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Research Article

Rapid detection assay for the invasive vase tunicate, *Ciona intestinalis*, using loop-mediated isothermal amplification combined with lateral flow dipstick

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Abstract

Invasive tunicate species threaten the shellfish aquaculture industry not only in Prince Edward Island (PEI) but also nationally and worldwide. Rapid screening tools for water samples are crucial for the efficient management of aquatic invasive species. This project aims to develop a rapid detection assay capable of identifying larvae of the vase tunicate, *Ciona intestinalis,* in seawater. In this study, a loop-mediated isothermal amplification (LAMP) method has been developed to detect the 18S ribosomal DNA extracted from *C. intestinalis.* This assay was performed in three steps: 1) a DNA extraction step using a direct lysis buffer, 2) an amplification step using a heating block, and 3) a detection step using a lateral flow dipstick. The sensitivity of the assay was estimated at one larva spiked in 100 µL of seawater and the turnaround time of the assay was assessed at 80 min including the lysis step. Given the advantages of this assay such as rapid amplification, ease of use and detection, it could be implemented for monitoring bays and estuaries. In the future, rapid diagnostic assays will constitute a new generation of diagnostic platforms enabling the early detection of non-indigenous invasive species.

Key words: *Ciona intestinalis*; loop-mediated isothermal amplification; probes; lateral flow dipstick

Introduction

In the past 30 years, the increase of international trade has resulted in the movement of non-indigenous species (NIS) around the world. Damage caused by NIS was estimated at US$137 billion per year in the United States (Pimentel et al. 2000) and more than US$314 billion per year in the following combined countries: US, UK, Australia, Brazil, South Africa and India (Pimentel et al. 2001). In Canada, NIS caused economic damage estimated at CAD$7.5 billion per year (Dawson 2002).

Invasive aquatic species are alien organisms that threaten the ecosystem as well as economic activities, particularly in the aquaculture industry of Prince Edward Island (PEI), Canada, but also nationally and worldwide. PEI represents an ideal environment for the establishment of invasive tunicate species mainly due to the high nutrient load (Locke et al. 2007). In addition, the large surface of artificial structures deployed in PEI seawater significantly contributes to the establishment of invasive tunicate species (Locke et al. 2007).

Invasive tunicates compete for food and space with filter feeders such as mussels and oysters thus affecting shellfish aquaculture by increasing its production costs (i.e., labour and equipment). Consequently, shellfish production decreases due to poor growth of the produced organisms. For instance, it is estimated that the solitary tunicate *Styela clava* caused the loss of CAD$88.4 million/year to the aquaculture industry in Atlantic Canada (Colautti et al. 2006). In PEI alone, the loss was estimated at CAD$3 million/year. Currently, three more invasive tunicate species (*Ciona intestinalis, Botrylloides violaceus* and *Botryllus schlosseri*) have colonized aquaculture farms in PEI. In addition, 17 tunicate species are

considered as potential future invaders for Atlantic Canada's aquatic area (Locke 2009).

Ciona intestinalis represents a real threat to PEI's aquatic ecosystem and mussel aquaculture (Ramsay 2009). It has been shown that *C. intestinalis* has extensively expanded in distribution and abundance in PEI seawater and become a dominant fouling species (Ramsay et al. 2008). *Ciona intestinalis* was observed for the first time in 2004 in Montague River, PEI. Two years after its detection, *C. intestinalis* had become established as the main invasive species in Montague River and spread to several estuaries and bays in PEI (Ramsay et al. 2008). The success of *C. intestinalis* establishment in these areas is mainly due to favorable environmental conditions in particular the availability of food sources (high phytoplankton productivity) as well as temperature levels which are suitable for its reproductive activity throughout the summer and fall seasons (Ramsay 2009).

In 2009, *C. intestinalis* was first detected in the Magdalen Islands (47°22′49″N, 61°54′03″W, Quebec, Canada) demonstrating the expansion of this species in Atlantic Canada (Willis et al. 2011). Although the pathways leading to successful invasions are not well understood, in this instance *C. intestinalis* had been initially observed at one port on the hull of a barge originating from PEI (A. Locke, pers. comm.), followed by searches of other high-risk sites. For this purpose, rapid detection assays are essential not only for the identification of infested areas but also for the prevention of the expansion of targeted species by mitigation.

