Specificity of PCR and *in situ* hybridization assays designed for detection of *Marteilia sydneyi* and *M. refringens*

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SUMMARY

Primers and DNA probes designed for use in the specific detection of the paramyxean parasites *Marteilia sydneyi* and *Marteilia refringens* were tested for their potential to cross-react with closely related species in Polymerase Chain Reaction (PCR) and *in situ* hybridization. PCR primers and a DNA probe designed within the ITS1 rRNA of *M. sydneyi* were specific for *M. sydneyi* when compared with related species of *Marteilia* and *Marteilioides*. PCR primers designed within the 18S rRNA of *M. refringens* were specific in the detection of this species in PCR while a DNA probe (named Smart 2) designed on the same gene cross-reacted with *M. sydneyi* in tissue sections of *Saccostrea glomerata* as well as *Marteilioides* sp. infecting *Striostrea mytiloides*. Though not species specific, the Smart 2 probe provided a stronger signal in detection of all stages of *M. sydneyi* than the ITS1 probe. The ITS probe is proposed for use as a confirmatory diagnostic tool for *M. sydneyi*.

Key words: *in situ* hybridization, *Marteilia refringens*, *Marteilia sydneyi*, *Marteilioides* spp., PCR, species specificity.

INTRODUCTION

DNA-based detection techniques offer many applications for the study of parasites and the diseases they cause. In particular, two DNA technologies, the Polymerase Chain Reaction (PCR) and *in situ* hybridization, have been used in scientific research as well as for disease management in aquaculture (Fong *et al*. 1993; Stokes & Burreson, 1995; Stokes, Siddall & Burreson 1995; Bartholomew *et al*. 1997; Antonio *et al*. 1998; Frasca *et al*. 1999; Le Roux *et al*. 1999; Moran *et al*. 1999; Berthe, Burreson & Hine, 1999; Kleeman & Adlard, 2000).

Molecular techniques are rapid and highly sensitive. Furthermore, these technologies enable the unambiguous detection of all life-cycle stages of a pathogen that are otherwise unrecognizable using traditional detection methods. Nonetheless, such tests must be validated before they can be used with confidence (Hiney & Smith, 1998). Such assays must prove (1) sensitive enough to detect low levels of infection and/or individual cells, (2) unable to cross-react with host tissue, (3) able to be repeated with consistent results and (4) their levels of specificity among closely related species must be qualified.

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In 2 separate papers, PCR and *in situ* hybridization assays developed for the specific detection of the paramyxean parasites *Marteilia sydneyi* (see Kleeman & Adlard, 2000) and *Marteilia refringens* (see Le Roux *et al*. 1999) in their respective oyster hosts, *Saccostrea glomerata* and *Ostrea edulis*, were shown to meet the first 3 of these requirements. While the fourth remained untested, the selection of variable regions in the nuclear ribosomal DNA for primer and probe design may confer inherent specificity to the assays: the highly mutable nature of the transcribed spacer regions (Li & Graur, 1991) constituted the rationale for the selection of the ITS1 sequence by Kleeman & Adlard (2000) for the design of PCR primers and a DNA probe specific for *M. sydneyi*; and Le Roux *et al*. (1999) identified highly variable regions on the SSU of the rRNA gene of *M. refringens*, following comparison with various organisms, for the design of specific PCR primers and DNA probes.

Marteilia sydneyi and *M. refringens* have caused serious mortalities among cultured oysters since the late 1960s. *Marteilia sydneyi* causes QX disease in the Sydney rock oyster in Australia (Lester, 1986) and *M. refringens* is the aetiological agent of Aber disease in Europe (Grizel *et al*. 1974). DNA tools developed for the detection of these pathogens will be invaluable for studying aspects of these diseases that have thus far eluded researchers, specifically the complete life-cycle of the organism and its early

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development in the oyster host. However, the possibility of cross-reaction with closely related species must first be assessed before these tests can be used with confidence. This paper describes an evaluation of cross-reactivity in the *M. sydneyi* and *M. refringens* specific primers and DNA probes in PCR and *in situ* hybridization.

MATERIALS AND METHODS

Specimen preparation

Marteilia sydneyi infected *Saccostrea glomerata* were obtained from the Pimpama River, Queensland, Australia. *Marteilia refringens* infected *Ostrea edulis* were obtained from La Tremblade, France. Oysters were tested for the presence of Marteiliosis by microscopical examination of Hemacolor (Merck) stained imprints of oyster digestive gland. Five oysters of each species were selected with high parasite load (> 100 parasites per 100× field of view) and a portion of the digestive gland stored in 100% alcohol. Tissue samples (of each oyster) were fixed for either 6–24 h or for 1–16 weeks in 10% buffered formalin, dehydrated in alcohol and embedded in paraffin. Histological sections were cut (6 μm) and stained in haemotoxylin and eosin (H&E) to confirm presence or absence of the parasite in the digestive tubules. Additional paramyxean species were supplied: *Marteilioides chungmuensis* infecting *Crassostrea gigas* ($n = 1$) and fixed in 100% alcohol were obtained from Japan; and *Marteilioides* sp. infecting the blacklip oyster, *Striostrea mytiloides* (junior synonym *Saccostrea echinata*), ($n = 1$) and fixed in 10% buffered formalin were supplied from Darwin Harbour, Australia.

