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Research Article

Development and application of a quantitative real-time PCR assay for the globally invasive tunicate *Styela clava*

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Abstract

Styela clava Herdman, 1881, is a solitary ascidian native to the Northwest Pacific, which has spread globally over the past 90 years, reaching pest levels and causing concern to the aquaculture industry in some regions. It has a relatively short-lived larval stage, spending only limited time in the water column before settling on a desirable substrate. Early detection of this species is an important step in both the prevention of its spread and of successful eradication. Here we report the development of a qPCR based assay, targeted to a region of the mitochondrial cytochrome oxidase I gene, using TaqMan® MGB, for the early identification of *S. clava*, post-settlement. The assay takes into account the moderately high level of haplotype diversity within this species, while also accommodating the need for species specificity. Using exemplars of closely related ascidian species, we show that our assay is specific for *S. clava* and is able to detect this species at low concentrations, equivalent to a single propagule. We also demonstrate, through "spike in" tests using environmental extracts, that our assay is robust to the potential inhibitors that might be found in environmental samples, which suggests this assay could have utility in the field, and may be developed further to detect larvae at an earlier stage of introduction.

Key words: ascidian, environmental monitoring, detection, mtDNA, cytochrome oxidase I, TaqMan® MGB

Introduction

Invasive marine species are frequently not detected until they have become well established. Post-invasion eradication is time-consuming, costly and ineffective, with the potential for eradication or elimination of the bio-invader predicted, in most instances, to be inversely proportional to the numbers of individuals and localities affected. Thus, any successful strategy for the control of marine invasive species requires early detection as part of ongoing surveillance procedures. A lack of tools and knowledge around the ability to distinguish invasive species from native species, or each other, their point of origin, and their transmission vector, makes the management of bio-invasions problematic (Ruiz et al. 2000; Bax et al. 2001; Darling and Blum 2007). It is therefore vital that we develop the scientific capacity required to identify and isolate invasive marine species prior to their widespread establishment.

The bio-invasive species we are investigating, *Styela clava* Herdman, 1881, is a solitary sea squirt (class Ascidiacea) native to the northwest Pacific that has spread globally over the past 90 years. It has dispersed sequentially into the coastal waters of North America (c.1920), Europe (1953), Australia (1978), New Zealand (2005) and the Mediterranean Basin in 2005 (Davis and Davis 2006; Davis and Davis 2008; Goldstien et al. 2011).

In the past, the invasion of *S. clava* had a devastating effect on the blue mussel aquaculture industry of Prince Edward Island (PEI), Canada, where fouling by this species on mussel lines resulted in an increase in production and processing

costs, and a potential loss in production of up to 50% to the shellfish industry (Colautti et al. 2006). Although, this species is no longer considered a threat to the PEI aquaculture industry, this is not a result of adequate detection and treatment methods, but rather it appears to have been outcompeted by other species, such as *Ciona intestinalis* Linnaeus, 1767 (Ramsay et al. 2008). The ecological impacts of *S. clava* in a bio-invasive context are largely unknown and, while it is currently being kept under control by the presence of other species, it still remains a potentially significant threat (Sievers et al. 2013).

Styela clava relies predominantly on anthropogenic transport of adults to disperse over larger distances (Dupont et al. 2009; Goldstien et al. 2010). Its larval stage is relatively short lived, persisting for approximately 12 hours in the water column before settling and attaching to a desirable surface (Minchin et al. 2006; Dupont et al. 2009). Therefore, the window for early detection, after the initial introduction of this species, is very small. However, once established, while the larvae produced by each individual are each in the water column for only a few hours, the population recruitment window can be months. Nonetheless, a study carried out in Prince Edward Island by Bourque et al. (2007), when the population was well-established, with highly dense, abundant adults, showed that the level of detection for larvae within the water column was less than one larva per litre. Post-settlement detection is likely to be more successful, although studies have shown that even on settlement plates and biofilms, *Styela clava* is still only picked up in low numbers, if at all (Bourque et al. 2007; McClary et al. 2008). If not detected quickly, and permitted to attach and establish, this species becomes difficult to control. Once attached, the most commonly used method of control is to handpick them from the surface, but this is both costly and time-consuming. Other methods used include exposure to air or extreme temperatures, pressure washing or topical application of sprays and dips of highly concentrated salt, hydrated lime, or acetic acid solutions, none of which are particularly effective (LeBlanc et al. 2007; Locke et al. 2009) and can be detrimental to the epifauna of the aquaculture species (Paetzold et al. 2008). It is for these reasons that we have sought to develop a molecular based assay that is more sensitive to small populations and small individuals than current visual survey methods, for the detection of *Styela clava*. Due to the difficulty in detecting small populations in the water column, we decided to initially focus our assay on post-settlement environmental samples.

