the oysters can produce pearls several times over their lifespan, they are a valuable livestock and understanding mortality issues for this industry is therefore of paramount importance.

The management of the industry in Western Australia is complex[4] and includes a diagnostic capacity to identify pathogens and the existence of passive and targeted surveillance programs aimed at identifying endemic diseases to ensure that only the movement of healthy oysters occurs[5, 6]. These controls have resulted in the detection of a haplosporidian parasite of pearl oysters on three occasions[6]. Haplosporidiosis is a notifiable disease in Western Australia and detection has resulted in compulsory destruction of affected stock. The locations of these infections are indicated in Figure 1.   The parasite was attributed to *Haplosporidium hinei*[7] and is considered to represent a serious concern to the industry[6, 8]. A recent study demonstrated a widespread infection by another haplosporidian parasite, *Minchinia occulta* in rock oysters (*Saccostrea cuccullata*) in pearl producing zones of Western Australia[9, 10]. The parasite was associated with 80 % mortality in rock oysters[11]. The differential diagnosis of the rock and pearl oyster haplosporidians is based on the ultrastructural characteristics of the parasite's spores since the non-spore forming life-stages appear very similar.

The phylum Haplosporidia comprises a small group of spore forming obligate protozoan parasites of a number of fresh water and marine invertebrates[12]. At present there are 39 recognised species in the phylum, however numerous others have been reported, but not specifically described. Haplosporidian parasites can be amongst the most dangerous of all molluscan pathogens particularly where naïve hosts are exposed to the parasite[13]. *Haplosporidium nelsoni* is the aetiological agent of the highly pathogenic Multinucleate Sphere X (MSX) disease in the eastern oyster *Crassostrea virginica* that along with *Perkinsus marinus*, has devastated wild oyster populations and hindered oyster aquaculture development on the eastern seaboard of the USA [14]. *Bonamia ostrea* has severely affected the European flat oyster *Ostrea edulis* industry in Western Europe [15]. Massive mortalities of over 90 percent of dredge oysters (*Ostrea chilensis)* have been recorded in a wild fishery in Foveaux Strait in New Zealand [16]. These mortalities were associated with *Bonamia exitosa* which may also be present in Europe [17]. Locally, *Bonamia roughleyi,* believed to be the aetiological agent of Winter Mortality, has had a considerable impact on the production of Sydney rock oysters.   The association of haplosporidian parasites with commercially significant mortalities means they are important parasites for study.

In an effort to determine whether batches of *Haplosporidium hinei*-infected pearl oyster spat from past outbreaks also contained *Minchinia occulta* previously detected in rock oysters (*Saccostrea cuccullata*) a polymerase chain reaction (PCR) and *in situ* hybridisation (ISH) assay for *Minchinia occulta* was used to analyse haplosporidians in archived tissues from *Haplosporidium hinei* infected pearl oysters.

Methods and materials

Representative histologically-positive paraffin-embedded pearl oyster spat infected with *Haplosporidium hinei* were obtained from archived cases of previously reported outbreaks[6]. Samples of *Minchinia occulta* in *S. cuccullata* were obtained from the Montebello Islands as described previously [10]. The original geographic location of the source material is shown in Figure 1.

In order to assess whether *Minchinia occulta* was present in batches of *Haplosporidium hinei* infected pearl oysters, archived formalin fixed samples of pearl oysters infected with the parasite were subjected to PCR and ISH. All histologically positive samples available were assessed including six samples from Carnarvon, four were from Cascade Bay and two from Willie Creek. In addition, thirty random pearl oysters from the Cascade Bay infection were assessed for *Minchinia occulta* through ISH.

DNA extraction: Archived samples.

Genomic DNA was extracted from the formalin-fixed paraffin-embedded pearl oysters using the freeze thaw method outlined previously[9]. The quality of the genomic DNA and absence of inhibitory factors from the extractions was verified using PCR with bivalve primers 16R3 5'-GCT GTT ATC CCT RNR GTA-3' and Proto 16'F'-AWK WGA CRA GAA GAC-3'[18].

Amplification by Polymerase Chain Reaction.

The Minch F1B/R2B primers (Table 1) encompassed a 144 bp variable section of the small ribosomal subunit (SSU) region of the parasite's rRNA gene. Each PCR was performed using the method outlined previously [10]. An additional measure was taken

to ensure the absence of contamination from genomic *Minchinia occulta* DNA in the F1B/R2B reactions. This measure involved the attempted amplification of a 754 bp section of the *Minchinia occulta* rRNA gene using the FSSUF/SSR69 primers (Table 1). A positive amplification from these primers would indicate contamination since formalin fixation results in the cross linking of histones and fragmentation of sample DNA making amplification of sequences larger than 300 to 500 bp difficult [19]. These reactions were performed in addition to the usual negative controls employed in all PCR reactions and were assessed using genomic rock oyster DNA to ensure successful amplification of the target sequence. The reaction conditions for the FSSUF/SSR69 reactions were as follows: Reaction mixtures had a total volume of 25 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 units of *Taq* polymerase and template DNA. Each of the reaction mixtures was subjected to (i) an initial denaturation phase of 5 min at 94°C, (ii) 35 amplification cycles, each cycle consisting of 30 sec of denaturation at 94°C, 30 sec of annealing at 59°C, 4 minutes of extension at 68°C and (iii) a final 7 min extension at 68°C. Polymerase chain reaction products were visualised by loading a 10 µL aliquot of the products on a 2% agarose gel and electrophoresing it for 20 min at 90 volts. Detection of the PCR products was performed using ethidium bromide staining.