DNA extraction and purification

Genomic DNA was extracted from infected oyster tissue stored in 100% alcohol following digestion in a solution of extraction buffer (100 mM Tris, pH 8·0, 100 mM EDTA, pH 8·0, 100 mM NaCl), 10% SDS and proteinase K at 56 °C for 4–24 h. Remaining proteins and polysaccharides were removed by phenol/chloroform/isoamyl alcohol extraction and nucleic acids recovered by ethanol precipitation (see Sambrook, Fritsch & Maniatis, 1989). DNA concentration and purity were estimated by measuring the 260/280 optical density ratio of a solution following RNase treatment (0·2 μg RNase/μl of genomic DNA).

PCR primer sequence and DNA probe production

The location in the rDNA of primers designed by Kleeman & Adlard (2000) and Le Roux *et al*. (1999) for use in the PCR assay and in production of the DNA probes are given in Fig. 1A. Primer sequences are given in Fig. 1B. The *M. sydneyi* ITS1 primers, LEG1 and PRO2, were designed for the specific detection of *M. sydneyi* in the PCR assay and in construction of DNA probe ITS1 for use in *in situ* hybridization. The *M. refringens* SSU primers, SS2 and SAS2, were designed for the specific detection of *M. refringens* in the PCR assay and the primers SS2 and SAS1 were used in construction of the DNA probe Smart 2 for use in *in situ* hybridization. The DNA probes were synthesized by incorporation of digoxygenin-11-dUTP during PCR and using the PCR DIG probe Synthesis Kit (Boehringer Mannheim) according to the protocol suggested by the manufacturer. Incorporation of digoxigenin (DIG) was indicated by an increase in molecular mass as analysed on ethidium bromide-stained agarose gels and the labelled PCR product purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim). Probe concentration was estimated by side by side comparison of diluted series of the probe and DIG-labelled control (Boehringer and Mannheim) in a spot test on nylon membranes according to methodology in the application manual.

Polymerase chain reaction

The primer pairs LEG1/PRO2 (ITS1) and SS2/SAS2 (SSU) were tested in PCR on 100 ng, 10 ng and 1 ng of genomic DNA extracted from *M. sydneyi* infected *S. glomerata*, *M. refringens* infected *O. edulis* and *M. chungmuensis* infected *C. gigas*. The universal primers CS2/CAS1 (SSU rDNA) designed by Le Roux *et al*. (1999) were tested on genomic DNA extracted from infected oysters to act as an internal control. PCR was carried out under the following reaction parameters, expressed as final concentrations: $MgCl_2$ 2 mM; buffer 67 mM Tris–HCl, 16·6 mM $(NH_4)SO_4$, 0·45% (v/v) Triton X-100; dNTP's 200 mM; primers 10 pmol each; *Taq* DNA polymerase (Promega) 3 Units; DNA 1–50 ng; ultrapure water to 20–50 μl total. Thermal cycling parameters were as follows: denaturation at 95 °C for 60 s; primer annealing at 55 °C for 60 s; chain extension at 72 °C for 60 s, repeated for 30 cycles with a final cycle incorporating a 7 min extension. 'No DNA' samples were included as negative controls.

Gel electrophoresis and Southern blot analysis

Electrophoresis of amplified products was conducted through submarine agarose gels (1·2% [w/v] agarose, 1·2 μl ethidium bromide [10 mg/ml w/v] in 20 ml total, for 30 min at 100 V/20 mA) and examined and photographed under ultraviolet light. A molecular weight standard (100-bp ladder, Gibco BRL) was used to estimate the size of products. For

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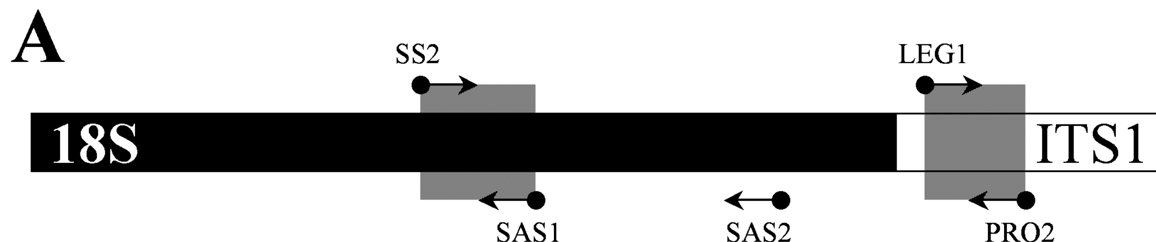

B

	Primer	Sequence 5'-3'	Purpose	Source
M. sydneyi	PRO2	TCA AGG GAC ATC CAA CGG TC	PCR and ITS1 probe	Kleeman & Adlard (2000)
	LEG1	CGA TCT GTG TAG TCG GAT TCC GA	PCR and ITS1 probe	Kleeman & Adlard (2000)
M. refringens	SS2	CCG GTG CCA GGT ATA TCT CG	PCR and Smart 2 probe	Le Roux et al. (1999)
	SAS1	TTC GGG TGG TCT TGA AAG GC	Smart 2 probe	Le Roux et al. (1999)
	SAS2	CGA ACG CAA ATT GCG CAG GG	PCR	Le Roux et al. (1999)

Fig. 1. Oligonucleotide primers designed in the *Marteilia refringens* (*M. refringens*) 18S and the *Marteilia sydneyi* (*M. sydneyi*) ITS1 region of the rRNA gene cluster for use in PCR and for production of the Smart 2 probe and ITS1 probe. (A) Annealing site. (B) Primer sequence.

