

Development of 18S rDNA and COI gene primers for the identification of invasive tunicate species in water samples

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Abstract

Invasive tunicates are creating costly fouling problems to the mussel aquaculture industry in many regions including Prince Edward Island, Canada. These invasive tunicates are also posing a threat to the ecosystem integrity of native species. There are currently four invasive tunicate species present in waters surrounding Prince Edward Island including: *Ciona intestinalis*, *Styela clava*, *Botryllus schlosseri*, and *Botrylloides violaceus*. Current monitoring practices for the presence of these species in PEI are conducted by costly and time consuming recruitment plates or via microscopy surveys for egg and larval stages using dissecting microscopes. However, these methods are inadequate for early detection. Recruitment plates only allow for the detection of tunicates once they have already established, and microscopic methods can be inaccurate since it is difficult to distinguish between eggs and larvae of different species of tunicate. In this study, we propose polymerase chain reaction (PCR) based detection as a means to overcome the limitations of current monitoring practices in water samples. For this purpose, oligonucleotide primers were generated to enable the rapid analysis of samples for the presence of invasive tunicates by PCR. Primers were designed from 18S rDNA and COI gene sequences specific to each species and were evaluated for efficacy, specificity, and sensitivity in detecting target tunicate species. Through efficient detection methods and careful monitoring it is hoped that further invasions of these tunicate species throughout Prince Edward Island waters can be prevented or managed in their early stage.

Key words: 18S rDNA, ascidians, COI, cytochrome oxidase, PCR, species identification, tunicates

Introduction

Invasive tunicate species are currently causing challenges in the mussel aquaculture industry in Prince Edward Island, Canada by fouling aquaculture gear, mussel lines, buoys, boat hulls, piers, and other artificial substrates (Thompson and McNair 2004). There are currently four invasive tunicate species on PEI including: *Styela clava* (Herdman 1881) (Club Tunicate), *Ciona intestinalis* (Linnaeus 1758) (Vase Tunicate), *Botryllus schlosseri* (Pallas 1766) (Golden Star Tunicate) and *Botrylloides*

violaceus (Oka 1927) (Violet Tunicate) (DFO 2006). A native tunicate, *Molgula citrina* (Alder & Hancock 1848) (Sea grape), is also present in PEI but has had less of an impact on aquaculture in this region. *Didemnum vexillum* (Kott 2002) is currently not present in Prince Edward Island, but has caused significant fouling problems elsewhere and is currently located on George's Bank, just south of the Canadian/US border.

Current monitoring techniques for tunicate species involve the use of recruitment plates which are placed in bays and rivers in Prince Edward Island, dive surveys of anecdotal areas

of infestation, and a public awareness program. Juvenile and adult tunicates obtained by these methods are then identified and, if possible, quantified. However, detecting early developmental stages of these tunicates is more challenging. Water samples are collected and examined under a dissecting microscope to scan for the presence of tunicate eggs and larvae. However, it is extremely difficult and time consuming to visually identify tunicate species at the egg and larval stages. In 2006, Bourque showed that millions of tunicate eggs and larvae were being released from mussel processing facilities via effluent outflow into adjacent waters. Current mitigation procedures are being evaluated to determine an efficient mechanism to reduce the load of tunicate propagules in processor outflow. It is clear that high throughput methods need to be developed to efficiently and accurately identify tunicate species at the egg and larval stages.

The objective of this study was to develop sets of specific, unique, and sensitive DNA primers to facilitate the rapid analysis of water samples for invasive tunicate detection by PCR (polymerase chain reaction). Primers specific to the tunicate species mentioned above were developed from published small subunit ribosomal DNA (18S rDNA) and cytochrome oxidase gene sequences. Both the 18S ribosomal gene and the cytochrome oxidase gene have been used in many studies for species identification because they include highly conserved regions at the species level (Wada et al. 1992; Bell and Grassle 1998; Le Roux et al. 1999; Hare et al. 2000; Mo et al. 2002; Stach and Turbeville 2002). Primers were first tested for efficacy using samples from as many locations as possible for each species. The specificity of primer sets was then evaluated against the other

tunicate species currently present in the area. Lastly, the primer sets were tested for sensitivity using set quantities of egg and free swimming larval life stages present in water samples. Through the development of efficient molecular detection methods and careful monitoring, it is hoped that further invasions of these tunicate species throughout Prince Edward Island waters can be prevented or managed in their earliest stage.