There are multiple detection technologies available (reviewed in Wood et al. 2013), but quantitative real-time PCR (qPCR) has been widely used for the detection and quantification of a variety of marine and freshwater invasive species, including *Asterias amurensis* Lutken, 1871 (Bax et al. 2006), *Karenia mikimotoi* Miyake and Kominami ex Oda (Smith et al. 2007) and *Corbula amurensis* Coan, 2002 (Smith et al. 2012). qPCR is fast, more sensitive, and affords greater specificity than conventional PCR due to the use of a highly specific internal probe together with the standard primer pair. With the use of appropriate reference standards it provides opportunity for detection, as well as quantification, enabling assessment of propagule pressure. In addition, results can be obtained immediately without the need for post-PCR processing.

The object of our study was to develop a qPCR assay for the identification of *Styela clava*, that is specific for this species alone and will not detect closely related native species, or other invasive species. In addition to this need for species specificity, the assay was also required to be flexible enough to enable the detection of the large number of haplotypes present in this global invader. A study by Goldstien et al. (2011) found 45 haplotypes across 554 individuals worldwide, based on a region of the cytochrome oxidase subunit I gene sequence, with New Zealand exhibiting a particularly high diversity of unique haplotypes. Our challenge was to design a qPCR assay that could accommodate this variation, while retaining species specificity and was robust enough to not be adversely affected by low DNA quality or environmental contaminants. Environmental samples rarely contain DNA of a single species that is of sufficiently high quality and purity to optimise the majority of qPCR assays. Rather, these samples are heterogeneous, consisting of those species present at a given time and place, which are often extracted with a high quantity of potentially inhibitory substances. These inhibitory substances are present in the samples themselves (e.g. humic acid in environmental samples), and can cause DNA or polymerase degradation, poor primer annealing, or they may interfere with the fluorescent probes used in qPCR, resulting in decreased sensitivity (Schrader et al. 2012). Therefore, it was also important to demonstrate that our assay could not only work under ideal conditions, but also on field samples.

	Forward Primer (Nucleotides 240-260)	TaqMan® Probe (Nucleotides 316-335)	Reverse Primer (Nucleotides 370-389)
S.c.	tccggcggtagtccttttatt	ttagctaggaacttggccca	ttacatttggcggggatctc
S.p.(A)	...t..tc.tt.tt.a..g..	..gt..g.a..t..a..a..	..g...c....aa..g.t..
S.p.(P)	g..c...c.t..tt....g..	c.....g.a...c.a.....	......c.a..ta.a..t..
D.v.	...a...t..t.tt.ac.t..	c....a..a.gta....a..	.....c......a.ag.a..
B.l.	...t..ac..t.tt..c.t..	..gt.a..a..t..a..t..	c.t........ta..g.t..
C.s.	...t..tt.tt.ta.g.....	..gt..gct.cgg.aaga..	........a..ta..g.t..
C.n.	......at..---t.......	c.tt..g.t..t..a..t..	........a.-caa..gt.t
C.sp.	......at..---t.......	c.ct..g.t..t..a..a..	..g.....a.-caa..gt.t

Figure 1. Sequence alignments for 5' primer, probe, and 3' primer regions for the COI region of all *Styela clava* haplotypes (*S.c.*) and the closely related species, *Styela plicata*, Auckland (*S.p.* A), *Styela plicata*, Portugal (*S.p.* P), *Distomus variolosus* (*D.v.*), *Botrylloides leachii (B.l.), Ciona savignyi (C.s.), Cnemidocarpa nisiotis (C.n.), and Cnemidocarpa sp. (C.sp.)* Nucleotides where other species differ from *S. clava* are highlighted in bold. Dots represent identical nucleotides between *S. clava* and other species. Sequences are shown 5'-3' with nucleotide positions indicated relative to the *S. clava* mtDNA COI sequence (Accession GU328006). Dashes in alignment represent missing bases.