The molecular technique, loop-mediated isothermal amplification (LAMP) is performed isothermally for nucleic acid amplification and therefore does not require any expensive equipment such as a thermal cycler (Notomi et al. 2000). LAMP reaction consists of three steps: an initial step, a cycling amplification step and an elongation step. A DNA polymerase with strand-displacement activity, as well as two inner (FIP, BIP) and outer (F3, B3) primers are required to amplify six regions of the target DNA with high specificity (for review see Mori and Notomi 2009). The amplification is performed under isothermal conditions and the reactions produce a series of stem-loop DNA with different sizes. At the detection level, a lateral flow dipstick (LFD) is combined with the LAMP for rapid detection. The approach is based on a miniaturized LFD, where the targeted sequences are visually detected. These strips detect the biotin labelled amplicon that has been hybridized with an FITC-labeled DNA probe. Therefore, the LFD enables sequence-specific detection, suitable for field application, which is crucial for the early detection of invasive aquatic species.

This study aims to develop a new screening assay that could be used in the field for early detection purposes. Our approach is to focus on the amplification of nucleic acids specific to *C. intestinalis* using an isothermal condition. To achieve our purpose, the loop-mediated isothermal amplification (LAMP) was identified as suitable.

Material and methods

2.1. DNA extraction

Genomic DNA from *C. intestinalis*, *S. clava*, *B. schlosseri*, *B. violaceus* and *Didemnum* sp. was extracted using Qiagen QIAamp DNA Mini kit (Toronto, ON, Canada) according to the manufacturer's instructions. Briefly, 30 mg of tissues were lysed using the lysis buffer with proteinase K for 3 hours at 56ºC. The DNA was eluted with 30 L of RNAse/DNAse free water.

DirectPCR DNA extraction sytem from Viagen Biotech (Los Angeles, CA, US) was used with some modifications for rapid extraction of DNA from seawater samples. Egg and sperm were collected from adult *C. intestinalis* and mixed in Petri dishes filled with filtered seawater. After incubation at room temperature for 16 hours, 1 and 10 free-swimming larvae were collected and spiked in 100 L natural seawater (collected from the field) in 1.5 mL microcentrifuge tubes. After centrifugation (500g for 10 min), direct lysis buffer (100 L) was added to the sample and mixed with a DNAse/RNAse free pastel (VWR, Canada). The mixture was incubated at 63ºC for 15 and 30 min respectively in order to determine the optimal conditions for DNA extraction.

2.2. Primer design

A set of four primers was designed from an 18S ribosomal RNA gene sequence (GenBank accession no. AB013017; Wada 1998) using PrimerExplorerV4 program (Fujitsu, Tokyo, Japan) (Figure 1). To ensure the specificity of the primers, they were blasted against the nucleotide GenBank database and assessed against *S. clava*, *B. schlosseri*, *B. violaceus* and *Didemnum* sp. The biotin-labeled forward inner

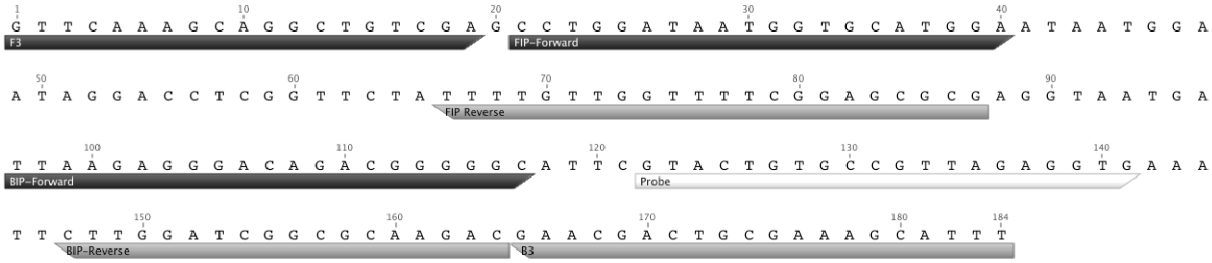

Figure 1. Localization of primers-probe set on the 18S ribosomal RNA amplicon target gene (accession # AB013017). The figure was made using Geneious version 5.5.

primer (Biotin-FIP: 5'Bio- CGCGCTCCGAAAA CCAACAAAATTTTCCTGGATAATGGTGCAT GGA-3'), backward inner primer (BIP: 5'-TTAAGAGGGACAGACGGGGGCTTTTGTCT TGCGCCGATCCAAG-3') and two outer primers (F3: 5'-GTTCAAAGCAGGCTGTCGA-3' and B3: 5'-AAATGCTTTCGCAGTCGTTC-3').

