

Research article**Survey of harbours in Newfoundland for indigenous and non-indigenous ascidians and an analysis of their cytochrome *c* oxidase I gene sequences**Ashley G. Callahan^{1*}, Don Deibel¹, Cynthia H. McKenzie², Jennifer R. Hall¹ and Matthew L. Rise¹¹Ocean Sciences Centre, Memorial University, St. John's NL, A1C 5S7, Canada²Fisheries & Oceans Canada, Northwest Atlantic Fisheries Centre, P.O. Box 5667, St. John's NL A1C 5X1, CanadaE-mail: ashleyc@mun.ca (AGC), ddeibel@mun.ca (DD), cynthia.mckenzie@dfo-mpo.gc.ca (CHM), jrhall@mun.ca (JRH), mrise@mun.ca (MLR)

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Abstract

Invasive, non-indigenous ascidians have been a significant biofouling problem for the aquaculture industry in Nova Scotia and Prince Edward Island since the mid-1990's. The problematic species in Atlantic Canada include *Styela clava*, *Ciona intestinalis*, *Botryllus schlosseri* and *Botrylloides violaceus*. Newfoundland harbour surveys that we have performed over the past three years revealed the presence of *B. schlosseri* and *B. violaceus*. As of yet, neither of these species has reached invasive abundance in Newfoundland.

Portions of the COI genes of two non-indigenous ascidians (*Botryllus schlosseri* and *Botrylloides violaceus*) and two indigenous ascidians (*Boltenia echinata* and *Halocynthia pyriformis*) were cloned and sequenced. We then determined intraspecific and interspecific COI sequence variation. The BLASTN results showed that *Botryllus schlosseri* and *Halocynthia pyriformis* match the other listings for these species in GenBank. However, the BLAST results from *Botrylloides violaceus* were more similar to *B. schlosseri* than *B. violaceus*. While there were no other entries for *Boltenia echinata*, it was similar to other species in the Pyuridae family.

Intraspecific similarity in the COI sequence for Newfoundland populations was >99.7% for *B. violaceus*, *H. pyriformis* and *B. echinata*, and 86.5-96.6% for *B. schlosseri*. Interspecific similarities among all four species were <80.3%. This indicates that the COI gene should be an effective species-specific molecular marker for the identification of eggs and larvae of these Newfoundland ascidians.

Key words: COI, invasive ascidians, mtDNA, *Botryllus schlosseri*, *Botrylloides violaceus*, nucleotide sequence**Introduction**

Bioinvasions are some of the leading marine environmental issues in the world and represent a serious global threat (Ruiz et al. 2000; Stachowicz et al. 2002; Occhipinti-Ambrogi and Galil 2004; Campbell et al. 2007). Non-indigenous marine species can threaten marine biodiversity, survival of native species and the economy (Blum et al. 2007). Non-indigenous ascidians are a significant biofouling problem for the aquaculture industry worldwide, especially on both the east and west coasts of Canada. Over the past ten years, four ascidian tunicates [*Styela clava* Herdman, 1881, *Ciona intestinalis* (Linnaeus, 1767), *Botryllus schlosseri* (Pallas, 1766) and *Botrylloides violaceus* Oka, 1927] have reached invasive levels at aquaculture sites in Nova Scotia and Prince Edward Island, Canada (Carver et al. 2003; Locke et al. 2007).

Impacts of invasive ascidians on the aquaculture industry include biofouling of the harvested species themselves as well as fouling of the growing and processing equipment (Carver et al. 2003). These impacts lead to decreased economic performance of the farms due to increased production costs (Carver et al. 2003). Newfoundland is considered to be a high-risk area for the introduction of these four non-indigenous species (Therriault and Herborg 2008a, b). This prediction is based on the fact that Newfoundland has many shipping links with the other Maritime provinces and that some areas of the coastline have environmental conditions within the tolerance limits of these non-indigenous ascidians (Locke et al. 2007; Therriault and Herborg 2008b).

There has been very little targeted research conducted on invasive ascidians in Newfoundland thus far. The only documented occurrence

of a non-indigenous ascidian in Newfoundland was *Botryllus schlosseri*, which was reported in 1975 on the west coast (Hooper 1975). Rapid assessment surveys (RAS) are an efficient and reliable way to look for indigenous and non-indigenous species occupying an area. They are particularly useful in identifying the presence or absence of rare species in a given area and are commonly conducted in harbours (Pederson et al. 2005; Campbell et al. 2007).