Materials and methods

Samples, DNA extraction and verification

Voucher DNA and tissue samples of *Styela clava* including a broad range of known haplotypes, covering the extremes in diversity, were selected from a previous study (Goldstien et al. 2010). These samples were obtained from various ports located in New Zealand, Australia and the United States (Table 1).

Additional tissue samples from several Styelids were tested using the assay to check for cross-species detection. *Styela plicata* Lesueur, 1823 (Nazare, Portugal) and *Distomus variolosus* Gaertner, 1774 (Plymouth Sound, UK) were provided by John Bishop (Plymouth), while a local sample of *Styela plicata* (Auckland, NZ) was obtained from Mike Page (NIWA). The two *S. plicata* individuals represent two haplotypic groups, so both were included in this study to ensure that the assay did not detect either of them. DNA from *Cnemidocarpa nisiotis* Sluiter, 1900 (Dunedin, NZ), and a sample of an uncharacterized *Cnemidocarpa* species (Dunedin, NZ), were obtained from Genievive del Mundo (Canterbury). A DNA sample of the Styelid *Botrylloides leachii* Savignyi, 1816 (Lyttelton, Christchurch, NZ), as well as a sample of an ascidian from the order Phlebobranchia, *Ciona savignyi* Herdman, 1882 (Dunedin, NZ), were obtained from Phoebe Heenan (University of Otago).

DNA was extracted from tissue samples using a lithium chloride salt extraction protocol (Gemmell and Akiyama 1996). We then used a standard PCR designed to amplify a region of the mitochondrial cytochrome c oxidase subunit I gene (Folmer et al. 1994; Goldstien et al. 2010) and sequenced the amplicon to confirm that amplifiable DNA was present in the samples post-extraction, and that the sequence generated was consistent with the voucher samples' taxonomic identification.

Primer and probe design

The qPCR assay was developed using TaqMan® minor groove binder (MGB) chemistry and was designed around part of the highly conserved mitochondrial cytochrome oxidase I (mtCOI) region using Primer Express version 2.0 (Life Technologies, Foster City, CA). The key criteria were to design primers that would generate species specificity while accommodating the haplotypic diversity observed within *S. clava* (Figure 1).

The forward primer used was SC1F 5'–TCCGG CGGTAGTCCTTTTATT–3'and the reverse primer was SC1R 5'–GAGATCCCCGCCAAATGTAA–3', which produces a 150bp product. The sequence for the TaqMan® probe, called SC1, was 5'–TTA GCTAGGAACTTGGCCCA-3' and was labelled at the 5' end with the fluorescent reporter dye FAM-6-carboxyfluorecein and at the 3' end with a non-fluorescent quencher (NFQ) MGB. All primers and probes were produced by ABI (Life Technologies, Foster City, CA).

qPCR assays

Quantitative PCR (qPCR) assays were performed on a MX3000P (Agilent Technologies). The standard reaction conditions were as follows: 5µl 2 x Bioline Sensi-Fast Probe LoRox Mix, 0.3µM

Table 1. *Styela clava* samples of varying haplotypes and other closely related species used in the development of the qPCR assay in this study; Total nucleotide changes and % nucleotide difference are calculated across the mtCOI region, + positive qPCR result, - negative qPCR result.

