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Elucidating the life cycle of *Marteilia sydneyi*, the aetiological agent of QX disease in the Sydney rock oyster (*Saccostrea glomerata*)

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ABSTRACT

Marteilia sydneyi (Phylum Paramyxea, Class Marteiliidea, Order Marteiliida) (the causative agent of QX disease) is recognised as the most severe parasite to infect *Saccostrea glomerata*, the Sydney rock oyster, on the east coast of Australia. Despite its potential impact on industry (>95% mortality), research towards lessening these effects has been hindered by the lack of an experimental laboratory model of infection as a consequence of our incomplete understanding of the life cycle of this parasite. Here, we explored the presence of this parasite in hosts other than a bivalve mollusc from two study sites on the Hawkesbury River, New South Wales, Australia. We employed PCR-based in situ hybridisation and sequence analysis of a portion of the first internal transcribed spacer of rDNA in an attempt to detect *M. sydneyi* DNA in 21 species of polychaete worm. *Marteilia* DNA was detected in 6% of 1247 samples examined by PCR; the analysis of all amplicons defined one distinct sequence type for first internal transcribed spacer, representing *M. sydneyi*. Of the polychaete operational taxonomic units test-positive in PCR, we examined 116 samples via in situ hybridisation DNA probe staining and identified *M. sydneyi* DNA in the epithelium of the intestine of two specimens of *Nephtys australiensis*. Two differing morphological forms were identified: a 'primordial' cell that contained a well-defined nucleus but had little differentiation in the cytoplasm, and a 'plasmodial' cell that showed an apparent syncytial structure. This finding represents the first known record of the identification of *M. sydneyi* being parasitic in an organism other than an oyster, and only the third record of any species of *Marteilia* identified from non-molluscan hosts. Future work aims at determining if *N. australiensis* and *S. glomerata* are the only hosts in the life cycle of this paramyxean, and the development of experimental models to aid the production of QX disease-resistant oysters.

1. Introduction

Since the mid-1970s, the protozoan *Marteilia sydneyi* (Phylum Paramyxea), the aetiological agent of QX disease, has been recognised as the most pathogenic parasite of the Sydney rock oyster (SRO), *Saccostrea glomerata*, particularly in estuaries of southern Queensland (Qld) and northern New South Wales (NSW), Australia (Adlard and Ernst, 1995). As a consequence of the significant impact the disease may have during outbreaks (i.e. ⩾95% mortality; (Bezemer et al., 2006)), control measures typically require quarantining entire estuaries in order to restrict the movement of infected stock. In the absence of data on transmission and the causative elements that promote these outbreaks, this has been the most conservative course of action and, until recently, the only management tool available to protect the SRO industry. However, with reports indicating that *M. sydneyi* is present in most estuaries in which major SRO culture is undertaken (Adlard and Wesche, 2005), even though many have never suffered significant disease events, research has turned to investigating the contribution of oyster immuno-competence to disease inhibition (Bezemer et al., 2006; Butt and Raftos, 2008; Green et al., 2009; Dang et al., 2011), the production of QX disease-resistant oysters (Nell et al., 2000; Nell, 2001; Green et al., 2008) and disease resistance biomarkers (Simonian et al., 2009).

One major obstacle to furthering these avenues of research is the lack of a laboratory or experimental model of infection, a consequence of our incomplete understanding of the life cycle of this parasite. The best known component(s) of the *M. sydneyi* life cycle (as with the pathogenic *Marteilia refringens* from *Ostrea edulis*

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in Europe) involve the definitive host (Perkins and Wolf, 1976). Similar to studies from Europe which postulate the existence of a complex life cycle for *M. refringens* (Berthe et al., 1998, 2004; Audemard et al., 2001, 2002; Arzul et al., 2013; Boyer et al., 2013), the suggestion that the life cycle of *M. sydneyi* is indirect originated in the mid-1980s when cross-infection experiments failed (Lester, 1986). Additionally, the discovery in vitro that spores of *M. sydneyi* have a limited viability in the marine environment (Wesche et al., 1999), implicated the existence of one or more intermediate host(s). Adlard and Lester (1996) postulated the existence of a direct correlation between the abundance of the major component of benthic organisms (i.e. polychaete worms) and the prevalence of QX disease during outbreaks, which spurred interest in investigating this group as possible alternate hosts. However, the use of classical laboratory techniques has inhibited the reliable and unambiguous identification of unknown morphological stages of *M. sydneyi* in alternate hosts (Kleeman and Adlard, 2000), indicating the clear need to undertake a systematic molecular investigation of a range of polychaetes in affected estuarine systems.

PCR-based techniques, employing suitable gene markers, coupled with diagnostic methods such as in-situ hybridisation (ISH), have been used to investigate parasitic life cycles (Fong et al., 1993; Stokes et al., 1995). Previously, we have shown this approach is highly sensitive and specific for the accurate identification of *Marteilia* infections in oysters (Anderson et al., 1995; Kleeman and Adlard, 2000; Kleeman et al., 2002a,b; Adlard and Worthington-Wilmer, 2003; Adlard and Wesche, 2005). To date, PCR-only based approaches have revealed the presence of *M. sydneyi* nucleic acids in polychaetes collected in QX-endemic areas; however, whether the *M. sydneyi* DNA detected in these samples is derived from developing infections or the result of accidental ingestion of the pathogen during feeding remains to be explored. Thus, in the present investigation we examined a range of polychaetes in the Hawkesbury River, NSW, Australia, employing a combined PCR/ISH-based approach in an attempt to identify and characterise previously unknown life cycle stages of *M. sydneyi* and link these genetically with those detected previously in *S. glomerata*.

