Development of a real-time PCR assay for the detection of the invasive clam, Corbula amurensis, in environmental samples

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ABSTRACT

The detection of invasive species soon after an incursion, when the population is confined to a small area and at a low density, maximizes the probability of successful eradication. In response a number of sensitive molecular methods have been developed for identifying the larvae of marine invertebrate pests at extremely low concentrations. In this study we developed a highly sensitive real-time PCR assay targeting the 18S ribosomal DNA for the rapid and accurate identification of the Asian clam Corbula amurensis in environmental samples. Larvae of C. amurensis were spiked into commonly encountered sampling matrices including benthic assemblages, biofilms, sediment grabs and plankton net hauls, and the sensitivity of the assay was assessed. In this study the assay reliably detected one larva in up to 10 g of sediment, and five larvae in 10 g of benthic invertebrate and macro-algal assemblages. Seawater and benthic assemblage samples were collected from four major ports around New Zealand and all were negative for C. amurensis using the real-time PCR assay. This assay has the potential to enhance current surveillance methods, especially regarding morphologically difficult to identify early life-stages. Real-time PCR can be used with high through-put platforms and is extremely sensitive, increasing detection potential during initial stages of incursions.

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1. Introduction

The Asian clam Corbula amurensis (Family Corbulidae, formerly known as Potamocorbula amurensis; Coan, 2002) was first discovered in San Francisco Bay in 1986. It was most likely transported from Asia as larvae in ballast water (Carlton et al., 1990). The range and biomass of the clam increased greatly after its discovery with large expanses of the benthos becoming C. amurensis monocultures. The ecosystem was also dramatically altered with the increase in filter feeders greatly reducing phytoplankton biomass in the bay (Alpine and Cloern, 1992). C. amurensis is highly eurytopic and can tolerate a wide range of salinities, temperatures and substrates (Carlton et al., 1990). Laboratory studies show that the minimal time for larval development, from fertilization to metamorphosis, is 17–19 days (Nicolini and Penry, 2000). This larval stage is longer than the shipping travel time between San Francisco Bay and many major ports worldwide (Aldworth, 1999). The length of the larval period coupled with highly euryhaline larval stages means this species has a high risk for introduction into new regions via ballast water discharge (Nicolini and Penry, 2000).

Initial morphological identification as C. amurensis (Carlton et al., 1990) from San Francisco Bay is now uncertain. Sato and Azuma (2002) claim that the San Francisco Bay sample is morphologically more similar to species present in the Ariake Sea, Japan (identified as Potamocorbula cf. laevis) than to native C. amurensis from northeast Japan. This hypothesis awaits confirmation by molecular data.

Surveillance is an important precursor to effective management of invasive species. Detection of invasive species soon after an incursion, when the population is confined to a small area and at a low density, will maximize the probability of successful eradication (e.g., the eradication of the black-stripped mussel, Mytilopsis sallei, from Darwin; Simberloff, 2001). Current surveillance techniques for marine invasive species include epibenthic sled tows, diver and drop camera searches (wharf piles, seafloor, etc.), as well as shoreline searches (Inglis et al., 2006). Few programs monitor for dispersive life-stages of invasive species in the water column. Morphological identification of early life-stages (gametes and larvae) is difficult for most marine organisms, yet recognition of these stages is critical for detecting and tracking invasions (Darling and Blum, 2007). This limitation may have prevented the early detection of past invasions, particularly those originating from ballast water.

Rapid and unambiguous identification of planktonic organisms is difficult and genetic approaches provide powerful options for detection (Deagle et al., 2003). Molecular methods have been developed for identifying the larvae of marine invertebrates, e.g., Mytilus spp., Geller et al. (1994); Asterias amurensis, Mountfort et al. (2007) and Smith et al. (2011); and various species, Harvey et al. (2009) and Jones et al. (2008). We have developed a sensitive real-time PCR assay for C. amurensis, targeting 18S ribosomal DNA (rDNA) present

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in environmental samples. The assay has been optimized for both adult tissue and larval samples and an internal reference real-time PCR assay was incorporated into the analysis to control for environmental inhibitors. A key consideration in the application of molecular methods for surveillance is whether newly developed assays are compatible across sampling methods (Bott et al., 2010). The utility of our *C. amurensis* real-time PCR assay for detection across common sampling matrices including benthic assemblages, biofilms, sediment grabs and plankton net hauls, was assessed.

2. Materials and methods

2.1. Specimen collection and larval rearing

Adult specimens of *C. amurensis* were collected from northern San Francisco Bay (California, United States) in April 2008. Clams were depurated overnight (ca. 18 h) in filtered seawater and then opened. Clam tissue was preserved in RNAlater® (Qiagen, Hilden, Germany). Two adult females and two males were retained for spawning at the West Coast Aquaculture laboratory (Bethel Island, California, United States). Eggs and sperm from the adults were collected and mixed. Sub-samples of the larvae were collected at ca. 24, 36, 48, 72, 96, 120, 144, 168 and 172 h post-fertilization. Subsamples were spilt and preserved in RNAlater®. Adult specimens from closely related species were collected from a variety of locations for DNA sequencing and/or cross-reactivity testing (Table 1).