Material and Methods

Primer Design

The 18S rDNA sequences for *Styela clava* (Genbank accession no. [L12442](#)), *Ciona intestinalis* (Genbank accession no. [AB013017](#)), *Botryllus schlosseri* (Genbank accession no. [AB211066](#)), and *Botrylloides violaceus* (Genbank accession no. [AY903927](#)) were retrieved from Genbank and aligned using ClustalW. Primers were manually designed from 18S rDNA regions that were unique to each tunicate species.

The COI gene sequences for *Ciona intestinalis* (Genbank accession no. [AJ517314](#)), and *Botryllus schlosseri* (Genbank accession no. DQ340224) were also aligned using ClustalW. Primers were manually designed from COI regions that were unique to each species.

To minimize false-positives, all primers were assessed to ensure specificity by NCBI-BLAST (National Centre for Biotechnology Information-Basic Local Alignment Search Tool) (Altschul et al. 1997). Primer suitability was further evaluated using IDT Oligoanalyzer 3.0. Although multiple primers were developed for each species, only the most sensitive and specific primers are presented in this paper (Table 1).

Table 1. Sequences of primers designed in this study

Primer	Sequence 5'-3'	Location
CIONAINTESTCOI-F1	GTCGTTGTTACTTCTCATGCAT	163-184
CIONAINTESTCOI-R1	CCGGATCAAAGAACGTAGTATTTAAA	643-666
STYCLAV18S-F1	GCAAGGCTGGTCACTGG	60-76
STYCLAV18S-R1	GACACTCGCTGCTTCACTC	874-892
BOTVIOLET18S-F1	GCGGTCTTGTGCCGGCACAACC	268-291
BOTVIOLET18S-R1	CAGACCTTTCGGCCAGG	1493-1513
BOTSCHLOCOI-F1	GATAATTAGGAGGTTTGGTAATTGGTTA	81-108
BOTSCHLOCOI-R1	GACCAATACAGTTCAACAAAAACAAAGAT	405-432

Table 2. Tunicate species and geographic origin of samples used in this study

<i>Ciona intestinalis</i>	<i>Styela clava</i>	<i>Botryllus schlosseri</i>	<i>Botrylloides violaceus</i>
Montague River, PEI, Canada (46°10'35"N; 62°34'15"W)	Montague River, PEI, Canada (46°10'35"N; 62°34'15"W)	Orwell River, PEI, Canada (46°08'56"N; 62°53'35"W)	Magdalen Islands, Quebec, Canada (47°24'22"N; 61°50'41"W)
Woods Hole MA, USA (41°31'25"N; 70°40'15"W)		Ladysmith, BC, Canada (Orange morph) (49°00'17"N; 123°48'57"W)	Woods Hole, MA, USA (41°31'25"N; 70°40'15"W)
Grevelinger Lake, The Netherlands (51°44'21"N; 3°49'35"E)		Salem, MA, USA (42°31'12"N; 70°52'55"W)	Nanaimo, BC, Canada (Purple morph) (49°12'37"N; 123°57'22"W) Salem, MA, USA (Purple and orange morph) (42°31'12"N; 70°52'55"W)

Tissue samples and DNA Extraction

Adult tunicate samples were obtained from geographic locations around PEI, within Canada, the USA, and Europe (Table 2). These samples were used for efficacy and specificity studies. DNA extractions were performed on fresh or ethanol fixed tissues using QIAamp DNA MiniKits (Qiagen Inc, Canada) according to manufacturer's instructions.