Given that the assessment of potential contamination of the PCR reactions may be confounded if the source of contamination were shorter gene fragments from the F1B/R2B PCR and cloning reactions, an alternative region was selected for amplification. Consequently, potential contamination by shorter rRNA gene fragments, such as those from the F1B/R2B PCR reactions were assessed by using

the primers designated SSF66 and SSR69 (Table 1). These primers were designed to target a 220 bp sequence so that amplification from formalin fixed tissues could be reliably performed. The SSF66/SSR69 primers target variable regions of the parasite's SSU rRNA gene but encompass a conserved region. Amplification conditions were the same as for the Minch primers except the annealing temperature was raised to 62°C. These primers were assessed previously and were found not to react to closely related haplosporidian species *H. nelsoni* and *H. costale* [20]. The specificity of the SSF66/SSR69 reactions was also monitored in this study with *H. nelsoni* and *H. costale* DNA.

Polymerase chain reaction was also used to attempt to amplify *Haplosporidium hinei* DNA from the histologically positive pearl oyster samples. These reactions were performed using all combinations of the HAP primers [21] and at least 10 other primer combinations of novel design utilising 16s, and protozoan (18S-EUK-581-F/18S-EUK-1134-R) [22] primers with proximate target regions suitable for amplification from formalin fixed material.

DNA sequencing

DNA cloning and sequencing was performed using the method outlined previously[20]. Three cloned DNA inserts were each sequenced for each positive PCR. Primer sequences were removed from each end of the resulting consensus sequence and the identity, position and direction of the PCR products were verified by directly comparing to the *Minchinia occulta* SSU rRNA sequence from *S. cuccullata*. Additional comparisons were also made using BLAST searches of GenBank database: (http://www.ncbi.nlm.nih.gov/blast/).

In-situ hybridisation

In addition to the precautions taken to ensure the absence of contamination in the PCR reactions, ISH was also performed. *In situ* hybridisation (ISH) was performed on histologically-positive formalin-fixed, paraffin-embedded tissue collected at the Carnarvon, Cascade Bay and Willie Creek infection sites and performed using the oligonucleotide probe SSRDb. In addition, ISH was performed on thirty random oysters from the Cascade Bay outbreak. The SSRDb probe targets a highly variable region on the *Minchinia*'s SSU rDNA sequence (positions 567-588) and was commercially synthesized and labelled with dioxigenin at the 5' end (Operon Technologies, Germany). Positive controls included sections of rock oyster infected with *Minchinia occulta* collected from the Montebello Islands in July 2005. Negative controls used sections containing no DNA in the hybridisation mix as well as uninfected pearl oyster spat obtained from the Broome hatchery. The *in situ* hybridisation procedure was performed using the method outlined previously[20]. The specificity of the SSRDb probe was assessed and monitored using *Haplosporidium costale*, *Haplosporidium nelsoni* and *Minchinia teredinis* sections.

Genealogical comparisons of the SSU rRNA sequences

The phylogenetic relationships between SSU rDNA sequences of *Minchinia occulta* were compared to other *Minchinia* spp. using distance based trees. The SSU region of the rRNA gene sequences used in the distance comparison were obtained from GenBank. The sequences used belonged to *Haplosporidium nelsoni* (U19538), *Minchinia teredines* (U20319), *Minchinia tapetis* (AY449710), *Minchina chitonis* (AY449711), *Minchinia* sp. from *Cyrenoida floridana* (AY449712), *Bonamia ostreae* (AF262995), *Bonamia exitiosa* (AF337563). Sequences were aligned using

CLUSTALW [23] in MEGA 3.1 software [24]. Distance based trees were constructed using the MEGA 3.1 computer program [24] with a neighbour-joining algorithm [25]. Nodal support was determined by 1000 replicate bootstrap analyses.

Relationships amongst the SSU rDNA sequences were explored using a minimum spanning network (MSN). An MSN, describing the nucleotide substitutions amongst the SSU rRNA alleles was constructed by statistical parsimony, as applied in the software TCS 1.18 [26]. Once the necessary input file was constructed for the TCS 1.18 software, a parsimony connection limit of $P = 0.05$ was used in the construction of the network. The output was untangled so that no SSU rDNA alleles crossed base substitutions. Nucleotide substitutions were displayed on the network along with the base change, type and location. The network was then applied to a map indicating the location from which the sequences were obtained.

Results

The results presented in this study indicate *Minchinia occulta* was present in batches

of pearl oyster spat affected by *Haplosporidium hinei*. The reactions with the Minch

F1B/R2B primers produced consistently positive results with no indication of

contamination (Figures 2 and 3). Of the twelve histologically positive pearl oyster

samples tested, five were positive by PCR (Table 2). Of the six histologically positive

samples from Carnarvon, three produced positive PCR results while both of the Willie

Creek samples produced positive PCRs (Table 2). No *Minchinia occulta* sequences

could be obtained from the four histologically positive Cascade Bay samples. While

these samples were negative by ISH, samples from Cascade Bay did produce positive

ISH results with the SSRDb probe at low levels of intensity (8/30; 26.7%). Overall,

three clones were obtained from each of the positive PCR samples to produce fifteen

sequences.