The cytochrome *c* oxidase I (COI) gene of mitochondrial DNA (mtDNA) is a common gene useful for molecular identification of species and for uncovering patterns of diversity within and among populations and in communities (Muirhead et al. 2008). As far as we know, there is no information in the literature on molecular analyses and gene sequencing of ascidian species collected from Newfoundland populations. The COI gene is a logical first choice for species identification and may be useful for early detection of propagules for management purposes. A relatively short (~ 650 base) fragment of COI has been used in the identification of species in many groups of animals (e.g. fish, birds, insects) because this size of DNA fragment allows the acquisition of reliable sequences from single reads using standard cycle sequencing, and this COI region is generally highly conserved within species (Hajibabaei et al. 2007).

The objectives of this study were: (a) to determine the presence of non-indigenous ascidians in harbours along the south and west coasts of Newfoundland, and (b) to determine the nucleotide sequence of a portion of the COI gene of mtDNA of indigenous and non-indigenous ascidian tunicates, to confirm taxonomic identity and to compare COI sequence similarity within and among species.

Methods

Rapid assessment surveys

Rapid assessment surveys (RAS) were carried out in a total of 37 Newfoundland harbours on the south and west coasts of insular Newfoundland (September 2006-October 2008) (locations not shown). The RAS followed existing protocols (Pederson 2005; Campbell 2007). During these RAS, SCUBA divers took a combination of underwater videos, a vertical series of still photographs, and visual transect surveys of the bottom in addition to the

collection of environmental data. Wharf pilings, boat hulls, seafloor and artificial structures were assessed for the presence of non-indigenous species. GPS coordinates were recorded of all wharves examined. The RAS were conducted by a team of 5-7 individuals, including two SCUBA divers. The RAS ranged from 1-3 hours per harbour based upon the size of the area to be assessed. The RAS team assessed all of the wharf pilings in each harbour.

Sample collection and study species

In 2006 and 2007, adult specimens of *Botryllus schlosseri*, *Botrylloides violaceus*, *Halocynthia pyriformis* (Rathke, 1806) and *Boltenia echinata* (Linnaeus, 1767) were collected for genetic analyses, from sites within Newfoundland, Prince Edward Island and Massachusetts, by SCUBA divers or by hand from wharf pilings. Samples were fixed in 95% ethanol and stored at room temperature. In this paper we report gene sequences from a subset of these samples (Table 1). *Botryllus schlosseri* is a colonial ascidian that originated from the Mediterranean Sea, while *Botrylloides violaceus*, also colonial, is thought to have originated from the Northwest Pacific Ocean (Berrill 1950; Carver et al. 2006). *B. echinata* and *H. pyriformis* are solitary ascidians, both having a northern boreal distribution (Plough 1978). Both are indigenous to Newfoundland.

DNA extraction, COI amplification and sequencing

Adult ascidians were visually identified based on morphology (Plough 1978; Pollock 1997) before genetic analyses were performed. For each of the colonial ascidians (*Botryllus schlosseri* and *Botrylloides violaceus*), several zooids (~5-10) from a system were removed and considered to be a single sample. Tissue was removed from the pharyngeal sac of the solitary ascidians (*Halocynthia pyriformis* and *Boltenia echinata*). The numbers of samples analyzed from each location are shown in Table 1.

Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Mississauga, ON) following the manufacturer's protocol. To remove residual ethanol prior to DNA extraction, ascidian tissue samples were soaked in the following series of ethanol/phosphate buffered saline (PBS) washes for five minutes each: 75% ethanol/25% PBS; 50% ethanol/50% PBS; 25% ethanol/75% PBS; 100% PBS.

Figure 1. Map of the region containing insular Newfoundland, Canada, indicating sampling locations for the molecular analyses (main map; letters corresponding to Table 1) and the locations of *Botryllus schlosseri* (Green) and *Botrylloides violaceus* (Red) from the Rapid Assessment Survey (RAS) (map inset).