For sensitivity studies involving solitary tunicates, both unfertilized eggs and free swimming larval stages were used. Unfertilized eggs were manually collected from *Ciona intestinalis* and *Styela clava* specimens in the lab. These eggs were then placed in microcentrifuge tubes filled with filtered seawater in quantities of 1, 5, 10, 20, 50, and 100. The samples were then centrifuged, water was removed and DNA was extracted from the eggs using the QIAamp DNA MiniKits (Qiagen Inc, Canada). Free swimming larvae were generated in the lab by removing egg and sperm samples from *Ciona intestinalis* and *Styela clava*. These gametes were then placed in Petri dishes with filtered sea water and left for 15-17 hours. Free swimming larvae were collected by pipette and placed in microcentrifuge tubes in 1, 5, 10, 20, 50, and 100 quantities. DNA extractions were then performed as described above.

Since the colonial tunicates used in this study spread most often via fragments of adult colonies, individual zooids and small fragments of colonies were used for sensitivity testing for *Botryllus schlosseri* and *Botrylloides violaceus*. Individual zooids and fragments were placed in microcentrifuge tubes filled with filtered seawater. The microcentrifuge tubes were then centrifuged, water was pipetted off and DNA extractions were performed using the QIAamp

DNA MiniKits (Qiagen Inc, Canada) according to manufacturer's instructions.

PCR

PCR was performed in 25 µl final volume containing 12.5 µl AmpliTaq Gold PCR Master Mix (Applied Biosystems manufactured by Roche, Branchburg, New Jersey), 10 pmol appropriate forward primer, 10 pmol appropriate reverse primer, 25-60 ng appropriate DNA and 9.5 µl sterile ddH₂O. Samples were denatured for 3 minutes at 92°C, amplified over 35 cycles consisting of 1 minute at 94°C for denaturation, 1 minute at 50°C (*C. intestinalis*), 53°C (*B. schlosseri*), 58°C (*S. clava*), or 62°C (*B. violaceus*) for primer annealing, and 3 minutes at 72°C for elongation. Following the last cycle, polymerization was extended for 5 minutes at 72°C to complete elongation.

Agarose Gel Electrophoresis

PCR amplicons were separated on 1% agarose gels containing 0.5 µg ml⁻¹ ethidium bromide and visualized using ultraviolet light. Images were captured using AlphaImager 1.0 (Alpha Innotech Corp., San Leandro, CA).

Cloning and Sequencing

PCR amplicons were cloned and sequenced to ensure that the correct target gene was being amplified. The amplicons were inserted into the pCR2.1-TOPO vector using the Invitrogen TOPO TA cloning kit following the manufacturer's instructions. Plasmids were amplified by transforming One shot® Electrocompetent TOP 10 *E. coli* cells that were then grown overnight in LB. Plasmids were purified using the Qiagen Plasmid Isolation Miniprep Kit and

followed by digestions with EcoRI (Fermentas) and 1% agarose gel electrophoresis to confirm that the plasmid insert was of similar size to the desired PCR amplicon. Plasmid inserts were confirmed by sequencing performed by ACGT Corp., Toronto, Canada.

Results

The expected 832 bp amplicon of *Styela clava* DNA was amplified by the STYCLAV18S primer set (Figure 1A). This primer set was highly specific and did not amplify DNA of any

other tunicate species tested. The primers were able to detect all quantities of both eggs and free swimming larvae tested right down to 1 egg and 1 larva (Figure 1B and 1C). Sequencing confirmed that the amplicon produced was from *Styela clava* 18S based on identity with the 18S rDNA sequence in Genbank ([L12442](#)).

The CIONAINTESTCOI primer set amplified the expected 503 bp fragment of *Ciona intestinalis* COI DNA, but did not amplify DNA from any other tunicate species tested (Figure 1D). These primers were able to detect as little as 1 egg and 1 free swimming larva (Figure 1E and 1F).