Attempts to detect contamination with genomic *Minchinia occulta* DNA by amplifying a

754 bp section of the *Minchinia occulta* rRNA gene using the FSSUF and SSR69

primers failed (Figure 3). This result is consistent with observations that amplification

of formalin fixed tissues above 200-500 bp is difficult. In addition, the SSF66/SSR69

primers that target a different region were successful in obtaining a positive reaction

from the Willie Creek and Carnarvon samples.

When the SSU rDNA sequences were compared to the sequences of *Minchinia*

occulta from *S. cucullata*, the genetic distances between the sequences were

considerably less than the distance between known species for this region (Figures 4

and 5). The region targeted by the Minch primers is a variable section of the

parasite's SSU rRNA gene. Four sites within the SSU rRNA sequence produced by the pearl and the archived 2002 rock oyster samples were different to that produced by the *Minchinia occulta* (GenBank accession number: EF 165631). Base 1389 on EF165631 was different in all parasite SSU rRNA sequences from Carnarvon which had a T at this site rather than the C present in the other samples. Both the Carnarvon and the sequence obtained from the archived 2002 rock oyster samples had a G substituted for an A at base 1359 on EF 165631. One of the Carnarvon sequences had a T at base 1311 rather than the C present in all other samples. Both Willie Creek samples were identical to the 2005 rock oyster samples except base 1315 had an A substituted for a G.

The sequences produced by the SSF66/SSR69 primers from the Willie Creek and Carnarvon samples were identical to that obtained from the *Minchinia occulta* infecting rock oysters from the Montebello Islands. Consequently, the sequences obtained from the Willie Creek samples had a known sequence of 364 bp with only a single base pair difference with the *Minchinia* parasite from the Montebello Islands. Attempts to obtain *Haplosporidium hinei* DNA from the histologically positive samples utilising the HAP [21], 16 s [27], Protozoan (18S-EUK-581-F/18S-EUK-1134-R) [22] and novel primers were unsuccessful in producing any *Haplosporidium* spp. sequences.

Interspecific and intraspecific comparison of the SSU rDNA sequences.
Overall, the genetic distance between the pearl oyster parasite PCR products was of the same magnitude as the genetic distance between the pearl oyster PCR products compared to the rock oyster PCR products (Figure 4). No sequences could be obtained from the Cascade Bay samples.

The distance based phylogenetic trees clearly show the pearl oyster sequences grouping closely together with the rock oyster samples and are distinct from the rest of the *Minchinia* and are even more distant from the *Bonamia* and the *Haplosporidium* (Figure 4).

The minimum spanning network (MSN) more clearly illustrates the nature of the relationships among the SSU rRNA alleles (Figure 5). Unlike the distance based tree (Figure 4), the MSN showed a tendency for the SSU rRNA sequences from the same geographic region to cluster together rather than sequences from each host to group together. The main findings of the MSN were:

- Sequences grouped on the basis of geographic region rather than by host species.
- Sequences were also loosely grouped according to the year of sampling.
- The rock oyster parasite SSU rRNA sequences were between the pearl oyster SSU rDNA sequences from the two different regions.

In situ Hybridisation

In situ hybridisation (ISH) was performed in order to validate the sequences obtained by PCR from the infected pearl oyster tissues and confirm the positive PCRs were not the result of contamination. Parasite identity and location in tissue sections was confirmed in haematoxylin-eosin sections cut adjacent to those tested with ISH (Figure 7). The SSRDb *in situ* hybridisation assay yielded positive reactions from all of the *Minchinia*-infected pearl and rock oysters (Figure 7). The assay did not hybridise to

Haplosporidium costale, H. nelsoni or *Minchinia teredinis* infected oyster tissues (Figure 8).

The cells reacting to the SSRDb probe were detected in the connective (Leydig) tissue surrounding the digestive gland and less commonly in the mantle and gills of infected oysters. The cells consisted predominately of single; bi- and tri- nucleate life stages and were approximately 7 µm in diameter. No phagocytosis of parasite cells or necrotic debris was observed despite the presence of granulocytes and hyalinocytes among the parasites. The intensity of infection of the SSRDb positive cells was considerably lower compared to the presumptive *Haplosporidium hinei* cells (Figure 7).

Discussion

The results presented in this study indicate pearl oyster spat are susceptible to infection by a *Minchinia* species most likely to be *Minchinia occulta* recently identified in rock oysters from the same coastline. *In situ* hybridisation of pearl oyster spat from Cascade Bay indicated a prevalence of infection of 26.7% although at low levels of intensity. The results of DNA-based diagnostic assays supported each other. The SSU rDNA sequences obtained from the Willie Creek samples encompass a variable region of the *Minchinia* SSU rRNA gene yet it differs from the rock oyster parasite by only one base pair. The results of the same assay from the Carnarvon sample differed by only two base pairs. Positive PCRs were obtained using the SSF66/SSR69 primers which target two other variable SSU rDNA regions. The SSF66/SSR69 PCR assay produced negative results when tested with *H. nelsoni* and *H. costale* infected oyster DNA. When the sequence obtained from the SSF66/SSR69 regions is added to the 144 bp sequence obtained from the F1B/R2B reactions, the Willie Creek sample has a total of 364 bp of known sequence with only a single base pair different from the rock oyster parasite. The PCR produced consistent results with no indication of contamination. Attempts to target a larger region with the primers FSSUF and SSR69 failed. This is consistent with observations that formalin fixation makes amplification of regions larger than 200-500 bp difficult due to fragmentation of DNA and crosslinking of histones to DNA [19]. The genetic distances obtained from comparing the *Minchinia* sequences from rock and pearl oyster samples were considerably less than the distance between known species for this region. In addition, the sequences acquired from the pearl oysters were not grouped together but were distributed between the rock oyster *Minchinia occulta* sequences when placed on the minimum spanning network.

