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**Research Article**

# A PCR-based assay to facilitate early detection of *Diplosoma listerianum* in Atlantic Canada

Jessica E. Willis[1,2,φ*], Sarah Stewart-Clark[2,3], Spencer J.Greenwood[2,4,φ], Jeff Davidson[3] and Pedro Quijon[1]

[1]*Dept. of Biology, University of Prince Edward Island, 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3*
[2]*Dept. of Pathology and Microbiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3*
[3]*Dept. of Health Management, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3*
[4]*AVC Lobster Science Centre, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3*

E-mail: *jwillis@upei.ca (JEW)*, *pquijon@upei.ca (PQ)*, *seclark@upei.ca (SSC)*, *sgreenwood@upei.ca (SJG)*, *davidson@upei.ca (NS)*

*Corresponding author

[φ]*Present address: Department of Biomedical Sciences, University of Prince Edward Island, 550 University Ave, Charlottetown, Prince Edward Island, Canada, C1A 4P3*

## Abstract

The recent detection of the invasive colonial tunicate *Diplosoma listerianum* in Havre- Aubert, Magdalen Islands (Quebec, Canada) in 2008, prompted the development of a molecular assay as a method to detect and monitor for the potential invasion of this species in Prince Edward Island. The aim of this study was to design a highly sensitive, species-specific Polymerase Chain Reaction (PCR) assay capable of detecting *D. listerianum* with a high efficacy in local water samples. To accomplish this, oligonucleotide primer sets were designed from the 18S rDNA gene of *D. listerianum*. Primer sets were evaluated for specificity using the GenBank database, followed by a series of spiked water sample trials involving various tunicate species. Assay efficacy was tested and then evaluated by conducting spiked water sample trials using *D. listerianum* samples from two different geographic locations (Japan and Canada). Primer sets that were shown to be species specific were then tested for their analytical sensitivity and environmental efficacy by spiking local water samples with various amounts of *D. listerianum* tissue. The primer set DlistF1/DlistR1 was found to be species specific and yielded no false positive results when tested with tissue from the four invasive tunicate species currently present on Prince Edward Island (PEI) (*Styela clava*, *Botryllus schlosseri*, *Botrylloides violaceus*, and *Ciona intestinalis*). This assay was also capable of detecting *D. listerianum* DNA from two different populations, demonstrating its potential for use in other geographic locations, which may possess different haplotypes of the species. As the results of this study demonstrate, the DlistF1/DlistR1 assay has a high analytical sensitivity, detecting DNA from as little as 1 zooid in a water sample, and was not inhibited when tested with water samples collected from various bays across both PEI and the Magdalen Islands. The DlistF1/DlistR1 molecular assay provides a monitoring tool for shellfish aquaculture regions and can be used to facilitate early detection of this species. This level of early detection is beneficial to facilitate the implementation of mitigation programs in time to prevent *D. listerianum* from reaching nuisance levels.

**Key words:** 18S rDNA, aquatic invasive species, aquaculture, *Diplosoma*, detection, PCR, tunicate

## Introduction

Several thousand aquatic invasive species (AIS) are transported globally in ship ballast water, on boat hulls and in sea chests every day (Smith et al. 1996; Lambert 2007). Once established, these species can be difficult if not impossible to eradicate, and can have severe consequences to marine aquaculture operations, fisheries, native biodiversity, and the integrity of coastal ecosystems (Anil et al. 2002; Hayes et al. 2005).

There are currently four invasive tunicate species that are causing challenges in Prince Edward Island (PEI) Canada: *Ciona intestinalis* Linnaeus, 1767, *Styela clava* Herdman, 1881, *Botrylloides violaceus* Oka, 1927, and *Botryllus schlosseri* Pallas, 1774 (Ramsay et al. 2008). These invasive tunicates attach to both natural and artificial substrates and are capable of fouling mussel lines and aquaculture gear including buoys, ropes, cages, and moorings (Astudillo et al. 2009; Darbyson et al. 2009).

Due to heavy fouling, these tunicates affect labour and costs associated with the mussel aquaculture industry in PEI (Thompson and McNair 2004; Locke et al. 2007; Ramsay et al. 2008; Arsenault et al. 2009; Gittenberger 2009).