Southern blot analysis, samples were transferred overnight to positively charged nylon membranes with 0·4 M NaOH, baked for 3 h at 80 °C and stored until hybridization. Membranes were pre-hybridized in 3× SSC (20× SSC = 3 M NaCl; 0·3 M Na-citrate, pH 7·0), 50% formamide, 1× Denhardts solution and 0·5 mg/ml heat-denatured herring sperm DNA) at 37 °C for 2 h. The pre-hybridization buffer solution was replaced with hybridization buffer (3× SSC, 50% formamide, 1× Denhardts solution and 0·5 mg/ml heat-denatured herring sperm DNA and 5% dextran sulfate) containing 50 ng/ml of DIG-labelled DNA probe (*M. sydneyi* ITS1 DNA probe (Kleeman & Adlard, 2000) for the LEG1/PRO2 assay and *M. refringens* Smart 2 DNA probe (Le Roux *et al*. 1999) for the SS2/SAS2 assay) and incubated overnight at 42 °C. Removal of unhybridized probe was achieved by 2×5 min washes in 2× SSC at room temperature and 2×15 min washes at 42 °C with 0·1× SSC. Following equilibration in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7·5), membranes were blocked for 30 min at room temperature in blocking buffer (maleic acid buffer plus 1% blocking reagent (Boehringer Mannheim)). Membranes were incubated at room temperature for 30 min with anti-digoxigenin-alkaline phosphatase antibody (Boehringer Mannheim) diluted 1:5000 in blocking buffer followed by removal of unbound antibody with 2×15 min washes in washing buffer (maleic acid buffer+0·3% Tween 20). After equilibration in detection buffer (100 mM Tris–HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9·5) the membrane was incubated at room temperature in the dark for 4–5 h in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) diluted in detection buffer. The reaction was stopped with a TE buffer wash.

In situ *hybridization assay*

The *M. sydneyi* ITS1 DNA probe and *M. refringens* Smart 2 DNA probe were tested in separate assays on tissue sections of *M. sydneyi* infected *S. glomerata*, *M. refringens* infected *O. edulis* and *Marteilioides* sp. infected *St. mytiloides*. Sections were cut 6 μm thick and placed on silanized slides (PROSCITEC) and baked for 45 min at 60 °C. Sections were deparaffinized and rehydrated in an ethanol series. Sections were permeabilized with 10 μg/ml proteinase K, for the 24 h-fixed sections, and 100 μg/ml, for sections fixed for ⩾1 week, in TNE (50 mM Tris–HCl, 10 mM NaCl, 1 mM EDTA.$2H_2O$, pH 7·4) for 30 min at 37 °C in a humid chamber. Proteolysis was inactivated by 2×1 min washes in PBS followed by equilibration in 2× SSC. Samples were pre-hybridized in 200 μl of pre-hybridization buffer in a humid chamber for 30 min at 37 °C. The pre-hybridization buffer solution was replaced with 100 μl of hybridization buffer containing 5–10 ng/100 μl DIG-labelled probe. Sections were covered with plastic cover-slips and placed in an oven at 85–95 °C for 5–10 min to denature the target DNA then immediately cooled on ice for 5 min and allowed

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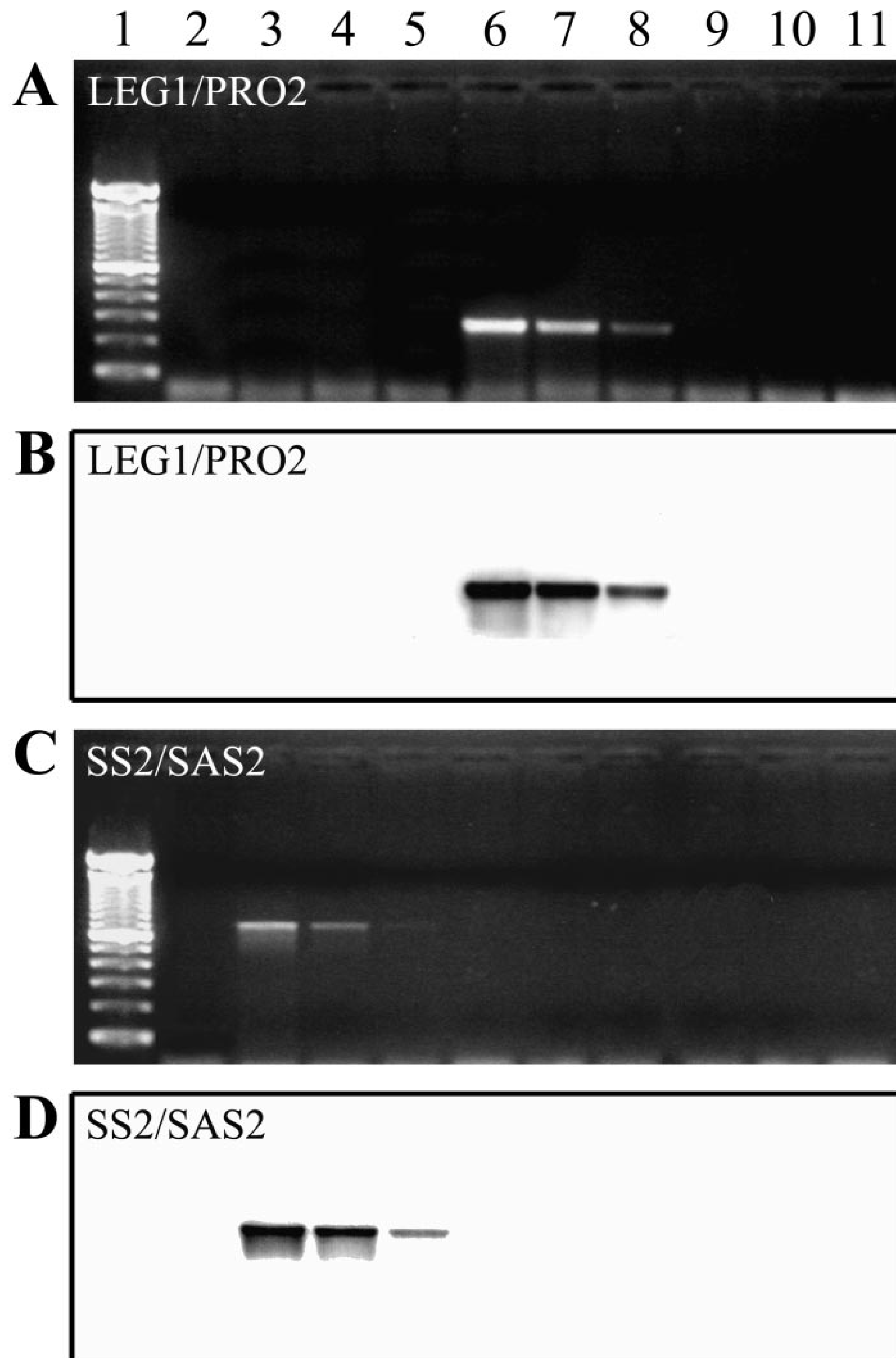