The PCR results were further validated by *in situ* hybridisation with a 25 base oligonucleotide probe (SSRDb). The probe did not detect closely related haplosporidians: *Haplosporidium costale, H. nelsoni* or *Minchinia teredinis* but did produce a positive reaction from some of the haplosporidian parasites in *Haplosporidium hinei* infected pearl oyster spat. Overall, three separate variable sections of the SSU region of the parasite's rRNA gene were tested by two different molecular methods and found to be consistent with a *Minchinia* species most likely *Minchinia occulta* detected previously in rock oysters.

Attempts to amplify *Haplosporidium hinei* DNA using a variety of degenerate primers have so far been unsuccessful. The primers used included all combinations of the HAP primers [21], protozoan [22] and others of novel design (unpublished observations) so as to target smaller DNA fragments from formalin fixed tissues. Specificity was lowered in some of these reactions but no *Haplosporidium hinei* amplifications could be obtained. It is likely problems with poor fixation combined with non-specific and degenerate primers prevented amplification. The F1B/R2B and SSF66/SSR69 primers were specifically designed to target *Minchinia occulta* DNA and perhaps for this reason were successful. Further research into the molecular characterisation of *Haplosporidium hinei* will require either frozen or ethanol stored samples.

The presence of co-infections of haplosporidian parasites is not unprecedented. Co-infections of *Haplosporidium nelsoni* and *Haplosporidium costale* have been previously observed in *Crassostrea virginica* on the eastern sea board of the United States [28]. Both *Haplosporidium hinei* and *Minchinia occulta* parasites appear to be

opportunistically parasitising weakened pearl oyster spat. Both parasites have only been detected in juvenile pearl oysters less than 12 mm in size. It appears likely that as the pearl oyster increases beyond this size it may be able to respond effectively to the parasites. Another haplosporidian parasite has been documented infecting pearl oysters in a similar fashion as *Minchinia occulta* and *Haplosporidium hinei*. There have been sporadic reports of *Bonamia roughleyi* like infections of pearl oysters [3, 29]. In the mid 1990s a *Bonamia roughleyi* like organism was found infecting pearl oyster spat in Carnarvon, Western Australia [30].

While *Minchinia occulta* has been associated with significant mortalities (80%) in rock oysters[11] there is not yet any evidence that it causes disease or mortality in pearl oysters. However, mass mortalities of juvenile pearl oysters are well recognised [3]. It appears pearl oysters below certain sizes are susceptible to infection by parasites that would otherwise be considered too host specific to be a threat. Indeed, there is a range of potentially serious pathogens in wild bivalves that juvenile pearl oysters are exposed to upon deployment [10]. Sequential examination of batches of juvenile oysters up to 23 weeks from deployment in the Northern Territory of Australia demonstrated progressive colonisation by a range of unidentified protozoan and metazoan organisms [3]. This may explain some of the mortalities experienced by the pearling industry on deployment of spat to grow-out leases. Further research is required to document the susceptibility of juvenile pearl oysters to infection and to characterise the age or size where an effective immune response commences. It is likely that this research may have significant implications for industry attempting to deploy hatchery bred pearl oyster spat to the wild. These issues will become more significant as the industry moves towards hatchery production of spat.

The uni-nucleated naked cells originally identified as the origin of the *Minchinia* sequence appear morphologically similar to a *Bonamia* species. It is possible the previously described *Bonamia* infections in pearl oyster spat are actually *Minchinia occulta* infections. The genus *Minchinia* is a sister taxon to the genus *Bonamia* and consequently the possible dominance of a uni-nucleated cell stage in the *Minchinia occulta* infected rock oysters described previously[9] may be a result of an evolutionary relationship between these two genera. There appears to be little information in the literature on the non-spore life stages of most *Minchinia* species; rather most authors report only spore morphology [31-33]. If the cryptic uni-nucleated stages detected previously[9] are a dominant part of the life-cycle of species within *Minchinia* then this may explain this lack of information. *Bonamia* infected pearl oyster spat are required for further analysis.

The results presented in this study show pearl oyster spat are susceptible to infection by a *Minchinia* species most likely to be *Minchinia occulta* recently identified in rock oysters and that juvenile pearl oysters may be susceptible to infection by pathogens that may normally be considered too host specific to be a threat. Further sequence analysis of *Minchinia occulta* from pearl oysters will require ethanol stored or frozen samples of the parasite. There are also several other areas of future research into both of these parasites that need to be pursued. Foremost amongst them is obtaining an SSU rRNA gene sequence for *Haplosporidium hinei* and attempting to determine the pathogenicity of the parasites. Any research should also determine whether the parasites are associated with disease and mortalities using Evans postulates [34] or a similar scheme.

Acknowledgements

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Tables and Figures

Table 1 Primer sequences employed in during the study.

Primer	Sequence (5' -3')	Position*	Reference
Minch F1B	ctc gcg ggc tca gct t	1295	Bearham et al (2007)
Minch R2B	ggc gct ttg cag att ccc ca	1439	Bearham et al (2007)
SSF66	ccg ccg atg ccc agc cgt at	551	Bearham et al (2008b)
SSR69	agc cca aaa cca aca aaa cgt cca ca	754	Bearham et al (2007)
FSSUF	ctc aaa gat taa gcc atg cat gtc caa gta ta	#	Bearham et al (2007)
SSRDb#	gtt agc ctt gcg cgc agc cga tac g	567	This study

*Position on the entire *Minchinia occulta SSU rRNA gene sequence Genbank accession: EF 165631.* SSRDb

was used as an ISH probe only. # FSSUF primer targets a region before EF165631.