In October 2008, the first official documented sighting of the invasive tunicate *Diplosoma listerianum* Milne-Edwards, 1841 in the Magdalen Islands in the Gulf of St. Lawrence was recorded. Species identity was confirmed using morphological and molecular methods (Nathalie Simard, Sarah Stewart-Clark, and Mary Carman-Personal Communication).

*D. listerianum* is widespread throughout Europe but has spread globally (Lambert 2001; Rocha et al. 2009) including areas such as Japan, Madagascar, Australia, South Africa, Peru, Brazil, Atlantic-Central America, the Netherlands, and the United Kingdom (Yamaguchi 1975; Millar 1988; Kott 2001; Monniot 2001; Sanamyan and Schories 2004; Rocha and Kremer 2005; Rocha et al. 2005; Gittenberger 2007; Vance et al. 2008). *D. listerianum* has also spread along the Eastern United States, including the Great Bay estuary in New Hampshire (Dijkstra and Harris 2007) and throughout New England within the Gulf of Maine (Harris et al. 1998; Harris and Tyrell 2001; Dijkstra et al. 2007; Carman and Grunden 2010).

*D. listerianum* has been documented as a highly successful invader, rapidly colonizing available primary space as well as outcompeting other neighbouring invasive tunicates, including *B. schlosseri* (Schmidt and Warner, 1986; Altman and Whitlatch 2007; Vance et al. 2008). Since *D. listerianum* has been found to colonize mussel lines (Gittenberger 2009; Rocha et al. 2009) there is concern that bivalve aquaculture farmers may have yet another tunicate species fouling their mussel lines should *D. listerianum* successfully establish itself in Atlantic Canada.

Taking into consideration factors such as climate, current distribution, and potential transport vectors, *D. listerianum* was recently classified as a potential invader to Atlantic Canada (Locke 2009), and thus its sighting in the region was not completely unanticipated. Nonetheless, based on the success of invasions in this region by other invasive tunicate species, *D. listerianum* poses an ecological threat to local benthic communities as well as a financial threat to the mussel aquaculture industry in Atlantic Canada.

Due to the close proximity of the Magdalen Islands to Prince Edward Island (PEI) there is concern that this AIS may spread to PEI via recreational boating, commercial fishing vessels, or the Souris/Cap-aux-Meules Cooperative of Transport Maritime and the Air (CTMA) Group ferry. Due to the risk that invasive species pose to ecosystems and the shellfish aquaculture industry, efforts should be directed to better predict and halt introductions of potentially harmful species (McKindsey et al. 2007; Mouland 2008). It is also important to monitor for seasonal sexual reproduction in currently invaded areas as this provides bivalve aquaculture farmers with an indication of when they should annually begin and finish treating their mussel lines in order to prevent excessive tunicate colonization. A common mitigation strategy for invasive tunicates fouling suspended mussel lines is to spray the lines with a high pressure water system (Paetzold and Davidson 2010). However, removal of the tunicates creates more available space on which new recruits may settle, and thus monitoring for tunicate reproduction can provide a good indication of when and how often mussel lines require treatment throughout the year.

Molecular tools have previously proven useful for the early identification of planktonic larvae and the monitoring of the new recruitment of various AIS (Burton 1996; Deagle et al. 2003; Patil et al. 2005; Boeger et al. 2007; Darling and Tepolt 2008; Jones et al 2008; Harvey et al. 2009). Recently, molecular assays have been developed for the early detection of the four invasive tunicates currently present on PEI (Stewart-Clark et al. 2009). In addition, these assays have been successfully used in rapid assessments conducted by the Department of Fisheries and Oceans Canada (DFO) and have been implemented in current monitoring efforts in many areas of PEI (our data).

The objective of this study was to design a highly sensitive, species specific DNA assay for the early detection of *D. listerianum* in water samples from across PEI and the Magdalen Islands, Quebec. The 18S rDNA gene was chosen as the target gene due to the evolutionarily conserved nature of the gene (Gonzalez and Schmickel 1986). The small number of variable regions in the 18S rDNA gene is helpful in designing primers to amplify different species where only subtle genomic differences exist (Stach and Turbeville 2002). The 18S rDNA has proven to be a very successful genetic marker as species specific molecular assays have been developed for many marine organisms including

surf clams, nematodes, and tunicates (Bell and Grassle 1998; Oliveira et al. 2005; Stewart-Clark et al. 2009). Primer sets were first screened for their efficacy and specificity in detecting *D. listerianum* against other local invasive tunicate species and various other *Diplosoma* species from other geographic regions.

