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Development of a qPCR assay for the Pacific Oyster, *Crassostrea gigas*

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Executive Summary

The *National System for the Prevention and Management of Marine Pests* requires tools for the detection and monitoring of marine pests. Specific, robust molecular assays for the identification and quantification of marine pests (including eggs and larvae) from environmental samples provide the rapid, low-cost surveillance required to support effective control of marine pests.

Marine pest surveillance is currently based on dive surveys, dredges, traps and plankton tows, with organisms being identified by traditional taxonomy. This process is slow and expensive. Quantitative polymerase chain reaction (qPCR) is suitable for marine pest surveillance because it can test very large numbers of samples and rapidly identify the genetic material of the targeted organism (referred to as high-throughput screening). PCR is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA), and qPCR is a PCR technique monitored in real-time through changes in fluorescence to allow quantification of the amount of target DNA in a sample. The development of assays for marine pests using this method will facilitate testing plankton samples to verify the presence of a potential pest species in marine waters. This surveillance method is faster and cheaper than traditional surveys.

In this study we developed a qPCR assay for the detection of the Pacific oyster, *Crassostrea gigas*. We have assessed the specificity of the assay with a range of other bivalve and marine species, and the assay is specific based on the DNA controls in our collection. We tested DNA extracted from plankton samples from Port Adelaide for *C. gigas* using the qPCR assay developed in this study and found no positives.

This assay, in conjunction with our other marine pest assays and plankton sampling methodologies provides a basis for ongoing routine surveillance of marine pests. There is still, however, a need to validate these methods in more localities and on different plankton assemblages. This broader validation will help to identify any issues with sampling procedures, and calibrate the DNA extraction process on a wider range of samples, allowing confirmation of the general applicability of the test.

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Introduction

Marine pests can cause substantial harm to endemic biodiversity and habitats (Galil, 2007; Wallentinus and Nyberg, 2007). Marine pests can be translocated and introduced by vectors including ship ballast, hull fouling, floating debris, transportable man-made structures and aquarium releases (Bax et al., 2003). Marine pest introductions continue to occur and threaten the marine environment, associated industries, communities and social amenity (Hayes and Sliwa, 2003). Increasing globalisation will intensify worldwide transport networks and air transport of live seafood and aquarium fish, and shipping will become faster and trips between ports will become more frequent. Propagule pressure is likely to increase unless effective strategies are employed for prevention, early detection and control. Central to such strategies is the ability to rapidly detect and identify the presence of a pest species.

The early detection of newly arrived pests is essential to facilitate an effective response or containment. The Australian, State and Territory governments, along with marine industries and researchers, are collaborating to implement Australia's *National System for the Prevention and Management of Marine Pest Incursions* (the National System). The National System aims to prevent new marine pests arriving, respond when a new pest does arrive or a significant range extension occurs, and minimise the spread and impact of pests already established in Australia.

The National System requires tools for the detection and monitoring of marine pests. Specific, robust molecular assays for identification and quantification of marine pests (including eggs and larval stages) from environmental samples facilitate rapid, low-cost surveillance, and inform effective control strategies where marine pest incursions and range extensions are detected. The development and implementation of rapid, sensitive and accurate diagnostic techniques for identification and surveillance of marine pests from environmental samples (e.g. sea water, sediments, and ship's ballast), particularly in areas that are currently pest free, is an essential step in early detection and control of marine pests.

Current marine pest diagnostics research at SARDI includes the development and refinement of qPCR assays for the detection of a number of marine pest species (see Bott et al; 2010a; Bott and Giblot-Ducray, 2011a; 2011b). PCR is an enzymatic technique used for the amplification of nucleic acids (e.g. DNA). qPCR is a PCR technique that enables quantification of target DNA through monitoring changes in fluorescence in the reaction in real-time. Consultation between SARDI Aquatic Sciences and Biosecurity SA determined

that a qPCR assay for the Pacific Oyster, *Crassostrea gigas,* should be developed*. Crassostrea gigas* was listed on the Consultative Committee on Introduced Marine Pest Emergencies (CCIMPE) Trigger List, which was endorsed by the former National Introduced Marine Pest Coordination Group (NIMPCG), now the Marine Pest Sectoral Committee (MPSC).