Fig. 2. Evaluation of the specificity of the primer pairs LEG1/PRO2 and SS2/SAS2 tested in PCR against genomic DNA extracted from *Marteilia refringens* infected *Ostrea edulis* (lanes 3–5), *Marteilia sydneyi* infected *Saccostrea glomerata* (lanes 6–8) and *Marteilioides chungmuensis* infected *Crassostrea gigas* (lanes 9–11), following detection by agarose gel electrophoresis (A and C) and Southern blotting (B and D). Lane 1, 100 bp ladder; lane 2, no DNA control; lanes 3, 6 and 9, 100 ng genomic DNA; lanes 4, 7 and 10, 10 ng genomic DNA; lanes 5, 8 and 11, 1 ng genomic DNA.

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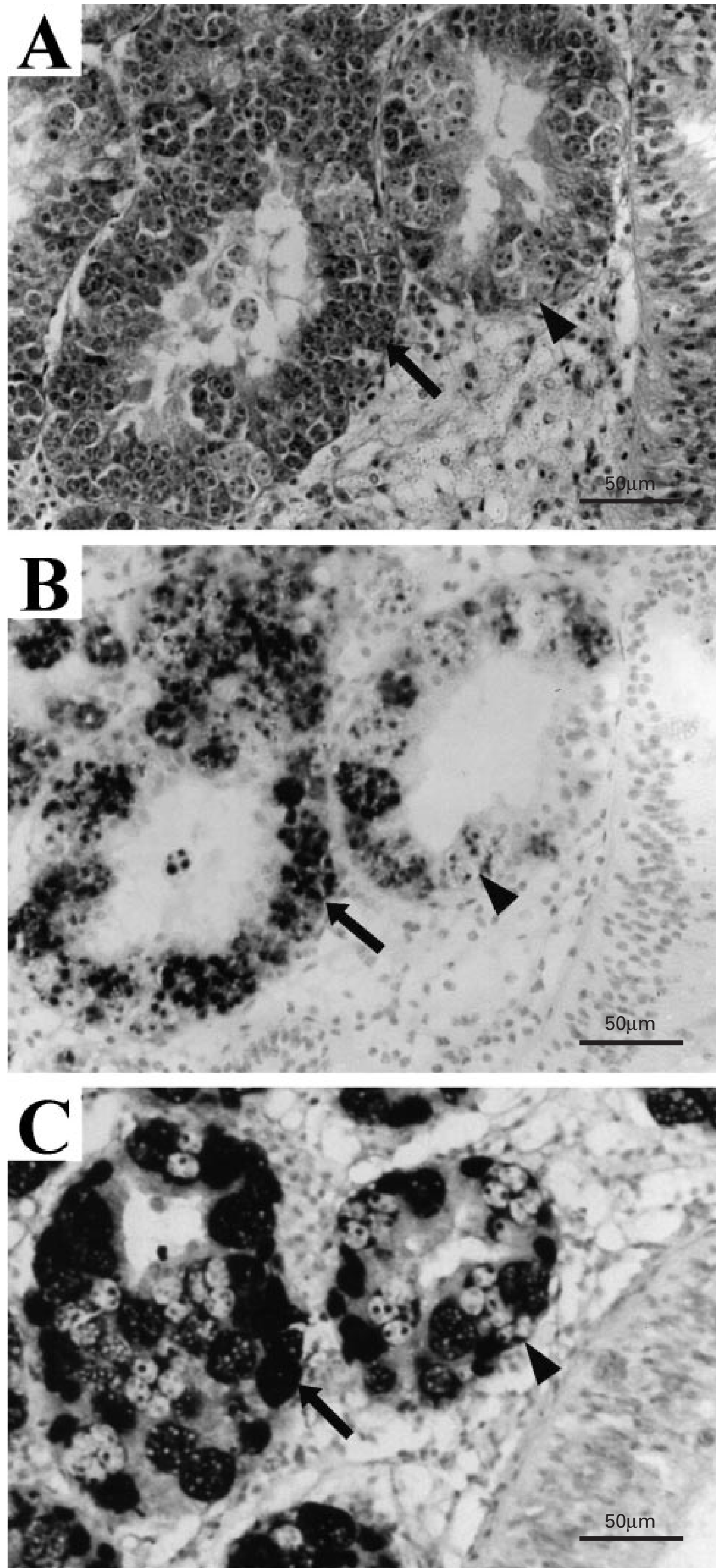

Fig. 3. Specificity of the ITS1 probe and Smart 2 probe in *in situ* hybridization as tested on *Marteilia sydneyi* in the digestive tubules of *Saccostrea glomerata*. (A) Detection by conventional histology, H&E stain. (B) Detection of mature (arrow head) and immature (arrow) sporont stages by the ITS1 probe. (C) Detection of mature and sporont stages by the Smart 2 probe.