Sample ID	Location	Acc No.	Hap No.	Total Nucleotide Changes (bp)***	% Nucleotide Difference***	qPCR
S. clava PS14	Puget Sound, USA	GU328006	1	0	0	+
S. clava OZ20	Melbourne, Australia	GU328008	3	3	0.5	+
S. clava VIA97	Auckland Viaduct, NZ	GU328028	2	5	0.8	+
S. clava AP13	Avery Point, USA	GU328024	9	1	0.2	+
S. clava PHBD5	Pine Harbour Marina, NZ	GU328020	27	1	0.2	+
S. clava L5A	Lyttelton Port, NZ	GU328022	29	3	0.5	+
S. clava MCD3	Mumford Cove, USA	GU328015	44	1	0.2	+
S. plicata	Nazare, Portugal	FJ528632	N/A	131	22.1	-
S. plicata	Auckland, NZ	FJ528634	N/A	145	24.5	-
D. variolosus	Plymouth Sound, UK	FJ528652	N/A	139	23.4	-
B. leachii	Lyttelton Port, NZ	FJ528645*	N/A	134	23.8	-
C. savignyi	Dunedin, NZ	JF919710*	N/A	205	34.5	-
C. nisiotis	Dunedin, NZ	XXXX**	N/A	126	25.8	-
Cnemidocarpa sp.	Dunedin, NZ	XXXX**	N/A	129	26.4	-

*Sequence similar to isolate in GenBank. Exact isolate unknown
***Cnemidocarpa* accession numbers pending
***Sequence lengths compared vary depending on data available in Genbank

each of primers SC1F and SC1R, 0.2µM SC1 probe, with DNA templates added at varying concentrations, as detailed below, depending on the assay purpose, to a final volume of 10µl. The cycling conditions involved an initial denaturation step at 95ºC for 10min followed by 40 cycles at 95ºC for 10sec then 53ºC for 50sec.

Assay flexibility

To confirm that the full extent of haplotypic diversity present in *Styela clava* could be detected by our assay, we undertook replicate qPCRs on seven different haplotypes including the most frequent haplotype and those that differed by 1bp, 3bp and 5bp compared to the most frequent haplotype. Assay conditions were as described above, with 10ng of sample template.

Species specificity

The specificity of our assay was examined by undertaking replicate qPCRs with *S. clava, S. plicata* (Portugal), *S. plicata* (Auckland), *Distomus variolosus*, *Botrylloides leachii*, *Ciona savignyi, Cnemidocarpa nisiotis,* and *Cnemidocarpa sp.* Initially, we used 10ng of purified template from each of the samples to examine assay performance under optimum conditions. We then repeated the assay using 8ng of the competing sample, spiked with 0.2ng *S. clava* to confirm that lower concentrations of *S. clava* can be detected even in the presence of larger quantities of competing DNA.

Sensitivity of detection

The whole organism may, or may not be present in environmental samples such as hull scrapings or ballast waters, thus it is important for a successful assay to be able to detect *S. clava* at low levels. We undertook a serial dilution of *S. clava* ranging from 0.1ng to 1fg to determine the sensitivity of our assay. We increased our qPCR cycles from 40 to 45 for this assay because we were working at the extremes of detection.

Environmental samples

Our assay was assessed on environmental samples in order to replicate the habitat from where *Styela clava* is most likely to be found. DNA samples were taken from wharf pile scrapings obtained from Cawthron Institute, Nelson, NZ. They were collected from several different ports around New Zealand, including New Plymouth, Picton, Wellington, and Nelson, preserved in RNALater and homogenized using a hand-blender. A PowerMax® Soil DNA isolation kit (Mo Bio Laboratories Inc, Carlsbad, CA) was then used to extract DNA from 10g sub-samples from each environmental isolate (Cawthron Institute, Nelson, pers. comm.). In addition to these environmental samples, we collected scrapings from a selection of wharves in Hobson Bay Marina, Hauraki Gulf, Auckland, and extracted DNA using the same process described above. Standard qPCRs were undertaken using DNA template purified from port samples to determine if there was any positive

signal for *S. clava* in the samples. Secondarily, as a form of positive control, some of the Hobson Bay wharf pile scrapings that produced a negative qPCR result were spiked with *S. clava* larvae, re-extracted and tested with the qPCR assay to determine if detection could be obtained in the presence of DNA of environmental origin.

Results and discussion

Styela clava is a genetically diverse species, thus a key requirement of this assay was the need to be able to detect a wide range of *S. clava* haplotypes while also distinguishing this species from closely related species that may be present in environmental samples. We examined assay performance across seven *S. clava* isolates representing cytochrome oxidase I haplotypes that differed from the most common haplotype by up to five base substitutions (Goldstien et al. 2011). We were able to successfully amplify each of these isolates individually with an amplification efficiency of 93% (Table 1, qPCR data shown in supplementary Figure 1S). An amplification efficiency of 100% indicates two copies are generated from each template molecule during the exponential phase of the PCR cycle, with amplification efficiencies between 90% and 110% generally considered acceptable for the purposes of qualification and comparison (Nordgard et al. 2006).