2. Materials and methods

2.1. Sample timing

The current study employed an established PCR protocol (Kleeman and Adlard, 2000; Adlard and Worthington-Wilmer, 2003) to detect the presence of *M. sydneyi* DNA in benthic macrofauna. We anticipated that PCR-positive samples would fall into two categories; 'false positives' that were the product of incidental ingestion of spores which remain in the digestive tract of benthic fauna, and 'real positives' that were the result of uptake and development of the parasite within 'true' alternate hosts. To minimise the likelihood of detecting false positives we sampled benthic organisms in November (late spring). We anticipated that sampling during this temporal window would mean that the majority of infected oysters had already shed spores and died and parasite development within an alternate host would be well advanced to allow infection of oysters during the following (mid-summer) infection period (Bower et al., 1994). Consequently, the probability of detecting developing *M. sydneyi* stages in alternate hosts would be maximised.

2.2. Sample collection on the Hawkesbury River, NSW

The New South Wales Department of Primary Industries (NSW DPI), Australia provided a geographic map of the upper Hawkesbury River region. Two areas, Cobar (33°32′37″S 151°08′17″E) and Kimmerikong (33°32′51″S 151°09′10″E), associated with current and former oyster leases and unfarmed neighbouring areas, were selected for sampling (Fig. 1). Both areas were overlaid with a numbered grid; 150 computer generated random numbers were then plotted across each area and the latitude and longitude determined for these (data not shown). GPS points for each sampled site were relocated on the Hawkesbury River using a hand-held GPS unit (see Fig. 2A, B). Benthic samples were collected using a van Veen grab sampler between 7 and 20 November 2006 from 50 sites across Cobar (Fig. 2A) and 64 at Kimmerikong (Fig. 2B). One sample was taken at each site and only five samples were collected at one time to prevent deterioration of the macrobenthic fauna. Samples were placed in separate 5 L containers for transport and labelled with the site number (1–150 for Cobar and 151–300 for Kimmerikong samples) before each was reduced in volume by washing it through a series of two stacked sieves (1 mm and 500 μm). 'Semi-clean' samples were then soaked for a further 45 min, followed by a second wash (500 μm sieve) to remove loosened sediment.

'Clean' samples were poured into Petri dishes and allowed to settle for 10–15 min before being scanned using a stereomicroscope. Polychaetes were removed from the sample using featherweight forceps and stored in hemagglutination trays in river water. Polychaetes were classified into operational taxonomic units (OTUs) (family; putative species) for each site. Such an approach to classification was felt appropriate because Australia has some of the highest diversity of polychaetes in soft sediments and a large number of taxa remain to be described (Beesley et al., 2000). Numbers of each OTU from each site were recorded before individual site OTUs were combined and half the specimens transferred to microcentrifuge tubes containing 95% ethanol (for DNA analysis) and half to 10% formalin (to represent an OTU or for ISH) (both at room temperate; RT). Specimens for ISH were changed from formalin to ethanol after 1–2 weeks. Formalin-fixed OTU specimens were photographed using a Nikon Digital Sight camera (DS-5M) attached to a stereomicroscope located at the Queensland Museum, Brisbane, Australia. Voucher specimens for all polychaete OTUs have been retained in the research collection at the Queensland Museum.

2.3. Genomic DNA extraction and PCR-coupled sequencing

Genomic DNA (gDNA) was extracted from single polychaete specimens using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The PCR protocol employed here essentially followed that reported by Kleeman and Adlard (2000) and Adlard and Worthington-Wilmer (2003). In brief, 195 nucleotides of the first internal transcribed spacer (ITS1) of rDNA were amplified using the primers LEG1 (forward: 5′-CGATCTGTGTAGTCGGATTCCGA-3′) and PRO2 (reverse: 5′-TCAAGGGACATCCAACGGTC-3′) (Kleeman and Adlard, 2000). PCR was carried out in a volume of 25 μl containing 2.5 μl of 10× HotMaster™ *Taq* buffer (Qiagen) with 25 mM magnesium chloride ($MgCl_2$), 200 μM of each dNTP, 50 pmol of each primer, 1.25 μl of DMSO (5.0% final concentration) and 0.75 U of HotStar*Taq* DNA polymerase (Qiagen) utilising a cycling protocol that consisted of 95 °C for 10 min (initial denaturation), followed by 35 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s (annealing) and 65 °C for 30 s (extension), with a final extension at 65 °C for 5 min.

Following PCR, all amplicons were run on a 1% TBE (0.89 M Tris base, 0.89 M boric acid, 0.5 M EDTA buffer; Sigma Aldrich, USA) agarose gel; amplicons indicated to be of the appropriate size (i.e. 195 nucleotides) and representing each distinct OTU from Cobar and Kimmerikong were purified using the QIAquick® PCR Purification Kit (Qiagen), according to the manufacturer's