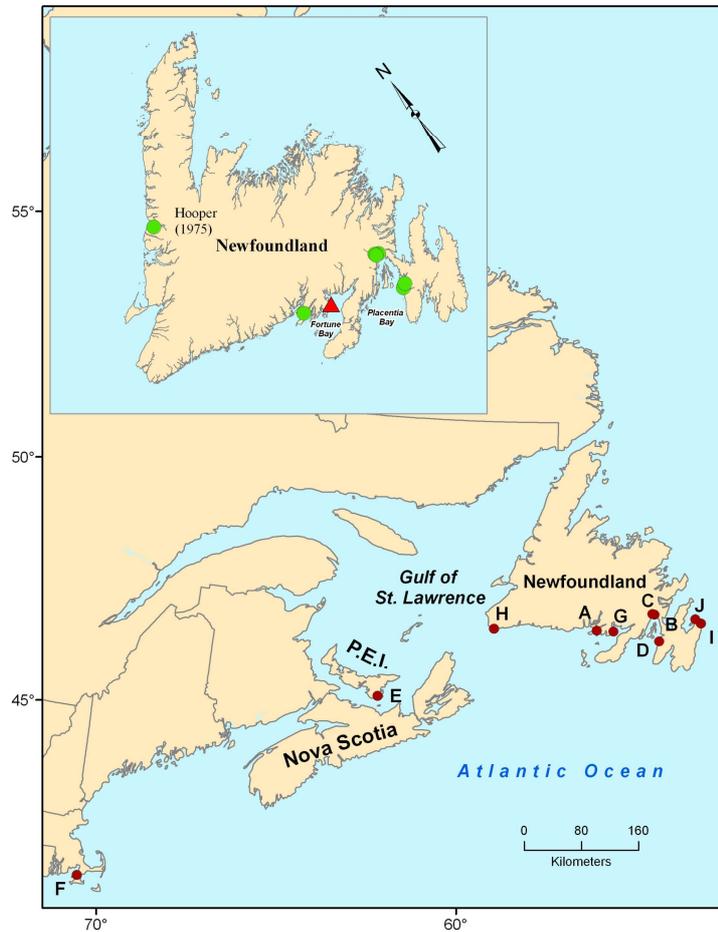


Table 1. Geographic location, map code for Figure 1, latitude and longitude (GPS), sample size (n) and GenBank accession numbers for the partial nucleotide sequences of the cytochrome *c* oxidase I gene. NL = Newfoundland, PEI = Prince Edward Island, MA = Massachusetts.

Species	Location	Map Code	Latitude	Longitude	n	GenBank Accession Numbers
<i>Botryllus schlosseri</i>	Hermitage, NL	A	47.5563	-55.9259	1	GU065354, DQ340216
	North Harbour, NL	B	47.8590	-54.1000	1	GU065353, DQ340205
	Arnold's Cove, NL	C	47.8747	-54.1682	1	GU065350
	Argentia, NL	D	47.2920	-53.9904	1	GU065349
	Murray River, PEI	E	46.0170	-62.6155	2	GU065351
<i>Botrylloides violaceus</i>	Woods Hole, MA	F	41.5170	-70.6683	2	GU065352
	Belleoram, NL	G	47.5272	-55.4092	3	GU065355, GU065356
	Murray River, PEI	E	46.0170	-62.6155	1	GU065357
<i>Halocynthia pyriformis</i>	Woods Hole, MA	F	41.5170	-70.6683	3	GU065358, GU065359
	Port-aux-Basques, NL	H	47.5751	-59.1402	4	EU178858
<i>Boltenia echinata</i>	Logy Bay, NL	I	47.6253	-52.6646	1	EU178861
	Port-aux-Basques, NL	H	47.5751	-59.1402	1	GU065360
	Logy Bay, NL	I	47.6253	-52.6646	1	GU065361
	Bauline, NL	J	47.7232	-52.8348	1	GU065362

Total DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Mississauga, ON) following the manufacturer's protocol. To remove residual ethanol prior to DNA extraction, ascidian tissue samples were soaked in the following series of ethanol/phosphate buffered saline (PBS) washes for five minutes each: 75% ethanol/25% PBS; 50% ethanol/50% PBS; 25% ethanol/75% PBS; 100% PBS.