Table 2. Summary of number of samples tested and a comparison of results from

PCR and ISH assays.

Location	n	PCR	ISH-SSRDb
Cascade Bay	4	0	0
Cascade Bay 2	30*	N/A	8
Carnarvon	6	3	3
Willie Creek	2	2	2

* Second batch of archived Cascade Bay oysters were histologically negative although retrospective

examination of the ISH (SSRDb) positive sections revealed haplosporidian parasites in the affected
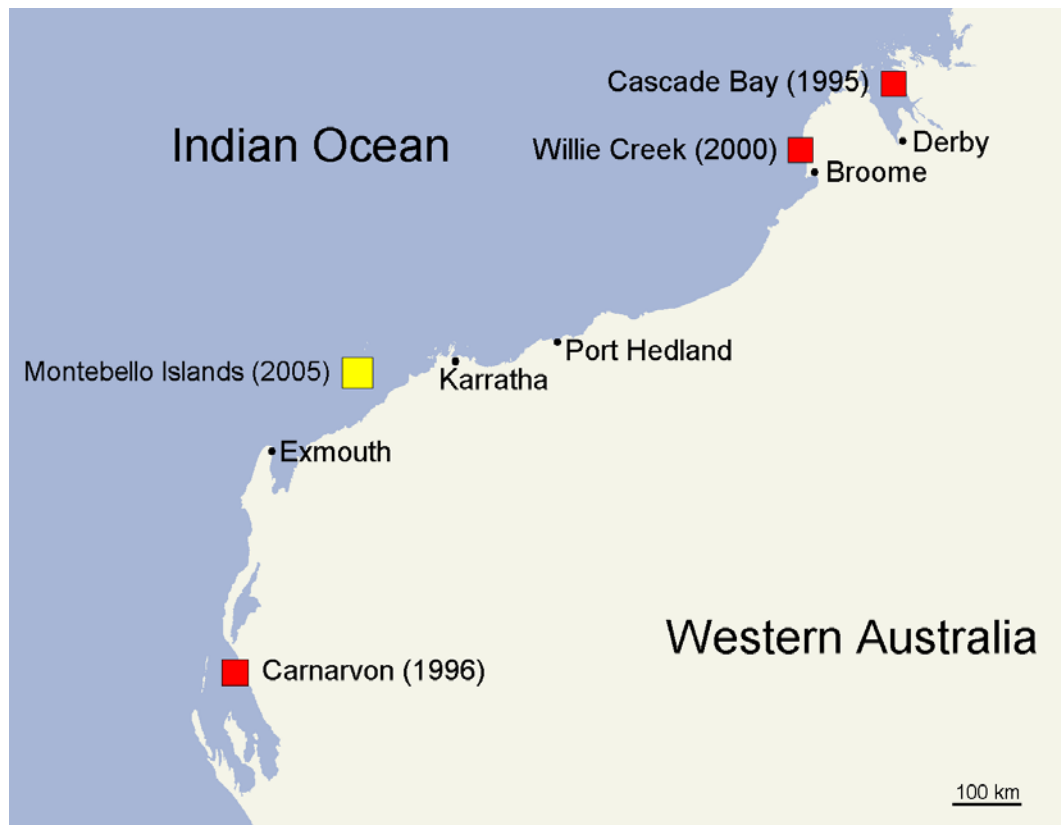
tissues.