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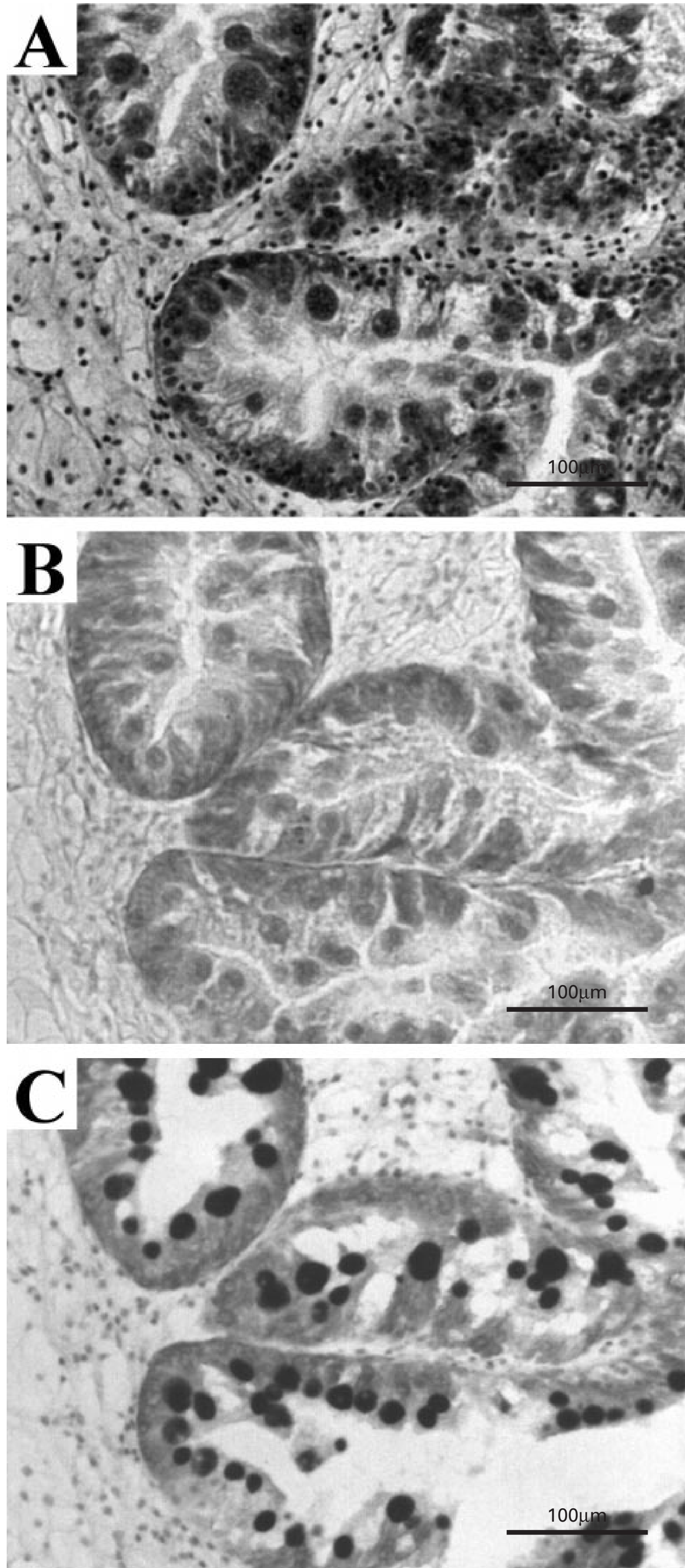

Fig. 4. Specificity of the ITS1 probe and Smart 2 probe in *in situ* hybridization as tested on *Marteilia refringens* in the digestive tubules of *Ostrea edulis*. (A) Detection by conventional histology, H&E stain. (B) Absence of reaction using the ITS1 probe. (C) Detection of sporonts by the Smart 2 probe.