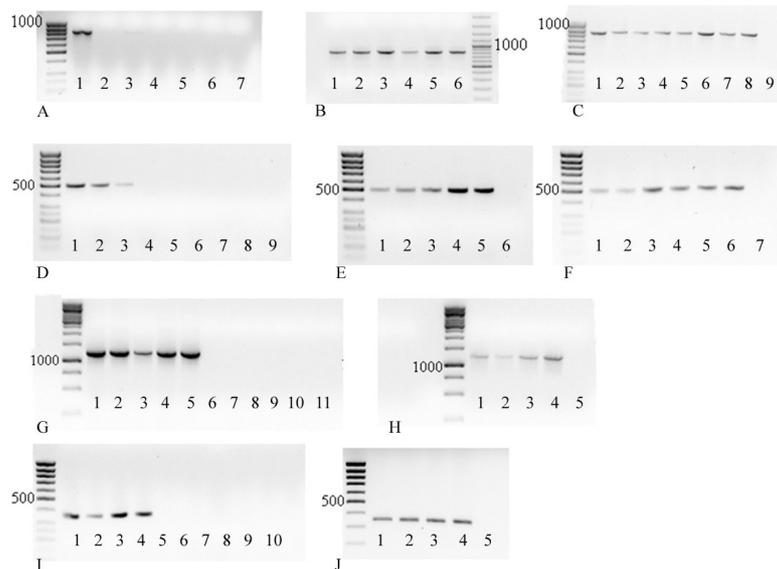


Figure 1. **A** - Specificity agarose gel of PCR products with STYCLAV18S-F1 & R1 primers with: Ln1 = *S. clava* DNA (PEI, Canada), Ln2 = *C. intestinalis* DNA (PEI, Canada), Ln3 = *B. violaceus* DNA (BC, Canada), Ln4 = *B. schlosseri* DNA (PQ, Canada), Ln5 = *Didemnum vexillum* DNA (BC, Canada), Ln6=*Molgula* sp. (PEI, Canada), Ln7 = Negative control; **B** - Sensitivity agarose gel of PCR products with STYCLAV18S-F1 & R1 primers with: Ln1 = 1 egg, Ln2 = 5 eggs, Ln3 = 10 eggs, Ln4 = 20 eggs, Ln5 = 50 eggs, Ln6 = 100 eggs; **C** - Sensitivity agarose gel of PCR products with STYCLAV18S-F1 & R1 primers with: Ln1 = 1 free swimming larvae, Ln2 = 5 free swimming larvae, Ln3 = 10 free swimming larvae, Ln4 = 20 free swimming larvae, Ln5 = 50 free swimming larvae, Ln6 = 100 free swimming larvae, Ln7 = Negative control; **D** - Specificity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1 = *C. intestinalis* DNA (PEI, Canada), Ln2 = *C. intestinalis* DNA (MA, USA), Ln3 = *C. intestinalis* DNA (Netherlands), Ln4 = *S. clava* DNA (PEI, Canada), Ln5 = *B. violaceus* DNA (BC, Canada), Ln6 = *B. schlosseri* DNA (PQ, Canada), Ln7 = *Didemnum vexillum* DNA (BC, Canada), Ln8 = *Molgula* sp. (PEI, Canada), Ln9 = Negative control; **E** - Sensitivity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1 = 1 egg, Ln2 = 5 eggs, Ln3 = 10 eggs, Ln4 = 50 eggs, Ln5 = 100 eggs, Ln6 = Negative control; **F** - Sensitivity agarose gel of PCR products with CIONAINTESTCOI-F1 & R1 primers with: Ln1 = 1 free swimming larvae, Ln2 = 5 free swimming larvae, Ln3 = 10 free swimming larvae, Ln4 = 20 free swimming larvae, Ln5 = 50 free swimming larvae, Ln6 = 100 free swimming larvae, Ln7 = Negative control; **G** - Specificity agarose gel of PCR products with BOTVIOLET-F1 & R1 primers and: Ln1 = *B. violaceus* (Purple morph) DNA (BC, Canada), Ln2 = *B. violaceus* (Purple morph) DNA (MA, USA), Ln3 = *B. violaceus* (Orange morph) DNA (MA, USA), Ln4 = *B. violaceus* DNA (PEI, Canada), Ln5 = *B. violaceus* (Purple morph) DNA (MA, USA), Ln6=*C. intestinalis* DNA (PEI, Canada), Ln7=*S. clava* DNA (PEI, Canada), Ln8=*B. schlosseri* DNA (PQ, Canada), Ln9=*Didemnum vexillum* DNA (BC, Canada), Ln10=*Molgula* sp DNA (PEI, Canada), Ln11=Negative control; **H** - Sensitivity agarose gel of PCR products with BOTVIOLET-F1 & R1 primers and: Ln1 = 1 zooid, Ln2 = 1 zooid, Ln3 = Fragment of zooid, Ln4 = Fragment of zooid, Ln5 = Negative control; **I** - Specificity agarose gel of PCR products with BOTSCHLOCOI-F1 & R1 primers with: Ln1 = *B. schlosseri* DNA (PEI, Canada), Ln2 = *B. schlosseri* DNA (PQ, Canada), Ln3 = *B. schlosseri* DNA (BC, Canada), Ln4 = *B. schlosseri* DNA (MA, USA), Ln 5 = *C. intestinalis* DNA (PEI, Canada), Ln6 = *S. clava* DNA (PEI, Canada), Ln7 = *B. violaceus* DNA (BC, Canada), Ln 8 = *Didemnum vexillum* DNA (BC, Canada), Ln9 = *Molgula* sp. DNA (PEI, Canada), Ln 10 = Negative control; **J** - Sensitivity agarose gel of PCR products with BOTSCHLOCOI-F1 & R1 primers with: Ln1 = 1 zooid, Ln2 = 1 zooid, Ln3 = fragment of zooid, Ln4 = fragment of zooid, Ln5 = Negative control