We were also able to demonstrate species specificity; none of our competing test samples amplified with our qPCR assay (Table 1, qPCR data shown in supplementary Figure 2S) despite positive results with COI test primers showing that DNA was present in the samples and able to be amplified in all samples (results not shown). "Spike in" tests of *S. clava* DNA into our trans-species assays demonstrated that the qPCR assay could detect the presence of *S. clava*, even in the presence of large quantities of competing DNA, that no PCR inhibitors were present, and that the assay is specific to *S. clava* (qPCR data shown in supplementary material, Figure 3S).

Using a dilution series ranging from 0.1ng through to 1fg of DNA we demonstrated that our assay was sensitive enough to detect small quantities of genetic material within a sample (Table 2, qPCR data shown in the supplementary Figure 4S). Our assay is able to detect as little as 0.1pg of DNA, approximately equivalent to a single diploid cell based on genome size estimates from a sister Styelidae (Fafanđel et al. 2008), i.e., our assay should be adequate for single cell detection. Previous studies have reported assays that can detect as little as 1fg of their target species, e.g., *Corbula amurensis* (Smith et al. 2012), so further optimisation of this assay may be possible; however, the biological limits of different systems will be dependent on the quantity of DNA present in a single cell. An increase in PCR cycle number was not sufficient to detect lower levels, and further increases in cycle number would likely lead to other artefacts and errors.

Table 2. qPCR results for *S. clava* sensitivity testing; + positive qPCR result, - negative qPCR result.

S. clava concentration	qPCR
0.1ng	+
0.01ng	+
0.001ng (1pg)	+
0.0001ng (0.1pg)	+
0.00001ng (0.01pg)	-
0.000001ng (1fg)	-

Table 3. qPCR results for environmental samples; + positive qPCR result, - negative qPCR result.

Port of Origin	Number of Samples	qPCR
Hobson Bay wharf pontoon	4	+
Hobson Bay wharf pile	4	-
Nelson	5	-
Wellington	5	-
Picton	5	-
New Plymouth	5	-
Hobson Bay wharf pile (spiked with *S.clava*)	2	+

Utilising our assay on environmental samples collected from a series of port surveys in New Plymouth, Picton, Wellington and Nelson, we found no evidence of *S. clava* among these samples. It has been confirmed by NIWA that this species had not been previously detected by them, at these particular sampling locations, prior to the date of collection, and was not visually observed during sample collection (NIWA, pers. comm.). Therefore, our assay supports this finding. Samples taken from wharf pile scrapings from four different locations at Hobson Bay Marina (wharf B, wharf E, wharf F and the Far Jetty), also produced a negative qPCR result, while those collected from the base of the pontoons from the same wharves, produced a positive qPCR result (Table 3, qPCR data shown in supplementary Figure 5S). The marinas of

Hauraki Gulf are known to have established populations of *S. clava*, and the Hobson Bay site is situated very close to several marinas that showed high abundance of this species when they were sampled in 2009 and 2010 (Goldstien et al. 2010). In addition, *S. clava* were visually observed on the Hobson Bay wharf pontoons during sample collection. Our assay has, therefore, confirmed the presence of this species in this location. Our results showing varying degrees of detection between different wharves, as well as between different structures of individual wharves, demonstrates the stratification and patchiness of larval transport and settlement success, and also highlights the need to optimize a sampling approach if this assay is to be used for routine detection.

As additional controls we retained untreated pile scrapings from Hobson Bay wharves B and E that had previously produced negative qPCR results, spiked these with *S. clava* larvae and extracted these samples afresh. These spiked-in controls gave a positive qPCR result, thus demonstrating positive detection from environmental samples (Table 3, qPCR data shown in supplementary Figure 5S). Because environmental samples have potentially high levels of contaminating DNA and potential inhibitors of PCR present this additional test provides support for the efficacy of this approach on real-world materials.