Pacific oyster, *Crassostrea gigas*

The Pacific oyster, *Crassostrea gigas* (Figure 1), is native to the Pacific coast of Asia, and has established outside its native range in North America, Europe, New Zealand and Australia (Smith et al., 1996; Hewitt et al., 2004; Troost, 2010). In Australia it is farmed commercially in South Australia, Tasmania and New South Wales, and as an invasive species it can modify estuarine habitats and out-compete native invertebrates (NSW Department of Primary Industries, 2012).

Figure 1*: Crassostrea gigas* in an intertidal zone

Molecular testing methods for marine pests

Development of rapid testing methods has recently focussed on molecular techniques, and a broad range of these techniques have been assessed for marine pests (see Bott et al., 2010b and references therein). PCR has revolutionised many areas of biological research including species and strain delineation. PCR can amplify minute amounts of template DNA, and its high specificity makes it effective for species and strain identification for a wide range of organisms. The relatively low cost of equipment and reagents makes PCR accessible to a wide range of laboratories. qPCR offers high-throughput screening of samples and allows amplification of target DNA to be monitored in real-time. It offers rapid analysis time (< 2 hours), linear quantification over a wide dynamic range (>6 orders of magnitude), and has the benefit of not requiring post-PCR handling ("closed-tube" format). It is now routinely used in numerous clinical applications for the detection of a wide range of bacterial, fungal, parasitic and viral diseases of humans (Espy et al., 2006). Several recent studies have utilised qPCR-based techniques for the identification of marine pests (see Galluzi et al., 2004; Pan et al., 2007).

Development of these tests requires that the target organism is taxonomically unambiguous. Testing species closely related to the target organism and testing environmental samples containing unknown taxa is required to assess assay specificity. Most test development achieves the first criterion, but for implementation, it is important to validate tests on samples exhibiting higher complexity such as water and sediment due to the high diversity inherent to these types of samples. These samples should be obtained from a variety of localities covering the biogeographic regions where the test will be used.

Many PCR-based tests are developed based on nuclear, ribosomal and mitochondrial gene sequences. A suitable DNA region should vary in sequence sufficiently to allow the identification of an individual organism to the taxonomic level required. For specific identification, the DNA marker should exhibit little or no genetic variation within a species but differ sufficiently between species so as to allow unequivocal delineation.

Genes evolve at different rates. In nuclear genes and spacers, there is typically little variation among individuals of a species within and between populations (Larsen et al., 2005; Livi et al., 2006). The ribosomal DNA (rDNA) genes, Internal Transcribed Spacers (ITS) and Intergenic Spacer (IGS)/ Non-transcribed Spacer (NTS) regions are particularly useful as species specific markers for marine pest assay development. The mitochondrial genome is also utilised for diagnostic purposes; mitochondria are generally inherited

maternally making them particularly useful as a species-specific marker for the delineation of closely related species (e.g. Blair et al., 2006, Kamikawa et al., 2008).

Methods

We modified a *Crassostrea gigas* qPCR assay based on mitochondrial cytochrome c oxidase 1 (cox1) sequences designed by CSIRO Marine Research to make the assay more sensitive in line with other qPCR assays used at SARDI. We have utilised the Forward primer (5'-TCT TAT TCG TTG GAG ACT TTA TAA CCC T-3') and TaqMan MGB probe (5'-CCC CGT GAC TTA TAA TG-3') from CSIRO and designed a new reverse primer (5'-ATA ACC AAC GCA TGC CTA GTT AC-3'). We conducted specificity testing of this new qPCR assay using a defined set of heterologous controls.