Figure 1 Map of north Western Australia indicating the locations of 3 past haplosporidian outbreaks in pearl oysters at Cascade Bay (1995), Carnarvon (1996), Willie Creek (2000) and the source of rock oysters infected with *Minchinia occulta* from the Montebello Islands (2005).
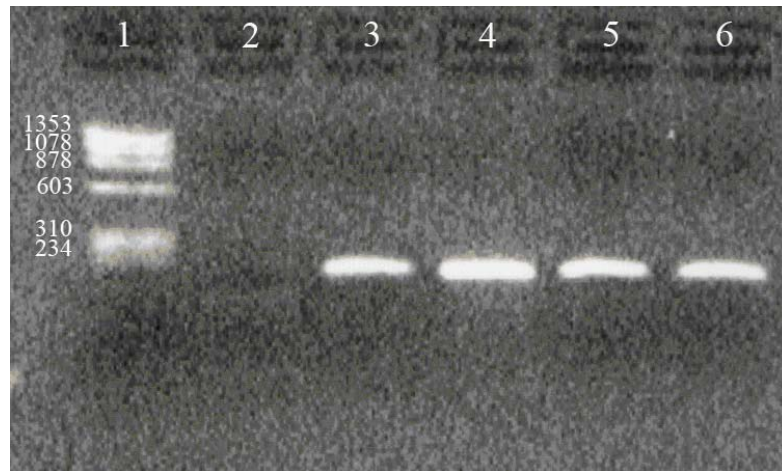
Figure 2. Agarose gel electrophoresis of formalin pearl oyster tissue from each of the infection sites. Samples were electrophoresed in a 2% agarose gel for 20 minutes at 90V. Lane 1 contains *Hin*dIII/Phi markers, size of markers are indicated on the left. Lane 2 is the negative control (no DNA), Lane 3 contains haplosporidian DNA from the Carnarvon site, Lane 4 contains haplosporidian DNA also from the Carnarvon site, Lane 5 contains haplosporidian DNA from the Willie creek site. Lane 6 contains haplosporidian DNA also from the Willie Creek site. DNA was stained with ethidium bromide and visualised using UV light.
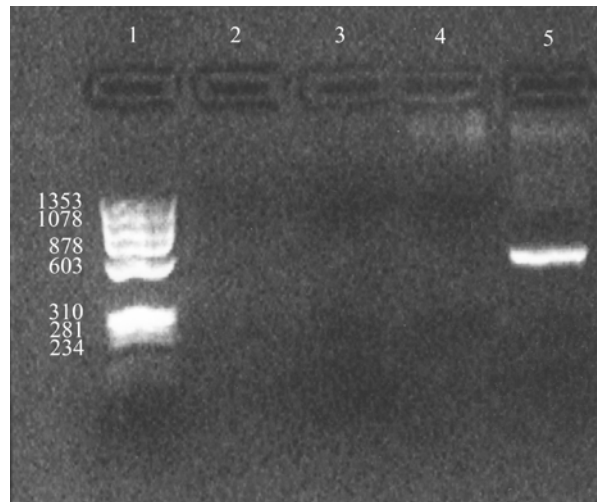
Figure 3. Agarose gel electrophoresis of the FSSUF and SSR69 primers and formalin fixed pearl oyster tissue from the Willie Creek infection site. Samples were electrophoresed in a 2% agarose gel for 20 minutes at 90V. Lane 