Primer sets were then evaluated to assess their analytical sensitivity and efficacy in environmental water samples. The lower limit of detection tested in this study was 1 single zooid, which is the smallest individual unit within the colony. The ultimate goal of this study was to develop an assay that can be used in future monitoring efforts for *D. listerianum* across PEI and the Magdalen Islands, or modified and optimized for use in other geographic regions.

## Materials and methods

### Primer Design

As there were no 18S rDNA sequences for *Diplosoma listerianum* available in GenBank, PCR amplification of the 18S rDNA gene was required for sequence alignment and initial primer design. To generate an 18S rDNA sequence for *D. listerianum*, DNA was extracted from a preserved tissue sample found in Havre Aubert, Magdalen Islands, Quebec (denoted MAG C) and a 502 base pair segment of the 18S rDNA gene was amplified using the universal 18S rDNA primers CASIS/CAS2 (Le Roux et al. 1999). PCR amplicons were sequenced in both directions at the Laboratory Services, University of Guelph. The *D. listerianum* 18S rDNA partial sequence from the Magdalen Islands (denoted MAG C) (GenBank accession no. HM641906) and Japan (GenBank accession no. HM641905) were aligned using Clustal W with the 18S rDNA sequences of *Diplosoma simileguwa* Hirose and Oka, 2005 (GenBank accession no. AB211108.1, AB211107.1), *Diplosoma simile* (GenBank accession no. AB211106.1, AB2111 05.1, AB211104.1, AB211103.1, AB211102.1, AB211101.1), *Diplosoma ooru* Hirose and Suetsugu, 2005 (GenBank accession no. AB2111 00.1, AB211099.1, AB211098.1, HM641902), *Diplosoma virens* (GenBank accession no. AB211109.1, AB211110.1, AB211111.1, AB211112.1, AB211113.1, AB211114.1, AB211115.1), *Diplosoma watanabei* Hirose and Oka, 2009 (GenBank accession no. HM641904), *Diplosoma sp*. (GenBank accession no. AB211118.1, AB211116.1, AB211119.1,

AB211121.1, AB211120.1, AB211117.1), *Diplosoma aggregatum* (GenBank accession no. HM641903), *Diplosoma variostigmatum* (GenBank accession no. HM641901), and *Diplosoma gumavirens* Hirose and Oka, 2009 (GenBank accession no. HM641900), as well as with 18S rDNA sequences of native and invasive tunicate species that are present on PEI. These included *S. clava* (GenBank accession no. FM897318.1, L12442.1), *B. schlosseri* (GenBank accession no. FM897303.1, AB211066.1), *C. intestinalis* (GenBank accession no. AJ250778.1, AB013017.1), and *Molgula* sp. (GenBank accession no. L12426.2, AY040738.1, L12434.2). Primers were then designed from these aligned 18S rDNA sequences based on regions that were unique to *D. listerianum*. Each 18S rDNA primer generated in this study for *D. listerianum* was entered into NCBI BLAST (National Centre for Biotechnology Information - Basic Local Alignment Search Tool) (Altschul et al. 1997) to observe their resemblance to genes of organisms contained in GenBank so as to ensure that they were only specific to *D. listerianum*.

Each primer set was screened for proper melting point range and the propensity to form hairpin structures, homodimers, and hetero-dimers using the IDT SciTools Oligoanalyzer 3.1 (*http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/*) to ensure that all primers were suitable candidates for PCR amplification. Four primer sets were chosen and used in further laboratory evaluations (DlistF1/DlistR1, DlistF2/DlsitR2, DlistF3/ DlistR1, and DlistF4/DlistR1) (Table 1).

### Specificity Testing

Various tissue samples were collected to ensure efficacy and specificity of the designed assay. Tissues included *D. listerianum* samples from both the Magdalen Islands (MAG C) and Japan as well as various other *Diplosoma* species collected from Japan: *D. aggregatum, D. watanabei, D. variostigmatum, D. ooru,* and *D. gumavirens* (Table 2). Extracted DNA of these various species underwent PCR with each of the four designed primer sets. All DNA extractions were performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Canada) following the manufacturer's protocol. The same procedure was repeated with samples of *S. clava, B. schlosseri, B. violaceus,* and *C. intestinalis* that were collected on PEI to ensure that the designed primer sets did not amplify other tunicate species.