All assays at SARDI Diagnostics (see Figure 2) are developed as qPCR, using TaqMan® minor groove binder (TaqMan MGB) chemistry. DNA sequences of the desired genetic marker of target and related organisms were imported into the sequence manipulation software Bioedit© (available from http://www.mbio.ncsu.edu/bioedit/bioedit.html), and aligned using Clustal W (Larkin et al., 2007). The genetic marker of choice is defined by the ability for that marker to be able to delineate the target from heterologous species and also the availability of sequences from publicly available databases. The National Centre for Biotechnology Information (NCBI), as a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH), has developed databases to deal with molecular data, and facilitates the use of molecular databases by the research and medical community, Genbank® (http://www.ncbi.nlm.nih.gov/genbank/) is one of these databases and is an annotated collection of all publicly available nucleotide and amino acid sequences. A range of DNA sequences of *C. gigas* and related taxa were obtained from GenBank.

Sequences of target and related taxa were aligned to infer sequence regions that appeared to be diagnostically useful. DNA sequences were identified which are common to the target taxa but where there are enough differences to distinguish target from related taxa. Specific reverse PCR primers to match the forward primer and TaqMan MGB probe already developed were identified by eye and checked using OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) (Kibbe, 2007) to ensure that they displayed suitable thermodynamic properties and nucleotide content for efficient amplification.

Samples

Samples of *C. gigas* from farms in South Australia were purchased in Adelaide from seafood shops, and immediately stored frozen, or preserved in ethanol, for gDNA extraction. Samples of Sydney Rock Oyster, *Saccostrea glomerata* and native flat oyster, *Ostrea angasi* were donated by Tom Madigan, SARDI Food Safety.

DNA extractions

Genomic DNA was extracted from target and non-target tissues using either of two methods. The first method was the Root Disease Testing Service (RDTS) commercial DNA extraction method, a service provided by SARDI Diagnostics, while the second method was the QIAGEN DNeasy Blood and Tissue kit, following the manufacturer's instructions. DNA concentration was estimated by fluorometry (Wallac® 1420 multilabel counter) using Quant-iT™ PicoGreen® (Invitrogen). gDNA for qPCR specificity experiments was diluted to 200 pg/µl.

Figure 2: SARDI Diagnostics Laboratory

Plankton sampling

Plankton samples were collected from Port Adelaide in November/December 2010 and May 2011 using the methodology developed by SARDI (Giblot-Ducray and Bott, in preparation). Two freeze dried brine shrimp, *Artemia* sp. were added to the sample at collection, and a brine shrimp qPCR assay was used as a control to monitor that the sample had been maintained appropriately and quality had not been significantly affected by handling. Briefly, plankton was collected in plankton nets, the contents transferred to a specimen container containing RNAlater, and brine shrimp added. The sample was later filtered through a 48μm qualitative paper filter (Filtech) in the laboratory and DNA extracted. Extracted plankton DNA was then tested using the *C. gigas* qPCR assay. The complete method is outlined in a commercial-in-confidence report to Biosecurity SA (Giblot-Ducray and Bott, in preparation).

Results

Development of Pacific Oyster, *Crassostrea gigas* qPCR assay

The TaqMan MGB assay for the detection of the Pacific Oyster, *Crassostrea gigas,* designed by CSIRO Marine Research was re-designed for increased sensitivity to suit our needs. Changing the reverse primer improved the assay with Ct values for *C. gigas* gDNA extracts at 200 pg/μl dropping from 22-24 with the original CSIRO reverse primer to 19-20 with the newly designed reverse primer. The Ct value represents the PCR cycle number at which the fluorescence signal passes a fixed threshold, displayed as a horizontal green line in plots showing number of qPCR cycles vs magnitude of the fluorescence signal intensity (ΔRn) (Figure 3). The lower the Ct value, the more target DNA has been detected.

Specificity of the *Crassostrea gigas* qPCR assay

The modified assay was tested on a range of related and unrelated non-target taxa to assess that it was specific and did not detect non-target species. This experiment, including the Sydney rock oyster (*Saccostrea glomerata*), the native flat oyster (*Ostrea angasi*), a range of other bivalve species as well as more distant taxa, showed that the modified assay is specific to *C. gigas* (Table 1).