PCR yielded the expected 1245 bp amplicon when *Botrylloides violaceus* 18S DNA was amplified with the BOTVIOLET18S primer set (Figure 1G). Sequencing confirmed that the amplicon produced was from *Botrylloides violaceus* 18S rDNA and was identical to the 18S rDNA sequence in Genbank ([AY903927](#)). The BOTVIOLET18S primer set did not amplify DNA from any other tunicate tissue tested. In sensitivity testing, the BOTVIOLET18S primers were able to detect one zooid, and even smaller fragments of one zooid (Figure 1H).

The primer set designed to amplify sections of the *Botryllus schlosseri* COI gene produced the expected amplicon (Figure 1I). The BOTSCH-LOCOI-F1 and R1 primers did not amplify any DNA from the other tunicates tested in this study. The BOTSCHLOCOI-F1 and R1 primers were able to detect as little as a fragment of one zooid present in a water sample (Figure 1J).

Discussion

Our results show that the primers designed in this study for PCR are suitable for species identification of invasive tunicates at many life stages, including eggs and free swimming larvae. Even when the primers differ by only one base pair from the target gene sequences of other species, as with the STYCLAV18S primers, they do not cross react in the presence of other tunicate DNA, as tested in this study. This specificity is particularly useful at egg and larval stages where current identification methods between species can be inaccurate and difficult. Although results are not shown in this study, these primer sets were also able to detect target species when mixed DNA samples from multiple tunicate species were added to PCR reactions. Despite the fact that some tunicate species, such as *B. violaceus* and *B. schlosseri*, have different colour morphs, the primers designed in this study are capable of identifying different morphs as one species. The primer sets for all four species in this study were able to detect tunicate DNA from all geographic regions sampled, indicating that the primer regions are not areas of high variability within each species. In addition to the geographic locations sampled in this study, these primer sets have also been successfully used to amplify tunicate DNA from Ireland, Northern Ireland, Spain, and multiple locations within Canada (Stewart-Clark, unpublished data). It is

possible that other unsampled geographic locations may have haplotypes with mutations in the 18S and COI genes at our primer regions. As a result, our primer sets would not detect the presence of these individuals. However this is a limitation of any PCR based assay (Darling and Blum 2007; Hare et al 2000). It is very important that molecular assays, such as those developed in this study, are validated in their area of use. The presence of PCR inhibitors, or of other species with similar sequences may impact the assay results.