1 contains *Hin*dIII/Phi markers, size of markers are indicated on the left. Lane 2 is the negative control (no DNA). Lanes 3 and 4 contains haplosporidian infected pearl oyster DNA from the Willie Creek site. Lane 5 contains *Minchinia occulta* infected genomic oyster DNA from the Montebello Islands (positive control). DNA was stained with ethidium bromide and visualised using UV light.
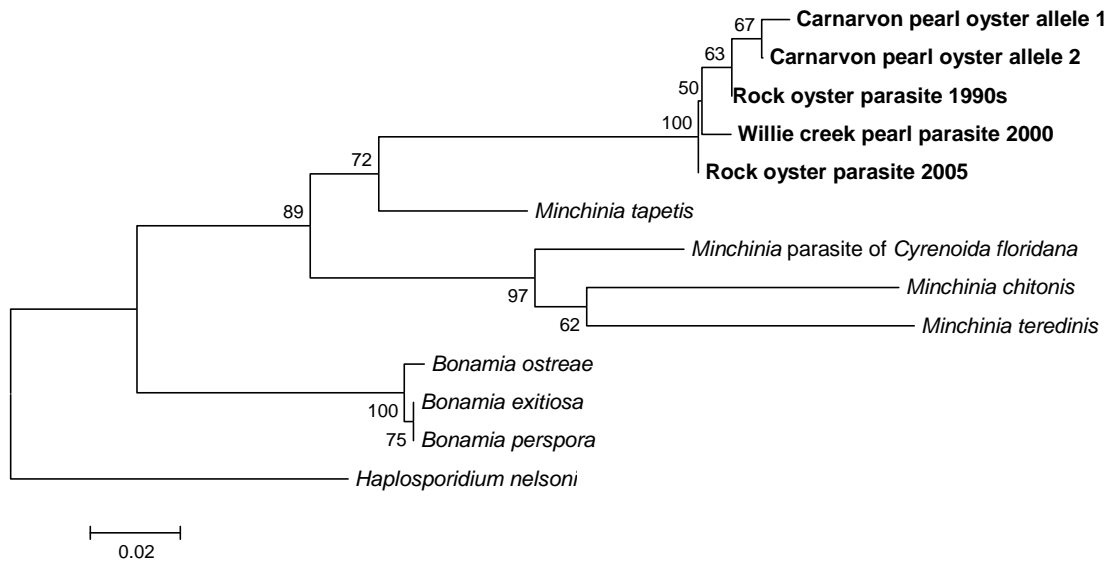
Figure 4. Neighbour Joining tree illustrating the relationships among the 144 bp SSU rRNA gene sequences from phylum Haplosporidia. Tree was constructed with a Kimura 2-parameter algorithm. The scale bar represents the equivalent of 0.02% nucleotide sequence divergence. Nodes are supported by a 1000 replicate bootstrap analysis.
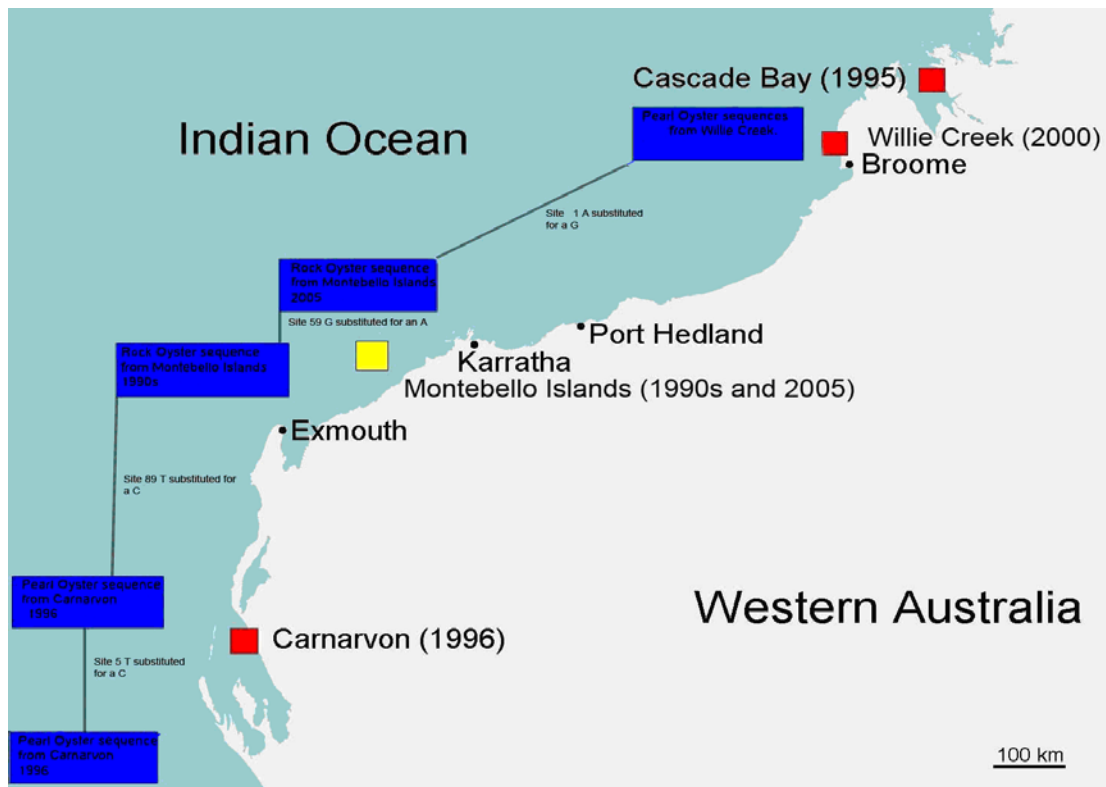
Figure 5. A minimum spanning network illustrating the relationships between the SSU rRNA gene sequences obtained from the rock and pearl oyster parasites. The blue rectangles represent extant parasite SSU rRNA alleles. Site indicates position on the Minch F1B/R2B sequence.
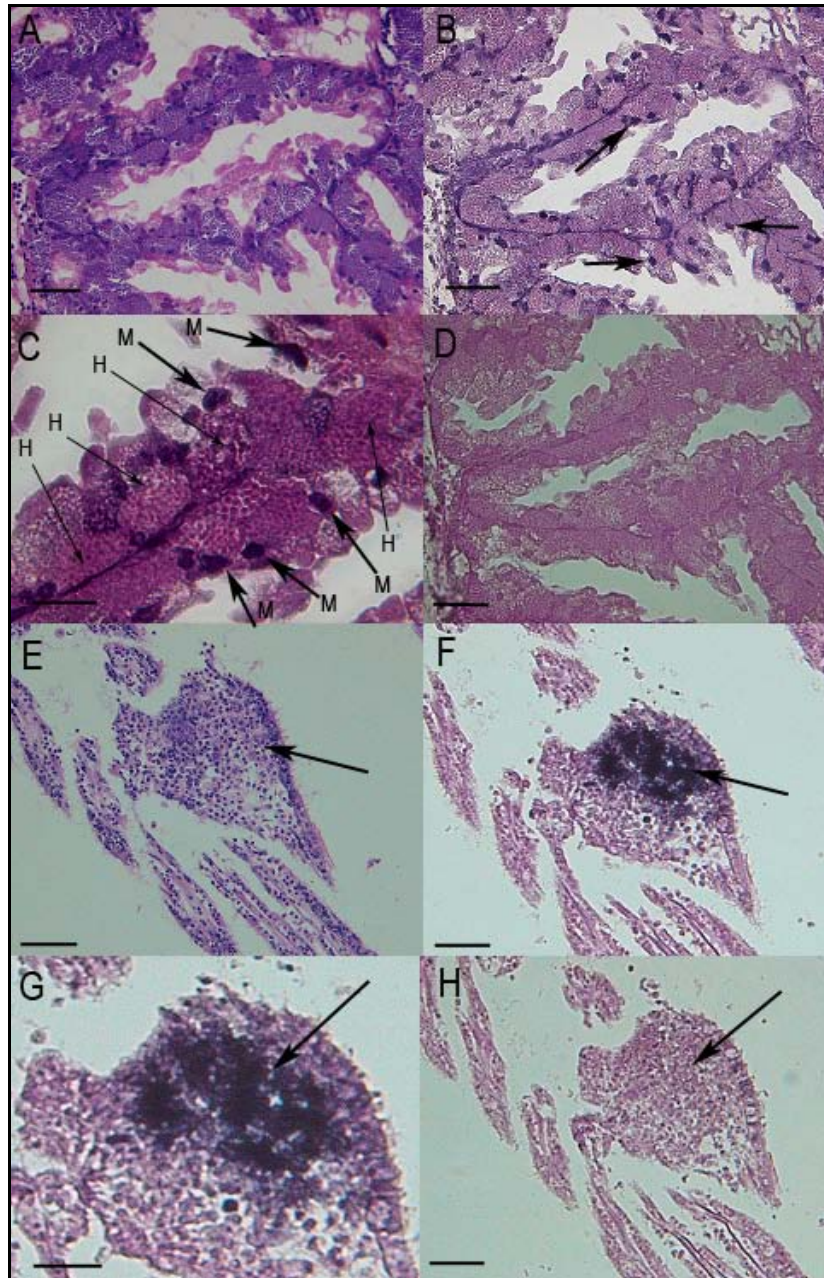