Table 1: Results of specificity testing for *Crassostrea gigas* qPCR assay

Phylum	Class	Genus	Species	Conc. (pg/ul)	RT-PCR Ct thold 0.2
Mollusca	Bivalvia	*Saccostrea*	*glomerata*	200	UD
		Ostrea	*angasi*	200	UD
		Crassostrea	*gigas*	200	19.8
		Limnoperna	*securis*	200	UD
		Musculus	*miranda*	200	UD
		Musculus	*cummingianus*	200	UD
		Perna	*viridis*	200	UD
		Modiolus	*micropterus*	200	UD
		Trichomya	*hirsutus*	200	UD
		Musculista	*senhousia*	200	UD
		Perna	*canaliculus*	200	UD
		Corbula	*gibba*	200	UD
Echinodermata		*Asterias*	*amurensis*	200	UD
		Asterias	*amurensis*	200	UD
		Asterias	*amurensis*	200	UD
Chordata		*Ciona*	*intestinalis*	200	UD
		Ascidiella	*aspersa*	200	UD
Heterokontophyta	Phaeophyceae	*Undaria*	*pinnatifida*	200	UD
Arthropoda		*Carcinus*	*maenas*	200	UD
Annelida	Polychaeta	*Sabella*	*spallanzanii*	200	UD
		ntc			UD

Key: NTC- No Template Control, UD-undetected

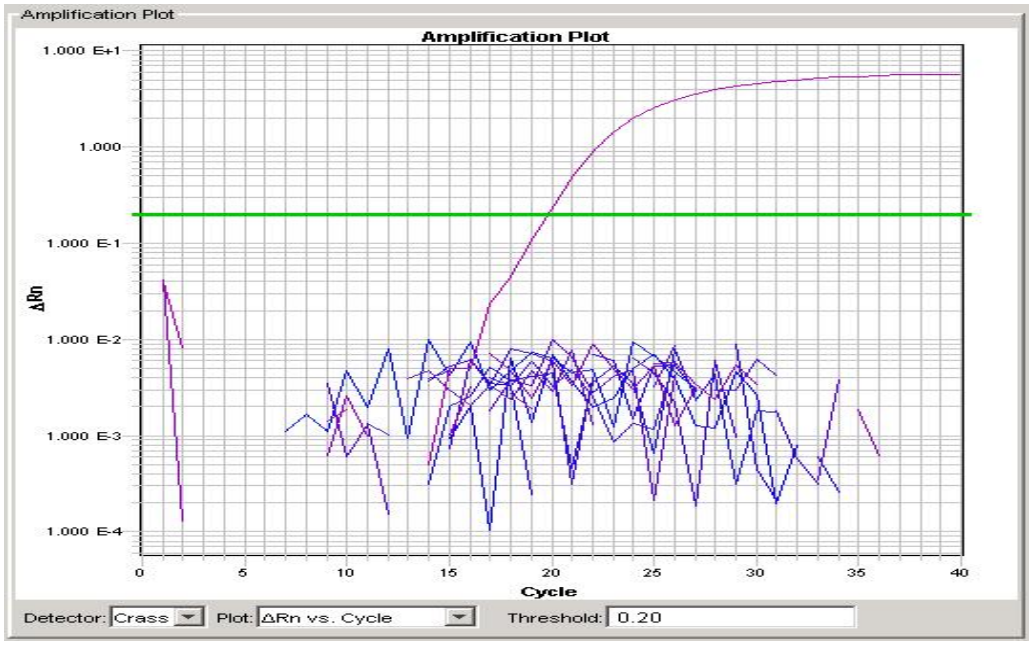

Figure 3: Amplification plot for specificity testing of *Crassostrea gigas* qPCR assay

Sensitivity of the *Crassostrea gigas* qPCR assay

The limit of detection of the *C. gigas* assay was determined using dilutions of target DNA ranging from 200 000 to 2fg/µl. For each level, a total of 32 replicate PCRs were conducted. The assay can reliably detect 20 fg/µl of *C. gigas* DNA, and more erratically as little as 2fg/µl (Figure 4). The assay proved to be linear over at least 6 orders of magnitude (R^2=0.995; Figure 5), and the efficiency of the assay calculated based on these calibration data is 94.5%.