**Table 1.** Sequences for primers designed in this study for *Diplosoma listerianum.*

| Primers | Sequence 5' -3' | Location | Annealing Temp (°C) |
|---------|-----------------|----------|---------------------|
| DlistF1 | CGA GGC GAA CCG AAA ACT TTG TAA ACT TG | 207-234 | 69.0 |
| DlistR1 | GGG GAA CGG TCT ACC AGT ATG AAC | 389-412 | 69.0 |
| DlistF2 | GGT CGC TTG CCG TTT CGC TTG AA | 173-195 | 55.0 |
| DlistR2 | CCA AGC GGA GCC GTC TCT TTT CAT T | 342-366 | 55.0 |
| DlistF3 | TTT CGC TTG AAT GCG CGG CAG | 185-205 | 69.0 |
| DlistF4 | AAA ACT TTG TAA ACT GGA CGG ACC GGC | 219-245 | 69.0 |

**Table 2.** Collection data for tunicate tissue samples used in this study.

| Species | Collection Date | Collection site | Geographic Coordinates |
|---------|-----------------|-----------------|------------------------|
| *Diplosoma gumavirens* | Oct 26, 2007 | Japan; Okinawa prefecture, Ishigakijima Is., Hirakubo lighthouse | N 24°36'45" E 124°18'56" |
| *Diplosoma ooru* | Oct 25, 2007 | Japan; Okinawa prefecture, Ishigakijima Is., Shiraho village | N 24°20'57" E 124°15'19" |
| *Diplosoma variostigmatum* | Oct 26, 2007 | Japan; Okinawa prefecture, Ishigakijima Is., Hirakubo lighthouse | N 24°36'45" E 124°18'56" |
| *Diplosoma aggregatum* | Aug 2, 2008 | Japan; Okinawa prefecture, Ishigakijima Is., Muiga cliff | N 24°43'31" E 125°22'50" |
| *Diplosoma watanabei* | Dec 10, 2008 | Japan; Okinawa prefecture, Ishigakijima Is., Ngamahama | N 24°43'38" E 125°14'24" |
| *Diplosoma listerianum* (MAG C) | Oct. 5, 2008 | Havre Aubert, Magdalen Islands, Québec, Canada | N 47°14'08.9" W 061°50'03.3" |
| *Diplosoma listerianum* | Jul 16, 2009 | Misaki Shipyard docks, Japan | N 35°10'25.8" E 139°37'03.6" |
| *Botryllus schlosseri* | Jun 26, 2009 | St. Peter's Bay, PEI | N 46°25'08.8" W 62°35'35.4" |
| *Ciona intestinalis* | Jul 20, 2009 | St. Mary's Bay, PEI | N 46°07'39.8" W 62°29'04.5" |
| *Styela clava* | Jul 20, 2009 | Savage Harbour, PEI | N 46°24'58.4" W 62°51'00" |
| *Botrylloides violaceus* | Jul 20, 2009 | St. Mary's Bay, PEI | N 46°07'39.8" W 62°29'04.5" |

*Analytical Sensitivity Testing*

The analytical sensitivity of the primer sets was evaluated using colonies (consisting of 5-6 zooids) and individual zooids of *D. listerianum* that were dissected from preserved tissue using a dissecting microscope. Five replicates of each quantity of tunicate material were tested. Individual zooids and colonies were placed into 1 mL of nuclease free water. Samples were then centrifuged at 10,000 x g for 2 minutes, after which all of the excess water was removed. A Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Canada) was used to extract DNA following the manufacturer's instructions until the final step, at which point only 20 μL of AE buffer was added (as opposed to 200 μL) for individual zooids, or 50 μL for single colonies. The quantity and purity of all extracted DNA (tissue samples as well as spiked and non-spiked water samples) was evaluated using a NanoDrop spectrophotometer.