Three primer sets were used to amplify partial sequences of the mtDNA COI gene. The primer sets included: the primers, LCO1490f, 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198r, 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3', (Folmer et al. 1994), ASC_COI_F, 5'-TCG ACW AAT CAT AAA GAT ATT AG-3' and ASC_COI_R, 5'-GTA AAA TAA GCT CGA GAA TC-3' (Vogler, per. comm.), and a species-specific primer set was developed for *Botrylloides violaceus*, Violet Forward, 5'-TTA GGT TTT GGT CTA GGT TTA TTG-3' and Violet Reverse, 5'-TAA ATG TTG ATA AAG TAC AGG GTC-3'.

Polymerase chain reaction (PCR) amplifications were performed using DyNAzyme EXT DNA polymerase (MJ Research, Waltham, MA). Briefly, 50 μ l reactions were prepared containing 100 ng of DNA, DyNAzyme EXT DNA polymerase (1U), the manufacturer's Optimized DyNAzyme EXT Buffer (1X final concentration), 0.2 mM dNTPs and 0.2 μ M each of forward and of reverse primer.

The PCR reaction conditions were 94°C for 2 min, followed by 40 cycles (94°C for 30 sec, 37°C for 30 sec, and 72°C for 1 min) and a final extension at 72°C for 7 min. The PCR products were electrophoresed on 1.5% agarose gels with 1 kb plus ladder (Invitrogen, Burlington, ON), and amplicons were excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). The PCR amplified COI was subcloned into pGEM T-Easy (Promega, Madison, WI). Transformations were performed using either Subcloning Efficiency DH5 α Chemically Competent Cells (Invitrogen, Burlington, ON) or JM109 High Efficiency Competent Cells (Promega, Madison, WI) following the manufacturers' instructions. Recombinant clones were screened for inserts of correct size by visual comparison of clone restriction fragments with a DNA size marker (1 kb plus ladder; Invitrogen, Burlington, ON) using 1.5% agarose gel electrophoresis.

Plasmid DNA from positive clones was isolated for sequencing using the QIAprep Spin Miniprep Kit (Qiagen). Each individual clone was sequenced in both directions (using M13 Forward and M13 Reverse primers). DNA was amplified from approximately 100 ng of recombinant plasmid DNA using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the unincorporated BigDye terminators were removed using the BigDye X Terminator Purification Kit (Applied Biosystems). The purified sequencing reactions were processed by capillary electrophoresis using the 3730xl DNA analyzer (Applied Biosystems).

Sequence comparisons

COI sequences from each individual were compiled and aligned using Vector NTI Advance 10 (Invitrogen) and a consensus sequence was determined for each individual and/or species. Compilation and alignments were performed using AlignX, which uses the CLUSTALW algorithm (Thompson et al. 1994).

To confirm taxonomic identity, the COI sequences generated for the four putative species in this study were compared with those in GenBank using a Basic Local Alignment and Search Tool Nucleotide (BLASTN 2.2.20) search of the non-redundant nucleotide collection (nr/nt). A BLASTN search can be run to determine the similarity of an unknown DNA sequence (query) with the collection of all known DNA sequences in GenBank. BLAST searches were performed between January 2009 and January 2010 and reflect the state of the database at that time. In this paper we focus only on those BLASTN hits with the highest percent identities and the lowest E-values).

To confirm high intraspecific similarity in the COI sequences for samples from the Newfoundland populations, we determined genetic distance (100 – the sequence dissimilarity score) pairwise for all haplotypes using AlignX. High intraspecific similarity and lower interspecific similarity are prerequisites for future use of the COI gene for species-specific markers for Newfoundland ascidians. All of our sequences have been deposited in GenBank and the GenBank accession numbers are shown in Table 1.

Results

Rapid assessment surveys

We initially discovered *Botryllus schlosseri* on the bottom of a small, local fishing vessel in Argentia harbour in December 2006, and *Botrylloides violaceus* on wharf pilings in Belleoram harbour in October 2007 (Figure 1). Subsequently, we have found *B. schlosseri* in several additional harbours, primarily on the east side of Placentia Bay and the west side of Fortune Bay (Figure 1). *B. schlosseri* has been found growing not only on boat hulls, but also on wharf pilings, kelp fronds, mussel shells and encrusting bryozoans. As of January 2010, we have found *B. violaceus* only in Belleoram, where it is growing on boat hulls, wharf pilings, as well as on mussel shells and natural substrates. In February 2008, a follow-up survey revealed that *B. violaceus* appeared to have died back substantially during winter. It is notable that all of the harbours in which non-indigenous ascidians were documented are on the south coast of Newfoundland.