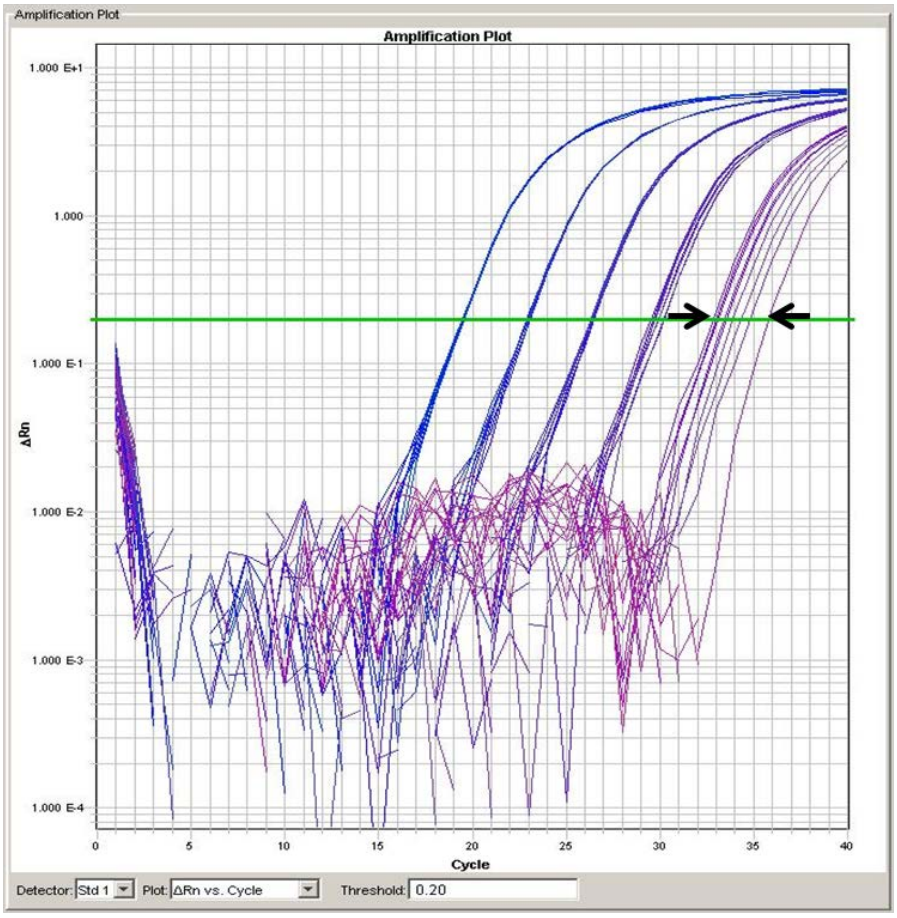

Figure 4: Sensitivity testing for *Crassostrea gigas* qPCR assay. Note: The arrows indicate the 2 fg/ µl replicates