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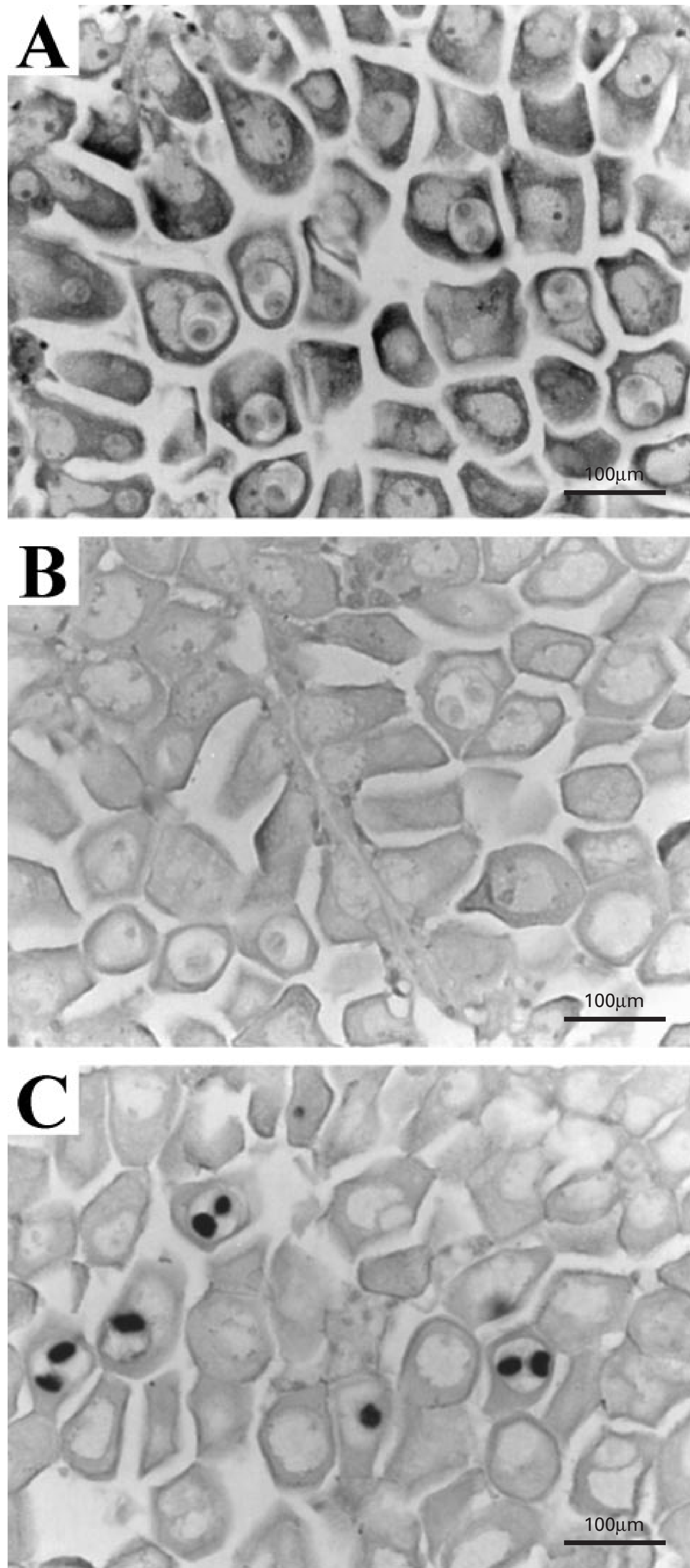

Fig. 5. Specificity of the ITS1 probe and Smart 2 probe in *in situ* hybridization as tested on *Marteilioides* sp. in the gonad of *Striostrea mytiloides*. (A) Detection by conventional histology, H&E stain. (B) Absence of reaction using the ITS1 probe. (C) Detection of sporonts by the Smart 2 probe.

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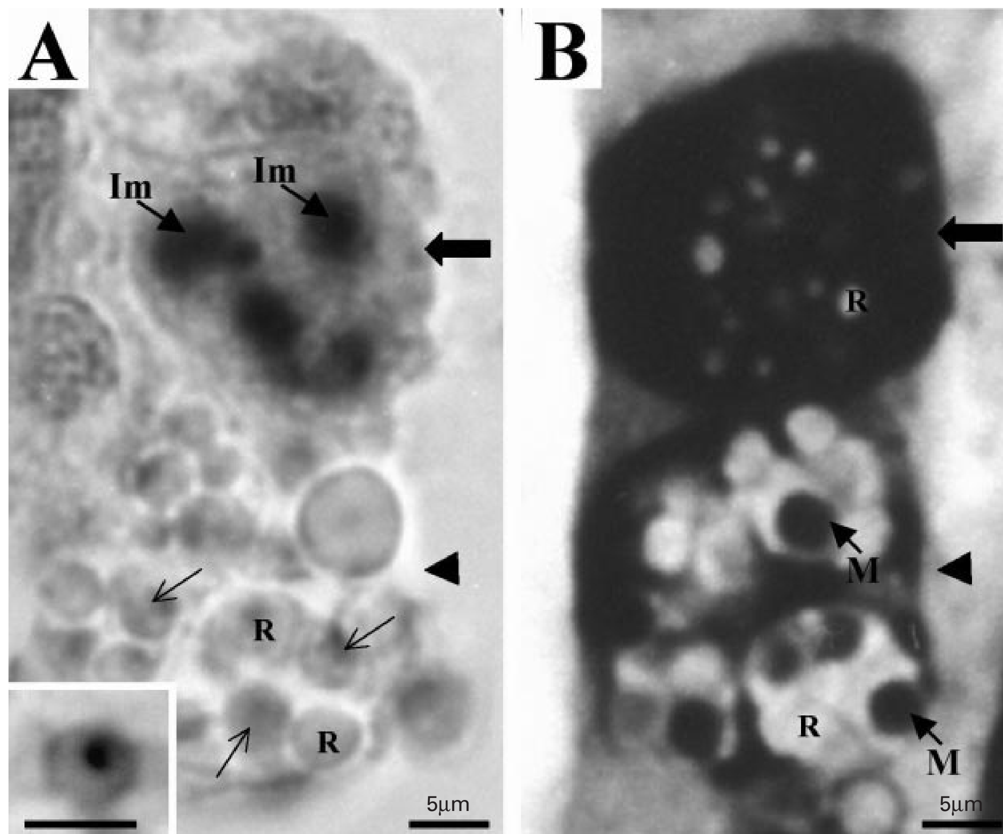