Cytochrome *c* oxidase I sequences

We obtained partial COI sequences from four species of Newfoundland ascidians from eight different harbours (Table 1). Initially, the primers developed by Folmer et al. (1994) for the amplification of a 709 bp fragment of the COI gene from phylogenetically diverse metazoan invertebrates were used on all four species in our study. These primers generated amplicons for *Halocynthia pyriformis* and *Botryllus schlosseri*, but yielded either no or incorrect amplicons for *Boltenia echinata* and *Botrylloides violaceus*. Therefore, another primer set developed by Vogler (pers. comm.) was applied to these two species. These Vogler primers were successful in the amplification of a COI fragment in *B. echinata* but still failed for *B. violaceus*. Therefore, we designed new species-specific primers for *B. violaceus* based on the limited sequences obtained from the Folmer primers. The *B. schlosseri* and *H. pyriformis* partial COI sequence we obtained was 658 bp (excluding primer sequences). The *B. violaceus* partial COI sequence was 590 bp, whereas that of *B. echinata* was 850 bp (excluding primer sequences). The sequences which were submitted to GenBank at the time of writing are indicated in Table 1. Our COI

sequences are the first in GenBank for *H. pyriformis* and *B. echinata*.

The number of haplotypes and polymorphic sites were much higher for *Botryllus schlosseri* from Newfoundland harbours than for any of the other three species examined in this study, including the samples from the Northwestern Atlantic (NWA) populations (Table 2). In comparison to *B. schlosseri*, the number of haplotypes of the non-indigenous *Botrylloides violaceus* was much lower, with only one haplotype in common amongst the Newfoundland and NWA populations (Table 2). Newfoundland populations of each of the two indigenous species had two haplotypes, but a low number of polymorphic sites (≤ 2).

Table 2. Population, sample size (n), number of haplotypes (Nh) and number of polymorphic sites (ps) of a segment of the cytochrome *c* oxidase I gene for the four ascidian species studied. NWA includes samples from Woods Hole, Massachusetts and Prince Edward Island (see Table 1). 'Newfoundland' includes samples from all of the harbours listed in Table 1.

Species	Population	n	Nh	ps
<i>Botryllus schlosseri</i>	Newfoundland	4	3	29
	NWA	4	1	0
<i>Botrylloides violaceus</i>	Newfoundland	3	1	0
	NWA	4	1	0
<i>Halocynthia pyriformis</i>	Newfoundland	5	2	2
<i>Boltenia echinata</i>	Newfoundland	3	2	1

Results from the BLAST analyses helped to confirm taxonomic identification of the four putative ascidian species in this study. Every sample from Newfoundland (Table 1) was BLASTN searched. The *Botryllus schlosseri* samples from this study matched other *B. schlosseri* GenBank submissions with similarities ranging from 95-100% (Table 3). Similarly, the *Halocynthia pyriformis* samples matched other *H. pyriformis* species in GenBank, with similarities $\geq 99\%$. There were no COI samples of *B. echinata* in GenBank prior to this study. Our Newfoundland samples were most similar to the pyrid ascidian *Microcosmus sulcatus* (accepted name: *Microcosmus vulgaris* Heller, 1877) (76% similarity, Table 3). The Newfoundland samples tentatively identified as *B. violaceus* based on morphological features had greatest similarity to several *B. schlosseri*

Table 3. Species names associated with the known sequences most similar to the cytochrome c oxidase I gene fragments of the four putative Newfoundland ascidian species, as shown by BLASTN searches of GenBank. % ID = % nucleotide sequence identity. E = expected value, a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size. NWA= (Prince Edward Island + Massachusetts).