*Water Sampling and Evaluation*

Water samples were collected from various locations to test the efficacy and analytical sensitivity of the primer sets in environmental water samples which may naturally contain PCR inhibitors (Wilson 1997; Radstrom et al. 2004; Mountfort and Hayden 2007) (Table 3). Water samples from Cardigan Bay, Marchwater Bay, and Montague River were collected from the surface using sterile 50 mL collection tubes for use in analyzing assay efficacy in local environmental conditions. Water samples for preliminary evaluation (3 from Souris Harbour and 1 from Havre Aubert) were collected using a 64 μm plankton sieve that was raised and lowered within the water column at a rate of 50 L/min for 3 minutes (Smith 2009) to depths ranging from 0.5 metres to 3 metres. This water sampling method was chosen as it is the industry

standard used in provincial rapid assessments for invasive tunicates, and it has been shown to be sufficient for collection of tunicate larvae and eggs (Gill et al. 2007). All water samples were returned to the lab and stored at -80°C. Water samples were thawed as needed for the experiment and aliquoted into a total of 11 sub-samples from each location, 5 of which were spiked with colonies and 5 spiked with indiv-dual zooids. These spiked water samples were centrifuged at a speed of 450 x g for 5 minutes to separate pelleted material from the supernatant. The supernatant was then removed and pelleted material of all samples was transferred to 1.5mL tubes. DNA was extracted following the procedure described above. The remaining sub-sample from each location was saved for use in negative controls during PCR reactions to demonstrate that water samples did not naturally possess any *D. listerianum* DNA.A preliminary evaluation of water samples from Souris Harbour and Havre Aubert for *D. listerianum* was conducted by performing DNA extractions as described above without spiking to screen for the presence of *D. listerianum*.

*PCR and Agarose Gel Electrophoresis* PCR were performed in a 25 µL volume containing 12.5 µL Amplitaq Gold Master Mix (Applied Biosystems manufactured by Roche, Branchburg, New Jersey), 9.5 µL Qiagen Nuclease Free Water, 1 µL forward primer (10µM), 1 µL reverse primer (10µM), and 1 µL of undiluted target DNA. In each reaction that was performed a series of negative controls were included, in which the 1 µL of DNA was substituted with an additional 1 µL of Nuclease Free Water. Positive controls that consisted of 1 µL of DNA extracted from a preserved tissue sample of *D. listerianum* from the Magdalen Islands (MAG C) were included in subsequent reactions following assay efficacy experiments. DNA was denatured for 3 minutes at 92°C, followed by 34 cycles consisting of denaturation at 94°C for 1 minute, a primer annealing period of 1 minute at 69°C (DlistF1/DlistR1, DlistF1/ DlistR3, DlistF1/ DlistR4) or 55°C (DlistF2/ DlistR2), and a 3 minute elongation period at 72°C. The reaction was completed with a 5 minute polymerization extension period at 72°C.

PCR amplicons were separated in 1 % agarose gels via electrophoresis. Gels were prepared using 1.25 g Electrophoresis Grade Agarose, 13.5 µL SYBR Safe DNA Gel Stain (Invitrogen), 125 mL 1X Tris/Borate/EDTA buffer, and were visualized under ultraviolet light. Images were viewed using Quantity One Software and a Gel Documentation (Gel Doc) System (BioRad).

## Results

Of the four primer sets that were designed and evaluated, DlistF1/DlistR1 was found to meet all the required criteria of specificity, analytical sensitivity, and efficacy. The forward primer (DlistF1) had maximum query coverage of 86% in NCBI-BLAST, with no tunicate species producing significant alignments. The reverse primer (DlistR1) yielded maximum query coverage of 83% with tunicates *D. simile* and *D. ooru* having only 79% coverage. The remaining three primer sets, though displayed as unique in NCBI-BLAST, were not found to be species specific when tested against other tunicate species (data not shown). The DlistF1/DlistR1 assay was capable of detecting *D. listerianum* DNA from different populations as it successfully amplified *D. listerianum* DNA from both Japan and the Magdalen Islands, with PCR yielding the proper amplicon size of 205 base pairs (Figure 1A). Species identity was confirmed by sequencing. The DlistF1/DlistR1 assay did not amplify the DNA of other invasive tunicate species currently present on PEI (*B. schlosseri*, *C. intestinalis*, *B. violaceus*, or *S. clava*) (Figure 1B). The DlistF1/DlistR1 assay was specific and only detected *D. listerianum* DNA versus other *Diplosoma* species including *D. gumavirens*, *D. ooru*, *D variostigmatum*, *D. aggregatum*, and *D. watanabei* (Figure 1C).