Figure 6. Haplosporidian parasites (arrows) identified in an *in situ* hybridisation of pearl oyster digestive gland. (A): H/E stained section of an infected pearl oyster digestive gland. (B) Serial *in situ* hybridisation containing the SSRDb probe. Scale bar = 65 μm (C): Higher magnification view of the same section. Arrow with an M denotes a *Minchinia*, Arrow with an H denotes *Haplosporidium hinei*. (D): Negative control serial section containing an irrelevant (MSX) probe. Scale bar = 65 μm. (E): H/E stained section of an infected rock oyster gill section. Scale bar = 40 μm. (F) Serial *in situ* hybridisation containing the SSRDb probe. Scale bar = 40 μm (G): Higher magnification view of the same section. (H): Negative control serial section containing an irrelevant (MSX) probe. Scale bar = 40 μm. Sections are counterstained in a brazilin hematoxylin.
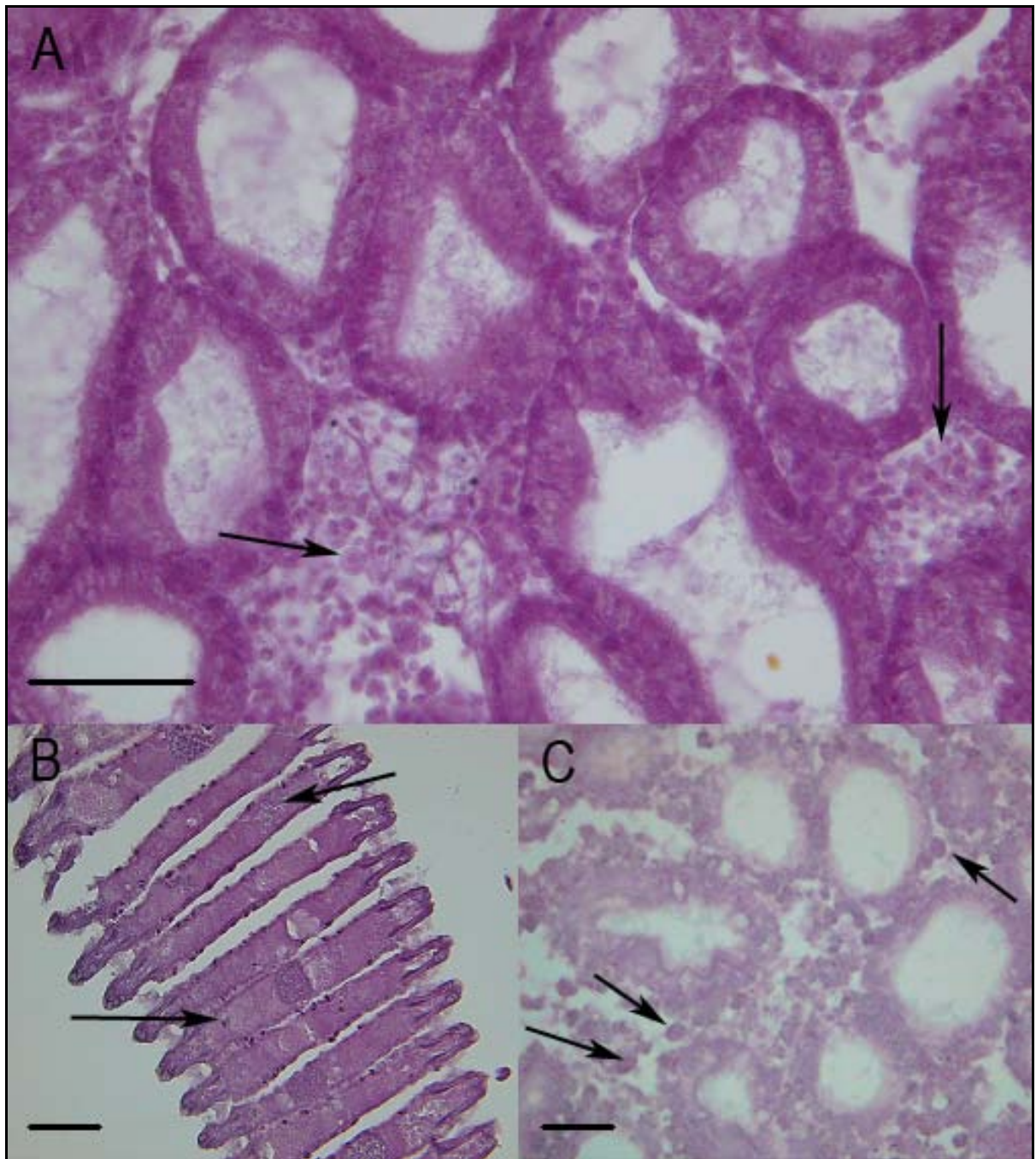
Figure 7. Bi-valve sections used to monitor the specificity of the SSRDb ISH assay. Arrows indicate example parasites. (A) *Haplosporidium nelsoni* infected Eastern oyster (*Crassostrea virginica*) digestive gland. Scale bar = 35 μm, *Minchinia teredinis* in *Teredo* sp. gill. Scale bar = 40 μm. (B) and *Haplosporidium costale* infected Eastern oyster digestive gland. Scale Bar = 20 μm.