Putative ascidian species	Location	BLASTN		
		Most homologous species name and GenBank Accession #	% ID (align)	E-value
<i>Botryllus schlosseri</i>	Newfoundland	<i>Botryllus schlosseri</i> ; FJ528641.1	626/653 (95%) to 653/653 (100%)	0.0
	NWA	<i>Botryllus schlosseri</i> ; FJ528641.1	627/651 (96%)	0.0
<i>Botrylloides violaceus</i>	Newfoundland	<i>Botryllus schlosseri</i> ; AY116601.1	481/588 (81%)	1e-161
	NWA	<i>Botryllus schlosseri</i> ; AY116601.1	481/588 (81%)	1e-161
<i>Halocynthia pyriformis</i>	Newfoundland	<i>Halocynthia pyriformis</i> ; FJ528610.1	656/658 (99%) to 658/658 (100%)	0.0
<i>Boltenia echinata</i>	Newfoundland	<i>Microcosmus sulcatus</i> ; AM292321.1	645/848 (76%) to	3e-172 to 6e-
			646/848 (76%)	174

Table 4. Nucleotide sequence identity table (%) among the four ascidian species studied from Newfoundland. The values were generated between all pairs of samples using AlignX.

	<i>Botrylloides violaceus</i>	<i>Botryllus schlosseri</i>	<i>Halocynthia pyriformis</i>	<i>Boltenia echinata</i>
<i>Botrylloides violaceus</i>	100	78.8-80.3	74.2-74.4	76.3-76.4
<i>Botryllus schlosseri</i>		86.5-96.6	75.6-76.3	76.9-78.6
<i>Halocynthia pyriformis</i>			99.7	74.9-79.0
<i>Boltenia echinata</i>				99.9

sequences from GenBank, but the similarities were low (i.e., 81 %, Table 3). The similarity to the single *B. violaceus* sequence in GenBank was even lower (80 %).

We then compared sequence similarities among our Newfoundland samples of the four species, to confirm that the COI gene qualifies as a species-specific marker for Newfoundland populations, i.e. low interspecific and high intraspecific similarity. Intraspecific similarity (the diagonal in Table 4) of all species except *B. schlosseri* was $\geq 99.7\%$. Intraspecific similarity for *B. schlosseri* was somewhat lower among Newfoundland populations (87-97 %, Table 4). The interspecific similarities among the four species (i.e., the off-diagonal elements in Table 4) were much lower than the above intraspecific values, ranging from 74-80 %.

Discussion

Rapid assessment survey

Our RAS revealed the presence of two non-indigenous ascidians in Newfoundland harbours, *Botryllus schlosseri* and *Botrylloides violaceus*. *B. schlosseri* was first reported in Newfoundland by Hooper (1975), while this is the first report of

B. violaceus. This successional pattern is similar to that reported from elsewhere in the north-western Atlantic. *B. schlosseri* has been present in the Gulf of Maine since 1870, while *B. violaceus* was detected there only 25 years ago (Gould 1870; Dijkstra et al. 2007). *B. schlosseri* also preceded the presence of *B. violaceus* in Prince Edward Island, Canada, in 2001-02 (Locke et al. 2007).

Botrylloides violaceus was most likely introduced into the Gulf of Maine via aquaculture activity and recreational boating (Dijkstra et al. 2007). Our first sighting of *B. violaceus* in Belleoram harbour was on the hull of several small fishing vessels. At least one of these vessels had been purchased within the previous five years from Nova Scotia, which is known to have *B. violaceus* (Andrea Locke, pers. comm.). There are no regulations regarding small vessel movement (except aquaculture related vessels) between Canadian provinces, or hull cleaning of small coastal boats, so the risk to Newfoundland from this vector is high.

The Newfoundland Department of Fisheries and Aquaculture took the discovery of *B. schlosseri* and *B. violaceus* seriously, using the same management protocols as are used for fish health issues, prohibiting the export of mussels from

Placentia Bay for processing elsewhere in 2006. This highlights the importance of non-indigenous species issues in Newfoundland, and the need for more scientific information to better inform management decision-making. The Newfoundland Department of Fisheries and Aquaculture has monitored Newfoundland aquaculture sites for aquatic invasive species since 2005. No non-indigenous tunicates have been found at any Newfoundland aquaculture site to date.