The primer set DlistF1/DlistR1 detected DNA from 1 zooid, the lower limit chosen for this study due to the colonial nature of *Diplosoma* (Figure 1D). Analytical sensitivity tests performed in environmental water samples demonstrated that 1 zooid was detectable in spiked grab water samples taken from Cardigan Bay, Marchwater Bay, and Montague River (Figure 1E), as well as from sieve concentrated spiked samples taken from Souris Harbour (Figure 1F) and Havre Aubert (Figure 1G). PCR performed for preliminary screening of water samples from Souris Harbour and Havre Aubert did not detect the presence of *D listerianum* other than the spiked positive control (Figure 1H).
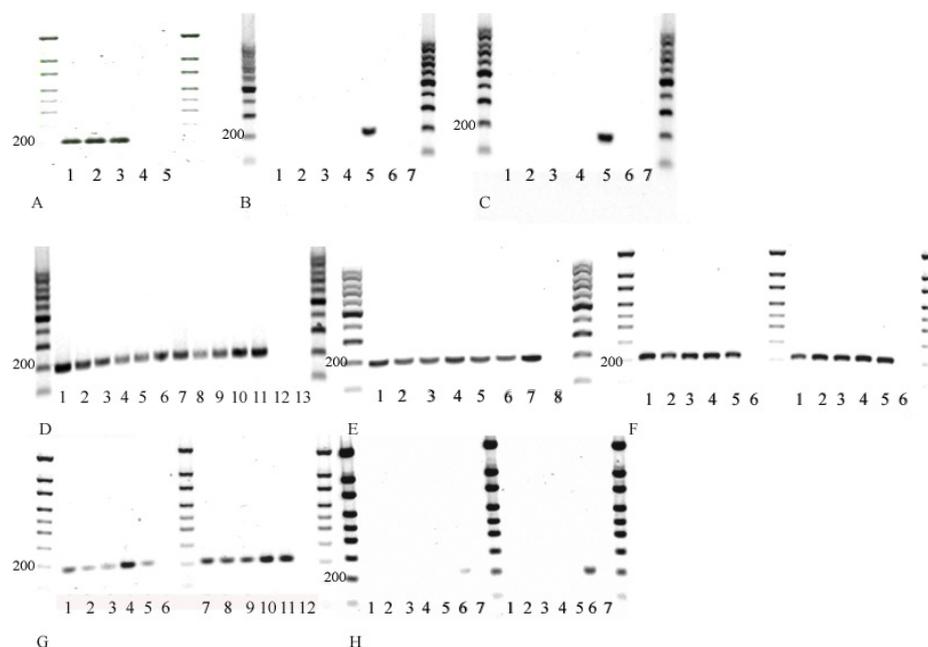
**Figure 1.** Agarose gel of PCR amplicons produced using the DlistF1/DlistR1 assay for:
**A** two *Diplosoma listerianum* samples from Masaki Shipyard docks, Japan and a *D. listerianum* sample from Havre Aubert, Magdalen Islands. Ln1= *D. listerianum* (MAG C), Ln2= Japan *D. listerianum* sample 1, Ln3= Japan *D. listerianum* sample 2, Ln4-5= Negative controls.
**B** genus specificity testing: Ln1= *B. schlosseri*, Ln2= *C. intestinalis*, Ln3= *B. violaceus*, Ln4= *S. clava*, Ln5= *D. listerianum* (MAG C), Ln6-8= Negative controls.
**C** species specificity testing for *D. listerianum* with water samples spiked with: Ln1= *D. gumavirens,* Ln2= *D. ooru,* Ln3= *D. variostigmatum,* Ln4= *D. aggregatum,* Ln5= *D. watanabei*, Ln6= *D. listerianum* (MAG C), Ln7-8= Negative controls.
**D** individual zooids and colonies for analytical sensitivity testing. Ln1-5= *D. listerianum* zooids, Ln6-10= *D. listerianum* colonies, Ln11= *D. listerianum* (MAG C), Ln12-13= Negative controls.
**E** field evaluation of various PEI water samples. Ln1= Montague River spiked with a single zooid, Ln2= Cardigan Bay spiked with a single zooid, Ln3= Marchwater Bay spiked with a single zooid, Ln4= Montague River spiked with colony, Ln5= Cardigan Bay spiked with colony, Ln6= Marchwater Bay spiked with colony, Ln7= *D. listerianum* (MAG C), Ln8= Negative control.
**F** field evaluation of water samples collected from Havre Aubert, spiked with individual zooids or colonies. Ln1-5= single colonies, Ln6= Negative control, Ln7-11= individual zooids, Ln12= Negative control.
**G** field evaluation of water samples collected from Souris Harbour, PEI, spiked with individual zooids or colonies. Ln1-5= single colonies, Ln6= Negative control, Ln7-11= individual zooids, Ln12= Negative control.
**H** water samples evaluated from (left) Havre Aubert, Magdalen Islands, and (right) Souris Harbour, PEI. Ln1-5= water samples, Ln6= *D. listerianum* (MAG C), Ln7= Negative control.