Conclusion and future directions

We have successfully designed a quantitative real-time PCR assay using TaqMan® MGB chemistry to target a 150bp region of the COI gene of the invasive ascidian *Styela clava*. This assay is flexible enough to accommodate the high intrinsic variability observed in *S. clava* haplotypes, and specific enough to ensure species' specificity, with no cross-reactivity observed. It is sensitive enough to detect to the level of a single cell, and although we have only tested it on post-settlement samples, we expect this protocol to be applicable to detection from the water column or ballast water with sampling modifications.

TaqMan® MGB has been widely used in other applications including the detection of *Lepeophtheirus salmonis* Krøyer, 1837 and *Caligus elongatus* von Nordmann, 1832, larvae (McBeath et al. 2006) and of the black-striped mussel, *Mytilopsis sallei* Recluz, 1849 (Bott et al. 2012). TaqMan® MGB chemistry increases the melting temperature (Tm) of probes, enhancing the differences in Tm between matched and mismatched probes to enable more accurate allelic discrimination than standard TaqMan® assays. In addition, the presence of a non-fluorescent quencher at the 3' end allows the reporter dye contributions to be measured more precisely, enhancing the sensitivity and precision of detection. These were useful features for our assay given the haplotypic diversity exhibited by *S. clava*. They allowed us to design shorter primers, without which we may not have achieved the desired level of detection and differentiation.

The target species was not detected in a variety of port samples tested, but a "spike in" test of environmental samples, prior to DNA extraction suggests that such detection is possible in field samples. This test of "field-utility" is vital, as there is likely to be DNA of other flora and fauna present as well as inhibitory compounds in any real world sample tested for the presence of a bio-invasive. Here, we show that we were able to detect *S. clava* DNA when combined with typical environmental samples, at low levels with high accuracy.

The prospect of linking our qPCR assay with portable qPCR platforms, such as the Spartan DX-12 (Spartan Bioscience Inc.) which is small enough to be taken into the field and can process 12 samples per run, provides a realistic approach for bio-invasive detection in the field. However, developing such an *in situ* method would rely on making other steps of the process portable such as the DNA extraction, unless a more sensitive assay could be developed to detect the species without the need for such purification. In addition to this, measures would have to be taken in order to address the significant quality control issues that are likely to arise with transferring laboratory systems into the field environment. Further field validation, along with increases in assay sensitivity, and platform optimisation, will increase the utility for *in situ* detection of larvae prior to species establishment. Used in conjunction with traditional morphological testing, the developed assay will likely add to the toolbox for "in-the-field" detection of *S. clava* and other marine bio-invasive species.

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Supplementary material

The following supplementary material is available for this article.

Figure 1S. qPCR data showing detection of a range of *S. clava* haplotypes.
Figure 2S. qPCR data showing that other species are not detected by this assay.
Figure 3S. qPCR data showing DNA from competing samples spiked with 0.2ng of *S. clava* DNA.
Figure 4S. qPCR data showing the detection limits of the assay.
Figure 5S. qPCR data showing the testing of the assay on a range of environmental samples.