The primers designed in this study for solitary tunicates are extremely sensitive as they are able to detect as little as one egg or larva present in a water sample. The primers designed to detect the colonial tunicates are also extremely sensitive as they are able to detect as little as one zooid. While sensitive PCR-based assays have been developed to identify larval forms of other species in water samples such as bivalves (Bell and Grassle 1998; Hare et al 2000; Wang et al. 2006) no such assays have yet to be developed to detect invasive tunicates at microscopic stages in water samples.

The species-specific primers generated in this study meet the criteria listed by Darling and Blum (2007) for ideal detection methods for aquatic invasive species: rapid, cost-effective, accurate and accessible. It is hoped that the primer sets designed in this study can become integrated into current monitoring practices for invasive tunicates in Prince Edward Island as well as in other jurisdictions, since the current detection methods of microscopy surveys of water samples is so time consuming and challenging. These primer sets could be used to screen mussel processing plant effluent outflow, mussel growing waters and ballast water for the presence of eggs and larvae of invasive tunicates.

Field trials are currently being conducted to ensure that PCR inhibitors, which may be present in water samples from processing facilities, do not limit the efficacy of these primer sets in detecting invasive tunicates. Specificity and sensitivity of these primer sets is being evaluated in field water containing various amounts of sediment and organic matter to determine the impact that these compounds have on the diagnostic capacity of these assays. In addition, sampling methods are also being evaluated in field conditions to determine the optimal water sampling techniques and locations

within processing facilities to correctly sample for these PCR based assays.

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References

- Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25: 3389-3402, doi:10.1093/nar/25.17.3389
- Bell JL, Grassle JP (1998) A DNA probe for identification of larvae of the commercial surfclam (*Spisula solidissima*). *Molecular Marine Biology and Biotechnology* 7: 127-137
- Bourque D (2006) Evaluating and managing the role of processing plants as vectors for aquatic invasive species. Oral Presentation at Aquaculture CanadaOM, November 19-22, 2006, Halifax, NS, Canada
- Darling JA, Blum MJ (2007) DNA-based methods for monitoring invasive species: a review and prospectus. *Biological Invasions* 9: 751-765, doi:10.1007/s10530-006-9079-4
- DFO (2006) Aquatic Invasive Species: Restricting a Body of Water. http://www.glf.dfo-mpo.gc.ca/ais-eae/2_2-e.html (Accessed February 2007)
- Hare MP, Palumbi SR, Butman CA (2000) Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. *Marine Biology* 137: 953-961, doi:10.1007/s002270000402
- Le Roux F, Audemard C, Barnaud A, Berthe F (1999) DNA probes as potential tools for the detection of *Martelia refringens*. *Marine Biotechnology* 1: 588-597, doi:10.1007/PL00011814
- Mo C, Douek J, Rinkevich B (2002) Development of a PCR strategy for thraustochytrid identification based on 18S rDNA sequence. *Marine Biology* 140: 883-889, doi:10.1007/s00227-002-0778-9
- Stach T, Turbeville JM (2002) Phylogeny of tunicata inferred from molecular and morphological characteristics. *Molecular Phylogenetics and Evolution* 25: 408-428, doi:10.1016/S1055-7903(02)00305-6
- Thompson R, MacNair N (2004) An overview of the clubbed tunicate (*Styela clava*) in Prince Edward Island. Fisheries and Aquaculture Technical Report # 234. PEI Department of Agriculture, Fisheries, Aquaculture and Forestry, 29 pp
- Wada H, Makabe KW, Nakauchi M, Satoh N (1992) Phylogenetic relationships between the solitary and colonial ascidians, as inferred from the sequence of the central region of their respective 18S rDNAs. *Biological Bulletin* 183: 448-455, doi:10.2307/1542021
- Wang S, Bao Z, Zhang A, Guo W, Wang X, Hu J (2006) A new strategy for species identification of planktonic larvae: PCR-FLRP analysis of the internal transcribed spacer region of ribosomal DNA detected by agarose gel electrophoresis or DHPLC. *Journal of Plankton Research* 28: 375-384, doi:10.1093/plankt/fbi122