**Table 3.** Location, date, depth, and sampling information for water samples used in this study. Plankton net not used for surface water samples.

| Collection Site | Collection Date | Depth (m) | Geographic coordinates | Plankton net (μm) | Total sample volume (mL) |
|---|---|---|---|---|---|
| Havre Aubert, Magdalen Islands | Aug 19, 2009 | 2-10 | N 47º14'08.9" W 61º50'03.3" | 64 | 500 |
| Havre Aubert, Magdalen Islands | Aug 19, 2009 | 2-10 | N 47º14'08.9" W 61º50'03.3" | 64 | 500 |
| Havre Aubert, Magdalen Islands | Aug 19, 2009 | 2-10 | N 47º14'08.9" W 61º50'03.3" | 64 | 500 |
| Souris Harbour, PEI | Aug 21, 2009 | 3-8 | N 46º21'03.01" W 62º15'30.52" | 64 | 30 |
| Marchwater Bay, PEI | Aug 12, 2009 | Surface | N 46º31'21.3" W 63º42'32.7" | - | 500 |
| Montague River, PEI | Aug 10, 2009 | Surface | N 46º10'32.8" W 62º35'36.4" | - | 500 |
| Cardigan Bay, PEI | Aug 12, 2009 | Surface | N 46º08'59.2" W 62º30'57.7" | - | 500 |

## Discussion

The DlistF1/DlistR1 assay was capable of amplifying target DNA from two different populations of *Diplosoma listerianum* (Magdalen Islands, Canada, and the Misaki Shipyard, Japan). This indicates that the assay developed in this study can potentially be used for early detection in the region and has the potential to be used in other geographic locations that may possess different haplotypes, which is an important criterion for invasive species molecular detection tools. Further validation, including testing *D. listerianum* samples from other geographic locations, would confirm the suitability of this assay for widespread geographic use.

Of the four primer sets designed in this study (Table 1), the DlistF1/DlistR1 assay was determined to be specific at the species level as it exclusively amplified *D. listerianum* DNA and did not amplify DNA from any other *Diplosoma* species or DNA from any other tunicate. In addition, the DlistF1/DlistR1 assay did not yield any false positives when used in PCR with environmental water samples, indicating that this assay does not amplify non-target marine organisms which are present in water samples. This level of specificity ensures that false positive results will not be expected when testing for the presence of *D. listerianum* in PEI due to the presence of other tunicate DNA in water samples.

Assay sensitivity is of particular importance in the initial detection of invasive species because low analytical sensitivity could limit the ability to detect minute quantities of DNA associated with early stages of invasion. Some molecular assays for various AIS have been shown to be highly sensitive, detecting as little as 1 larva spiked in 1 mL of ballast water for the Pacific oyster *Crassostrea gigas* Thunberg, 1793 (Harvey et al. 2009), and as little as one egg or larva for the solitary tunicates *Ciona intestinalis* and *Styela clava* (Stewart-Clark et al. 2009). As do most colonial ascidians, *D. listerianum* reproduces sexually via the release of mature larvae that were brooded within the adult colony (Pemberton et al. 2003) as well as asexually by budding as cells either divide and regenerate themselves at the margin of the attached colony or naturally fragment away from the main colony and divide upon settlement on a suitable substrate (Berill 1935; Bullard et al. 2007; Vance et al. 2008).