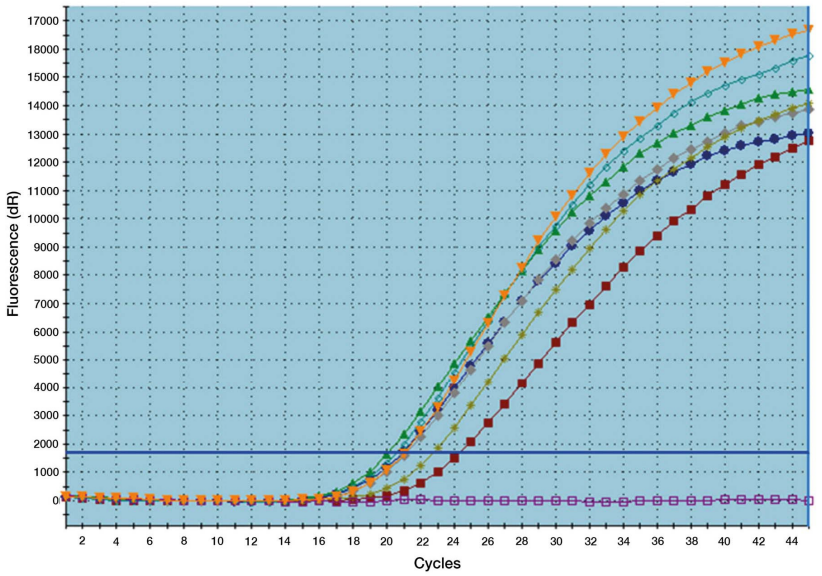

Figure 1. qPCR data showing detection of a range of *S. clava* haplotypes (listed in Table 1). Most samples are detected around the same cycle threshold. Differences in crossing points are most likely due to differences in pipetting. The sample below the threshold line is the non-template control containing no DNA. (Amplification efficiency 93%.).

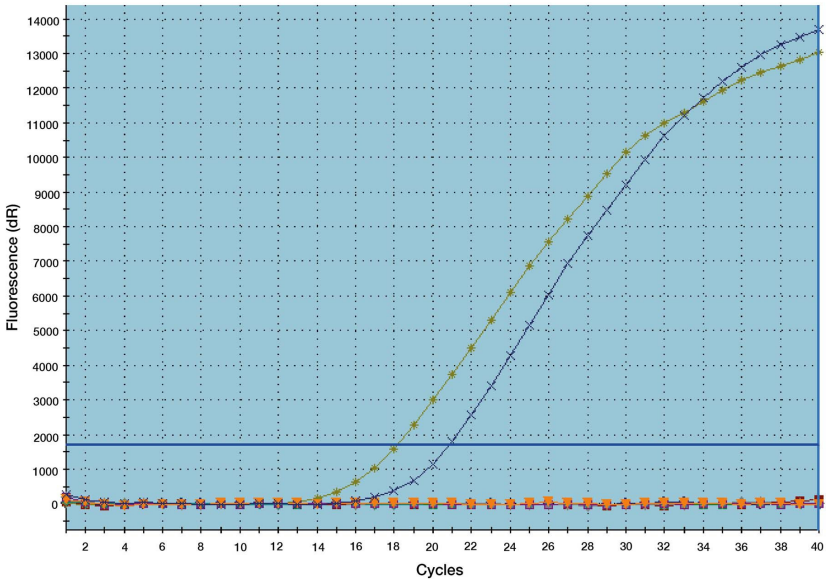

Figure 2. qPCR data showing that other species are not detected by this assay. All competing samples tested (*S. plicata*, Auckland, *S. plicata*, Portugal, *D. variolosus, B. leachii, C. savignyi, C. nisiotis, and Cnemidocarpa sp.*) do not cross the detection threshold while two different *S. clava* haplotypes (hap1 and hap2) are detected. (Amplification efficiency 93.9%).

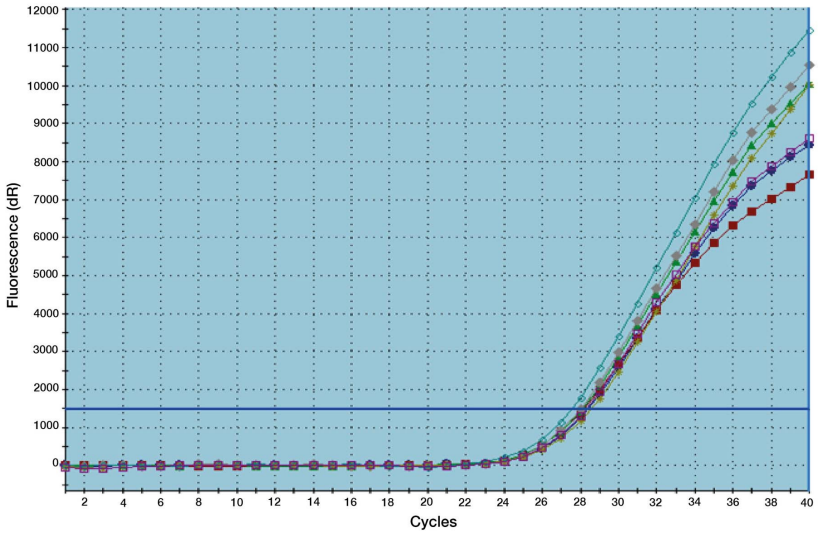

Figure 3. qPCR data showing DNA from competing samples spiked with 0.2ng of *S. clava* DNA. *S. clava* is detected in all samples. (Amplification efficiency 94.6%).