2.3. Loop-mediated isothermal amplification conditions

LAMP reaction mixture contained 2 mM of each primer Biotin-FIP and BIP, 0.2 mM of each outer primer F3 and B3, 1.4 mM of dNTP mix (Promega, Madison, WI, USA), 0.3 M betaine (Sigma–Aldrich, St. Louis, MO, USA), 6 mM $MgSO_4$, 8 U of *Bst* DNA polymerase (large fragment; New England Biolabs Inc., Beverly, MA, USA) along with 2.5 l of the 10X supplied buffer and the specified amount of template DNA in a final volume of 25 l. Negative and positive controls were performed using RNAse/DNAse free water and extracted DNA from *C. intestinalis* tissues respectively. The amplification step was performed at 63ºC for 60 min.

2.4. Probe design and hybridization conditions

A DNA probe (5'FITC-GTACTGTGCCGTTA GAGGTG-3') was designed from the targeted sequence using Visual OMP program (DNA Software, Michigan, USA). The probe was labeled with FITC at the 5' end and localized between FIP and BIP primers (Figure 1). The FITC-DNA probe (20 pmol) was added to the amplified sequence and the hybridization step was performed at 63ºC for 5 min.

2.5. Lateral flow dipstick (LFD) detection system

The LFD strip was purchased from Milenia Biotec GmbH (Gieben, Germany). Streptavidin anti-biotin molecules are coated on the nitrocellulose membrane. After the hybridization step, 150 l of the buffer was added to 8 l of the hybridization solution. After homogenization by mixing, the LFD was dipped into the mixture for 5 min. The hybridized FITC-DNA probe was revealed by binding the FITC to its antibody coated on gold-red beads, which could be visualized as a band where biotin-FIP was complexed to streptavidin.

Results and discussion

The focus of this study is to develop a rapid detection assay capable of identifying the presence of *Ciona intestinalis* larvae. This species is recognized as an invasive species competing for food and space with other filter feeders, thus affecting the ecosystem equilibrium and the local aquaculture economy (Ramsay et al. 2009). For instance, in PEI, the mussel aquaculture market dramatically suffered from the introduction of invasive species such as *C. intestinalis.*

In this study, the rapid detection was performed on *Ciona intestinalis* larvae's DNA in the laboratory. The sensitivity and specificity of the assay were assessed using DNA extracted from various tunicate species. For the specificity, DNA was extracted from *Didemnum* sp., *C. intestinalis*, *Styela clava*, *Botryllus schlosseri* and *Botrylloides violaceus*. The assay was performed on the extracted DNA and the data