2.2. DNA extraction and 18S rDNA sequencing

Tissue from adult specimens was removed and genomic DNA was extracted using i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea) following the manufacturer's animal tissue protocol. An approximately 1.7 kb region of the 18S rDNA was amplified using the eukaryote-specific primers EukA (5′ AACCTGGTTGATCCTGC-CAGT 3′) and EukB (5′ TGATCCTTCTGCAGGTTCACCTAC 3′) (Medlin et al., 1988). PCR amplifications were carried out in 50.0 μL reaction volumes containing; 25.0 μL of i-Taq 2× PCR master mix (Intron, Gyeonggi-do, Korea), 0.4 μM of both primers and 1.0 μL of template DNA (concentration range ca. 20–180 ng). Thermocycling conditions consisted of: 94 °C for 3 min, 1 cycle; 94 °C for 45 s, 55 °C for 1 min; 72 °C for 3 min; 30 cycles; 72 °C for 10 min, 1 cycle. Amplified products were purified using AxyPrep PCR cleanup kits (Axygen, California, United States) and sequenced using the forward primer EukA and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, United States) at the Waikato University DNA Sequencing Facility (Hamilton, New Zealand). Sequence chromatograms were examined visually and base-calling errors corrected manually using BioEdit Sequence Alignment Editor (Hall, 1999).

Table 1
Bivalve species used in this study for cross-reactivity testing; + positive real-time PCR result, − negative real-time PCR result.

Species	Collection site	Sample number	Real-time PCR
Corbula amurensis	San Francisco Bay, United States	10	+
Potamocorbula cf. *laevis*	Ariake Bay, Japan	2	+
Corbula zelandica	Picton Harbour, New Zealand	2	−
Corbula gibba	Bay of Morlaix, France	2	−
Mya arenaria	San Francisco Bay, United States	4	−
Mytilus galloprovincialis	Pelorus Sound, New Zealand	2	−
Perna canaliculus	Pelorus Sound, New Zealand	2	−
Crassostrea gigas	Pelorus Sound, New Zealand	2	−
Pecten novaezelandiae	Pelorus Sound, New Zealand	2	−

2.3. Primers and probe design for real-time PCR assay

The target positions for forward and reverse primers and the Taq-Man probe were designed using a multiple 18S rDNA alignment (ClustalW; Thompson et al., 1994) of *C. amurensis* sequences, closely related species sequenced in this study and sequences from GenBank (http://www.ncbi.nlm.nih.gov/) including *Corbula zelandica* (JF947193), *Corbula gibba* (AY192691), *Corbula sinensis* (AM774545) *Mya truncata* (AY570556), *Mya arenaria* (AF120560), *Corbicula fluminea* (EF613239), *Notocorbula coxi* (AY192684), *Varicorbula dissimilis* (AF120561), *Sphenia perversa* (AM774544), *Pisidium obtusale* (AM774539), *Nausitora fusticula* (AY192697), *Lyrodus pedicellatus* (AM774540), *Pholas dactylus* (AY070122), *Leukoma staminea* (AM774570), *Musculium lacustre* (AM774538), *Bankia carinata* (AF120564), *Callista chione* (AJ007613), *Sphaerium striatinum* (AF120558) and *Sphaerium corneum* (AM774537). Primer and probe sequences were checked in silico for potential cross-reactivity in GenBank using BLAST online software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers and probe were synthesized by Geneworks (Thebarton, South Australia). The TaqMan probe was synthesized with 6-FAM reporter dye at the 5′-end and Black Hole Quencher 1 at the 3′-end.

2.4. DNA extraction efficiency

The DNA extraction efficiency from adult tissue (three replicates of 50 mg) was assessed using six methods; i-genomic CTB DNA extraction mini kits (Intron, Gyeonggi-do, South Korea), PureLink™ genomic DNA mini kit (Invitrogen, California, United States), PowerSoil® DNA isolation kit (Mo Bio, California, United States), Mollusc DNA kit (Omega, Georgia, United States), ISOLATE genomic DNA mini kit (Bioline, London, United Kingdom), and a lysis buffer method (500 mM KCl, 200 mM tris pH 8.0, 15 mM MgCl$_2$, 80 ng proteinase K). All DNA extractions were quantified on NanoPhotometer (Implen, Munich, Germany) to check for DNA quantity and quality (A_{260}/A_{280} ratio). PCR assays were run using the above protocol with 1 and 10 ng of DNA. The DNA extraction efficiency from larvae (three replicates of ten individuals, 92 h) was assessed using four methods; i-genomic CTB DNA extraction mini kits (Intron), PureLink™ genomic DNA mini kit (Invitrogen), PowerSoil® DNA isolation kit (Mo Bio), and ISOLATE genomic DNA mini kit (Bioline). All DNA extractions were quantified on a NanoPhotometer to check for DNA quantity and quality. The efficiency of each DNA extraction used in the real-time PCR assay was assessed with 1 and 10 ng of DNA.

2.5. Real-time PCR assay optimization and sensitivity

The real-time PCR assay was optimized and carried out on a Rotor-Gene 6000 (Corbett, Australia), using adult *C. amurensis* genomic DNA. The optimized assay consisted of a 25 μL reaction containing; 12.5 μL of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, California, United States), 200 nM of forward and reverse primers, 160 nM probe, 0.8 μg non-acetylated bovine serum albumin (BSA; Sigma-Aldrich, Auckland, New Zealand), and 10 ng of DNA template. All PCR reactions in this study were set up manually and all included no template control samples. Assays were run in clear 0.2 mL thin-wall PCR tubes (Axygen, California, United States). PCR cycling used the following conditions: 50 °C for 2 min, 95 °C for 2 min and 45 cycles of 95 °C for 15 s and 60 °C for 45 s.

The sensitivity of the real-time PCR assay was evaluated with genomic DNA extracted from *C. amurensis* adult tissue and larval samples. The amplification efficiency of the assay for extracted adult tissue (ISOLATE genomic DNA mini kit, Bioline) was determined by using serially diluted DNA samples (analyzed in triplicate) and the corresponding cycle threshold (Ct) data. *C. amurensis* larvae (92 h) were isolated by micro-pipette and the genomic DNA of one, two, three and five larvae (three replicates of each) were extracted