Fig. 6. Hybridization of the ITS1 probe (A) and Smart 2 probe (B) to immature (block arrow) and mature (block arrow head) sporont stages within sporangiosori of *Marteilia sydneyi* in tissue sections of *Saccostrea glomerata*. (A) Insert. Mature spore showing hybridization localized in the sporoplasm. Unlabelled arrows indicate some spores that have failed to hybridize. Im, immature spore contained within a sporont; M, mature spore contained within a sporont; R, refringent granule.

to hybridize overnight in a humid chamber at 42 °C. Post-hybridization washes included 2 × SSC at room temperature, twice for 5 min, and 0·1 × SSC at 42 °C, once for 10 min, followed by equilibration in maleic acid buffer. DIG-labelled probe detection included blocking sections with 200 μl of blocking buffer at 37 °C for 15 min followed by incubation for 1 h at 37 °C with 200 μl of dilute anti-digoxigenin-alkaline phosphatase conjugate (1:500 in blocking buffer). Unbound antibody was removed with 2 × 1 min washes in washing buffer and 1 × 5 min wash in detection buffer. BCIP/NBT was diluted in detection buffer and 200 μl of the colour solution added to the tissue and incubated at room temperature in the dark for 1–24 h. Signal intensity was assessed microscopically before stopping the reaction with a 5 min TE buffer wash. Slides were washed in ddH_2O and stained for 2 min in Bismark brown Y followed by ethanol dehydration and mounted in DPX via toluene.

RESULTS

The PCR assay

The quality of the genomic DNA and absence of inhibitory factors from infected oyster samples was verified following the amplification of all isolates by the universal primers CS2/CAS1 as well as detection of *M. sydneyi* DNA using the primers PRO2/LEG1 (Fig. 2A, lanes 6–8) and *M. refringens* DNA using the primers SS2/SAS2 (Fig. 2C, lanes 3–5). The *M. sydneyi* primers PRO2/LEG1 did not cross-react with either *M. refrigens* (Fig. 2A, lanes 3–5) or *M. chungmuensis* (Fig. 2A, lanes 9–11). Likewise, the *M. refringens* primers SS2/SAS2 did not cross-react with either *M. sydneyi* (Fig. 2C, lanes 6–8) or *M. chungmuensis* (Fig. 2C, lanes 9–11). Results assessed on agarose gels were confirmed following Southern blotting (Fig. 2B, E) which enables more sensitive detection of amplified fragments.

In situ *hybridization*

Parasite identity and location in tissue sections were confirmed in sections cut adjacent to those tested in *in situ* hybridization and after staining with H&E (Figs 3A, 4A, 5A). The ITS1 DNA probe recognized *M. sydneyi* DNA in tissue sections of *S. glomerata* (Fig. 3B) but did not hybridize to *M. refringens* in infected *O. edulis* tissue sections (Fig. 4B) or *Marteilioides* sp. in tissue sections of *St. mytiloides* (Fig. 5B). The Smart 2 probe hybridized to *M. refringens* in *O. edulis* (Fig. 4C) and cross-reacted

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with *M. sydneyi* in tissue sections of *S. glomerata* (Fig. 3C). Detection of *Marteilioides* sp. in tissue sections of *St. mytiloides* was also possible with the Smart 2 probe (Fig. 5C). The ITS1 probe required at least 4 h development to achieve a satisfactorily detectable signal. Precipitates were first detectable following 20 min using the Smart 2 probe which were suitably intense after 1–2 h.

The Smart 2 probe gave a more intensive signal in detection of *M. sydneyi* than the ITS1 DNA probe. Hybridized cells were easily detected at high power magnification with the use of both probes. At low power magnification, hybridized cells were easily overlooked when labelled by the ITS1 probe but still clearly observed when detected by the Smart 2 probe. Furthermore, different life-cycle stages showed different levels of detection for each probe. Fig. 6 compares the detection signal for each probe for immature and mature sporonts, contained within sporangiosori, of *M. sydneyi*. In immature sporont stages, the ITS1 probe hybridized to the spores but did not recognize superseded cells (Fig. 6A) while the Smart 2 probe hybridized to spores, sporonts and sporangiosori (Fig. 6B). In mature sporonts, the ITS1 probe inconsistently detected spores and where hybridization occurred it was localized in the innermost sporoplasm (Fig. 6A), while the Smart 2 probe showed strong, consistent hybridization to spores within sporonts as well as the sporangiosoral cell enclosing the sporonts (Fig. 6B). No precipitate was noted in the extraspore cytoplasm of mature sporonts as the refringent granules dominate the cell and are not accessible to the probes.

Where no signal was detected by the ITS1 probe, the quality and accessibility of targeted nucleic acids in tissue sections of infected oysters was verified following labelling of *M. refringens* within *O. edulis* as well as *Marteilioides* sp. in tissue sections of *St. mytiloides* by the Smart 2 DNA probe. No background hybridization was detected in surrounding oyster tissue, although colour precipitation extending beyond 4 h resulted in blue colouration, ranging from diffuse (4 h substrate incubation) to concentrated (24 h substrate incubation), in regions of the gills of some samples run with and without the addition of the DNA probes in the assay. Adjacent sections stained with H&E determined that the area was cartilaginous. No signal was detected in tissue regions carrying *Marteilia* or *Marteilioides* infections (digestive gland and gonad) and where the digoxigenin-labelled probe was omitted.