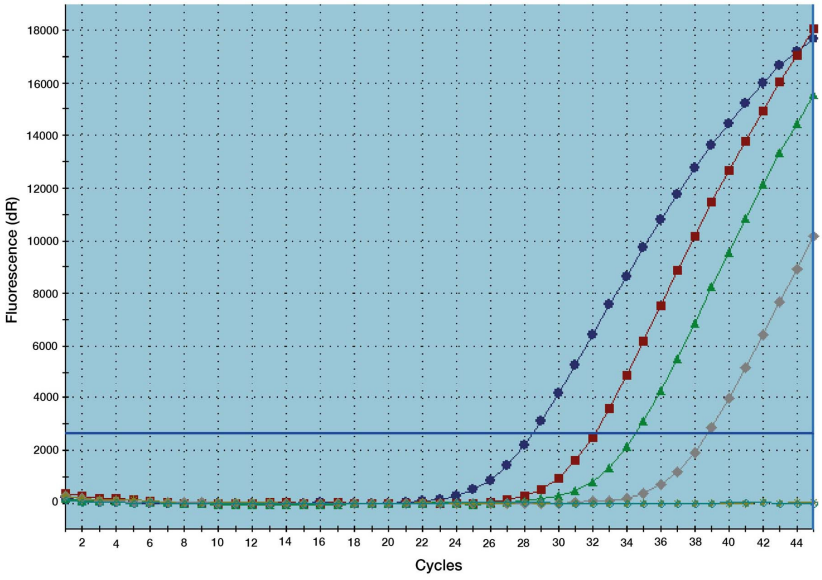

Figure 4. qPCR data showing the detection limits of the assay. The samples giving positive results have *S. clava* quantities (from crossing points left to right) of 0.1ng, 0.01ng, 0.001ng (1pg) and 0.0001ng. (Amplification efficiency 100.1%).

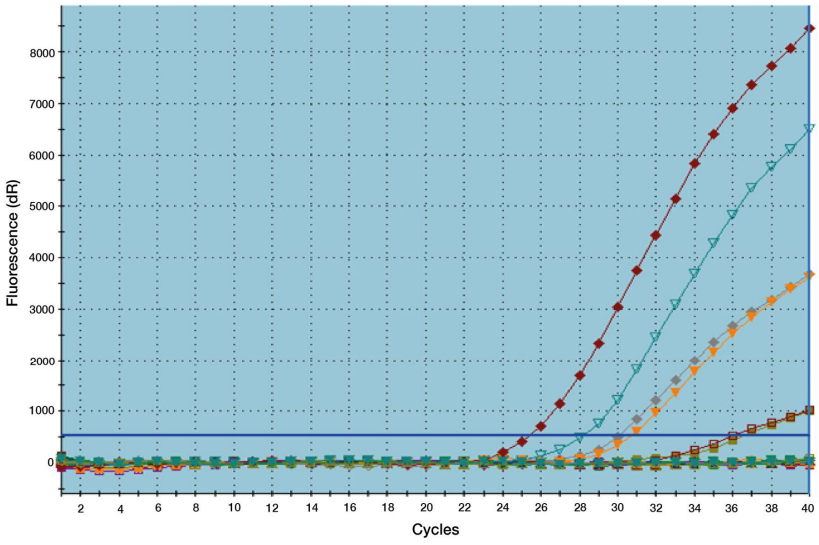

Figure 5. qPCR data showing the testing of the assay on a range of environmental samples. From left to right, the first two positive results are DNA from scrapings taken from two different wharf piles at Hobson Bay (wharf B and wharf E), which initially gave negative qPCR results before being spiked with S. clava larvae and re-extracted, and the next four positives are DNA from wharf scrapings taken from the base of the pontoon from four wharves at Hobson Bay (wharf B, wharf E, wharf F, and the far jetty wharf). DNA taken from wharf pile scrapings at New Plymouth, Wellington, Picton, Nelson, and Hobson Bay all produced negative results. (Amplification efficiency 94.6%).