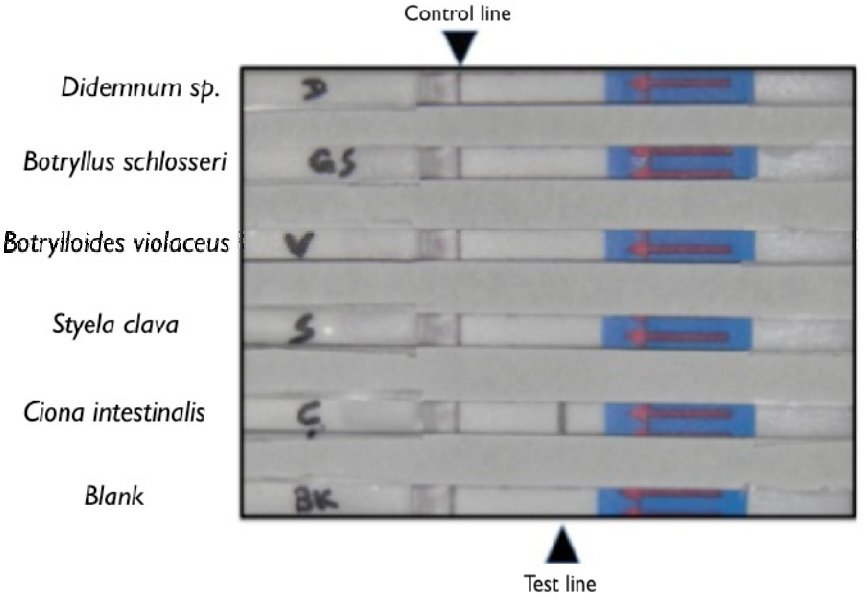

Figure 2. Assay specificity DNA was extracted from *Didemnum sp.*, *Ciona intestinalis, Styela clava*, *Botryllus schlosseri* and *Botrylloides violaceus*. The assay showed a specific band related only to vase (*Ciona intestinalis*) tunicates. The control line corresponds to the binding reaction between the antibody coated on the nitrocellulose membrane and the equivalent antibody coated on gold-red beads.

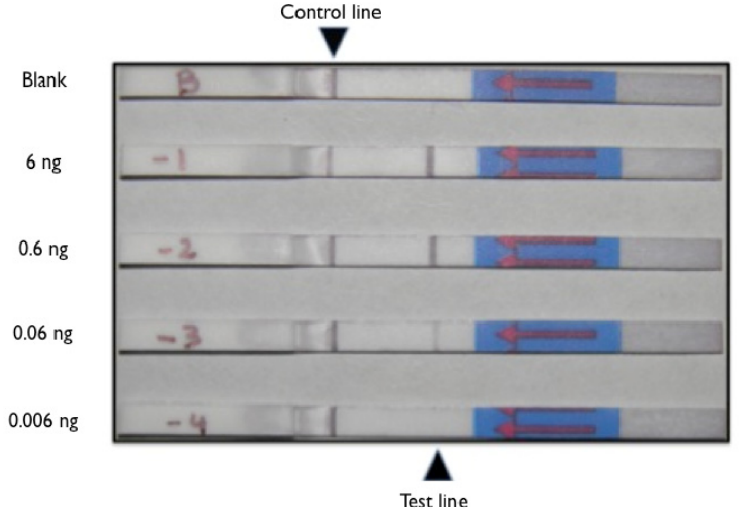

Figure 3. Assay sensitivity using extracted DNA A 10-fold serial dilution of extracted DNA from *Ciona intestinalis* was performed using the assay. The lower limit of detection of the assay is 0.06 ng.

showed a specific band for DNA extracted from *C. intestinalis* (Figure 2). The sensitivity of the assay was assessed using a 10-fold serial dilution of the extracted DNA (60 ng) and was estimated at 0.06 ng of extracted DNA (Figure 3). In addition, the sensitivity of the assay was assessed using larvae. Different quantities of larvae were spiked in filtrated seawater. The assay, which was able to detect as little as 1 larva (Figure 4), was performed in 3 steps: lysis, amplification and hybridization/detection. The turnaround time for the amplification is about 45 min and the hybridization/detection step is about 5–10 min long. One larva spiked in 100 µL of seawater was lysed for 15 and 30 min, before the assay was performed. A 30-min-lysing time sufficed to detect a band (Figure 5). The total turnaround time of the assay from collection to detection is approximately 80 min.

Currently, the main method for the identification of invasive tunicate species is morphological taxonomy (Lambert 2001). For