As live larvae were unavailable in this study, analytical sensitivity was measured only by the detection of individual zooids (groups of which are embedded in tunic to comprise a fragmented or established colony) (Goodbody 1974; Mackie and Singla, 1987). As larvae spend little time in the water column before settling and meta-morphosing (Cloney 1982) zooids may be preferred targets for detection of colonial tunicates due to the likelihood of finding colony fragments dispersed in the water column and their indefinite survival. The molecular assay developed in this study was capable of ampli-fying 1 zooid of *D. listerianum* in spiked water samples. This was chosen as the unit of highest analytical sensitivity in this study due to the likelihood of fragmentation, a common method of dispersion in colonial tunicates. Fragments can drift in the water column; therefore they are likely to be the smallest detectable measurement of DNA for the invading organism.

Analytical sensitivity of the DlistF1/DlistR1 assay was not impacted by the presence of compounds such as sediment present in water samples or by effluent from nearby processing plants, two potential PCR inhibitors that are often present in environmental water samples (Wilson 1997). Neither was analytical sensitivity impeded by the presence of non-target DNA in environmental water samples, which often contain microscopic and macroscopic non-target biomass. This can be concluded as NanoDrop results demonstrated that the amount of DNA extracted in spiked water samples was far beyond the levels of DNA that could be acquired from the single colony or zooid that was present in the samples (data not shown). Assay analytical sensitivity remained at 1 zooid in spiked water samples taken from various locations across PEI (Marchwater Bay, Montague River, Cardigan Bay, and Souris Harbour) and in Havre Aubert, Quebec. It is important to note that while the assay was not inhibited during PCR using water samples from these locations, results may vary in other locations and during different seasons in the same location. There are many organic and inorganic compounds that can potentially inhibit the Taq polymerases used in PCR (Queiroz et al. 2001), and thus PCR optimization may be required when adapting the use of this assay in different locations. These potential issues with PCR inhibition from environmental samples are

a well known limitation for any PCR based assay but can be controlled for by using positive and negative controls in each assay reaction.

Preliminary evaluation of water samples from Havre Aubert and Souris Harbour using the DlistF1/DlistR1 assay were negative for *D. listerianum* DNA. These results are consistent with the outcome of extensive rapid assessments by visual inspection that was conducted in the Magdalen Islands during July of 2009 by DFO, Quebec region (Jeff Davidson, Personal Communication). This rapid assessment failed to detect *D. listerianum* colonial growth in these harbours despite initial positive detection in October, 2008.

Due to the breadth of factors that influence invasion success there are many potential explanations for the apparent unsuccessful colonization of *D. listerianum* in the Magdalen Islands. Water temperatures are a known influence in seasonal reproduction as water temperatures that fall below 8°C have been shown to affect gonad ripening in this species (Brunetti et al. 1988). Furthermore, winter water temperatures below 4°C inhibit recruitment of *Diplosoma* during the following spring (Osman and Whitlatch 2007) due to altered testicle development (Brunetti 1988). Water conditions also determine the time required for reproduction to resume, with delayed recruitment found in summer seasons which had been preceded by cold winters (Stachowicz et al. 2002). High summer temperatures may not affect reproduction but do alter the mean life span of colonies by affecting rates of maturation. However, due to the recorded success of this invader in cold climates (such as Northern Europe), water temperature in the littoral zone is unlikely to be the sole contributing factor. The roles of many other environmental conditions, including salinity, trophic resources, predation rates, spatial competition, species diversity, and genetic variability of the colonists remain unexamined and should be taken into consideration (Carlton 1996).

Despite the current absence of this nuisance species in either of the areas originally evaluated, the DlistF1/DlistR1 assay developed in this study can be used to develop a program to monitor for and detect the arrival of *D. listerianum* in the waters surrounding PEI, the Magdalen Islands, as well as other areas in the region. However, further field validation of this assay as well as optimization of water sampling methodologies are required in large scale water samplings to ensure its efficacy as a high throughput screening tool in future monitoring programs. Integration of the DlistF1/DlistR1 assay into future monitoring programs to screen waters surrounding mussel leases, mussel processing plant effluent, as well as ship ballast water will allow for early detection and provide an opportunity for quick response to this potential invader

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