Cytochrome c oxidase I sequences

The four ascidian species differed markedly in the success of COI sequence amplification using universal primers (Folmer et al. 1994) that have been used to amplify COI sequences from over 80 taxa, including ascidians and bivalves (Folmer et al. 1994). However, Pérez-Portela et al. (2009) reported failure of amplification in the ascidian *Stolonica socialis* (Hartmeyer, 1903) using the Folmer primers, but no problem in the amplification of *B. violaceus*. Dan Bock (pers. comm.) also report amplification failure of *B. violaceus* using the Folmer primers. In our study, the Folmer primers either failed to generate COI amplicons (*Botrylloides violaceus* and *Boltenia echinata*) or generated non-ascidian COI sequences (*Mytilus trossulus* Gould, 1850). The most likely explanation for the failure of the Folmer primers to amplify COI is the divergence between species at this particular locus. In the case of incorrect amplicons being generated, i.e. the amplification of COI from non-target organisms, many of the ascidians we collected were attached to the surfaces of other organisms (i.e. kelp, mussels and bryozoans). To circumvent these issues, we used the Vogler primer set (a modification of the Folmer primers), which was designed to be more specific for the amplification of COI in ascidians (Vogler, pers. comm.). This set has been successful in the amplification of several ascidians species from the Mediterranean Sea (Vogler, per. comm.) and also worked well for our *B. echinata*. In the case of *B. violaceus*, we had to develop a species-specific primer by aligning the partial sequences of *B. violaceus* and other closely related species to identify regions in which the sequences were different, thereby eliminating the generation of COI sequences from contaminating organisms.

Although our sample size was relatively small, our estimates of the number of haplotypes for *B. schlosseri* fall within the range of that

published by López-Legentil et al. (2006) of 2-4 population-1 based on larger sample sizes of 11-25. In addition, they found three haplotypes from only four samples at Woods Hole, Massachusetts, U.S.A., as we did for *B. schlosseri* in Newfoundland (Table 2). The number of polymorphic sites found for *B. schlosseri* within Newfoundland (29) was much higher than the polymorphic sites in other three ascidians (0-2) from this study. However, they fall within the range of polymorphic sites for *B. schlosseri* (12-89) reported by López-Legentil et al. (2006). High numbers of polymorphic sites have also been reported for other ascidian species (Turon et al. 2003; Tarjuelo et al. 2001; Lopez-Legentil and Turon 2005; Silva and Smith 2008) including the invasive ascidians *Ciona intestinalis* and *C. savignyi* from California (Dehal et al. 2002; Vinson et al. 2005). Silva and Smith (2008) reported recently that ten different ascidian species show substantially different levels of genetic polymorphism and exceptions to the assumption that invasive species start with a low level of genetic polymorphism that increases over time.

In contrast to *Botryllus schlosseri*, the other non-indigenous ascidian, *Botrylloides violaceus*, showed only one haplotype within Newfoundland and the NWA. These results are comparable to those of Dan Bock (pers. comm.), who also found very low haplotype and nucleotide diversity in *B. violaceus*. Within a total of 192 samples from North America and Japan, there were only five haplotypes, and the individuals from the NWA all shared the same haplotypes (Dan Bock, pers. comm.).

The BLAST results provided confirmation of our taxonomic identifications for three out of the four species that we studied. Since there were no other COI sequences available in GenBank for *B. echinata*, our sequences matched most closely with *Microcosmus vulgaris*, also in the Pyuridae family to which *B. echinata* belongs. The case for *Botryllus violaceus* was more complex. The most similar sequence to our samples of *B. violaceus* was *Botryllus schlosseri*, and the single GenBank entry for *B. violaceus* was slightly less homologous. Given this uncertainty, we sent a type specimen to an independent authority who confirmed *B. violaceus* (Gretchen Lambert, pers. comm.).

We determined that the COI gene can be used to differentiate among indigenous and non-indigenous ascidians species from Newfoundland. However, levels of intraspecific similarity

were not the same for all four species. With the exception of *B. schlosseri*, intraspecific similarity was > 99 %. Although intraspecific variability was much higher in *B. schlosseri* compared to the other species, similarity within this species (87-95%) was still greater than all of the interspecific similarities, which were < 85 %. It will be important in future research to sequence more individuals from several Newfoundland populations to expand knowledge of haplotype diversity of populations of *B. schlosseri* and *Botrylloides violaceus* in Newfoundland. This question has important management implications, because regulation of invasion vectors is often the most effective way to control the spread of invasive marine invertebrates (Bax et al. 2003). Ascidians are difficult to identify with morphological methods, especially when they are in the egg, larval and juvenile stages of development (Darling and Blum 2007). Therefore, molecular methods for identifying ascidian gametes and early life stages are needed. This paper presents the preliminary work necessary to develop molecular markers for rapid identification of early life stages of these ascidians.

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