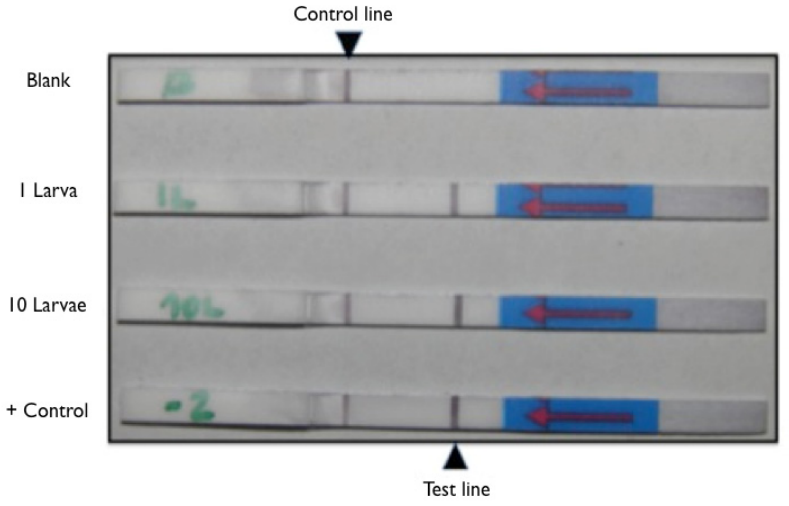

Figure 4. Assay sensitivity using larvae spiked in seawater 1 and 10 larvae were added to seawater and the assay was performed to detect the DNA. The lower detection limit of the assay is one larva.

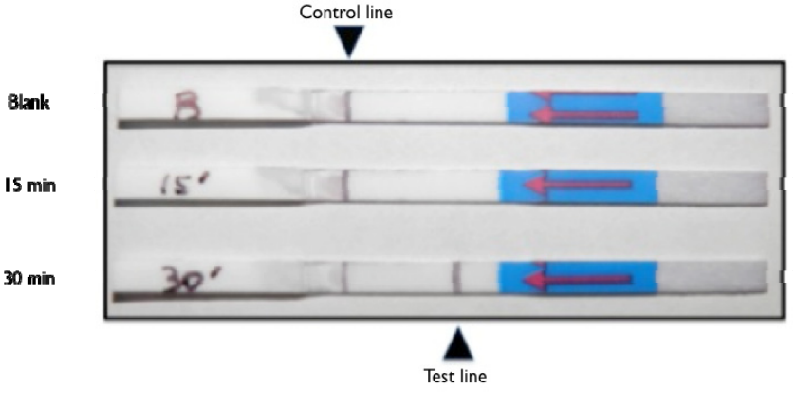

Figure 5. Turn-around time for the lysis step The time required for complete lysis of the sample was estimated at 30 min.

the efficient management of bay and aquaculture infrastructure, early warning detection tools are needed (see review Bott et al. 2010). To overcome this, primers were developed for Polymerase Chain Reaction (PCR) detection assays using 18S and COI as sensitive and specific target genes to detect invasive tunicate species from Atlantic Canada seawater (Stewart-Clark et al. 2009; Willis et al. 2011). Although PCR-based technologies currently represent the "golden standard" in screening assays, these technologies are not generally suitable for field detection. PCR-based technologies require sophisticated equipment and skilled technical personnel.

Since this assay is currently performed under isothermal conditions, therefore no sophisticated equipment is required, it would be suitable for use in the field. In addition, the assay is performed in 80 minutes with high sensitivity (1 larva) and a specificity comparable to PCR-based assays.

However, DNA amplification techniques such as PCR and LAMP do have some limitations. Due to the high amount of amplified material, contamination is likely to occur during the amplification steps. To avoid cross contamination between samples, molecular devices present an interesting avenue for field screening (Harvey et al. 2009). Interestingly, micro-fluidic chips integrating LAMP technology have recently been developed in the field of pathogen detection (Fang et al. 2010). These new technologies will provide interesting tools for invasive tunicate species detection in the field.

Conclusion

In the last decade, much effort has been put into the development of rapid molecular assays to detect the presence of invasive species in seawater at an early stage. The suggested diagnostic tool does not require special instruments since the user simply has to use a heat block or incubator for the LAMP step and dip the LFD in appropriately buffered LAMP reaction solution. Using the combined LAMP and LFD system (LAMP–LFD), the total assay interval is approximately 80 min and the assay can specifically detect *Ciona intestinalis* in seawater.

The suggested assay could be adapted for the detection of multiple invasive aquatic species present in different biological matrices such as tissues and seawater. The test is expected to produce a screening assay that could be used in the field. Further investigations should be conducted in the field to validate the accuracy of the assay.