DISCUSSION

Levels of cross-reactivity in molecular detection assays are influenced by a variety of factors including assay conditions and DNA extraction or tissue preparation methods, but pivotal to developing specific tests is the appropriate design of primers and probes in regions of a well-characterized gene. Nuclear ribosomal DNA (rDNA) is widely recognized as a useful target for the definition of genetic markers informative at several levels (Gasser & Zhu, 1999). Variation in the rate of evolution of different regions of the rDNA results in target sequences ranging from highly conserved to highly mutable (Hillis & Dixon, 1991). The recognition of variable regions within the rDNA gene following comparison with gene sequences of other species allows the design of molecular detection tools of various levels of specificity. However, the rDNA sequences generated for the paramyxean parasites *M. sydneyi* and *M. refringens* did not span the same gene region: Anderson, Adlard & Lester (1995) provided putative ITS1 gene sequence for *M. sydneyi*, its location in the rDNA gene confirmed by Kleeman & Adlard (2000); and Berthe *et al*. (2000) provided the SSU rDNA sequence for *M. refringens*.

While the SSU sequence for *M. sydneyi* and ITS1 sequence for *M. refringens* are being generated and will be provided in future publications, this study has determined that both of these regions, particularly the ITS1, can be useful for the development of specific tests to detect paramyxean species in the absence of comparative sequence data from closely related organisms. It has been shown that the primer pair LEG1/PRO2, designed by Kleeman & Adlard (2000), and the region they amplify, constituting the ITS1 DNA probe, are useful for the development of DNA-based diagnostic systems for the specific identification of *M. sydneyi* among related species. Likewise, the PCR primers SS2/SAS2, designed on the SSU rRNA of *M. refringens* (see Le Roux *et al*. 1999), were found to be specific to *M. refringens* when tested against at least 2 different paramyxean species. However, the SSU Smart 2 probe (Le Roux *et al*. 1999) was found to hybridize with all *Paramyxea* species tested.

The rDNA SSU contains several variable motifs, interspersed within highly conserved regions, which often have a high degree of sequence similarity between closely related species within a putative genus. While this has allowed the phylogenetic interpretation of congeneric species (Blair & Barker, 1993), it makes difficult the development of species specific primers and probes. Present results suggest that the 265 bp region spanning the Smart 2 probe is relatively homologous between Paramyxea species. In turn, the ITS1 region spanning the 195 bp *M. sydneyi* specific DNA probe is likely to have low sequence similarity with *M. refringens* and *Marteilioides* sp. which suggests uniqueness of this motif. The internal transcribed spacers within the rDNA gene cluster typically evolve more rapidly than coding regions as they are less likely to be constrained functionally (Gasser, 1999) and are therefore more likely to provide species-specific regions.

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The PCR primers and DNA probes are yet to be tested on other paramyxean species. The potential for cross-reaction of the ITS1 primers and probe with *M. branchialis* is of interest as this species has been found in the same geographical location and host as *M. sydneyi* (see Anderson & Lester, 1992). Failure to find infections confirmed as *M. branchialis* and inability to receive material from previous workers, have hindered such tests. Le Roux *et al.* (1999) determined that the Smart 2 probe was able to hybridize to both *M. refringens* and *M. maurini* in tissue sections of infected mussels, *Mytilus galloprovincialis*, and considered that the Smart 2 probe was likely to be able to detect European *Marteilia* species. Present results suggest that the probe is able to detect Paramyxea species from at least two different genera and is likely to be a phylum specific test. While it needs to be established that other taxonomic groups are not detectable with this probe, incidental parasites that did not belong to the Paramyxea found in *S. glomerata* (ciliates, trematodes) and polychaetes (microsporidians, coccidians, gregarines, haplosporidians) did not cross-react (unpublished observations).

In *in situ* hybridization, the Smart 2 probe provided a stronger signal in detection of sporont stages of *M. sydneyi* than the ITS1 probe. In addition, the Smart 2 probe was more reliable in the detection of mature spores. Kleeman & Adlard (2000) noted the inconsistent and low signal in detection of mature spores of *M. sydneyi*, as well as the localization of specific cells (predominantly newly cleaved cell forms), by the ITS1 probe. Current findings suggest that the lower signal detection of the ITS1 probe in comparison to the SSU Smart 2 DNA probe reflects the availability of the target sequence. Internal transcribed spacer regions are excised from the mRNA in the cell cytoplasm prior to ribosomal construction (Campbell, N., personal communication). While the ITS target is still available in the nucleus (DNA), the RNA in mature cells would have few or no ITS regions, hence fewer hybridized probes and less precipitate. ITS regions would be expected to be present in larger quantities in the cell cytoplasm of early developmental stages that are rapidly dividing or replicating.

In conclusion, it has been revealed in the present study that while the ITS1 provides the best region for the production of species specific PCR tests, the 18S is the preferred region for probes designed for use in *in situ* hybridization. It is considered that short oligonucleotide probes designed in highly specific regions of the SSU may provide a better option than ITS probes for use in the development of species specific *in situ* hybridization assays. DIG-labelled oligonucleotides designed in the SSU of *Haplosporidium nelsoni* and *Minchinia costalis* have been used successfully to provide strong signals in the specific detection of these pathogens in *in situ* hybridization (Stokes & Burreson, 1995; Stokes *et al.* 1995). Nevertheless, the DNA probes developed thus far are considered valuable tools. A highly sensitive and reliable detection assay able to locate all paramyxean species, and a probe able to confirm the specific identity of detected cells, will be useful given the impact these parasites have on commercial fisheries.

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