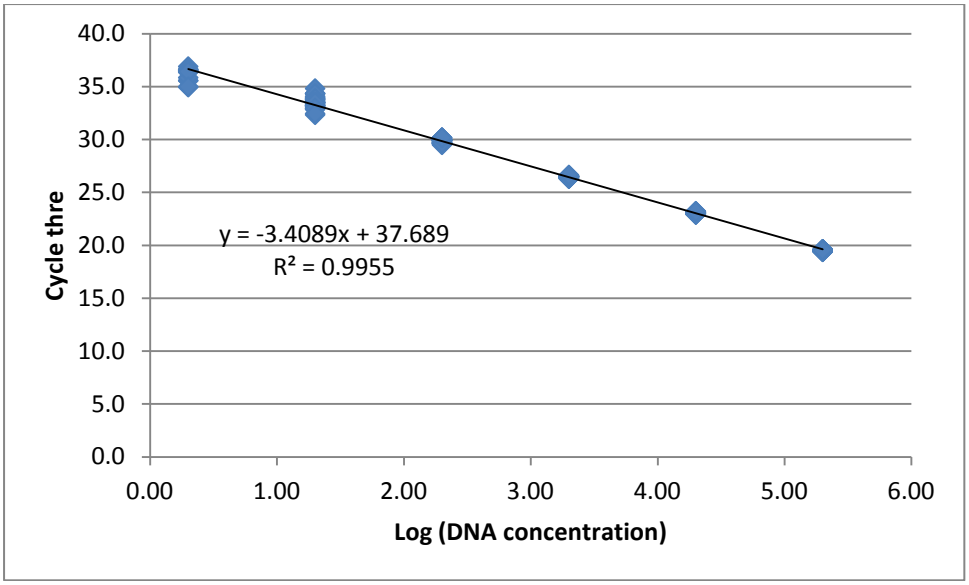

Figure 5: Standard curve of serial dilutions of *C. gigas* gDNA (200,000 to 2 fg/µl)

Plankton Sampling

Crassostrea gigas was not detected in either the November/December 2010 or May 2011 samples from the Port of Adelaide marine pest survey.

Discussion

We have developed and lab validated a qPCR assay for the detection of the Pacific oyster, *Crassostrea gigas.* The assay has a limit of detection between 2 and 20 fg/µl and quantification is linear over six orders of magnitude. The specificity testing conducted using SARDI's range of heterologous controls shows that this assay currently satisfies the requirements for a specific qPCR assay. Field samples from Port Adelaide were negative for *C. gigas* as expected; this species was not found by traditional methods and is not known to occur in the area (Wiltshire and Deveney, 2011). It should be noted, however, that qPCR assays require rigorous field proofing for specificity by being applied to field samples from a wide variety of localities with different endemic fauna and flora. This is needed to further check the assays against a wider range of DNA from non-target species present in plankton samples. The ability to conduct plankton sampling and correlate positive qPCR results with introductions and/or discovery of pest species detected by traditional means is an important process to undertake because it builds confidence in qPCR assay utility. It may also identify specificity problems with DNA of species that are not available on Genbank and which were not used during laboratory validation. Recent advances in high-throughput pyrosequencing offer the ability for future projects to pre-screen samples from various localities to better understand the sequence diversity in the environment, which will ultimately aid in test

development, sampling design and understanding of baseline aquatic biodiversity. We anticipate 454-based technology will assist us to understand and identify problems with assay specificity. 454-based or similar technologies may also provide the basis of a new platform for marine pest surveillance.

Conclusions and future approaches

It is vital to have access to marine pest larvae and gametes to more accurately validate marine pest qPCR assays, given that this is the life stage that these assays target. A better understanding of biological processes, including spawning timing and cues of marine pests in their invasive range is required to optimise surveillance using molecular methods. The Marine Pest Monitoring Manual (see http://www.marinepests.gov.au/marine_pests/publications/monitoring/monitoring_manual) plankton sampling guidelines are designed to detect listed phytoplankton species, not the larvae of benthic or pelagic pest species. The Monitoring Manual needs to be amended to take into account the spawning seasons of non-planktonic pest species. Future marine pest projects must improve our understanding of invasion biology, catalogue marine pest DNA sequence data to aid understanding phylogenetic relationships, and validate existing assays and sampling strategies in a wide range of localities.

This qPCR assay for *C. gigas*, in conjunction with assays already developed for other marine pest species, is a step toward implementation of a comprehensive, rapid, cost-effective surveillance system for marine pests in Australia. With continued development, such a surveillance system for monitoring marine pests in plankton using rapid, specific, sensitive qPCR assays will become the platform to inform managers of the presence of marine pests in a cost-effective manner.

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