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References

Bott NJ, Ophel-Keller KM, Sierp MT, Herdina, Rowling KP, McKay AC, Loo MGK, Tanner JE, Deveney MR (2010) Toward routine, DNA-based detection methods for marine pests. *Biotechnology Advances* 28: 706–714, http://dx.doi.org/10.1016/j.biotechadv.2010.05.018

Colautti R, Bailey S, Overdijk C, Amundsen K, MacIsaac H (2006) Characterised and Projected Costs of Nonindigenous Species in Canada. *Biological Invasions* 8: 45–59, http://dx.doi.org/10.1007/s10530-005-0236-y

Dawson M (2002) Plant Quarantine: a tool for preventing the introduction and spread of alien species harmful to plants. In: Claudi R, Nantel P and Muckle-Jeffs E (eds), Alien invaders in Canada's waters, wetlands and forests. Canadian Forest Service, Ottawa, Canada, pp 243–251

Fang X, Liu Y, Kong, J, Jiang X (2010) Loop-Mediated Isothermal Amplification Integrated on Microfluidic Chips for Point-of-Care Quantitative Detection of Pathogens. *Analytical Chemistry* 82: 3002–3006, http://dx.doi.org/10.1021/ac1000652

Harvey JBJ, Hoy MS, Rodriguez R (2009). Molecular detection of native and invasive marine invertebrate larvae present in ballast and open water environmental samples collected in Puget Sound. *Journal of Experimental Marine Biology and Ecology* 369: 93–99, http://dx.doi.org/10.1016/j.jembe.2008.10.030

Lambert G (2001) A global overview of ascidian introductions and their possible impact on endemic fauna. In: Sawada H, Yokosawa H, Lambert CC (eds), The Biology of Ascidians. Springer-Verlag, Tokyo, pp 249–257

Locke A, Hanson J, Ellis K, Thompson J, Rochette R (2007) Invasion of the southern Gulf of St. Lawrence by the clubbed tunicate (*Styela clava* Herdman): Potential mechanisms for invasions of Prince Edward Island estuaries. *Journal of Experimental Marine Biology and Ecology* 342: 69–77, http://dx.doi.org/10.1016/j.jembe.2006.10.016

Locke A (2009) A screening procedure for potential tunicate invaders of Atlantic Canada. *Aquatic Invasions* 4: 71–79, http://dx.doi.org/10.3391/ai.2009.4.1.7

Mori Y, Notomi T (2009) Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *Journal of Infectious Chemotherapy* 15: 62–69, http://dx.doi.org/10.1007/s10156-009-0669-9

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T (2000) Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research* 28: e63, http://dx.doi.org/10.1093/nar/28.12.e63

Pimentel D, Lach L, Zuniga R, Morrison D (2000) Environmental and economic cost of nonindigenous species in the United States. *Biosciences* 50: 53–65, http://dx.doi.org/10.1641/0006-3568(2000)050[0053:EAECON]2.3.CO;2

Pimentel D, McNair S, Janecka J, Wightman J, Simmonds C, O'Connell C, Wong E, Russel L, Zern J, Aquino T, Tsomondo T (2001) Economic and environmental threats of alien plant, animal and microbe invasions. *Agriculture, Ecosystems and Environment* 84: 1–20, http://dx.doi.org/10.1016/S0167-8809(00)00178-X

Ramsay A, Davidson J, Landry T, Arsenault G (2008) Process of invasiveness among exotic tunicates in Prince Edward Island, Canada. *Biological Invasions* 10: 1311–1316, http://dx.doi.org/10.1007/s10530-007-9205-y

Ramsay A (2009) Recruitment patterns and population development of the invasive ascidian *Ciona intestinalis* in Prince Edward Island, Canada. *Aquatic Invasions* 4: 169–176, http://dx.doi.org/10.3391/ai.2009.4.1.17

Stewart-Clark S, Siah A, Greenwood S, Davidson J, Berthe F (2009) Development of 18S rDNA and COI gene primers for the identification of invasive tunicate species in water samples. *Aquatic Invasions* 4: 575–580, http://dx.doi.org/10.3391/ai.2009.4.4.2

Wada H (1998) Evolutionary history of free-swimming and sessile lifestyles in urochordates as deduced from 18S rDNA molecular phylogeny. *Molecular Biology and Evolution* 15: 1189–1194, http://dx.doi.org/10.1093/oxfordjournals.molbev.a026026

Willis J, Stewart-Clark S, Greenwood SJ, Davidson J, Quijon P (2011) A PCR-based assay to facilitate early detection of *Diplosoma listerianum* in Atlantic Canada. *Aquatic Invasions* 6: 7–16, http://dx.doi.org/10.3391/ai.2011.6.1.02